Prof. Proud’s laboratory studies the signalling pathways by which hormones, growth factors and nutrients regulate the function of mammalian cells, especially protein metabolism. The proper control of these pathways plays an important role in cell growth and proliferation, the storage and utilisation of nutrients, and in neurological processes. Defects in their control contributes to tumorigenesis, type 2 diabetes, dyslipidaemias, cardiovascular disorders and neurodegenerative disease.

A major nutrient-sensitive signalling pathway involves the mammalian target of rapamycin, complex 1, mTORC1. mTORC1 is activated by hormones and amino acids, and regulates multiple steps in protein synthesis including the initiation and elongation stages. We are also currently investigating the role of several protein kinases closely related to the mTORC1 pathway, all of which directly impact the mRNA translation machinery. As direct regulators of key protein synthesis factors, these kinases, which include MNK1, MNK2 and eEF2K, play a central role in cellular metabolism. MNK1 and MNK2 phosphorylate the key translation initiation factor eIF4E.

Projects 1, 3 & 4 are available for Honours projects and Project 2 is available for PhD or Honours projects.

1. **Prevention of cancer cell migration and invasion by inhibiting eukaryotic elongation factor 2 kinase (eEF2K)**

**BACKGROUND:** Tumour metastasis is a hallmark of malignant carcinomas and is associated with poor prognosis and survival rates among high-risk cancer patients. Most cancer patients die of the resulting secondary tumours rather than the primary one. According to the US National Cancer Institute Surveillance, Epidemiology and End Results (SEER) registries, in 2016, the average survival rates from diagnosed metastatic cancer patients are only 25% or less compared to patients suffering localized (organ-defined) or regional (lymph node invasion) cancers. This has not improved much during the last decade.

In recent years, regulatory mechanisms driven by diverse molecular signalling pathways controlling protein synthesis have been revealed to play central roles in tumour metastasis and progression. Thus, the capacity of the translation machinery of cancer cells to “fine-tune” gene expression has attracted increasing attention in both basic and translational cancer research fields.

**Fig 1.** Cancer cells survive and gain the ability to spread under microenvironmental stress conditions through the inhibition of mTORC1 and subsequent activation of eEF2K.
Although global protein synthesis is generally increased in cancer cells, accumulating data indicate that the translational regulatory networks somehow steer events to adapt to microenvironmental stress conditions and satisfy the survival and spreading requirements of such cells. Regulation of mRNA translation is essential for reshaping the cellular translatome, and thus the proteome, to initiate tumorigenesis and metastasis. However, the underlying mechanisms are incompletely understood. Our recent data show that control of translation elongation plays roles in tumour cell migration and invasion by regulating the proteome of cancer cells.

Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) is a very specific, atypical protein kinase whose only known and validated substrate is eEF2. eEF2 mediates the codon-by-codon translocation of ribosomes along mRNAs and the concomitant movement of aminoacyl-tRNA from A into P site. Phosphorylation of eEF2 by Thr56 by eEF2K impedes the binding of eEF2 to ribosomes and hence impairs translation elongation. Interestingly, eEF2K is highly expressed in some cancers such as medulloblastoma, glioblastoma, breast cancer and pancreatic tumours. It is activated under several stress conditions typically present in the tumour microenvironment, e.g. lack of nutrients, hypoxia, acidosis and/or energy deprivation. We have shown that eEF2K activity can regulate the expression of proteins that important in cell migration and thus tumour metastasis. eEF2K also helps cancer cells survive stress conditions such as nutrient starvation or hypoxia, perhaps by regulating cell metabolism. As it is not essential, on-target side-effects are not expected, so eEF2K may be a valuable new target for preventing cancer metastasis. We are working with drug discovery companies to study this.

AIMS: Recently we have evidence that pharmacological or genetic blockage of eEF2K prevents cell migration and invasion in a variety of cancer cells including lung, breast and prostate cancer cells. In this project, we will explore the molecular mechanisms by which eEF2K activation encourage cancer cell spreading. We will use the state-of-the-art CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) gene editing technology to delete eEF2K in different cancer cell lines as models to explore the role of eEF2K in cancer cell biology including their migration and invasion. The migratory potential of these cells will be tested by two-dimensional wound-healing and three-dimensional transwell invasion assays.

