

Nutrition & Metabolism

Cell Signalling & Gene Regulation

Honours Projects, 2019

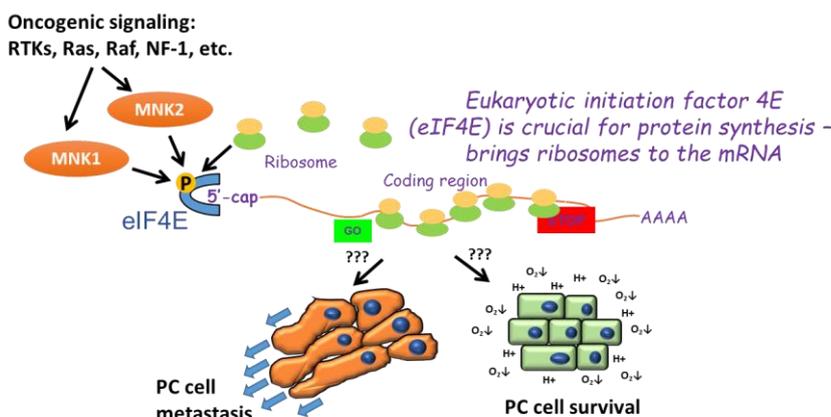
Prof. Proud's laboratory studies the signalling pathways by which hormones, growth factors and nutrients regulate the function of mammalian cells, especially protein metabolism. The proper control of these pathways plays a crucial role in cell growth and proliferation, the storage and utilisation of 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 currently studying the role of several protein kinases closely related to the mTORC1 pathway and which directly impact the mRNA translation machinery. As direct regulators of key protein synthesis factors, these kinases, MNK1, MNK2 and eEF2K, play a central role in cellular metabolism. MNK1 and MNK2 phosphorylate the key translation initiation factor eIF4E, which is strongly implicated in cancer.

1. Targeting MAP kinase-interacting kinases (MNKs) as future treatment for prostate cancers.

BACKGROUND: In Australia, prostate cancer (PC) is the most common male cancer. According to GLOBOCAN, it is ranked 4th in terms of total cancer incidence. Over 1 million cases of PC and 300,700 related deaths were recorded worldwide in 2012. The main cause of mortality in PC is metastasis, a hallmark of malignant carcinomas that has poor prognosis and survival rates. The main clinical interventions for metastatic PC primarily relies on androgen deprivation therapies, designed to suppress testosterone synthesis. Despite being initially effective, patients generally develop castration-resistant cancers and currently there is no effective treatment at this stage.

In recent years, regulatory mechanisms driven by diverse molecular signalling pathways controlling protein



synthesis have been revealed to play central roles in tumour growth and metastasis. 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. Eukaryotic initiation factor 4E (eIF4E) plays a crucial role in protein synthesis by binding the 5'-terminal cap structure of mRNAs and the

scaffold protein eIF4G. eIF4E is phosphorylated by the MAP kinase-interacting protein kinases (MNKs), which also bind eIF4G. Enhanced levels of eIF4E and of its phosphorylation are linked to oncogenesis in

Fig 1. The best characterized substrate of the MNKs – eIF4E has been implicated in protein synthesis and the progression of prostate cancer (PC).

PC. Increased association of eIF4E with eIF4G enhances the ability of MNK to phosphorylate eIF4E which has been shown to promote tumorigenesis in PC (1).

Global protein synthesis is generally increased in cancer cells, and 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 proteome to initiate and drive tumorigenesis and metastasis. However, the underlying mechanisms are incompletely understood. Better understanding is essential to guide development of novel therapies for PC. Our recent data show that the MNKs play an essential role in cancer cell migration/invasion in breast cancer cells (2). As the MNKs are not essential, we do not expect on-target side-effects of drugs that inhibit MNKs, so they may be a valuable new target for preventing PC progression. We are working with drug discovery companies to study this.

AIMS: In this project, we will explore the molecular mechanisms by which MNK/eIF4E 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 the MNKs in PC cell lines as models to explore the role of MNKs 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 MNKs on cell growth, proliferation and cell cycle, migration, invasion, metabolism and on protein synthesis in these cancer cells. The function of the MNK-mediated phosphorylation is currently not known: to address this key question, we are using a novel high-throughput technique to identify mRNAs whose translation is affected by the MNKs. This involves using RNA-eIF4E cross-linking combined with next-generation sequencing technology to pinpoint those mRNAs regulated by the phosphorylation of eIF4E.

To complement this approach, we will also compare the spectrum of proteins being synthesized in wild-type (WT) and *MNK1/2*^{-/-} cells, by using the BONCAT (bioorthogonal non-canonical amino acid tagging)-pSILAC (pulsed stable isotope-labelling with amino acids in cell culture) technique. These methods are already fully established in the host lab.

