RNA Binding Proteins, Ageing and Caloric Restriction

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Abstract

A globally ageing population means an increase in age-associated diseases with many facing a lower quality of life and an increased strain on the world's economy. It is therefore paramount that the ageing process is more greatly understood to reduce these detrimental effects. Senescence, the process by which cells permanently cease replication is suggested as a driving force for the ageing process. While caloric restriction has been considered as the only robust environmental method available to increase longevity and reduce age associated disease in a range of model organisms since the pioneer experiment by McKay et al in 1935. This project seeks to explore the effect of both ageing and calorie restriction further by investigating changes to the gene expression profile of the cell using samples from mouse liver tissue. The mice in this study were fed an *ad libitum* or calorie restricted diet before being euthanized at different ages. RNA sequencing data is available from the mouse liver and has here been probed with the aim of providing insight into the ageing process. The results shown include the downregulation of Major Urinary Proteins as the only clear trend in genes showing the most altered expression and yields some interesting questions as to the true function of major urinary proteins in mice. However, the project then seeks to understand what is modulating the changes to gene expression, theorising that subtle changes to RNA binding proteins could allow these diverse proteins to generate the changes in gene expression seen in the RNA Sequencing analysis. Human dermal fibroblasts are also used as a model to begin to understand more greatly the role of SRSF1, a splicing factor, in senescence.

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List of Abbreviations	
Abbreviation	**Explanation**
CR	Caloric Restriction
DDR	DNA Damage Response
DR	Dietary Restriction
MUP	Major Urinary Protein
NMD	Non-sense Medicated Decay
GO	Gene Ontology
NIA	National Institute for Ageing
WNPRC	Wisconsin National Primate Research Centre
mTOR	Mammalian Target of Rapamycin
IGF	Insulin Growth Factors
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
RBP	RNA Binding Protein
snRNP	small nuclear ribonucleo proteins
ESE	Exonic Splicing enhancer
ISE	Intronic Splicing Enhancer
ESS	Exonic Splicing Silencer
ISS	Intronic Splicing Silencer
SR	Serine-Arginine
TARDBP	TAR DNA-binding protein 43
PSI	percentage of the functional transcript present

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Chapter 1 Introduction

1.1 Introduction to Ageing and Senescence

Due to advances in healthcare the maximum human life span has continued to increase resulting in a globally ageing population with increased incidence of age-associated diseases and therefore a reduced quality of life in old age (Kirkwood, 2005). Ageing is accompanied by a decline or loss in tissue functions and the inability to maintain homeostasis leading to age-associated diseases such as diabetes or Alzheimer's disease. Conversely, ageing may also result in a lethal gain in function where cells proliferate uncontrollably forming cancers (Manuscript 2010; C. J. Kenyon 2010; Wheeler and Kim 2011). The subsequent increase in number of individuals requiring medical care and treatment for age-associated diseases, particularly those which require full time care such as sufferers of Alzheimer's disease, has the potential to place a large strain on the world's economy (Lutz, et al; 2008). To increase the quality of life of an ageing population and reduce strain on the global economy it is important that much ageing research takes a strong focus on not just understanding the ageing process or increasing lifespan further but also on reducing the onset of age-associated diseases.

The study of ageing is challenging due to a variety of different factors including the lack of universally agreed physiological and biochemical markers leading mortality to be the only robust marker for ageing (Roth et al. 2006). For example, microarray analysis of the transcriptional profile of skeletal muscle in Rhesus macaques after a 9-year experiment focusing on ageing revealed 107 genes up regulated by CR and 93 genes down regulated. This study highlighted the need for suitable biomarkers to be found as although there was no observed change in traditionally used age-related markers the primates showed clear physiological differences (Kayo et al. 2001; Roth et al.). Studying ageing is only further complicated by the fact that there is a large variation in the longevity of different species therefore the use of model organisms may not always be applicable to understanding ageing within a human population (Kirkwood and Rose 1991; Fontana and Klein 2007). Evidence suggests that environmental factors such as nutrition can alter the average life span of model organisms (Fontana and Klein 2007; Zimmerman et al. 2003). While this is important in understanding the ageing process there is also clearly heritable content (Wheeler and Kim 2011; C. J. Kenyon 2010; Fontana and Klein 2007) for example, a single gene alteration can greatly alter longevity in *C.elegans* which must also be given careful consideration when

studying potential ageing interventions (Hansen et al. 2007; Greer et al. 2007; C. Kenyon et al. 1993; C. J. Kenyon 2010; Weinkove et al. 2006).