We will study the effects of genetic deletion of eEF2K on cell growth, proliferation and cell cycle, migration, invasion, metabolism and on protein synthesis in these cancer cells. We will also explore how eEF2K promotes cancer cell migration by comparing the spectrum of proteins being synthesized in wild-type (WT) and eEF2K<sup>−/−</sup> cells, by using the BONCAT (bioorthogonal non-canonical amino acid tagging)-pSILAC (pulsed stable isotope-labelling with amino acids in cell culture) technique coupled with mass-spectrometry. These methods are already fully established in the host lab. The project will provide new insights into the potential of eEF2K as a target in anti-cancer therapy.

Key reference:

2. **Systematic analysis of the elf4E interactome: understanding the distinct functional roles of the three mammalian elf4E proteins in translation initiation**

BACKGROUND: One of the most intriguing findings to arise from the recent availability of genome-wide datasets measuring the rates of synthesis and turnover of both mRNA and protein is that cellular protein abundance is determined mainly by controls at the level of translation. Indeed, the majority of variance (~55%) in protein levels is determined by the rates of translation of their mRNAs. Translational control of protein amount has several advantages: for example, one, production of a given protein can be quickly activated at the ribosome, without the need to first transcribe, process and export the template mRNA, and two, a protein’s synthesis can be restricted to specific subcellular locales, which is essential in polarised cells such as neurons or (cancer) cells undergoing directed migration.
While we now understand a great deal about the general mechanisms of translation, our insights into the broader picture of how both cellular environment and sequence-specific translational regulatory mechanisms cooperate to control the translational efficiency (T_i) of a given mRNA under specific cellular states are poor. However, it is clear that translation initiation, i.e., the step where ribosomes are recruited onto the mRNA and locate the start codon, is a key site of regulation. Initiation involves the association of the mRNA with a set of proteins termed eukaryotic initiation factors, eIFs. There are 3 core eIFs (which together form ‘the eIF4F complex’): the mRNA 5’ cap-binding protein eIF4E (which occurs as three different forms encoded by different genes); the RNA helicase eIF4A; and the scaffold eIF4G, which interacts with eIF4E and eIF4A as well as PABP (which in turn also binds the 3’ poly(A) tail of the mRNA). The eIF4F:mRNA assembly – the classic ‘closed-loop’ structure – provides a platform onto which the 43S small ribosomal subunit, already bound to other translation factors and termed the 43S pre-initiation complex (PIC) docks, putting in motion the process of translation.

Interestingly, while the vast majority of translation initiation research has studied the eIF4E-type protein eIF4E1, there are two other mammalian 4E isoforms (arising from the other two genes); we henceforth refer to them as 4E1, 4E2 and 4E3 (the 4Es). We recently made the significant discovery that 4E2 may act as the key mediator of a stress response that achieves selective translational inhibition of mRNAs involved in cellular growth. As the field lacks even basic experimental data on 4E2 and 4E3, understanding our important and novel findings requires a research strategy that yields comprehensive data on the mRNA and protein cohorts of each 4E, and uses the data to build and test models of 4E function.

**AIMS:** In this project, we will probe how changes in the cellular environment affect the individual functions and macromolecular interactions of 4E1, 4E2 and 4E3. In brief, we plan to investigate, using human cell lines grown in culture, how alterations to the cellular environment that have been designed to alter mTORC1 (mammalian target of rapamycin complex 1) signalling affect both the mRNA and protein partners of the three 4Es. As mTORC1 activity has been shown to play a major role in the regulation of translation initiation (partly by mechanisms which are well understood and partly through unknown means) we plan to identify the individual mRNAs and protein partners of each 4E under each of the five cellular growth conditions.

Next, to understand how these cellular environments alter translation, we will use polysome profiling to determine the translational efficiency (T_i) of individual mRNAs, combined with pulsed, isotopic labelling (BONCAT, see above) of cellular proteins to allow a direct readout of the synthesis rates of individual types of proteins. Finally, by integrating the information we will obtain about the translational activity of individual mRNAs with our knowledge of each message’s 4E isoform association, across five different cell contexts, we will gain a number of novel and comprehensive insights into the functional roles of each 4E in translation initiation which can then be tested by experiment.

This project will involve a range of state-of-the-art techniques and will require contributions from a number of researchers, and we expect there will be major roles for both PhD and MPhil/Honours students across this ambitious proposal.

