UNIVERSITY OF BIRMINGHAM

Project 1: **Role of cap 1 and cap 2 2'-O-ribose methylation in Drosophila development and nervous system function.**

and

Project 2: **LIM Domain Only Proteins, LMO1 and LMO4 in Hematopoiesis**

by

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A combined research thesis submitted to the University of Birmingham as part of the requirement for the degree of MASTER OF RESEARCH in Molecular and Cellular Biology.

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Summary

Role of cap 1 and cap 2 2'-O-ribose methylation in Drosophila development and nervous system function (Page 1) - The presence of cap 1 and cap 2 2'-O-ribose methylation on the second and third nucleotide of mRNA has been known for more than 30 years however studies into the role of these methylations are very limited. Research has shown that Cap 1 methylation may be related to translational efficiency however no studies have shown a role for Cap 2 methylation. This study aims to extend the understanding of the function of these methylations in the neural development of Drosophila.

LIM Domain Only Proteins, LMO1 and LMO4 in Hematopoiesis (Page 72) - The LIM Domain Only (LMO) proteins have been shown to play a major role in cellular differentiation and development at the embryonic stage and in many human cancers. The aim of this study was to develop tools to perform overexpression and knockdown experiments with LMO1 and LMO4 proteins in murine early stage hematopoietic progenitors and ES cells in an attempt to identify their function. Preliminary results show a possible apoptotic effect of LMO4 knockdown in myeloid progenitor cells. This study provides the basis for future research into these proteins.

Role of cap 1 and cap 2 2'-O-ribose methylation in Drosophila development and nervous system function.

by

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Project 1 - MRes in Molecular and Cellular Biology

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ABSTRACT

The presence of cap 1 and cap 2 2'-O-ribose methylation on the second and third nucleotide of mRNA has been known for more than 30 years however studies into the role of these methylations are very limited. Past studies have shown a potential link to increase in translational efficiency by the presence of cap 1 methylation however there is little information for the role of cap 2 methylation. This study attempts to contribute to the understanding of these functions through the study of cap methylation in D.*melanogaster*. A number of trials were carried out looking for a link to neural plasticity through the use of ion channel blocking compounds and interaction with the TOR pathway through dietary restriction. When the ion channel blockers, Ethosuximide and Propranolol , were applied the survival rate of $Cap2^{null}$ mutants was decreased more than in $Cap1^{null}$ mutants, however $Cap2^{null}$ mutants were least effected by nutrient depravation. The results show some potential areas for further research to further elucidate the roles of cap 1 and 2 mRNA methylation.

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INTRODUCTION

In 2000 the sequencing of the entire human genome was completed and was heralded as the answer to many biological and genetic questions. This information however serves little purpose without understanding how the expression of these genes is controlled (Orphanides & Reinberg, 2002). Control of gene expression is an essential process for both prokaryotes and eukaryotes and is particularly important in complex multi-cellular organisms. Almost every cell in an organism has the potential to express every gene within its genome however this has to be controlled to allow the creation of the many specialised cells found throughout a complex organism. Gene expression is also controlled by environmental factors, particularly in single cellular organisms but also in more complex organisms. Understanding the different pathways for gene expression control is essential for making use of the information obtained from genome sequencing projects and has the potential for development of new medicines and treatments.

Central to this whole regulatory process is the way in which proteins are produced from DNA. There are two basic stages in this process, transcription, the conversion of genes in the DNA sequence to mRNA and translation, the conversion of the mRNA sequence into amino acids which then form a polypeptide chain which can fold to produce the tertiary structure of a protein (Campbell, *et al.* 2008). Transcription is a three stage process, in the first stage, initiation, RNA polymerase binds to a specific promoter site on the DNA sequence. In eukaryotes this binding is mediated by a group of proteins known as transcription factors. Transcription factors have to be present and bind to the promoter site to allow RNA polymerase to bind, this is

known as the transcription initiation complex. Once bound, RNA polymerase begins to unwind and separate the double DNA strand to allow access to the nucleotide bases. During the next stage, elongation, the RNA polymerase moves along the DNA strand, from the 3' to 5' end, creating a complementary RNA strand and allowing the DNA to rewind behind it. In the final stage, termination, the RNA polymerase disassociates from the DNA strand releasing the completed RNA strand (Campbell, *et al.* 2008). During the transcription process the newly produced RNA strand undergoes pre-mRNA processing to modify the RNA ready for translation outside the nucleus at ribosomes in the cytoplasm.

Translation then takes place at ribosomes within the cell cytoplasm. During translation the mRNA passes through a ribosome which enables tRNA interpreters to bind to complimentary sequences in the mRNA. Each of the tRNA molecules has an anticodon on one end and the complimentary amino acid on the other, as these bind to the mRNA they leave behind the amino acids producing a polypeptide chain. Once complete the polypeptide chain will then be able to fold into a protein (Campbell, *et al.* 2008).

Gene Expression Control

There are a number of points throughout this DNA to Protein pathway at which gene expression can be regulated. These include transcriptional initiation, post transcriptional modification, translational initiation and post translational modification (King, 2013). The majority of expression control in eukaryotes is carried out at the transcriptional initiation stage this either occurs through limiting access to the DNA

by either chromatin modifications or DNA methylation or during recruitment of transcription factors (Hoopes, 2013). Transcription factors are required in eukaryotes to allow for the binding of RNA polymerase II to the DNA strand and by modifying the availability of transcription factors this can control initiation of transcription (Campbell, *et al.* 2008).

If transcription is able to take place the next gene expression control points are in the RNA processing stages prior to translation. There are a number of processes which occur to prepare an mRNA strand for translation. These include capping, splicing and packaging, which are important processes to protect the mRNA from degradation, for its successful transportation to the ribosomes and to allow initiation of translation (Soller, 2006). By limiting any of these modifications the gene expression process can be slowed or completely halted. If the mRNA has been fully processed and it successfully reaches the ribosomes controls can also be applied at the translational initiation stage. The mRNA has to bind to the translation initiation complex, this binding can be limited by both the modifications applied to the mRNA during processing and the presence of 4E-BP's (4E Binding Proteins) which act as inhibitors on the translation initiation complex (Penney, *et al*. 2012). The final stage of gene expression regulation acts following translation on the amino acid sequence and the folding and transportation of the protein, this can include methylation, acetylation and disulphide bond formation amongst many other processes (King, 2013).

Pre-mRNA Processing

This study focuses on the controls in the post-transcription and translation initiation stage of the gene expression process. Before RNA can be transported from the nucleus for translation it has to undergo a number of modifications to become a mature mRNA strand and be packaged into a ribonucleoprotein complex (mRNP) to allow it to pass through the pores in the nuclear membrane. There are four main modifications which occur during this processing, 5' end capping, splicing, polyadenylation and packaging (Carmody & Wente, 2009). The first modification to occur is capping of the 5' end which happens after the first 20-30 nucleotides have been added to the RNA Strand (Moore & Proudfoot, 2009 & Carmody & Wente, 2009). This cap serves a number of purposes, in particular the prevention of degradation by nuclease enzymes within the cell (Orphanides & Reinberg, 2002). The basic cap is a 7-methyl guanosine cap on the first nucleotide in the transcript, this is referred to as cap 0 and found in animals, plants and yeast. In animals there is also potential for methylation of the two following nucleotides, known as cap 1 and cap 2 methylation (Kruse, *et al*. 2011).

The second modification which occurs to pre-mRNA is splicing which involves the removal of introns, leaving only the exons which code for the required amino acids. This is done by an assembly known as the spliceosome, which is composed of small nuclear ribonucleoproteins (snRNPs) (Campbell, *et al.* 2008). The pre-mRNA molecule also undergoes polyadenylation, gaining a poly-A-tail of between 50 and 250 adenine molecules on the 3' end. This partially serves a similar purpose to the 5' capping, acting to protect the RNA from degradation (Campbell, *et al.* 2008). If this

processing isn't completed successfully the mRNA molecule will be degraded by processes such as non-sense mediated decay (Cheng, *et al*. 2006).

In the final stage of mRNA processing it is packaged into messenger ribonucleoprotein (mRNP) complexes which are composed of the mRNA and a number of proteins to assist with transport out of the nucleus and the initiation of translation (Hieronymus & Silver, 2004). The mRNP complexes are transported out of the nucleus through nuclear pore complexes (NPCs) (Carmody & Wente, 2009).

5' Cap Methylation

The particular area of interest for this study is the 2'-O-ribose methylation which can occur as part of 5' capping on the second and third neucleotides of mRNA, known as cap 1 and cap 2 (Kruse, *et al*. 2011). As previously explained all mRNA gains a 7-methyl guanosine cap on its first nucleotide at the 5' end (cap 0) (fig 1.1). This is essential in mammalian cells as without this cap the RNA strand would be degraded (Werner, *et al.* 2011). This cap is formed by the process of three enzymes, a triphosphatase which causes hydrolysis of 5' γ-phosphate, a guanyltransferase which adds a guanine monophosphate nucleoside and a methyl transferase which methylates the N7 position of the guanine molecule (Moore & Proudfoot, 2009, Soller, 2006 & Ghosh & Lima, 2010). Often in higher eukaryotes the triphosphatase and guanyltransferase are combined into one enzyme with 2 active sites (Belanger, *et al*. 2010). It is believed that cap 0 formation is a co-transcriptional process with the methyltransferase enzyme binding to the c-terminal domain of RNA polymerase II during transcription meaning that this is the first modification which occurs to the RNA strand (Belanger, *et al*. 2010). It has been suggested that as well as preventing

degradation by exonucleases, this cap effects the efficiency of translation, the initiation of translation and splicing, 3' end processing and neucleocytoplasmic transport (Soller, 2006, Belanger, *et al*. 2010, Werner, *et al.* 2011, Kruse, *et al*. 2011). It is thought that the cap 0 methylation interacts with the nuclear binding complex which is why it influences the formation of the 3' end and also splicing (Kruse, *et al*. 2011). The influence of cap 0 methylation on translation efficiency mainly stems from its interaction with eIF4E (Eukaryotic translation initiation factor 4E) which is part of the eIF4F cap binding complex (Kruse, *et al*. 2011 & Costa-Mattiolo, *et al*. 2009).

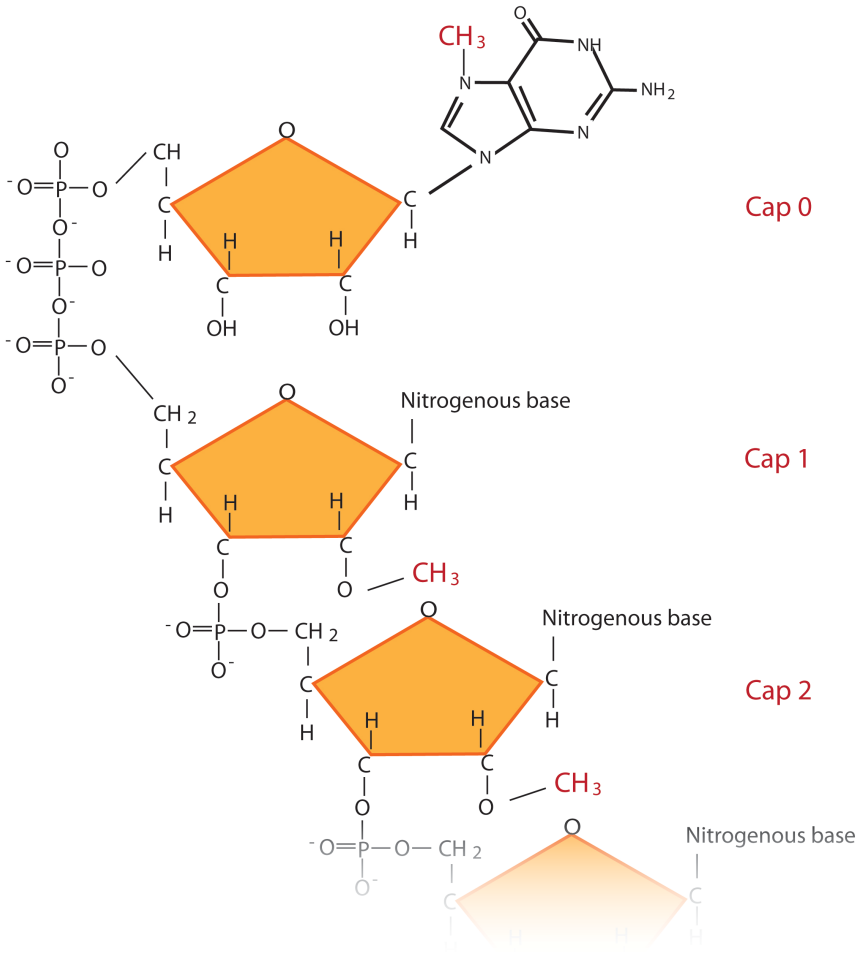

Figure 1.1: 5' end of mRNA molecule showing cap 0, 1 and 2 methylation on the first second and third nucleotides respectively (Kruse, *et al*. 2011 & Werner, *et al.* 2011).

Some studies suggest that cap 1 and cap 2 methylation are formed sequentially after the cap 0 formation, however both cap 1 and cap 2 methylation can occur without the presence of other methylation (Werner, *et al.* 2011). It has however been shown that the presence of cap1 increases the efficiency of cap 2 methylation (Werner, *et al.* 2011). It is thought that in Humans cap 1 methylation is found on all mRNA molecules and cap 2 is found on around half, however there is no study of the frequency of its occurrence in other organisms (Belanger, *et al*. 2010 & Werner, *et al.* 2011).

Cap 1 and Cap 2 are 2'-O-methylations on the first and second nucleotides respectively and are produced by two different methyltransferase enzymes. In humans these are know as hMTr1 and hMTr2 and were first identified in HeLa cells over 30 years ago by Langberg & Moss (1981) however there is a lack of research into the exact functioning of these proteins. hMTr1 is found only within the nucleus meaning that cap 1 methylation must occur before the mRNA strand is transported out of the nucleus and may occur co-transcriptionally in the same way as cap 0 formation (Werner, *et al.* 2011 & Belanger, *et al*. 2010). Some studies have shown that the presence of cap 1 promotes binding to ribosomes and therefore increased translational efficiency in vitro, there is however no evidence in current studies that this causes any developmental or phenotypic effects in vivo (Kruse, *et al*. 2011 & Belanger, *et al*. 2010).

The hMTr2 protein is found throughout the cell including the nucleus, however it is mainly active in the cytoplasm with <5% activity in the nucleus (Werner, *et al.* 2011). It has been shown that hMTr2 can function without cap 1 methylation being present

but at much reduced efficiency (Werner, *et al.* 2011). The purpose of cap 2 methylation is largely unknown however a study by Werner, *et al.* (2011) has shown that mRNA strands with all 3 caps present have a higher affinity to ribosomes indicating increased transcriptional efficiency. There is also currently no study to suggest why cap 2 methylation is present on some mRNA strands and not others and whether this is just randomly occurring.

Another type of methylation is possible when the second nucleotide is adenosine which can become methylated at the N^6 position, known as m^6Am. This methylation occurs alongside regular cap 1 methylation and is produced by a unique methyltransferase which is mainly active in the cytoplasm. It has had very limited study and the purpose and frequency of it is currently unknown (Kruse, *et al*. 2011).

5' Cap Methylation in Viruses

Kruse, *et al*. (2011) suggest that cap 1 methylation must be important as many viruses encode their own methylase enzymes to form cap 1 methylation. This is particularly seen in viruses which are active in the cytoplasm such as west nile virus and vesicular stomatitis virus supporting the theory that cap 1 formation is usually carried out in the nucleus (Daffis, *et al.* 2010). It is thought that these viruses encode methyltransferase enzymes to produce cap 1 methylation to reduce the host cells immune response (Kruse, *et al*. 2011). Daffis, *et al.* (2010) showed that the presence of cap 1 in viral mRNA reduced the activation of IFIT genes which are known to restrict translation. This possibly evolved as an immunity method to allow cells to differentiate between host and viral mRNA. No viruses have been reported which

encode for cap 2 or m^6Am methylases, however viral mRNA can gain this methylation through action of the host cell methylases.

mRNA cap methylation in Drosophila *Melanogaster*

The orthologs to the human *hMTr1* and *hMTr2* genes in D. *melanogaster* are know as *CG6379* and *aft*, they have amino acid similarities of 30% and 40.27% respectively (GeneCards, 2013a & GeneCards, 2013b). This suggests that D.*melanogaster* is a good model organism to relate any findings back to humans. They are also shown to be close evolutionary relatives for these genes on the phylogenetic tree (fig 1.2).

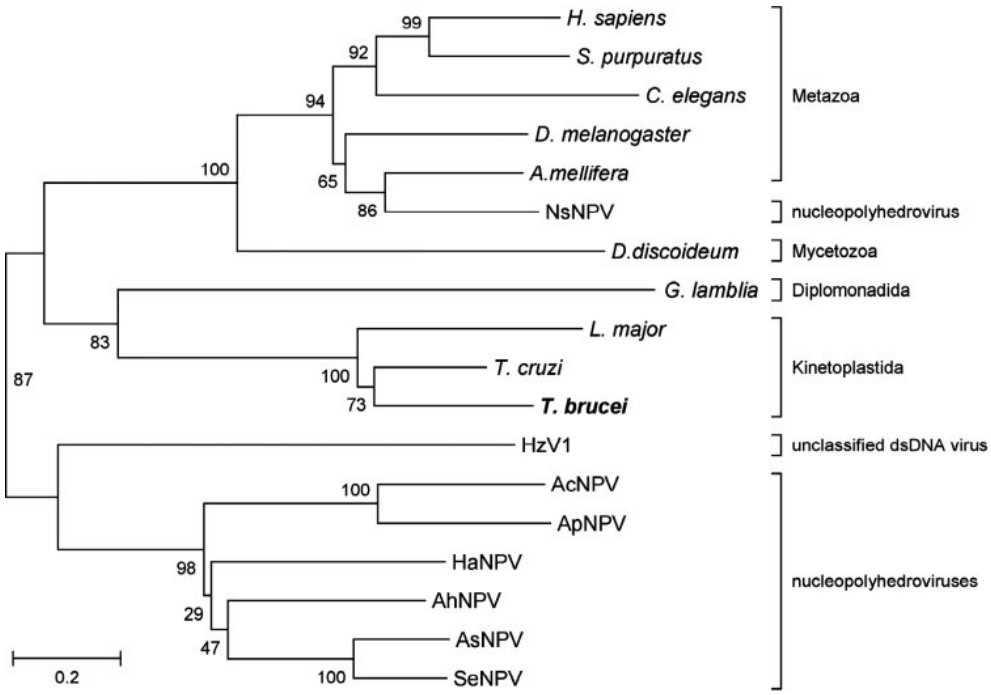

Figure 1.2 Phylogenetic tree for hMTr1 gene, numbers show the percentage of bootstrap support (Zamudio, *et al*. 2007).

Translation Efficiency and Synaptic Homeostatic Control

To allow the nervous system to have plasticity which is required during development, growth, memory formation and learning, synapses need to have a system of homeostatic control (Turrigiano, 2008). A number of different routes for this control have been proposed, the most significant of which are changes in the expression of post-synaptic receptors and retrograde control of neurotransmitter release (Penney, *et al*. 2012). It is believed that these are mainly regulated by translation (Costa-Mattiolo, *et al*. 2009).

Most regulation of translation in eukaryotes occurs at the initiation stage where a ribosome is recruited to an mRNA strand. Initiation is a 3 stage process, firstly a 43S ribosomal pre-initiation complex is formed, mRNA is then bound to this complex before the 80S ribosomal complex formation (Costa-Mattiolo, *et al*. 2009). The most common control point in this process is at the cap binding protein eIF4E. This protein works in a complex known as eIF4F which also comprises an RNA helicase enzyme to unwind the secondary structure of the 5' UTR (eIF4A) and a pair of bridging proteins to bind the mRNA to the 43S pre-initiation complex (Costa-Mattiolo, *et al*. 2009). eIF4E can be inhibited by 4E-BP's (4E Binding Proteins) and will also not bind to an mRNA molecule without the cap 0 methylation being present (Penney, *et al*. 2012). Some studies have also suggested that the presence of cap 1 may increase the recognition and binding ability of the mRNA to eIF4E (Kruse, *et al*. 2011). However there are currently no studies to suggest what, if any effect, the presence of cap 2 methylation has on the efficiency of this binding.

The TOR (Target of Rampmyecin) pathway is also thought to play an important role in regulation at the translation initiation stage and therefore potentially synaptic homeostasis (Penney, *et al*. 2012). Studies have shown that organisms which receive reduced nutrient intake tend to experience an increase in life span as a result of reduced activity in the TOR pathway (Layalle, *et al.* 2008). This is thought to be partially due to the absence of TOR pathway activity reducing the rate of translation (Penney, *et al*. 2012). Under normal circumstances TOR interacts with the eIF4F cap binding complex in two ways. Firstly TOR phosphorylates 4E-BP's which prevents them from being able to bind to eIF4E. This reduces the level of inhibition taking place and leaves more available protein to bind to mRNA 5' Caps to initiate translation (Teleman, *et al. 2005*). TOR also phosphorylates S6K, which amongst other actions results in the phosphorylation of eIF4B, which in turn promotes the helicase function of eIF4A increasing its ability to unwind the 5' UTR section of the mRNA (Penney, *et al*. 2012). This means that in a condition of reduced nutrients 4E-BP's and the absence of S6K phosphorylation would result in much reduced translational efficiency (fig 1.3).

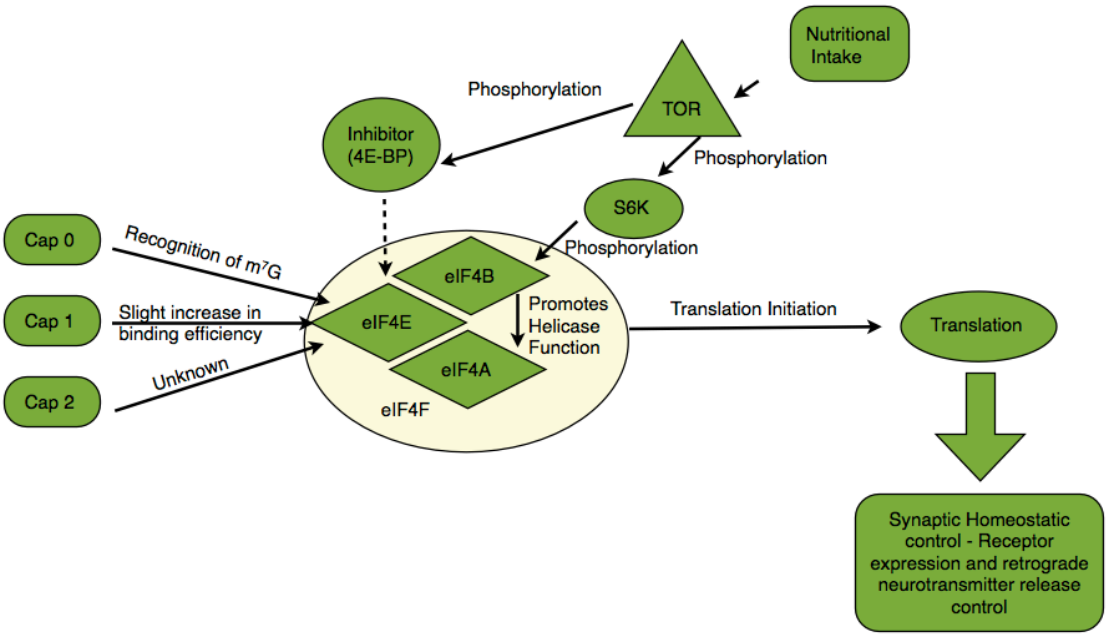

Figure 1.3 Interactions between mRNA cap methylation, the translation initiation complex and the TOR pathway (Penney, *et al*. 2012, Kruse, *et al*. 2011, Li, *et al*. 2010 & Costa-Mattiolo, *et al*. 2009).

To assess the influence which cap 1 and 2 methylation have on drosophila development and nervous system function through the regulation of translation this research follows several paths. As it has been shown that reduced nutrient intake results in less activity in the TOR pathway and therefore an increase in translational efficiency a study of CG6379^{null} and aft^{null} mutant flies with reduced nutrient intake should demonstrate whether these methylations result in increased translational efficiency. Trials were also carried out using ion channel blocking compounds and neurotransmitters to establish whether cap mutants would be able to homeostaticly balance their synapses to live and grow normally. The most likely root for any effect would be if the absence or presence of cap 1 or 2 methylation increased or decreased translational efficiency therefore having a similar effect on life span to the TOR pathway.

MATERIALS AND METHODS

Stocks and Maintenance

Stocks of flies of each genotype were created in the lab as part of a previous project. The four genotypes used were a *CG6379*^{null} mutant which results in an absence of cap 1 methylation, an aft^{null} mutant preventing cap 2 methylation and a double mutant with neither gene so with an absence of both cap 1 and 2. The flies used as a control in all experiments were yellow/white mutants as this was the background genotype of the cap mutants. The Cap mutants were produced as part of a previous project having deletions starting at the P-element in the promoter and deleting the catalytic domain of the genes. After production of these flies their genotype was checked by PCR (results not shown).

All stocks were stored at room temperature on either standard or +20% yeast fly food medium as detailed below. All experimental work, unless otherwise stated, was carried out incubated at 25°C in a humidity controlled incubator with a 12:12 hour light:dark cycle. When anesthetisation was required for counting or sorting of flies a CO_2 jet or pad was used and the time kept to a minimum to prevent any long term damage to the flies.

Fly Food Medium

Medium for all trials was produced using a standard method and recipe although some trials used an increased nutrient food with 20% additional yeast (table 2.1).

Table 2.1 Ingredients for standard drosophila food medium.

Ingredients	Quantity for 1.75 litre batch
Water	1700 ml
Agar	18 g
Nipagin	50 ml
Dextrose	150 g
Cornmeal	170 g
Yeast	30 g (+20% = 36g)

The medium was produced by boiling the water, agar and nipagin until all agar was dissolved. The dextrose, cornmeal and yeast were then added and the mixture was stirred continually and heated until a smooth consistency was achieved. The medium was then dispensed into plastic and glass vials with approximately 10ml in each vial. Once cooled the vials were then plugged with cotton wool and refrigerated until needed. When used for most purposes a small amount of dry yeast was added to the top of the medium to provide additional food for the adult flies.

Nutrition Trial

A study was carried out on the effect of varying the nutritional components supplied to the flies of the four different genotypes. Five different food types were setup, two with reduced yeast at 20% and 2%, one with no dextrose, one with all nutritional components reduced to 40% and finally a control of standard food (Table 2.2).

Table 2.2 - Alternate fly food medium recipes for nutrient deprivation trials.