The general decrease in the functionality of tissues and organs seen during the ageing process is most likely driven by cellular senescence (Kirkwood and Rose 1991). Senescence is the irreversible arrest in the cell cycle that may be induced by a variety of factors and pathways including; telomere erosion, DNA damage, oncogene induction and persistent cellular stresses (Greider 2001; Campisi 2005). The stability of the genome is monitored by the cell continually and as the DNA becomes unstable or damaged the cell will temporarily halt division. Should this damage be irreparable and persist the cell will enter a state of permanent cell cycle arrest. During senescence, the cell undergoes large changes in gene expression as well as changes to cell and nuclear morphology. Cellular senescence has been proposed as an anti-cancer mechanism, whereby the cell prevents tumorigenesis by detecting DNA damage which could result in oncogenic mutations; a theory supported by oncogene induced senescence (Campisi 2005). Once cells have entered a senescent state they display a senescence associated secretory phenotype (SASP) as well as persistent DNA damage foci (Nelson et al. 2012). SASP means the cell secretes a mixture of cytokines and growth factors in order to communicate with their environment and this has been demonstrated to have both pro and anti-tumorigenic effect in the surrounding cells (Nelson et al. 2012; Campisi 2005).

1.2 Caloric Restriction

Currently Caloric Restriction (CR) (sometimes known as dietary restriction (DR)) is the only robust, environmental method shown to increase longevity and attenuate age-associate diseases across a range of model organisms and can be defined as a reduction in calorie intake without malnutrition (Fontana and Klein 2007; Mair and Dillin 2008). The increase in life span mediated by a 30% reduction in food intake was first discovered in 1935 by Mckay et al in a mouse model (McCay, Crowell, and Maynard 1935). Since then CR has been demonstrated to increase longevity and decrease age associated biomarkers in a range of model organisms, for example decreased insulin resistance in mice (Mair and Dillin 2008). While there is a good volume of evidence to suggest CR increases life span and attenuates age-associated diseases in model organisms there is very limited and sometimes conflicting evidence of this in human or primate models. Longitudinal CR studies in humans are economically very demanding due to the required length of the study as well as the availability of willing volunteers to consume a carefully controlled and restricted diet over

their lifetime (Holloszy and Fontana 2007). Similarly it is rare to find a human population which has experienced a shortage in food availability without experiencing malnutrition, an exception to this being the Okinawan population in Japan (Willcox et al. 2007). The Okinawan population consistently consumed less calories than other Japanese or Western populations without malnutrition according to a census, and this community has the greatest number of centenarians than any other (Willcox et al. 2007). While the Calorie Restriction Society has provided many volunteers for short-term studies this is often difficult data to interpret for many reasons. For example, age-matched comparisons are said to consume a 'typical western diet' (Fontana et al. 2004) which contains a large amount of processed food while those in the CR group are consuming unprocessed food therefore any increase in longevity could be attributed to an inherent toxicity within a 'typical western diet'. While there are studies comparing a calorie restricted group to lean athletes, there is again a large difference in the life styles of such groups (Cangemi et al. 2010) and it would be difficult to attribute any changes in longevity to the effect of calorie intake alone.