**Key references:**
3. **Cellular-stress induced activation of non-TFEB regulated lysosomal genes**

**BACKGROUND:** Imperative to the survival of the mammalian cell is the ability to catabolise and recycle molecules that are detrimental to its function, whether they be intrinsically toxic at any level, or otherwise harmless or even necessary until a threshold has been reached [1]. Lysosome biogenesis (the formation of a new lysosome, the organelle that is primarily responsible for the degradation of biomolecules) ensures that this occurs. Lysosomal dysfunction is linked to a range of diseases, including neurodegenerative disorders such as Alzheimer’s Disease, and is also important in cancer. Thus, a better understanding of the control of lysosomal gene expression is essential.

Lysosomes are generated at a steady rate in normal conditions. However, during cellular insults (such as an accumulation of toxic products, or nutrient depletion) there is an increase in the expression of lysosomal genes, and in the number and size of lysosomes. Cellular stress-induced transactivation of most lysosomal genes is mediated by the transcription factor EB (TFEB) [2]. Normally, TFEB resides in the cytoplasm in an inactive form, although during cellular stress, TFEB translocates to the nucleus, thus allowing it to increase the expression of its target genes. The DNA sequence to which TFEB binds is known as the Coordinated Lysosomal Expression and Regulation (CLEAR) element, of which most lysosomal genes have at least one copy [2; 3]. Autophagy (the regulated destruction of cellular molecules and structures) is a process that is intricately linked with lysosomal biogenesis and function, and a cohort of autophagy related genes is also transactivated by TFEB during cellular stress [3].

The cellular insults that activate TFEB also increase the expression of lysosomal genes that are not regulated by TFEB, although the means by which this occurs remain elusive. One mechanism which could be involved is the eukaryotic initiation factor (eIF) 2α/activating transcription factor (ATF) 4 pathway. Here, in cases of amino acid deprivation or endoplasmic reticulum stress, eIF2α is phosphorylated by a specific kinase (GCN2 or PERK), which in turn increases levels of ATF4, a transcription factor that, directly or indirectly, increases the transcription of a subset of genes implicated in autophagy [4;5].
project will address the question ‘could this pathway also be involved in the transcription of lysosomal genes that are not regulated by TFEB?’ Red/green refers to inactive/active components.

**HYPOTHESIS:** the eIF2α/ATF4 pathway contributes to the control of lysosomal gene expression under specific conditions.

**AIM:** to investigate the role of the eIF2α/ATF4 pathway in the cellular-stress induced increase in non-TFEB gene transcription.

**PROJECT OUTLINE:** Initially, human cell lines (including 293T and A549) and mouse embryonic fibroblasts (MEFs) will be treated with pharmacological activators of the eIF2α/ATF4 pathway. These include brefeldin A (which promotes endoplasmic reticulum stress) and histidinol (which mimics amino acid starvation). Both can induce expression of ATF4. Quantitative real-time polymerase chain reaction (qRT-PCR) and/or immunoblotting will be then be used to determine the levels of candidate lysosomal mRNA and proteins, respectively. As controls, levels of ATF4 and C/EBP homologous transcription factor (CHOP, a direct target of ATF4) will concurrently be determined, as these are expected to increase in these circumstances.

To further confirm the responses to the chemical treatments, the previous experiments will be repeated using MEFs where stress-inducible eIF2α phosphorylation has been genetically inactivated by knocking out GCN2 or PERK. If levels of a candidate ATF4-regulated lysosomal gene are increased by brefeldin A or histidinol in normal cells, but not in the genetically modified lines, this will further implicate the eIF2α/ATF4 pathway in non-TFEB driven lysosomal gene expression.

Luciferase reporter assays will then be used to delineate the specific DNA sequence (known as the response element) to which the trans-activating protein associates. Finally, to identify which candidate transcription factors (such as ATF4 or CHOP) bind to the response elements of the genes of interest, chromatin immunoprecipitation (ChIP) assays will be employed.

During this project, you will learn a range of up-to-date molecular biology techniques and the data will contribute to understanding the control of autophagy in relation to human disease.

