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Identification of the components of the Eukaryotic Queuine Transglycosylase Complex and the effect of its substrate Queuine on Metabolism

Patti Hayes

A Thesis Submitted to
Trinity College Dublin
for the degree
of
Doctor of Philosophy

School of Biochemistry and Immunology
Trinity College Dublin
2010
# Table of Contents

Declaration of Authorship .................................................. i
Acknowledgements .............................................................. ii
Abstract ................................................................................. iii
Publication ................................................................................ iv
Abbreviations ....................................................................... v

## Chapter 1 Introduction
1.1 Queuine, a tRNA modification ........................................... 1
1.1.1 The cellular function of tRNA ....................................... 1
1.1.2 An overview of tRNA modification .............................. 3
1.1.3 Introduction to Queuosine ........................................... 3
1.1.4 Modifications of the Queuosine Molecule ...................... 5
1.2 The Queuine Insertion Activity ........................................ 6
1.2.1 The properties of the transglycosylase complex ............. 6
1.2.2 The significance of the tRNA\textsuperscript{Tyr} intron with respect to transglycosylase activity ...................... 7
1.2.3 The presence of tRNA molecules within the Mitochondrion 7
1.2.4 Queuine Salvage ....................................................... 8
1.3 The Function of Queuine .................................................. 9
1.3.1 The Physiological Relevance of Queuine in bacteria ....... 9
1.3.2 The Physiological Relevance of Queuine in Eukaryotes .... 9
1.3.3 The Link between Queuine and Cancer ...................... 10
1.3.4 The effects of phosphorylation upon queuine insertion .... 12
1.3.5 The Role of Queuine in Metabolism ............................. 14
1.4 Metabolic Alterations and the Cancer Phenotype ............... 15
1.4.1 The Warburg Effect .................................................. 15
1.4.2 Glutamine Addiction ................................................ 18
1.5 Project Overview ......................................................... 19
Chapter 2 Materials and Methods

2.1 Chemicals, Reagents and Enzymes

2.2 Cell culture

2.2.1 Culturing of cell lines

2.2.2 Queuine supplementation of serum free medium

2.2.3 Passage of cell lines

2.2.4 Cryopreservation of cell lines

2.2.5 Re-establishing cell lines from frozen stocks

2.3 Cloning of the *qtrt1* and *qtrtd1* cDNA

2.3.1 Reverse transcription polymerase chain reaction (RTPCR) with specific Primers

2.3.2 Reverse transcription polymerase chain reaction (RTPCR) with random Primers

2.3.3 Amplification of the cDNA product using Polymerase chain reaction technique

2.3.4 Agarose gel electrophoresis and DNA extraction

2.3.5 Restriction Enzyme digestion of DNA for ligation into plasmids

2.3.6 DNA Ligation

2.3.7 Non-denaturing polyacrylamide gel electrophoresis

2.3.8 Site Directed Mutagenesis

2.3.9 Preparation of competent cells

2.3.10 Transformation of plasmid DNA into competent bacterial cells

2.3.11 Screening of competent cells screened for positive transformation

2.4 Purification of anti-TGT and anti-QTRTD1 anti-sera

2.5 Protein determination

2.6 Isolation of RNA from cultured cell lines

2.7 Transfection of COS-7 cells with queuine transglycosylase coding plasmids

2.8 Preparation of cells for Fluorescence Imaging

2.8.1 Staining of cells with Mitotracker dyes

2.8.2 Fixation of cells for Confocal Microscopy

2.9 Western blotting analysis

2.9.1 Isolation of total protein from cultured cells

2.9.2 Isolation of cellular extracts from cultured cells

2.9.3 Isolation of Nuclear and Cytoplasmic fractions using NE-PER Reagent

2.9.4 Isolation of Membrane fractions using Mem-PER Reagent

2.9.5 Isolation of Mitochondrial fractions using Mitochondria Isolation Kit for Culture
Cells

2.9.6 RNase digestion of protein cellular extracts 33
2.9.7 Competition Assay involving substrates of TGT 33
2.9.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 33
2.9.9 Transfer of proteins to PVDF membrane 34
2.9.10 Antibody probing 33

2.10 Spectrophotometric Assays 35
2.10.1 Citrate Synthase Assay 35
2.10.2 Cytochrome c Oxidase Assay 36
2.10.3 Lactate Dehydrogenase Assay 35

2.11 Metabolite Analysis 37

2.12 Polarographic Assays 37
2.12.1 Measurement of Respiration through Complexes of the Electron Transport Chain 38
2.12.2 Investigation of Capacity of Electron Transport Chain 38

2.13 Genomic DNA extraction from adherent cultured cells 39

2.14 Comparison of mitochondrial DNA copy number to nuclear copy number using qPCR 39

2.15 Determination of Mitochondrial Membrane Potential 40

2.16 Detection of ROS using Flow Cytometry 40

2.17 Preparation of samples for Transmission Electron Microscopy 41

2.18 Animals 41
2.18.1 Isolation of genomic DNA from mouse ear punches 42
2.18.2 Screening of TGT genetrap animals using PCR from genomic DNA 43
2.18.3 Whole Murine Spleenocyte Culture 43
2.18.4 Determining total live cell count using Ethidium Bromide/Acridine Orange 43
2.18.5 T lymphocyte specific stimulations 43
2.18.6 Preparation of tissue homogenates 44
2.18.7 Isolation of Mitochondria from Brain using Percoll density gradient centrifugation 45

2.19 Statistical Analysis 46
Chapter 3

3.1 Introduction

3.2 Results

3.2.1 Examination of the genetic and protein sequences of TGT
3.2.2 Homologue of TGT are found in eukaryotes
3.2.3 Expression and sub-cellular localization of TGT and QTRTD1

3.3 Discussion

Chapter 4

4.1 Introduction

4.2 Results

4.2.1 TGT and Qv1 associate in vivo
4.2.2 TGT and Qv1 are directed to the mitochondria
4.2.3 TGT is associated with RNA in vivo

4.3 Discussion

Chapter 5

5.1 Introduction

5.2 Results

5.2.1 The proliferation of primary and transformed cells is unaffected by queuine
5.2.2 Queuine is observed to affect the metabolism of both primary and transformed cells
5.2.3 The Effect of Queuine Deficiency on Mitochondrial Number and Morphology
5.2.4 Queuine has no effect on Mitochondrial Function
5.2.5 The effects of Queuine are dependent on substrate supply

5.3 Discussion

Chapter 6

6.1 Introduction

6.2 Results

6.2.1 The qtrtl−/− genotype occurs in accordance with Hardy-Weinberg Equilibrium
6.2.2 Animals which carry at least one copy of the qtrtl knockout gene are
predisposed towards weight gain

6.2.3 The bioenergetic flux of TGT animals is abnormal

6.2.4 The βF1 subunit of ATP synthase is up-regulated in the brains of knockout animals

6.3 Discussion

Chapter 7 Discussion

7.1 Discussion

7.2 Future Work

Appendix

Chapter 8 References

Publication
Declaration of Authorship

This thesis is the sole work of the author and has not been submitted in whole or in part to this or any university for any other degree. The author gives permission for the library to lend or copy this work upon request.
I would like to thank my supervisor Dr. Vincent Kelly for his valuable advice and guidance. I'd also like to credit the other members of the lab for the support they've provided me over the four years of this project; Cóilín, Ilana, Takafumi-san, Tanya and Sreeja.

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Lastly, I must mention my family who contributed in a substantial way towards the completion of this work.
Abstract

Queuine is a 7-deazaguanine derivative of guanine, which replaces guanine in position 34 of the anticodon loop of the transfer RNA for the amino acids, asparagine, aspartic acid, histidine and tyrosine. This base or related analogues are found within eukaryotes, eubacteria and archaebacteria. The physiological relevance of queuine has yet to be determined. The enzyme responsible for the base-exchange reaction is called tRNA guanine transglycosylase (TGT). While the eubacterial and archael forms of TGT have been well characterized, very little is known about the eukaryotic enzyme. This study identified the presence of two mammalian homologues, TGT and a more distantly related paralogue which is named queuine tRNA ribosyltransferase domain containing 1 (QTRTD1). Subsequent analysis of expressed sequence databases, revealed two additional QTRTD1 splice variants. The TGT and QTRTD1 variant 1 proteins were found to be capable of interacting with each other and co-localized to the mitochondria. In order to further elucidate the function of queuine in eukaryotes, a transformed tissue cell line was analyzed for any differences between queuine lacking and queuine supplemented cells. Differences in the activity of lactate dehydrogenase (LDH) were observed as were changes in the levels of metabolites integral to glycolysis and glutaminolysis. These changes in metabolism were independent of any change in mitochondrial volume or number although less cristae were observed in the queuine lacking cells using transmission electron microscopy. Oxidative phosphorylation and the membrane potential of the cells remained unaffected by the absence of queuine. When the glucose of the cell culture medium was substituted with galactose so the cells were less reliant on glycolysis to fulfil their bioenergetic requirements, changes were observed with regard to mitochondrial function. These cells appeared to use the ATP synthase to maintain their membrane potential. When these galactose adapted cells were exposed to conditions of substrate limitation, the activity of complexes II and III was markedly down-regulated in the queuine containing cells. The development of a gene trap mouse for TGT permitted in vivo analysis. Preliminary data from these animals demonstrated that these knockout animals for TGT have a predisposition towards weight gain as they age. Complexes II and III of the electron transport chain were also affected in an age dependent fashion in TGT knockout animals. The LDH activity was depressed in these knock-out animals from birth. These results indicate that the physiological relevance of queuine is worthy of further analysis.
Publication

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<tr>
<td>[³H]</td>
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<td>TBE</td>
<td>Tris-borate/EDTA buffer</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamide</td>
</tr>
<tr>
<td>TGT</td>
<td>tRNA Guanine Transglycosylase</td>
</tr>
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<td>transfer RNA</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-(hydroxymethyl)-propane-1,3-diol</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<td>---------------------------</td>
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<tr>
<td>Q</td>
<td>queuine</td>
</tr>
<tr>
<td>QTRTD1</td>
<td>queuine tRNA ribosyltransferase domain containing 1</td>
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Chapter 1

Introduction

1.1 Queuine, a tRNA modification

1.1.1 The cellular function of tRNA
The role of RNA in the cell is not restricted to the transfer of information. It has been observed to act in a catalytic and structural capacity which is reflected in the increased dynamism of RNA compared to DNA- there are many types of RNA moieties in the cell. The tRNA molecule is transcribed in a precursor form which is eventually processed to yield a mature tRNA molecule of between 73 and 93 nucleotides long. All tRNAs will assume a secondary canonical cloverleaf structure which is allowed by the conservation of base-pairing nucleotides situated in the stem-loop regions of the tRNA. Each tRNA is capable of binding an amino acid before entering the ribosome complex where codon : anticodon binding between the charged tRNA and mRNA is essential for translation. The structure of the tRNA is integral to the functionality of the molecule and this imposes limits on the variation which can occur along its sequence. Furthermore, there are 45 tRNA molecules for 64 codons. However, the presence of modifications at any one of these bases provides a means to circumvent this problem.

1.1.2 An overview of tRNA modifications
Between 10-25% of the nucleosides of any given tRNA are subject to post-transcriptional modifications. To date, over one hundred different types of tRNA modification have been identified; ranging from methylation to base isomerization (Garcia et al., 2005) (Figure. 1.1). Many of these modifications are carried out by a single enzyme while other modifications are so complex as to require a multi-enzymatic pathway. RNA-modifying enzymes represent approximately 10% of the coding capacity of the genome across all three kingdoms (Ferré-D’Amaré et al.,
The modification of tRNA occurs towards the end of tRNA maturation which is a tightly controlled nuclear event occurring with a conveyor-like processivity.

Figure 1.1 The canonical 2-d structure of tRNA and the range of potential modifications it can carry. The nucleotides are numbered from 5' to 3'. The queuine modification is indicated in red on position 34. Many of the modifications indicated are methylations (m). There are also thiolations (t), pseudouridylation (ψ), selenation (s) and acetylation (a). It can be seen that some modification only affect a particular nucleotide (i.e. methylation of an adenosine at position 25).

The location of the modification within the tRNA molecule will determine the effect that modification will have upon functionality; those occurring within the anticodon loop are typically associated with codon recognition and are often required to ensure translational fidelity (Hopper et al., 2003). In a small number of cases the loss of a tRNA modification in the anticodon loop has been linked to human disease. For example, individuals afflicted with the disease MERRF (myoclonus epilepsy associated with ragged-red fibers) carry a mutation within the T-loop of the mitochondrial tRNA_Lys which coincides with an absence of a taurine modification from the wobble position of the anticodon loop. Furthermore, disruption of the genes coding for the enzymes responsible for this modification causes a MERRF-like...
phenotype. This phenotype is believed to arise, in both cases, because of an inability of the anticodon loop to decode its cognate codon efficiently (Umeda et al., 2005).

A further role for tRNA modification within the anticodon loop is the ability to increase the range of codon recognition of certain tRNA species. Within *S. cerevisiae*, a carboxymethylaminomethylation at the wobble position of the nuclear encoded tRNA$_{\text{LYS}}^{\text{CUU}}$ (tRK1) allows this tRNA to decode the AAA and AAG codons under conditions of cellular stress (Kamenski et al., 2007). Additionally, modifications can also function to stabilize a tRNA molecule by inhibiting its degradation (Engelke et al., 2006) as well as optimizing its interaction with the ribosomal complex (Alexandrov et al., 2006) both of which processes are aided by inducing the tRNA molecule to adopt a canonical cloverleaf structure. As an example of the stabilising effects that modification can bestow upon the tRNA structure, mitochondrial tRNA$_{\text{LYS}}^{\text{A}}$ presents itself again. Within mammalian systems, human mitochondrial tRNA$_{\text{LYS}}^{\text{A}}$ adopts a non-cloverleaf structure when it lacks a methyl modification at position A9-N$^1$ (Helm et al., 2004). A tRNA that cannot attain the correct three-dimensional structure will not be optimally recognised by the translation apparatus.

Given their widespread occurrence and the significant number of enzymes dedicated to their production it is somewhat surprising that many tRNA modifications do not appear to be essential to the viability of the cell as gene deletion studies, principally in bacteria, for many of these tRNA modification enzymes resulted in only minor phenotypic effect (Engelke et al., 2006).

1.1.3 Introduction to Queuosine

Queuosine (Q) is a tRNA modification found in the anticodon loop of tRNA isoacceptors for the amino acids asparagine, aspartate, tyrosine and histidine. The structure of queuosine consists of a secondary amine attached to a cyclopentenyldiol ring structure (Figure 1.2). Q is found exclusively in the wobble position (base 34) of the aforementioned tRNA isotypes in all eukaryotic and eubacterial species, with the exception of yeast and plant leaf cells (Kersten et al., 1988).

Eubacteria are capable of synthesizing Queuosine *de novo*. The synthesis of Q by eubacteria occurs through a multi-step pathway, beginning with the nucleotide triphosphate GTP (Figure 1.3). Eukaryotes are incapable of queuosine
biosynthesis. Higher eukaryotes can salvage both the queuosine nucleoside and the free base of queuosine, known as queuine or Q-base, from enteric flora or from ingested food. The queuine base is then directly inserted into tRNA by a base-for-base exchange reaction with guanine, i.e. during the exchange process the phosphodiester backbone of the tRNA molecule is not broken.

Figure 1.2 The chemical structure of the queuosine modification. Queuosine is a modified 7-deazaguanine nucleoside that replaces guanine at position 34 of the anticodon loop.

Figure 1.3 Queuosine biosynthesis by eubacteria. The Q biosynthetic pathway is similar in all the eubacteria species, relying on the enzymatic activity of at least 5 proteins (QueC, QueF, TGT, QueA and an unidentified coenzyme B$_{12}$ dependant enzyme) NADPH, reduced nicotinamide cofactor; SAM, S-adenosylmethionine; B$_{12}$, vitamin B$_{12}$ (Roth et al., 2007). In bacteria, it is the preQ$_{1}$ nucleotide which is inserted into the tRNA and further modified to become the Q-tRNA.
1.1.4 Modifications of the Queuosine Molecule

Queuine, once incorporated into the tRNA molecule, can be subject to further modification by the addition of monosaccharide residues. Of the four Q-tRNA species, only tRNA$^{Asp}$ and tRNA$^{Tyr}$ are modified further by the addition of a mannose (ManQ) and galactose (GalQ) residue, respectively. These sugars are covalently appended to the hydroxyl group at position two of the cyclopentenyldiol ring. The physiological purpose of these secondary modifications is currently unknown.

![Mannosyl Queuosine](image1)

**Figure 1.4** The structure of mannosyl queuosine and galactosyl queuosine. Typically the distinction between manQ and galQ is not made within the literature with both being denoted as Q*. ManQ and galQ are modifications found only within tRNA$^{Asp}$ and tRNA$^{Tyr}$, respectively (Limbach *et al.*, 1994).

The commonality between tRNA$^{Asp}$ and tRNA$^{Tyr}$ is the presence of either a cytosine or adenosine at position 36. This correlation prompted an investigation of the influence of the loop sequence upon glycosylation of the queuosine tRNA molecule (Haumont *et al.*, 1987). A series of chimeric tRNAs were produced. For example a tRNA$^{Ser}$, was constructed to bear the anticodon of tRNA$^{Tyr}$ which failed to support glycosylation, indicating the importance of the sequence context for the sugar modification. A series of yeast tRNA$^{Tyr}$ species were also made with changes to key residues within the anticodon loop and were injected into *Xenopus* oocytes. It was noted that changes at position 36, 37 and 38 had the greatest effect upon glycosylation activity with no concomitant effect observed upon transglycosylase activity. Of those tRNA species that are capable of being modified in this manner, approximately half carried these residues (Gündüz *et al.*, 1984). Research suggests
that the removal of the mannose and galactose residues is necessary for recycling of
the queuosine molecule back to usable queuine as less than 5% of degraded
intermediates were found to contain these forms (Gündüz et al., 1984).

Curiously, it has also been found that the tRNA\textsuperscript{ASP} queuosine residue within
\textit{E. coli} is glutamylated by a unique glutamyl tRNA synthase like enzyme, known as
YadB, on the hydroxyl position of the cyclopentenyldiol ring (Blaise et al., 2004).
The YadB enzyme has a high Km for glutamate, suggesting that the reaction is
specific to occasions where the cellular concentration of glutamate increases (Salazar
\textit{et al.}, 2004). This modification could also potentially exist within eukaryotes having
avoided detection due to the prevailing method of tRNA isolation using basic
conditions which would result in hydrolysis of the glutamyl-queuosine moiety.

1.2 The Queuine Insertion Activity

1.2.1 The properties of the transglycosylase complex

A form of the queuine insertion activity is present within archaea, eubacteria and
eukaryotes. In bacteria, the queuine insertion activity was initially termed as a
guanine transglycosylase in bacteria until its true substrate, preQi was discovered.
The enzymatic reaction responsible for this insertion has been found to observe a
ping-pong reaction mechanism (Garcia \textit{et al.}, 2003) where the tRNA is bound first
followed by cleavage of the guanine from the wobble position. The active site of the
transglycosylase then binds preQ\textsubscript{1} before its insertion into the TGT-tRNA
intermediate. The related enzyme in Archaea inserts an analog of queuine called
preQ\textsubscript{0} into the position 15 of the D-loop of the tRNA. The crystal structure of this
protein has been solved and many of the residues involved in catalysis have been
observed to be conserved (Ishitani \textit{et al.}, 2002, Stengl \textit{et al.}, 2005).

Much of the work on characterizing the properties of the transglycosylase
complex was the result of purification attempts of the activity. The molecular weight
of the activity in \textit{E. coli} was determined, by way of SDS-PAGE to be approximately
46 kDa (Shindo-Okada \textit{et al.}, 1980). Initial attempts to purify the eukaryotic
transglycosylase activity utilised rabbit reticulocytes and were unsuccessful in
identifying the protein responsible for the reaction (Farkas \textit{et al.}, 1973). A second
attempt by the same group using rabbit erythrocytes was successful and a putative
molecular weight was assigned to the protein responsible for queuine insertion into tRNA (Howes et al., 1978). Gel filtration and DEAE-cellulose chromatography yielded an approximately 104kDa band when electrophoresed on a non-denaturing acrylamide gel. This band resolved into two smaller bands of 43 and 60kDa, with the larger band being further separated into two bands of nearly identical size when the sample was S-aminoethylated. The amino acid sequence of this purified activity was not determined.

The transglycosylase activity has also been purified from wheat germ, rat and bovine liver. The plant activity yielded a molecular weight of 104 kDa which was found to be a homodimer composed of 68 kDa subunits (Walden et al., 1982). The purification attempt from bovine liver was the source of the first partial amino acid sequence for the transglycosylase activity (Slany et al., 1995). However, none of the attempts at obtaining a pure isolate of the transglycosylase activity were successful, a fact which will be discussed further in Chapter 3.

1.2.2 The significance of the tRNA^{Tyr} intron with respect to transglycosylase activity

Within higher eukaryotes, only two tRNA molecules contain an intron; tRNA^Leu within Drosophila and tRNA^{Tyr} in humans (van Tol et al., 1988). The intron is located between nucleotides 37 and 38 in the anticodon loop and may either impede or hasten the functioning of tRNA modifying enzymes that are dependent upon the presence of an intron within the tRNA molecule (Grosjean et al., 1997). For instance, the modifying enzyme pseudouridine synthase, which modifies position 35 of the tRNA^{Tyr} molecule, is absolutely dependent on the presence of the intron (Choffat et al., 1988).

The presence of an intron in tRNA^{Tyr} provides a valuable marker for the pseudouridine synthase enzyme. This does not seem to be the case with the queuine transglycosylase enzyme. In fact, a necessity for intron removal has been observed. In 1983, Nishimura established that the queuine modification could only be detected in the 78 nucleotide long tRNA transcript, that version of the tRNA molecule in which the intron had been removed.
1.2.3 The presence of tRNA molecules within the Mitochondrion

In many species, for example the trypanosomal parasites that cause African Sleeping Sickness, the import of nuclear-encoded tRNA into the mitochondria is a common occurrence, attendant with the loss of cognate genes from the mitochondrial genome. While the mammalian mitochondrial genome has been subject to a similar level of genetic reduction, capable of producing only thirteen proteins, it produces a minimal, yet sufficient set of 22 tRNAs hence negating any requirement for the import of tRNAs from the cytoplasm. The genes coding for this small cadre of proteins continue to be maintained within the mitochondrial genome as they are composed of highly hydrophobic residues. Furthermore, these mitochondrial tRNAs are post-translationally modified with queuine which has been detected within rat and opossum mitochondrial tRNA\(^{\text{Asp}}\) (Randerath et al., 1984; Mörl et al., 1995).

1.2.4 Queuine Salvage

As mentioned earlier, eukaryotes do not possess the means to synthesize queuine \textit{de novo} and instead must retrieve it through dietary means or from the gut micro-flora. Mammals are however capable of salvaging queuine (Gündüz et al., 1982) through tRNA turnover. The as yet unidentified salvage enzyme activity promotes queuine formation through cleavage of the molecule from Q-5'-phosphate (Gündüz et al., 1984). Within mammals, a putative queuine salvage enzyme was tentatively identified through gene similarity with the TGT sequence (Vandenbergh et al., 2003). This gene was observed to contain a CA dinucleotide repeat polymorphism.

The use of a radioactively labelled queuine analogue, \(^{3} \text{H}\)dihydroqueuine, known to be incorporated into tRNA, provided the avenue to determine the presence of a salvage mechanism within eukaryotes. Vero cells, a monkey kidney cell line, were grown in a medium containing serum stripped of queuine (Gündüz et al., 1982) before being treated with dihydroqueuine. HPLC analysis revealed that the level of radioactivity remained constant and concentrated within a single peak corresponding to the size of dihydroqueuine. The turnover of dihydroqueuine containing tRNA would be expected to degrade the radioactive base into a series of detectable breakdown products which would be unable to act as a substrate for TGT. This was not the case, as an additional activity was recycling it back to an active substrate for the transglycosylase. To further understand the pathway through which salvage of
the queuosine analogue was occurring, cell extracts from a cell line unable to salvage Q base, mouse fibroblast L-M cells, were chromatographed to determine the identity of degraded intermediates. HPLC analysis indicated that a Q-monophosphate molecule was present. This intermediate was capable of acting as a substrate for salvage activity when incubated with Vero cell extract and through use of appropriate HPLC markers was identified as queuosine 5'-phosphate.

The queuine salvage activity has also been demonstrated to occur in plants; with both the algal species *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* being capable of cleaving queuosine to queuine for insertion into cognate tRNA species (Kirtland *et al.*, 1988). The mechanism by which this occurs in plants is thought to be different from that of higher eukaryotes since the epoxy-queuosine intermediate, which is readily salvaged by Vero cells, is not a substrate for the putative salvage enzyme within these algal species. Based on this difference, the further observation within this paper that the enzyme activity did not require phosphate may not be applicable to the study of the salvage activity within mammals.

1.3 The Function of Queuine

1.3.1 The Physiological Relevance of Queuine in bacteria

As a tRNA modification, queuosine would naturally be assumed to affect the charging ability of the tRNA molecules it is found in. However, the amino-acyl charging ability of *E. coli* mutants carrying a deletion of the *tgt* gene remained intact (Noguchi *et al.*, 1982) and overall protein synthesis increased (Nishimura., 1983). However, when these cells were mixed with a wild-type strain containing TGT, competitive growth experiments revealed a marked reduction in viability of the TGT knockout strain. Research carried out by Frey in 1989 examined the translational fidelity of an *E. coli tgt* mutant and found that the read-through of a nonsense codon increased two-fold. However this only occurred when the Q-tRNA codon was preceded on the ribosome by another Q-tRNA codon. This being the only discernible phenotype, it was hypothesized that queuine was acting in the capacity of a signal for stress-related protein expression.

Within *Shigella flexneri* the expression the transcription factor *virF*, whose downstream targets include a range of virulence genes was negatively affected by a mutation occurring within the *tgt* gene (Durand *et al.*, 2000). The effect upon
expression was attributed to impaired translation as levels of \( \text{virF} \) mRNA remained unchanged. Furthermore, as reported by Hurt \textit{et al.}, (2007), the \( \text{virF} \) mRNA is recognised by TGT and as such has been postulated to contain a riboswitch binding motif. If one is to assume that TGT is catalysing the modification of this mRNA with pre\( \text{Q}_1 \), it would signify a role for queuine outside of the tRNA anticodon loop.

1.3.2 The Physiological Relevance of Queuine in Eukaryotes

In the 1980s, Walter Farkas was the first to move the study of queuine into the \textit{in vivo} mammalian model, breeding germ free mice and maintaining them on a chemically defined diet which also lacked queuine for four weeks. It was observed that while the queuine modification of tRNA\(^{\text{His}} \) and tRNA\(^{\text{Asp}} \) decreased to 15 % of normal levels within this time period, the tRNAs for tyrosine and asparagine remained at levels approximately 85% that of their fully modified levels, perhaps related to the glycosylation of their queuine residues. These mice manifested no ill effects from a diet lacking queuine and were actually observed to gain a little weight, a fact which was attributed to increased water retention. By 1981, he had maintained a cohort of germ free mice on the queuine lacking diet for one year, the period required to observe all tRNA species as being unmodified by queuine. This study confirmed that mice wholly lacking Q-tRNA had no ill effects nor any increase in the incidence of cancer (see below). However, this study hinted at the existence of a mammalian queuine salvage activity as mice fed queuine modified tRNA (Q-tRNA) were able to incorporate the queuine into their tRNA. It was not until 1997 that Marks and Farkas were able to demonstrate pathological implications of a lack of queuine through removal of both tyrosine and queuine from the diet of the germ free mice. Within a short period of time, eighteen days, every animal within the cohort demonstrated symptoms ranging from squinting, stiffness, staggered movement and seizures leading eventually, in all cases, to death.