Interestingly two longitudinal studies carried out on rhesus macaques showed contradictory results, while the National Institute for Ageing (NIA) study suggested CR does not significantly increase longevity in non-human primates a study carried out at the Wisconsin National Primate Research Centre (WNPRC) showed that a calorie restricted diet does increase average life span in non-human primates (Mattison et al. 2012; Colman et al. 2009). These primates make a good human model organism due to their substantial number of similarities, therefore a more in depth comparison is needed between these two studies to determine why such differences in outcomes should arise. This should help to discern the suitability of a CR regime or mimetic in humans. Research has also suggested that the severity of the restriction has an important effect on longevity. Mair et al (2008) suggest there is a 'sweet spot' to a calorie restricted diet which will produce a maximum longevity increase while a very severe restriction in calorie intake actually causes a reduction in life-span (Mair and Dillin 2008). It also well documented that obesity has a detrimental effect on longevity and increases the onset of age associated diseases therefore it has been suggested that the longevity increase seen under CR is simply the result of rescuing laboratory animals from obesity (Harper, Leathers, and Austad 2006). This is supported by data which suggests wild-caught mice placed on a CR diet do not show an increased average life span (Harper, Leathers, and Austad 2006) perhaps also providing evidence that while CR has resulted in a consistent increase in longevity in in-bred lab model organisms the results may not be replicated in a natural, genetically varied population.

A definitive molecular mechanism which underpins the longevity increase mediated by CR has not yet been determined and while there are many proposed pathways, the actuality is likely a combination of these (Mair and Dillin 2008). The pathway activated may also depend on the age the CR diet is initiated and the severity of the restriction. For instance, there is much experimental evidence that mammalian target of rapamycin (mTOR), Sirtuins, AMP kinase and insulin growth factor (IGF) signalling (C. J. Kenyon 2010; Mair and Dillin 2008) are involved in nutrient sensing pathways effected by a CR diet. However, AMP kinase is activated particularly by CR at middle age(C. J. Kenyon 2010). Long lived *C. elegans* mutants (daf-2/daf-16 mutants) show that a single genetic mutation is capable of greatly increasing average lifespan, this pathway is comparable to the AMP kinase, FOXO pathway in mammals (Weinkove et al. 2006; C. J. Kenyon 2010). It is the effects downstream of these metabolic and hormonal pathways which continue to remain elusive (Plank et al. 2012). The discovery of mTOR as a nutrient sensing receptor activated by CR has led to the discovery of Resveratrol, a compound thought to be a mimetic for the effects of a CR diet (Baur et al. 2006; Timmers et al. 2011). While the successful use of Resveratrol is not yet widely accepted and has been disputed among different research groups there is some evidence that this compound is able to act on the mTOR pathway while other research suggests that only rapamycin is able to act on mTOR in this way (Miller et al. 2011). The discovery of a pharmaceutical mimetic is one of the crucial long-term goals in determining the molecular pathways affected by CR. This would ideally allow the population to experience the beneficial reduction in age-associated diseases without consuming a CR diet, which would likely not be successfully introduced into modern society. The following chapters throughout this thesis will introduce and discuss in more detail some of the molecular components which may contribute to the ageing process including RNA binding proteins and splicing factors.

1.3 Background

The data presented in this paper from the analysis of mouse liver tissue has originated from a study at Newcastle University from the group of Prof. Thomas Von Zglinicki who originally conducted the experiments and provided the materials. More information is available in previous papers by Cameron et al (2012) and Miwa et al (2014). The male C57BL/6 mice were randomly assigned to a DR or an AL group at 3 months of age (day 0 of the experiment). DR was initiated at 3 months and lasted for either 12 months, Mice had AL access to water in both groups but the mice in the DR group received 60% of the food of those in the AL group. These pellets were also smaller to reduce competition for food (Cameron et al., 2012;

Miwa et al., 2014). This is further described in chapter 7 section 7.1. Mice were housed individually and were euthanized at 3 months, 15 months, 24 months and 30 months. The total RNA from liver tissue was extracted and sequenced using 100 base, paired end sequencing (Illumina) as described in materials and methods. Analysed data (DESeq and DEXSeq) was made available as spreadsheets including the following information: gene name, Ensambl ID (http://www.ensembl.org/index.html), log fold change and statistical testing for differences in expression. Raw gene count files were also made available. This analysis was carried out by Dr David Dolan and Albert Lahat, colleagues in the Grellscheid lab group,

1.4 Project Aims

This project seeks to investigate changes to the gene expression profile of the cell during the ageing process and to gain an understanding of how a calorie restricted diet might affect the expression profile of the cell. In doing so the project sought to investigate any gene families which showed significant changes in this analysis whilst also seeking to gain and understanding of the mechanisms which mediate these changes in gene expression.