**KEY REFERENCES:**

4. Exploring the functions of MAPK-interacting kinases (MNKs) in metabolic disease

**BACKGROUND:**

**Metabolic disease:** In Australia and many other countries, the prevalence of overweight and obesity have increased significantly over the past two decades. The most recent population data indicate almost two-thirds of Australian adults are overweight or obese. Being overweight or obese increases the risk of developing long-term comorbidities including cardiovascular disease, hypertension and type-2 diabetes. These diseases present an enormous burden to patients, their carers, health systems, and communities. There is therefore an urgent need to understand the mechanisms underlying obesity and weight gain and to explore novel therapeutic strategies to tackle the obesity epidemic.

The mitogen-activated protein kinase (MAPK)-interacting kinases (MNKs) are a family of kinases activated by signalling through MAPK (ERK and p38) pathways. The best-studied and only validated in vivo substrate of the MNKs is eukaryotic initiation factor 4E (eIF4E), which binds the 5’-m7G cap structure of eukaryotic mRNAs to facilitate the recruitment of ribosomes and their subsequent scanning to initiate mRNA translation. eIF4E phosphorylation is not critical for general mRNA translation and its biological significance is yet to be fully
resolved (Ueda et al., 2004). It is suggested however, that phosphorylation may cause the release of eIF4E from the initiation complex, enabling it to initiate another round of mRNA translation and/or to recruit additional mRNAs into active polyribosomes. The phosphorylation of eIF4E may also be important for regulating the translation of specific mRNAs in response to certain stimuli.

**MNKs are novel potential targets in metabolic disease:** Recently, the MNKs were identified as a novel potential therapeutic target for managing obesity and improving metabolic health (Moore et al., 2016). Eliminating MNK activity was shown to protect animals fed a high-fat diet (HFD) from weight gain, adipose tissue (AT) inflammation and the onset of glucose intolerance, which leads to type-2 diabetes. It is hypothesised the MNKs play a crucial role in the biology of AT and muscle, which are important sites of insulin-regulated metabolism.

Adipose tissue is a dynamic, hormone-responsive tissue that adapts to energy requirements, storing excess energy in times of energy abundance (fed state) and releasing energy in times of energy deprivation (fasting) to maintain systemic energy homeostasis. Our data show that MNKs play a key role in the differentiation of fat cells (adipocytes) which may explain why MNK knock-out mice gain less weight on a HFD.

The major tissue responsible for insulin-stimulated glucose uptake from the blood is skeletal muscle. Insulin stimulates glucose uptake by eliciting the mobilisation of the glucose transporter 4 (GLUT4) from intracellular vesicular compartments to the cell membrane. A key player here is protein kinase B (PKB, also called Akt) which is activated by insulin and controls GLUT4 localisation. Insulin resistance is a major characteristic of type-2 diabetes whereby the capacity of insulin to increase glucose uptake is significantly reduced. Our data indicate that MNKs regulate the ability of insulin to switch on PKB and glucose transport. This may explain why HFD-fed MNK-KO mice retain better glucose tolerance that WT animals.

**Figure 1:** (A) The MNKs play a key role in promoting adipocyte differentiation and this will be explored using the 3T3-L1 system; (B) MNKs may impair the ability of insulin to switch on PKB and thus affect glucose transport by controlling the translocation of GLUT4 to the cell membrane – to be studied in C2C12 cells.

**Cell systems to be used in this project:** We have shown that knock out mice lacking MNKs fed a high fat-diet gain less weight and display improved glucose metabolism such as lower circulating blood glucose levels and greater insulin sensitivity compared to WT mice. We hypothesise that the MNKs play a role adipose biology and insulin-regulated metabolism in skeletal muscle. For this project the murine 3T3-L1 pre-adipocyte and C2C12 murine myoblast (muscle) lines will be the primary models of investigation. The 3T3-L1 model is the ‘gold-standard’ for studying adipocyte differentiation and biology, while C2C12 myoblasts can be differentiated into contracting myotubes upon serum depletion and express proteins characteristic of functionally mature muscle cells.

**AIMS:** To use specific pharmacological MNK inhibitors to investigate the roles of these protein kinases in fat cell biology, insulin signalling and glucose metabolism, using the 3T3-L1 and C2C12 cell systems.
You will learn and apply a range of molecular biology techniques including reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and Western blot to assess changes in mRNA and protein expression, respectively.

You will not be working with animal models in this project.

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