1.3.3 The Link between Queuine and Cancer

Towards the ends of the 1970s, a number of groups observed that a variety of tumour cell lines were deficient in Q-tRNA species. This was why the mice which Farkas had bred and maintained on a queuine free diet were monitored for the development of cancer. The molecular mechanisms driving this change are opaque and efforts to
clarify queuine’s role, if any, in cancer progression have focused upon differentiation and proliferation. From a reductionist viewpoint, cancer can be said to be characterized by poor differentiation and enhanced proliferation.

In experiments where undifferentiated murine erythroleukemic cells are induced to differentiate into mature erythroid cells, a significant increase in the Q-tRNA species was found to occur during the early stage of this differentiation (Shindo-Okado et al., 1981). Likewise, when the human K562 erythroleukaemic cell line was treated with the irreversible inducer Ara-C the cells were found to undergo a significant irreversible increase in Q-tRNA in line with the development of a differentiated phenotype, as measured by the number of benzidine positive cells, an assay which measures haemoglobin which is only present in mature erythrocytes (Chen et al., 1994). When the cells were treated with a drug whose effects were transient, the increase in queuosine modification was also found to be as short-lived as the drug’s effect upon differentiation. Furthermore, in both Chinese hamster ovary cells and fibroblasts, the transformed versions of these cell lines were found to contain significantly less queuine within their tRNA despite increased levels of the transglycosylase activity (Muralidhar et al., 1989, Morgan et al., 1996). In the case of human leukaemic and lymphmic cells the lack of Q-tRNA was found to be independent of substrate limitation (Emmerich et al., 1985). In fact, the relationship between Q-tRNA deficiency and poor differentiation has been put forward by two independent groups in 1992 and 1994, as a means of malignancy grading for lung and ovarian cancer patients (Huang et al., 1992, Baranowski et al., 1994) (Figure 1.5).

Figure 1.5. Queuine deficiency in tRNAs from normal myometrium (I), myomas (II), well differentiated (III), moderately differentiated (IV) and moderately to poorly and poorly differentiated (V) ovarian tumours (Baranowski et al., 1994).
In contrast, queuine’s effect upon proliferation varied depending upon the cell type under examination. Helga Kersten’s group carried out the majority of the work, culturing a wide range of primary cells, oncogene transformed cells and cancer cell lines in a bid to quantify the effect upon proliferation of queuine (Langgut et al., 1993). While her group demonstrated that queuine had a small effect, doubling the rate of proliferation at most, the effect was dependent upon the atmospheric culture conditions and cell type. Within primary cell lines supplemented with physiological concentrations of chemically synthesized queuine, a stimulatory effect upon proliferation under normoxic conditions became inhibitory when oxygen became limiting. However, within the transformed cell lines, varying effects were noted depending on the particular cell line under consideration. They supposed that this variance arose due to the difference in the damage in cellular signalling which each cancer cell line carried and endeavoured to confirm that queuine’s effect was dependent on particular signalling pathways. The neuronal cell line NIH-3T3 was transformed with the ras, raf and erb oncogenes. Those cells transformed with ras and erb demonstrated decreased proliferation in the presence of queuine when compared to the queuosine lacking counterparts. Chronic exposure of primary human cells to tumour promoting agents such as phorbol esters causes these cells to develop the characteristics of a transformed cell line with the concurrent development of queuosine hypomodification (Elliott et al., 1984). When co-treated with queuine, the degree of queuosine modification and rate of proliferation only changed marginally from the more normal phenotype. Additionally, the expression of the oncogenes c-fos and c-myc, as investigated using Northern blot analysis was found to exhibit differences between HeLa cells grown in the absence and presence of queuine (Langgut et al., 1990). In DLAT mice the protein expression of c-fos and c-myc were found to decrease to levels observed in healthy mice when they were fed a queuine rich diet (Pathak et al., 2008).

1.3.4 The effects of phosphorylation upon queuine insertion.

Queuine is unusual in that it is the only nucleotide whose presence within the cell is solely reliant upon a cellular uptake mechanism. This uptake has been found to be sensitive to treatment with phorbol esters which are tumour promoting agents. Treatment of human fibroblasts with PDD and teleocidin, both of which compounds
demonstrate little structural similarity to each other was seen to cause an increase in the uptake of dihydroqueuine, a radioactively labelled queuine analogue (Elliott and Crane, 1990). The structures of these phorbol esters are unrelated which is significant as it indicates that their effect upon queuine uptake is indirect, possibly dependent upon a signalling pathway. Further experiments using a range of PKC agonists and antagonists resulted in an increase and decrease in uptake being observed, respectively. However the effects of phosphorylation are not restricted to just the queuine uptake apparatus.

The PKC agonists used in the queuine uptake studies were the growth factors EGF and PDGF, ligands for cognate receptor tyrosine kinases. This is significant as Langgut demonstrated in 1993 that queuine was capable of modulating the activity of the EGF receptor in two carcinoma cell lines, A431 and HeLa-S3. Langgut continued these studies in HeLa cells, finding that administration of queuine caused a decrease of phosphorylation of downstream effector proteins for EGF such as pp110 and pp16 (Langgut et al., 1995). The phosphorlyated residues affected were alkali stable indicating queuine influenced tyrosine phosphorylation as phosphoserine and phosphothreonine residues are alkali labile. The pp110 is a protein which is frequently found to be mutated within cancer and forms the catalytic subunit of the PI3K protein, implicating a role for queuine within a specific signalling pathway. This result was repeated in vivo when Pathak et al. moved the investigation into mouse models. Mice in which Dalton’s lymphoma ascites had been transplanted (DLAT) were compared with non-lymphoma induced mice as regards the phosphorylation of the two tyrosine phosphoproteins; the cytosolic pp110 and the cytosolic and membranous pp52. The increased phosphorylation of these proteins observed within DLAT mice subsided significantly, in a dose dependent fashion, when mice were fed queuine (Pathak et al., 2007).

The transglycosylase activity is also sensitive to changes in phosphorylation status. The work of Elliott (1990) implicated at least two proteins in the transglycosylase activity, the expression of both being up- or down-regulated in response to increased phosphorylation. During the isolation of the queuine transglycosylase enzyme from rat liver, a manifold increase in the transglycosylase activity was observed upon addition of phosphatase inhibitors or PKC activators to the isolation buffers (Morris et al., 1995). When the purified fractions were run on an
SDS-PAGE gel, two bands were visible whose intensity relative to each other was dependent on the transglycosylase activity. Those samples which had been treated with phosphatase inhibitors and consequently demonstrated increased activity ran with a weaker band at approximately 60kDa and a stronger band running at approximately 35kDa while the converse was seen in those samples with a low level of transglycosylase activity. The enzyme represented by the 60kDa band was cloned by Deshpande (1996) but had no detectable transglycosylase activity. Whereas, in separate work undertaken by the same group, the approximately 35kDa size protein, on being cloned into an expression vector and transfected into a cell line lacking the TGT gene, allowed these cells to insert queuine into the anticodon loop.

1.3.5 The Role of Queuine in Metabolism

It has been well documented that the activity of lactate dehydrogenase changes during cancer formation and under conditions of hypoxia (Walenta et al., 2004). There is strong evidence documenting the effect of queuine upon lactate dehydrogenase activity. The earliest recorded attempt at quantifying this change was in 1984 and occurred within Dictyostelium (Schachner et al., 1984). Slime mould grown under conditions of starvation were observed to accumulate less D(-)-lactate when queuine was absent from the growth medium and were found to be missing an isoform of lactate dehydrogenase present in slime mould grown in the presence of queuine. The situation in mammalian tissue is reassuringly similar to that found in Dictyostelium. Helga Kersten’s group focused their efforts on examining the situation in HeLa cells, finding that the expression of the LDHk isozyme, involved in the anoxic stress response, was returned to levels observed under normal conditions when serum starved cells were treated with queuine. The same group then found that the A4 isoform, the predominant LDH activity under hypoxic conditions and therefore associated more with glycolysis was up-regulated in the absence of queuine (Reisser et al., 1994).

When the same group carried out an MTT assay on HeLa cells under aerobic and hypoxic conditions, they found that those cells lacking queuine under conditions of plentiful oxygen had a slight increase in mitochondrial electron flow, whereas under hypoxic conditions, the electron flow was marginally reduced in the presence of queuine. Differences in superoxide dismutase (SOD) and components of the
cellular antioxidant stress apparatus have strengthened this putative link of queuine to mitochondrial function. Murine fibroblasts cultured without queuine were found to contain half the amount of manganese SOD, a mitochondrial localised enzyme (Szabo et al., 1988). Research was also conducted to measure the amount of lipid peroxidation in liver and kidney of queuine lacking mouse. Increased lipid peroxidation is an indicator of increased oxidative stress within the cell and in this instance cells maintained in queuine containing medium appeared to have a higher amount of thiobarbituric acid reactive material indicating increased oxidative stress. Administration of queuine to DLAT mice has also been found to increase the activity of antioxidant stress enzymes (Pathak et al., 2008).

1.4 Metabolic Alterations and the Cancer Phenotype

1.4.1 The Warburg Effect

More than eighty years ago, it was observed that ascites tumour cells demonstrated a significant increase in glucose consumption coupled with increased lactate production. This perturbed metabolism was found to be common among cancerous tissues and was termed the Warburg effect after Otto Warburg who made that initial observation (Warburg, 1925). While glycolysis is far less efficient than oxidative phosphorylation at producing ATP, the glycolytic flux of the cancer cell is so high that aerobic glycolysis is capable of satisfying the cell’s bioenergetic requirements (Guppy et al., 1993).

The increased proliferation which is a hallmark of cancer necessitates a substantial increase in the flux through several biosynthetic pathways, for which the carbon skeleton of glucose provides ample fodder. The ribose sugars are used to meet the increased demand for nucleotide synthesis while citrate and glycerol—both products of glycolysis—are used to form lipids, a substrate which cancer cells are forced to synthesize since the rate of growth outstrips the extracellular supply available (deBerardinis et al., 2008) (Figure 1.6).
Figure 1.6. The primary metabolic pathways within the cancer cell, the Warburg effect and glutamine consumption, feed into many biosynthetic pathways, of which lipid biosynthesis is included. Glc, glucose; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehydes 3-phosphate; PK-M2, Pyruvate kinase isoform 2; LDHA, Lactate dehydrogenase A; FAS, fatty acid synthase; ACL, ATP citrate lyase; OAA, Oxaloacetate; ME, Malic Enzyme; GLS, Glutaminase; Gin, Glutamine; Glu, Glutamate (DeBerardinis and Cheng., 2010)

Clearly, when the metabolism of a cell is skewed so far away from what is considered normal, there are knock-on effects. In this instance, a consolidation of the Warburg effect is observed as the now acidic micro-environment of the cell combined with increased oxidative stress act to perturb signalling within the cell, which ultimately causes an up-regulation in glucose uptake, glycolysis and lactate
production. A component of the hypoxia inducible factor (HIF-1) transcription factor finds itself stabilized under such conditions and acts to increase the expression of a range of glycolytic proteins (Mansfield et al., 2005). The myc oncogene, discussed earlier as being deregulated under conditions of queuine hypomodification and frequently so in cancerous tissues, encodes a transcription factor known to affect glucose metabolism. It affects the expression of LDH-A, whose increase in expression in transformed fibroblasts and Burkitt’s lymphoma has been found to be reliant upon c-myc overexpression (Shim et al., 1997). Furthermore, components of signalling pathways become targets for mutation – as discussed previously the PI3K pathway carries the heaviest mutation load across all cancers (Bachman et al., 2004).

While the above description seems to indicate that signalling within the cancer cell and hence metabolism is out of control, in fact, the system achieves an equilibrium for which the increased glycolytic flux also appears to be responsible. It has been suggested that since glycolysis, in this case running at an extremely high rate, acts as a nexus feeding into many other biosynthetic and bioenergetic pathways, it can exert control over these processes (Newsholme et al., 1985). If glycolysis slows down, then by necessity biosynthesis of nucleotides will subside to a proportional degree.

This increase in glycolysis fed biosynthesis results in a depletion of essential metabolites within the cell. In normal somatic tissue, glucose is typically oxidised to pyruvate and the majority of this pyruvate is converted to acetyl coenzyme A to enter the tricarboxylic acid (TCA) cycle where the reducing equivalents necessary to power the electron transport chain are produced. As the importance of oxidative phosphorylation diminishes within the cancer cell, it could be assumed that the TCA cycle becomes extrinsic to the cellular machinery. However, this is not the case as the function of the TCA cycle changes to meet the demands of the cancer phenotype – renewal of those metabolites which are heavily in demand, such as the increased levels of citrate required for lipid synthesis. The phenomenon is known as anapleurosis and is not restricted to glucose metabolism. Not only do cancer cells exhibit a massive increase in glucose consumption but many are also found to be dependent on glutamine, an amino acid whose requirement in the cell is as high as that of glucose in the aberrant Warburg phenotype.
1.4.2 Glutamine Addiction

Glutamine is the most abundant amino acid within the body and accounts for nearly 20% of the amino acid content in plasma (deBerardinis et al., 2010). The increased demand that transformed tissue demonstrates for glutamine arises as its metabolic intermediates are easily funneled into a range of anabolic pathways such as nucleotide biosynthesis, the production of non-essential amino acids, regeneration of reducing equivalents within the cytosol, production of anti-oxidants to combat the increased toxicity of the cancer cell environment and indirectly feeding all of the above through anapleurosis. This increase in glutamine consumption has also been found to be regulated by the \textit{myc} oncogene as it targets and represses and the expression of two microRNA species, miR23a and b, who ordinarily function to repress the activity of glutaminase, an enzyme integral to glutamine’s increased importance in the cancer cell environment (Gao \textit{et al.}, 2009).

The nitrogens, both amido and amino, of glutamine can be used to build the ring structures of purines and pyrimidines and can be donated to the production of nonessential amino acids (deBerardinis \textit{et al.}, 2010). However, in the latter instance, this nitrogen is not directly diverted to amino acid production – glutamine is converted to glutamate through the action of glutaminase and from there further converted, most usually, to alanine and aspartate. The conversion of glutamine to glutamate is the entry point for the carbons of glutamine to enter the TCA cycle, which is truncated in anapleurosis and devoted almost solely to replenishment of those intermediates required to fuel the increased biosynthetic flux of the cell. The regeneration of the NAD+ species necessary for glycolysis are dependent on the malate-aspartate shuttle, as the malate coming from the aspartate, a break-down product of glutamine, is reduced by cytosolic NADH before it enters the mitochondria to pass these electrons into the electron transport chain.

Glutamine is also instrumental in combating the increased oxidative stresses encountered within the cancer cell. A specific antiporter in the plasma membrane swaps extracellular cysteine for intracellular glutamate providing an excess of the latter exists. This is important as cysteine and glutamate together with glycine form glutathione (GSH), a critical anti-oxidant molecule. Glutamine is also partially responsible for maintaining a reduced cellular GSH pool through glutaminolysis – the partial oxidation of glutamine to lactate occurring through the TCA cycle. The malic
enzyme which aids in the conversion of glutamine to pyruvate reduces NADP+ to NADPH, which provides the reducing equivalents necessary for reducing GSH and the reductive reactions of nucleotide and lipid biosynthesis. From this, it can be seen that the cancer cell provides a larger role for glutamine as a respiratory substrate as opposed to being involved primarily in protein synthesis. In fact, within the cancer cell, approximately half of the carbons of glutamine have been traced to lactate.

1.5 Project Overview
For the above discussed reasons, it was decided to investigate the significance of queuine to the unique cancer phenotype. However, it was first necessary to

- Identify the components of the eukaryotic queuine tRNA insertase activity.
- Once these enzymes had been found, further characterization of the identified enzymes was carried out as regards their tissue expression profile and with regard to availability of their substrate queuine.
- This led to the examination of the intracellular distribution of these enzymes and their relationship with each other.
- Much of the literature has reported differential effects on cellular proliferation in response to queuine. Different transformed cell lines carry different oncogenic insults affecting different signaling pathways. Queuine has been linked to the Protein Kinase C (PKC) pathway and so it was decided to investigate the effect of growth promoting agents on the localization and expression of the queuine tRNA insertase enzymes.
- Further investigation of the correlation between cancer grading and QtRNA modification was warranted and focused on the effect of queuine on cancer metabolism.
- Finally, the function of queuine was investigated and the effect of queuine deficiency on cellular metabolism was studied using both in vitro and in vivo methods.
Chapter 2

2.1 Chemicals, enzymes and reagents
Chemicals and enzymes used were readily available from commercial sources. Restriction enzymes were purchased from Roche Applied Science (West Sussex, UK). T4 DNA ligase, Taq DNA polymerase, cloned PfuTurbo DNA polymerase, dNTPs, Bacterial Alkaline Phosphatase were obtained from Takara (Otsu, Shiga, Japan). N,N,N',N'-tetramethylene diamine, sodium dodecyl sulphate, molecular weight protein marker, agarose, 1 Kb DNA ladder, Sodium Chloride, Glycerol, Bromophenol Blue, Kanamycin, Ampicillin, secondary antibodies and all reagents for respirometric analysis were purchased from Sigma-Aldrich Co. Ltd (Sigma-Aldrich, Dublin, Ireland). M-Per protein isolation kit, cellular fraction isolation kits were from Pierce (Thermo Scientific, Rockford, IL, USA). NADH was bought from Calbiochem (Merck, Darmstadt, Germany). Nova Biopacks were from Nova Biomedical Corporation (MA, USA). 1.5 ml RNase-free tubes, RNase Zap and SUPERase-In RNase Inhibitor were purchased from Ambion (Austin, TX, USA). Superscript III RT, Ultrapure phenol, Mitotracker dyes, Lipofectamine 2000 reagent, Alexa fluoros, Prolong Gold, DAPI, PBS, TrypLE Select and DMEM were purchased from Invitrogen (Carlsbad, California, USA). 30 % Acrylamide solution and scintillation fluid were from National Diagnostics (Atlanta, Georgia, USA). Ultraculture medium and custom Ultraculture medium were from Lonza (Lonza, Basel, Switzerland). All plasticware was purchased from Corning. PCR QIAquick kit, QIAquick gel extraction kit, QIAquick miniprep and Endofree Plasmid MaxiKit were purchased from Qiagen (Hilden, Germany). Bradford Reagent (5X) was obtained from Bio-Rad (Hercules, California, USA). Immobilon-P transfer membrane, enhanced chemiluminescence solutions and the Milli-Q Direct 3 water filtration system were purchased from Millipore (Billerica, Massachusetts, USA). Recombinant murine TGT and Qvl were produced in lab and sent to Harlan where antisera were raised against them in rabbit (Harlan Laboratories, Indianapolis, IN). Antibodies against IF1 and βF1 were a kind gift from Prof. José Cuezva. [H^+]TPMP^+ was a kind gift from Dr. Derek Nolan, Queuine was a kind gift from Professor Susumu Nishimura.
Materials and methods

2.2 Cell culture

2.2.1 Culturing of cell lines

During the course of this study, two cell lines were used, COS-7 cells, an adherent monkey kidney epithelial cell line and HeLa cells, an adherent human epithelial cervical cancer cell line. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. COS-7 cells were routinely grown in DMEM medium supplemented with 2 mM L-glutamine, 0.1 mg/ml penicillin streptomycin and 10% Foetal Bovine Serum (FBS) 10% Horse Serum (HS). HeLa cells were maintained in EMEM supplement with 2mM L-glutamine, 0.1 mg/ml penicillin streptomycin and 10% FBS. Queuine deficiency was induced in these cell lines by growing them in serum free Ultraculture medium supplemented with 2 mM L-glutamine and 0.1 mg/ml penicillin-streptomycin.

2.2.2 Queuine supplementation of serum free medium

A 100 μM stock solution of chemically synthesised queuine (provided by Dr. Nishimura) was prepared in ultrapure H₂O and stored at 4°C. Serum free Ultraculture medium was made queuine sufficient by the addition of 0.2% (v/v) of queuine stock solution, giving a final concentration of 200 nM.

2.2.3 Passage of cell lines

Cells were passaged upon reaching 80% confluence. Growth medium was removed from the cells, which were then washed once with PBS. TrypLE, a non-animal alternative to porcine trypsin, was added to the cells (1.5 ml per 75 cm² surface area) and detachment induced by incubating the cells at 37°C for approximately 5 minutes. Subsequently, fresh medium was added to the dish (4 ml per 75 cm² surface area) and a single cell suspension achieved by pipetting the medium a number of times. The cells were pelleted by centrifugation at 300 x g for 4 minutes, and reseeded at a ratio of 1:3 to 1:6, depending upon the length of time needed before the cells are used for experiments.
2.2.4 Cryopreservation of cell lines

Cells were cultivated in T175 flasks until they reached 80% confluence. They were detached from the flask using TrypLE, as described above, and centrifuged at 300 x g for 5 minutes. The cell pellet was resuspended in 9 ml of fresh freezing mix (95% growth medium, 5% Ultrapure DMSO). Aliquots of 1 ml, containing approximately 2x10^6 cells, were placed into cryotubes. These were then placed in a cell freezing device which contained isopropanol and transferred to -70 °C for 24 hours before being moved to a liquid nitrogen container for long-term storage.

2.2.5 Re-establishing cell lines from frozen stocks

Cryotubes that had been maintained in liquid nitrogen, containing ~2x10^6 cells/ml, were thawed by partially submerging them in a 37°C water bath. Once the ice-crystals had melted, the cells were placed in 12 ml of fresh medium and pelleted by centrifugation at 300 x g for 5 minutes. The pellet was resuspended in 10 ml of medium and plated onto a 10 cm diameter cell culture dish or T75 flask.

2.3 Cloning of the qtrt1 and qtrtd1 cDNA

2.3.1 Reverse transcription polymerase chain reaction (RTPCR) with specific Primers

cDNA inserts were cloned from a RNA pool isolated from mouse using a specific primer designed from the gene of interest. RNA work must be free of Ribonuclease (RNase) enzymes that catalyze the hydrolysis of RNA into smaller units. RNases are found on skin and surface tops and therefore gloves were worn, RNase free tips and containers were used.

Mouse kidney RNA was used in a reverse transcription reaction which contained; 2 pmol of reverse primer, 1 μg Mouse kidney RNA, 4 μl of Takara dNTP mix (2.5 mM). The tube was heated to 65° C for 5 minutes and immediately placed on ice for 2 minutes. The reaction mix contained 1 μl of RNase inhibitor (Superase In), 4 μl of 5x First-Strand Buffer, 1 μl of 0.1 M DTT and 2 μl of Superscript III RT. This was incubated at 55°C for 1 hour. The enzymes were inactivated by heating to 70°C for 15 minutes. RNA was removed from the reaction by adding RNase A (7 units/μl), 1 μl was added to the reaction and incubated for 10 minutes at 37°C. The
cDNA product was purified using the QIAquick PCR purification kit according to the manual supplied by the manufacturer. Columns in the kit were used to remove enzymes and salts from the sample. The cDNA was eluted using 30 µl of Nuclease Free Water.

2.3.2 Reverse transcription polymerase chain reaction (RTPCR) with random Primers

cDNA inserts were cloned from an RNA pool isolated from mouse using random primers. Reaction mix: 1 µl of 200 ng/µl random primers, 5 µl of 20 ng/µl total RNA, 4 µl Takara dNTP mix (2.5 mM each nucleotide), nuclease free water up to 13 µl. The tube was heated for 5 minutes at 65°C then immediately transferred onto ice for 2 minutes. To the tube was added 4 µl of 5x First strand buffer, 1 µl of 0.1M DTT, 1 µl SuperInasin (20U/µl), 1 µl of superscript III RT (200U/µl). The tube was incubated at 25°C for 5 minutes to allow random primers to anneal, and then 1 hour at 50°C. Enzymes were inactivated by heating the tube for 15 minutes at 70°C. RNA was removed from the reaction by adding 1 µl of RNase A (7 units/µl) and incubated for 10 minutes at 37°C. The cDNA product was purified using the QIAquick PCR purification kit according to the manual supplied by the manufacturer. The cDNA was eluted using 30 µl of Nuclease Free Water.

2.3.3 Amplification of the cDNA product using Polymerase chain reaction technique

PCR reaction was preformed using; 2 µl of the reverse transcription reaction, 2.5 µl of 10 x PfuTurbo reaction buffer, 2 µl of Takara dNTP mix, 1 µl of the forward primer (10pmol/µl), 1 µl of the reverse primer (10pmol/µl), 0.5 µl of PfuTurbo DNA polymerase and 16 µl of Nuclease Free water. The cDNA sequences were amplified according to the PCR conditions shown in Table 2.1 using the primers in 2.2 and 2.3.

2.3.4 Agarose gel electrophoresis and DNA extraction

Agarose gels were made with 1 x TAE buffer (0.04 M Tris, 1.1% (v/v) acetic acid, 1 mM EDTA, pH 8.5), 1% agarose and 5 µl Ethidium bromide. DNA Samples were prepared by
the addition of a 1/10 volume of 10 X DNA loading dye (15% (w/v) ficoll; 0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF; 1 mM EDTA in distilled H2O) and resolved at 100 V for 1 hour with 1 x TAE as running buffer. The desired bands were excised from the gel under minimum UV light exposure and purified using the Qiagen gel extraction kit. The DNA was routinely eluted using 30 µl of Nuclease Free Water.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
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<td>68°C</td>
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Table 2.1: Optimized PCR conditions for amplification of cDNA

* Time is optimized based on length of DNA.
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<tr>
<th>Gene</th>
<th>Vector</th>
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<th>Sequence</th>
<th>Cut Site</th>
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<tr>
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<td>EcoRI</td>
</tr>
<tr>
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</tr>
<tr>
<td>qtrtd1</td>
<td>pcDNA3.1</td>
<td>mQtrtd1-</td>
<td>GCCTCGAGAGAAAACATCTTG</td>
<td>XhoI</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>2-pCMV</td>
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<td></td>
</tr>
<tr>
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<td>pCMV.HA</td>
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Table 2.2 Primer sequences for cloning of qtrtd1 variants into various expression vectors.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Vector</th>
<th>Primer Name</th>
<th>Sequence</th>
<th>Cut Site</th>
</tr>
</thead>
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<tr>
<td>Full <em>qtrtl</em></td>
<td>pcDNA3.1</td>
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<td>pcDNAF</td>
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<td></td>
<td></td>
<td>GTTC</td>
<td></td>
<td></td>
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<tr>
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<td>pcDNA3.1</td>
<td>mTGTFull-</td>
<td>CACCTCGAGAGATGTGAGC</td>
<td>XhoI</td>
</tr>
<tr>
<td></td>
<td>myc/his</td>
<td>pcDNAR</td>
<td>ATGATTTCCCACAGAG</td>
<td></td>
</tr>
<tr>
<td>Truncated</td>
<td>pCMV.Myc</td>
<td>mTGT-</td>
<td>CGAGAATTCCCGATGCAGCT</td>
<td>EcoRI</td>
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<td><em>qtrtl</em></td>
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<tr>
<td>Truncated</td>
<td>pCMV.Myc</td>
<td>mTGT-</td>
<td>CACCTCGAGATGTGAGC</td>
<td>XhoI</td>
</tr>
<tr>
<td><em>qtrtl</em></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>pCMVMyc</td>
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<td></td>
</tr>
<tr>
<td>Full <em>qtrtl</em></td>
<td>pCMV.Myc</td>
<td>mTGT-</td>
<td>CACCTCGAGATGTGAGC</td>
<td>XhoI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCMVXhoIR</td>
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<td></td>
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<td>Full <em>qtrtl</em></td>
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<td>SfuI</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>CA</td>
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<tr>
<td></td>
<td>HA</td>
<td>ATTC</td>
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<td></td>
</tr>
</tbody>
</table>

Table 2.3 Primer Sequences for cloning full and truncated *qtrtl* into various expression vectors.