Ingredients (grams/ litre)	F (standard food)	y20% (20% yeast content)	y2% (2% yeast content)	S0 (no dextrose)	F40% (40% nutrient content)
Technical Agar	10	10	10	10	10
Dry Yeast	20	4	0.4	20	8
Cornmeal	100	100	100	100	40
Dextrose	85	85	85	0	34
Nipagin (ml)	20	20	20	20	20

Each food type was made using the standard procedure of heating the water and agar until boiling and fully dissolved. Then adding the other ingredients before reheating and mixing thoroughly. The food was then dispensed into plastic vials with 10ml in each vial. Once cooled and solidified 50 embryos were added to each vial and this was repeated in triplicate for each genotype on each food type.

To allow for the embryos to be collected cages were set up for each genotype. Each cage was fitted with a plate with a red grape juice gel (table 2.3) and a small amount of yeast paste in the centre. These plates were then changed twice a day to allow for the collection of fresh embryos. Embryos were collected by washing the plates with distilled water and then filtering the solution which was produced. The residue was then removed from the filter paper and resuspended in water to allow for the embryos to be counted and placed into the vials.

Table 2.3 - Components of red grape juice gels for cage plates.

Ingredients	Quantity for 250ml
Technical agar	5.6g
Tap Water	200ml
Red grape juice concentrate	50ml

The vials were then incubated at 25°C and the flies which emerged were counted at 10, 12, 14, 16 and 18 days. After counting on each occasion the flies were removed and stored in vials containing standard food to allow for further phenotypic study.

Phenotypic Studies - Negative Geotaxis

Negative geotaxis is a method for assessing the motor abilities of flies by assessing their climbing ability. This was used to study any effect on motor ability resulting from adjusted nutrient intake across the studied genotypes. This method has been published previously by Kerr, *et al.* (2009) and also in numerous other papers. Flies were briefly anaesthetised using CO_2 before being separated by gender with 10 flies placed in each of a number of vertical column measuring 25cm in length and approximately 1.5cm in diameter (modified from plastic 25ml pipettes). Dependent on the number of flies available this was repeated in triplicate for each genotype on each food type. The columns were then placed in a 25°C incubator for 30 minutes to allow the flies to fully recover from the anaesthetic. After this the flies were given a trial run of negative geotaxis by tapping them to the bottom of the columns and allowing them to climb for 45 seconds. Once this was complete flies in each column in turn were

tapped to the bottom and then allowed to climb for 45 seconds before observing and recording the number at the top and bottom of the column. This was repeated 3 times for each column allowing a gap of around 1 minute between trials. An effort was also made to try and maintain a similar number and intensity of taps between columns and trials.

The results of this study were analysed by calculating the performance index. This was achieved by taking averages from the repeats to give a single result for, total number of flies, number of flies at the top and number of flies at the bottom for each genotype on each food type. The performance index was then calculated as follows, $PI = 0.5 \times ({}^{n}total + {}^{n}top - {}^{n}bottom) / {}^{n}total$.

Phenotypic Studies - Adult Body Length

Another phenotypic analysis carried out was the measurement of overall body length in adult flies. This was carried out on both the nutrient restricted flies and also flies which had been treated with propranolol as these appeared to visually show a slight size difference between genotypes. To achieve these measurements flies were anaesthetised with CO_2 before being separated by gender and then fixed to microscope slides by means of clear PVA glue. An effort was made to try and align the flies on their side and in a straightened body position to assist with accurate measurement. These slides were then photographed using a microscope fitted with a camera and measurements were carried out using the software 'ImageJ' which was calibrated with a millimetre square grid.

Compound Assay

Compound assays were setup to determine the effect of a range of ion channel blocking compounds on the cap mutant flies. The first compound assays used a fairly broad range of compounds known to block various ion channels and included a common neurotransmitter (table 2.4)

Table 2.4 Preliminary compound trials showing solvents and required dilution from dry form.

Compound	Solvent	Molecular Weight	Quantity of compound to make 10ml - 500mM Solution (mg)
Acetylcholine Chloride	Water	188.66	908.3
Ethosuximide	Water	141.17	705.85
GABA	Water	103.12	515.6
Carbamazepine	Ethanol	236.27	1181.35
Na Valporate	Water	166.19	830.95
Propranolol	Water	295.8	1479

To trial the compounds 10 vials were setup for each genotype, each of which contained 3 male and 3 female flies. These were then flipped into new vials every 24 hours with the aim of getting a comparable number of eggs laid in each vial. At 2 days of larval development the 6 vials with most comparable larval activity from each genotype were chosen and had 500 µl of one of the compounds applied in a 500mM and 200mM concentration with each repeated in triplicate. This resulted in final concentrations of approximately 25mM and 10mM once diluted by the food. Controls were also setup for each compound using 500 µl of the solvent in which the

compound was diluted. The only exception to this protocol was carbamazepine which does not dissolve well in any solvent which can be applied to drosophila. Therefore this was applied as a partial suspension in 50% ethanol as this was the strongest solvent which was determined not to have a significantly negative impact on the larvae.

Ten days after egg laying counts were taken of the number of flies hatched in each vial, this allowed for a quick determination of which compounds had an effect and were worth repeating. Flies from this initial count were also kept to use for further phenotypic study to assess whether any of the compounds had a significant effect of the size of the flies.

This protocol was repeated for the compounds ethosuximide, propanolol and carbamazepine based on the results of the preliminary trials. On the repeated trial the number of flies hatched at day 10 were counted and removed from the vials and further counts were carried out on days, 12, 14, 16 and 18 to look for signs of developmental delay (Fig 2.1).

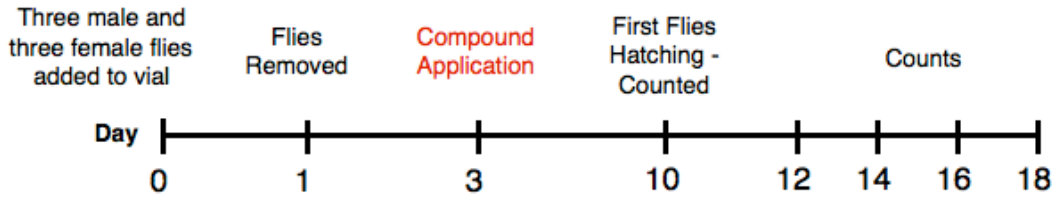

Figure 2.1 - Experimental timeline for application of compounds and counting of hatched flies.

Compound Assay - with set number of embryos

The results from the second compound assay showed some interesting findings, however the results for developmental delay were shown to be very unreliable due to variation in the number of eggs laid in each vial. This was particularly notable in the cap 1 & 2 double mutants which appeared to lay very unreliably. To combat this a final assay was set up using 50 embryos per vial rather than trusting every fly to lay an average number in a vial. This was carried out using the method previously described for embryo collection for the nutrition trials.

In the same way as the previous assays compounds were applied to the day 2 larvae produced from the embryos. In this final trial the compounds were applied in a wider range of concentrations to achieve results appropriate for the creation of dose response curves (Table 2.5). Counts of hatched flies were then carried out on day 10, 12, 14, 16 and 18 and flies disposed of after each count.

Table 2.5 - Compounds and concentrations used in final trials showing both concentration before addition to food and afterwards.

Compound	Initial Concentrations (before application to food)	Final Concentrations (after application to food)
Ethosuximide	1.25M, 0.5M, 0.2M, 0.08M	62.5mM, 25mM, 10mM, 4mM
Propranolol	0.5M, 0.2M, 0.08M	25mM, 10mM, 4mM
Carbamazepine	0.2M, 0.08M, 0.032M	10mM, 4mM, 1.6mM

RESULTS

Nutrient Restriction Trials

Total Adult Fly Emergence

Trials were carried out to ascertain the effects of various types of nutrient deprivation on cap methylation mutant drosophila. For the purposes of simplifying the annotation in the results, the genotypes will be described as follows, C or control (Yellow White Mutant), Cap1^{null} (yw CG6379$^{null)}$, Cap 2^{null} (aft^{null}) and Cap 1^{null} Cap 2^{null} (yw CG6379^{null}; aft^{null}). The first results to consider are the overall number of flies emerging over the 18 day period from egg laying. The control flies showed a reduction in numbers in all nutrient restriction conditions when compared to normal food. The most reduction was seen in the reduced yeast conditions at 20% and 2% yeast with only 40% of the flies reaching adult hood. The Cap1^{null} mutants showed very little variation in emergence across all food types except on the 2% yeast food where only a small percentage emerged. The Cap2^{null} mutants also showed a similar pattern with little variation between food types except for the 2% yeast food where none emerged (fig 3.1). Finally the Cap1^{null} Cap2^{null} mutants showed a reduction in emergence on all food types, however interestingly remained at a similar level to the control on the 2% yeast food.

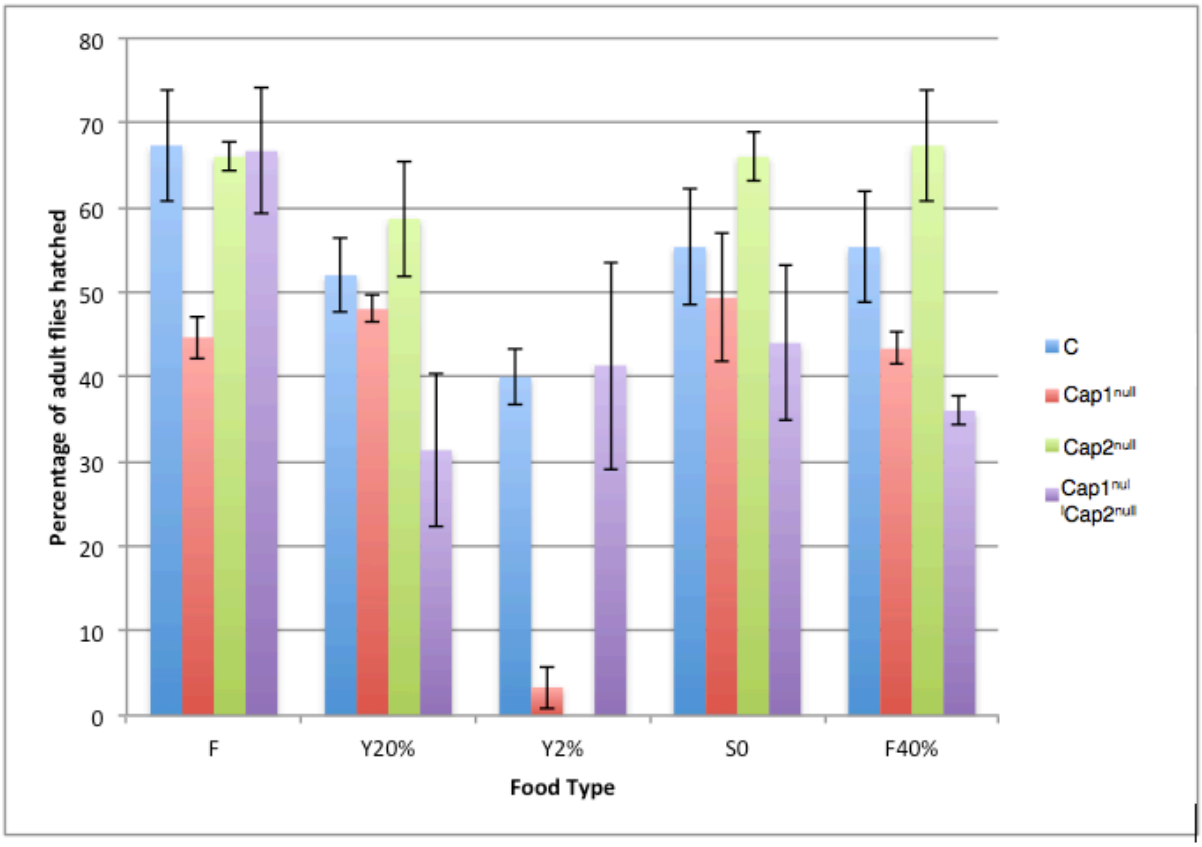

Figure 3.1 - Total percentage of adult flies emerging from 50 embryos in an 18 day period after egg laying. Comparing nutrient restricted food types to cap methylation mutant genotypes. There were no survivors for the $Cap2^{null}$ flies on Y2% food. Error bars show standard deviation. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, Cap 2^{null} - aft^{null} and Cap 1^{null} Cap 2^{null} ($CG6379^{null}$; aft^{null}).

Developmental Delay

Comparison of the time taken for flies to emerge was also carried out, counting the flies at 2 day intervals to look for any developmental delay. As expected on standard food the majority of flies emerged on day 10 for all genotypes and after day 12 no more flies emerged. There was however a significantly smaller percentage of flies for the $Cap1^{null}$ mutants which showed a smaller number on day 10 and a slightly larger number emerging on day 12 (fig 3.2a). The food with an absence of sugar (dextrose) also showed a similar pattern with the majority of flies hatching on days 10 and 12. It does however show a developmental delay particularly in $Cap1^{null}$ and $Cap12^{null}$

mutants with a greater percentage of flies emerging on day 12 (fig 3.2d). All other food types have a reduction in the amount of yeast supplied at 40%, 20% and 2%, which show no emergence of flies at 10 days in any genotype. These results also appear to show that the greater the reduction in yeast the greater the developmental delay and the smaller overall percentage of flies emerge by day 18. This is particularly notable in the 2% yeast food where no emergence is seen in Cap 2^{null} and only 2% in Cap 1^{null}. Interestingly however the Cap 1^{null} Cap 2^{null} mutant doesn't show any reduction compared to the other 2 yeast reduced food types.

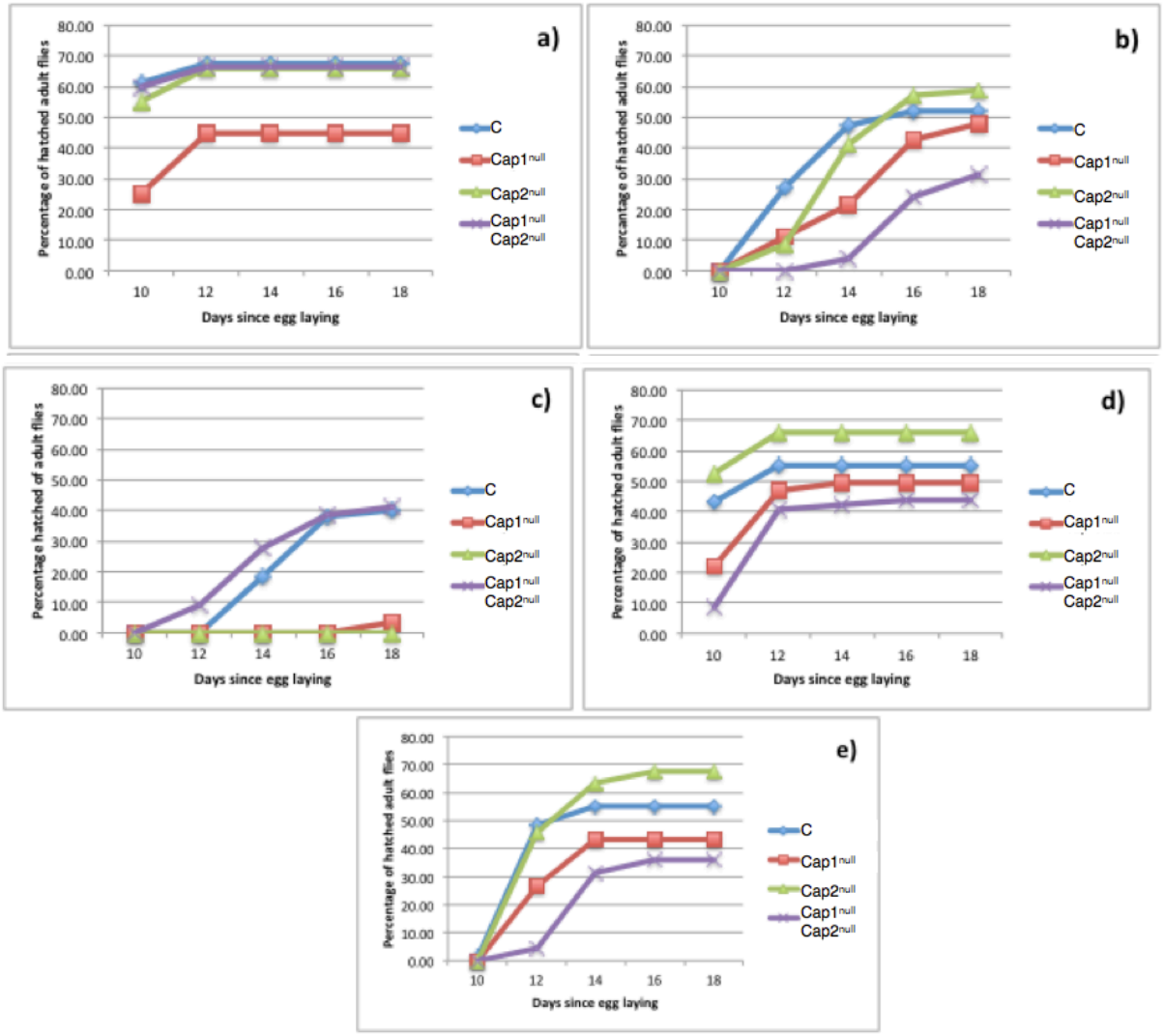

Figure 3.2 Developmental delay graphs showing emergence of adult flies from 50 embryos over an 18 day period from egg laying on nutrient restricted food types. a) Normal food, b) yeast reduced to 20 %, c) yeast reduced to 2%, d) no dextrose, e) all nutritional constituents reduced to 40%. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, $Cap\ 2^{null}$ - aft^{null} and $Cap\ 1^{null}\ Cap\ 2^{null}$ - yw $CG6379^{null}$; aft^{null}.

Negative Geotaxis - Motor Control

Negative geotaxis was carried out on the flies from each food type to establish whether there was any locomotor dysfunction. This was not possible on any $cap1^{null}$ $cap2^{null}$ mutants except standard food and only on the control flies for 2% yeast as not enough of the emerging flies were surviving by the point at which negative geotaxis was carried out. The least reduction in motor ability occurred in $cap\ 1^{null}$

females across all analysed food type. Interestingly the motor ability of all mutants appears to increase under 20% yeast conditions (Fig. 3.3). The male flies show a very similar pattern to the females suggesting little difference between gender.

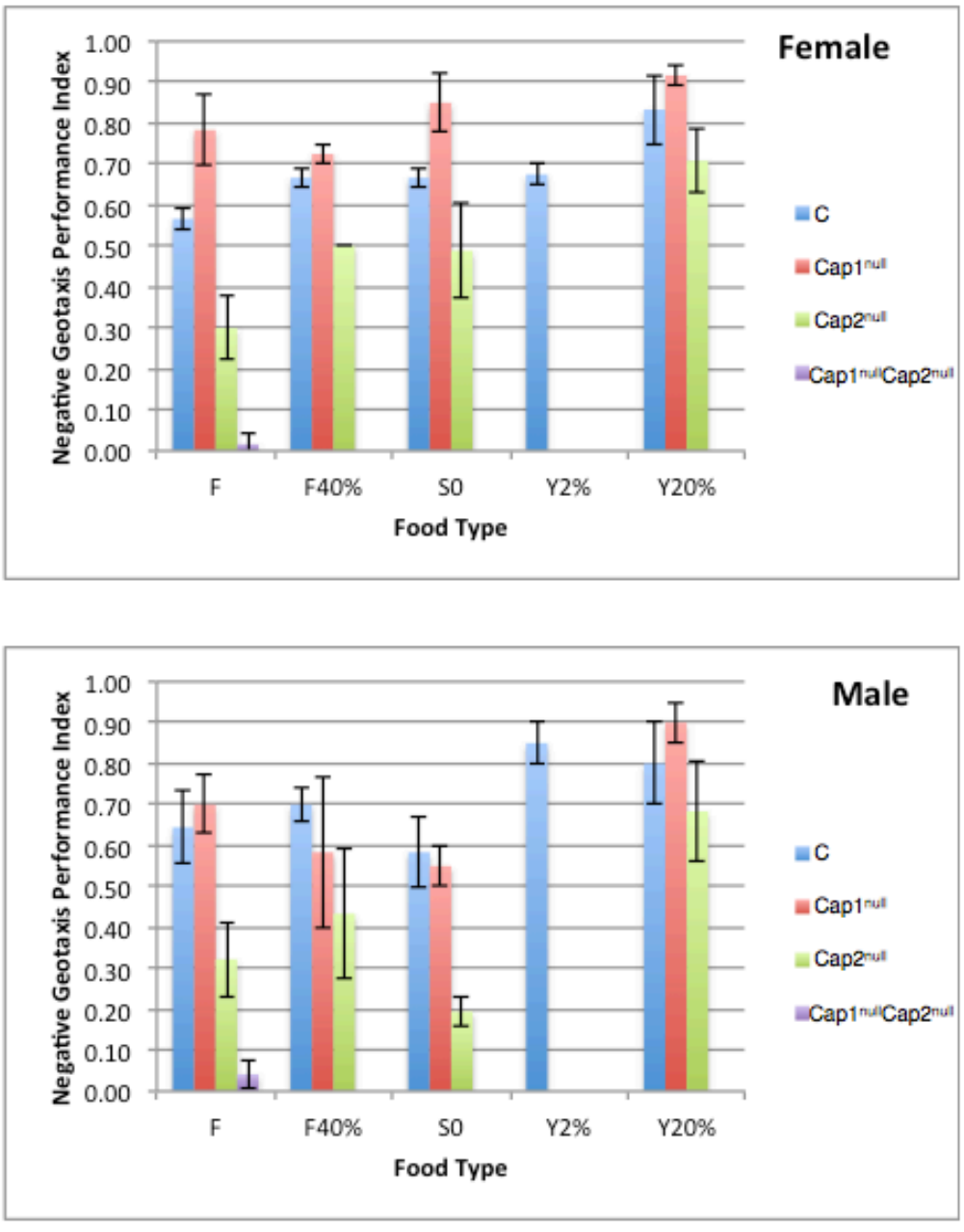

Figure 3.3 - Negative geotaxis performance index for female and male cap mutant D. *melanogaster* on nutrient reduced food types. Error bars show standard deviation. The genotypes not assayed due to lack of flies were $Cap1^{null}$ Y2%, $Cap2^{null}$ Y2% and $Cap12^{null}$ F40%, S0,Y2% and Y20%. F - Normal food, Y20% - yeast reduced to 20 %, Y2% - yeast reduced to 2%, S0 - no dextrose, F40% - all nutritional constituents reduced to 40%. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, Cap 2^{null} - aft^{null} and Cap 1^{null} Cap 2^{null} - $CG6379^{null}$ & aft^{null}.

Developmental Restriction - Body Size

Measurement of body size was also carried out on flies from each genotype and food type with the exception of Cap 1^{null} and Cap 2^{null} on the 2% yeast food and Cap 1^{null} Cap 2^{null} on the 20% yeast food. Measurements on these flies could not be carried out either because no flies emerged or a very limited number emerged. Flies were measured by means of fixing to a microscope slide and then measuring a straight line from the head to the end of the abdomen (Fig 3.4) Once standard deviation was taken into account there was very little significant difference between genotypes and food types (Fig 3.5). Analysis was also carried out on total numbers across all genotypes on each food type, this also showed no significant differences between the food types (Fig. 3.6).

Figure 3.4 - Example of body length measurement technique of D. *melanogaster*

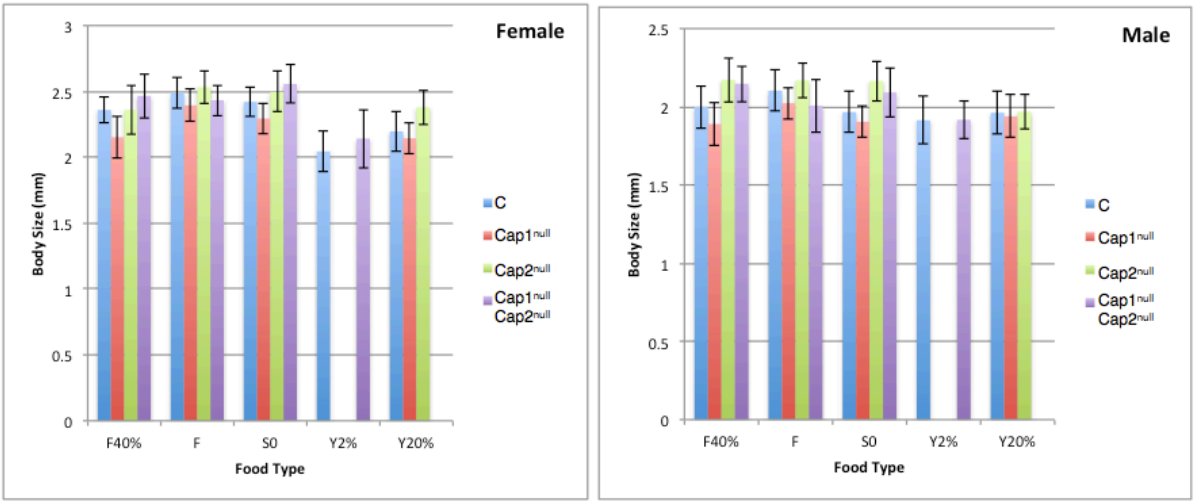

Figure 3.5 - Sizes of D.*melanogaster* cap mutant flies on reduced nutrient food types. Error bars show standard deviation. Assays were not carried out on Y2% Cap1^{null} and Cap2^{null} and Y20% Cap12^{null} due to a lack of surviving flies. F - Normal food, Y20% - yeast reduced to 20 %, Y2% - yeast reduced to 2%, S0 - no dextrose, F40% - all nutritional constituents reduced to 40%. C - Yellow White Mutant, Cap1^{null} - CG6379^{null}, Cap 2^{null} - aft^{null} and Cap 1^{null} Cap 2^{null} - CG6379^{null} & aft^{null}.