Chapter 2: Trends in the Gene Expression Profile

2.1 Trends in the Gene Expression Profile-Introduction

Many studies have shown that ageing is associated with a general decrease in tissue function, reduced capacity for cells to maintain homeostasis and altered metabolism. Conversely, consuming a calorie restricted diet has been shown to alter metabolism as well as mitochondrial biogenesis and autophagy (Wheeler and Kim 2011; Mair and Dillin 2008; Fontana and Klein 2007). In order to discern whether similar patterns were seen in mouse liver tissue the gene expression data produced during RNA Sequencing analysis was analysed to look for patterns in the changes of transcript abundance in genes associated with specific functions. The development of high throughput sequencing (HTS) such as RNA Sequencing analysis allows the transcriptional profile of the cell to be examined closely and in comparison to micro arrays this method allows a greater depth to the analysis (Z. Wang, Gerstein, and Snyder 2010). This technology is relatively newly developed and is beginning to replace traditionally used microarray experiments however there is likely still much work to be done regarding the down-stream computational analysis of this data and there is little information available which concisely summarises how RNA-Seq data is best validated (Rajkumar et al. 2015). Many changes to the transcriptome detected in such analysis are tissue specific and comparing these changes throughout a range of tissues and species should elucidate core transcriptional changes throughout ageing (Wheeler and Kim 2011). The data available was DESeq data from an RNA sequencing analysis carried out on mouse liver tissue. DESeq analysis was used to align the gene reads measured during the RNA sequencing to a reference genome and compare gene expression levels between two groups (see materials and methods for more information).

When selecting a single age group from the mouse samples to use in the analysis to gain a better understanding of the overall ageing process the 24-month group was selected. 24 month is likely the sample which best represents an aged group as although data was available for 30 month mice these mice are likely displaying ‘survivor effects’ which could lead to false conclusions (Robins 1987). Survivorship bias results in error as it concentrates on those examples which survived a process, since this is a study looking at the process of ageing in the average population concentrating on the 30-month example would likely lead to false conclusions and mortality studies such should always be mindful of this bias. The gene expressions in the 24-month age groups were compared to the ‘young’ or ‘3 month’ samples which are used as a control throughout this study. The DESeq analysis is carried out

by comparing gene counts for two different age groups or diet type (each group has a triplicate repeat from three different mice) which has been normalised according to the size of the genome to determine which mRNA transcripts have undergone the greatest changes in abundance between two groups ($p<0.05$). This analysis produces lists of genes containing data about over 5000 genes therefore it is not a simple task to ascertain some clear meaning from this data and there is likely a variety of approaches which could be taken.

Gene lists were analysed using Panther (http://www.pantherdb.org/) to classify all genes which showed a statistically significant change in expression according to biological process using Gene Ontology (GO) IDs to highlight over all trends. This analysis uses the gene names of all genes which show a significant change when mice of different age and diet types are compared from the DESeq results. Genes are then grouped according to GO annotation to give insight into any patterns which may be present in the genes altered by the ageing process. While a more specific analysis of the genes which shows the largest changes in transcript abundance should provide in tables 1 and 3 to show more specific information around genes which may be important in the ageing process or in the longevity increase seen in caloric restriction.