2.3.5 Restriction Enzyme digestion of DNA for ligation into plasmids

DNA PCR products and plasmids were cut using identical restriction enzymes at 37°C for 1 hour (3-5 µl DNA, 3 µl compatible Buffer, 1.5 µl restriction enzyme 1, 1.5 µl restriction enzyme 2, nuclease free water to a final volume of 30 µl). In order to prevent plasmid re-ligation in the absence of insert, the plasmid was dephosphorylated by incubation with alkaline phosphatase at 55°C for 2 hours (1 µl buffer, 7.5 µl nuclease free water and 1.5 µl BAP). The DNA and plasmid were gel purified on a 1% agarose gel, using a Qiagen gel extraction kit.
2.3.6 DNA Ligation

Ligation of DNA insert and plasmid were setup as shown on Table 2.3. Two controls were set-up to ensure proper identification of positive colonies. Ligation was incubated overnight at 16°C.

**Table 2.3: Components mixed for ligation.** Samples 1 and 2 are experimental controls, no ligation should occur. Sample 3 contains the three components required for ligation, DNA insert should ligate with plasmid.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Plasmid</th>
<th>Insert</th>
<th>Ligase</th>
<th>Water</th>
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<tr>
<td>1</td>
<td>1.1 μl</td>
<td>-</td>
<td>-</td>
<td>9 μl</td>
</tr>
<tr>
<td>2</td>
<td>1.1 μl</td>
<td>-</td>
<td>5 μl</td>
<td>4 μl</td>
</tr>
<tr>
<td>3</td>
<td>1.1 μl</td>
<td>4 μl</td>
<td>5 μl</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.7 Non-denaturing Polyacrylamide Gel Electrophoresis

A non-denaturing 5% polyacrylamide gel is useful for separating DNA fragments between 100-500 bp long. It was made up using 1.5 ml 10X TBE Buffer (900 mM Tris-Base, 900 mM Boric Acid, 20 mM EDTA), 1.875 ml 40% acrylamide/Bis 19:1, 11.575 ml ddH₂O, 25 μl TEMED and 25 μl 25% Ammonium persulphate. DNA Samples were prepared by addition of a 1/10 volume of 10 X DNA loading dye (15% (w/v) ficoll; 0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF; 1 mM EDTA in distilled H₂O) and resolved at 100V for 1 hour with 1 x TBE as running buffer. The desired bands were excised from the gel under minimum UV light exposure.

2.3.8 Site Directed Mutagenesis

For the purposes of correcting a single base change mutation, primers were designed carrying the corrected sequence. A reaction was set up carrying 5 μl of 10 x PfuTurbo reaction buffer, 1 μl of the DNA template, 4 μl of Takara 2.5 mM dNTP mix, 2 μl of the forward primer (10 pmol/μl), 2 μl of the reverse primer (10 pmol/μl) as shown in Table 2.5, 1 μl of PfuTurbo DNA polymerase and 35 μl of Nuclease Free water. Samples were run through PCR conditions shown on Table 2.4. The reaction was
then placed on ice for 2 minutes to allow it to cool to below 37°C before adding 1 μl of the restriction enzyme DpnI. The reaction was incubated for 1 hour at 37°C.

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>1 minute/kb of plasmid length.</td>
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</tbody>
</table>

Table 2.5 PCR conditions for site directed mutagenesis

<table>
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<th>Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>QTRTD1_sdm_for</td>
<td>GCAACAAAAATGGAATCCAAAGAAAAATAAAAAGGCTTGGAATC</td>
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<tr>
<td>QTRTD1_sdm_rev</td>
<td>GATCCAAAGCCTTTTATTTTTTCTTGGATGTTCCATTTTTGTTGC</td>
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</table>

Table 2.6 Primer sequences for site directed mutagenesis of qtrtd1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qtrtd1screenfor</td>
<td>CCAGCTCACACTCTCATCCCTAGCA</td>
</tr>
<tr>
<td>Qtrtd1screenrev</td>
<td>GACGTCATCTCTTGGTTCGCAACCAGTTG</td>
</tr>
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</table>

Table2.7 Primer sequences for screening of qtrtd1 splice variants

2.3.9 Preparation of competent cells

A flask holding 3.5 ml of LB broth (10g bacto-tryptone, 5g yeast extract, 10g NaCl, and adjusted to pH 7.0 with NaOH) was inoculated with appropriate E. coli strain and grown overnight. A 1 ml volume of overnight culture was centrifuged at 600 x g for 5 minutes, the medium was discarded and the cell pellet was resuspended in 1 ml of fresh LB. The cell suspension was transferred to a flask containing 100 ml of LB broth and incubated at 37°C for approximately 2 hours until the optical density reached 0.2 to 0.4 at 600 nm. Once optical density was reached the culture was placed
on ice for 15 minutes before centrifugation in a GSA rotor at 2,500 x g for 10 minutes at 4°C. The pellet was resuspended in 5 ml of 75 mM CaCl₂ and incubated on ice for 20 minutes. Subsequently, the cell suspension was centrifuged in a GSA rotor at 2,500 x g for 10 minutes at 4°C and resuspended in 4 ml of TFB2 buffer (10 mM MOPS pH 7.0, 75 mM CaCl₂, 10 mM rubidium chloride, 15% (v/v) glycerol). The cells were kept on ice for 20 minutes before being snap-frozen in liquid nitrogen and stored in 200 µl aliquots at -70°C.

2.3.10 Transformation of plasmid DNA into competent bacterial cells

The total 10 µl ligation mix (Table 2.3), was added to an eppendorf containing 200 µl of TMC buffer [10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 10 mM CaCl₂] and 100 µl of competent TOP10 cells and placed on ice for 20 minutes. Pipette tips were always cut when using competent cells. Transformation was carried out by heat shock, by incubating the cells and DNA in a 42°C water bath for exactly 80 seconds and then placed immediately on ice for 2 minutes. Liquid LB was preheated to room temperature and 1 ml was added to the transformation before incubation at 37°C for 1 hour. Cells were centrifuged at 5,000 x g for 2 minutes, medium was discarded and cells were resuspended in remaining medium and plated on an LB agar plate containing the appropriate selection antibiotic for the plasmid. Colonies were grown overnight at 37°C.

2.3.11 Screening of competent cells screened for positive transformation

Colonies which grew on antibiotic agar plates were selected and screened by a PCR method. Eight colonies were picked and re-plated onto a fresh agar plates for permanent plates of each clone, which were grown overnight at 37°C. The next day single colonies from permanent plates were used to inoculate 200 µl LB with 2 µl of selected antibiotic. Inoculated tubes were put into the orbital shaker at 37°C for 4 hours. 2 µl of each culture was removed for the PCR reaction with Go Taq polymerase, using specific primers for the cloned gene. PCR product was run out on an agarose gel and visualised with ethidium bromide. A permanent stock was made from a positive colony and stored at -70°C in 15% glycerol.
2.4 Purification of anti-TGT and anti-QTRTD1 anti-sera

Anti-sera was raised in rabbits by Harlan Bioproducts against recombinant TGT and QTRTD1 produced within the lab by Mr. Colin Boland. The anti-sera was purified using Pierce Nab Protein A Plus Spin Kit. Protein A binds to IgG from a broad range of species, including rabbit. Once purified, the anti-sera was loaded on a spin column loaded with recombinant protein bound Ultralink Biosupport Resin. Recombinant protein was dialysed into coupling buffer (0.1 M MOPS, 0.6 M sodium citrate, pH 7.5) before being bound to resin at a concentration of 88 µg of protein per 1 mg of beads. Coupling was allowed to continue overnight at room temperature before the reaction was quenched with 3 M ethanolamine. The resin was washed with 1 M NaCl and equilibrated with 10 mM Tris, pH 7.4 before the purified anti-sera was negatively selected against the column. Antisera was eluted with 1 mM NaCl, 10 mM Tris, pH 7.4.

2.5 Protein determination

Bradford assay was used to determine protein concentration. A 10 µl volume of protein sample and 200 µl of Bradford reagent (BioRad) were aliquoted together and the absorbance was read on the Spectra max 340PC machine. Bovine serum albumin (BSA) was used for protein concentration standards which ranged between 50-500 µg/ml.

2.6 Isolation of RNA from cultured cell lines

Cells were cultured in 6 well plates to a confluence of 80%. For RNA isolation, cells were washed with PBS and lysed directly on the culture dish with an RNA isolating product TRI reagent (1 ml per 10 cm²), which was used according to the manufacturer’s instructions. RNA isolated from the cultured cells was reverse transcribed with random primers to produce a cDNA pool as previously described. The RNA concentration was determined spectrometrically using the equation: Absorbance at 260 nm x the dilution factor x 40 = Concentration of RNA µg/ml.
2.7 Transfection of COS-7 cells with queuine transglycosylase coding plasmids

Cells were seeded on 4-well culture slides or 6-well dishes so as to be approximately 80% confluent on the day of transfection. Transfections were carried out according to the Lipofectamine 2000 protocol using a ratio of 1 µg of DNA to 4 µl of Lipofectamine. Depending on the purpose of the transfection, the transfection was carried out for either 3 or 5 hours; the former for those cells from which protein was to be isolated and the latter for cells that were to be fixed for imaging. Cells were then left for 48 hours before use in downstream applications.

2.8 Preparation of cells for Fluorescence Imaging

2.8.1 Staining of cells with Mitotracker dyes

Cells were washed with pre-warmed growth medium before being incubated in either Mitotracker Red CMXRos at 50 nM or Mitotracker Green dye at 40 nM for 15 minutes at 37 °C. At the end of this incubation period, the cells were washed with pre-warmed growth medium again before being fixed with 4% paraformaldehyde.

2.8.2 Fixation of cells for Confocal Microscopy

To the medium covering the cells, 8% paraformaldehyde (8% w/v paraformaldehyde, 30% v/v H₂O and Phosphate Buffered Saline (PBS) to total volume, pH 7.5) was added in a 1:1 ratio so as to render the paraformaldehyde at a 4% working concentration. All steps were carried out while the cells were being agitated gently. The cells were incubated for 15 minutes and then washed three times for 15 minutes apiece with PBS. The cells were then permeabilized with 0.1% Triton-X100 in PBS for 5 minutes before being washed three more times with PBS. The cells were then blocked for 30 minutes with 10% FBS in PBS before being incubated with primary antibody diluted in 10% FBS in PBS for an hour. After three x 5 minute PBS washes, the secondary antibody also diluted in 10% FBS in PBS was placed on the cells for a further hour. The cells were washed and then fixed as before. After another round of washes, the cells were stained with 0.2 µg/ml DAPI for 2-3 minutes.
2.9 Western blotting analysis

2.9.1 Isolation of total protein from cultured cells

Cells were cultured in 6 well plates to a confluence of 80%. Cells were washed with PBS and all remaining PBS was removed with a fine pipette. To isolate protein, 200 μl mammalian protein extract reagent (M-PER) and 1 μl of protease inhibitor (Sigma) was added to each well. The plate was gently shaken for 5 minutes and placed on ice. Cell lysate was transferred into an eppendorf and centrifuge at 14,000 x g for 10 minutes at 10°C. Supernatant was collected and placed on ice. Bradford assay was used to measure the protein concentration of each sample. Samples were prepared for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) by standardising protein concentration to 1 μg/μl with 2 x SDS-loading buffer [0.1 M Tris-HCL (pH 6.8), 4% SDS, 20% Glycerol, 2-Mercaptoethanol, Bromophenol Blue (1%) and H2O]. The loading buffer was always used at a 1:1 volume ratio or higher to protein extract. Samples were stored at -70°C, if not used immediately.

2.9.2 Isolation of cellular extracts from cultured cells

Cells were cultured in 6 well plates to a confluence of 85-90%. Cells were washed with PBS which was then removed using a fine pipette. A 200 μl volume of TrypLE, 1 μl of protease inhibitor and 1 μl phosphate inhibitor were added to each well and the plate was incubated at 37°C for 10 minutes. Subsequently, 700 μl of PBS was added to the plate and the cells collected in an eppendorf which was spun at 850 x g for 15 minutes at 4°C. The remainder of the protocol was carried out according to manufacturer’s instructions (Pierce).

2.9.3 Isolation of Nuclear and Cytoplasmic fractions using NE-PER Reagent

The protocol was carried out using solutions at 4°C. The cell pellet isolated in section 2.9.2 was dried and resuspended in 200 μl of Cerl reagent before being vortexed for 15 seconds and incubated on ice for 10 minutes. This was to induce swelling of the plasma membrane before lysing the cells with the addition of 11 μl of CerII reagent. The cells were vortexed for 5 seconds and incubated on ice for a further 1 minute before being vortexed for a further 5 seconds. The cells were spun at 16,000 x g for 10 minutes and the supernatant was removed as the cytosolic fraction. The pellet was resuspended in 100 μl NER reagent the purpose of which was to remove the nuclear
proteins from the pellet. The suspension was vortexed for 15 seconds and incubated on ice for 40 minutes, being vortexed vigorously every 10 minutes. At the end of this incubation period, the suspension was centrifuged at 16,000 x g for 10 minutes and the supernatant removed as the nuclear fraction.

2.9.4 Isolation of Membrane fractions using Mem-PER Reagent
The cell pellet was homogenized in 150 µl of Reagent A and incubated at room temperature for 10 minutes to allow cell lysis to occur before the addition of 450 µl of diluted C reagent. This was kept on ice for 30 minutes after vigorous dilution. The suspension was centrifuged at 10,000 x g for 3 minutes at 4°C and the supernatant was incubated at 37°C for 10 minutes and then centrifuged at 10,000 x g for 2 minutes at room temperature to allow for a phase separation. The bottom hydrophobic fraction contained the majority of membrane proteins and was isolated for further analysis.

2.9.5 Isolation of Mitochondrial fractions using Mitochondria Isolation Kit for Culture Cells
The cell pellet was resuspended in 800 µl of ice cold reagent A and the tube vortexed for 5 seconds before being kept on ice for 2 minutes. At the end of this incubation period, 10 µl of reagent B was added to the mixture to lyse the cells. The solution was kept on ice for 5 minutes before the addition of 800 µl Reagent C. The mixture was centrifuged at 700 x g for 10 minutes at 4°C to remove cellular debris and nuclei. The supernatant was spun at 12,000 x g for 15 minutes at 4°C and the resulting pellet washed with a further 500 µl Reagent C before being spun again for a further 5 minutes at 12,000 x g to isolate the mitochondrial fraction in the pellet.

2.9.6 RNase digestion of protein cellular extracts
To 10 µg of freshly prepared nuclear and cytoplasmic extracts was added 6µl of RNase A and the sample incubated at 25°C for 15 minutes. Samples were heated to 98°C for 2 minutes then immediately placed on ice for 2 minutes before being loaded.
2.9.7 Competition Assay involving substrates of TGT

To 10 μg of a fresh nuclear extract, 50 μM of Queuine, Guanine and 9-deazaguanine was added to three separate reactions which were carried out at 25°C for 15 minutes. Samples were heated to 98°C for 2 minutes then immediately placed on ice for 2 minutes before being loaded on an SDS-PAGE gel.

2.9.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were routinely resolved on a 12% Sodium Dodecylsulphate (SDS) polyacrylamide gel. Before loading, samples were mixed with an equal volume of gel loading buffer (0.5 M Tris-HCl (pH 6.8), 4.4% (w/v) SDS, 20 % (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and bromophenol blue) and heated to 98°C for 2 minutes before being immediately placed on ice for 2 minutes. Pre-stained markers were loaded along with samples for molecular weight standards. Samples were run in 1 x running buffer (50 mM Tris, pH 8.3, 400 mM Glycine, 0.1 % (w/v) SDS) at 35 mAmp for approximately 1 hour.

Table 2.8 Components of an SDS-PAGE Gel

<table>
<thead>
<tr>
<th>Components</th>
<th>12 % Stacking Gel (ml)</th>
<th>5% Resolving Gel (ml)</th>
</tr>
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<tbody>
<tr>
<td>H2O</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>30 % Acrylamide/Bis 37.5:1</td>
<td>4</td>
<td>0.67</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>1.0 M Tris pH 6.8</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>10 % ammonium persulphate</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

2.9.9 Transfer of proteins to PVDF membrane

The resolved proteins were transferred from a polyacrylamide gel to an Immobilon Polyvinylidene diflouride (PVDF) membrane using a semi-dry transfer system. The
SDS-PAGE gel was removed from the glass plates and immersed in 20 ml of cathode buffer [25 mM Tris, 40 mM 6-amino-n-caproic acid, 10% (v/v) methanol, (pH 9.4)] for 15 minutes. The membrane was prepared by soaking in methanol for 15 seconds, nuclease free water for 2 minutes and anode buffer II (25 mM Tris, pH 10.4, 10% (v/v) methanol) for at least 5 minutes. Six pieces of 3MM Whatman paper were cut to the appropriate size to cover membrane and gel. Two pieces of filter paper were soaked in anode buffer I (0.3 M Tris, pH 10.4, 10% (v/v) methanol), 1 piece in anode buffer II and 3 pieces in cathode buffer for 2 minutes. The transfer was stacked in the following order; 2 pieces of filter paper in anode buffer I were placed onto the anode electrode, 1 piece of filter paper soaked in anode buffer II, PVDF membrane, polyacrylamide gel, 3 pieces of filter paper soaked in cathode buffer and the cathode electrode on the top of the stack. The transfer was run for 1 hour at 120 V.

2.9.10 Antibody probing

The PVDF membrane was blocked for non-specific binding by incubating overnight in TBST buffer (20mM Tris HCL (pH7.4), 150mM NaCl, 0.1% Tween) containing 10% (w/v) dried skimmed milk (marvel). The membrane was incubated with antisera in TBST buffer containing 10% (w/v) dried skimmed milk (marvel). Specific primary antisera dilutions can be seen on Table 2.5 The immobilised proteins samples were allowed incubate with the primary antisera for 1hour with constant shaking at room temperature. The membrane was washed 3 x 15 minutes with TBST to remove any unbound antisera. The secondary antisera was incubated with the membrane in TBST buffer containing 10% (w/v) dried skimmed milk for 1 hour at room temperature with constant shaking. The membrane was washed 3 x 15 minutes in TBST buffer to remove any unbound secondary antisera before the antisera complex was visualised using enhanced chemiluminesence according to the manufacturer’s instructions.
Table 2.9 Primary and secondary antisera dilutions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>1° Antisera Dilution</th>
<th>2° Antisera Dilution</th>
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<tbody>
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<td></td>
<td></td>
<td>(Anti Rabbit from donkey)</td>
<td>(Anti mouse from sheep)</td>
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<td>TGT</td>
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</tr>
<tr>
<td>Qv1</td>
<td>1:1000</td>
<td>1:20,000</td>
<td></td>
</tr>
<tr>
<td>c-myc</td>
<td>1:500</td>
<td></td>
<td>1:2,000</td>
</tr>
<tr>
<td>Actin</td>
<td>1:3,000</td>
<td></td>
<td>1:2,000</td>
</tr>
</tbody>
</table>

2.10 Spectrophotometric Assays

2.10.1 Citrate Synthase Assay

The activity of citrate synthase was measured using a coupled reaction, where CoASH, a product of the citrate synthase catalysed reaction becomes a substrate in a reaction with 5, 5-dithio-bis-nitrobenzoic acid (DTNB) to yield 5-thionitrobenzene (TNBH), a coloured compound whose absorbance can be measured at 412 nm. The assay was carried out in acrylic cuvettes which contained a 2.8 ml volume of 0.1 M Tris, 0.15 M Sucrose, pH 8.1. The absorbance was set to zero before 50 μl DTNB (4mg/ml), 50 μl acetyl CoA (2 mM) and 20 μg total protein isolated from cultured cells. The contents of the cuvette were agitated and the reaction allowed to proceed for 3-4 minutes. At this stage, the assay was started by the addition of 75 μl oxaloacetate (60 mM). The assay was repeated in triplicate for each sample. The DTNB, Acetyl CoA and oxaloacetate were all freshly prepared.

2.10.2 Cytochrome c Oxidase Assay

The cytochrome c oxidase activity was determined using a spectrophotometric assay by measuring the rate of oxidation of reduced cytochrome c at 550 nm. Cytochrome c
(sigma) was reduced by addition of several crystals of ascorbic acid (Fluka) until a colour transition from red to brown was observed. The excess ascorbic acid was removed by passing the reduced cytochrome c through a PD10 gel filtration column (GE healthcare) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The concentration of the reduced cytochrome c was determined spectrophotometrically using cytochrome c oxidised with 1 M potassium ferricyanide as a blank. The reaction mixture was carried out in a 2.8 ml volume of 10 mM potassium phosphate buffer and 10 µM reduced cytochrome c. The rate was observed for 1 minute before addition of 200 µg protein. The initial rate was used to calculate activity.

2.10.3 Lactate Dehydrogenase Assay
The activity of lactate dehydrogenase was measured by measuring the decrease in absorbance at 340 nm which results from the oxidation of NADH. The assay was carried out in acrylic cuvettes which contained a 2.8 ml volume of 200 mM Tris-HCl, pH 7.3, 6.6 mM NADH and 30 mM sodium pyruvate. The absorbance was set to zero and the rate was read for four minutes to establish a blank rate. The reaction was initiated by the addition of 20 µg total protein isolated from cultured cells and the initial linear portion measured.

2.11 Metabolite Analysis
A Nova Bioprofile 400 Analyzer (Nova Biomedical, MA, USA) was used to measure the levels of glucose, lactate, glutamine, glutamate and ammonia. The Nova Analyzer does this through an assortment of amperometric and potentiometric electrodes. Glucose, lactate, glutamine and glutamate are detected using amperometric electrodes with each containing an immobilized enzyme within the membrane which will produce H₂O₂ from the specific substrate being measured and oxygen. The evolved H₂O₂ will then be oxidised at a platinum anode whose potential is held constant. The electron flow generated is proportional to the concentration of the substrate being measured. Ammonia is measured using a potentiometric electrode which contains a sensor specific to the ion. Cells were seeded at a density of 7 x 10⁴ cells/cm² in medium lacking sodium bicarbonate and cultivated with no medium change for 72 hours. The medium was removed and passed through a 0.22 µm filter before being
run over the Nova analyzer. The medium used was also examined to ensure that the levels of metabolites would fall within the detection limits of the machine.

2.12 Polarographic Assays

An Oroboros oxygraph was used for respirometric analysis. The Oroboros converts the negative time derivative of oxygen consumption into a respirometric rate. The Oroboros was calibrated with respiration medium MiR05 (500 μM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1g/L fatty acid free BSA, adjusted to pH 7.1 with KOH) at 37°C for approximately 1 hour. The medium was stirred at 540 rpm until calibration at air saturation was attained, as evidenced by a stable oxygen flux. Cells were introduced into the 2cm³ chamber at a density of at least 2.10⁶ cells/ml. Approximately ten minutes is required for the cells to establish a basal rate of respiration.

2.12.1 Measurement of Respiration through Complexes of the Electron Transport Chain

Any measurement of the activity of the complexes of the electron transport chain using respirometric analysis on cells requires the cell membrane of those cells be permeabilised so that any substrates added can access the components of the electron transport chain. This can be done by the addition of a detergent such as digitonin which will bind to and replace cholesterol which is found within the plasma membrane, but not the mitochondrial outer membrane. Digitonin does not affect mitochondrial respiration if used at the correct concentration. This varies between cell lines with primary cell lines requiring smaller doses of digitonin with HeLa cells requiring 0.005% (w/v) to enable full permeabilization of the cell membrane without being deleterious to the mitochondria. Digitonin was introduced into the chamber and the basal rate of respiration observed to decrease before stabilizing due to dilution of substrates. The addition of substrates of complex I, 10 mM glutamate and 5 mM malate, were used to measure the rate of state 2 respiration. To achieve state 3 respiration, a saturating concentration of ADP, 2 mM, was introduced into the chamber. Complex I was inhibited with 500 nM rotenone before succinate, 10 mM, was added to induce complex II supported respiration and re-establish state 3
respiration. Inhibition of complex III with 5 \( \mu M \) antimycin a was followed by stimulation of complex IV with 100 \( \mu M \) TMPD and 400 \( \mu M \) ascorbate. The mitochondrial membrane integrity was checked by the addition of 10 \( \mu M \) cytochrome c. Damage to the mitochondrial membrane would result in loss of cytochrome c placing a restriction upon the rate of electron transport. Titration of cytochrome c should cause little or no increase in rate of respiration.

2.12.2 Investigation of Capacity of Electron Transport Chain

Once a basal rate of respiration was established, Oligomycin was added at 1\( \mu g/ml \) using a Hamilton microsyringe. Once a stable decrease in respiratory rate was observed for five minutes, a step-wise titration of FCCP (carbonyl cyanide \( p \)-trifluoromethoxyphenylhydra zone) was started, in 500 nM increments every 180 seconds until 4.5 \( \mu M \) final concentration had been attained. The addition of rotenone and antimycin A at 0.5 \( \mu M \) and 2.5 \( \mu M \) respectively was necessary to inhibit respiration so that the contribution of non-oxidative side reactions to the rate of oxygen consumption could be ascertained.

2.13 Genomic DNA extraction from adherent cultured cells

Cells were collected from the surface of the dish and the pellet washed twice with PBS. The pellet was resuspended in 504 \( \mu l \) of digestion buffer, which was composed of 450 \( \mu l \) of 1 X SSC (20 mM Tris-HCl, pH 7.5, 15 mM trisodium citrate, 150 mM NaCl and 1 mM EDTA), 50 \( \mu l \) of 10 % SDS and 4 \( \mu l \) of Proteinase K was added and the tube incubated at 55 °C, with gentle agitation, overnight. The next day 500 \( \mu l \) of a 50:50 mixture of phenol:chloroform was added and the tubes mixed by inversion for 5 minutes. The tube was centrifuged at 16,000 x g for 5 minutes and upper aqueous layer removed and transferred to a new 1.5 ml tube. Then 500 \( \mu l \) of isopropanol was added to the tube and the DNA precipitated by inversion of the tube until the DNA fibres became visible. The DNA was then pelleted by centrifugation at 5,900 x g for 30 seconds and the pellet washed with 70% ethanol and resuspended in 100 \( \mu l \) of water. The tube, with the lid open, was incubated at 65 °C to allow any excess ethanol to evaporate. The genomic DNA was stored at -20 °C.
2.14 Comparison of mitochondrial DNA copy number to nuclear copy number using qPCR

Quantitative PCR (qPCR) was performed on an Applied Biosystems 7500 System using SYBR green PCR. Sybr green fluoresces on binding to double stranded DNA so the fluorescence is directly proportional to the level of DNA amplified. The reaction mixture is detailed in Table 2.10 and the conditions detailed in Table 2.1 although the annealing temperature was changed to 60 °C.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>5</td>
</tr>
<tr>
<td>10 Sybr Green Mix</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>dNTP (2.5 mM)</td>
<td>4</td>
</tr>
<tr>
<td>Forward Primer (10 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer(10 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>GoTaq DNA polymerase</td>
<td>0.25</td>
</tr>
<tr>
<td>Water</td>
<td>9.25</td>
</tr>
</tbody>
</table>

Table 2.10 qPCR Reaction Components

2.15 Determination of Mitochondrial Membrane Potential

The radiolabelled cationic molecule triphenylmethylphosphonium bromide was used to estimate the membrane potential. This lipophilic molecule will accumulate against the mitochondrial membrane potential according to the Nernst equation.

Cells were cultured in 10cm² culture dishes to approximately 90% confluency before being trypsinized and counted. Ten million cells were resuspended in DMEM lacking FBS, antibiotics and phenol-red and incubated for 80 minutes with 5 nM TPMP⁺ spiked with 100 nCi of [H³]TPMP⁺ and 5 nM tetraphenylboron (TPB), an anionic compound which would aid equilibration of TPMP⁺ into the cell. The cells were layered on top of 100 ul of di-iso-octyl phthalate: di-iso butyl phthalate (1:2) before being spun at 10,000 x g at 4 °C for 30 seconds and 100 µl of the supernatant collected. The pellet was resuspended in 100 µl 20 % Triton X-100 (vol/vol) and 1ml
of Ecoscint scintillation cocktail was added to both supernatant and pellet before the samples were counted on a scintillation counter. The mitochondrial membrane was collapsed by addition of 1μM FCCP 10 minutes prior to addition of TPMP+/TPB. The energization dependent TPMP+ uptake was expressed as an accumulation ratio calculated using the equation; [(TPMP/mg protein)/(TPMP/μl supernatant)] (Hyslop et al., 1997, Appleby et al., 1999).