The data will provide crucial new insights into the potential of MNKs as targets for anti-PC therapy.

Key references:

1. Furic L, Rong L, Larsson O, Koumakpayi IH, Yoshida K, et al. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *PNAS*. 107(32): 14134-9, 2010.
2. Tian S, Wang X, **Proud CG**. Oncogenic MNK signalling regulates the metastasis suppressor NDRG1. *Oncotarget*. 8(28):46121-35, 2017.
3. Bramham CR, Jensen KB, **Proud CG**. Tuning Specific Translation in Cancer Metastasis and Synaptic Memory: Control at the MNK-eIF4E Axis. *Trends Biochem Sci*. 41(10):847-858, 2016.

2. Novel pharmaceutical inducers of the integrated stress response (ISR)

BACKGROUND:

Imperative to the survival of the mammalian cell is the ability to catabolise and recycle molecules under conditions of acute stress, such as nutrient deprivation. Autophagy (the regulated destruction of cellular molecules and structures) can thus serve as a pro-survival process (at least in the short-term) that permits the cell to achieve break down unwanted or defective components (e.g., proteins) to provide building blocks for synthesis or energy production (such as amino acids).

One pathway which promotes autophagy is the integrated stress response (ISR), central to which is the eukaryotic initiation factor (eIF) 2 α /activating transcription factor (ATF) 4 axis. Here, in cases of amino acid deprivation or endoplasmic reticulum stress, eIF2 α is phosphorylated by a specific kinase (GCN2 or PERK, respectively). To conserve the cell's resources, this event results in the decreased translation of most messenger (m) RNAs. However, it also causes the increase in translation of a few mRNAs, including that for ATF4, a transcription factor. In turn, ATF4 increases transcription of a distinct subset of genes implicated in autophagy [1] (Figure 1).

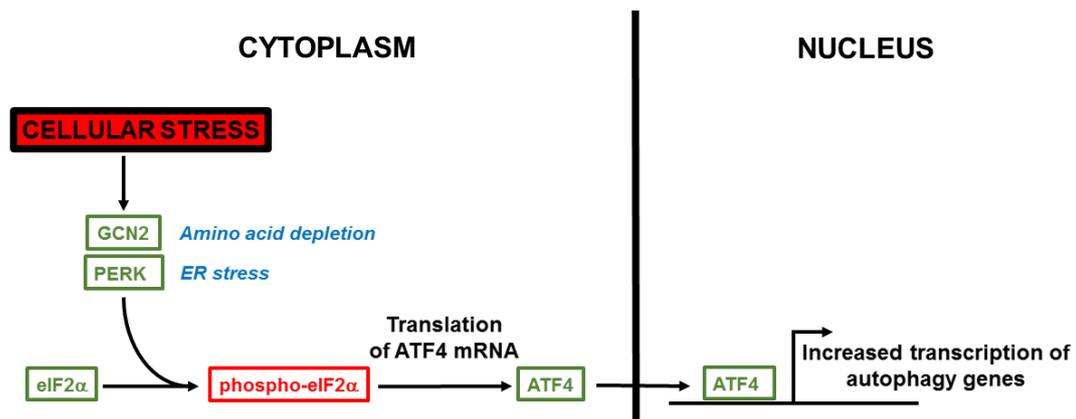


Figure 1. Schematic of the integrated stress response (ISR).

Cyclosporin (CSA) is an antibiotic that inhibits the phosphatase activity of the protein phosphatase calcineurin by forming a complex with cyclophilin (CpN). The immunosuppressant function of CSA, which provides its pharmaceutical benefit, is believed to be elicited by inhibiting the activity of calcineurin (CaN) towards nuclear factor of activated T-cells (NF-AT) in T-lymphocytes, thus decreasing its translocation to the nucleus and subsequent transcription of genes for inflammatory cytokines (Figure 2). CaN is now also reported to regulate Gcn2 in yeast [2]. However, the role of CaN in the mammalian ISR is yet to be investigated in detail. Given its widespread use as an anti-inflammatory agent in humans, e.g., to prevent organ transplant rejection, it is crucial to learn more about the other effects of CSA. Our initial data demonstrate that CSA can increase eIF2α phosphorylation, ATF4 protein levels and ATF4 target gene mRNA levels in mammalian cell culture [Fedele et al., *unpublished*].