2.2 Trends in the Gene Expression Profile-Results

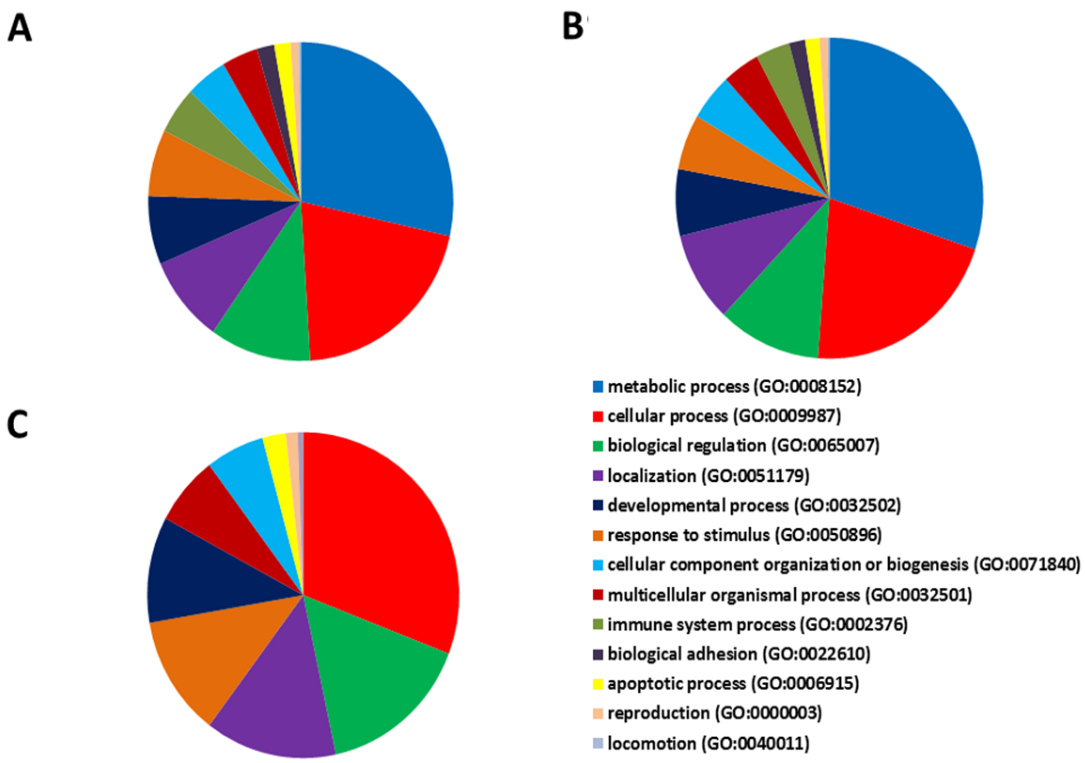


Figure 1: PANTHER analysis of all genes which showed statistically significant changes in transcript abundance during ageing and CR in mouse liver tissue. Analysed using PANTHER (http://www.pantherdb.org/) to classify each gene according to Biological Process. A) Genes significantly altered in expression when 24 month AL was compared to 3 month. B) Genes significantly altered in expression when 24-month CR was compared to 3 month. C) Genes significantly altered in expression when 24 month AL was compared to 24-month CR.

Table 1: Genes showing largest increase in transcript abundance when 3 month and 24-month mouse liver tissue were compared Gene short names are shown. Log 2-Fold change is the logarithm (base 2) of the change between the two values when the first is divided by the second. For example, when abundance at 3 months is divided by abundance at 24 months. Table headings show how each fold change was calculated. The Log 2-fold changes shown in this table are all positive as these genes show an increase in abundance in the comparison. Table heading 3AL/24AL indicates genes which have increased in transcript abundance in the 24 month aged mice fed an AL diet when compared with the 3 month mice. Table heading 3AL/24CR indicates genes which have increased in transcript abundance in the 24 month aged mice fed a CR diet when compared with the 3 month mice. Table heading 24AL/24CR indicates genes which have increased in transcript abundance in the 24 month aged mice fed a CR diet when compared with the 24 month mice fed an AL diet.