2.16 Detection of ROS using Flow Cytometry
The fluorometric dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was used for the detection of ROS in cultured whole cells using flow cytometric means. The H2DCFDA dye is nonfluorescent until the acetate groups are removed through intracellular esterases and the remaining conjugate is oxidised rendering it detectable using excitation and emission wavelengths of 492-495/517-527 nm. Cells were washed with PBS before being incubated for 30 minutes in 40 μM H2DCFDA in PBS prewarmed to 37 °C. The PBS was removed and the cells further incubated in prewarmed growth medium for 20 minutes before cells were collected by trypsinization and 2.5 x 10⁴ events were analysed per sample using flow cytometry. The forward and side scatter of the cells were examined to confirm the cells were undamaged by the dye loading and trypsinization. Negative (unstained) and positive (100 μM H2O2) were also included during analysis.

2.17 Preparation of samples for Transmission Electron Microscopy
A total of 1-2 x 10⁷ HeLa cells were fixed with 4 % glutaraldehyde solution in situ for 1 hour. Cells were scraped off the surface of the dish and washed twice with 50 mM phosphate buffer. An equal volume of 2 % agarose was added to the cell pellet and allowed to solidify at 4 °C for 30 minutes before being cut into approx. 4 mm² slices. These slices were washed for 10 minutes in 50 mM phosphate before 2 % osmium tetroxide (OsO4) in 50 mM phosphate was added. Cells were incubated for 45 minutes before the OsO4 was aspirated off and the samples dehydrated using an increasing percentage alcohol series (30-95 % aqueous ethanol solutions for 10 minutes intervals). Samples were then left in 100 % ethanol overnight before being washed twice for 15 minutes each with 100 % propylene oxide at room temperature. An equal volume of epoxy resin was then added to the propylene oxide suspended
slices and allowed to incubate at room temperature for 3 hours on a rotor. The 50 % epoxy resin solution was replaced with a 100 % solution and allowed to incubate on a rotor at room temperature for a further 3 hours. Slices were then removed from the resin solution before being placed in a mould and covered with fresh 100 % resin. The mould was placed in an oven set at 50 °C and degassed for 1 hour before the temperature was increased to 60 °C and left overnight. Ultrathin sections were cut on an ultramicrotome and collected on copper grids. Each grid was counterstained with uranyl acetate and lead citrate before being examined using a JEOL 1210 electron microscope.

2.18 Animals
Specific pathogen-free mice were maintained according to the regulations and guidelines of the Irish Department of Health and Children.

2.18.1 Isolation of genomic DNA from mouse ear punches
Ear punches were taken from 21 day old mice to obtain a tissue sample from which genomic DNA was isolated. Tissue samples were transferred to a 1.5 ml tube and digested immediately or stored at -20 °C. To each ear punch 504 μl of digestion buffer, which was composed of 450 μl of 1 X SSC (20 mM Tris-HCl, pH 7.5, 15 mM trisodium citrate, 150 mM NaCl and 1 mM EDTA), 50 μl of 10 % SDS and 4 μl of Proteinase K was added and the tube incubated at 55 °C, with gentle agitation, overnight. The next day 500 μl of a 50:50 mixture of phenol:chloroform was added and the tubes mixed by inversion for 5 minutes. The tube was centrifuged at 16,000 x g for 5 minutes and upper aqueous layer removed and transferred to a new 1.5 ml tube. Then 500 μl of isopropanol was added to the tube and the DNA precipitated by inversion of the tube until the DNA fibres became visible. The DNA was then pelleted by centrifugation at 5,900 x g for 30 seconds and the pellet washed with 70% ethanol and resuspended in 100 μl of water. The tube, with the lid open, was incubated at 65 °C to allow any excess ethanol to evaporate. The genomic DNA was stored at -20 °C.
2.18.2 Screening of TGT genetrap animals using PCR from genomic DNA

The primers used to amplify each sequence are presented in Table 2.10 and the PCR components and conditions in Table 2.11 and 2.12 respectively.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGTFor</td>
<td>GAGGCCGCGTGTGGATTCGATCTG</td>
</tr>
<tr>
<td>TGTRev3</td>
<td>CAGAGCATTCTGGATCTCCACCG</td>
</tr>
<tr>
<td>BGeoRev</td>
<td>TCTAGCCTCGAGGTGACGGGTATCG</td>
</tr>
</tbody>
</table>

Table 2.11 Primers for genotyping wild type, heterozygous and homozygous animals

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>1</td>
</tr>
<tr>
<td>5x GoTaq buffer</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>dNTPs (2.5 mM)</td>
<td>4</td>
</tr>
<tr>
<td>Forward Primer (10 pmol/μl)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer (10 pmol/μl)</td>
<td>1</td>
</tr>
<tr>
<td>GoTaq DNA polymerase</td>
<td>0.25</td>
</tr>
<tr>
<td>Water</td>
<td>10.75</td>
</tr>
</tbody>
</table>

Table 2.12 PCR Reaction Components

<table>
<thead>
<tr>
<th>Temperature (C)</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>5 mins</td>
<td>1</td>
</tr>
<tr>
<td>98</td>
<td>10 seconds</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>30 seconds</td>
<td>35</td>
</tr>
<tr>
<td>72</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>8 minutes</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.13 PCR Reaction conditions
2.18.3 Whole Murine Spleenocyte Culture

All mice used were males aged 12 weeks or older. Whole spleens isolated from male C57BL/6 mice were extruded through a 40 μm nylon mesh using a 3 ml syringe plunger. The cells were washed in a total volume of 50 ml of sterile PBS and centrifuged at 300 x g for 5 minutes to discard supernatant. The cell pellet was re-suspended in 20 ml PBS to determine total cell count.

2.18.4 Determining total live cell count using Ethidium Bromide/Acrildine Orange

Total cell density was calculated by staining a 10 μl aliquot of the cell suspension with Ethidium Bromide/Acrildine Orange (EBAO) in a 1:1 ratio. A 10 μl aliquot of this mixture was applied onto a disposable haemocytometer and counted under a fluorescence microscope. EBAO is a combination of two individually used intercalating agents: ethidium bromide (EB) and acridine orange (AO) which stain nucleic acid content. In apoptotic cells the membrane is permeable to both EB and AO which then bind DNA and give an orange colour fluorescence. In non-apoptotic cells however, only the cell-permeable dye AO is able to penetrate the cell and stain the DNA content green. Therefore, their combination can distinguish between green/viable cells and orange/apoptotic cells. The total live cell count per ml was calculated using the following formula:

\[
\text{Cell Number/ml (x10^6)} = \text{Live cell count x Dilution factor x Total volume of suspension}
\]

2.18.5 T lymphocyte specific stimulations

Cell culture 24 well plates were coated with 2 μg/ml of αCD3ε in warm PBS for 2 hours at 37°C. Prior to plating, the unbound αCD3ε antibodies in the wells were washed off with sterile PBS. Between Day 0 – 5 post-plating, the cells were also stimulated with 5 ng/ml of soluble Interleukin-2 (IL-2) every 48 hours.

2.18.6 Preparation of tissue homogenates

Animals were starved overnight and sacrificed by cervical dislocation. The brain and liver were extracted and washed three times with ice-cold homogenization buffer (250 mM sucrose in 2.5 mM Tris-HCL, pH 7.4). The tissues were diced into small
pieces using a fine scissors before being homogenized with a Dounce homogenizer. The homogenate was centrifuged at 2200 rpm for 3 minutes at 4 °C and the supernatant removed and retained.

2.18.7 Isolation of Mitochondria from Brain using Percoll density gradient centrifugation

Animals were starved overnight before being sacrificed by cervical dislocation. The brain was removed from the skull cavity, weighed and placed in approximately 30 ml of ice cold isolation buffer (10 mM Tris, 1 mM EDTA, 160 mM sucrose, pH 7.4). The tissue was washed twice with 30 ml isolation buffer and the excess buffer removed so the remaining buffer just covered the tissue. The tissue was minced with a blade before being washed twice using approximately fifteen times the volume of minced tissue. The excess buffer was decanted before the tissue was transferred to a 40 ml Dounce homogenizer and isolation buffer containing protease inhibitors was added to achieve 10% tissue weight per volume. The tissue was homogenized using four strokes with the loose pestle and eight strokes with the tight pestle. The homogenization was carried out on ice. The homogenate was centrifuged at 1,300 \( \times g \) at 4 °C for 3 minutes. The supernatant was carefully decanted, taking care not to transfer any of the loose pellet, and retained on ice in a new centrifuge tube. The pellet and small amount of supernatant associated with it was placed back in the Dounce homogenizer. Isolation buffer was added to approximately half the previous volume and the suspension was homogenized using the tight pestle before being centrifuged as before. The resulting supernatant was added to previously retained supernatant and centrifuged at 21,000 \( \times g \) at 4 °C for 10 minutes. Discard the supernatant and resuspend the pellet in 10 ml of ice cold 15% Percoll solution per gram of tissue by homogenizing with the loose pestle. The Percoll gradient was constructed of a 40% Percoll layer introduced slowly beneath a 23% layer so as to introduce a sharp interface between the two. The Percoll suspended extract was carefully overlaid on the top of the gradient. The tube was spun at 30,000 \( \times g \) at 4 °C for 5 minutes. Three distinct bands were produced and the bottom band contained the purified mitochondria. This was removed, taking care to remove as little surrounding Percoll as possible. Four volumes of isolation buffer was added to the mitochondrial fraction and centrifuged at 16,700 \( \times g \) at 4 °C for 10 minutes. The supernatant was
aspirated, taking care not to dislodge the loose pellet and 500 µl fatty-acid free BSA (10 mg/ml) was added to the tube before 3 ml of cold isolation buffer was added. The tube was spun at 6,900 x g at 4 °C for 10 minutes. A firm pellet should be produced and the pellet transferred to a 2 ml Dounce homogenizer. The pellet is resuspended with four strokes of the loose pestle in 300 µl cold isolation buffer. The prepared mitochondria were used within four hours of preparation for oxygraph analysis and frozen at -80 °C for further spectrophotometric assays.

2.19 Statistical Analysis

All average results presented as mean ± S.E.M were calculated using Microsoft Excel and Prism Graphpad software.
Chapter 3

The qtrtl gene

3.1 Introduction

The bacterial enzymes responsible for queuosine biosynthesis have been extensively investigated (Iwata-Reuyl et al., 2003). It is from these efforts that the tRNA insertion step of the queuine precursor molecule preQ₁ is known to occur through a single enzyme species, the tRNA guanine transglycosylase (TGT) enzyme. Despite knowing the identity of the bacterial enzyme, numerous efforts over the past 30 years to indentify the related eukaryotic activity have met with failure.

In contrast to the bacterial enzyme, which uses preQ₁ as substrate, the eukaryotic activity inserts queuine base into tRNA, which is recovered from bacteria resident in the gut or from ingested food. There is little understanding of the how queuine uptake is achieved and it may rely on known purine nucleotide transporters or have its own dedicated transporter. Studies by Walter Farkas on germ-free mice demonstrated that whole tRNA, in addition to queuine, can reconstitute queuosine modified tRNA when given to the mice thus proving the existence of a salvage activity (Reyniers et al., 1981). This salvage activity was further investigated using the Vero and L-M cell lines, the latter lacks the queuine salvage activity. Treatment of both cell lines with the radioactive queuine analog [³H]dihydroqueuine demonstrated that when the Vero cell line was deprived of queuine in the medium, the insoluble half life of the radioactivity was 52 days whereas the half life in the L-M cells was only 1.2 days. Addition of queuine back to the medium obviously competed against the intrinsic salvage activity in the Vero cells as the half life diminished to 3.1 days whereas it remained unchanged in the L-M cells (Gündüz et al., 1982). This salvage activity has also been found to proceed through the intermediate queuosine-5'-phosphate as Vero cells were found to be capable of recycling this nucleoside but not the 3'-phosphate form into queuine (Gündüz et al., 1984). The same activity has been found to exist in the algal plants Chlorella...
pyrenoidosa and Chlamydomonas reinhardtii and the recycling is reliant on the formation of the 5' phosphate of the nucleoside (Kirtland et al., 1988).

Intriguingly, mitochondrial aspartyl tRNA of rat and opposum has been shown to contain queuosine nucleoside, an observation made by direct sequencing of the tRNA (Randerath et al., 1984; Mörl et al., 1995). This raises the question of how these tRNA species are being modified since the mitochondrial tRNA pool of higher eukaryotes does not freely exchange with the cytoplasmic tRNA pool. Mitochondria possess a small level of autonomy in the cell as they have their own genome which encodes a full set of tRNA molecules and many of the rRNAs necessary for protein translation. However, the mitochondria are still reliant on the nuclear genome for the component proteins of the translational machinery and it is extremely important that the mitochondria are capable of importing these proteins. This is achieved through a range of channel proteins embedded in the mitochondrial membranes. It is conceivable that queuine could exert an influence on this system.

Initial attempts to purify the eukaryotic queuine tRNA transglycosylase activity were unaware that the true substrate of the enzyme was queuine and for this reason the activity became known as a guanylating activity due to its ability to insert guanine into tRNA; hence the name tRNA guanine transglycosylase. These studies were carried out on rabbit reticulocytes, cells that lack a nucleus and as a result are unable to synthesise nucleotides de novo (Hankins et al., 1970, Farkas et al., 1973). Thus, it was argued that the incorporation of radiolabelled guanine into the RNA of these cells could not be the result of nascent tRNA synthesis. When it was discovered that guanine was being incorporated into a specific position of the endogenous tRNA further work to characterize this enzymatic activity led to it being the first tRNA modifying enzyme to be biochemically characterized (Farkas et al., 1973).

Attempts to identify the tRNA modifying activity in rabbit reticulocytes and Ehrlich ascites tumour cells (a tumourigenic cell line) proved problematic as only low transglycosylase activity was present in both (Farkas et al., 1973, Itoh et al., 1977). It was only when Farkas turned his attention towards isolating the transglycosylase activity from rabbit erythrocytes that a small measure of success was had; an approximate 3,000 fold purification that provided an isolate with a specific activity of 153 pmol/h/mg protein. At the time, this was the highest reported specific activity for a eukaryotic tRNA transglycosylase (Howes et al., 1978). When the fraction
containing the putative activity was resolved by SDS-PAGE, two bands of 60 kDa and 43 kDa were apparent. Almost all subsequent attempts to purify the transglycosylase reported that it comprised two subunits, a ~60 kDa protein and a smaller ~30-40 kDa partner. This can easily be discerned in Table 3.1 where all the attempts at purifying the transglycosylase activity have been summarised. The activity from wheat germ proved an exception where both proteins were found to be of identical weight (Walden et al., 1982). As the size of the smaller sub-unit was found to vary in size between studies, an assumption was made that the primary catalytic activity was located within the larger subunit and the smaller partner protein harboured a chaperone function (Deshpande et al., 1996).

<table>
<thead>
<tr>
<th>Source</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>No. of Subunits</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
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<td>E.coli</td>
<td>46</td>
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</tr>
<tr>
<td>Z.mobilis</td>
<td>45</td>
<td>1(46)</td>
<td>preQ&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>H.volcanii</td>
<td>78</td>
<td>1(78)</td>
<td>preQ&lt;sub&gt;0&lt;/sub&gt;</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>140</td>
<td>2(68+68)</td>
<td>Queuine</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>104</td>
<td>2(60+34.5)</td>
<td>Queuine</td>
</tr>
<tr>
<td>Rat liver</td>
<td>80</td>
<td></td>
<td>Queuine</td>
</tr>
<tr>
<td>Rabbit erythrocyte</td>
<td>104</td>
<td>2(60+34.5)</td>
<td>Queuine</td>
</tr>
<tr>
<td>Bovine liver</td>
<td>100</td>
<td>2(60+31)</td>
<td>Queuine</td>
</tr>
<tr>
<td>Human liver</td>
<td>104</td>
<td>2(60+34.5)</td>
<td>Queuine</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of the properties of the purified transglycosylase activities from various organisms. Adapted from Morris et al., 2001

The studies mentioned above relied on radioactive guanine incorporation into tRNA as a means to trace the enzymatic activity and on electrophoresis to visualize the purity of the fractions. Curiously, despite obtaining good amino-acid sequence data from the purified preparations none of the peptide sequences aligned with the bacterial TGT protein. One such preparation from bovine liver yielded an active fraction containing proteins of 66 and 32 kDa and peptide sequence data was successfully recovered (Slany et al., 1995). At that time, genomic and transcript data were insufficiently detailed to allow the recovered peptide sequence data to be assigned to a protein, however our own recent sequence analysis definitively shows that the peptides belong to an asparaginyl-tRNA synthetase and 2,4-dienoyl CoA reductase, the latter enzyme being involved in fatty acid metabolism. The following year Farkas and his group published a paper detailing the purification of transglycosylase activity from rabbit erythrocytes and reticulocytes (Deshpande et al.,
1996). They found that the purified fractions from each source yielded a different banding pattern on SDS-PAGE and focused on a band of 60 kDa which was common to both preparations; assuming it to be the TGT activity. Upon obtaining peptide sequence information, they could show that it bore significant similarity to members of the deubiquitinating enzyme family. In fact, the protein they had sequenced was later shown to be ubiquitin specific protease 14 (USP14); a protein involved in deubiquitination of proteins. It is perhaps worth mentioning that this assignment of the queuine tRNA insertase activity to USP14 has since given rise to significant confusion in the literature and sequence databases. Two additional proteins that Farkas’s group successfully obtained sequence from, one of 99 kDa and another of 57kDa, showed substantial similarity to a translational elongation factor and an immunophilin protein (Katze et al., 1996).

It was in 2001 that the human orthologue of the bacterial TGT was found by using the *E.coli* TGT sequence as bait to search the human EST database (Deshpande et al., 2001). This human homologue, when cloned into the cell line GC(3)/c1, lacking the ability to insert queuine into its tRNA was found to reconstitute that activity. The molecular weight of this human TGT was found to be 44 kDa (Deshpande et al., 2001). Although this work provided some clues as to the involvement of TGT in the tRNA transglycosylase step it inadequately defines the activity since i) the mutation in the GC(3)/c1 cell line is unknown and the TGT enzyme could as likely be reconstituting the queuine salvage activity, ii) the transglycosylase activity could be reliant on a number of unidentified protein, TGT being just one member and iii) the eukaryotic TGT protein alone was never shown to be active for queuine insertion into tRNA.

The above discussions serve to illustrate the pitfalls encountered when investigating the eukaryotic transglycosylase activity. These included the incomplete annotation of the eukaryotic genome and the inability to link the eukaryotic transglycosylase activity with the well-defined bacterial TGT orthologue. This problem may have arisen in part due to the ‘stickiness’ of the transglycosylase activity which was seen it co-purifying with a diverse array of proteins, some of whose functions could provide tantalizing clues about the function of the queuine transglycosylase and by extension its substrate. Briefly, the aims of this chapter are to
address some of the issues mentioned above and to use a genetic approach to help identify the queuine insertion apparatus of the eukaryotic cell.
3.2 Results

3.2.1 Examination of the genetic and protein sequences of TGT

In an effort to explore the identity of the transglycosylase activity, the most logical approach was through recourse to the eubacterial TGT enzyme which has been well characterized in *E. coli* and *Z. mobilis*. Already the gene coding for TGT from *E. coli* had been successfully used to find the human ortholog (Deshpande et al., 2001). Both these bacterial sequences were used as bait sequences in protein blast (pblast), nucleotide blast (nblast) and translated nucleotide blast (xblastx) searches of genome and EST databases. It quickly became apparent that every eukaryotic species carried a gene coding for a TGT orthologue, which was not unexpected as queuine has long been known to be present within the somatic tissue of all species examined. The only exception to this, and in agreement with the literature, was *S. cerevisiae* (Walden et al., 1982), which was lacking the TGT gene. Curiously though, other yeast species examined were found to contain TGT.

In higher eukaryotes, the gene encoding TGT is referred to as *Qtrtl* and in a bid to further characterize the *Qtrtl* gene and its protein product TGT, the genetic and protein sequence of a handful of species was compared using Genedoc, a multiple sequence alignment programme (Nicholas et al., 1997). Sequences were chosen from all branches of the tree of life and included examples of plant, arthropod, amphibian, protozoan, fish, fungus, nematode and mammals. By basing the comparative study on a diverse array of organisms, the conserved nature of the TGT protein could be demonstrated. The sequences of the *E. coli* and *Z. mobilis* gene and protein were also included for comparison purposes (Figure 3.1). The protein sequences of the mouse and *Z. mobilis* TGT were found to share 42% identity. When the crystal structure of the *Z. mobilis* TGT was solved, it was observed to adopt the most common protein fold, a TIM barrel, so called after the first enzyme shown to contain this fold the triosphosphate isomerase enzyme (Romier et al., 1996). From this, it was possible to annotate the protein sequence with the α-helices and β-strands necessary for it to assume this conformation. Comparison against the ten other sequences chosen by us demonstrated that these tertiary structural elements of the enzyme are conserved. Additionally, the active site nucleophile Asp 280 (according to *Z. mobilis* numbering) has also been highly conserved. Inclusion of the *E. coli* ortholog in the comparative
analysis allowed for the immediate visual identification of an extension of the N terminal region of the eukaryotic TGT protein in comparison to the E.coli version. The mammalian TGT extension is sixteen amino acids long. The length of the TGT protein between the species examined was between 350 and 450 amino acids long, a range which is not far removed from the average length reported in the eukaryotic proteome (Wong et al., 2008). It has been observed that proteins which fit within this range also demonstrate a higher level of conservation (Lipman et al., 2002).

A comparison of the Qtrtl sequences demonstrated that the degree of identity between the genes coding for TGT was not as high as that observed in the protein sequence analysis. However, the degree of identity was similar and this decrease in identity seems to be the cause of synonymous nucleotide substitution, showing that while the genes are subject to drift, a positive selective pressure is maintaining the Qtrtl gene within all eukaryotic genomes examined (Figure 3.2).

One anomaly encountered during this search was the protein sequence of the TGT retrieved from the phytoplankton Ostreococcus tauri which was found to have an abnormally long protein sequence. Ostreococcus tauri are the smallest known eukaryotes and their genome has excited interest as it contains many adaptations in its packaging of DNA and the unusual genetic arrangements it uses to enable its small cell size (Palenik et al., 2007). One of these is the use of genetic fusion proteins, a concept similar to that of operon controlled gene expression in bacteria. In this instance, the TGT protein was found to be in frame with genes coding for a transcription factor called TAF12, a ubiquitin specific protease and an enoyl-coenzyme A (Fig 3.3). The fact that the gene fusion identified in Ostreococcus contained the sequences coding for a ubiquitin specific protease, a transcription factor and dienoyl reductase was interesting since, as mentioned earlier, the protein purification attempts to indentify TGT in eukaryotes lead to the co-purification of homologous of these proteins.

### 3.2.2 Homologues of TGT are found in eukaryotes

During the search for eukaryotic homologues of TGT, another related protein was encountered called queuine tRNA ribosyltransferase domain containing 1 (QTRTD1). This protein was observed to share 23% identity and 41% similarity with the TGT
enzyme. Furthermore, the encoding gene was found to be invariably present in those species which also carried TGT, and never present by itself.

A distance tree was constructed using the bacterial and eukaryotic TGT sequences and the newly discovered QTRTD1 sequence and it was demonstrated that the sequences of the bacterial and eukaryotic TGT were more similar to each other than either was to QTRTD1 (Figure 3.4). This hints to the possibility that Qtrtd1 has been present in the eukaryotic genome for longer than Qtrtl; both genes could be the result of a gene duplication in a common ancestor but in the case of Qtrtd1, this ancestor is likely to have preceded that of the eukaryotic in which Qtrtl first appeared. The implications for the functionality of this evolutionary relationship will be dealt with in greater detail in a later chapter.

What also became apparent was that there appeared to be other TGT related proteins within the eukaryotes examined. This number varied depending on the species and some of the proteins were substantially shorter than expected for a functional protein. On closer inspection, all these protein variants were found to be the result of alternative splicing of the Qtrtd1 gene. This gene, in mice, was found to be transcribed from the minus strand of chromosome 16 and contained nine exons which through alternative splicing was responsible for two proteins which were found through pblast and tblastn searches of the mouse non-redundant and est databases – NM029128 and BC017628. A third variant was found when total kidney RNA was reverse transcribed from a 4 week old male mouse. These three splice variants were named according to standard convention – Qv0, Qv1 and Qv2, it was Qv1 which was discovered in lab. On examination of the genetic structure of these variants, Qv0 was found to be encoded without exon five while Qv1 and Qv2 utilized all nine exons. However Qv2 retained a third of intron six through use of a cryptic donor site that engaged further downstream with exon seven. Furthermore, there was an inverse relationship in the length of the mRNA transcript and the protein product (Figure 3.5 a,b). On consultation of the Alternative Splicing Database (Thanaraj et al., 2004), Qv0 and Qv2 were present in complete form Qv1 was present within the database as partial 5’ and 3’ sequences.

The expression pattern of the three splice variants was investigated by reverse transcription-PCR using a set of primers that had been designed to recognise and distinguish between the cDNA of all three variants. The RNA from a variety of
tissues was isolated – kidney, lung, spleen and also from COS-7 cells (this cell line was chosen to allow further analysis of the TGT and QTRTD1 proteins). The expected size of the amplification products for variant 0, variant 1 and variant 2 were 562 bp, 775 bp and 1617 bp respectively. From each tissue, only one amplification product was observed, which corresponded to the size of Qv1 (Figure 3.5 c).

3.2.3 Expression and sub-cellular localization of TGT and QTRTD1
Antisera had been produced against murine recombinant truncated TGT and Qv1 cloned and purified in lab by Coilin Boland. These antisera were purified using a proprietary purification kit from Pierce which utilized the IgG binding properties of protein A isolated from S. Aureus. Total protein was isolated from COS-7 cells grown in fetal bovine serum (FBS) containing medium and immunoblotted using the purified antisera. Both antisera produced a single band of approximately 62 kDa (Figure 3.6 a). While this demonstrated that the antisera gave when cell extract is resolved by SDS-PAGE, it did not eliminate the possibility of cross-reactivity of the antisera especially since the TGT and Qv1, according to the immunoblotting results, were of nearly identical molecular weight.

To rule out the possibility of cross-reactivity, purified recombinant murine TGT and Qv1 were bound to an azlactone based agarose resin which was then packed on a column. Azlactone will bind almost instantaneously with primary amines such as those found on the side chains of some amino acids. This bond can be broken using a buffer containing a secondary amine. Over each column, the cognate anti-serum was run and then the flow-through of each column was used in immunoblotting experiments of total protein. When the antiserum was run over a column bound to its target protein, the epitopes capable of recognising the protein were stripped away and no band appeared on the blot. When the opposing antiserum was run over each column, any idiotypes capable of recognising the opposing member protein were removed. When the counter-selected antisera were immunoblotted, a single band of the correct size appeared. These experiments proved that that purified antisera to both proteins was highly specific to the antigen to which it was raised (Figure 3.6 b).

Subsequent experiments were carried out using antisera that had been purified by counterselection i.e the anti-TGT sera was passed through a column of resin bound to Qv1 and *vice versa*. The expression of TGT and Qv1 were investigated under
conditions of serum supplementation and without animal serum. In addition, since the transglycosylase activity inserts queuine into tRNA and we sought to determine whether the expression of TGT and Qvl was dependent on the presence of its substrate. Queuine is a component of foetal bovine serum but, for unknown reasons, is absent from horse serum. Cells were also grown in Ultraculture medium, a proprietary medium containing no serum and shown by Tatsiana Rakovich, in our laboratory, to be devoid of queuine. Total protein was isolated from COS-7 cells which had been cultured in the above growth conditions and the expression of TGT and Qvl examined (Figure 3.7 a). It was observed that queuine status had no effect on either protein.