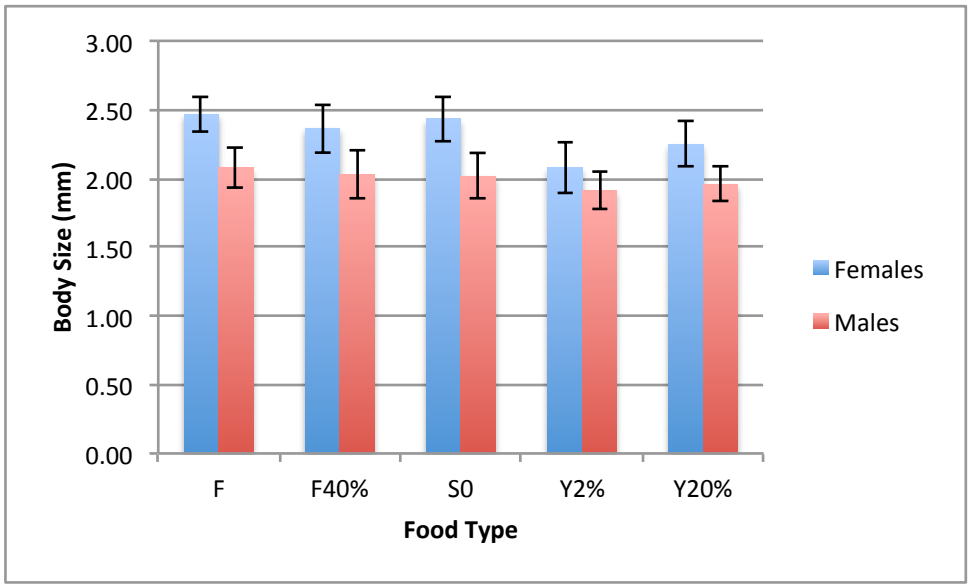

Figure 3.6 - Average size across all mutant genotypes on each nutrient restricted food type. Error bars show standard deviation. F - Normal food, Y20% - yeast reduced to 20 %, Y2% - yeast reduced to 2%, S0 - no dextrose, F40% - all nutritional constituents reduced to 40%.

Compound Trials

The compound trials used a selection of ion channel blockers to look for effects of mRNA cap methylation on neural development. Ion channels were blocked and the phenotypic effects of this studied in each genotype.

The experimental procedure had three stages, preliminary trials using a large number of compounds, followup trials with the compounds showing a potential phenotypic effect and finally trials using a defined number of embryos.

Preliminary Trials

A preliminary trial was carried out to establish any variation in the effect of a range of compounds between the different genotypes (Fig 3.7). Controls were also put in place using the solvents in which the compounds were diluted. The first result to consider is that a significantly smaller number of the Cap 1^{null} Cap 2^{null} mutants emerged under all compound conditions including H_2O suggesting that the results for these mutants may not be accurate to compare to other genotypes. GABA and Acetylcholine appeared to have little effect when compared to the H2O control in all genotypes and increased concentration of these compounds didn't show a significant reduction survival.

From the preliminary trial 3 compounds were chosen for further trials based on their significant overall effect of survival rates in all genotypes and interesting increase in survival rate of some genotypes over the control. The first of these was Ethosuximide

which at 10mM concentration showed a greater number of Cap 2^{null} mutants hatching compared to Cap 1^{null} mutants. However when the concentration was increased to 25mM the Cap 2^{null} mutants dropped off and the Cap 1^{null} mutants increased. Both carbamazepine and propranolol were also chosen as they were shown to have a significant impact on the number of flies emerging and a significant discrepancy between the control genotype and the cap mutant genotypes. Some interesting results were also seen in GABA, flunarizine and acetylcholine however these would require further trials to assess their true effect (fig. 3.7).

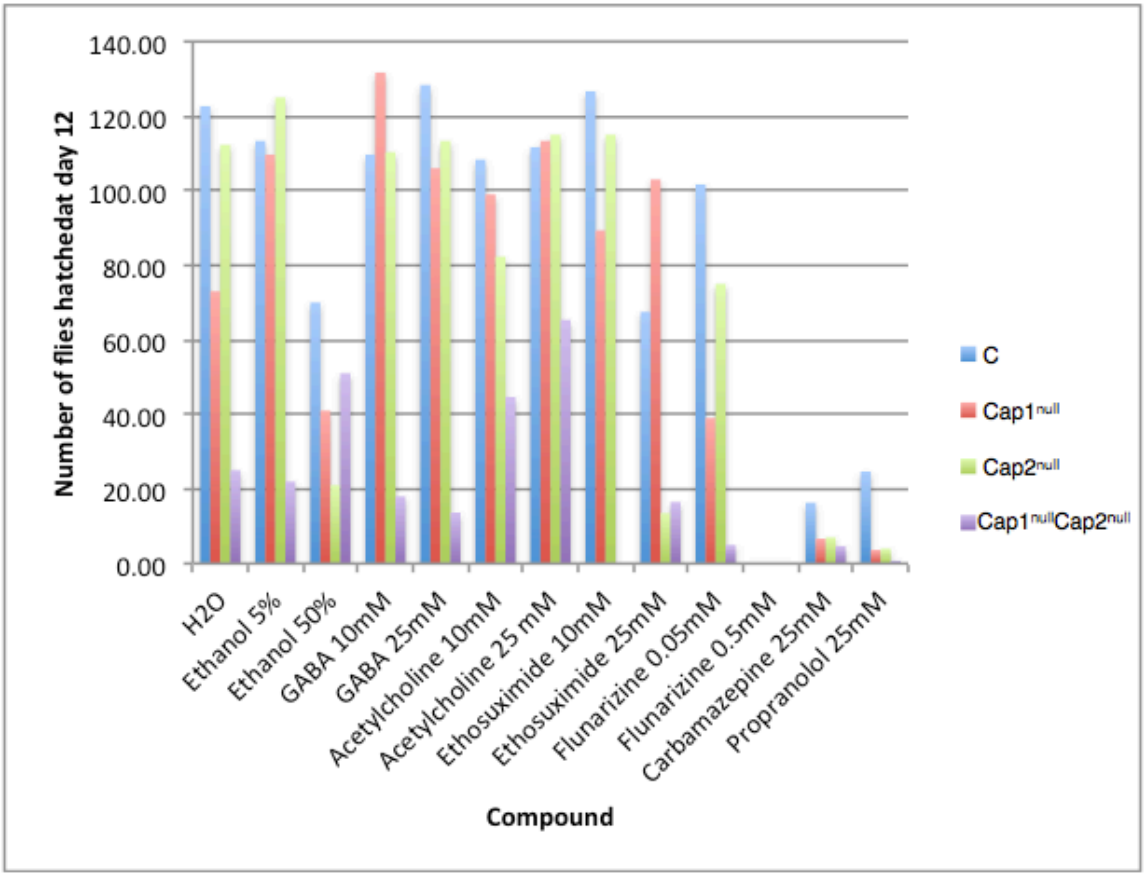

Figure 3.7 - Trial of D.*melanogaster* mRNA cap methylation mutants with various compounds applied to larvae, 2 days post egg laying. C - Yellow White Mutant, Cap 1^{null} - $CG6379^{null}$, Cap 2^{null} - aft^{null} and Cap 1^{null} Cap 2^{null} - $CG6379^{null}$ & aft^{null}.

Follow-up Compound Trials

The results of the followup trials were first analysed by dose response, to ascertain the effect of increasing concentration of each compound on the flies. The Cap 1^{null} and Cap 1^{null} Cap 2^{null} larvae treated with ethosuximide both showed a reduction in the overall number of flies emerging as the concentration increased. However the control and Cap 2^{null} flies both showed an increase suggesting that ethosuximide may promote growth and survival in these flies (fig. 3.8a). This however is not supported by the preliminary trials which showed the opposite result (fig. 3.7).

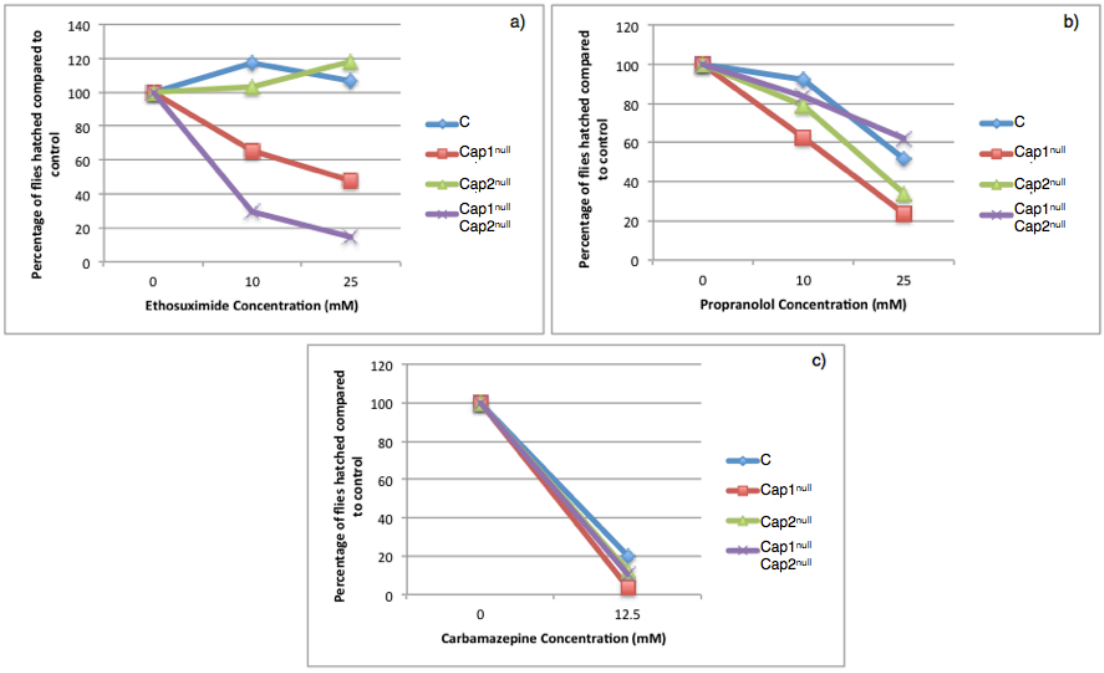

Figure. 3.8 - Percentage emergence of mRNA cap mutant D.*melanogaster* treated with varying concentrations of the ion channel blocking compounds a) Ethosuximide. b) Propranolol c) Carbamazepine. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, Cap 2^{null} - aft^{null} and Cap 1^{null} Cap 2^{null} -$CG6379^{null}$ & aft^{null}.

The flies hatching from larvae treated with propranolol showed a decrease in percentage emerging for all genotypes, however Cap 1^{null} Cap 2^{null} mutants showed a more gradual decline than the other mutants or the control, which suggests a

possible significant interaction when no additional cap methylation is present (fig. 3.8b).

Carbamazepine unfortunately couldn't be trialed at the same concentrations as the other compounds as it does not dissolve sufficiently in any solvent suitable for use with drosophila. It was applied in a 50% ethanol solution which resulted in a proportion of it dissolving. It was therefore applied as a suspension of approximately 12.5mM. This showed a clear effect on all genotypes with the Cap 1^{null} mutants being most effected and the control flies being least effected (fig. 3.8c).

A comparison of developmental delay (late emergence) was also attempted which showed some potentially significant results, however, once standard deviation was calculated this removed any significance of these results. It is thought that the error was probably introduced as a result of the experimental method, relying on the flies within each vial to lay a comparable number of eggs on a daily basis. This was particularly noted as a problem with the Cap 1^{null} Cap 2^{null} mutants which showed much reduced fertilised egg production in all trials. This was also mirrored in the results of the preliminary trials (Fig 3.9).

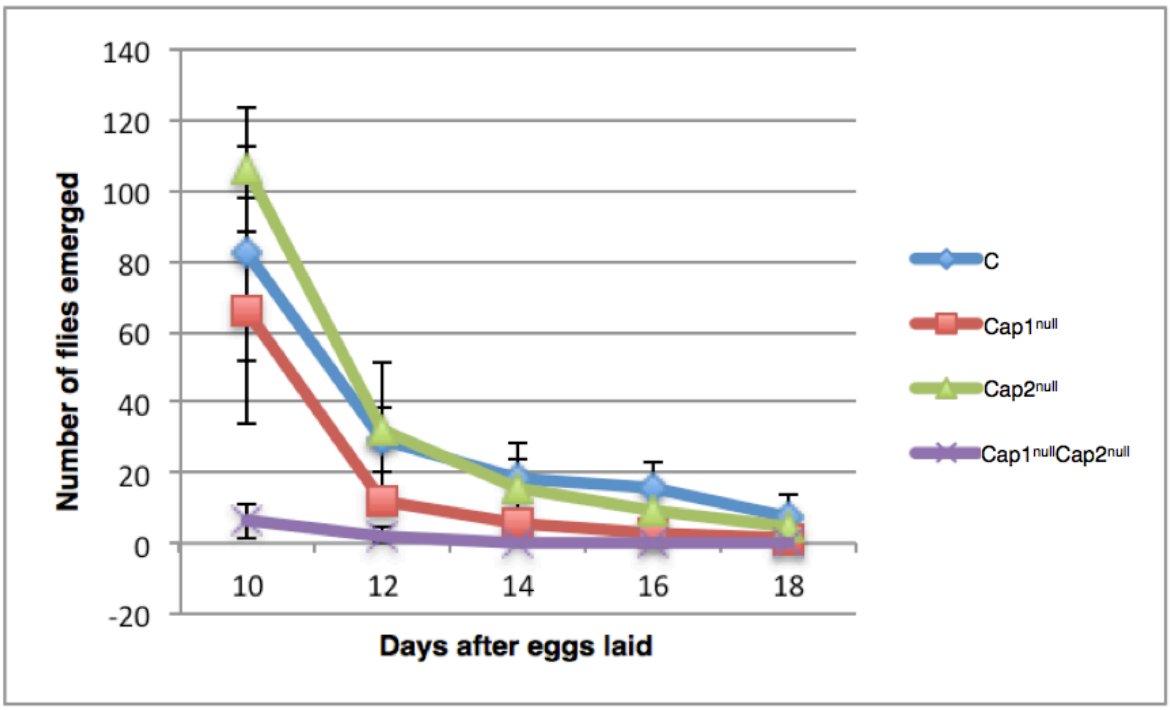

Figure 3.9 - Number of mRNA cap mutant D.*melanogaster* treated with 25mM Ethosuximide hatching over period up to 18 days from initial egg laying. Error bars show standard deviation. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, $Cap\ 2^{null}$ - aft^{null} and $Cap\ 1^{null}\ Cap\ 2^{null}$ - $CG6379^{null}$ & aft^{null}.

In an attempt to rectify this problem and reduce the error a final set of trials were carried out, placing 50 embryos in a vial to ensure identical starting numbers before treatment with compounds.

Compound Trials using a Defined Number of Individuals

The results of the final compound trials were analysed for dose-response as a much larger range of concentrations had been used, particularly with ethosuximide. The control flies showed a similar result to the previous trials with a slight increase in the percentage hatching between 0mM and 10mM and then a gradual drop as the concentration increases. The mutant flies however did not respond in a similar way to

the previous trial, Cap 1^{null} mutants followed a similar pattern to the control flies whereas in the previous trial they had not shown this rise in hatching in the presence of the compound. Cap 1^{null} Cap 2^{null} mutants also showed a similar pattern with an increase in hatching up to 10mM before rapidly falling off. This however is seen at a much smaller overall percentage emergence than the control and cap 1^{null} mutants (Fig 3.10a).

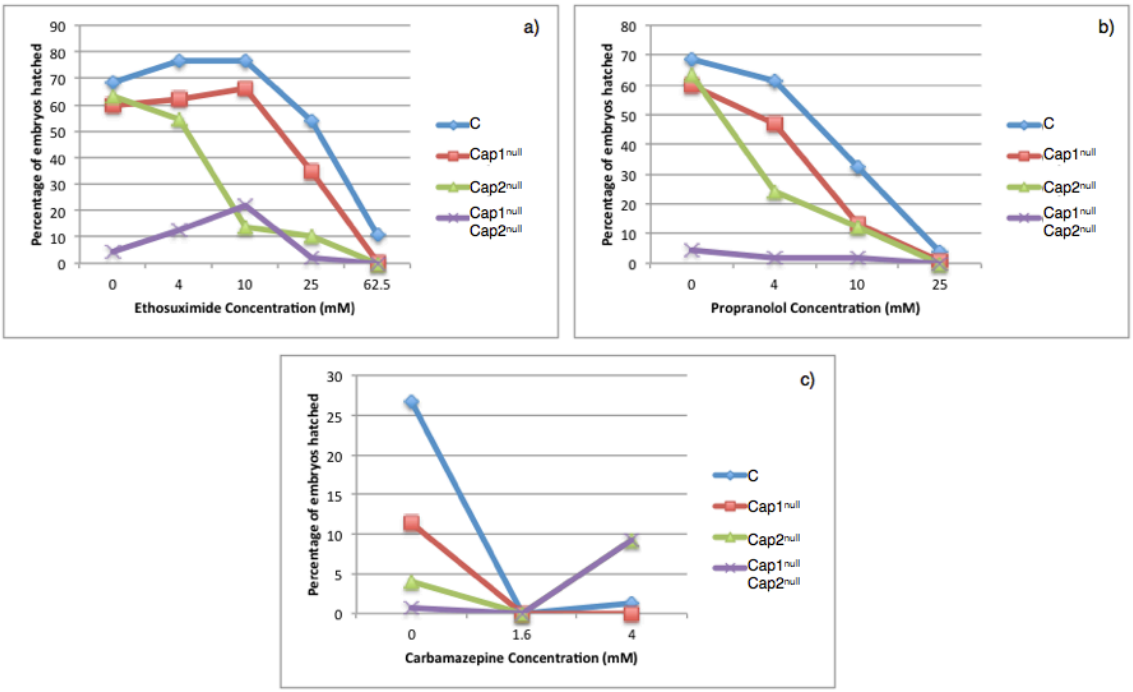

Figure 3.10 - Average percentage emergence from 50 mRNA cap mutant D.*melanogaster* embryos treated with varying concentrations of the ion channel blocking compounds a) Ethosuximide b) Propranolol and c) Carbamazepine. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, Cap 2^{null} - aft^{null} and Cap 1^{null} Cap 2^{null} - $CG6379^{null}$ & aft^{null}.

Increasing propranolol concentration appeared to show a similar decline in flies emerging across all genotypes as concentration increases. The only exception to this is that in a similar way to ethosuximide concentration the cap 2^{null} mutant falls off more rapidly on introduction of the compound than any others (Fig 3.10b).

The final compound in these trials was carbamazepine, in a similar way to the pervious trials this had to be tested at a smaller range of concentrations due to the use of a suspension rather than a dissolved solution. The solvent used with carbamazepine was 50% ethanol solution which explains the more widely varied and reduced hatching in all genotypes at 0 mM carbamazepine concentration. The results also show a potential anomaly with no flies emerging in any genotype at 1.6mM concentration yet flies emerging in all but one genotype at 4mM (Fig 3.10c). It should however be noted that the very low percentage of overall emergence limits the reliability of these results.

Due to the increased accuracy which could be achieved by controlling the initial of number embryos it is possible to analyse graphs of developmental delay for each genotype. The most significant results of this analysis can be seen in flies treated with ethosuximide, at 4mM there is a clear developmental delay shown in Cap 1^{null} Cap 2^{null} mutants with no flies emerging until day 12 and at this point only a comparable number to other genotypes at day 12 (Fig 3.11 a). The most significant difference when the concentration is increased to 10mM is the reduction in Cap 2^{null} mutants. At this point the control flies also come in line with the emergence pattern of cap 1^{null} mutants (Fig. 3.11 b). At 25mM there is a clear developmental delay in all genotypes with cap 2^{null} and Cap 1^{null} Cap 2^{null} having a slightly greater delay than the other genotypes (Fig. 3.11 c).

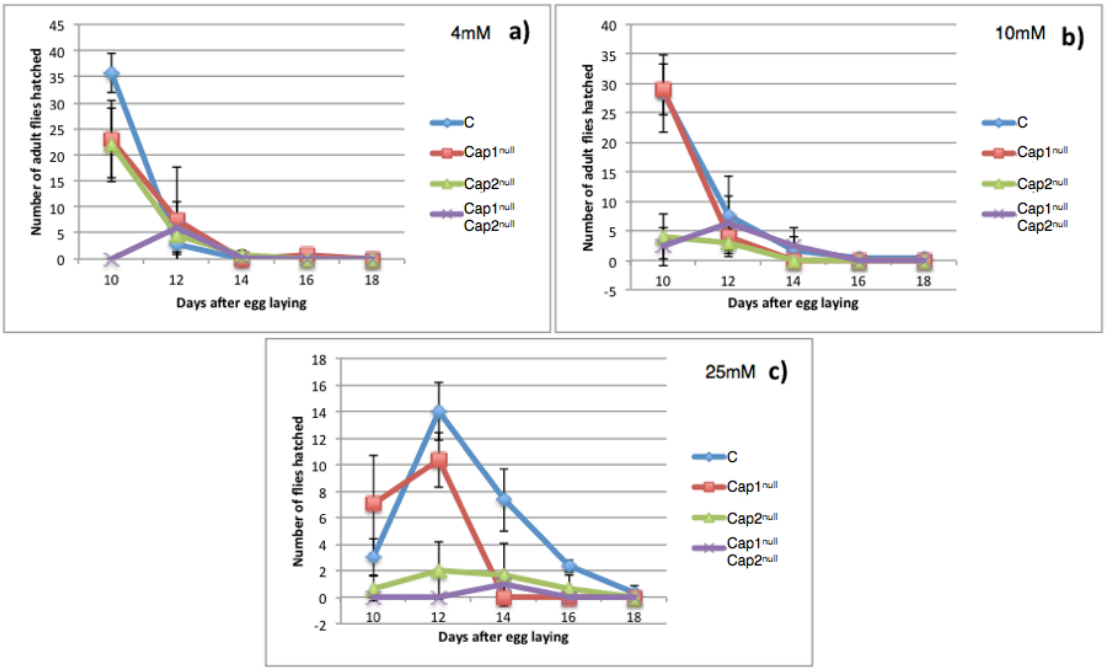

Figure 3.11 - Number of mRNA cap mutant D.*melanogaster* hatching over period up to 18 days from initial egg laying after treatment with the ion channel blocking compound ethosuximide. a) 4mM, b) 10mM, c) 25mM Error bars show standard deviation. C - Yellow White Mutant, $Cap1^{null}$ - $CG6379^{null}$, $Cap\ 2^{null}$ - aft^{null} and $Cap\ 1^{null}\ Cap\ 2^{null}$ - $CG6379^{null}$ & aft^{null}.

Propranolol was only analysed at 4mM for developmental delay due to the very small number of flies emerging at higher concentrations. All genotypes showed a comparable drop from the initial number of flies emerging at day 10 suggesting its only effect at this concentration is to reduce the overall number of flies rather than induce a developmental delay effect (Fig. 3.12).

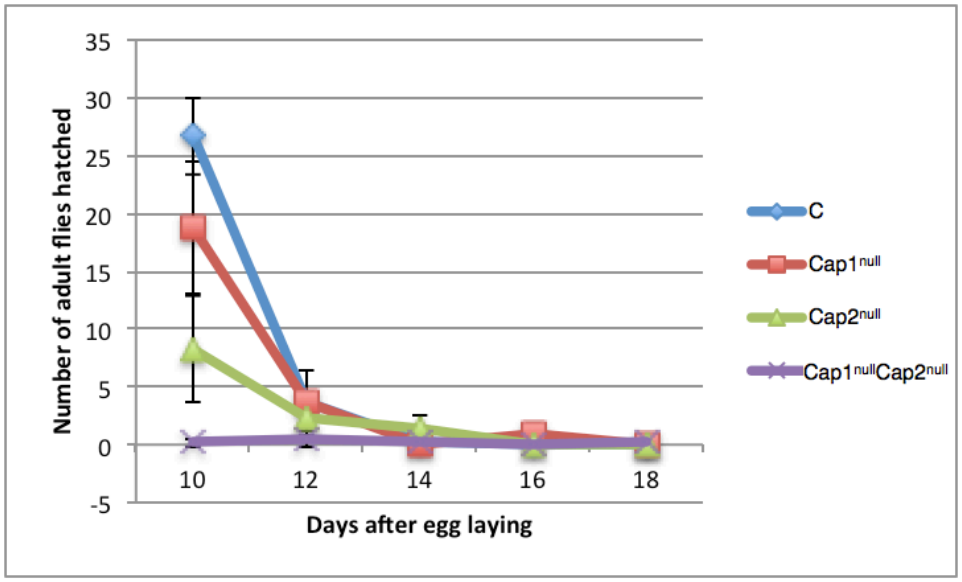

Figure 3.12 - Number of mRNA cap mutant D.*melanogaster* treated with 4mM propranolol hatching over period up to 18 days from initial egg laying. Error bars show standard deviation. C - Yellow White Mutant, Cap1^{null} - CG6379^{null}, Cap 2^{null} aft^{null} and Cap 1^{null} Cap 2^{null} - CG6379^{null} & aft^{null}.

Preliminary Cap 1^{null} Cap 2^{null} Breeding Trials

Following the observation that Cap 1^{null} Cap 2^{null} mutants didn't produce as many adult flies as the other mutants a preliminary trial was setup to assess the fecunduity of these flies. An initial trial was set up with mutant virgin females and control males. This trial suggested a potential mating deficiency in these flies as in a 2 hour period only one out of the 10 flies mated. Further study would be required to confirm this and to produce results suitable for statistical analysis.

Results Summary

Nutrient Deprivation

- Nutrient restriction reduces overall number of flies emerging.
- Yeast reduction has the most significant effect.
- Cap 2^{null} mutants overall suffer least from nutrient deprivation except at 2% yeast.
- Absence of both Caps simultaneously however seems to rescue these nutrient deprived flies
- Removal of dextrose from food resulted in a slight developmental delay in Cap 1^{null} and Cap 1^{null} Cap 2^{null} mutants.
- Decreasing yeast concentration increases developmental delay in Cap 1^{null} and Cap 2^{null} mutants but not Cap 1^{null} Cap 2^{null}
- 20% yeast appears to increase motor ability in negative geotaxis
- No significant effect was found on body size of any genotype on any food type.

Compound Trials

- Ethosuximide
    - Preliminary trials showed a fall in the number of Cap 2^{null} mutants with increase in concentration but an increase in Cap 1^{null} mutants.
    - These initial results were contradicted by the follow-up trial.
    - The final results however show much closer alignment with the preliminary trial results with a decrease in Cap 2^{null} mutants with increase in concentration but an increase in Cap 1^{null} mutants.
    - The Cap 1^{null} Cap 2^{null} mutants also follow the same pattern as Cap 1^{null}
    - Increasing concentration results in developmental delay in all genotypes but is more pronounced in Cap 2^{null} and Cap 1^{null} Cap 2^{null} mutants.