Upregulated

3AL/24 AL			3AL/24CR			24AL/24AL		
Gene name	**Log 2-Fold Change**	**P Value**	**Gene name**	**Log 2-Fold Change**	**P Value**	**Gene name**	**Log 2-Fold Change**	**P Value**
Igkc	6.96	2.80E-158	*Fmo3*	9.18	2.31E-67	*Acot3*	6.70	2.14E-133
Gm24105	5.91	1.19E-34	*Cyp2b13*	8.76	2.88E-58	*Cyp2a4*	6.66	5.27E-116
Gm14667	5.40	2.27E-21	*Cyp2b9*	8.58	1.16E-124	*Fmo3*	6.07	8.10E-33
Ngp	5.37	1.42E-20	*Slc22a26*	8.22	4.04E-76	*Cux2*	5.91	2.85E-39
Slc22a26	5.16	2.22E-28	*Ppp1r3g*	7.28	1.89E-40	*Cyp2b13*	5.81	1.12E-62
Camp	5.05	7.66E-20	*Myh1*	6.75	4.45E-24	*Ppp1r3g*	4.63	2.79E-15
Ltf	4.90	1.90E-16	*Gm24105*	6.61	9.10E-40	*Usp2*	4.51	3.17E-82
Pou2af1	4.45	9.41E-22	*Slc22a27*	6.30	3.42E-70	*Cyp4a14*	4.43	1.97E-50
Mpo	4.45	1.79E-15	*Cyp2a4*	6.27	5.00E-110	*Pdk4*	4.28	3.01E-51
Cyp2b9	4.42	2.95E-20	*Rgs16*	6.25	8.47E-42	*Cyp2a5*	4.11	4.98E-56
Igkv5-39	4.40	1.15E-12	*Gm14667*	5.95	1.05E-19	*Gm15441*	4.10	1.30E-13
C130026I21Rik	4.38	7.35E-17	*Tnnc2*	5.60	1.70E-15	*Btbd19*	4.05	1.49E-21
Esm1	4.36	1.03E-23	*Gm13855*	5.55	1.09E-88	*Angptl4*	4.00	6.76E-69
Napsa	4.33	7.47E-16	*Mfsd2a*	5.54	2.84E-79	*Cox6a2*	3.85	3.52E-10
S100a8	4.31	2.61E-48	*Per1*	5.13	1.84E-80	*Cyp4a31*	3.64	8.61E-20
Irg1	4.29	1.88E-14	*Cux2*	5.06	4.04E-30	*Acot2*	3.60	3.38E-59
Ighg1	4.23	3.03E-13	*Sult3a1*	4.97	7.04E-13	*Gm15611*	3.59	6.72E-10
Gm9844	4.21	5.23E-41	*Acot2*	4.70	1.84E-44	*Fmo2*	3.47	1.97E-50
S100a9	4.06	3.71E-16	*Cox6a2*	4.51	1.12E-09	*Nup62cl*	3.47	1.76E-10
Irf4	4.05	3.58E-15	*Ppp1r3a*	4.47	2.17E-09	*Ciart*	3.46	1.30E-11

Table 2: Genes showing largest decrease in transcript abundance when 3 month and 24-month mouse liver tissue were compared. Gene short names are shown. Log 2-Fold change is the logarithm (base 2) of the change between the two values when the first is divided by the second. For example, when abundance at 3 months is divided by abundance at 24 months. Table headings show how each fold change was calculated. The Log 2-fold changes shown in this table are all negative as these genes show a decrease in abundance in the comparison. Table heading 3AL/24AL indicates genes which have decreased in transcript abundance in the 24 month aged mice fed an AL diet when compared with the 3 month mice. Table heading 3AL/24CR indicates genes which have decreased in transcript abundance in the 24 month aged mice fed a CR diet when compared with the 3 month mice. Table heading 24AL/24CR indicates genes which have decreased in transcript abundance in the 24 month aged mice fed a CR diet when compared with the 24 month mice fed an AL diet.