After ascertaining that both TGT and Qvl demonstrate measurable expression in a range of tissue types, the cellular localization of both proteins was investigated. Cell fractions were isolated and run on an SDS-PAGE gel before being transferred to a nitrocellulose membrane and immunoblotted using the purified and counter-selected anti-TGT and anti-QTRTD1 antisera. These blots revealed that both TGT and QTRTD1 appear to occupy different sub-cellular locations. TGT was present primarily in the cytosol and nucleus whereas Qvl was to be found to be associated primarily with the mitochondria (Figure 3.7 b).
Figure 3.1 Protein sequence alignment of TGT from a range of species. Alignment of the eubacterial TGT protein (E.coli and Z.mobilis) against TGT from a range of eukaryotes using the multiple alignment program GeneDoc (Nicholas et al., 1997). Each eukaryotic species was chosen from a different branch of the phylogenetic tree as represented on the top left. Conserved residues are shown in white against a dark background. The numbering refers to the TGT from the slime-mould Dictyostelium discoideum. The secondary structure of the TGT from Z.mobilis is shown above the sequences. The residue Asp280 (according to Z.mobilis numbering) is highlighted in red in all species.
Figure 3.2 Gene sequence alignment of Qtrl from a range of species. Alignment of the eubacterial tgt gene (E.coli and Z.mobilis) against Qtrl from a range of eukaryotes using the multiple alignment program GeneDoc (Nicholas et al., 1997). Each eukaryotic species was chosen from a different branch of the phylogenetic tree as represented on the top left. Conserved residues are shown in white against a dark background. The numbering refers to the very first species, the TGT from the slime-mould Dictyostelium discoideum.
Figure 3.3 The fusion protein structure of *Ostreococcus taurii* contains TGT in frame with other proteins. The protein sequence of *Ostreococcus taurii* was analysed using the conserved domains program available on the NCBI website. The sequence was searched for regions of similarity to other proteins and was found to contain conserved regions from the ubiquitin specific proteases (blue), the coenzyme A hydratase superfamily (red) and the transcription factor TAF12 (yellow). The e-values returned from the conserved domain program is presented in table format for each of the recognised domains.
Figure 3.4 Distance tree of eubacterial TGT and eukaryotic TGT and QTRTD1. Constructed using MEGA 4.0 (Tamura et al., 2007). The blue grouping represents the eubacterial TGT. The green grouping represents the eukaryotic TGT and the orange grouping represents eukaryotic QTRTD1. The eukaryotic QTRTD1 appears as an offshoot of the eubacterial and eukaryotic TGT, indicating an earlier arrival on the evolutionary tree. Additionally, neither TGT nor QTRTD1 occur in isolation in any eukaryotic species. Scale bar is 0.1 million years (mya).
Table 3.5 Examination of the splice variants of QTRTD1 and their expression.
The top line represents the genomic structure of qtrtd1, with the various alternative spliced products underneath. The sizes of the boxes are only broadly proportional to the actual length of the sequences. Exon 5, illustrated by a blue box within the schematic, is not transcribed in Qv0 and intron 6 can be seen to be partially retained in the Qv2 transcript. Qv0, BC083089; Qv1, NM029128; Qv2, BC017628. Below that is a protein alignment of Qv0, Qv1 and Qv2 showing that the Qv2 protein is truncated. Primers were designed against common exonic regions in all three variants. The length of the amplified product allowed the identification of a particular variant; v0, 562bp; v1, 775bp; v2, 1617bp. RNA was isolated and reverse transcribed before amplification with the QSR and QSF primers. Products were run on a 2.5 % agarose gel.
Figure 3.6 Purification and Counter-Selection of Antisera against TGT and QTRTD1. (a) Antisera which had been raised against recombinant tTGT and Qvl were purified on column bound Protein A according to manufacturer's instructions (Pierce). (b) The antisera were then selected on resin bound to the opposing member protein or to the protein against which the antisera was raised. The total cell extracts of COS-7 cells were resolved using SDS-PAGE before being transferred to membrane and subjected to immunoblot analysis. Qvl antisera selected on resin bound recombinant tTGT, which would remove any antibody idiotypes capable of recognising TGT, gave a band of 62 kDa (left hand side, upper panel). This was indicative of exclusive recognition of the endogenous Qvl protein. When selected on Qvl bound resin, the Qvl antisera did not recognise proteins in COS-7 protein extract, so demonstrating specificity of the antisera (left hand side, lower panel). Similarly, TGT antisera selected on resin bound recombinant Qvl (leading to the removal of antibody idiotypes capable of binding Qvl) gave an expected band of 62 kDa (right hand side, lower panel). However, when TGT antisera was selected against resin bound tTGT no band was observed (right hand side, upper panel). Results presented are representative of three independent experiments.
Figure 3.7 Examination of the expression and localization of TGT and QTRTD1.
(a) COS-7 cells were grown in three different serum conditions; UL ultraculture medium, HS horse serum containing medium and FBS foetal bovine serum containing medium. (b) Cell extracts were prepared according to manufacturer’s instructions (Pierce). Protein extracts were prepared with protease inhibitors and run on a 12% SDS-PAGE gel before being transferred to PVDF membrane and immunoblotted using antisera specific for TGT, Qv1 and Actin.
3.3 Discussion

In bacteria, tRNA transglycosylase activity has been unequivocally associated with the TGT enzyme, however identification of such activity in eukaryotes proved problematic. In fact, somewhat disconcertingly, the transglycosylase activity isolated from rat liver (Shindo-Okada et al., 1980) demonstrated substantially higher affinity for guanine than queuine. The various studies that attempted to isolate the queuine transglycosylase activity never successfully proved the purity of their preparations. The activity has always been reported to co-purify with other seemingly unrelated and functionally dissimilar proteins. In one instance, the identity of the transglycosylase activity was equated to that of a de-ubiquitin protease. This uncertainty in the literature raised the possibility that the eukaryotic transglycosylase activity may not be associated with the TGT protein, or at least not exclusively.

A thorough interrogation of Genbank demonstrated that every eukaryote contained an orthologue of the bacterial TGT. Furthermore, a substantial level of identity, observable in both gene and protein comparisons is generally illustrative of a high degree of conservation in the activity of a protein. This fact, coupled to the retention of the Qtrt1 gene across almost all eukaryotic species implies an important role for the TGT protein. While only a handful of sequences were chosen to be displayed, it is important to point out that the gene coding for TGT is found in all eukaryotic species with the exception of S. cerevisiae. Among these proteins is the algal protozoan, Ostreococcus tauri, a species which has been called the smallest eukaryote. The genome of this species is reduced in scale with the size of the organism and the genetic structure resembles the operon structure of the eubacterial genome as functionally related genes or genes whose protein products will be required consecutively are clustered together and transcribed as fusion proteins. Interestingly, the fusion protein of which TGT is a part of in Ostreococcus tauri also includes those coding for a USP, a transcription factor and a dienoyl reductase, members of protein families which the transglycosylase has been co-purified with in the past.

During our database searches, it came to light that a homologue of TGT was to be found in each eukaryote. This protein was called QTRTD1 and was coded for by the Qtrtd1 gene. There has been only one mention of this protein within the literature where it is designated as being a putative queuine salvage activity.
The protein sequence of two variants which were the product of alternative splicing from the mouse Qtrtdl gene were found in the EST databases. A third splice-variant was then found when the Qtrtdl cDNA was reverse transcribed from mouse kidney and this was named Qvl. The only hints of the existence of Qvl were to be found in partial 5' and 3' sequences within the Alternative Splicing Database (Thanaraj et al., 2004). In general, alternative splicing of a gene provides isoforms that are specific to a particular tissue or developmental stage. In the case of the Qtrtdl gene, the Image-clones for the Qv0 and Qv2 splice-variants were found in murine day eight embryonic germ cells and in adult retinal tissue, respectively. Our analysis of the qtrtdl splicing pattern in adult somatic tissue demonstrated that Qvl is the sole variant expressed in these tissues.

Using antisera generated against recombinant TGT and Qvl, which had been purified and tested for specificity of recognition of target proteins, the size of both proteins was ascertained to be 62 kDa using immunoblot analysis. This also demonstrated that TGT and Qvl were expressed at a sufficient level for detection. Additionally, the protein expression of both TGT and Qvl was found to be independent of substrate concentration. However, this may have been a cell-line specific effect as the rat hepatoma cell line H4-II-E-C3 demonstrated an increase in the expression of TGT when cultivated in FBS containing medium. This increase was not observed when these cells were grown in medium containing horse serum meaning it was likely to be a queuine specific effect.

Finally, the cellular localization of both proteins was examined using cellular fractions and immunoblot analysis. The finding was that both occupied different compartments in the cell. Where TGT was demonstrated to reside in the cytosol and nucleus, Qvl was found to be primarily mitochondrial. The association of Qvl with mitochondria is not surprising as tRNA isolated from mitochondrial of opossum and rat was found to be modified for queuine (Randerath et al., 1984, Mörl et al., 1995). Since the mammalian genome encodes a set of tRNA sufficient to meet its protein translation requirements, this would indicate that the mitochondrial tRNA is modified for queuine in situ. What is puzzling is that, according to the immunoblot results, there appears to be no overlap in the localization patterns of these two proteins, which is unusual in that both could be expected to be involved in the queuine modification of tRNA. Our results to this point led us to speculate that Qvl is responsible for the
queuine modification of mitochondrial tRNA whereas cytosolic tRNA was the substrate for the TGT enzyme.
Chapter 4
TGT and Qv1 are directed to the mitochondria

4.1 Introduction
Prior to carrying out this study, details of how the eukaryotic cell inserts queuine into tRNA remained unknown. Likewise, the operation of a number of other steps in the queuine utilisation pathway, including uptake and salvage, remained understood only in broad terms. A number of studies in the literature suggested that queuine modification of tRNA could be enhanced by growth stimuli, and in particular through protein kinase C (PKC) activation, leading to increased queuine uptake and increased transglycosylase enzyme activity. This fact, coupled with the observation that tRNA of neoplastic transformed cells is unmodified with respect to queuosine (Okada et al., 1978) raised the question of how cellular transformation and cellular growth stimuli relate to queuine modification of tRNA.

In the first study to examine the effect of growth signals on queuine, chronic treatment of human fibroblasts with the tumour promoter PDD (Phorbol 12,13-didecanoate) were found to cause a decrease in queuine uptake that correlated with the saturation density of the cells (Elliott et al., 1984). Unfortunately, the results of this work were complicated by the fact that chronic treatment with PDD can have an inhibitory effect on growth. Therefore, this relationship was again investigated with lower, non-chronic, levels of PDD and this time the treatment was found to stimulate cellular queuine uptake (Elliott et al., 1986). This effect was linked to the activation of PKC (Langgut et al., 1995, Morris et al., 1996) as treatment with epidermal growth factor (EGF) could produce similar effects as TPA (12-O-tetradecanoylphorbol-13-acetate) (another small molecule activator of PKC) treatment on tRNA modification (Langgut et al., 1996). By contrast, activators of cAMP and cGMP kinases were found to have no effect on QtRNA status (Morris et al., 1996).

The above results demonstrate that the activation is specific to PKC and is consistent with the fact that agonists and antagonists of PKC exert stimulatory and
inhibitory effects upon the rate of queuine uptake respectively. Interestingly, although PKC activation could stimulate queuine uptake, inhibitors of PKC were unable to prevent queuine influx into the cell and instead only slowed down the rate at which the cell reached saturating levels of queuine from two hours to six hours; a three fold reduction in uptake (Langgut et al., 1996). This result revealed that a basal (stimulus insensitive) uptake mechanism for queuine exists. Intriguingly, not only can PKC signalling positively affect queuine uptake but the activity of the queuine RNA transglycosylase itself can also be enhanced. Two studies have reported that treatment of cells with either PDD or TPA caused an increase in transglycosylase activity which was not dependent on an increase in RNA or protein synthesis (Langgut et al., 1995). This effect was verified to be dependent on PKC since treatment with phosphatases caused a decrease in queuine incorporation into cellular tRNA.

It is likely that the effect of PKC on QtRNA formation is a widely conserved mechanism since purified transglycosylase activity from rat liver can be directly phosphorylated by purified PKC, leading to increased activation and conversely, phosphatase treatment has the ability to inhibit the activation (Morris et al., 1995). The original studies with chronic treatment with tumour promoters helped discover a queuine salvage pathway since the depressive effects of PDD and TPA on queuine uptake in fibroblast cells was found to be ameliorated by a salvage activity which developed with increased passage number (Elliott et al., 1985). Although the work cited above served to outline various aspects of the queuine system in eukaryotes, it provided few clues as to the molecular identity of the participating proteins.

For this reason, our efforts to understand the eukaryotic system directed us to studies on the bacterial TGT enzyme in the expectation that a comparative analysis would help to determine the function of the eukaryotic TGT and Qvl enzymes. The crystal structure of the *Zymomonas mobilis* TGT and the kinetic data obtained from *E.coli* TGT mutants provided significant data on the functional residues and the reaction mechanism of the bacterial enzyme. The first reported structure of the *Z.mobilis* TGT demonstrated that the enzyme formed a \((\beta/\alpha)_8\) barrel structure whose carboxy-terminal helix comprised a zinc ion binding domain (Romier et al., 1996). The authors of the study deduced that the U\(_{33}\)G\(_{34}\)U\(_{35}\) sequence of the tRNA anticodon loop is recognised by the barrel portion of the protein while the phosphate backbone associates with the zinc binding domain, which has a conserved consensus
motif of CxCx2Cx29+H. Subsequent crystallisation of a covalently trapped reaction intermediate of Z. mobilis TGT with 9-deazaguanine provided definitive evidence that residues Asp 280 and Asp 102 are responsible for nucleophilic attack of the guanine nucleotide and deprotonation of Nγ respectively (Xie et al., 2003). This study helped highlight the importance of Asp 280, since the tRNA molecule was observed to be covalently attached to the TGT enzyme via the side chain of this amino acid. The crystal structure data also provided valuable information on the sequence determinants required for tRNA recognition. Earlier HPLC analysis had shown there were strong contacts made between the protein and the tRNA anti-codon stem-loop structure with bases 34 and 35 acting as identity elements (Mueller et al. 1995). This data could be confirmed from the crystal structure, but in addition, it was found that four of the seven nucleotides of the anticodon loop region were flipped out (Xie et al., 2003).

The work on the bacterial enzymes also provided suggestive evidence that TGT was capable of dimerization. When TGT was purified from E.coli, it was found to assume a trimeric quaternary structure (Garcia et al. 1993). It is possible that dimerization was misreported as trimerization due to the elongated nature of a crystallographic dimer causing it to appear as a higher molecular weight (Romier et al., 1996). This was later demonstrated to be a concentration dependent effect as TGT only oligomerized in solution at concentrations of 5mg/ml or above (Reuter et al. 1995). The residues responsible for oligomerization were thought to reside next to the active site of the protein as mutation of catalytic residues reduced the ability of E.coli TGT to oligomerize. Similarly, binding of tRNA substrate by the wild type E.coli enzyme abolished oligomeric formation (Reuter et al., 1995, Reuter et al., 1994). In fact, it was the crystal structure data from Z. mobilis which pinpointed the residue involved in formation of the dimer interface. The histidine at the end of the zinc binding motif had wrongly been assigned as a zinc ligand since mutation caused the TGT to lose activity. In fact, it was found to be necessary for tethering of the C-terminal subdomain to the rest of the protein which allowed the protein to adopt the correct tertiary structure. Furthermore, the side chain of the histidine residue forms a hydrogen bond with the oxygen of another TGT molecule (Romier et al., 1996). This dimer interface was seen to be in good agreement with other proteins of similar size. Furthermore, residues situated in the C and N-termini of the protein have been linked
to dimer formation. These residues are situated in the catalytic domain, Zn binding
domain and tRNA binding domain and have been demonstrated be conserved across
eukaryote and eubacterial species (Stengl et al., 2007).

The previous chapter provided data that in eukaryotes TGT protein localises
to the nucleus and cytoplasm whereas Qv1 co-purifies with the mitochondria. This
raises the question of why these homologous proteins should occupy non-overlapping
regions of the cell. Further investigation of the localization of both proteins was
deemed necessary for this reason and from the simple point of view that the cellular
address of a protein has a strong bearing on its function.

The typical mitochondrion contains between 1000-2000 types of protein
molecule at any one time, but less than one percent of these proteins are
mitochondrial in origin. The mitochondrial genome exhibits a vastly increased
mutation load compared to the nuclear genome. Over time, this has resulted in the
loss of genes from the mitochondrial genome and it is only those proteins which are
too hydrophobic to be imported from the cytosol which have remained in the reduced
mitochondrial genome (Spinazzola et al., 2009). In mammals, only thirteen proteins
are mitochondrial in origin although the mammalian mitochondrial genome encodes a
full set of twenty-two tRNAs meaning that they do not require the import of nuclear
encoded tRNA. Accordingly, the pathways responsible for mitochondrial protein
import have a high degree of sophistication which directs them to the four distinct
regions of the mitochondria—the matrix, the inner mitochondrial membrane (IMM),
the intermembrane space (IMS) and the outer mitochondrial membrane (OMM). The
mechanism to direct proteins to these different organellar compartments is dependent
on targeting elements found in the protein’s sequence and each region has its own
semi-dedicated import pathway (Figure 4.1) — the best characterized being the
classical import pathway or presequence pathway which typically directs proteins to
the matrix, with a smaller proportion ending up in the IMM or the IMS.
Figure 4.1 The protein import pathways of the mitochondria. All nuclear encoded mitochondrial proteins pass through the TOM complex (light blue) before following the route particular to their final destination. Those proteins bound for the matrix follow route 1, route 2 is for those proteins which will end up in the inner mitochondrial membrane, route 3 is for the outer mitochondrial membrane proteins, the proteins of the inter membrane space follow route 4 whereas mitochondrial encoded inner membrane proteins are directed there via route 5 (Endo et al., 2009).

The proteins which pass through the classical import pathway have their sequencing determinant within an amino-terminal cleavable pre-sequence. This amino terminal cleavable pre-sequence is between twenty and fifty amino acids long and contains a higher than average number of positive residues which enable it to adopt an amphipathic α-helix. For those proteins lacking a cleavable presequence but instead containing internal determinants specific for the IMM, there is the carrier precursor route. The mitochondrial intermembrane space assembly (MIA) complex is responsible for targeting proteins to the IMS and the sorting and assembly machinery (SAM) pathway is required for correct integration of proteins in the OMM. The SAM pathway processes β-barrel proteins which pass through the TOM40 complex into the IMS where they are guided to the SAM complex by small Tim protein chaperones.

All of these import pathways rely upon the translocase of the outer membrane (TOM) complexes (van der Laan et al., 2010, Milenkovic et al., 2007). There is also a translocase of the inner membrane (TIM) complexes but the subunits utilized in this translocase complex vary depending on the mitochondrial compartment the protein is
being directed to. The classical import pathway is currently the best characterized pathway as approximately 70% of mitochondrial targeted proteins contain the amino terminal presequence which funnels them through this pathway. These amino-terminal precursor proteins are initially bound by the Tom20 subunit of the TOM complex, a receptor which then acts to pass the protein through Tom40, the central pore subunit of the TOM complex. For those proteins with a tendency towards aggregation, the Tom20 receptor is assisted by Tom70, another receptor more typically associated with binding those proteins containing internal targeting sequences.

Once the precursor has been passed through Tom40, it will remain bound to the *trans* side of the subunit Tom22 until it is then passed to the TIM23 complex. This is a membrane potential dependent process with the proton motive force providing the directional force towards the matrix. The precursor protein is displaced from Tom22 through competitive binding with the positively charged Tim21. This frees the precursor to bind to the receptor complex of the Tim23 complex, which can act in a dual capacity to either further direct the precursor protein to the matrix where a mitochondrial precursor peptidase will cleave the amino terminal sequence or direct it to the IMM. This latter process is dependent on a membrane anchor sequence being adjacent and downstream of the amino terminal presequence. What distinguishes TIM23<sup>sort</sup> (the IMM directing complex) from TIM23<sup>motor</sup> (the matrix directing form) is the presence of the presequence translocase-associated motor (PAM) which contains Hsp70, capable of providing a driving force through the IMM by the binding and hydrolysis of ATP. Those proteins destined for the matrix require both a functioning membrane potential and sufficient ATP, whereas those which will become embedded in the IMM will not utilise ATP as PAM is not associated with the TIM23<sup>sort</sup> machinery. In fact, the TIM23<sup>sort</sup> complex has been found to associate with complexes III and IV of the electron transport chain in order to maintain the proton gradient which powers it. Instead, the hydrophobic anchor sequence will cause the processing of the precursor protein to stall where it will then be laterally released into the IMM.

While eukaryotes have retained only one step of the bacterial queuosine biosynthetic pathway—the insertion of queuine into tRNA (analogous to preQ1 incorporation into tRNA)—it would be wrong to assume that this step comprises the
full extent of the cellular queuine apparatus. In fact, it appears eukaryotes have, by necessity developed a queuine salvage activity not present in bacteria which is evidenced by the detection of queuine intermediates in animal cells (Gündüz et al., 1984) and algal species (Kirtland et al., 1988). As has been mentioned in Chapter 3, no research has ever been able to definitively establish that the eukaryotic TGT is responsible for the transglycosylase activity, despite the fact that TGT must be fulfilling a significant function which led to its retention within the proteome. It is likely that the role of TGT within the cell has developed or changed from that of its bacterial counterpart. This chapter will provide a comparison to the known protein domains of the bacterial TGT relative to the eukaryotic TGT and Qv1 proteins, provide additional data on their intracellular localisation and examine some of the functional aspects of the TGT enzyme.
4.2 Results

4.2.1 TGT and Qvl co-associate in vivo

Various kinetic studies on mutants of the E.coli TGT enzyme have helped to clarify the mechanism of the transglycosylase reaction. Briefly, the reaction relies upon the recognition and binding of the tRNA substrate by TGT, this being dependent on the correct sequence determinants located in the anticodon loop. The subsequent removal of the guanine base is a result of nucleophilic attack after which the queuine is inserted into the tRNA by the enzyme. The domains necessary for all the above steps have been identified. Therefore, the protein sequences of murine TGT and Qvl were aligned using Genedoc and the domains discussed above were highlighted (Figure 4.2). It is interesting to note that TGT and Qvl have retained the regions necessary for substrate and tRNA recognition; the asparagine residue responsible for nucleophilic attack of the phosphoribosyl bond connecting guanine to the tRNA and the cysteine rich motif responsible for correct protein-tRNA binding. Another noteworthy conservation is the C-terminal histidine zinc-binding motif as this is thought to be responsible for dimer formation of the bacterial enzymes, as mentioned in the introduction. It was decided therefore to examine the ability of the eukaryotic enzymes to form homo- or hetero-meric structures.

To carry out this work c-myc and HA tagged versions of the murine TGT and Qvl proteins were created and used in immunoprecipitation pull-down experiments. The expression vectors were transiently transfected into COS-7 cells (which were shown earlier to contain TGT and Qvl (Figure 3.6, panel a) and after a period of 48 hours, the total protein was extracted from the cells and run over an anti-HA antibody conjugated column. The eluate from the column was then probed with anti-HA and anti-myc antibodies. The protein extract which had not been run over the HA-pull-down column was also probed using the same antibodies to ensure that the transfected genes were being expressed. The experiment was also repeated with encoded TGT and Qvl labelled with both tags in a bid to ascertain if any self-association of the proteins occur. From these experiments, it was observed that TGT and Qvl associate with each other in vivo whereas Qvl only weakly self-associates and TGT is not capable of self-association (Figure 4.3).
4.2.2 TGT and Qvl are directed to the mitochondria

Although the previous immunoblotting experiments of purified cellular extracts (Chapter 3) had demonstrated that TGT and Qvl occupied different and non-overlapping areas of the cell, the co-immunoprecipitation experiments revealed a clear association between TGT and Qvl. In order to resolve this contradiction, it was decided to investigate the localization of the TGT and Qvl proteins using confocal analysis, where the proteins could be examined in situ, avoiding possible artefacts arising from protein isolation which could, for example, easily remove proteins only tentatively associated with cellular organelles.

Before embarking on this work, it was necessary to firmly establish that the antisera against TGT and Qvl were suitable for confocal analysis i.e. capable of recognising only the protein to which the anti-sera was raised against. The monkey kidney cell line, COS-7 cells were chosen for this analysis due to their large cell volume and good intracellular morphology which can be easily visualised. The cells were cultured to approximately 60-70% confluence before being probed with either pre-immune serum or antisera which had been pre-incubated with its cognate protein (rTGT or rQvl) (Figure 4.3, panel b). No signal was observed in either control demonstrating that, to the best of our knowledge, no off-target recognition of epitopes is occurring (Figure 4.4, panel a).

The localization pattern observed when COS-7 cells were probed with anti-TGT and anti-Qvl antisera was distinctive (Figure 4.5). In fact, the localization pattern for both proteins appeared to be very similar although Qvl was seen to be more definitively excluded from the nucleus. Importantly, both TGT and Qvl demonstrated a reticular pattern of localization within the cytosol that was clearly reminiscent of mitochondrial staining. This was verified further using Mitotracker Red, a dye that stains only actively respiring mitochondria as its fluorescence is dependent on the oxidation of its chloromethyl moiety. Co-localization of the Mitotracker dye—detected using the red channel—with the green of the Alexa-fluor 488 conjugated secondary antibody yielded an orange-yellow overlay. This result confirmed that substantial amounts of both TGT and Qvl are localised to the mitochondria (Figure 4.6).

The possibility that TGT and Qvl are located in other regions of the cell can not be ruled out based on our confocal data alone since the results are not quantitative.
and may only reflect a concentration of protein within or associated with an intracellular structure. However, coupled to the earlier immunoblotting results it can be stated that Qv1 is stability associated with the mitochondria. The results also seem to indicate that while TGT associates with the mitochondria in vivo, the handling necessary to make mitochondrial extracts causes TGT to be released from the organelle and therefore it does not form a stable interaction.

Given the above results, the gene sequences of TGT and Qv1 were examined for known mitochondrial targeting sequences using Mitoprot (Claro et al., 1996), a computer programme that examines the sequence for published consensus residues associated with the classical mitochondrial protein import pathway (Figure 4.7, panel a). An N-terminal region of Qv1 was found to contain just such a cleavable consensus sequence of 42 amino acids long. Mitoprot reported a 96.85 percentage probability that this peptide sequence was responsible for direction of the protein to the mitochondria. Proteins containing such a precursor sequence can be directed to the matrix or the IMS; targeting to the latter being dependent on an additional sequence determinant being adjacent to the cleavable precursor. This sequence determinant is usually referred to as a membrane anchor sequence and is composed of hydrophobic residues which will cause the Tim complex machinery to stall and the protein to be laterally released into the IMS. A search of the downstream region of the precursor region of Qv1 yielded no such residues so it was concluded that Qv1 did not contain the bipartite sequence determinants necessary for direction to the IMS but was instead a matrix protein.

The sequence of TGT was analysed using Mitoprot in a similar manner but the values reported indicated that the mechanism of its targeting to the mitochondria was independent of any amino terminal cleavable presequence (Figure 4.7, panel a). This excluded TGT from containing any upstream sequence determinants that would direct it to the mitochondria via the classical import pathway. This was confirmed through transient transfection of the myc-tagged mouse Qtrt1 gene into COS-7 cells. These cells were then probed using a monoclonal anti-myc antibody and visualized using confocal microscopy. This TGT also demonstrated mitochondrial staining when stained with Mitotracker Red indicating that the sequence determinants necessary for its direction to the mitochondria were embedded within the sequence itself (Figure 4.7, panel b).
4.2.3 TGT is associated with RNA *in vivo*

The activity of the queuine transglycosylase enzyme has been shown to be dependent on the phosphorylation status of the cell. To further investigate this, COS-7 cells were treated with the tumour-promoting agent (and PKC activator) TPA for 48 hours and the location and expression of TGT and Qv1 were investigated using confocal analysis of paraformaldehyde fixed cells and immunoblotting of cell fractions.