- Propranolol
    - All genotypes show a decrease with increasing concentration
    - Cap 1^{null} Cap 2^{null} mutants show a more gradual decline in numbers compared to the other genotypes.
    - Cap 2^{null} mutants decline more quickly with increased concentration
    - No sign of developmental delay
- Carbamazepine
    - Unreliable results probably due to application as a suspension and the use of 50% ethanol solution

DISCUSSION

The 7-methyl guanosine cap on the first nucleotide of mRNA has previously been the focus of much research. It has been shown to be one of the first modifications to the RNA strand in the mRNA pre-processing stage and is thought to be important in triggering the formation of the poly-a tail on the 3' end of the mRNA strand. It has also been shown to be important for the protection of mRNA from degradation by endonucelases and initiation of translation through binding with the translation initiation complex (Soller, 2006). Studies of Cap 1 and 2 methylation, however, are much more limited as knockout organisms have not been described yet. It is however important to also note that studies have shown cap 1 methylation to be present on all mRNA strands in humans and to be specifically formed by some viruses suggesting that it must have an important role (Kruse, *et al*. 2011). It has been suggested in some research that the presence of cap 1 may increase translational efficiency and may also interact without proteins to suppress any immune response to the mRNA (Daffis, *et al.* 2010 & Belanger, *et al*. 2010). However there is very limited understanding of the route by which this methylation acts to increase efficiency. Cap 2 methylation has undergone even less research than cap 1 and there is no current information to suggest what if any phenotypic effects this causes. As cap 2 methylation is not present on all mRNA strands this would suggest that it is not essential for efficient translation, however, as it is formed by a specific methyltransferase enzyme and is present on approximately half of all mRNA which would suggest that it isn't simply an accidental addition (Belanger, *et al*. 2010 & Werner, *et al.* 2011).

In an attempt to further define the role of cap 1 methylation and to identify a role for cap 2 methylation a series of trials were carried out focused on the effects of nutrient deprivation on these mutants. Nutrient deprivation is known to act on two pathways within cells, the insulin PI3 kinase pathway and the TOR (Target of Rapamycin) pathway (Li, *et al*. 2010). These pathways can act independently of each other or in conjunction with the insulin PI3 kinase running into the TOR pathway (Li, *et al*. 2010). Nutrient deprivation has now been shown in a number of studies to increase life span at a cost to developmental rate mainly through interactions in the TOR pathway (Layalle, *et al.* 2008). It is believed that amongst other control mechanisms this may be as a result of modification to the rate of translation which would result in slower production of proteins so a slower overall growth rate (Penney, *et al*. 2012). This may also have an effect on synaptic homeostasis and plasticity as reduced translational rates slow the ability for increases in the number of post-synaptic receptors and effect the retrograde control of neurotransmitter release (Penney, *et al*. 2012). As cap 1 methylation has been shown to influence translational efficiency this is a good initial model as theoretically the presence of cap 1 methylation should partially rescue the nutrient deprived flies in comparison to those with no cap 1 methylation.

The initial results for these trials were focused on overall emergence of flies from each genotype on each food type. Although this does not show delayed development it can be used to assess complete limitation of development. On standard food all genotypes showed a similar percentage emergence of around 68% with the exception of the cap 1^{null} mutant which only showed emergence of 44%. This would immediately suggest a developmental problem induced by the absence of cap 1

methylation, however, this is not then reflected in Cap 1^{null} Cap 2^{null} mutants. This result is also supported by the developmental delay analysis which shows slightly slower emergence of the cap 1^{null} mutants compared to the other genotypes. This would almost suggest that the presence of cap 2 methylation on its own is a limiting factor for translation. This finding also appears to be supported in the 20% yeast food type, which shows an absence of cap 1 methylation results in delayed development compared to the control flies. An absence of cap 2 results in an initial delay in development followed by an increase in developmental rate above the control flies, however, this is again contradicted by the double mutant genotype.

The cap 1 methylation effects in this study can be compared to pervious studies to help ascertain the reliability of the results. Although there are no other studies which have used nutrient deprivation, Kuge, *et al*. (1998) showed that the rate of translation was increased in the presence of cap 1 methylation and therefore should result in developmental delay and potentially reduced numbers emerging as adults. This supports the finding that on standard food the cap 1^{null} mutant has reduced numbers and slightly delayed development. There is also some evidence from crystallographic analysis that cap 1 methylation may strengthen binding to eIF4E (Kruse, *et al*. 2011).

The effect of the absence of cap 2 methylation is much harder to define. When compared to control flies on all nutrient reduced food types a significantly increased developmental rate is seen. This is quite unexpected as it can be assumed that all of these mutants would have cap 1 methylation which would imply a limiting effect of cap 2 methylation, however, there is currently no evidence as to how this functions. It

was suggested by Belanger, *et al*. (2010) that as removal of cap 1 methylation had only very limited effect but removing both cap 1 and 2 methylation may have a more obvious phenotypic effect. It is clear from the results that the double mutant flies do respond differently to those with methylation. Notably they tend to have slightly slower and reduced development with nutrient restriction, however when the level of yeast is reduced to 2% the decrease in flies emerging is marginal in comparison to the other mutants. This again may support the theory of cap 2 methylation slowing the rate of translation.

To understand the effect of the different food types it is important to understand how the different nutrients are used by the Drosophila and their potential effect. It has been shown by Bass, *et al.* (2007) that in terms of life span the need for sugar is very limited and above 50g/l of sucrose in food the flies do not modify their eating behaviour but still undergo fecundity reduction suggesting a toxic effect of high sugar concentration. This study uses dextrose as the sugar source as this reduces bacterial growth, however, it is assumed that this will have the same effect on flies as sucrose (Ashburner & Roote, 2007). Previous studies have shown that yeast has the main dietary restriction effect when it comes to increased life span and that is reflected in this study with the slowing of development alongside reduction is yeast concentration in food (Chippindale, *et al*. 2002). It has also been shown that the effects of yeast are not related to caloric provision but the quality of yeast used and its nutritional composition (Ashburner & Roote, 2007). Grandison, 2009 has shown that the nutritional component within yeast which has this effect on lifespan and development is amino acid content and that other components have little effect on life span. This

also supports the theory that dietary restriction would interact with the TOR pathway as this is very sensitive to amino acid intake and therefore potentially translational initiation efficiency (Grandison, 2009).

This evidence supports the results that decreasing yeast concentration in food would result in more developmental delay for cap 1^{null} mutants if cap 1 influences translation efficiency as some studies suggest. As developmental delay is also induced in cap 2^{null} mutants this may suggest that they also have a role in translational efficiency. However this is not supported by the results of the Cap 1^{null} Cap 2^{null} mutants who appear to be less effected by the reduced yeast than the other genotypes except the control. This may either imply an experimental error or some unknown reaction resulting for the absence of any cap methylation.

The analysis of body size of the nutritionally deprived flies showed very little significant variation between genotypes on each food type. Layalle, *et al.* (2008) suggest that reduced nutrition results in overall larger flies due to an increased growth period this is not reflected in the results of the current study. The reason for this may however be related to the inherent problems of measuring full body length of flies, there is potential for the flies body shape to be curled or contracted which when looking for a small amount of variation introduces a greater margin of error. A more accurate strategy may have been to measure head capsule width as although this varies less than full body size it is possible to carryout much more accurate measurement.

Negative geotaxis assays were carried out to establish any motor dysfunction within the dietary restricted cap methylation flies. Due to the relationship previously described between the homeostatic control at synapses and translational efficiency this should demonstrate the influence of cap methylation on translational efficiency. It would be expected that if cap 1 or 2 methylation increased efficiency that at a restricted level of yeast those flies with cap methylation would perform better. This however is not reflected in the results as at 20% yeast all genotypes performed better particularly the Cap 1^{null} mutants. This may be a reflection of a toxicity effect of the higher concentration of yeast in the standard food and not related to the dietary restriction (Ashburner & Roote, 2007). Unfortunately at 2% yeast concentration the number of flies emerging was reduced below the number required for negative geotaxis so analysis of this effect at a lower concentration can not be carried out. Further research to look at a wider range of yeast concentrations on cap mutants may resolve the reason for this effect.

The other trials carried out looked more specifically at neural and synaptic development of cap methylation mutants through treatment with compounds known to effect neural and synaptic signalling. Synaptic plasticity has been shown to have a strong link to translational efficiency potentially through regulation at the mRNA processing stage, post-transcription (Costa-Mattiolo, *et al*. 2009). Synapses use either G-protein-coupled receptors or ionotropic receptors to sense neurotransmitter release (Voglis & Tavernarakis, 2006). By introducing an ion-channel blocking compound onto the larvae this would theoretically mean that development would be slow or halted in those mutants which had less efficient translation to produce extra

ion channels and restore homeostasis. The initial range of compounds in preliminary trials also included acetylcholine a neurotransmitter to look for any effects of this destabilising synaptic homeostasis.

The preliminary trial showed some interesting results, firstly it should be noted that the accuracy of results related to the double mutant (Cap 1^{null} & Cap 2^{null}) are very poor in the preliminary trial due to their unreliable mating and laying of fertilised eggs, this can be seen particularly in those flies treated with H_2O which should have had very little effect. The preliminary trial also only accounts for flies hatched on day 10 so does not show any flies which may have been developmentally delayed and hatched at a later point.

The compound ethosuximide appears to have a more significant effect on some genotypes than others, in both the preliminary and final trials those genotypes with an absence of cap 2 methylation were most greatly effected by the compound. Ethosuximide is an ion channel blocker which works on a wide range of ion channels but particularly T-type calcium channels (Collins, *et al.* 2008). T-type calcium channels are known to be very sensitive and are the first ion channels to respond to weak stimulus (Cain & Snutch, 2010). This would suggest that the effect of inhibiting a number of these channels would result in a decrease of response to weak stimulus within the neurones. There is an apparent interaction between cap 2 methylation and the effects of this compound as greater developmental delay was seen in cap 2^{null} and Cap 1^{null} Cap 2^{null} flies. This may suggest that cap 2 has involvement in increasing the efficiency of the translation process, however, this is contradicted by

the results from the nutrient restricted flies. The alternative explanation for this is that cap 2 methylation or ethosuximide has some other unknown interaction which generates this effect.

Propranolol another trialed compound, is a non-selective beta blocker, blocking adrenergic g-protein coupled receptors (Dzialowski, *et al*. 2006). Propranolol acts to block the g-protein coupled rectors in synapses which is the root cause of any developmental effects related to cap methylation. This explains the overall decrease in the emergence of flies with increasing concentration of propranolol. It would again be expected that if cap methylation promoted translational efficiency that those flies without cap methylation would experience more effect than those with. The results also support a role for cap 2 methylation in the translation process as cap 2^{null} mutants were most greatly effected by the increasing concentration of propranolol. Propranolol, however, did not show any developmental delay for any genotype, suggesting that the inhibition of adrenergic g-protein coupled receptors above a certain level can not be corrected by synaptic homeostasis processes.

The final compound carbamazepine is known to block the functioning of voltage gated sodium channels which are often present in synapses (Sheets, *et al*. 2008). The results for this compound appear very unreliable, the root cause of this is two-fold, partially as a result of the compounds inability to dissolve in water and partially due to the effects of the ethanol solution used as a solvent. Even when applied as a suspension in 50% ethanol solution carbamazepine becomes very sticky and it is not possible to accurately distribute an exact concentration to vials. Along with this the

high level of ethanol required also has an adverse effect on the flies as seen in the significant reduction in control fly hatching in all genotypes. It is, however, an interesting result that the ethanol solution appeared to have an interaction between the genotypes with those having the cap 2 methylation in place being more successful than those without. This may suggest a protective role of cap 2 methylation in the presence of toxic levels of compounds such as ethanol. This is an area which would benefit from further study.

There is suggestion from studies of viruses that cap 1 methylation interacts with interferon induction which has an anti viral role in cells (Daffis, *et al.* 2010). It is suggested that this is the reason for viruses which encode their own methyltranferase enzymes to produce this methylation as it prevents the release of interferons as part of an immune response to foreign RNA. If this is the case this suggests an entirely different pathway by which cap methylation could be influencing translation rather than just simply through the efficiency of binding. It should, however, also be noted that this is less likely to be a root of action for cap 2 methylation as it doesn't occur on all mRNA and viruses do not encode their own cap 2 methyltransferases although can use those already present within the cell (Kruse, *et al*. 2011).

There are a number of problems within this study which have put the reliability of some results into doubt. As mentioned above the compound carbamazepine could not be applied accurately and if a new method for the application of this compound could be developed then this may show more significant and reliable results. An ongoing issue in the initial compound trials was the unreliability of egg laying

particularly by the Cap 1^{null} Cap 2^{null} mutants. This was rectified in later trials and it is important to note that the use of specific numbers of embryos in future studies would significantly reduce error in the results. It should also be noted that all of the compounds used have a significant number of reported effects which are often subject to limited study and not well understood. This means that caution should be taken when using them to directly look for effects of cap methylation on translation efficiency as other chemical interactions may also be taking place. Further study is also required into the breeding of Cap 1^{null} Cap 2^{null} mutants as preliminary findings suggest that they may have a breeding deficiency, which if studied further may demonstrate a further role of cap 1 and 2 methylation.

There are, however, some interesting findings from this study which have potential to generate further research. Some of the results within the study seem to backup theories that cap 1 methylation may cause a slight increase in translational efficiency. It also opens up some interesting avenues for further study into the role of cap 2 methylation as in the nutritional trials, this appeared to have a limiting effect on translation, whereas in the compound trials the opposite effect seemed to be demonstrated.

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Appendix 1
Raw Data

Nutrition Trials

F	Day 10	Day 12	Day 14	Day 16	Day 18	Total
C1	34	2	0	0	0	36
C2	27	2	0	0	0	29
C3	31	5	0	0	0	36
PC1 1	14	7	0	0	0	21
PC1 2	9	13	0	0	0	22
PC1 3	15	9	0	0	0	24
PC2 1	28	4	0	0	0	32
PC2 2	30	3	0	0	0	33
PC2 3	25	9	0	0	0	34
PC12 1	25	4	0	0	0	29
PC12 2	36	2	0	0	0	38
PC12 3	29	4	0	0	0	33
Y20%						
C1	0	23	0	0	0	23
C2	0	18	7	2	0	27
C3	0	0	23	5	0	28
PC1 1	0	0	5	13	5	23
PC1 2	0	17	5	2	0	24
PC1 3	0	0	5	17	3	25
PC2 1	0	9	14	5	0	28
PC2 2	0	1	16	7	2	26
PC2 3	0	3	19	12	0	34
PC12 1	0	0	4	16	2	22
PC12 2	0	0	1	9	3	13
PC12 3	0	0	1	5	6	12
Y2%						

C1	0	0	13	9	0	22
C2	0	0	3	14	3	20
C3	0	0	12	6	0	18
PC1 1	0	0	0	0	3	3
PC1 2	0	0	0	0	0	0
PC1 3	0	0	0	0	2	2
PC2 1	0	0	0	0	0	0
PC2 2	0	0	0	0	0	0
PC2 3	0	0	0	0	0	0
PC12 1	0	10	9	2	0	21
PC12 2	0	3	9	12	4	28
PC12 3	0	1	10	2	0	13
S0						
C1	19	4	0	0	0	23
C2	22	7	0	0	0	29
C3	24	7	0	0	0	31
PC1 1	12	9	1	0	0	22
PC1 2	10	10	2	0	0	22
PC1 3	11	18	1	0	0	30
PC2 1	24	7	0	0	0	31
PC2 2	29	5	0	0	0	34
PC2 3	26	8	0	0	0	34
PC12 1	2	24	1	0	0	27
PC12 2	6	14	1	2	0	23
PC12 3	5	10	0	1	0	16
F40%						
C1	0	28	4	0	0	32
C2	0	22	5	0	0	27
C3	2	21	1	0	0	24
PC1 1	0	15	6	0	0	21
PC1 2	0	13	8	0	0	21

PC1 3	0	12	11	0	0	23
PC2 1	0	22	14	2	0	38
PC2 2	0	21	7	2	0	30
PC2 3	0	26	5	2	0	33
PC12 1	0	5	13	1	0	19
PC12 2	0	1	15	2	0	18
PC12 3	0	1	12	4	0	17

Negative Geotaxis

Food	Genotype	Gender	Above	Below	Total	P
F	C	F	6	3	15	0.6
F	C	F	6	4	15	0.566667
F	C	F	3	2	15	0.533333
F	C	M	8	0	15	0.766667
F	C	M	5	3	15	0.566667
F	C	M	5	2	15	0.6
F	PC1	F	9	1	10	0.9
F	PC1	F	6	1	10	0.75
F	PC1	F	4	0	10	0.7
F	PC1	M	3	1	10	0.6
F	PC1	M	6	1	10	0.75
F	PC1	M	6	1	10	0.75
F	PC12	F	0	9	10	0.05
F	PC12	F	0	10	10	0
F	PC12	F	0	10	10	0
F	PC12	M	0	9	10	0.05
F	PC12	M	0	8	10	0.1
F	PC12	M	0	9	10	0.05
F	PC12	M	0	9	10	0.05
F	PC12	M	0	10	10	0
F	PC12	M	0	10	10	0
F	PC2	F	2	4	10	0.4
F	PC2	F	1	3	10	0.4
F	PC2	F	1	4	10	0.35

F	PC2	F	1	4	10	0.35
F	PC2	F	0	7	10	0.15
F	PC2	F	2	7	10	0.25
F	PC2	F	0	5	10	0.25
F	PC2	F	0	5	10	0.25
F	PC2	F	0	4	10	0.3
F	PC2	M	0	2	10	0.4
F	PC2	M	0	4	10	0.3
F	PC2	M	0	5	10	0.25
F	PC2	M	0	6	10	0.2
F	PC2	M	0	5	10	0.25
F	PC2	M	1	6	10	0.25
F	PC2	M	1	4	10	0.35
F	PC2	M	2	2	10	0.5
F	PC2	M	1	3	10	0.4
F40%	C	F	7	1	10	0.8
F40%	C	F	6	2	10	0.7
F40%	C	M	5	1	10	0.7
F40%	C	M	6	3	10	0.65
F40%	C	M	6	1	10	0.75
F40%	PC1	F	7	2	10	0.75
F40%	PC1	F	6	2	10	0.7
F40%	PC1	M	5	3	10	0.6
F40%	PC1	M	8	2	10	0.8
F40%	PC1	M	2	5	10	0.35
F40%	PC2	F	1	1	10	0.5
F40%	PC2	F	1	1	10	0.5
F40%	PC2	F	2	2	10	0.5
F40%	PC2	M	2	5	10	0.35
F40%	PC2	M	1	5	10	0.3
F40%	PC2	M	1	6	10	0.25
F40%	PC2	M	2	4	10	0.4
F40%	PC2	M	4	1	10	0.65
F40%	PC2	M	4	1	10	0.65

S0	C	F	5	1	10	0.7
S0	C	F	5	2	10	0.65
S0	C	F	4	1	10	0.65
S0	C	M	3	2	10	0.55
S0	C	M	3	3	10	0.5
S0	C	M	4	0	10	0.7
S0	PC1	F	9	0	10	0.95
S0	PC1	F	7	1	10	0.8
S0	PC1	F	7	1	10	0.8
S0	PC1	M	2	2	10	0.5
S0	PC1	M	4	2	10	0.6
S0	PC2	F	1	2	10	0.45
S0	PC2	F	1	3	10	0.4
S0	PC2	F	0	5	10	0.25
S0	PC2	F	3	1	10	0.6
S0	PC2	F	1	1	10	0.5
S0	PC2	F	1	2	10	0.45
S0	PC2	F	3	1	10	0.6
S0	PC2	F	3	0	10	0.65
S0	PC2	F	1	1	10	0.5
S0	PC2	M	0	7	10	0.15
S0	PC2	M	2	7	10	0.25
S0	PC2	M	0	6	10	0.2
S0	PC2	M	1	7	10	0.2
S0	PC2	M	0	7	10	0.15
S0	PC2	M	0	6	10	0.2
S0	PC2	M	1	6	10	0.25
S0	PC2	M	0	7	10	0.15
S0	PC2	M	0	6	10	0.2
Y2%	C	F	6	3	10	0.65
Y2%	C	F	5	1	10	0.7
Y2%	C	M	6	0	10	0.8
Y2%	C	M	8	0	10	0.9
Y20%	C	F	6	0	10	0.8

Y20%	C	F	9	0	10	0.95
Y20%	C	F	6	1	10	0.75
Y20%	C	M	5	1	10	0.7
Y20%	C	M	8	0	10	0.9
Y20%	PC1	F	9	1	10	0.9
Y20%	PC1	F	8	0	10	0.9
Y20%	PC1	F	9	0	10	0.95
Y20%	PC1	M	7	0	10	0.85
Y20%	PC1	M	9	0	10	0.95
Y20%	PC2	F	6	0	10	0.8
Y20%	PC2	F	4	0	10	0.7
Y20%	PC2	F	6	1	10	0.75
Y20%	PC2	F	5	1	10	0.7
Y20%	PC2	F	6	1	10	0.75
Y20%	PC2	F	3	2	10	0.55
Y20%	PC2	M	6	1	10	0.75
Y20%	PC2	M	7	0	10	0.85
Y20%	PC2	M	6	1	10	0.75
Y20%	PC2	M	4	0	10	0.7
Y20%	PC2	M	3	2	10	0.55
Y20%	PC2	M	4	4	10	0.5

Compound trials with defined number of Individuals

Genotype	Compound	Day 10	Day 12	Day 14	Day 16	Day 18	Total
C	50% Ethanol	11	0	0	0	0	11
C	50% Ethanol	8	0	0	0	0	8
C	50% Ethanol	19	1	0	0	1	21
PC1	50% Ethanol	5	0	0	0	0	5
PC1	50% Ethanol	5	0	0	0	0	5
PC1	50% Ethanol	7	0	0	0	0	7
PC2	50% Ethanol	4	0	0	0	0	4
PC2	50% Ethanol	2	0	0	0	0	2

PC2	50% Ethanol	0	0	0	0	0	0
PC12	50% Ethanol	0	0	0	0	0	0
PC12	50% Ethanol	0	1	0	0	0	1
PC12	50% Ethanol	0	0	0	0	0	0
C	C1.6	0	0	0	0	0	0
C	C1.6	0	0	0	0	0	0
C	C1.6	0	0	0	0	0	0
PC1	C1.6	0	0	0	0	0	0
PC1	C1.6	0	0	0	0	0	0
PC1	C1.6	0	0	0	0	0	0
PC2	C1.6	0	0	0	0	0	0
PC2	C1.6	0	0	0	0	0	0
PC2	C1.6	0	0	0	0	0	0
PC12	C1.6	0	0	0	0	0	0
PC12	C1.6	0	0	0	0	0	0
PC12	C1.6	0	0	0	0	0	0
C	C10	0	0	0	0	0	0
C	C10	0	0	0	0	0	0
C	C10	0	1	0	0	0	1
PC1	C10	0	0	0	0	0	0
PC1	C10	0	0	0	0	0	0
PC1	C10	0	0	0	0	0	0
PC2	C10	2	2	0	0	0	4
PC2	C10	3	0	0	0	0	3
PC2	C10	0	0	0	0	0	0
C	C4	0	0	0	0	0	0
C	C4	0	0	1	0	0	1
C	C4	0	1	0	0	0	1
PC1	C4	0	0	0	0	0	0
PC1	C4	0	0	0	0	0	0

PC1	C4	0	0	0	0	0	0
PC2	C4	1	1	4	0	0	6
PC2	C4	2	1	1	0	0	4
PC2	C4	3	0	1	0	0	4
PC12	C4	0	3	3	0	0	6
PC12	C4	0	0	0	0	0	0
PC12	C4	0	8	0	0	0	8
C	E10	32	3	0	0	0	35
C	E10	34	3	0	0	1	38
C	E10	19	17	5	1	0	42
PC1	E10	27	5	0	0	0	32
PC1	E10	35	2	0	0	0	37
PC1	E10	25	5	0	0	0	30
PC2	E10	3	6	0	0	0	9
PC2	E10	0	0	0	0	0	0
PC2	E10	9	3	0	0	0	12
PC12	E10	0	8	7	0	0	15
PC12	E10	0	0	0	0	0	0
PC12	E10	7	11	0	0	0	18
C	E25	4	16	9	2	0	31
C	E25	4	15	4	3	1	27
C	E25	1	11	9	2	0	23
PC1	E25	8	8	0	0	0	16
PC1	E25	2	10	0	0	0	12
PC1	E25	11	13	0	0	0	24
PC2	E25	0	0	0	0	0	0
PC2	E25	2	1	0	0	0	3
PC2	E25	0	5	5	2	0	12
PC12	E25	0	0	0	0	0	0
PC12	E25	0	0	1	0	0	1

PC12	E25	0	0	2	0	0	2
C	E4	40	0	0	0	0	40
C	E4	36	0	0	0	0	36
C	E4	31	8	0	0	0	39
PC1	E4	31	0	0	1	0	32
PC1	E4	25	0	0	0	0	25
PC1	E4	13	22	0	1	0	36
PC2	E4	26	6	2	0	0	34
PC2	E4	28	8	0	0	0	36
PC2	E4	12	0	0	0	0	12
PC12	E4	0	4	1	0	0	5
PC12	E4	0	13	0	0	0	13
PC12	E4	0	1	0	0	0	1
C	E62.5	0	0	0	0	1	1
C	E62.5	0	4	0	1	0	5
C	E62.5	0	3	5	2	0	10
PC1	E62.5	0	0	0	0	0	0
PC1	E62.5	0	0	0	0	0	0
PC1	E62.5	0	0	0	1	0	1
PC2	E62.5	0	0	0	0	0	0
PC2	E62.5	0	0	0	0	0	0
PC2	E62.5	0	0	0	0	0	0
PC12	E62.5	0	0	0	0	0	0
PC12	E62.5	0	0	0	0	0	0
PC12	E62.5	0	0	0	0	0	0
C	H2O	29	0	0	0	0	29
C	H2O	38	0	0	0	0	38
C	H2O	33	1	0	0	0	34
C	H2O	31	0	0	0	0	31
C	H2O	32	1	1	0	1	35

C	H2O	39	0	0	0	0	39
PC1	H2O	36	1	0	0	0	37
PC1	H2O	32	0	0	0	0	32
PC1	H2O	27	3	0	0	0	30
PC1	H2O	24	0	0	0	0	24
PC1	H2O	27	0	0	0	0	27
PC1	H2O	28	0	0	1	0	29
PC2	H2O	2	0	0	0	0	2
PC2	H2O	37	3	0	0	0	40
PC2	H2O	36	3	0	0	0	39
PC2	H2O	26	3	0	0	0	29
PC2	H2O	5	0	0	0	0	5
PC2	H2O	23	1	0	0	0	24
PC12	H2O	6	0	0	0	0	6
PC12	H2O	1	0	0	0	0	1
PC12	H2O	0	0	0	0	0	0
C	None	39	0	0	0	1	40
C	None	32	0	0	1	0	33
C	None	38	0	0	0	0	38
PC1	None	22	1	0	0	0	23
PC1	None	25	2	0	0	0	27
PC1	None	25	0	0	0	0	25
PC2	None	18	1	0	0	0	19
PC2	None	43	0	0	0	0	43
PC2	None	43	1	0	1	0	45
C	P10	1	11	5	0	0	17
C	P10	2	10	2	0	0	14
C	P10	4	7	3	0	4	18
PC1	P10	0	5	8	3	0	16
PC1	P10	0	0	1	0	0	1

PC1	P10	0	2	1	0	0	3
PC2	P10	0	8	3	3	0	14
PC2	P10	0	3	1	0	0	4
PC2	P10	0	0	0	0	0	0
PC12	P10	0	0	1	0	0	1
PC12	P10	0	0	2	0	0	2
PC12	P10	0	0	0	0	0	0
C	P25	0	0	1	0	0	1
C	P25	0	1	0	0	0	1
C	P25	0	2	1	0	1	4
PC1	P25	0	0	0	1	0	1
PC1	P25	0	0	0	0	0	0
PC1	P25	0	0	0	0	0	0
PC2	P25	0	0	0	0	0	0
PC2	P25	0	0	0	0	0	0
PC2	P25	0	0	0	0	0	0
PC12	P25	0	0	0	0	0	0
PC12	P25	0	0	0	0	0	0
PC12	P25	0	0	0	0	0	0
C	P4	29	3	0	0	0	32
C	P4	29	3	1	0	0	33
C	P4	22	5	0	0	0	27
PC1	P4	25	4	0	1	0	30
PC1	P4	20	7	0	0	0	27
PC1	P4	11	0	0	2	0	13
PC2	P4	5	3	3	0	0	11
PC2	P4	15	4	1	0	0	20
PC2	P4	5	0	0	0	0	5
PC12	P4	0	0	0	0	0	0
PC12	P4	1	1	0	0	0	2

PC12	P4	0	0	0	0	0	0
PC12	P4	0	0	0	0	0	0
PC12	P4	0	0	1	0	0	1
PC12	P4	0	2	0	0	1	3

LIM Domain Only Proteins, LMO1 and LMO4 in Hematopoiesis

by

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ABSTRACT

The LIM Domain Only (LMO) proteins have been shown to play a major role in cellular differentiation and development at the embryonic stage and in many human cancers. There is a large amount of evidence to show the crucial role of LMO2 within the early stages of hematopoiesis and due to the similarities of proteins in this family it would be logical to expect that LMO1 and LMO4 may also be involved. Current studies show that LMO1 has an involvement in some forms of T-ALL (T-Cell Acute Lymphoblastic Leukaemia) and LMO4 has a major role in cellular proliferation in some breast cancers. The aim of this study was to develop tools to perform overexpression and knockdown experiments with these proteins in murine early stage hematopoietic progenitors and in ES cells in an attempt to identify the function of the LMO1 and LMO4 proteins. Short Hairpin RNA (shRNA) constructs were produced and transfected into the cells and shown to work effectively in the knockdown of the proteins by up to 50%. Preliminary results regarding possible apoptotic effects of the LMO4 knockdown in myeloid progenitor cells were shown and a potential link to CDK9 suggested. This study provides the basis for future research into these proteins through the use of the tools created and starts to suggest possible roles for LMO1 and LMO4 in early stage hematopoiesis.