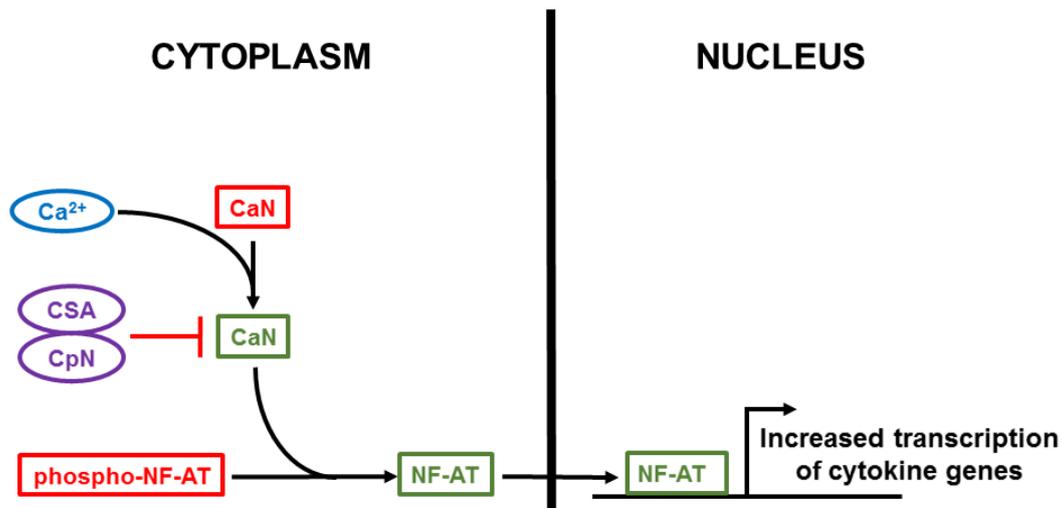


Figure 2. Schematic of the mode of action of cyclosporin A (CSA) in inhibiting NF-AT by blocking the activity of the protein phosphatase, CaN (calcineurin) by binding to cyclophilin.

HYPOTHESIS: CSA induces the ISR in mammalian cells.

AIM: To investigate the molecular mechanism by which CSA induces the phosphorylation of eIF2α and subsequent increase in ATF4 translation.

PROJECT OUTLINE:

Initially, to test the hypothesis, in addition to CSA, cells 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, whose levels will be determined by immunoblotting. Quantitative real-time polymerase chain reaction (qRT-PCR) and/or immunoblotting will be then be used to determine the levels of known downstream targets of ATF4, such as C/EBP homologous transcription factor (CHOP, another transcription factor).

To elucidate how CSA induces the ISR, the previous experiments will be repeated using cells where stress-inducible eIF2 α phosphorylation has been genetically inactivated by reducing the expression of GCN2 or PERK and/or where CSA's partner, CpN, or CaN have been knocked-down. If CSA's ability to induce the ISR is ablated by any of the knock-downs, this will implicate the targeted protein in the molecular mechanism.

Finally, combined treatments of physiologically relevant cells (such as T-lymphocytes) with CSA and inhibitors of the ISR (such as ISRIB, which inhibits the downstream consequences of eIF2 α phosphorylation) will be performed to determine whether the immunosuppressant nature of CSA can be maintained, whilst eliminating the capacity to induce the ISR. If so, this could form the basis of a treatment regimen that provides the desired immunosuppression without the coincidental chronic induction of the ISR.

During this project, you will learn a range of up-to-date molecular biology techniques. Furthermore, the data will contribute to understanding the control of the ISR, as well as the possible role it may perform in side-effects, experienced by the not insignificant number of patients whose disease treatments require the administration CSA for immunosuppression.

KEY REFERENCES:

1. Pakos-Zebrucka K, et al. 2016. EMBO Rep. 17: 1374-1395. 2. Vlahakis A, et al. 2014. Proc Natl Acad Sci U S A. 111: 10586-10591.

3. 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 protects 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. We hypothesise that MNKs play a key role in the biology of AT and muscle.

Adipose tissue is a dynamic, hormone-responsive tissue that adapts to energy requirements, storing excess nutrients in times of energy abundance (fed state) and releasing them 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) by regulating the induction of genes that are key players in adipogenesis. MNKs likely control the expression and/or function of the transcription factors that drive adipogenesis. This 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 may 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 than WT animals.

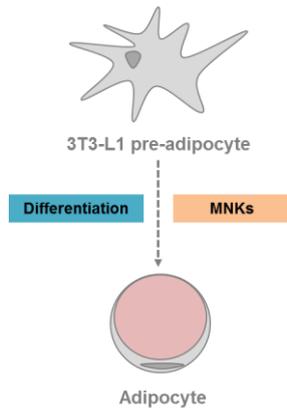


Figure 1: The MNKs play a key role in promoting adipocyte differentiation and this will be explored using the 3T3-L1 system.

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 in 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.

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