The Qv1 protein demonstrated no discernible difference in either expression or localization according to the confocal analysis. Cells were treated with 20 nM TPA for three and twenty-four hours in order to examine any immediate effects arising from stimulated signalling pathways, which would likely be a change in localization or an increase in protein expression which would require a longer period of time before being observable. In this regard, neither time-point had any effect on the localization of the Qv1 protein as visualized using confocal analysis. The Western blotting of cellular fractions demonstrated a similar result as regards expression of Qv1 with no demonstrable difference in expression seen.

Neither the expression nor the localization of the TGT protein was seen to be affected when examined using confocal microscopy (Figure 4.8). However, when protein fractions were electrophoresed and probed with anti-TGT antisera, two unexpected effects were observed. These appeared to be the formation of multiple bands on immunoblots (around the expected 60 kDa band) where before, a single band had been observed (Figure 4.9, panel a). It is possible that these weight changes indicate the presence of phosphorlyated forms of the protein, causing them to run marginally higher than they would normally. Furthermore, a higher molecular weight band was distinctly observable in the nuclear fraction where before this band was only weakly detectable in the cytosolic fraction. The size of this band was approximately 83 kDa, nearly 23 kDa larger than that of the TGT band. As stated earlier, during the transglycosylase reaction the bacterial TGT is known to form a covalent intermediate with its tRNA substrate. It is curious that the prominent band at 83 kDa corresponds in size to TGT bound to a tRNA molecule. In order to probe this possibility the nuclear fraction was treated with RNase A and immunoblotted as before (Figure 4.9, panel b). As stated earlier, the tRNA bound within the TGT intermediate complex is known to have four of its seven nucleotides within the anticodon loop flipped out, meaning that only three single stranded non-consecutive
nucleotides remain available for digestion by RNase A (Xie et al., 2003). For this reason, it was chosen to use an RNase A from Sigma, a preparation known to contain other RNases which are capable of attacking dsRNA. A total disappearance of the 83 kDa band was observed in the RNase treated fraction confirming that it is an RNA molecule. An increase in the detected amount of the 62 kDa band would be expected but is not observed, but this may be due to minor protease contamination of the RNase A. A control sample comprising a nuclear fraction treated with DNase demonstrated no diminution in the upper band (Figure 4.9, panel b). These results seem to indicate that the levels of TGT bound to tRNA increases in response to TPA treatment.

The next step was to determine whether this nuclear TGT was catalytically active. This was tested by treating nuclear fractions with TPA as before but with the addition of excess (millimolar amounts) of nucleotide substrate. If this nuclear TGT was capable of catalyzing queuine insertion into tRNA, treatment with queuine or guanine would be expected to produce a decrease in the size of the upper band. The control for this reaction was to incubate the nuclear fractions with the trapping substrate 9-deazaguanine. It was found that there was no difference between all four conditions; untreated, queuine, guanine and 9-deazaguanine. This result provides evidence that the nuclear localized TGT is capable of binding tRNA, but is not catalytically capable of inserting queuine into tRNA (Figure 4.9, panel c).

Although not investigated any further in this thesis, the results of this work together with recombinant studies carried out by Cólín Boland in our laboratory definitively showed that TGT and Qvl physically associate to reconstitute a queuine tRNA guanine transglycosylase complex. The results of this work can be found in the published paper included in the appendix of the thesis.
Figure 4.2 Protein alignment of mouse TGT and QTRTD1 with attention to residues vital for mutual association. The protein sequences were aligned using Genedoc (Nicholas et al., 1997). Those residues suspected to be involved in dimer formation are highlighted in red while those residues which comprise the cysteine rich motif which is integral to Zn binding are highlighted in blue. The crystal structure of the putative Z.mobilis TGT dimer is shown below the alignment (Stengl et al., 2007). The dimer interface is indicated in the black oval.
Figure 4.3 TGT and Qv1 demonstrate co-association. COS-7 cells were transiently transfected with constructs that express TGT with a C-terminal Myc (TGT-Myc) or HA epitope (TGT-HA) and with constructs that express Qv1 with an N-terminal HA epitope (HA-Qv1) or a C-terminal Myc epitope (Qv1-Myc), as indicated above each panel. Lysates were immunoprecipitated (IP) and immunoblotted (WB) using antibodies to Myc (α-Myc) or HA tag (α-HA). Total cell lysates western-blotted directly served as transfection controls (Input). Results presented are representative of three independent experiments.
Figure 4.4 Ascertainment of the suitability of the antisera against TGT and QTRTD1 for confocal analysis. (a) COS-7 cells were fixed using 4% paraformaldehyde. The left hand panels were treated with pre-immune sera taken from animals prior to injection with recombinant TGT or Qvl. The right hand panels were treated with antisera which had been pre-incubated with the cognate recombinant protein to prevent the relevant antibody idiotypes from recognizing the pertinent protein. (b) GST-tagged full length mouse TGT purified from E.coli BL21(DE3) tgr::Kmr cytosolic extracts by glutathione affinity chromatography. The SDS-PAGE gel shows uninduced cells before the addition of IPTG, induced pellet and supernatant after cell lysis and purified full length TGT after cleavage of the GST moiety with PreScission protease. (c) His-tagged mouse Qvl purified from E.coli BL21(DE3) tgr::Kmr cytosolic extracts by nickel-chelate chromatography. The SDS-PAGE gel shows uninduced cells before the addition of IPTG, induced pellet and supernatant after cell lysis and purified Qvl. (Coomassie stained blots provided by Cóilín Boland).
Figure 4.5 Confocal Analysis of the Cellular Location of TGT and QTRTD1. COS-7 cells were fixed in 4% paraformaldehyde. Cells were then probed with either anti-TGT (A-C) or anti-QTRTD1 (D-F) antisera, before being stained with the nuclear specific dye DAPI (B, E). A merge of antisera and DAPI is shown (C, F). Scale bar represents 20 μm. Results presented are representative of three independent experiments.
Figure 4.6 Demonstration of mitochondrial localization of TGT and QTRTD1. COS-7 cells were stained with 50 nM Mitotracker Red CMXRos dye for 15 minutes at 37°C (B, E) before being fixed with 4% paraformaldehyde and probed with either anti-TGT(D) or anti-QTRTD1(E) antisera. Panels C and F demonstrate a merge of the above. Scale bar is 20 μm. Results presented are representative of three independent experiments.
Figure 4.7 Investigation into the means by which TGT and QTRTD1 are directed to the mitochondria. (a) The protein sequences of Qvl (including an additional N-terminal region) and TGT were analysed using Mitoprot (Claros et al., 1996). (b) COS-7 cells were transiently transfected with constructs expressing a C-terminal Myc tag (A,B) and stained with Mitotracker Red CMXRos (C). Panels D shows a merge of the transfected TGT (green) with Mitotracker Red. Scale bar is 20 μm. Results presented are representative of three independent experiments.
Figure 4.8 The increased transglycosylase activity coincident with increased phosphorylation through the PKC pathway is independent on a change of localization of the TGT protein. COS-7 cells were grown in FBS containing medium and treated with 20 nM TPA for times indicated. Cells were stained with Mitotracker Red (sub-panels C,G and K) before being fixed, probed with anti-TGT antisera (B,F and J) and stained with DAPI (A,E and I). Sub-panels D, H and L demonstrate a merge of the above. Results presented are representative of three independent experiments.
Figure 4.9 Increased transglycosylase activity coincides with the appearance of a higher molecular weight form of TGT in the cytosol. (a) Cellular fractions were prepared according to manufacturer’s instructions (Pierce) as discussed in text. The top immunoblot shows those fractions prepared without phosphatase inhibitors. The blot on the bottom was loaded with fractions prepared with phosphatase inhibitors. (b) Cytoplasmic (C) and nuclear (N) fractions were prepared and the nuclear fraction treated with 6 μl of either DNase or RNase for 15 minutes at 25°C. (c). Cytoplasmic (C) and nuclear (N) fractions were prepared and the nuclear extracts were pre-incubated with 50 μM queuine, guanine and 9-deazaguanine for 15 minutes at 25°C. Results presented are representative of three independent experiments.
4.3 Discussion

A definitive function for either TGT or QTRTD1, beyond their involvement in queuine modification of tRNA, has never been shown. Therefore, the differing and non-overlapping cellular addresses of both proteins seen in Chapter 3 gave rise to a number of possibilities; that the proteins function independently in the cell, that they have roles unrelated to queuine modification, or that both proteins are involved in tRNA queuine modification but in different cellular locations.

The focus of this chapter was to resolve these possibilities and further characterize these proteins beginning with a direct sequence comparison of these proteins with the bacterial TGT. An alignment of the protein sequences of murine TGT and Qvl was informative in that both proteins were found to retain the regions necessary for recognition of the queuine and tRNA substrate and those catalytic domains which are responsible for inserting the former into the latter. Interestingly, two overlapping motifs designated as being involved in formation of a dimer interface were also seen to be conserved, indicating the TGT and Qvl proteins could potentially interact. However, this analysis presented no information regarding the roles of either protein in the cellular queuine-centric machinery.

The suggestion that these proteins contain regions that enable them to oligomerize was of interest, particularly as both proteins are invariably found together in all eukaryotes—a likely indication that their function is interdependent. Our immunoprecipitation experiments confirmed this assumption and showed a clear interaction between TGT and Qvl and that Qvl is capable of weakly interacting with itself. This finding did not correlate with the immunoblot results of the previous chapter where TGT and Qvl were seen to occupy non-overlapping regions of the cell. This is likely an artefact from preparation of the cellular fractions which were prepared through the use of proprietary reagents from Pierce. Isolation of the total protein using M-Per reagent requires lysis and centrifugation to remove cellular debris. The preparation of the mitochondrial fractions requires lysis of the cells using an unspecified detergent likely to be digitonin before multiple centrifugations at high speeds which incorporate wash steps. This indicates that the interaction between the two proteins is weak, a fact which has been backed up by gel filtration analysis carried out in lab (Boland et al., 2009) (Appendix Figure 1).
However, to clarify this issue the localization of both proteins was investigated using confocal immunofluorescence, for which the antisera against both proteins was found to be suitable. This method avoids the handling inherent to cellular fractionation. By confocal analysis, both TGT and Qvl were found to be associated with the mitochondria. However it cannot be said definitively that both these proteins are localized within the mitochondria as this is beyond the resolution of confocal microscopy. It may be that either or both of these proteins are associated with the mitochondria instead of inside it. The use of FRET (fluorescent resonance energy transfer) could be used to determine whether the TGT and Qvl proteins are truly mitochondrially localized. However, it can be inferred from the immunoblot and confocal studies that Qvl occupies a region of the mitochondria that remains intact throughout preparation of cellular fractions whereas TGT does not. The conclusion drawn from this result was that Qvl is a matrix or IMM protein whereas TGT is most likely associated with the OMM. Further recourse to sequence analysis was helpful in this regard as Qvl was observed to contain an amino-terminal presequence characteristic of mitochondrial directed proteins. The absence of a membrane anchor sequence meant that Qvl is likely directed to the mitochondrial matrix. The sequence of the TGT protein was then analysed in a similar manner but found not to contain a cleavable presequence signal. To ensure its localization was not under the control of further upstream elements which had not been included in the signalling determinants analysis, the expression of a transiently transfected TGT was investigated using confocal immunofluorescence. This TGT was also directed to the mitochondria, most probably through residues found with the protein coding sequence. However, the staining was not as intense as observed with the endogenous TGT. Only those cells with healthy mitochondria will demonstrate staining with the Mitotracker Red dye. It is possible that overexpression of the TGT protein has a detrimental affect on mitochondrial function making it more difficult to observe co-association of the transfected protein with this organelle. The likely mitochondrial sub-compartment candidates remaining were the IMS and the OMM. TGT was unlikely to be an IMS protein as these are typically small and carry a Cys-X$_3$-Cys or Cys-X$_9$-Cys motif (Milenkovic et al., 2007). Proteins which are directed to the OMM through the TOM complex are β-barrel containing proteins. Alternatively, a subset of OMM localized proteins are directed there independent of the TOM complex, but like their TOM
localized counterparts, these proteins also contain regions of hydrophobicity long enough to act as transmembrane segments, in fact the characteristics of all OMM proteins are similar enough to render a discussion of their targeting immaterial. In fact, what is important is that the functional amino terminal regions of those proteins not directed to the OMM via the TOM complex protrude into the cytosol (Setoguchi et al., 2006). Unfortunately, there is presently not enough data to fully ascertain the manner by which TGT is directed to the mitochondria and no means to explore the implications for the orientation that it adopts for its physiological function.

The transglycosylase activity has frequently been reported to be sensitive to phosphorylation and so, the response of TGT and Qvl expression and localization to increased global phosphorylation was investigated. This was done using TPA, a chemical that mimics the structure of DAG, a second messenger in PKC signalling. Neither TGT nor Qvl demonstrated any change in their cellular expression or localization when treated with this compound. However, Western blots probed with anti-TGT demonstrated bands indicative of phosphorylation sites within the protein. Furthermore, a high molecular weight band which was only weakly detectable in the cytoplasm under normal circumstances (i.e. non-stimulated) was observed in the nuclear fraction when it was probed with the anti-TGT antisera. The size of this band corresponded to a tRNA bound TGT molecule and treatment with RNase A caused its disappearance confirming the presence of an associated RNA molecule. The nuclear fraction of TGT, which importantly contains no Qvl, possessed no catalytic activity as treatment of the fraction with an excess of substrate caused no change in the appearance of the band. All of the above was interesting as it was previously demonstrated during the isolation of the transglycosylase activity that two bands were visible, an upper and a lower band, the latter being sensitive to treatment with PKC or phosphatase inhibitors while the former demonstrated no such effect (Morris et al., 1996).

In conjunction with Col lain Boland, a former student in our laboratory, it was verified that TGT and Qvl must physically associate to generate a queuine tRNA transglycosylase enzyme (Boland et al., 2009). From these results and those presented in Chapter 3, a model of TGT and Qvl functioning could be constructed whereby Qvl is found principally at the mitochondria and TGT is cytosolic and nuclear in location (Figure 4.5). Since, by itself, TGT has no catalytic activity it may be have a
chaperone function whereby newly synthesised tRNA is bound to the protein and directed to the mitochondria to be modified by a transient TGT-Qv1 complex. This is borne out by the fact that both TGT and Qv1 demonstrate a strong association in vivo. As other studies have shown TGT is sensitive to increased signalling through the PKC pathway, and we have found increased RNA bound in the nucleus, it is possible that increased phosphorylation causes TGT to bind more tRNA.

The necessity for queuine modification of mitochondrial tRNA could potentially answer questions regarding the purpose of queuine insertion. After all, the importance of the mitochondria to the correct overall functioning of the cell cannot be overstated. The mammalian mitochondrial genome produces the tRNAs necessary for translation of the thirteen proteins contained in its genome. The mitochondrial aspartyl tRNA has been found to be modified by queuine from direct sequencing experiments (Randerath et al., 1984). There are few reported instances of mitochondrial tRNAs of higher eukaryotes being exported to the cytoplasm. The mitochondrial tRNA^Met of rat cells has been found to be associated with a purely cytosolic protein Ago2 (Maniataki et al., 2005). However it seems unlikely that those tRNAs subject to queuine modification are being exported to the cytosol for modification before being re-imported, especially as it can now be seen that both TGT and Qv1 have a mitochondrial address. The relevance of queuine to mitochondrial function is certainly worthy of further investigation.
Chapter 5
The link between Queuine and Metabolism in vitro

5.1 Introduction
Recently, a great deal of research has been directed towards understanding how a typical cancer cell perverts its own metabolic flux to feed its increased biosynthetic and bioenergetic needs (deBerardinis et al., 2008). The increased biosynthetic demand arises in part from the increased proliferation that is characteristic of cancer cells. The two principle substrates that cancer cells exploit to satisfy their metabolic demand are glucose and glutamine. It has been well established that cancer cells show increased glucose consumption in the presence of oxygen leading to the production of lactic acid, commonly referred to as aerobic glycolysis or the Warburg effect. However, this increased consumption of glucose is reliant on the replenishment of TCA cycle intermediates through glutamine metabolism, a process called anaplerosis. In fact, cancer cells demonstrate a massively increased demand for glutamine to the extent that they are commonly referred to as having a ‘glutamine addiction’.

It is a misconception that the mitochondria of cancer cells become vestigial organelles. While the contribution of oxidative phosphorylation towards meeting the bioenergetic requirements of the cell becomes smaller, the TCA Cycle, though changed, remains just as necessary to the proper functioning of the cell. The TCA cycle acts as a reservoir of metabolic intermediates which can be shunted into those metabolic pathways that have been up-regulated to cope with the increased demands that the cancer phenotype imposes on them. For example, increased nucleotide biosynthesis is necessary for the doubling of the cellular DNA and de novo synthesis of fatty acids for the production of lipid membranes (Ookhtens et al., 1984).

One of the hallmarks of the Warburg effect is an increased production of lactate, one of the possible terminal points of the glycolytic pathway. This is important as it means that the pyruvate produced from glycolysis is not being converted to acetyl-CoA and, as a consequence, not entering into the TCA cycle. The manner of functioning which the TCA cycle has adapted in the cancer cell has led to it being referred to as a truncated TCA cycle. Glutamine is deamidated to glutamate
before entering a TCA cycle whose primary focus is the production of citrate and malate. The citrate produced is exported to the cytoplasm where it is converted to fatty acids through the action of the ATP-citrate lyase and fatty acid synthase. This series of reactions also serves the dual purpose of removing citrate from the cytoplasm where it would inhibit phosphofructokinase, a key enzyme in glycolysis (Yalcin et al., 2009). Like citrate, malate is exported to the cytoplasm where malic enzyme converts it to lactate and in the process produces the NADPH necessary for the synthesis of lipids and DNA. This pathway is believed to be the primary source of the reducing equivalent NADPH in cancer cells (deBerardinis et al., 2007). While oxidative phosphorylation within the cancer cell is down-regulated, it cannot be turned off completely as it is required to form the mitochondrial membrane potential. This potential is responsible for the import of substrates into the mitochondria, the continued importance of which has already been discussed.

The link between queuine deficiency and cancer has remained an unexplained curiosity. Decreased Q-tRNA status in various cancer types including lung cancer, leukaemia and lymphoma is a poor prognostic marker and correlates with the histopathological and the malignancy grade of the tumour (Huang et al., 1992, Emmerich et al., 1985). This observation lead to the Q-tRNA status being associated with aberrant signalling pathways in cancer, enhanced cellular proliferation and metabolic changes concomitant with the altered capacity of cancer cells for oxidative phosphorylation (Langgut et al., 1995, Langgut et al., 1993, Reisser et al., 1994).

Interestingly, the guanine analogue 7-methylguanine (7-meG) was found to act as a tumour promoter in a two-stage initiation-promoter experiment, most notably increasing the incidence of anchorage independent growth (Muralidhar et al., 1987, Muralidhar et al., 1989). The only biochemical effect which treatment of 7-meG was found to have upon cells was to cause Q-tRNA hypomodification, thereby linking the absence of queuine from the anticodon loop of tRNA with increased propensity for transformation. Another way in which queuine and its presence in tRNA was linked to the switch between proliferation and differentiation was a study which sought to examine the effects of the guanine analogs 6-thioguanine and 8-azaguanine on the differentiation on the promyelocytic HL-60 cell line (Kretz et al., 1987). This particular cell line lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) which would ordinarily metabolize the 6-TG to cytotoxic nucleotides. In the
absence of this pathway, the 6-TG was instead found to be incorporated into tRNA with the effect of causing an induction of differentiation. As queuine is irreversibly incorporated into tRNA, concurrent treatment of these HPRT<sup>+</sup> HL-60 cells with 6-TG and queuine (even at a concentration nearly a 1000-fold less than that of the 6-TG) resulted in a reversal of differentiation and an inhibition of growth. It was later shown that the granulocytic differentiation resulting from 6-TG treatment was irreversible except when the cells were treated concurrently with queuine (French et al., 1991).

This research was restricted to examining the effects of the absence of queuine using the analogues mentioned above. However, the group of Helga Kersten used chemically synthesized queuine and were able to examine the direct effects of its presence or absence independently of the complicating factors inherent to the use of the analogues. Addition of 300 nM queuine, the reported physiological concentration found in serum, to the growth medium of HeLa S3 cells was observed to cause an approximately two-fold increase in proliferation (Langgut et al., 1990). A more extensive study from the same group found that the proliferation of primary cells responds positively to queuine supplementation, whereas the growth of a range of transformed cell lines was found to exhibit less consistent results, i.e. sometimes queuine enhanced growth whereas in other cases growth was inhibited (Langgut et al., 1994). For instance, the NIH-3T3 cell line had been shown to demonstrate a positive correlation to queuine supplementation but when it was transformed with one of the discrete oncogenes ras, erb or raf, the effect of queuine on proliferation was reversed or disappeared in an oncogene specific manner. From this, the conclusion was that the effect of queuine upon proliferation is not a universal phenomenon and is dependent on the oncogenic insult carried by the transformed tissue.

The more interesting result from this study was the differential effects of queuine on proliferation in response to atmospheric culture conditions. HeLa cells cultured in the absence of queuine were demonstrated to exhibit no difference in proliferation under either aerobic or hypoxic culture conditions. However, with the addition of queuine, proliferation was stimulated in aerobic conditions but decreased under hypoxic conditions. This observed sensitivity of the effects of queuine to oxygen concentration is interesting as numerous sources have reported that the
transglycosylase activity is dependent on oxygen (Szabo et al., 1988, Langgut et al., 1993).

These results indicated that queuine was involved in the unique metabolism favoured by cancer cells, a metabolism maintained through aberrant signaling from those oncogenes so often mutated in tumourous tissues. This seems even more likely when the effects of queuine upon the activity and expression of lactate dehydrogenase (LDH) are considered. Cancer cells typically have increased lactate dehydrogenase activity which is apparent in the preferential oxidation of pyruvate to lactate as opposed to acetyl-CoA. It is has repeatedly been demonstrated that queuine can cause a suppression of the expression levels of those LDH isoforms associated with a more transformed phenotype (Reisser et al., 1993, Reisser et al., 1994). The LDH activity reported to be increased in Dalton’s Lymphoma transplanted mice (DLAT) decreased in response to dietary supplementation with queuine (Pathak et al., 2005, Pathak et al., 2008). This link to LDH activity is perhaps the most convincing of all the data linking queuine to cancer metabolism as it has also been observed in the amoeba species Dictyostelium discoideum (Schachner et al., 1984).

The purpose of this chapter was to investigate more fully the function of queuine, especially with regard to its effects upon the unique metabolism of the cancer cell.
5.2 Results

5.2.1 The proliferation of primary and transformed cells is unaffected by queuine

Helga Kersten’s group was responsible for the data showing queuine’s effect on cellular proliferation. This research was extensive in that it attempted to examine the effect in a variety of cell lines, including both primary and transformed cell lines (Langgut et al., 1994). The work produced in the Kersten lab led them to conclude that queuine had a definite effect on proliferation which was dependent on the predilection of the cell towards aerobic glycolysis. Specifically, the growth of primary cells, which would be expected to exhibit a dual metabolism incorporating the bioenergetic pathways oxidative phosphorylation and glycolysis were seen to respond positively to queuine supplementation. However, those transformed cells whose oncogenic fault led to erroneous signalling through the PI3K/Akt pathway responded in the converse manner to the addition of queuine to the medium (i.e. a decrease in proliferation). However, closer inspection of these results revealed that in many of the cell lines examined, the proliferation was not examined beyond three days in culture and any difference exhibited extended at most to a two-fold difference and in many cases less.

For the purpose of our studies we chose the parental HeLa cell line (note: these are not the S3 subtype) and activated primary T-cells from mouse spleen for further studies on queuine’s effect on proliferation. These cell types are representative of transformed and primary cell lines. The HeLa cell line, derived from a cervical carcinoma, has been used for many studies into queuine and its function, not least of all the research into the proliferation and the activity of the lactate dehydrogenase enzyme. Furthermore, the HeLa cell line was one of the first cell lines to be cultured on a continuous basis within a lab setting and its bioenergetics and metabolism are very well understood. Likewise, the proliferative and metabolic control of T-cells is very well studied. Proliferation can be induced rapidly by the addition of interleukin-2 (IL-2) and an activatory anti-CD3 antibody. This is especially important as the metabolism of these cells at this stage resembles that of nascent tissue. It must be remembered that embryonic and neonatal tissues are hypomodified with respect to QtRNA and their metabolism, in many respects, is similar to that of cancer cells.
To examine the effect of queuine on HeLa cell proliferation, five cultures of both queuine containing and queuine lacking cultures were set up in triplicate. The cells were seeded at a density of $5 \times 10^3$ cells per cm$^2$. At day 0, 300 nM queuine was added to half of these cultures and on each day from Day 1 onwards. Three of the queuine containing and queuine lacking cultures was counted using a haemocytometer. The seeding density chosen allowed the cells to be cultured for five days without reaching 100% confluency. When the results were plotted, it could be seen over the five days of growth that the effect of queuine was minimal. A transient increase in proliferation could be discerned at days 3 and 4 but the difference had disappeared by day 5 (Figure 5.1, panel b).

5.2.2 Queuine is observed to affect the metabolism of both primary and transformed cells

The data linking queuine to effects on metabolism have focused on the differential response of cells to oxygen limitation with or without queuine. This included the positive effect queuine was found to have on mitochondrial electron flow in HeLa cells, as determined using an MTT assay (Langgut et al., 1994). This positive correlation was abolished when the cells were cultured under hypoxic atmospheric conditions. Furthermore, it has been repeatedly demonstrated both in vitro and in vivo that the absence of queuine causes an increase in the expression and activity of LDH isoforms associated with a glycolytic phenotype, and that this change is entirely reversible upon supplementation with queuine (Langgut et al., 1993, Pathak et al., 2005). From this, it can be concluded that queuine may be involved in modulating the glycolytic flux in the cell.

HeLa cells were cultured for a period of 48 hours in serum free medium under aerobic conditions and 300 nM queuine. It has been demonstrated that this length of time is sufficient for approximately 80% of the tRNA to be modified by queuine (Langgut et al., 1995). A protein lysate was obtained from these cells and the LDH activity quantified by measuring the oxidation of NADH. The addition of queuine to these cells caused the LDH activity to decrease to approximately 75% of the activity present in the cells cultured without queuine (Figure 5.2, panel a). This result is in agreement with the literature.
An increase in the cellular LDH activity is most commonly associated with the Warburg effect. The Warburg effect does not occur in isolation and the typical cancer cell will demonstrate other aberrations of metabolism, as detailed in section 5.1. For this reason, an investigation into the sensitivity of significant metabolites to queueine in the cancer cell would be informative. The metabolites chosen for further examination were glucose, lactate, glutamate, glutamine and ammonia. The Nova Bioprofiler 400 Analyzer is capable of detecting and quantitating all these metabolites in the cell culture medium using enzyme based potentiometric sensors. Glucose and lactate were chosen for obvious reasons, as both of these metabolites book-end glycolysis or more specifically aerobic glycolysis. A correlation between these two levels would be extremely informative; increased glucose consumption without a concomitant increase in the level of lactate production would mean that the increased carbon from the glucose was instead finding its way into the TCA Cycle via acetyl-CoA. The levels of the glutamine, glutamate and ammonia were a way of investigating the metabolism of these cells from the other side of the TCA cycle. Much of the glutamine consumed by the cell is not destined for protein production but instead is instrumental in allowing the cell to meet the increased demand for anaplerosis and NADPH cofactor (DeBerardinis et al., 2008).

The medium was processed as described in section 2.11 for both HeLa and T-cells. Both cell types demonstrated changes in the amounts of these metabolites remaining in the cell culture medium, and more importantly the changes had an identical pattern; an increased consumption of glucose and glutamine and an increased production of lactate and ammonia (panels b, c). In the case of the T-cells (panel b), the differences were more pronounced, perhaps a result of the greater metabolic demands imposed by the cytokine driven proliferative push. A further difference which was not apparent in HeLa cells (panel c) was increased levels of glutamine consumed in those T-cells lacking queueine.