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It gives me great pleasure to thank Dr. Maarten Hoogenkamp and Dr. Vesna Stanulovic for welcoming me into their wonderful lab and for all their invaluable help and support. I would also like to thank the other members of my course in particular Panchali Kanvatirth, Melanie Schneider, Monika Köpf and Teja Bajt for their endless enthusiasm and support. My final thanks go to my family and girlfriend, Jen, for their encouragement and help throughout the year.

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INTRODUCTION

Although studies into the early stages of hematopoiesis have been carried out for many years, detail of the transcription factors and pathways involved is still lacking. During early embryonic development there are 2 clear types of hematopoiesis, primitive referring to the production of primitive or embryonic progenitor cells and definitive referring to the production of adult hematopoietic progenitor cells (Sturgeon, 2013). The primitive progenitor cells produce primitive erythroblasts with both embryonic and adult hemoglobin along with macrophages and megakaryocytes and are unable to produce lymphoid cells and hematopoietic stem cells (Sturgeon, 2013 & Lux, *et al*. 2008). During definitive hematopoiesis the cells produced contain only adult hemoglobins and hematopoietic stem cells (HSCs) are also produced. HSCs are able to seed the liver, bone marrow and other organs to continue life long blood cell production (Sturgeon, 2013 & Lux, *et al*. 2008).

Primitive hematopoiesis occurs mainly within the yolk sac forming a belt of primitive blood cells around the embryo (Lux, *et al*. 2008). The process first starts with the differentiation of pluripotent stem cells (known as embryonic stem (ES) cells in *in vitro* systems). The stem cells contained within blastocysts go though a number of stages of differentiation to form a colony, known as a hemangioblast, capable of differentiating into hematopoietic, endothelial and smooth muscle cells (Medvinsky, Rybtsov&Taoudi, 2011). The initial stages of differentiation were demonstrated by Fehling *et al.* 2003 showing that the cells go through a pre-mesoderm and a pre-hemangioblast stage defined by differences in expression of cell surface markers (fig 5.1). The main defining markers differing between the ES cell and hemangioblast is the expression of Flk-1 and Brachyury (Bry) (Fehling *et al.* 2003 & Gordon-

Keylock&Medvinsky, 2011). The hemangioblast then goes on to form an intermediate endothelial stage known as hemogenic endothelium, which is defined by being $Tie2^{hi}$, $cKit^{+}$ and $CD41^{-}$ (Lancrin, *et al*. 2009). It has been shown that the transcription factor Scl (also known as Tall) is critical for the differentiation from the hemangioblast (Lancrin, *et al*. 2009 & Sturgeon, 2013). Once formed, the hemogenic endothelium is able to produce the primitive hematopoietic cells.

The primitive hematopoietic system has been demonstrated *in vitro* in a number of studies and is commonly used in the study of early hematopoietic cells (Lancrin, *et al*. 2009). The early stages of differentiation in definitive hematopoiesis are less well understood and there is controversy around the in vitro system because there is currently no way to maintain the stem cells (Sturgeon, 2013). Hematopoietic stem cells (HSCs) are the key progenitor for definitive hematopoiesis and are found in the embryo from E10.5-11.5 (Sturgeon, 2013). In the same way as primitive hematopoiesis it is thought that a hemogenic endothelium is formed which is found in the yolk sack, dorsal aorta and placenta (Antas, *et al*. 2012). The definitive progenitor cells then bud from the surface of the endothelium where they can be sheared by blood flow and used to seed bone marrow and organs with HSCs (Antas, *et al*. 2012). There are also some interesting studies looking at the effect of the shear force of blood flow on the chemical signals within the hemogenic endothelium and whether this may affect the type of cells produced (Antas, *et al*. 2012 & Wolfe &Ahsan 2013). There has been disagreement about the exact origins of HSCs however Boisset, *et al*. (2010) used time lapse confocal microscopy to image the production of HSCs in slices through the embryonic aorta of mice and showed budding off of HSCs which they defined as being $Sca1^{+}$, C-Kit^{+}, $CD41^{+}$. The key difference which has been

identified in the formation of HSCs is the requirement for transcription factors Runx1 and Sox17 which are not required in primitive hematopoiesis (Sturgeon, 2013 & Lancrin, *et al*. 2009).

The exact differences between primitive and definitive hemogenic endothelium are still to be identified as HSCs are yet to be produced in vitro. It is likely that increasing knowledge about the transcription factors involved in the process would be the key to progressing this.

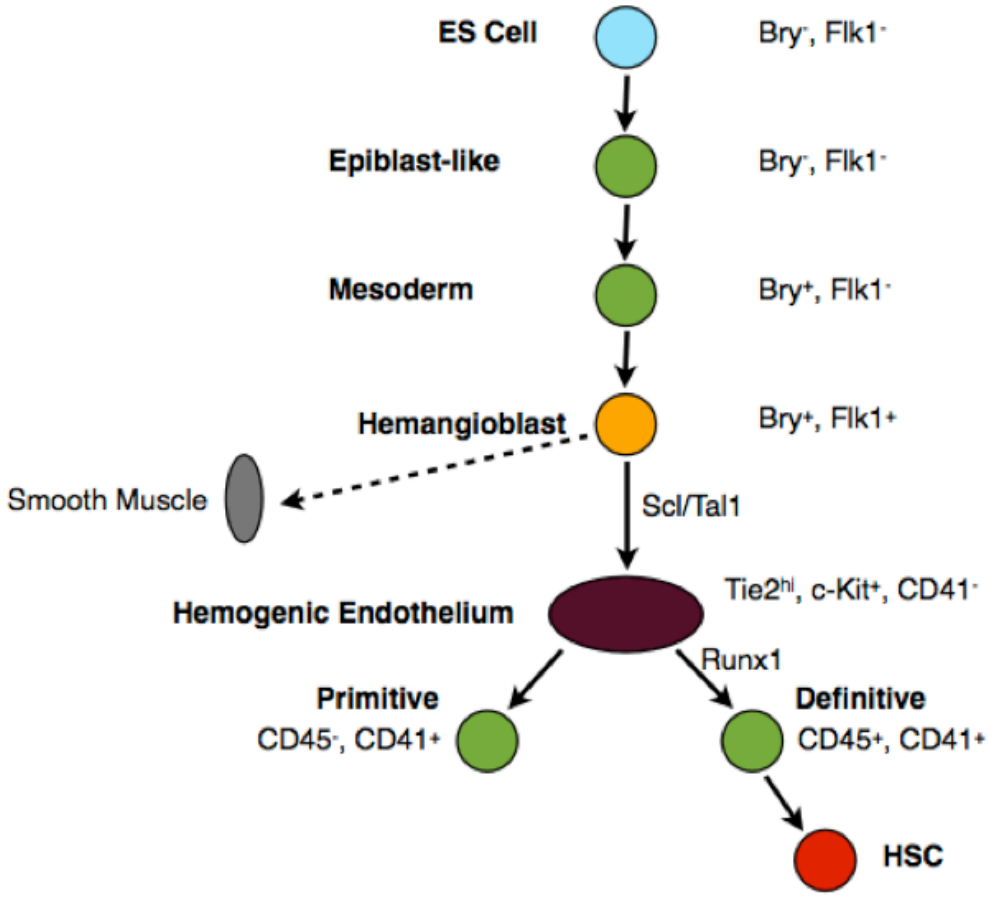

Figure 5.1 Early hematopoetic development showing some of the cellular markers and transcription factors involved (Fehling *et al.* 2003, Lancrin, *et al*. 2009, Sturgeon, 2013 & Gordon-Keylock&Medvinsky, 2011).

Relationship of the LMO Proteins to Early Stage Hematopoiesis

What relationship do the LMO family of proteins have to the early stages of hematopoiesis? The LMO (LIM Domain Only) family of proteins is of particular interest in relation to early hematopoiesis as they are thought to be involved in the regulation of transcription, through nucleating the formation of transcription factor complexes. This potentially gives them key importance in cell differentiation and cell fate (Matthews, *et al.* 2013). There is also evidence linking proteins within the family to development and diseases including some forms of T-ALL (T-cell Acute Lymphoblastic Leukemia), neuroblastoma and neural tube defects (Matthews, *et al.* 2013 & Lee *et al*. 2005).

The LMO Protein Family

LIM Domains

The family of LMO proteins are characterised by the presence of just two LIM domains and no other domains. Protein domains are individual units within a protein which are responsible for specific functions or interactions of the protein. A protein domain can be found across multiple proteins with different functions but with the structure of the domain remaining the same (EMBL-EBI, 2011). A LIM domain is a protein-protein interaction site made up of two zinc finger domains and usually has a size of around 50-60 amino acids (Zheng&Quanhui, 2007). The zinc finger domains are folded into the 'treble cleff' formation, are separated by 2 amino acids and contain 8 conserved residues (Zheng&Quanhui, 2007). Zinc finger domains have been related to a diverse range of functions including DNA recognition, acting as transcription factors, regulating apoptosis and protein structure formation (Laity, *et al*.

2001). Zinc fingers folded in the treble cleff formation are most commonly associated with binding directly to nucleic acids, however this has never been demonstrated for the LIM domain, which has only been shown to bind other proteins (Kadrmas&Beckerle, 2004).

The LIM domain is named after the proteins in which it was first discovered Linl-1, Isl-1 and Mec-3 and has been shown to be present in proteins of all well studied eukaryotic organisms (Kadrmas&Beckerle, 2004). Proteins containing LIM domains have been found in both the cytoplasm and nucleus and can be linked to other types of domain or can be the only domain a protein has, for example in the case of the LMO proteins. Proteins with LIM domains can be broadly classified into four groups, nuclear only, LIM only, LIM actin associated and LIM catalytic, the LMO proteins are all members of the LIM only group (Kadrmas&Beckerle, 2004). As mentioned earlier the main function which has been demonstrated for the LIM domain is to bind to other proteins. Protein binding interfaces are crucial for the formation of complexes with other proteins which are required for many biological processes (Kadrmas&Beckerle, 2004). As a result of the large amount of variability within the non-conserved regions of the LIM domain different LIM domains are able to specialise, meaning that as a family LIM domains have a wide range of binding partners (Kadrmas&Beckerle, 2004). Although LIM domains do not bind DNA they have consistently been shown to be critical in regulating gene expression, often through transcriptional regulation. Even proteins with LIM domains located in the cytoplasm have been shown to move between the cytoplasm and the nucleus allowing involvement in transcription factor complexes (Zheng&Quanhui, 2007).

The LMO Family

There are 4 proteins within the LMO family, LMO1, LMO2, LMO3 and LMO4.All of these proteins have been shown to be implicated in development and in a range of human cancers (Wang *et al.* 2010). They all generally function within transcription factor complexes in the nucleus and are expressed in a large number of embryonic and adult tissues. Particularly high expression is seen in the nervous system especially during early development. The proteins share a fairly high level of similarity, with LMO1 and LMO3 sharing the highest similarity at 89% and LMO4 being the least similar to others in particular LMO2 with a similarity of only 34% (Fig 5.2) (Matthews, *et al.* 2013).

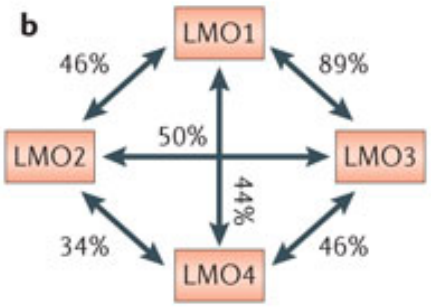

Figure 5.2. Similarities between the proteins in the LMO family (Modified from Matthews, *et al.* 2013).

LMO1

LMO1 has been shown to be mainly expressed within the nervous system however it has also been found in the thymus and kidneys of mice (Huret, 2013a). It was first discovered along with LMO2 when investigating chromosomal translocations resulting in a specific sub-type of T-ALL (Gill, 1995). LMO1 was identified near the break point of the t(11;14)(p15;q11) translocation in the T-ALL human cell line RPMI

8402 (Boehm, *et al*. 1988 & Matthews, *et al.* 2013). LMO 1 has also been implicated as an oncogene for neuroblastoma. Wang, *et al.* (2010) carried out a genome wide association study on neuroblastoma patients and identified four associated SNPs within the *LMO1* gene. They also showed that over-expression of the LMO1 protein results in the increased proliferation of neuroblastoma cells and that knock-down of the protein using shRNA inhibited growth.

An important role for LMO1 has also been shown in the homeostasis of gastric epithelial cells through regulation of apoptosis . Saeki, *et al*. (2007) showed that LMO1 has a regulatory effect on the *GSDM* gene which is known to be involved in apoptotic activity within pit cells and is often silenced in gastric cancer cells. LMO1 is not normally expressed within the hematopoeitc system and most of its functions appear to be shared with LMO2. As LMO2 is highly expressed and known to be critical for hematopoiesis, the great majority of research in this area is focused on LMO2.

LMO2

The LMO2 protein is probably the most researched of all the proteins within the LMO family due to its significant involvement in hematopoiesis. It is important during embryonic development as it is required for the production of primitive erthythreocytes and haemogenic endothelium which leads to HSCs and the definitive hematopoietic system (Huret, 2013b). LMO2 is also important for the early hematopoietic progenitors and the differentiation from HSCs to committed blood cells including the erythrocyte lineage in both embryos and adults.

A study of LMO2 knock-out mice showed that LMO2 is needed for erythropoiesis

within the yolk sack and knock-out mice died at E9-10 (Warren, *et al.* 1994). In 1997 Wadman, *et al*. showed that LMO2 formed a transcription factor complex with GATA-1, TAL1 (Scl) and Ldb1 in erythroid cells and that this complex was able to bind DNA, thereby regulating transcription. It has since been confirmed in other studies that this transcription factor complex is essential for erythropoiesis which is why knock-out mice did not survive past the embryonic stage (Kerenyi & Orkin 2010). In addition to the involvement of LMO2 in erythropoiesis, LMO2 has a very significant involvement in T-ALL in a similar way to LMO1. Translocations resulting in LMO2 overexpression include t(11;14)(p13;q11) and t(7;11)(q35;p13) which fuse genes highly expressed in T-cells upstream of the *LMO2* gene resulting in its overexpression (Matthews, *et al.* 2013). Along with its involvement in T-ALL LMO2 has also been implicated in B cell lymphomas. LMO2 is expressed in both normal B cells and B-ALL cells, it has been shown that an increased level of expression in B-ALL cells is correlated with better clinical outcomes (Malumbres, *et al*. 2011). Involvement in a large transcriptional binding complex has been shown in these cells which interestingly differs from complexes seen in erthyropoiesis and T-cell development due to the absence of GATA1-3. Overexpression of LMO2 however is not a sign of better clinical outcome in all types of B-cell lymphoma. It has been shown in ~1% of DLBCL (Diffuse Large B Cell Lymphoma) cases a translocation takes place which results in the expression of an extra transcription factor leading to LMO2 upregulation (Matthews, *et al.* 2013). The presence of LMO2 in this case is a sign of poor clinical outcome and it has been shown that LMO2 promotes proliferation of these cells. This shows that the background of other transcription factors within the cell also has a significant impact on the action of LMO2 reinforcing

the fact that due to its role in protein-protein interaction the actual outcome of inappropriate regulation of LMO2 can vary greatly.

LMO2 has also been implicated in solid tumors, including prostate cancer. LMO2 has been shown to be upregulated in the later stages of prostate cancer and in experiments of forced overexpression it has been shown to increase the invasive abilities and spread of cancer cells (Ma, *et al.* 2007). It is suggested that this results from the disruption of normal transcription factor complexes causing the repression of E-cadherin, a protein important in cell adhesion, which restricts the spread of cancerous cells (Matthews, *et al.* 2013). There is no evidence to suggest that overexpression of LMO2 alone can induce prostate cancer, however, it is clear that in a lot of cancers a number of mutations and deregulations can build up over time to progress the tumor growth.

LMO1 and LMO2 in T-ALL

LMO1 and LMO2 share a relatively high level of similarity, as do all proteins in the LMO family and as mentioned previously both LMO1 and LMO2 have been implicated in T-ALL. In ~50% of cases either LMO1 or LMO2 is upregulated and LMO2 upregulation occurs in ~9% of pediatric cases as a result of chromosomal translocations or a cryptic deletion (Matthews, *et al.* 2013). The reason for the upregulation is thought to be as a result of translocation of genes which are highly expressed in T-cells near to the *LMO1* or *LMO2* gene resulting in increased expression (Matthews, *et al.* 2013). In the case of the cryptic deletion Vlierberghe, et al. 2006 showed that this occurs in a negative regulatory region upstream of the LMO2 gene inactivating the regulatory effects of this region on LMO2 expression.

The development of T-ALL as a result of LMO2 upregulation was confirmed as a result of a side effect of gene therapy trials for x-linked SCID (Severe Combined Immunodeficiency Disorder). In the gene therapy the *IL2RG* gene was inserted using a retroviral vector to replace the mutated version causing the condition. Although the gene therapy was relatively successful in increasing the number of T-cells, after several years some of the patients developed T-ALL. This was caused by the insertion of the IL2RG gene upstream of the LMO2 gene resulting in overexpression of LMO2 and the eventual development of T-ALL (Hacein-Bey-Abina, *et al.* 2008 & Hacein-Bey-Abina, *et al.* 2003).

The upregulation seen in T-ALL without translocations is thought to result from the activation of a feedback loop which promotes expression of LMO2. The LMO2 protein has been shown to form a complex with ETS transcription factors which can then bind to the LMO2 promotor and upregulate expression (Matthews, *et al.* 2013 &Oram, *et al.* 2010). This means that a small increase in the expression of LMO2 or other related transcription factors may be able to unbalance the homeostasis of the system and form a large excess of LMO2. This is important because in the normal T-cell differentiation pathway LMO2 is downregulated throughout the process to a point where it is undetectable in the mature cells (Matthews, *et al.* 2013). McCormack, *et al.* (2010) demonstrated that forcing expression of LMO2 at the DN3 stage of the pathway resulted in self-renewal of these cells, effectively giving them a property of stem cells, which appears to be a first step in the progression to leukemia. Although this is a clear route implicating LMO2 in disrupting normal functioning in the thymus, T-ALL takes a significant period of time to develop after this extra proliferation of cells, between 2 and 6 years in humans (Hacein-Bey-Abina, *et al.* 2008). It is thought

that this is because the self renewal of cells at the DN3 stage is not directly responsible for leukemia as these cells are still able to develop into functional T-cells (McCormack, *et al.* 2010). It has been suggested that the pool of extra cells formed at this stage increases the likelihood of mutagenesis, inactivating tumor suppressors such as Cdkn2a and disruption of the NOTCH pathway resulting in the final high proliferation with low differentiation seen in T-ALL (Matthews, *et al.* 2013). This pool of cells represents 'leukemic stem cell' like cells which are a separate population to the leukemic cells. McCormack, *et al.* 2010 showed, in mice, that this pool of cells in very hard to target therapeutically resulting in a high percentage of relapse. This may partially be because the cells exhibit senescence whereas most cancer therapies target rapidly dividing cells.

LMO3

LMO3 is the least researched of the LMO family, however, as with the other members of the family it has been implicated in development and in cancer. Isogai, *et al.* 2011 showed that LMO3 and HEN2 (a neuronal transcription factor) are significantly up-regulated in unfavorable neuroblastoma tumors and that cells which over-expressed LMO3 had an increased growth rate and rapidly developed into tumors in mice. Further studies have shown that LMO3 and HEN2 in complex reduces the ability of another transcription factor HES1 to negatively regulate Mash1 (a proneuronal protein) which is known to be critical for neuronal development and is highly expressed in neuroblastoma cells (Isogai, *et al.* 2011). Studies have also suggested that LMO3 may form complexes with CIB (a calcium and integrin binding protein) which results in LMO3 activity moving from the nucleus to the cytoplasm and possibly

inhibiting cell proliferation (Hui, *et al*. 2009).

LMO1 and LMO3 exhibit the highest similarity within the LMO family and have been shown to be able to compensate for each other. Tse, *et al*. (2004) demonstrated that when knocking-down LMO1 and LMO3 separately, in mice, no discernible phenotype could be found. Although LMO1 is much more highly expressed during neural development than LMO3 the knockdown of LMO1 had no apparent neural defects. When both proteins were simultaneously knocked down this resulted in death shortly after birth. No morphological defects were found.However, it was hypothesised that death probably resulted from neural defects due to the lack of expression of LMO1 and LMO3, which would usually be found within the developing neural system. The compensatory effect of these two members of the LMO family raises potentially interesting questions regarding the extent to which other members of the family may compensate and this may be important to remember in studies such as this, involving the knockdown of just one member of the family.

LMO4

LMO4 has the least similarity to the other members of the LMO family and was first discovered in the cDNA library for a human breast tumor (Racevskis, *et al.* 1999). The majority of research around LMO4 is linked to its role in breast cancer, however, it has also been shown to be implicated in squamous cell carcinoma of the oral cavity, neural tube development and a survival advantage in pancreatic cancer (Matthews, *et al.* 2013 &Tse, *et al*. 2004). Unlike other members of the LMO family, LMO4 has been shown to be widely expressed across tissues in both embryonic and adult mice with highest expression in proliferating epithelial tissue (Sum, *et al.*

2005a). LMO4 is essential in the early stages of development. Knockout mice have shown embryonic lethality due to failed neural tube closure resulting in exencephaly and anencephaly (Tse, *et al*. 2004). Kwong, *et al.* 2011 showed an upregulation of LMO4 and Ldb1 in the nuclei of squamous cell carcinoma cells, particularly at the 'invasive front', supporting a significant role in cancer progression.

LMO4 was first identified in breast cancer as an auto-antigen (a protein generated by the tumor which evokes an immune response).It is associated with a poor prognosis (Matthews, *et al.* 2013) and in murine systems it was shown that over expression of LMO4 can result in tumor induction within mammary glands. This is also supported by siRNA knock-down of LMO4 in human cancer cell lines which shows a reduction in cell proliferation (Sum, *et al.* 2005b).The exact route by which LMO4 results in tumor induction is largely unknown, however, a number of potential routes have been identified in different cancer sub-types.

Unlike LMO1 and LMO2 there is no evidence to suggest that LMO4 induces stem cell like self renewal properties in cells (Matthews, *et al.* 2013). A major route by which changes in LMO4 expression act is via the cell cycle. Montañez-Wiscovich, et al. (2010) showed that knock-down of LMO4 in all examined sub-types of breast cancer resulted in G2/M arrest in the cell cycle. They also showed that other effects included increased cell death, amplification of centromeres and faults in spindle formation. Similar phenotypes also occur when LMO4 is over-expressed suggesting that for normal functioning a highly controlled level of LMO4 is required.

Studies have shown that LMO4 has a significant role in transcriptional regulation in breast cancer and a number of different interactions and binding partners have been identified. One of the most significant of these interactions is probably with the

BRCA1 protein which is known to be an active tumor suppressor in both breast and ovarian cancers. Sum, *et al.* (2002) showed that LMO4 is able to form a complex with CtIP and BRCA1, repressing the transcriptional activation of BRCA1 and therefore promoting cancerous cell growth in breast tissue. LMO4 has been shown to interact with repressor proteins such as MTA1 and RBBP8 which can act to repress ERα a common phenotype in the development of breast cancer(Singh, *et al*. 2005, Zheng&Quanhui, 2007 & Matthews, *et al.* 2013). It has also been shown that through this same pathway LMO4 can activate transcription by preventing binding of HDAC2 to chromatin and possibly also through the recruitment of GATA6 (Wang, *et al.* 2007 &Setogawa, *et al*. 2006). LMO4 has also been suggested to interact with the transcription factor DEAF1, where LMO4 may be responsible for localisation of DEAF1 in the nucleus (Matthews, *et al.* 2013).

The final way in which LMO4 overexpression may promote cancerous cell proliferation is through enhancing the TGFβ growth factor. It is known that TGFβ is involved in mesenchymal-epithelial interaction in cancer and development so its interaction with LMO4 is another potential pathway for the induction of tumor growth (Lu, *et al.* 2006).