The above data indicate that in the absence of queueine, cells more readily adopted a metabolism favouring increased biosynthesis while also meeting the bioenergetic requirements of the cell, a type of metabolism most commonly found in tumour tissue or rapidly proliferating cells. However, this type of metabolism typically coincides with a down-regulation of the mitochondrially localized metabolic pathway, oxidative phosphorylation. This, coupled with the fact that mitochondrial
tRNA is found to be modified for queuine, led to speculation that the absence of queuine could potentially have a deleterious effect upon mitochondrial function thereby necessitating the switch to glycolysis. This putative down-regulation in oxidative phosphorylation could be a result of changes in mitochondrial number of morphology, defects in the individual complexes comprising the electron transport chain or indeed a compound effect of the above.

5.2.3 The Effect of Queuine Deficiency on Mitochondrial Number and Morphology

The qualitative of mitochondrial number was ascertained using protein and DNA based methods. The citrate synthase protein is a mitochondrial matrix protein and a clear linear relationship has been demonstrated to exist between the amount of this protein and the number of mitochondria (Trounce et al., 1996). The activity of this protein was measured indirectly using a coupled spectrophotometric assay where the production of reduced acetyl-CoA provided a correlation to the amount of the enzyme present. The cytochrome c oxidase activity was also measured and normalised against the citrate synthase activity. The ratio of both these activities to each other provided an index against which the size of the mitochondria relative to the activity of its electron chain could be compared between queuine containing and queuine deficient cells. Both these assays used protein lysates from cells cultured for 48 hours in the presence or absence of queuine. No significant queuine dependent difference in either of the activities of these two proteins was detected, indicating that the mitochondrial number and the proportion of the mitochondria dedicated towards oxidative phosphorylation underwent no queuine dependent change (Figure 5.3, panel a).

The lack of variation between queuine and queuine lacking cells with regard to mitochondrial number was also confirmed using real-time PCR methods. Each mitochondrion contains its own genome so while a cell will contain only one copy of each nuclear gene, the number of copies of those genes of the mitochondrial genome reflects the number of mitochondria present in a cell. Cells were grown in culture as described above and the genomic DNA, both nuclear and mitochondrial isolated using phenol/chloroform extraction. This DNA was amplified using primers designed against two genes—both of which encoded proteins involved in mitochondrial function; one from the nuclear genome, Ndufv1 and the other from the mitochondrial
genome, *Cox1*. Both primers were designed to yield amplification products of 176 bp and 189 bp respectively. Visualization of the amplification products on a 1.5% agarose gel gave products of the expected size that were free of non-specific bands (Figure 5.3, panel b). Overlaying the amplification curves of both genes shows there is no difference in the mitochondrial gene copy numbers in cells lacking queuine relative to queuine sufficient cells (panel b). This can be readily demonstrated by comparing the ratio of nuclear to mitochondrial gene copies (panel b).

The morphology of cells grown with and without queuine was examined using electron microscopy (Figure 5.4). Samples were stained, fixed, dehydrated and embedded in resin before sections were cut and stained with uranyl acetate and lead citrate. The sections were viewed at 100 kV under a Jeol 2100 transmission electron microscope. From this preliminary analysis, the number and size of cristae appeared to be lower in queuine deficient cells relative to controls whereas the volume and number of mitochondria was deemed to be similar regardless of the presence of queuine. It should be stressed that at present this result is only subjective and will require further analysis of numerous electron micrographs to be quantitatively valid.

This work is currently underway.

### 5.2.4 Queuine has no effect on Mitochondrial Function

The mitochondrial function of those cells lacking queuine was compared to their queuine containing counterparts by using the oxygen consumption of the cells as a measure of oxidative metabolism. An Oroboros Oxygraph™ was used for this purpose since it is far more sensitive than the classic Clark electrode oxygraph. It also has the added benefit of providing simultaneous calculation of the respiratory rate.

The overall function of the electrode transport chain was investigated through the use of oligomycin and serial titrations of the ionophore FCCP, which acts as an uncoupler of mitochondrial respiration. Cells were placed in a basal medium containing abundant phosphate but no other energy substrate. As the cells were whole, they would contain their own internal supply of energy substrates and the observed oxygen consumption was referred to as the basal level of respiration. The addition of oligomycin inhibits the ATP synthase complex and the rate of consumption is seen to drop to what is referred to as the leak rate of respiration as oligomycin prevents the proton-motive force from being channelled through the ATP
However all mitochondria, no matter how well coupled and efficient lose a small percentage of their proton-motive gradient through their membrane. The most well known example of this is the UCP1 protein, which generates heat through loss of the $\Delta p$ (mitochondrial protomotive force). Therefore, the rate of leak respiration provides a good indication of the 'leakiness' of the membrane with regards to the proton gradient. The titration of FCCP at non-inhibitory concentrations causes the electron transport chain to proceed at full capacity as it attempts to maintain the rapidly dissipating proton-motive force. Separately, all these values provide a good indication of the overall working order of the electron transport chain and the ratio of the full capacity of the chain to the leak rate, the RCR value, provides a measure of how coupled the electron transport chain is to the ATP synthase.

From the traces obtained, it was possible to see that queuine had no statistically significant effect on either the basal or leak rate (Figure 5.5, panel a). Similarly, when the electron transport chain was forced to work at maximum capacity no significant differences could be observed in the cells grown in the presence or absence of queuine. Both queuine containing and queuine lacking cells had RCR values which, when compared to the literature values indicated that the methods of collecting and processing the cells was not damaging the mitochondria (Lartigue et al., 2009). Mitochondria which have suffered damage would typically be expected to have an RCR value of 4 or lower.

The individual electron carrying complexes of the electron transport chain were examined sequentially by measuring the sensitivity of the oxygen consumption to various substrates and inhibitors. This requires the permeabilisation of the cells using digitonin which replaces membrane cholesterol and so specifically targets the plasma membrane and not the mitochondrial outer membrane. This allows the addition of substrate to the intact and undamaged mitochondria while removing the barrier of the plasma membrane. The oxygen consumption after addition of complex specific substrates followed by an inhibitor to abolish the contribution of that particular complex to the respiratory rate was used to obtain an indication of the capacity of each individual complex within the electron transport chain (Figure 5.5, panel b). Complex I was stimulated with the NADH-linked substrates glutamate and malate and provided with an excess of ADP before being cancelled using rotenone. The FADH-linked substrate succinate fed complex II driven oxygen consumption
before it too was abolished using malonate. Complex III was targeted using glycerol-3-phosphate and then inhibited with antimycin A. The oxygen consumption of Complex IV was dependent on the addition of ascorbate and TMPD, the latter an artificial electron donor which can feed electrons directly to cytochrome c from ascorbate. However, this can result in autoxidation which is tempered by the addition of potassium cyanide, the remaining oxygen consumption being the non-specific oxygen consumption within the cell and any autoxidation. From the traces obtained it could be clearly seen that queuine has no statistically significant effect upon any particular part of the electron transport chain.

Lastly, the P/O ratio for NADH and FADH linked compounds was ascertained. The P/O ratio is a measure of the stoichiometric efficiency of oxidative phosphorylation or more simply, it allows the relationship between the electron transport chain and the ATP synthase to be quantitatively determined. This was determined by charting the change in oxygen consumption in permeabilised cells. The electron transport chain is modulated by substrate availability and this can be observed by the change in oxygen consumption under conditions of limited substrate supply. Those mitochondria which have access to an excess of phosphate, electron donor and ADP are said to respire at State 3. However, as the pool of ADP is converted to ATP, the oxygen consumption drops as the mitochondria enter State 4 respiration. The point at which State 3 degrades to State 4 is the point at which the ADP supply has become limiting and it is the difference in oxygen consumption between the addition of ADP and its consumption to ATP that is used to calculate the P/O ratio. However the demarcation between these two states is difficult to pinpoint in permeabilised cells without the addition of an inhibitor of adenylate kinase, P1,P5-di(adenosine-5’) pentaphosphate (Ap5A) which will prevent interconversion of the two nucleotides. For both NADH and FADH linked substrates, no difference in P/O ratio was observed between either queuine containing or queuine lacking cells (Figure 5.6). However, the rate of oxygen consumption was higher in queuine containing cells when they were fed glutamate and malate, NADH linked substrates, as sources of reducing equivalents, whereas there was no observable difference in the rate of oxygen consumption when the only source of electrons was FADH, a complex II specific substrate.
As a final test of the functioning of the electron transport chain, the production of reactive oxygen species (ROS) was determined. A mitochondrion which is malfunctioning is likely to produce higher levels of reactive oxygen species. Indeed, it has been reported that murine fibroblasts grown under queuine deficient conditions contain half the levels of SOD of their queuine cultured counterparts, an enzyme instrumental in mopping up these dangerous radicals. The measurement of ROS was assayed using 2',7'-di-chlorofluorescein acetate (H$_2$DCFDA), a reagent which fluoresces once oxidised. HeLa cells were cultured for 48 hours before cells were treated with the dye, harvested and analysed using flow cytometry (Figure 5.7, panel a). There was no difference in levels of superoxide radicals as detected using this dye.

The membrane potential of the cell is important not only for its role in providing a proton-motive force for production of ATP through ATP synthase but is necessary for import of proteins and substrate into the mitochondria and has a role in apoptotic signalling in the cell. The small difference observed with regard to the functioning of ATP synthase and the fact this was only apparent with NADH linked substrates rather than FADH linked substrates was significant. The transport of glutamate into the mitochondria is reliant on a functional membrane potential. The membrane potential was measured using the radiolabelled cation methyltriphenylphosphonium bromide (TPMP$^+$). The uptake of this cation increases logarithmically for every 61.5 mV of membrane potential. So, while the large surface area of this lipophilic molecule ensures its passage through lipid membranes, its uptake increases up to a ten fold in the cytoplasm, according to the plasma membrane potential and over 100 fold in the mitochondria (Murphy., 2008). This makes it ideal for providing accurate quantitation of mitochondrial membrane potential. Cells were cultured as detailed before and the accumulation ratio of the TPMP calculated as detailed in Section 2.15. There was no difference observed in the membrane potential that was dependent on queuine (Figure 5.6, panel b). It was also observed that the membrane potential of both queuine containing and queuine lacking cells responded in a similar manner to the addition of oligomycin, which would cause the membrane potential to increase, reflected in an increase in the calculated TPMP accumulation ratio.
The effects of Queuine are dependent on substrate supply

In the absence of queuine, cells consumed more glucose and glutamine and produced more lactate, without any observable deleterious effect on mitochondrial function. The presence of abundant glucose (20 mM) in the culture medium would provide the cell with a substrate supply buffer and potentially mask effects which would be apparent in vivo where glucose would be limiting. HeLa cells grown in culture medium in which the glucose has been substituted for galactose have a higher respiratory rate, as galactose cannot be metabolised through the glycolytic pathway as efficiently as glucose (Rossignol et al., 2004), although it can be used for nucleotide biosynthesis.

HeLa cells were adapted to growth in a specialised serum free medium containing galactose in place of glucose. The proliferation of these cells was not observed to change in response to the addition of queuine to the growth medium. Similarly, the difference in the metabolic trend of glutamine, glutamate and ammonia between queuine supplemented and non queuine supplemented cells was as observed in those cells grown in 20 mM glucose. The oxygen consumption of these cells was increased, indicating an increase in oxidative phosphorylation but addition of queuine had no effect on respiratory rate (Figure 5.8, panel b). The same was true when the overall capacity of the electron transport chain and the degree of coupling between the electron transport chain and ATP synthase was measured (panel a, c).

The membrane potential of these cells was then examined using Mitotracker Red CMXRos dye. Mitotracker Green was also used in a control capacity as it is unaffected by changes in membrane potential and so any effects observed with the Mitotracker Red dye would be independent of increases in mitochondrial volume. For use as an indicator of membrane potential, the concentration of the Mitotracker Red CMXRos dye has to be used in what is termed 'non-quench mode'. Mitotracker Red is targeted preferentially to the mitochondria where it will accumulate according to the gradient. However, if there is too much dye introduced into the cell, it will cause an increase in apparent dye concentration relative to untreated conditions when the ionophore FCCP is added. Further, the suitability of the Mitotracker Green dye for control purposes was demonstrated in a similar manner to ensure that there was no increase in mitochondria accounting for any differences observed with the Mitotracker Red CMXRos. This was done by showing that the Mitotracker Green dye
underwent no changes in intensity when cells stained with it were treated with agents which would affect the membrane potential whereas Mitotracker Red CMXRos, at the optimal concentration, demonstrated the changes in intensity expected (Figure 5.9).

There was no difference in membrane potential between queuine lacking and queuine containing cells as measured using the Mitotracker Dye. When 5 mM glucose, the physiological concentration, was added to the medium, an increase in membrane potential was observed within thirty minutes in the queuine lacking cells (Figure 5.10, panel H). This increase peaked at two hours and continued to be observed at twenty-four hours. The intensity of Mitotracker Green remained unchanged in response to glucose (Figure 5.11). The sensitivity of the membrane potential of those cells lacking queuine to addition of glucose could have been an effect of the membrane potential being maintained through reversal of the ATP synthase complex, feeding protons through this complex in a bid to maintain a membrane potential. The sudden increase in glucose in the cell overwhelmed the lower flux observed with galactose. In order to verify this mechanism, cells were treated with 5 mM glucose and 1 µg/ml oligomycin concurrently (Figure 5.12). The increase in membrane potential which queuine lacking cells demonstrated in response to glucose was abolished with the addition of oligomycin, verifying that the ATP synthase was working in reverse in order to maintain membrane potential. Those cells cultured with queuine demonstrated an increase in membrane potential in response to the treatment with oligomycin and glucose. This was independent of the addition of glucose and was as expected as the blocking of the ATP synthase in a normal functioning cell would result in an increase in membrane potential.

Furthermore, when these cells were cultured for twenty-four hours under conditions of low L-glutamine supply (≥1 mM), a difference was observed in the response of complexes II and III to substrate stimulation. Those cells grown in the absence of queuine demonstrated a rate of oxygen consumption no different to that observed under conditions of plentiful L-glutamine supply. However, the activity of complexes II and III in queuine containing cells was markedly depressed (Figure 5.13).
Figure 5.1 Queuine supplementation has no effect on the proliferation of transformed and primary cells. (a) T-cells were prepared as in section 2.18.3. (b) HeLa cells were seeded at 3 x 10^4/cm^2 in Ultraculture. For both cell types, half of the cultures were cultured in (▲) 1 μM queuine and half without queuine (■). Those cells grown in the presence of queuine were supplemented at 24 hour intervals from day of seeding. On each day, three dishes of both queuine supplemented and non-supplemented cells were removed from the experiment and their cells counted using a haemocytometer. The result shown is representative of three independent experiments.
Figure 5.2 A Metabolic Difference is observed that is dependent on the presence of queuine. (a) HeLa cells were cultured for 48 hours in Ultraculture with (grey) or without (white) 1 μM queuine before the total protein was extracted and the activity of lactate dehydrogenase was determined by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH. (b) T cells were grown in Lonza X-Vivo 15 medium on plates coated with αCD3ε. Cells were supplemented with IL-2 every 48 hours. Half of the culture dishes were supplemented with 1 μM queuine on a daily basis. After 5 days, the medium was removed, spun through 0.45 μm centrifugal filters to remove any protein and particulate matter and analysed using the Nova Bioprofile 400. (c) Hela cells were grown in Ultraculture medium. Half of the culture dishes were supplemented with 1 μM queuine on a daily basis. After 5 days, the medium was removed, spun through 0.45 μm centrifugal filters to remove any protein and particulate matter and analysed using the Nova Bioprofile 400. Results shown are representative of three independent experiments. The levels of metabolites consumed or produced was determined through comparison to levels in fresh medium (Appendix, Figure 2).
Figure 5.3 The effects upon metabolism are not coupled to mitochondrial mass or number. (a) HeLa cells were cultured for 48 hours in Ultraculture medium with or without 1 μM queuine before total protein was extracted. The activity of citrate synthase (left) and cytochrome c oxidase (middle) were determined using spectrophotometric assays. The ratio of the cytochrome c oxidase activity to the citrate synthase activity (right) provided an index of the activity of the electron transport chain to the mitochondrial volume. (b) HeLa cells were grown in Ultraculture medium. Cells were plated with 1 μM queuine for 48 hours and then the total DNA of the cell was pheno-chloroform extracted and amplified using primers designed against the mitochondrial coxl gene and the nuclear ndufvl gene. Amplification curves based on measurements of the fluorescent DNA binding dye Sybr-green allowing qualitative measurements of the final amplified product (left). The amplification curves for both mitochondrial (black) and nuclear (red) DNA can be seen to be closely overlaid in both queuine containing and queuine lacking cells. (B). Products of the reactions were run on a 2.5 % agarose gel in order to verify correct sizing of gene product. M; mitochondrial, N, nuclear (C) The ratio of mitochondrial to nuclear DNA was calculated based off the data from the amplification curves.
Figure 5.4 Queuine supplementation has minor effects on mitochondrial morphology. HeLa cells were cultured for 48 hours in Ultraculture medium with or without 1 μM queuine. Cells were fixed, dehydrated, embedded in resin and sectioned before being counterstained with uranyl acetate and lead citrate. Samples were examined using a JEOL 1210 electron microscope. Those samples cultured under queuine lacking conditions (A,B,E,F,I,J) are shown on left with samples supplied with queuine (C,D,G,H,K,L) are on right. The top row (A-D) shows cells viewed under a 10,000 x magnification. The middle row (E-H) shows cells under a 15,000 x magnification and the bottom row (I-L) shows cells under a 20,000 x magnification.
Figure 5.5 Examination of any correlative effects between queuine and the overall function of the electron transport chain. HeLa cells were cultured in Ultraculture with (red) or without (blue) 1 μM queuine for 48 hours. Cells were removed from the surface of the dish, resuspended in MiR05 and counted and 4 million cells introduced into each chamber of the Oroboros oxygraph-2k™. (a) The basal, leak and maximum capacity of the electron transport chain was determined. The degree of uncoupling or RCR value was calculated as the excess capacity of the electron transport chain over the leak value. (b) Cells were permeabilized with 0.005% digitonin. Once a stable rate of respiration had been established, each complex was stimulated and inhibited consecutively. Cells were permeabilized with 0.005% digitonin and 75 μM Ap5A added to each chamber. Once a stable rate of respiration had been established, the P/O ratio was measured using NADH-linked substrates glutamate and malate followed by 50 μM ADP. The rate of oxygen consumption was calculated from the oxygraph trace and data. Results presented are representative of three independent experiments.
Figure 5.6 Determination of the P/O ratio in cells cultured in the absence of queuine.
Cells were permeabilized with 0.005% digitonin and 75 μM Ap5A added to each chamber. Once a stable rate of respiration had been established, the P/O ratio was measured using NADH-linked substrates glutamate and malate followed by 50 μM ADP. The rate of oxygen consumption was calculated from the oxygraph trace and data. Results presented are representative of three independent experiments.
Figure 5.7 The absence of queuine has no effect on the mitochondrial membrane potential. (a) HeLa cells were cultured in Ultraculture medium with or without 1 μM queuine for 48 hours. Cells were washed and incubated in pre-warmed PBS containing 40 μM H2DCFDA for 30 minutes before addition of pre-warmed Ultraculture medium. Cells were trypsinized and analysed using flow cytometry and analysis was gated on single cells. (b) HeLa cells were cultured in Ultraculture medium with (grey) or without (white) 1 μM queuine for 48 hours before being trypsinized, counted and treated as detailed in section 2.15. The mitochondrial membrane potential was collapsed by addition of 1μM FCCP 10 minutes prior to addition of TPMP^+/TPB. The energization dependent TPMP^+ uptake was expressed as an accumulation ratio calculated using the equation: [(TPMP/mg protein)/(TPMP/μl supernatant)]. Results shown are representative of three independent experiments.
Figure 5.8 The mitochondrial function of galactose adapted HeLa cells was investigated using polarography. HeLa cells grown in Ultraculture medium lacking glucose and supplemented with galactose were grown with (red) or without (blue) 1 μM queuine for 48 hours. Cells were harvested, suspended in MiR05, counted and an appropriate amount was introduced into each of the chambers of the Oroboros oxygraph-2k™. (a) The overall rate and degree of uncoupling was ascertained as described previously. (b) The activity of each of the complexes I-IV of the electron transport chain was determined consecutively using substrates and inhibitors of each complex. (c) The NADH linked P/O ratio of these cells were determined by measuring the oxygen consumption after a known amount of ADP was introduced into the chamber. Results shown are representative of three independent experiments.
Figure 5.9 Determination of the non-quench concentration of Mitotracker Red dye. Galactose adapted Hela Cells were cultured in Ultraculture medium lacking glucose and supplemented with 10 mM galactose. Cells were stained with either 50 nM Mitotracker Red CMXRos dye or 40 nM Mitotracker Green dye for 15 minutes at 37°C before being fixed with 4% paraformaldehyde. Cells were treated with 2.5 µM oligomycin or 5 µM FCCP concurrently with the Mitotracker dye prior to fixation. Results shown are representative of three independent experiments.
Figure 5.10 Cells lacking queuine demonstrate a rapid increase in the \( \Psi_m \) in response to glucose. Galactose adapted HeLa cells adapted to the substitution of 10 mM galactose in place of glucose were stained with 50 nM Mitotracker RedCMXRos at 37°C for 15 minutes before being fixed with 4% paraformaldehyde. In those instances where 5 mM glucose was added to the culture medium, this was done the stated time before fixation. Result is representative of at least three independent experiments.
Figure 5.11 The apparent increase in $\Psi_m$ in response to glucose in cells lacking queuine is independent of changes in mitochondrial content. Galactose adapted HeLa cells adapted to the substitution of 10 mM galactose in place of glucose were stained with 40 nM Mitotracker Green at 37°C for 15 minutes before being fixed with 4% paraformaldehyde. In those instances where 5 mM glucose was added to the culture medium, this was done the stated time before fixation. Result is representative of at three independent experiments.
Figure 5.12 The glucose mediated increase in $\Psi_m$ is mediated through a reversal of the ATP synthase which is fed by glycolysis. Galactose adapted HeLa cells adapted to growth in 10 mM galactose were stained with 50 nM Mitotracker RedCMXRos for 15 minutes at 37°C. Cells were treated with 5 mM glucose 30 minutes prior to fixation and 2.5 $\mu$M oligomycin concurrently with Mitotracker dye. Result shown is representative of three independent experiments.
Figure 5.13 The mitochondrial function of galactose adapted HeLa cells exhibits a difference compared to their queuine lacking counterparts when substrate supply is limited. Galactose adapted HeLa cells grown in Ultraculture medium lacking glucose and supplemented with galactose were grown with (red) or without (blue) 1 uM queuine for 48 hours. Cells were harvested, suspended in MiR05, counted and an appropriate amount was introduced into each of the chambers of the Oroboros oxygraph-2k. The activity of each of the complex I-IV of the electron transport chain was determined consecutively using substrates and inhibitors of each complex. Results shown are representative of three independent experiments.
5.3 Discussion

The fact that queuine is found within the mitochondrial tRNA of mammals reveals much about the potential function of queuine. This has been corroborated by the published results of this project which demonstrated that the enzymes comprising the eukaryotic cellular queuine transglycosylase activity were localized to the mitochondria (Boland et al., 2009). The most parsimonious conclusion that one could arrive at from these two pieces of data would be that queuine is instrumental in the translation of the thirteen proteins within the mitochondrial genome. The absence of queuine has also been linked to increased proliferation in some cancer cells, a difference which was highly responsive to oxygen concentration (Langgut et al., 1994) and to an increase in the expression and activity of LDH isoforms associated with a cancer phenotype (Pathak et al., 2008).

One aspect of the metabolism peculiar to cancer cells is referred to as the Warburg metabolism, but the increased lactate production characteristic of the Warburg effect is only a small portion of the altered tumour metabolism. In fact, when considering the metabolism of transformed cells, it is important to bear in mind the reasons for the drastic alterations in their metabolic flux. These cells are the victims of their own hyper-proliferation and as such, have a greatly increased requirement for nucleotide and fatty acid biosynthesis. The effect of queuine on proliferation (the reason for the increased biosynthetic flux observed in cancer cells) and the bioenergetic state of the cell was investigated in this chapter.

Queuine exerted no statistically significant effect on proliferation and the results which Helga Kersten had published were not replicable in either transformed or primary cell lines. This may be because we used adherent parental HeLa cells as opposed to the non-adherent HeLaS3 subtype used by Kersten's group or perhaps because the use of Ultraculture™ Serum Free medium is masking the metabolic insufficiency of queuine deficient cells by providing large amounts of growth factors, the identify of which is not disclosed by the manufacturers. Interestingly though, the enhanced activity of lactate dehydrogenase was confirmed and cells supplemented with physiological levels of queuine were found to have an LDH activity which was 75 % of that found in queuine lacking cells. The measurement of metabolites integral to the metabolism of cancer cells confirmed that the absence of queuine caused cells, both primary and transformed, to adopt a glycolytic predisposition. In those cells
lacking queuine, it can be seen that glucose and glutamine consumption are being upregulated with a proportional and concomitant increase in the production of lactate. The increased production of glutamate and ammonia observable in queuine lacking T-cells but less obvious in HeLa cells, provide another interesting footnote in the manner in which glutamine is being consumed in these cells. Significantly, it provides a strong indication that the uptake of glutamine is not simply being increased to accommodate an increased demand for protein synthesis. When glutamine enters the TCA cycle, it is deamidated to glutamate, accounting for the increased production in glutamate and ammonia which is seen. This was interesting for another reason as glutathione (GSH) is dependent on glutamine for its synthesis. It has been documented that murine fibroblasts cultured without queuine contained half the level of manganese dependent superoxide dismutase and were subject to less oxidative stress according to a thiobarbituric acid reactive substance (TBARS) assay (Szabo et al., 1988). However, measurement of the cellular production of ROS species using the H2DCFDA dye demonstrated no difference between queuine containing and queuine lacking cells. Since the intensity of the H2DCFDA dye is dependent on oxidation by H2O2 and free radicals downstream of H2O2 it may be useful to use an alternative method in the future. For example, the MitoSOX Red dye which is sensitive to oxidation by superoxide (O2−) but which is relatively insensitive to oxidation by H2O2.

As the absence of queuine is instrumental in causing the cellular metabolism to adopt a more glycolytic metabolism, it seemed likely that the primary means of energy production in a normal cell (i.e. oxidative phosphorylation) would be affected. Helga Kersten's group had observed increased electron flow in those cells grown in medium supplemented with queuine, according to the results of an MTT assay (Reisser et al., 1994). It was first ascertained that the metabolic differences observed were not a result of decreased mitochondrial number or volume in response to queuine deficiency. This proved to be the case as demonstrated using protein activity assays and quantitative PCR. When the mitochondria of queuine containing and queuine lacking cells was visualized using transmission electron microscopy, preliminary evidence was found of less cristae being present in the queuine deficient mitochondria.
The respiratory capacity of these cells was investigated and compared against their queuine containing counterparts. The function of each complex of the electron transport chain was investigated using oxygen consumption measurements. Furthermore, it allowed for inclusion of what was assumed was a control, the functioning of complex II, the only complex of the electron transport chain whose components are solely encoded from the nucleus. However, none of the complexes of the electron transport chain were adversely or otherwise affected by the absence of queuine. The mitochondria of these cells were equally efficient, displaying respiratory control ratios which were compatible with well functioning mitochondria. This efficiency between the electron transport chain and the ATP synthase was also tested through measurement of the P/O ratio in response to NADH and FADH linked substrates. In both instances, queuine had no effect on the P/O ratio. However, an anomaly was encountered as it was consistently found that stimulation with NADH-linked substrates caused an increased rate of oxygen consumption in the cells grown with queuine. This was perplexing as no such difference had been observed during the sequential testing of the complexes function. The only difference between the experiments that could possibly explain this discrepancy was the addition of the inhibitor of adenylate kinase, Ap5A, which would prevent recycling of ATP to ADP. This indicated that complex I in cells cultured in the absence of queuine was sensitive to the levels of ADP present in the mitochondria. It was also noteworthy that this difference was only apparent with the addition of glutamate/malate and not succinate. Perhaps this is an indication that queuine dependent differences do exist in the functioning of the mitochondrially encoded complexes, complex II is wholly nuclear encoded, but that they become apparent under certain limiting conditions. Or it is possible that the activity of the individual complexes remain unchanged but that queuine affects mitochondrial nucleotide transport. The adenine nucleotide transporter (ANT) is the most highly expressed protein in the inner mitochondrial membrane and four different isoforms exist which are subject to tissue and developmental specific expression (Chevrollier et al., 2010). In cancer tissue, the predominant isoform is ANT2 which is responsible for importing glycolytically produced ATP into the mitochondrial matrix. The ATP synthase will then work in reverse and hydrolyse this ATP in order to maintain the mitochondrial membrane. As the mitochondria of cells lacking queuine appeared to have less cristae than those
mitochondria whose tRNA contained queuine, the link to the mitochondrial membrane potential appeared worthy of investigation. There was no evidence of a queuine sensitive difference in this mitochondrial membrane potential as determined through calculation of accumulation ratio between the cells and their supernatant of the radiolabelled lipophilic cation TPMP+. The membrane potential of both queuine containing and queuine lacking cells increased to a similar amount in response to the addition of oligomycin.

The proprietary serum free medium in which the HeLa cells had been cultured up until this stage contains an abundance of substrate of which are relevant include glucose, glutamine and nucleotides. HeLa cells are extremely malignant and carry so much genetic damage as to be irreversibly transformed. For this reason, their metabolic predisposition would be towards aerobic glycolysis. By depriving them of glucose, they can be coaxed to return to a metabolism which incorporates more oxidative phosphorylation (Rossignol et al., 2004). Although the respiratory rate was seen to improve when glucose was removed from the culture medium, no difference was observed between queuine containing and queuine lacking cells. The mitochondrial membrane potential of these cells was then investigated using Mitotracker Red CMXRs dye. The mitochondrial membrane potential of queuine lacking cells was seen to increase significantly in response to re-addition of glucose to the medium. The induction of this response was rapid and sensitive to treatment with oligomycin. This seemed to indicate that in those cells lacking queuine the mitochondrial membrane potential was being maintained through hydrolysis of glycolytically produced ATP by the ATPase activity of the F0F1 subunit.

What this also meant was that the response of the cells to queuine was dependent on substrate supply. The cells had already demonstrated a queuine dependent difference in response to the levels of ADP in the mitochondria. Perhaps a fault in adenine nucleotide transport within the cell was corrected in the queuine lacking cells through increased import of ATP produced from glycolysis. It was only the absence of glucose that had allowed differences in the response of the membrane potential between queuine containing and queuine lacking cells to become apparent. The reaction of the cells when they were placed under conditions of stress resulting from lack of substrates was examined and it was seen that the activity of complexes II and III were down-regulated in cells supplemented with queuine. From these results,
it seems that the effects of queuine are only noticeable in a stressful cellular environment. Under conditions of substrate limitation, those cells containing queuine acted to conserve scarce resources.
Chapter 6

The link between Queuine and Metabolism in vivo

6.1 Introduction

Much of the research on queuine has relied on in vitro methods of examination. However, this situation is not ideal and this has been reflected to some extent in the results produced using this methodology. The exhaustive enquiry into the effects of queuine on proliferation has produced much inconsistent and underwhelming data (Langgut et al., 1993). It must be remembered that cells grown in culture seek to replicate conditions in vivo but frequently fall short of true physiological conditions. For instance, the medium they are cultured in frequently contains an excess of metabolic substrate. This can obfuscate matters, especially when metabolism is being examined. In fact, the fault of cell culture is that it seeks to make the cells as comfortable as possible, thus ignoring the difficulties which the tumour tissue faces in vivo, the most relevant in this situation being difficulties in accessing sufficient levels of substrate. Furthermore, the contribution of the surrounding non-transformed tissue may be significant. The reverse Warburg theory suggests that the metabolism of the surrounding non-transformed tissue is corrupted towards an aerobic glycolytic metabolism and the energy rich metabolites produced are then transferred into the cancerous tissue, entering the TCA cycle and allowing the high production of ATP required via oxidative phosphorylation (Bonuccelli et al., 2010).

Of course, it has been demonstrated that both Drosophila melanogaster and Dictyostelium discoideum, with their short generational times, can be cultivated very rapidly to become totally queuine deficient with minimal expense (Ott et al., 1982, Jacobson et al., 1981). While queuine is present in all eukaryotes, thus implicating its significance in all forms of life, its particular relevance to the mammalian system can best be answered by moving the question into the mouse model. Prof. Walter Farkas was responsible for the establishment of the only reported mouse colonies known to be totally lacking in queuine (Farkas., 1980, Reyniers et al., 1981). This was accomplished by housing the animals under strict germ-free conditions and feeding
them a custom made liquid diet lacking in queuine. Eukaryotes are incapable of synthesizing queuine and are reliant on ingested queuine or the flora of the gut for bacterially derived queuine. Queuine is a resilient tRNA modification and one year is required to completely eliminate it from the tRNA (Reyniers et al., 1981), approximately 120 murine tRNA half-lives. This all illustrates the difficulties inherent in moving the study of queuine and its attendant transglycosylase activity to an in vivo setting.

The only difference reported between these queuine deficient animals and their queuine containing counterparts was a slight increase in weight gain which was attributed to increased fluid consumption—the effect of a solely liquid diet (Farkas., 1980). Certainly, these queuine free animals demonstrated no ill effects over the first year of their life (Reyniers et al., 1981). When tyrosine was removed from the diet of these germ free mice, the consequences were fatal with the death of every animal in the cohort occurring within only two weeks of removal of tyrosine (Marks et al., 1997). The symptoms that these animals demonstrated included staggered walking, squinting and finally seizures, all of which could potentially be linked to a neurological defect. Furthermore, these symptoms could be reversed by the addition of queuine or tyrosine alone which demonstrated that the tyrosine deficiency effect relates to its biosynthesis rather than utilisation.

With the identification of the eukaryotic genes responsible for coding the queuine transglycosylase machinery, disrupting these genes became possible and a gene-trap TGT knockout mouse line was successfully generated and validated to be QtRNA deficient by other members of our laboratory, manuscript in preparation. Transcription of the qtrtl gene, which encodes for TGT, was disrupted by insertion of a ROSFARY cassette into the second exon. Genetic disruption of TGT circumvents the expense and time involved in maintaining a queuine free colony. Although these gene trap animals contain queuine nucleobase, they are lacking one of the enzymes integral to the tRNA insertion process. This chapter focused on examining the effect of TGT deficiency on the animals.
6.2 Results

6.2.1 The \textit{qtrtl}\textsuperscript{+/–} genotype occurs in accordance with Hardy-Weinberg Equilibrium

Animals with a 129/C57/BL6 background and which were heterozygous for the \textit{qtrtl} gene were bred and the resulting litters genotyped (Table 6.1). The ratios of wild type, heterozygous and homozygous animals were found to obey Hardy-Weinberg equilibrium as approximately half the animals in each litter were heterozygous for the \textit{qtrtl} gene whereas the wild type and homozygous animals constituted a quarter of the litter each. This is significant information as it demonstrates that the removal of the \textit{qtrtl} is not embryonic lethal. This would be expected as it has already been established that embryonic development is not modified by queuine.

6.2.2 Animals which carry at least one copy of the \textit{qtrtl} knockout gene are predisposed towards weight gain

Three male and three female animals of each genotype, \textit{qtrtl}\textsuperscript{+/+}, \textit{qtrtl}\textsuperscript{+/-} and \textit{qtrtl}\textsuperscript{-/-}, were set aside and the weighed every 72 hours from two months until they were six months of age (Figure 6.1). In both male and female animals, a divergence in weight gain was observed to occur from three months onward. In those animals which were heterozygous for the \textit{qtrtl} knockout gene, the presence of a wild type allele had no mitigating effect on this observed weight gain. The weight gain observed appeared to be the result of increased fatty layers as opposed to a general increase in the size of the animals' vital organs. The liver and brains of these animals was unchanged regardless of their genotype.

6.2.3 The bioenergetic flux of TGT animals is abnormal

The LDH activity of brain and liver tissues from one week old and adult (four month old) animals was determined (Figure 6.2). In both neonatal and adult tissues, the wild type animals demonstrated a three times higher level of activity over that found in knock-out animals. The LDH activity of the heterozygous animals was found to be on a par with or below the level observed in knock-out animals, which is perhaps evidence for a dominant negative effect.
The mitochondrial volume was investigated in a similar manner in which it had been done in the cells. The citrate synthase activity was assayed, as well as the activity of cytochrome c oxidase (Figure 6.3, Figure 6.4). There was no difference observed between the different genotypes with regard to the range of activities found for either of these two enzymes. Furthermore, the ratio of cytochrome c oxidase activity to citrate synthase activity was also found to be identical between the different genotypes. From these data, it could be assumed that the mitochondrial number and volume and the activity of complex IV is unaffected by the absence of TGT protein. These assays were done in both the brain and liver.

The activity of each individual mitochondrial complex was then tested in percoll purified mitochondria from brain tissue of six month old animals using oxygen consumption as a valid parameter of respiratory rate. The activity of each complex was tested sequentially as detailed in section 2.11.1. From these oxygraph traces, it was possible to see that the activity of complexes II and III was adversely affected by the absence of at least one copy of the qtrtl gene (Figure 6.5). The range of activity in both heterozygote and knock-out animals was between a half and a quarter of that found in wild type animals. This difference was present in both male and female animals although it was found to be an age-specific effect. When the brains of two month old animals were processed in a similar manner, only the heterozygote demonstrated a similar decrease in the activity of complexes II and III, illustrating again the dominant negative effect present in heterozygote animals (Figure 6.6). The P/O ratio of these aged animals was also tested but no significant differences between the different genotypes was observed.

6.2.4 The βF1 subunit of ATP synthase is up-regulated in the brains of knockout animals

The expression of the ATPase Inhibitory Factor 1 (IF1) peptide and the βF1 subunit of the ATP synthase were investigated in brain and liver preparations from 3 month old male animals. While no change was observed in the expression levels of the IF1 protein, the brain tissue of the heterozygous and knockout animals demonstrated a marked increase in the levels of the βF1 subunit, which plays a role in the hydrolysis of ATP (Figure 6.7). This increase was not seen in the liver samples of these animals.
Table 6.1 The ratio of wild type, heterozygous and homozygous animals obeys Hardy Weinberg equilibrium. 13 litters (totalling 75 pups) from crossing heterozygous parents were genotyped and the ratio of wild type, heterozygous and homozygous animals calculated.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
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<tr>
<td>+/-</td>
<td>28.21</td>
<td>27.78</td>
<td>28</td>
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<tr>
<td>+/-</td>
<td>51.28</td>
<td>52.78</td>
<td>52</td>
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<td>-/-</td>
<td>20.51</td>
<td>19.44</td>
<td>20</td>
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Figure 6.1 Increased weight gain was observed in those animals lacking even one functional copy of the *qrtzl* gene. Three animals of each genotype, wild type (red), heterozygous (green) and homozygous (blue) animals and of each gender were weighed every three days between the ages of 2 to 6 months and their weights plotted.
Figure 6.2 The activity of lactate dehydrogenase is down-regulated in the brain and liver of +/- and -/- animals. Liver (A,C,E) and brain (B,D,F)tissue homogenates were isolated from 7 day (A,B), 21 day (C,D) and 3 month old animals (E,F). The activity of lactate dehydrogenase was determined by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH. Results shown are representative of three independent experiments.
Figure 6.3 The activity of citrate synthase is unaffected by the absence of TGT. Liver (a,c,e) and brain (b,d,f) tissue homogenates were isolated from 7 day, 21 day and 3 month old animals. The activity of citrate synthase was determined using spectrophotometric assays. Results shown are representative of three independent experiments.
Figure 6.4 The activity of cytochrome c oxidase is unaffected by the absence of TGT. Liver (g,i,k) and brain (h,j,l) tissue homogenates were isolated from 7 day, 21 day and 3 month old animals. The activity of cytochrome c oxidase was determined using spectrophotometric assays. Results shown are representative of three independent experiments.
Figure 6.5 The oxidative phosphorylation of +/- and -/- animals is deleteriously affected. Mitochondria was isolated from the brains of 6 month old male (a) and female (b) animals and purified using percoll. The protein concentration was determined using a Bradford assay and 1 mg of mitochondria was introduced into each chamber of an Oroboros oxygraph-2k™. Once a stable rate of respiration had been established, each complex was stimulated and inhibited consecutively in wild type (red), heterozygous (green) and homozygous (blue) animals. (c) These results were plotted. Results shown are representative of three independent experiments.
Figure 6.6 The difference which manifests in oxidative phosphorylation activity is age dependent. Mitochondria was isolated from the brains of 2 month old animals sacrificed using cervical dislocation. The mitochondria were purified using percoll density ultracentrifugation. (a) The protein concentration was determined using a Bradford assay and 1 mg of mitochondria was introduced into each chamber of an Oroboros oxygraph-2k™. Once a stable rate of respiration had been established, each complex was stimulated and inhibited consecutively in wild type (red), heterozygous (green) and homozygous (blue) animals. (b) These results were plotted. Results shown are representative of three independent experiments.
Figure 6.7 The expression of the βf1 subunit of the ATP synthase is upregulated in the brains of -/- animals. Liver and brain tissue homogenates were isolated from 3 month old animals and immunoblotted with a monoclonal antibody against the IF1 peptide and antisera against the βf1 subunit of the ATP synthase. Control protein homogenates from HeLa and HCT116 cells were included.
6.3 Discussion

It is a mistake to regard the lack of queuine as synonymous with the deletion of the \textit{qtrtl} gene. It has been demonstrated in previous chapters that there are two eukaryotic genes coding for components of the queuine transglycosylase machinery. The only enzymatic requirements for queuine in the typical eukaryotic cell are the insertion of the modified nucleotide in the tRNA or its retrieval and salvage (Günduz \textit{et al.}, 1984). While it is possible that these proteins are dedicated solely to either task, it has been demonstrated in this lab that the insertion of queuine into tRNA requires both proteins (Boland \textit{et al.}, 2009). However, in light of the fact that animals lacking TGT demonstrate differences in both weight gain and mitochondrial function which cannot be directly traced back to an absence of queuine, it does provide evidence that queuine is functionally relevant within the cell and interfering with the queuine apparatus produces observable consequences.

These include a down-regulation of LDH in the brain and liver tissue of those animals lacking even one functional copy of the \textit{qtrtl} gene. This is the opposite of what has been observed in this and other studies (Pathak \textit{et al.}, 2008). This demonstrates that the situation engineered in these animals is not replicating the state of the gnotobiotic mice bred by Walter Farkas (Farkas., 1980). What this result confirms is the importance of queuine to cellular metabolism and its putative link to cancer metabolism—lactate is a metabolite central to the difference between the metabolism of a normal somatic cell and a transformed cell. The activity of the citrate synthase and the cytochrome c oxidase enzymes confirms that this difference is not affiliated with the mitochondrial number or volume.

The differences observed on complexes II and III mirror those results seen in the starved HeLa cells. In the galactose adapted cells, the down-regulation of complexes II and III was observed in those cells supplemented with queuine whereas in the \textit{qtrtl} knockout mice, the activity of these same complexes was found to be markedly down-regulated. In the cells, it was assumed that queuine was acting in a regulatory capacity to conserve the limited metabolic substrates in the cell during a time of extreme cellular stress. This manufactured situation was extreme and its only value lay in outlining the parameters of queuine involvement. Comparison to the situation \textit{in vivo} would be limited value for this reason.
The choice of the brain for further examination of oxidative phosphorylation was predicated on the fact that the symptoms Farkas observed in his germ free tyrosine deprived mice were neurological in nature (Marks et al., 1996). The blood-brain barrier presents no impediment to queuine as pig brain tRNA has been found to be fully modified with respect to this particular modification (Siard et al., 1989). However, it must also be considered that the brain is an atypical tissue. It is not yet apparent that Qv1 is the predominant splice variant in this tissue, especially as the source tissue of the Qv2 IMAGE clone was from retinal tissue, an extension of the brain. However, the observation of a similar trend in brain and liver as regards LDH, citrate synthase and cytochrome oxidase activity is reassuring.

As regards to the mechanism of the observed effects of queuine on metabolic enzymes and oxidative phosphorylation, it is unlikely to be a result of a deleterious effect on mitochondrial protein translation as complex II is affected, the only complex of the electron transport chain which is wholly encoded by the nuclear genome. In fact, it is unknown whether the above demonstrated differences result from reduced activity of the enzymes examined or a decrease in the abundance of the Complex II and III enzymes or both. In fact, the finding that complexes II and III are affected is interesting as it has been observed previously that cytochrome b559 in Dictyostelium discoideum was down-regulated in amoeba grown under queuine deficient conditions. Cytochrome b559 is functionally related to the b type cytochromes present in complex III.

One must also consider these results in concert with each other. The weight gain exhibited by these animals coupled to the down-regulation in the activity of complexes II and III almost certainly has the same source defect. The role of complex III in fatty acid oxidation may be of relevance to the gain in weight although these mice did not present with fatty livers. It is likely that the effects observed on mitochondrial function are secondary knock-on effects of a primary fault resulting from queuine deficiency.

There is also the issue of the increased βF1 expression in the brains of the knockout animals. This would suggest that these animals have a greater capacity for hydrolysing ATP than their wild type counterparts. It is also significant that no change was observed in the levels of the IF1 peptide as this has been found to be upregulated in human cancer tissue (Sanchez-Cenizo et al., 2010).
The dominant negative effect observed must also be acknowledged. In our knockout animals the \textit{qtrtl} gene has been disrupted in its second exon producing a non-functional fusion with a β-galactosidase-neomycin. As a result a protein lacking its C-terminal tRNA binding domain would be produced. From the previous chapters, it is likely TGT functions in part as a chaperone of tRNA, ferrying the nuclear encoded tRNA into the cytoplasm whereas the mitochondrial TGT may fulfil a similar function in the matrix. From our published data it is most probable that TGT is only catalytically active when in association with Qv1. Therefore, the dominant negative effects seen here arise as a result of a truncated protein which is still capable of binding to Qv1 but not tRNA.
Chapter 7

7.1 Discussion

Over three decades worth of work have been devoted to the study of queuine and the enzymes responsible for its synthesis and insertion into tRNA. Although much progress has been made in the eubacterial system as regards identifying and characterizing the enzymes involved in the queuine biosynthetic pathway, the research generated in eukaryotes has been less straightforward (Phillips et al., 2008, Todorov et al., 2006, Kittendorf et al., 2003). While the focus of much of this research has been directed towards defining the function of this unusual nucleotide, it seems to have been a premature approach when one considers that the gene and protein identity of the transglycosylase activity was never initially solved. While the presence of queuine in both eukaryotes and eubacteria is compelling enough to suggest it fulfils a significant role in the cell, disappointingly little progress has been made in identifying what this role might be.

Many attempts at purifying and identifying the transglycosylase protein have been made but none could be qualified as a complete success. While many of the preparations did retain a high level of transglycosylase activity, they were frequently found to contain proteins whose association with the transglycosylase activity was considered unusual. In fact, in some instances, these proteins were erroneously identified as being responsible for the transglycosylase activity. It was only through comparison with the published sequence of the E.coli tgt gene that the human version of this protein was finally identified (Deshpande et al., 2001).

It was a similar genetic approach that formed the basis of this study and allowed the gene identities of the queuine transglycosylase activity to be established. The qtrl gene was found to code for the eukaryotic TGT protein and it demonstrated a level of conservation across a wide and varied range of species indicating its value within the cell. Furthermore, the genomes of all qtrl carrying species carried a second related gene called qtrtl1. Sequence analysis of this gene appeared to confirm
it had been in the eukaryotic genome longer that qrtl, probably introduced by the proto-mitochondria. The qtrtdl gene was subject to alternative splicing with three protein products found within the murine alternative splicing database. These three products were designated QTRTD1.V0, QTRTD1.V1 and QTRTD1.V2 but an investigation into their expression found that QTRTD1.V1 (Qvl) was the predominant splice variant in all of the tissues tested. This hinted at a complexity in the regulation of the queuine transglycosylase cellular machinery. Neither qrtl nor qtrtdl were found by themselves (i.e. only one or other of the proteins) in any genome examined, it seemed natural to assess their associativity. Co-immunoprecipitation experiments and confocal imaging confirmed that they were capable of physically associating with each other but it was also demonstrated by Cóilín Boland that the transglycosylase activity could be reconstituted when both proteins were incubated together (Boland et al., 2009). The finding that both these proteins shared a mitochondrial address provided a tentative link from queuine to the bioenergetic status of the cell, something which had been hinted at by much of the literature on queuine (Langgut et al., 1993).

It was in this manner that the focus of this research came to bear on the effect of queuine on the cellular metabolism. It has been reported numerous times that the tRNA of transformed tissue is frequently hypomodified for queuine. The fact that the QtRNA of cancer cells is frequently under-modified for this modification should not be dismissed as a side-effect of increased proliferation. It has been established that the QtRNA^{asp} and QtRNA^{Tyr} require a year to become fully unmodified in mouse liver. Even after four weeks on a chemically defined diet under germ free conditions, 88 % of these two tRNA isoacceptors were found to be modified for queuine (Farkas., 1980). For this reason, those metabolites which were of especial importance to the perturbed metabolism characteristic of cancer were analyzed. The levels of glucose, lactate, glutamine, glutamate and ammonia were all found to be responsive to queuine supplementation in a transformed cell line (HeLa) and a primary cell type (activated T cells). In both these cell types, the levels of the named metabolites in those cells cultured without queuine indicated that increased glycolysis and glutaminolysis was occurring. Both cell lines are subject to a massive proliferative impetus and favour glycolysis over oxidative phosphorylation in order to maintain their increased proliferation. In the case of the HeLa cells, this unique metabolism is
referred to as the Warburg effect and incorporates increased glutaminolysis. The metabolic situation in the T-cells may resemble the Warburg effect but is an example of the aerobic glycolysis favoured by cells which are undergoing rapid proliferation (immune associated cells and neonatal tissue) and the increased biosynthesis necessary in such an instance (Marko et al., 2010). In this instance, an over-reliance on glycolysis is favourable as it circumvents the increased ROS production attendant to increased electron transfer through oxidative phosphorylation. Interestingly, it has also been reported that neonate tissue is hypo-modified for queuine (Nishimura et al., 1983, Singhal et al., 1981).

The reason a cancer cell will adopt such a metabolism is that it enables it to engage in a higher rate of proliferation (Guppy et al., 1993). The effect of queuine on proliferation had been examined numerous times in the literature (Langgut et al., 1993) but in our studies, no real difference was observed in the effect of queuine on the proliferation of the cells. This result also demonstrated that the increased levels of metabolites observed was not because of an increased number of cells in the queuine lacking cultures but was the result of increased glycolytic flux. As the TGT and Qvl enzymes had been found to localize to the mitochondria and the mitochondrial tRNA^asp had been found to be modified for queuine (Randerath et al., 1984), a comprehensive analysis was undertaken in which all aspects of the mitochondria were examined; number, morphology and function. Preliminary results showed a minor decrease in the number of cristae by electron microscopy in queuine lacking cells but the number and function of the mitochondria appeared unaffected by the absence of queuine. However, the response of the queuine lacking cells differed to their queuine containing counterparts once these cells were exposed to conditions where substrate limitation became a real issue for the cell. HeLa cells will grow in the absence of glucose as long as it is substituted with either fructose or galactose, but these sugars are not as well metabolized through glycolysis and instead they enter the pentose phosphate pathway allowing the cellular demand for nucleotides to be met (Reitzer et al., 1979). The membrane potential of these galactose adapted HeLa cells, as analyzed using Mitotracker Red, was found to increase significantly in queuine lacking cells in response to the addition of 5 mM glucose to the medium. Furthermore, when the nutrient availability of these cells was limited, the activity of complexes II and III of the queuine supplemented cells were down-regulated within
twenty four hours. Intriguingly, this result was mirrored in the brains of the animals but it was the qtrtl knockout and heterozygous animals that demonstrated this down-regulation in these complexes. The distinction between the work in vitro and in vivo is important to stress as the lack of queuine is not necessarily the same as the absence of TGT.

It seems unlikely that queuine is affecting mitochondrial translation as complex II is encoded from the nuclear genome and only twenty four hours of starvation are required to elicit a response from the cells. Furthermore, the activity of ATP synthase has been demonstrated to be affected in the cells while the expression of a component of this complex, βF1, has been seen to be up-regulated in knock-out animals.

7.2 Future Work

The discovery of a eukaryotic homologue of TGT which itself is subject to transcriptional regulation through alternative splicing hinted that the expression of the queuine related cellular machinery is subject to tissue and developmental specific expression. The use of RNA-Seq technology enables the level of gene expression to be accurately determined. In fact, this technology allows the detection of gene expression within a very wide range, from single copies to multiple fold mRNA copies present within the cell. It does this through generation of millions of short fragments from the total mRNA of the cell (Trapnell et al., 2009). Furthermore, by mapping all these reads, it is possible to build a picture of any splice junctions within the gene and the predominant variant being produced within a particular cell type under certain conditions. Certainly, this would be valuable information.

As the glycolytic and glutaminolytic metabolites provided the most definitive and easily understood results, it is worth examining these metabolic aspects in more detail. The metabolic flux through both these pathways can be further delineated using stably labelled isotopes of glucose and glutamine and carrying out NMR analysis. It is also important to determine the cause of the increased aerobic glycolysis observed in queuine lacking cells. The Nova analyser results point to a gross overproduction of lactate and it would be interesting to examine the respirometric response of these cells to pyruvate. Perhaps, this excess production of lactate is entering the mitochondria through the lactate shuttle in order to compensate.
for a defect in the pyruvate uptake of the mitochondria of queuine lacking cells (Gladden., 2007). If these cells are producing an excess of lactate, it would also be worth examining the cytosolic NADH/NAD+ ratio as an excess production of lactate in the cell would cause this to decrease.

As a difference has been observed in the reaction of complex II and III to the absence of queuine and it has been reported that cytochrome b of Dicytostelium discoideum was adversely affected in the absence of queuine, it may be worthwhile to examine the cytochrome spectra of those cells cultured in the presence and absence of queuine.

However, before commencing further study, the growth conditions of the cultured cells require further consideration. The proprietary serum free medium they are grown in is known to contain extremely high levels of glucose (20 mM). The levels of other constituent nutrients in this medium remain unknown but it can be assumed the medium has been supplied with every possible substrate a transformed tissue may require in the absence of the growth factors present in FBS. The Ultraculture medium may be exerting a masking effect by rendering the deficiency in the queuine lacking cells irrelevant. The cells could be cultured in a basal medium supplemented with recombinant produced growth factors thus avoiding excessively high nutrient levels and queuine containing serum.
Appendix

Appendix Figure 1. Size-exclusion gel-chromatography of rTGT and Qv1. (A) rTGT and (B) Qv1 were chromatographed on a TSK-Gel G3000SWXL column by HPLC (Shimadzu) in 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 10% glycerol, individually giving sizes of 26 kDa and 37.5 kDa, respectively, or (C) pre-incubated and run together yielding two peaks identical to individual runs. Calibration of the column was made with alcohol dehydrogenase (150 kDa), albumin fraction V (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), as indicated by the black dots. The elution of protein was recorded at A220. (Boland et al., 2009)
Appendix Figure 2. The levels of metabolites in (a-d) Lonza Ultraculture Medium and (e) Lonza X-Vivo 15 Medium was determined using the Nova Bioprofle 400 analyser. The levels of glutamine, glutamate and ammonia was identical in both Ultraculture and X-Vivo 15 Medium and results were pooled. Medium was spun through 0.45 μm centrifugal filters to remove any protein or particulate matter.
Chapter 8

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152


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