Although most research looks at the negative clinical outcome of LMO4 overexpression there is also evidence to suggest that its overexpression can result in a positive outcome. In patients with pancreatic cancer it has been shown that tumors expressing higher levels of LMO4 give an improved outcome when surgically removed than those which do not (Murphy, *et al.*2008 & Yu, *et al*. 2008). It is unclear why this is and again potentially shows a role of LMO4 in cancer development and also identifies it as an important biomarker.

It is clear that none of the LMO family of proteins is able to function alone in disease or normal conditions. All proteins within the family are essential mediators in the formation of transcription factor complexes and through this route their deregulation has a key influence on both leukemias and solid tumors. Understanding the function of these proteins is therefore very important as this will increase our knowledge of tumor biology in general and more particularly they may prove to be therapeutic targets.

The LMO Family as Therapeutic Targets

Due to the oncogenic properties of LMO2 efforts have been made to develop ways of targeting and repressing LMO2 expression. Currently two methods for LMO2 targeting have been demonstrated, the first involves the introduction of an intrabody, an intracellular antibody which is able to bind to LMO2. This was shown to disrupt LMO2 function in T-ALL cells making it the basis for a potential therapeutic agent (Nam, *et al.* 2008). The second method involves the introduction of a peptide aptamer which binds to the second LIM domain of LMO2 and has again been shown to successfully disrupt the activity of LMO2 within T-ALL cells (Appert, *et al.* 2009). Both of these methods have been shown to halt tumor cell growth in T-ALL which not only confirms the role of LMO2 in T-ALL but also increases the possibility of drug development. Similar targeting strategies have been suggested for LMO4 in breast cancer, however, as the LMO proteins all have essential roles in normal cellular function as well as cancer general disruption of these proteins is not therapeutically viable (Matthews, *et al.* 2013). Methods would be required to make these inhibitors

specifically target cancerous cells and use of other therapeutics to form a combination therapy would be necessary.

Aims and Objectives

The aim of this study is to look for functions of LMO1 and LMO4 within hematopoiesis, particularly at the early stages. This is attempted through the development of shRNA knockdowns and overexpression constructs for the two proteins in PUER cells which are early myeloid progenitor cells in mice and A2lox, a doxycyclin inducible mouse embryonic stem cell system.

MATERIALS AND METHODS

The overall experimental method was to clone overexpression and shRNA sequences into PUER cells and A2lox ES cells to achieve knockdown of the LMO1 and LMO4 proteins. This was then followed up by studies of the phenotype of the cells to assess whether knockdown had been successful and whether the knockdown had any significant effect on the cell function or phenotype.

Cell Lines

All cell lines were cultured in humidified incubators at 37°C with 2% CO2 using Corning tissue culture flasks. Plat-E cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FCS, Penicillin/Streptomycin and Glutamax. Cells were split regularly before full confluence to ensure that the cell line remained healthy. PUER cells were cultured in IMDM with 10% FCS, Pen/Strep, Glutamax and 5ng/ml recombinant mouse IL-3 and were split when cells reached >1million per ml. When necessary ,*e.g.* after thawing out cells from liquid nitrogen, excess debris was removed from the cells by differential density centrifugation using Lympholyte-M and centrifuging at 1,000g for 20 minutes with reduced deceleration. The suspension of healthy cells was then removed and washed in PBS before returning to growth medium.

A2Lox ES cells were cultured based on Fehling, *et al.* (2003) in DMEM with recombinant mouse LIF on a layer of inactivated MEF (Mouse embryonic fibroblast) cells until differentiation was required.

Media composition for ES Cell Culture

FCS (15%)	75.0 ml
100x Pen/Strep	5.0ml
100x L-glu	5.0ml
Recombinant Mouse LIF	5.0ml
MTG (0.15mM)	0.5ml
Hepes Buffer 1 M stock	12.5ml
Invitrogen KO DMEM	to 500ml

Two days prior to differentiation the cells were transferred to gelatinized plates in the same media to allow them to adjust to the new type of plate and then one day before, transferred into IMDM still containing recombinant LIF. At day zero the cells were transferred to low adherence plates with differentiation medium so that they could grow into embryonic bodies. The cells were then cultured for between 3.2 and 3.75 days to allow development of hemangioblasts. At this point Flk1+ cells were separated by MACS separation and were then returned to culture for four days in blast medium which includes VEGF allowing the development of CD41+ haematopoietic precursor cells.

Production of shRNA sequences

The shRNA sequences were designed using RNAi Central shRNA designer from Hannon Lab based on LMO1 and LMO4 sequences sourced from NCBI BLAST. The sequences were also tested against NCBI BLAST to ensure that they were only found in the desired protein and any which were present in other proteins were discounted. Based on these generated sequences the oligonucleotides were ordered from Sigma-Aldrich. Initially three oligonucleotide sequences were designed for each protein however this was later increased to seven for LMO1 (Table 6.1)

Table 6.1 Oligonucleotide sequences for LMO1 and LMO4 shRNA's designed using RNAi Central shRNA designer from Hannon Lab

Name	Sequence (5' to 3')
shLMO1 **#1**	TGCTGTTGACAGTGAGCGAATGCTCTCCGTCCAACCTAAGTAG TGAAGCCACAGATGTACTTAGGTTGGACGGAGAGCATCTGCCT ACTGCCTCGGA
shLMO1 **#2**	TGCTGTTGACAGTGAGCGATGTGACTGTCGCCTGGGCGAGTAG TGAAGCCACAGATGTACTCGCCCAGGCGACAGTCACAGTGCCT ACTGCCTCGGA
shLMO1 **#3**	TGCTGTTGACAGTGAGCGAGGCTCCACTCTCTACACCAAGTAG TGAAGCCACAGATGTACTTGGTGTAGAGAGTGGAGCCCTGCCT ACTGCCTCGGA
shLMO1 **#4**	TGCTGTTGACAGTGAGCGAACTCTCTACACCAAGGCCAACTAG TGAAGCCACAGATGTAGTTGGCCTTGGTGTAGAGAGTGTGCCTACTGC CTCGGA
shLMO1 **#5**	TGCTGTTGACAGTGAGCGAAGACAAATTCTTCCTGAAGAATAG TGAAGCCACAGATGTATTCTTCAGGAAGAATTTGTCTCTGCCTACTGCCT CGGA
shLMO1 **#6**	TGCTGTTGACAGTGAGCGCCTACACCAAGGCCAACCTCATTAG TGAAGCCACAGATGTAATGAGGTTGGCCTTGGTGTAGATGCCTACTGC CTCGGA
shLMO1 **#7**	TGCTGTTGACAGTGAGCGCTCTCAATGGCACCTTTGAATCTAG TGAAGCCACAGATGTAGATTCAAAGGTGCCATTGAGATTGCCTACTGCC TCGGA
shLMO4 **#1**	TGCTGTTGACAGTGAGCGCCTACATTAGGTTATTTGGGAATAG TGAAGCCACAGATGTATTCCCAAATAACCTAATGTAGTTGCCTACTGCCTCGGA
shLMO4 **#2**	TGCTGTTGACAGTGAGCGACGGCATGATCCTTTGCAGAAATAG TAAGCCACAGATGTATTTCTGCAAAGGATCATGCCGCTGCCTA CTGCCTCGGA
shLMO4 **#3**	TGCTGTTGACAGTGAGCGCTCATCTCAAGTGTTTCACATGTAGT GAAGCCACAGATGTACATGTGAAACACTTGAGATGATTGCCTA CTGCCTCGGA

Cloning of LMO1 and LMO4 shRNAs into the pMSCV cloning vector

The shRNA sequences were first cloned into the pMSCV cloning vector which is a modified version of the MigRI vector based on the protocol by Stegmeier, *et al.* (2005) and includes a gene for hygromycin and ampicillin resistance. The MigRI vector has an IRES GFP between two viral LTRs and can be used to clone a gene in front of the IRES GFP and then using Plat E cells produce viral particles. The pMSCV vector has the IRES GFP sequence in MigRI replaced with the miR-30 sequence. This then means that the hairpin in the miR-30 sequence can be replaced with a designed shRNA sequence to silence a gene of interest.

To begin the cloning process the oligos first had restriction sites added by carrying

out a PCR using pSM2C primers which add the XhoI and EcoRI restriction sites (Table 6.2). An optimised PCR reaction was developed for the shLMO1 cloning to ensure that maximum efficiency was achieved (Fig 6.1). A column based PCR clean up was performed on the products using the 'NuceloSpin' PCR clean up kit protocol. The cleaned up samples were digested using EcoRI and XhoI restriction enzymes and then separated by gel electrophoresis on a 3% agarose gel. The pPrime plasmid was also digested using EcoRI and XhoI and separated on a 1% agarose gel. The size of the bands produced was confirmed against the ladder and they were cut from the gel. The DNA was eluted from the gel using the 'NuceloSpin' gel extraction protocol and DNA concentration was measured using a NanoDrop. The shRNA insert was ligated into the pPrime backbone using 0.5µl quick T4 DNA Ligase, 2µl 5x buffer, 100ng pPrime Backbone and 12.5ng of insert, the reaction was then made up to 10µl with water. The ligation was incubated at room temperature for 1 hour prior to use for transformation of competent *E.coli*. Unless otherwise stated all digestions were carried out using ThermoScientifc Fast Digest enzymes and Fast Digest Buffer at 37°C for 30 minutes.

Optimised sh Cloning PCR Program

- 92°C - 1min
- 6x
  - 94°C - 20 Seconds
  - 60°C - 15 Seconds
  - 72°C - 15 Seconds
- 36x
  - 94°C - 20 Seconds
  - 68°C - 20 Seconds
  - 72°C - 20 Seconds
- 72°C - 2 minutes
- 4°C - 10 minutes

Figure 6.1 Optimised PCR program for cloning of shLMO1 sequences.

Table 6.2 Primer sequences for pSM2C - Restriction sites highlighted in bold.

Primer	Sequence (5' to 3')
pSM2C Fwd - (**XhoI restriction site**)	GATGGCTG**C/TCGAG**AAGGTATATTGCTGTTGACAGTGAGCG
pSM2C Rev- (**EcoRI restriction site**)	GTCTAGAG**G/AATTC**CGAGGCAGTAGGCA

Transformation of Competent *E.coli*

Transformation of chemically competent *E.coli* (Bioline CH3-Blue) was carried out based on the Bioline protocol. In short, 2.5µl of the ligation was added to 25µl of competent cells, which after a short incubation on ice underwent heat-shock at 42°C for 30 seconds before returning to ice. LB medium was then added and the samples were incubated at 37°C for 1 hour. Finally the cells were spread onto LB plates containing 100µg/ml Ampicillin and allowed to grow at 37°C overnight. Colonies from these transformations were picked and cultured for mini-preps based on the protocol by Zhou, Yang & Jong, 1990. A sample of the DNA produced from the mini-preps was then digested with MfeI and XhoI to check for the correct insert size. MfeI was used instead of EcoRI as this produces a slightly larger fragment size which is easier to identify when separated by gel electrophoresis. Once separated on an agarose gel each mini-prep sample was checked for the correct band size to show that the insert was present. For all those where the insert was present a sample was sent for sequencing using the MSCV Fwd primer, which helped to ensure that the shRNA insert sequence was correct before transfecting it into cells or further subcloning.

Transfection of Plat-E cells

To successfully clone the shRNA sequences into PUER cells it was first necessary to transfect it into Platinum-E (Plat-E) retroviral packaging cells. Maxi-preps were carried out to produce larger quantities of the plasmids with the correct insert. To carry out the transfection Plat-E cells were cultured to between 70% and 80% confluent and transferred to fresh medium several hours prior to transfection. The transfections were carried out using Mirus TransIT transfection reagent following the Mirus protocol. For 15cm (176cm2) dishes this involved mixing 21ng of the plasmid DNA with 2.1ml of serum free medium and adding 63µl MirusTransIT. This was then incubated at room temperature for 15 - 30 minutes before adding it drop-wise to the Plat-E cells. The cells were then returned to incubate at 37°C overnight. After overnight incubation the cultures were then moved to a 30°C incubator for 3 days, allowing the viral particles produced by the cells to reinfect the cells significantly increasing the viral particle yield.

Infection of PUER cells

Using the viral particles produced by the Plat-E cells, PUER cells were infected to insert the shRNA sequences. PUER cells were infected by spin infection on 6-well plates. Between $0.5x10^6$ and $0.75x10^6$ cells per well were suspended in the supernatant from the Plat-E cells and centrifuged for 2 hours at 2,200 RPM. Once centrifuged, the plates were returned to a 37°C incubator to allow recovery for 1 hour before washing in IMDM medium and returning in their own culture medium at 37°C. Following this, after 24 hours hygromycin selection was started by addition of 10µl 50mg/ml hygromycin per 1ml of media. Cells were then checked daily against a

control to look for cell death and outgrowth of targeted cells.

Re-cloning

- pPrime/MSCV GFP

Due to a possible apoptotic phenotype of shLMO4 in PUER cells one of the shRNA sequences was re-cloned into an MSCV plasmid which contained GFP rather than hygromycin resistance. This allowed for targeted cells to be monitored more rapidly, both visually and by flow-cytometry. The original shLMO4 pPrime plasmid was digested with BglII and MfeI and MSCV GFP was also digested as a new backbone. The fragments were separated on agarose gel and the DNA extracted from the gel for ligation. The ligation, transfection, transformation and infection protocols were repeated as before, with the omission of the hygromycin selection.

- p2lox

To achieve insertion of the sequence into the ES cell A2lox inducible system it was necessary to re-clone the insert, together with the surrounding miR-30 sequence into the p2lox plasmid. By initially using the MigRI plasmid IRES GFP gets inserted behind the cDNA before the re-cloning into p2lox. The digestions with EcoRV and EcoRI for this re-cloning were performed sequentially, because of the presence of an extra EcoRI restriction site immediately next to the EcoRV site. Firstly p2lox was digested with EcoRV for 1 hour, after which EcoRI was added and the digest was allowed to continue for a further hour to ensure the plasmid was fully digested. The pPrime plasmid containing the shLMO insert was first digested with BglII for 1 hour and then cleaned using the 'NucleoSpin' PCR column clean up protocol. Following

this large fragment DNA polymerase I enzyme was used along with dNTPs to create blunt ends compatible with the EcoRV site, a further PCR cleanup step was carried out prior to digestion with MfeI. The products of these digestions were again separated by gel electrophoresis and the eluted DNA ligated to make the final plasmid.

Transfection of p2lox into A2lox ES cells

The A2lox cells were transfected with the p2lox plasmids via electroporation. After washing with PBS $1.2x10^6$ cells were mixed with 150µl of PBS, 20µg of the sh p2lox plasmid and 20µg of Cre-expressing plasmid. This was then elctroporated for 7 milliseconds at 240volts before being returned to growth medium. Following electroporation the cells contain both the p2lox plasmid including the sh construct and IRES GFP and a general CRE expressing plasmid. CRE-mediated recombination then places the cDNA and IRES GFP behind a dox-inducible promoter, which is already in the A2ox cells. A PFK promoter also contained within the plasmid is inserted in front of the Neomycin resistance gene which is already present in the cell meaning that neomycin resistance would be present in successfully transfected cells. After 7 days of selection individual colonies were picked of which 6 were frozen and 1 was used for initial tests.

Testing the constructs

Once produced it was necessary to test the constructs to ensure that the shRNAs had the desired effect of knocking down the proteins. This was first tested at the Plat-E transfection stage, Plat-E cells were transfected with both an shRNA and an over-

expressing construct for the protein containing GFP. This technique made it possible to compare fluoresence using fluorescence microscopy to see if the over-expressing construct was knocked down by the shRNA construct. The shLMO1 constructs were also tested in PUER cells by infecting PUER cells which already had an over expression construct for LMO1 with the sh construct. Fluoresence of these cells could then be analysed via Flow cytometry to identify the functional constructs. Flow cytometry was also used to analyse the shLMO4 GFP PUER cells to assess the infection efficiency and to monitor their survival rates.

Apoptosis Assay

An apoptosis assay was used on the shLMO4 GFP and the MigRI PUER cells to look for an apoptotic effect of the protein knock down. The assay used Annexin V and PI (Propidium Iodide) staining to identify apoptotic cells by flow cytometry. This was carried out using the eBioscience (2010) protocol which involves washing a sample of cells firstly in PBS and then in Annexin V binding buffer. Annexin V antibody is then added at 5µl per 100µl and incubated at room temperature for 15 minutes. After incubation the cells underwent a further wash in Annexin V buffer before being resuspended in 350µl and having 10µl of PI added immediately prior to analysis. The cells were analysed by flow cytometry detecting APC for the Annexin V, PE for the PI staining and FITC to detect GFP to be able to identify the successfully infected cells.

Western Blotting

Western blotting was used to determine the protein levels in both normal and transfected cells to assess the effect of the construct. Protein extract from both Plat-E

and PUER cells was produced by suspending the cells in loading buffer and boiling at 95°C for 5 minutes to denature proteins. SDS gels were loaded with up to 20µl of sample and run at 75V until the samples were in the running gel and then 150v until the smallest band of the ladder reached the end of the gel. Gels were blotted onto nitrocelulose membranes using an Invitrogen iBlot.Prior to the addition of antibodies ponceau-s stain was used to check for the presence of protein and equal loading on the membranes. Membranes were blocked using an appropriate blocking agent depending on the antibody (5% Milk in PBS-T or 5% Horse Serum in PBS-T). The secondary antibodies used were LiCor 800 antibodies which allowed imaging of the membranes on a LiCor infrared scanner.

qPCR

To carry out QuantitativePCR (qPCR), RNA extractions were performed on a range of cells. All RNA extracts were carried out using the QiagenRNeasy Mini Kit and final samples were eluted in 30µl of buffer. From these cDNA was produced using 1µl BiolineTetro Reverse Transcriptase, 1µl oligo(dT), 0.5µl 25mM dNTPs, 4µl Reverse Transcriptase Buffer, 1µl RNAseOut (RNase inhibitor), 10µl water and 2.5µl (1µg) RNA. This was then incubated at 45°C for 30 minute and 85°C for 5 minutes before storage at -20°C.

QuantitativePCR was then carried out using this cDNA to assess the expression of LMO1, LMO2 and LMO4 proteins in the cells used within the study. It was also used to assess the efficiency of the knockdown of LMO1 and LMO4 by the shRNA constructs. qPCR was carried out in 96-well plates with 5µl SybrGreen PCR master mix, 0.25µl (10 uM each) primers, 2.5µl cDNA and 2.25µl of water in each well. A

standard curve was produced for each set of primers using serial dilutions of the cDNA. Primers for the *GAPDH* gene were used for normalisation. The plates were run using an ABI 7500 Real-Time PCR System with a standard program and melting curve (Fig 2.2). Calculations were carried out to normalise the expression against GAPDH and then plots of the expression levels were produced.

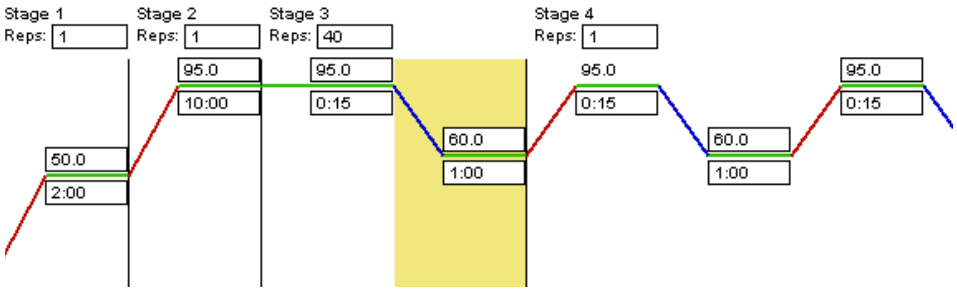

Figure 6.2. qPCR Program for RNA expression analysis.

Immuno-staining and Confocal Microscopy

To confirm the location of the LMO proteins within the cells immuno-staining and confocal microscopy of PUER and Flk+ ES cells cells was carried out. Cells were spun down at 300g before being washed in PBS with 2% FCS, 2% PFA and 0.05% tween. This was left to incubate for 5 minutes at room temperature and then washed twice with PBS+ 2% FCS + 0.05% tween. After washing the cells were resuspended in 100µl of the PBS solution and each sample had 1µg of primary antibody for one of the LMO proteins added. This was incubated at 4°C for 30 minutes before a further three washes and the addition of 1µg of secondary antibody. After a further incubation period three more washes were carried out and then the cells were transferred to slides using a cytospin. The final slides had Gold anti-fade regent with

DAPI applied to prevent the antibodies fluorescence from fading and also to stain the DNA within the cell. The slides were then analysed using an Zeiss LSM 510 confocal microscope.

Flow Cytometry and FACS

Flow cytometry was used to check the functioning of the LMO1 and LMO4 knockdown constructs in Plat E, PUER and Induced A2Lox ES cells. Plat E cells were prepared by first washing with PBS and then trypsinising prior to resuspension in PBS containing 10% FCS. PUER and A2Lox cells were prepared in a similar way but without the use of trypsin. The prepared cells were then run on a Cyan Flow Cytometer and the results analysed using Summit 4.3. FACS (Florescence Assisted Cell Sorting) was used to select cells containing the LMO4 knockdown construct for further analysis. Cells were prepared in a similar way to the flow cytometry analysis and returned to their standard growth media.

RESULTS

Expression of LMO Proteins

The expression of LMO1, LMO2 and LMO4 were demonstrated by qPCR within cells related to the early stages of hematopoiesis. Prior evidence suggested that LMO1 was only expressed in Flk+ cells so to confirm this two ES cell lines (CCB and A2Lox) with independent backgrounds were used. The results showed only very low levels of LMO1 expression in both ES cell lines and a clear upregulation in Flk+ cells. The expression levels in these cells prior to the Flk+ stage are very low and the expression within the myeloid progenitor PUER cells is also very low suggesting LMO1 is mainly needed at the intermediate Flk+ stage of the differentiation process. The levels of LMO2 are very low within the undifferentiated ES cells (CCB and A2lox) similar to LMO1. A higher level of expression is present in both the Flk+ cells and PUER cells. It is notable that the level in PUER cells is comparably higher than LMO1 and drops when the PUER cells undergo further differentiation. LMO4 is expressed at a low level in the undifferentiated ES cells (CCB and A2lox) and increases to a somewhat higher level in Flk+ haemangioblast cells, which was similar to the level observed in both undifferentiated and differentiated PUER cells (fig 7.1). Western blots using LMO1, LMO2 and LMO4 antibodies were also carried out across these cell types, however, the presence of the proteins could only be distinguished in over expressing cells for each protein. As it had been previously suggested that the LMO proteins were mainly located within the nucleus confocal microscopy was carried out on cells stained for DAPI and each of the LMO proteins. Along with this further western blots were also carried out using nuclear extract from A2Lox cells and A2Lox Flk+ cells. These results showed that only LMO2 was present in nuclear

extract from Flk+ cells and this localisation of LMO2 to the nucleus can also been seen in the confocal microscopy images. A low level of LMO4 was also shown in the ES cell nuclear extract but not in the Flk+ cells. From these westerns it was not possible to determine if this was also the case for LMO1 as the antibody bound unspecifically to degraded proteins in the ES nuclear extract sample (FIG 7.2).

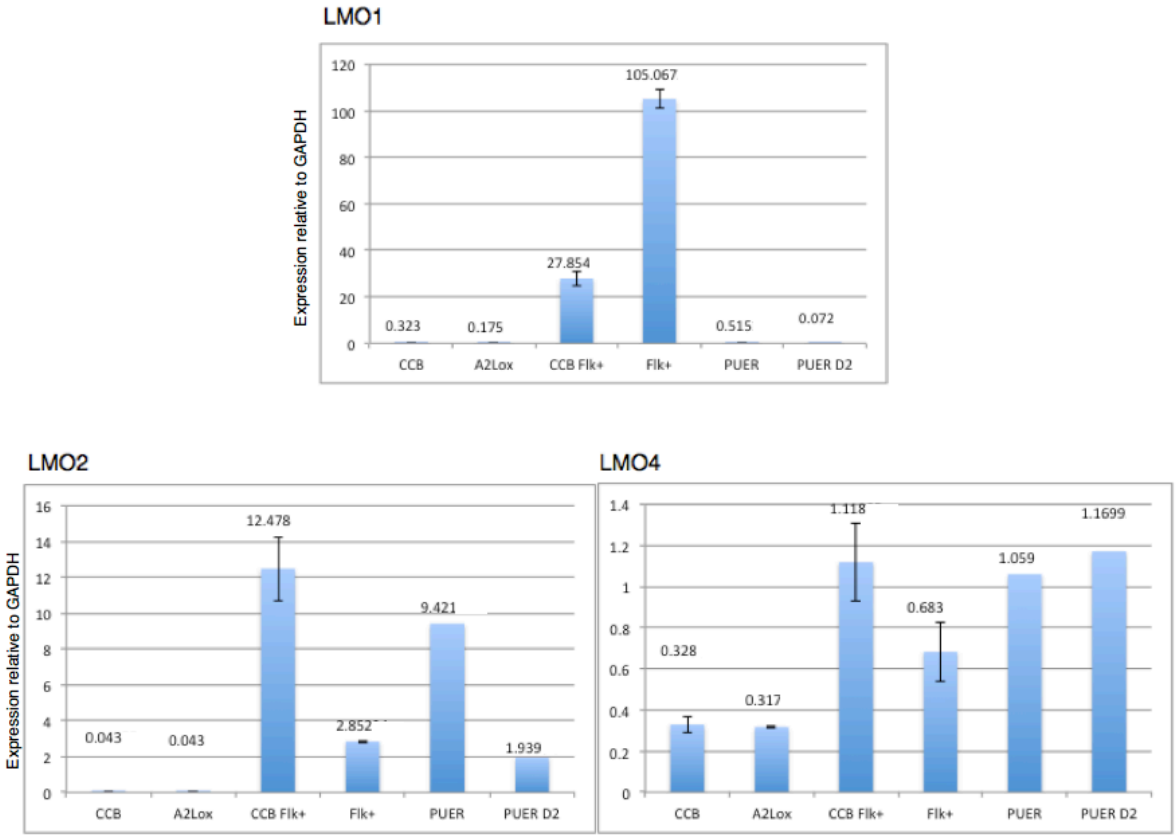

Figure 7.1. qPCR results comparing expression levels of LMO1, LMO2 and LMO4 to GAPDH on cDNA from cells at varying differentiation stages from the earliest stage ES cells (CCB and A2Lox) to Flk+ Hemangioblast type cells and finally myeloid progenitor cells (PUER) and differentiated progenitors.

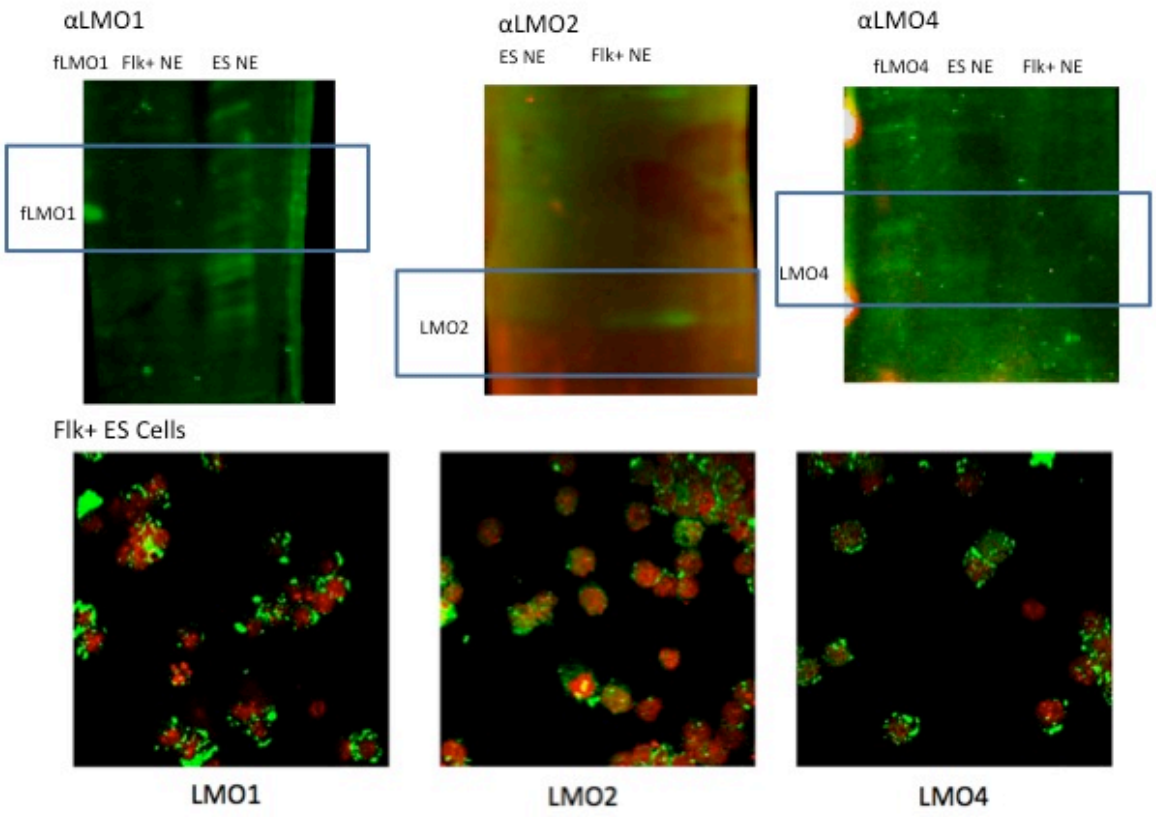

Figure 7.2. Western blots using αLMO1, αLMO2 and αLMO1 antibodies on A2Lox ES cell nuclear extract (ES NE) and Flk+ cell nuclear extract (Flk+ NE). Flag LMO1 and Flag LMO4 in PUER cells were also used as positive controls for LMO1 and LMO4. Confocal microscopy images show Flk+ ES cells stained for LMO1, LMO2 or LMO4 (Green) and DNA/DAPI showing the nucleus (RED). This shows LMO1 to be mainly cytoplasmic, LMO2 to be mostly nuclear and LMO4 to be both in Flk+ ES Cells.

Cloning shLMO1 and shLMO4 - pMSCV (pPrime)

The first stage of the cloning process required the addition of restriction sites to the shRNA oligonucleotides using PCR. All six initial reactions (3 x shLMO1 and 3 x shLMO4) showed a product of expected size (Fig 7.3). The products were then digested with EcoRI and XhoI for insertion into the pMSCV vector, this produced fragments of ~120bp which could then be eluted from the gel for use in the ligation reaction.

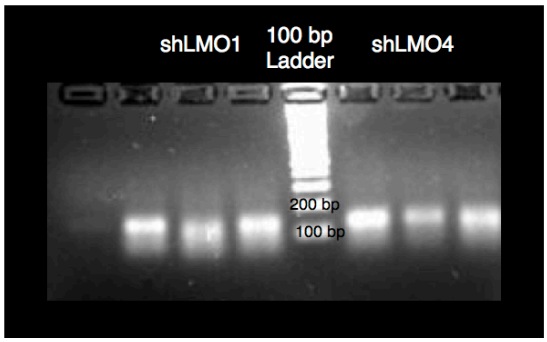

Figure 7.3. Agarose gel image showing PCR products from shLMO1 and shLMO4 reactions with pSM2C primers.

Following ligation and transformation into competent *E.coli* the DNA was extracted and digested with BglII and MfeI enzymes to check for the successful insertion of the insert into the plasmid and its amplification within the *E.coli*. Most samples for both shLMO1 and shLMO4 showed a band at ~350bp which was compared to a positive control (Flg 7.4). DNA from some of these samples was sequenced and showed at least one correct sequence for each of the 6 shRNAs with the exception of shLMO1 #2 which did not show a complete sequence due to a problem in the sequencing reaction. This was probably caused by a high GC-rich internal complementarity within the designed part of the shRNA sequence, which can prevent proper denaturation during the sequencing reaction.

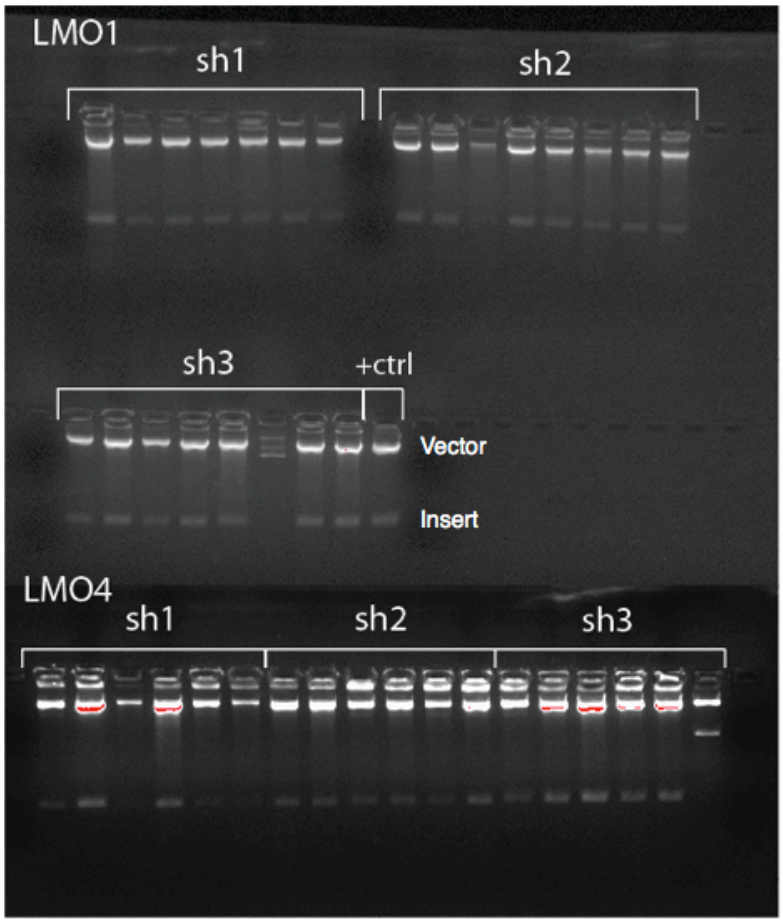

Figure 7.4. Digestions with BglII and MfeI of ligated shLMO1 and shLMO4 inserts after mini-preps. +ctrl shows the expected vector and insert sizes of positive clones, all clones showing an insert band of this size were regarded as positive.

After the functional validation of the shRNA constructs (descirbed below) we designed a further four constructs for shLMO1. Initially a number of failed attempts to produce a successfully ligated plasmid for these occurred, so optimisation of the initial PCR reaction was carried out. By creating a two stage PCR program with an initial 6 cycles with a 60°C annealing temperature and then a further 36 cycles with the annealing temperature increased to 68°C. This resulted in much cleaner bands which could be easily extracted from the gel with less chance of other fragments being included (fig 7.5).

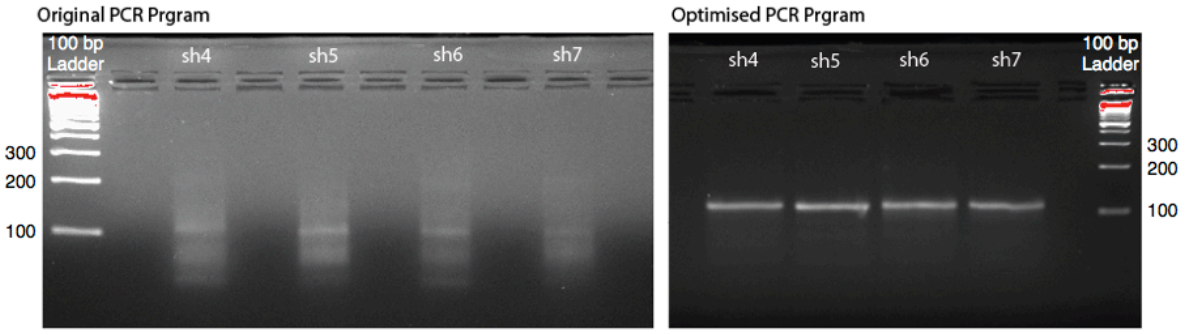

Figure 7.5. PCR optimistation for cloning of the shLMO1 constructs. Products after digestion with EcoRI and XhoI.

Functional Validation of shRNA Constructs

To test the shRNAs a co-transfection of Plat-E cells was carried out with each of the shLMO1 and shLMO4 constructs and plasmids for the over expression of the LMO1 and LMO4 proteins with IRES GFP. These cells were analysed visually by fluorescence microscopy and by flow cytometry which showed that constructs #2 and #3 for LMO4 were functioning correctly and knocking down the over expressed protein and that none of the shLMO1 constructs were working effectively. Based on these results shLMO4 #2 was used for further study (Fig 7.9) and the further four shLMO1 constructs were designed.

Once the extra shLMO1 constructs were successfully produced and sequenced these were transacted into Plat-E cells using the same co-transfection technique as previously described. The resulting cells showed a more significant reduction in GFP level of construct #6 than the control and other constructs when compared visually by fluorescence microscopy and this is supported by the flow cytometry results. These results show a clear shift to the cells being less intensely GFP positive for shLMO1 #6 when compared to shFF3, an shRNA directed towards the firefly luciferase gene, which is not present in normal cells so acts as a negative control (Fig 7.6a). This was

replicated in LMO1 GFP over-expressing PUER cells which were infected with the shLMO1 constructs. These results show a clear shift to being less GFP positive in both constructs #4 and #6 when compared to the normal overexpressing PUER cells (Fig 7.6b).

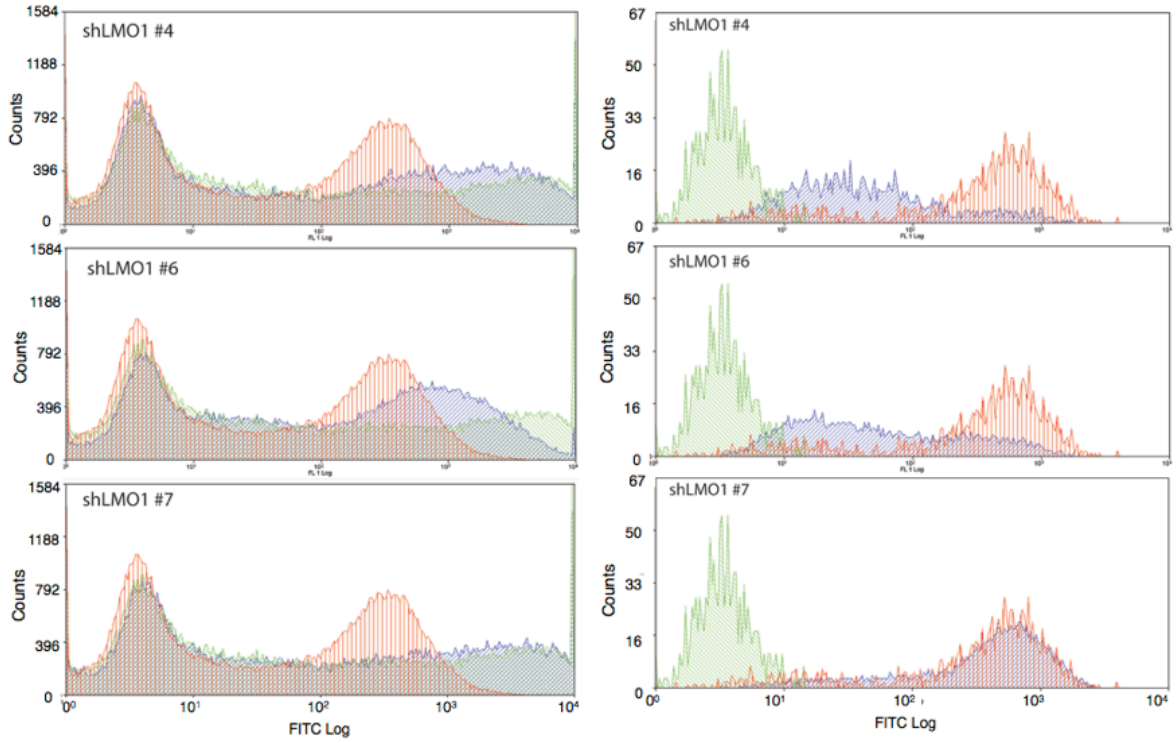

Figure 7.6. **a)** Flow Cytometry analysis of plat-e cells co-transfected with an LMO1 GFP expression construct and shLMO1 constructs (Red - shGFP, Green - shFF3, Blue - shLMO1 constructs). **b)** Flow Cytometry analysis of LMO1 GFP expressing PUER cells infected with shLMO1 constructs (Red - LMO1 GFP, Green - Uninfected cells, Blue - shLMO1 constructs.)

FlagLMO1 and FlagLMO4

Over-expressing PUER clones for LMO1 and LMO4 were also remade with the addition of the flag sequence so that the reliability of specific antibodies for LMO1 and LMO4 could be assessed. The overexpression flag constructs were first tested in Plat-E cells and analysed by western blotting using α-Flag, α-LMO1 and α-LMO4.

This showed that the flag constructs worked successfully against the Plat-E negative control (Fig 7.7).

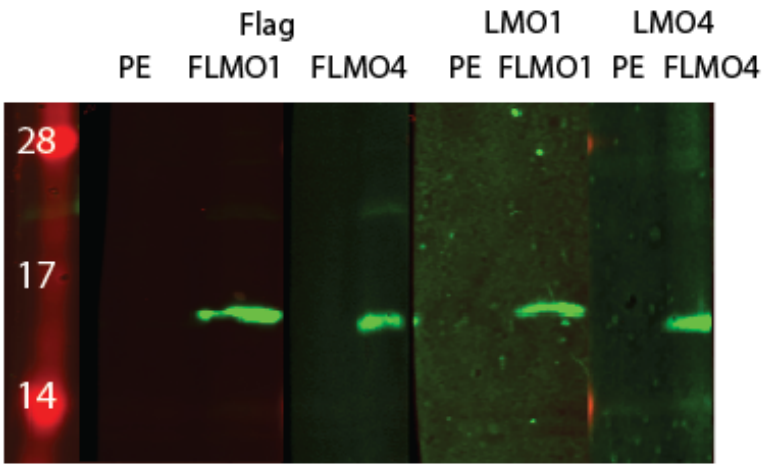

Figure 7.7. Western blot showing protein extract from Plat-E cells (PE) and Plat-E cells transfected with over expression constructs for Flag-LMO1 (FLMO1) and Flag-LMO4 (FLMO4).. Using both LMO and Flag primary antibodies to demonstrate the presence of both constructs and li-cor 800 secondary antibodies.

After transfection of PUER cells with the Flag constructs western blots were carried out using PUER nuclear extract to test the constructs. In western blots the flag constructs were shown to work with the LMO2 antibody and the use of anti-flag antibody demonstrated the functioning of the constructs in PUER cells. The use of the LMO2 antibody and construct acted as a control as the expression of LMO2 is well documented. The LMO4 antibody was also shown to work with flag-LMO4 in both PUER nuclear extract and Plat-E cells, however the level of LMO4 in normal PUER nuclear extract was not high enough to be detected (Fig 7.8).

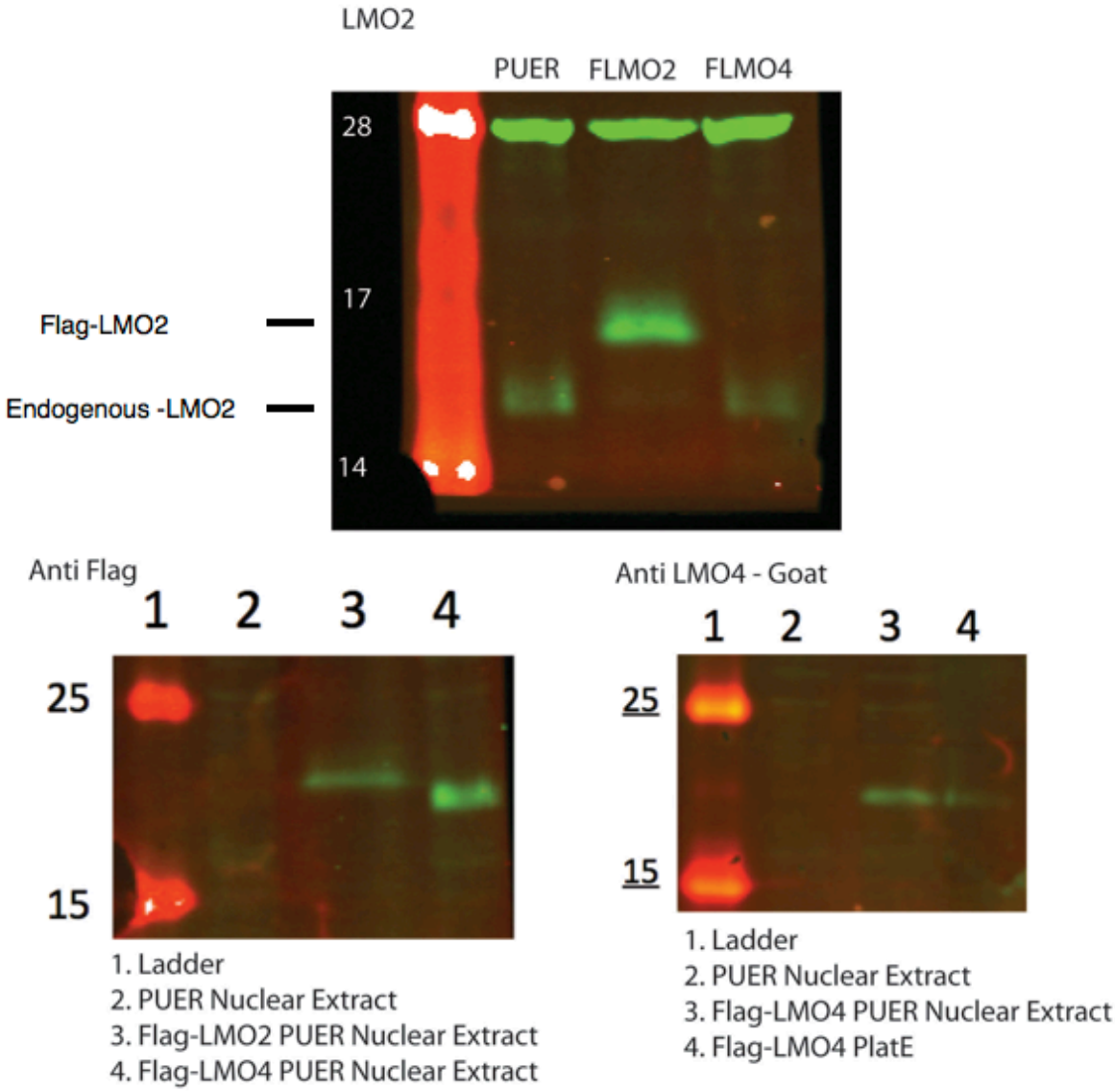

Figure 7.8. Western blots showing LMO2, LMO4 and Flag antibodies on Flag-LMO2, Flag-LMO4 and PUER cell nuclear extract.

shLMO4 PUER cells

PUER cells were infected with the shLMO4 construct to knock-down LMO4 production and an infection of PUER cells with MigRI was also carried out as a negative control, giving only GFP expression. The initial construct used a hygromycin resistance gene to allow for selection of successfully infected cells. In all replicates of this experiment all cells died within one week of infection and outgrowth of resistant

clones was unsuccessful. To ascertain whether this was a result of poor infection efficiency or a phenotype of the knock-down a new shLMO4 construct was produced, containing the GFP gene instead of the hygromycin resistance gene. One day after the infection the cells were analysed visually by fluorescence microscopy. This analysis showed at this time point a relatively low (~15%) proportion of GFP+ cells, indicating that a number of cells were becoming GFP positive. Further infections were carried out to allow for investigation into a possible phenotype. Following infection, a proportion of the cells were sorted at day 2 after infection by FACS into GFP positive and negative fractions. In this initial sort 30.59% of the MigRI cells were GFP-positive and 29.16% of the shLMO4 sorted cells were GFP–positive, showing a relatively high infection efficiency and an equivalent percentage to the control (Fig 7.9). At day 3 a sample of the unsorted cells was analysed by flow cytometry and this showed the GFP-positive fraction for the MigRI transfected cells to be still at 27.97%, however, for the shLMO4 transfected cells this had dropped to only 15.06% (Table 7.1). Further measurements were taken on days 4, 5, 6 and 8 and on each occasion the percentages remained at a similar level (Table 7.1, Fig 7.9).

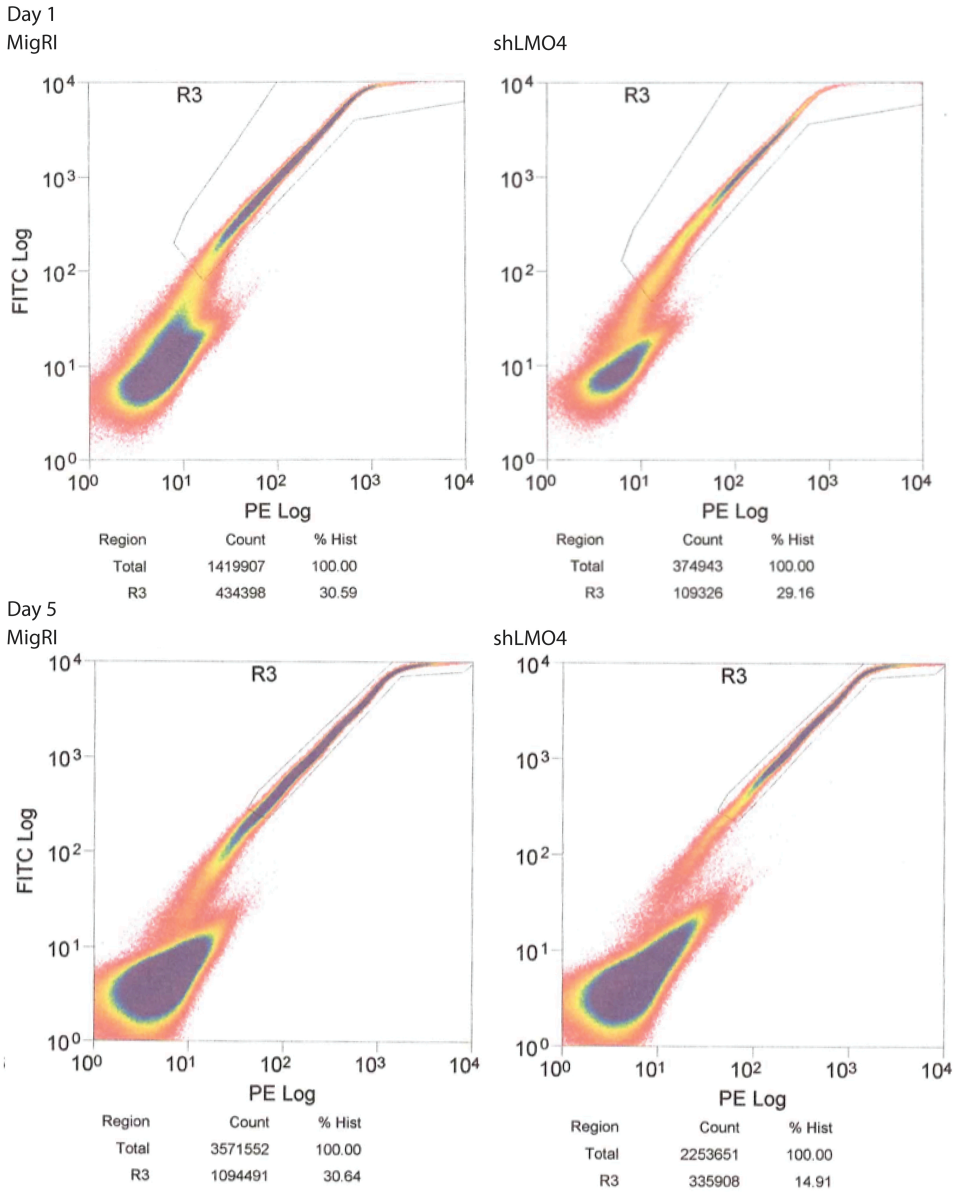

Figure 7.9. FACS plots for sorting of MigRI infected PUER cells and shLMO4 infected PUER cells on days 1 and 5 post infection. The sorting uses a combination of FITC and PE detection to detect and sort the GFP positive cells. R3 shows the percentage of GFP positive cells present within the population. The gating on Day 5 was stricter than Day 1 to ensure that only the most GFP positive cells were selected.

Table 7.1. Percentage of GFP positive cells within an unsorted population of infected MigRI and shLMO4 cells.

Day	MigRI	shLMO4
1	30.59%	29.16%
2	27.97%	15.06%
4	30.12%	14.28%
5	30.64%	14.91%
6	31.65%	14.47%
8	31.07%	14.24%

To confirm that the knockdown of LMO4 had been successful RNA extractions were carried out on sorted cells and cDNA produced for qPCR. The qPCR results showed a knockdown efficiency of 51% comparing PUER cells to shLMO4 PUER cells and 55% comparing the MigRI control to shLMO4 PUER cells (Fig 7.10) Following the discovery that shLMO4 PUER cells had a significant survival problem apoptosis assays were carried out. A comparison was carried out between MigRI PUERs and shLMO4 PUERs looking at Annexin V and PI staining. The results showed a higher percentage 14% of shLMO4 cells in an apoptotic/necrosis state when compared to 3% of MigRI cells (Fig 7.11).

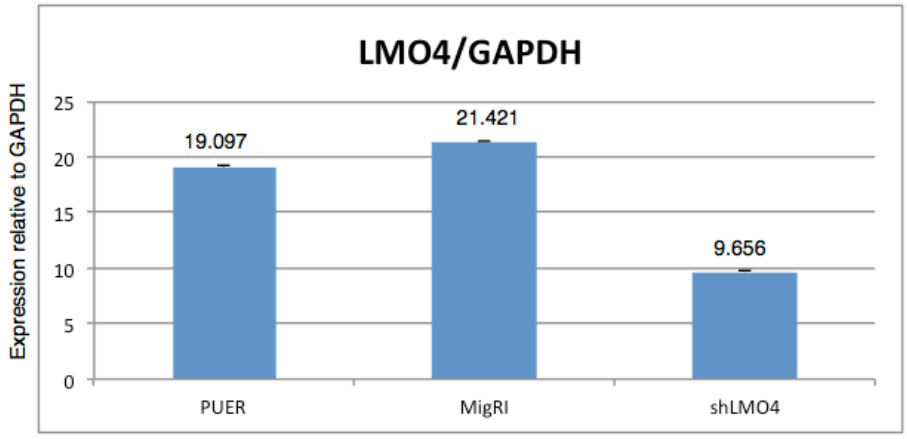

Figure 7.10. RNA Expression of shLMO4 PUER cells versus uninfected cells and MigRI infected cells.

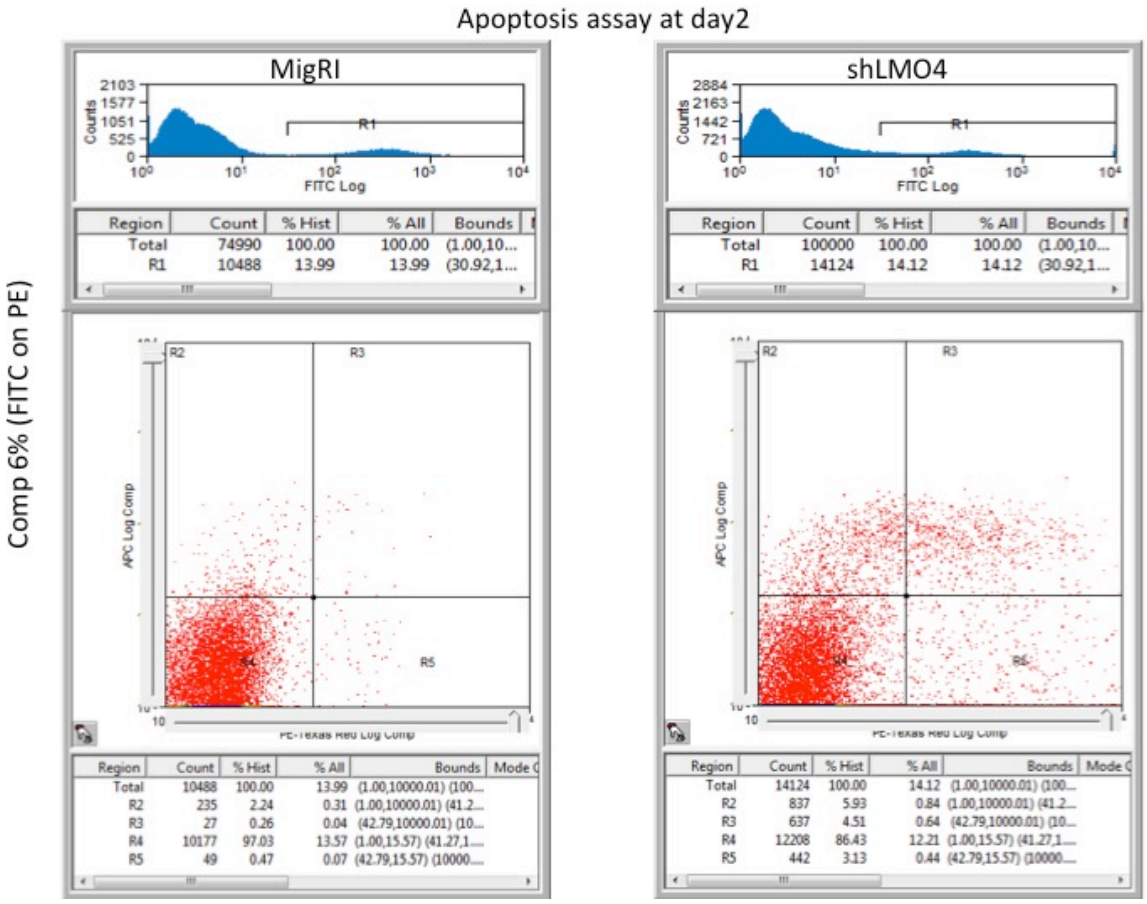

Figure 7.11. Apoptosis assay at day 2 following infection using Annexin V (APC staining) and PI (PE channel). R4= Healthy cells, R2= cells committed to apoptosis, R3= apoptotic cells with a permeable membrane and R5 = dead cells/necrotic cells.

shLMO1 and shLMO4 A2lox cells

A2lox cells are inducible ES cells which means that the Flag-LMO1 and shLMO1 constructs can be activated at different time points through differentiation by the addition of doxycyclin. Initial trials showed that in uniduced Flk+ cells containing the Flag-LMO1 construct a high level of LMO1 was expressed when compared to the Flk- undifferentiated cells. When induced the cells showed a 276% increase in LMO1 expression demonstrating that the induction system worked effectively (Fig 7.12).

The shLMO1 construct was analysed by FACS looking at the cKit (APC), CD41 (PE-

Cy7) and Tie2 (PE) markers. Gating on the c-kit+ cells, there appeared to be a decrease in the percentage becoming CD41+ in the induced LMO1 overexpressing (8%) and more so in the knockdown (5%) when compared to the uninduced cells, which were remarkably constant between the two cell lines (both 11%) (Fig 7.13).

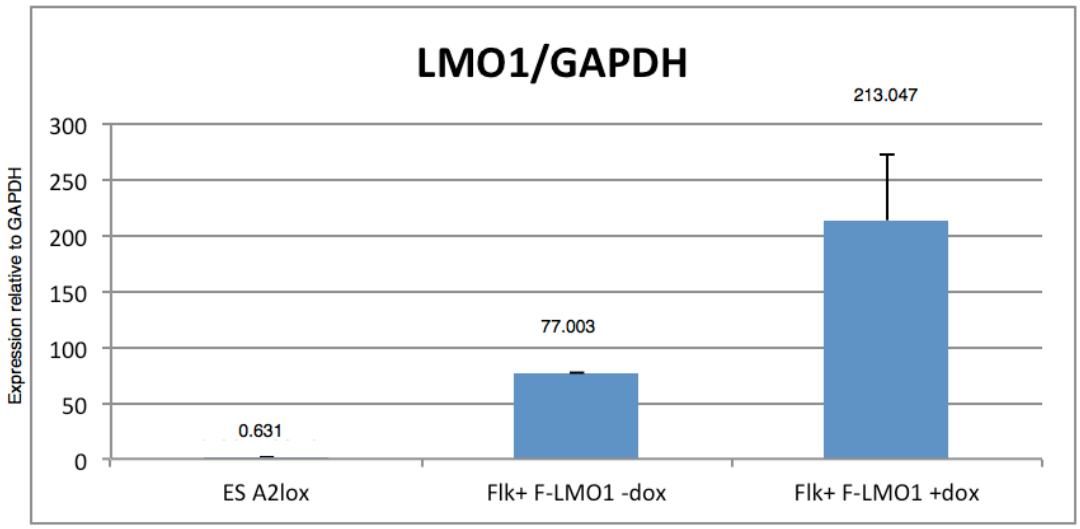

Figure 7.12. qPCR expression profile for LMO1 in Flk+ cells containing the Flag-LMO1 construct before and after induction with doxycyclin.

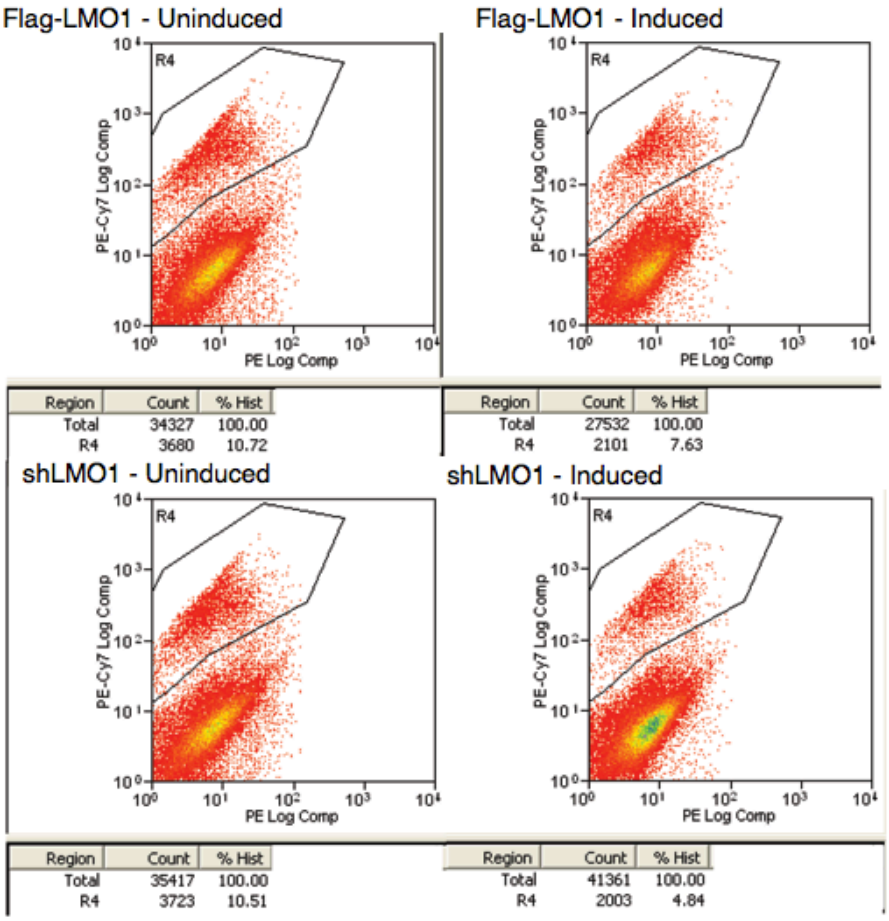

Figure 7.13. Flow cytometry results showing the percentage of A2Lox cells differentiating to Tie2+ CD41+ during induction of Flag-LMO1 and shLMO1 (R4). On induction of LMO1 over-expression (Flag-LMO1) the percentage of cells drops from 10.72% to 7.63% and on induction of the LMO1 knockdown (shLMO1) the percentage reduces from 10.51% to 4.84%.

We then investigated the efficiency of the shLMO4 construct in the A2lox clones. The level of LMO4 expression in shLMO4 A2lox ES cells was shown to be significantly lower than in control A2lox ES cells and unexpectedly remained unchanged after induction (Fig 7.14).

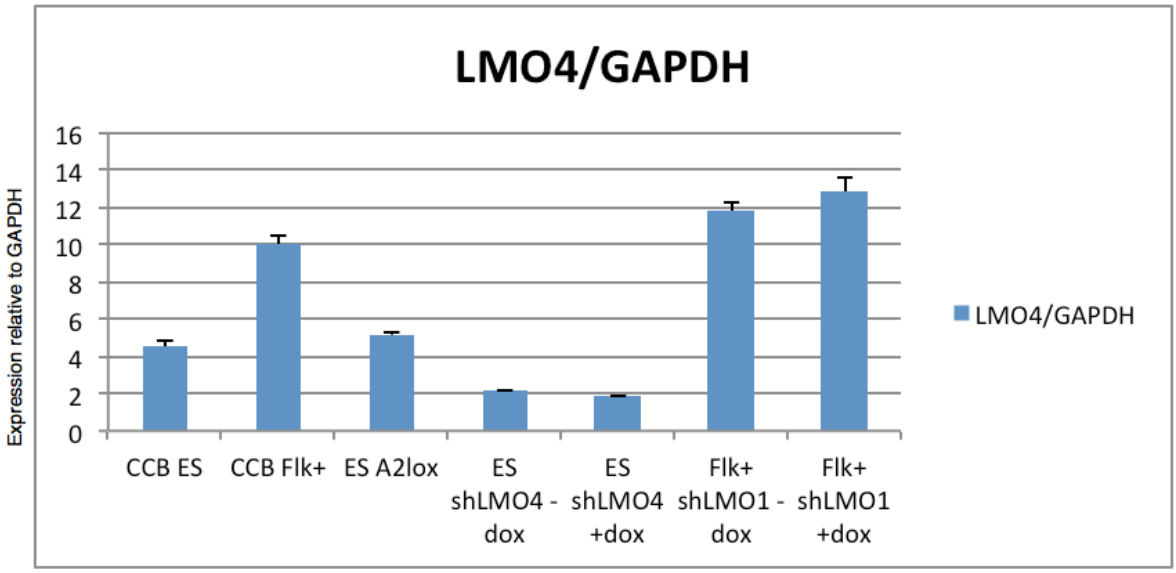

Figure 7.14. qPCR expression profile for LMO4 in ES cells and in shLMO4 A2lox cells before and after induction with doxycyclin. shLMO1 Flk+ A2lox cells were also included as a control to check that shLMO1 did not influence LMO4 expression.

DISCUSSION

The LMO family of proteins have all been linked with both embryonic development and a range of cancers which makes them very relevant to study in relation to hematopoiesis. It has been shown that LMO2 is crucial in the early stages of hematopoeisis and as a result of the high similarity between members of the LMO family it is logical to assume that LMO1 and LMO4 may also be involved in this process. LMO1 has been directly linked to hematopoiesis through its involvement in T-ALL and has been shown to be expressed only at the haemangioblast stage, however its exact role in non-T-ALL cells is not well understood. LMO4 has not been directly linked to hematopoiesis, but has been linked to neural development and is expressed in many tissues including those at the developmental stages of hematopoiesis.

LMO1 and LMO4 Expression

The expression was analysed by qPCR using the cDNA from the various cells used in this study. LMO1 showed the highest level of expression of the LMOs within the Flk+ cells with background levels in undifferentiated ES cells and PUER cells. Previous studies have also shown the expression levels of LMO1 to be very low within the hematopoietic development lineage except in the case of T-ALL where it can be upregulated and act in a similar way to LMO2 (Oram, *et al.* 2013). It is interesting that a temporary upregulation may occur at the Flk+ stage which suggests a role for LMO1 at this stage. This may mean that LMO1 is linked to differentiation of haemangioblasts into different lineages. As LMO1 and LMO2 act in a similar way it is

also possible that overexpression of LMO1 may prevent the developmental block seen with LMO2 knockdown. LMO4 overall showed less dynamic changes in its expression levels than the other LMO proteins, however, the results do demonstrate a rise in expression level between the undifferentiated ES cells and the Flk+ cells. A continued level of expression is then also seen into the myeloid lineage through PUER cells and differentiated PUER cells. Kenny, *et al*. (1998) showed a similar maintained level of expression through the lymphoid lineage supporting a role for LMO4 within hematopoiesis. LMO4 is also know to be much more widely expressed than the other LMO proteins so may have a more general developmental role across many cell types (Sum, *et al.* 2005a).

Finally, the involvement of LMO2 in hematopoiesis is very well documented.The mRNA expression results show a peak in expression at the Flk+ stage and a significant drop in expression by the differentiated PUER stage. Liu, *et al*. (2012) indeed showed that LMO2 was significantly upregulated in Flk+ cells heading towards a hematopoietic fate. It has also been shown that the level is downregulated again throughout myeloid differentiation (Natkunam, *et al.* 2007). In the lymphoid lineage LMO2 is also downregulated. In particular in T-cells LMO2 is completely silenced during the double negative stages and overexpression can lead to increased cell proliferation followed by T-ALL (Matthews, *et al.* 2013). Neither the myeloid nor lymphoid lineage shows any LMO2 expression by the fully differentiated cell stage and this can also be seen in the results of this study with the significant down regulation between the myeloid progenitor (PUER cells) and their 2 day differentiated state (Kenny, *et al*. 1998).

LMO1 and LMO4 Knockdown Constructs

To achieve knockdown of LMO1 and LMO4 small hairpin (sh) constructs were produced based on the siRNA principle. Small interfering RNAs (siRNAs) are a naturally occurring method of downregulating expression of a gene. They act by forming a double stranded RNA fragment which is cut into smaller fragments by the enzyme Dicer. It is thought this probably originally developed as a defence against RNA viruses. The RNA fragments then bind to complementary mRNA strands resulting in the digestion of these strands and mRNA being degraded and therefore not translated (NIGMS, 2013). siRNAs can also function without full complementarity by inhibition of RNA translation. Using shRNA is more effective than using siRNA because the shRNA is introduced into the cell in DNA form and integrated into the hosts genome before being expressed at high levels and forming RNA hairpins which can be cleaved by the Dicer enzyme. This is advantageous because a much smaller quantity of DNA is required than siRNA and also DNA is much more stable and constitutively present within the cells (Rao, *et al.* 2009).

The shRNAoligos for LMO1 and LMO4 were produced and the vector used to introduce these into the cells was the pPrime (MSCV) vector which is based on the MigRI vector. The PCR stage of the protocol had to be optimised due to poorly separating fragments causing the supposedly removed fragment to be ligated back in at a higher frequency than the insert. The constructs were then transfected into Plat-E retro-viral packaging cells in preparation for infection of PUER cells. To test the success of the constructs before moving into another cell type a co-transfection with a previously developed overexpression vector for each protein was carried out. For the shLMO1 constructs transfection of PUER cells expressing LMO1 were also

carried out. When these were analysed by FACS a clear shift from the highest GFP intensity was seen in construct #6 indicating that this construct was most effective at knocking own the LMO1 protein.

The main cells used in this study were PUER cells which are myeloid progenitors with inducible PU.1 expression. These cells were developed by Laslo *et al*. (2007) who showed that in the myeloid lineage PU.1 upregulation is required for progression from a multipotent precursor cell to myeloid cells. By making a construct where the PU.1 proteins merged to the estrogen receptor (ER) ligand binding domain, functional PU.1 protein is induced by addition of the synthetic estrogen OHT.This created a cell line which remains as a multipotent myeloid precursor until differentiation is required. These cells are not very early in the hematopietic lineage so are only able to differentiate into myeloid cells but they are still a useful starting point for testing new constructs as they are easy to culture and maintain and can be induced to differentiate.

shLMO1

The initial three shLMO1 constructs which were successfully produced were unsuccessful in their ability to knockdown LMO1. However two constructs out of the second batch of four showed a clear knockdown when infection of LMO1 expressing PUER cells was carried out. One of these, shLMO1 #6, was then used to reclone into p2lox and was electroporated into the A2lox ES cells.

The A2lox system is an inducible system allowing a construct to be induced at desired time points throughout the cell differentiation process by addition of

doxycyclin. Initial trials were carried out with Flag-LMO1 which showed an almost 3 fold increase when induced in Flk+ cells. This confirmed that the system functions correctly and these cells can be later used to study the effects of increased levels of LMO1.

A potentially interesting result was shown for shLMO1 when induced it showed a clearly lower percentage of cells becoming CD41 positive. The CD41 marker is generally associated with hematopoietic progenitor cells so this result suggests a possible involvement of LMO1 in the differentiation from haemangioblasts to early stage hematopoietic progenitors. This could suggest that the expression of LMO1 is necessary for the transition from haemangioblast to hemogenic endothelium. However, as this can only be assessed in the small fraction of the population which is also c-Kit positive the result is based on a very small change in the number of shLMO1 cells. It therefore needs to be repeated several times before conclusions can be made. There is also a question about these results due to the unsuccessful induction of shLMO4 constructs. This could suggest an overall problem with using this inducible system for sh knockdown. Despite the Hoogenkamp group successfully using it for the knockdown of LMO2 at the haemangioblast stage, further controls will need to be performed to be certain about the knockdown efficiency in these cell lines.

As demonstrated in the qPCR expression results PUER cells do not express appreciable levels of LMO1, so it was not expected to see a phenotype in these cells. Previous studies have shown a clear involvement of LMO1 overexpression in T-ALL which appears to be mostly attributed to its close similarity to LMO2, the two proteins almost being interchangeable in the pathways involved in T-ALL development. There

is evidence to suggest that LMO1 may be involved in hematopoiesis due to its binding partners. During thymic T-cell development LMO1 has been shown to form a complex with SCL which can inhibit the transcriptional activation action of E2A. It has been shown that the presence of the LMO1-SCL complex is necessary in the early stages of T-cell differentiation but is downregulated throughout the differentiation process, in cases where it is not downregulated T-ALL develops (Herblot, *et al.* 2000). This demonstrates a clear involvement of LMO1 in hematopoiesis, however there is still no real evidence of an exclusive role which is not also fulfilled by LMO2. The initial results in this study have shown a potential for LMO1 having an exclusive role in early hematopoiesis. To further these findings follow up studies are required to assess the effect of shLMO1 when it is induced at various stages throughout the differentiation process. It may also be important to look at the presence of any compensatory mechanism between LMO1 and the other members of the LMO family as this has already been shown between LMO1 and LMO3 in neural development (Tse, *et al.* 2004).

shLMO4

Functional sh constructs for LMO4 were produced and this was demonstrated in co-transfected Plat-E cells. When PUER cells were infected with the initial hygromycin resistance construct selection with hygromycin failed on each occasion due to the death of all cells. This meant that it was impossible to differentiate between the infection efficiency and any cell death phenotype of the construct. To solve this problem a construct with GFP rather than hygromycin resistance was produced. This meant that selection could then be carried out by FACS and a visual indicator could

also be seen to show that the infection was successful. The cells resulting from this were sorted showing around a 30% infection efficiency for both MigRI and shLMO4 from the Plat-E cell supernatant. The ~30% efficiency of infection achieved was much lower than that which would be expected in adherent cells however was very good for non-adherent cells and as this was carried out on a quantity of $4.5x10^6$ cells this gave an acceptable number to study (Cell Biolabs, 2012).

The cell death phenotype which was seen in the hygromycin selection was partially confirmed in the GFP cells. When analysed there was a clear fall of ~50% in the percentage of GFP cells by the second day in the shLMO4 cells as opposed to the MigRI cells. The percentage after this first drop however remained relatively constant for both genotypes. This suggested that the lack of LMO4 may be activating an apoptotic pathway or disrupting the cell cycle in some way. In an attempt to further elucidate the reason for this, apoptosis assays were carried out using PI and Annexin V. The Annexin V apoptosis assay works by antibody binding to the phosphatidylserine which is normally present on the inner surface of the cell membrane and becomes exposed on the outer cell surface during apoptosis. At later stages of apoptosis and during necrosis the cell membrane breaks down, allowing PI into the cells' nucleus, staining the DNA (Vermes, *et al.* 1995). It was shown that at day 2 after infection there were clearly higher levels of apoptotic activity in shLMO4 cells when compared to MigRI control cells. This result would suggest a crucial role for LMO4 within myeloid progenitor cells at this stage.

LMO4 is highly expressed in most proliferating epithelial tissue and in cells derived from epithelial tissue and has been shown in many studies to be linked to breast cancer with overexpression being linked to poor prognosis (Sum, *et al.* 2005a & Sum,

et al. 2005b). It has also been shown that LMO4 is critical for definitive erythropoiesis and is highly expressed in other definitive hematopoietic progenitors, particularly early T-cell progenitors. In a similar way to LMO1 and LMO2, no expression is seen in fully differentiated T-cells. In hematopoiesis LMO4 has been shown to form a complex with ldb1, eto-2 and cdk9, which is necessary for the transcriptional activation of Runx1, which is a required transcription factor for definitive hematopoiesis (Romano & Giordano, 2008, Lancrin, *et al*. 2009 & Sturgeon, 2013). From these studies it is clear that LMO4 has a role in hematopoiesis as well as epithelial cell proliferation and cancers. This does not however fully explain the reason for the apparent apoptotic affect seen in PUER cells. A potential route for this is through the interaction identified by Meier, *et al.* (2006) with cdk9 which is involved in many processes including cell growth, proliferation and anti-apoptotic protection (Romano & Giordano, 2008). Wang, *et al*. (2012) showed that CDK9 inhibition is a key regulator in the apoptosis of neutrophils, a later cell in the myeloid lineage. It was shown that when CDK9 was inhibited the level of Mcl-1, an anti-apoptotic protein, reduced causing rapid apoptosis. It is possible therefore that a drop in the level of LMO4 could reduce expression of CDK9 and lead to apoptosis within PUER cells. Cell cycle disruption and apoptosis by LMO4 has also been shown in breast cancer cells at the G2/M stage (Montañez-Wiscovich, et al. 2010). A reduction in the level of LMO4 resulted in amplification of centromeres, abnormal spindles and increased cell death. Again this could be a route for the apoptotic effect seen in PUER cells and further study is required to establish whether this is a possibility.

There are however complications to this finding. Firstly, the increased apoptotic level demonstrated here is measured at the second day by which time the main fall in cell

population has already occurred. It is therefore likely that this is the remaining fraction of apoptotic cells following the initial phase of cell death. Measurements at day1, when not all infected cells fully express the transgene yet, should give more insight. A further complication is that in a later flow cytometry analysis of sorted shLMO4 cells it was shown that after more than one week, there was a significant population of GFP negative. A possible cause for this would be the loss of transgene expression however this seems unlikely as the shRNA and GFP have independent promoters. Another possibility is that this population is apoptotic cells which have lost their GFP before falling apart and this is supported by the high proportion of cells in this population which were dead or dying, this argues against the possibility that these are healthy cells outgrowing the GFP positive cells.

Unfortunately, when introduced into the A2lox ES cell system the expression levels for the shLMO4 cells did not show a reduction in LMO4 expression when induced. They did however show a significantly lower level of LMO4 than untargeted cells. This suggests that the construct in this ES cell clone is constitutively active.. Alternative clones, with the same shRNA, have been frozen at an early stage and will be checked for their functionality. Further studies will therefore be required to establish whether the A2lox system is fully appropriate for sh knockdown.

Overall this study forms the basis for a large amount of future work into the roles of LMO1 and LMO4 within hematopoesis. A set of useful and functional tools have been developed which can now be used to study and attempt to understand the roles which these proteins play. Initial results within PUER cells show a definite involvement of LMO4 in cell function, at least partially through apoptosis. Future work

to identify the partners of LMO4 would help to understand whether there is a link to CDK9 and apoptosis by this route. The ES cell constructs are also exciting tools for future study, however, confirmation that the inducible system functions correctly with sh constructs will be required.

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