Downregulated

3AL/24AL			3AL/24CR			24AL/24CR		
Gene name	Log 2-Fold change	P Value	Gene name	Log 2-Fold change	P Value	Gene name	Log 2-Fold change	P Value
Mup17	-6.51	8.35E-231	*Mup17*	-11.33	1.08E-164	*Mup15*	-79.47	1.59E-43
Gm12744	-5.43	1.52E-25	*Mup15*	-11.31	1.80E-146	*Mup1*	-61.69	1.76E-52
Mup7	-5.29	2.95E-107	*Mup1*	-10.06	5.48E-175	*Mup19*	-35.21	2.18E-31
Gm15884	-5.14	1.84E-17	*Hsd3b5*	-9.97	2.32E-106	*Hsd3b5*	-29.81	3.10E-20
Cdh1	-5.11	1.92E-98	*Mup12*	-9.56	1.59E-118	*Mup8*	-29.26	1.48E-22
Mup-ps17	-5.06	3.55E-50	*Mup19*	-8.92	6.72E-111	*Npas2*	-29.05	3.96E-22
Selenbp2	-4.92	3.03E-87	*Mup8*	-8.50	1.63E-69	*Ngp*	24.37	7.33E-18
Fam221b	-4.84	1.72E-15	*Mup-ps17*	-7.21	2.04E-33	*Mpo*	23.36	8.21E-16
1700022I11Rik	-4.80	6.63E-15	*Selenbp2*	-6.80	4.50E-261	*Mup17*	22.92	7.38E-20
mt-Te	-4.80	9.00E-19	*Mup7*	-6.32	5.66E-18	*Mup12*	22.38	1.31E-19
Mup14	-4.70	1.11E-144	*Ces4a*	-6.16	7.52E-24	*Ltf*	22.02	3.64E-15
Gm20721	-4.65	4.13E-15	*Mup16*	-6.15	3.60E-23	*Camp*	20.86	7.19E-17
Gm26825	-4.64	1.87E-13	*Serpina4-ps1*	-6.07	8.17E-19	*Chil3*	19.02	8.77E-15
Mup15	-4.46	9.00E-83	*Snx22*	-6.01	7.93E-21	*Pou2af1*	17.54	7.43E-18
Gm29236	-4.39	3.63E-12	*Npas2*	-5.80	1.70E-24	*Cd79a*	11.99	1.93E-13
Sycp3	-4.39	7.74E-13	*Cyp4a12a*	-5.74	7.92E-85	*Blk*	11.87	4.66E-09
4930551O13Rik	-4.38	6.90E-12	*Ces2b*	-5.69	1.54E-20	*Gm15401*	11.29	1.75E-14
Dlec1	-4.38	2.80E-13	*Gm26631*	-5.65	4.30E-24	*Capn8*	11.04	6.33E-09
Mup-ps22	-4.36	2.42E-13	*Gm20721*	-5.63	2.40E-19	*Sult5a1*	10.82	4.57E-08
Gm38316	-4.34	1.52E-12	*Sult5a1*	-5.62	6.79E-17	*Cd177*	9.81	8.99E-08

2.3 Trends in Gene Expression-Discussion

Overall Figure 1 parts A and B show changes in the expression of genes associated with metabolic process, cellular process and regulator processes suggesting that genes associated with these functions undergo the most changes in transcript abundance during the ageing process regardless of diet type. Figure 1 C is important as this highlights differences between age-matched comparisons with different diet types. It is interesting to note that when age matched diets are compared in Figure 1 C we do not see substantial changes in metabolic processes but continue to see changes in the cellular and regulatory processes. This is a particularly important comparison to consider for two main reasons. Firstly, GO-slim biological process IDs have been used in Figure 1 and these encompass a subset of terms within the whole of the GO consortium and are designed only to give a broad overview. Secondly, this analysis does not give an indication of *how* these groups change; for example, Figure 1 shows a change in metabolic processes in 24 month AL and CR mice when compared to 3 month but these genes could show increased abundance in one instance and decreased abundance in the other. Therefore, when we consider that the very broad category of 'metabolic process' shows large changes in the AL and CR mice when compared to young and we do not know if these are the same subset of genes however when the aged matched comparison in Figure 1 does not show any changes in these categories this allows us to ascertain that these changes are most likely the same between the 3 month and 24 moth mice regardless of diet type. While this is a very simplified way at looking at a very large and complicated data set (DESeq comparisons result in over 5000 genes significantly altered between the RNA-Seq data sets) it allows a broad overall view of the trends in RNA transcript abundance between age and diet type. There is clearly not a change in abundance of a large array of mRNA transcripts associated with metabolic process between the age-matched 24 month dietary comparisons but there may still be a few large changes in key genes not highlighted by this analysis which would be worth following up.

Biological and cellular processes are very broad ranging terms and encompass many aspects of cellular processes such as RNA and DNA binding as well as regulation of redox homeostasis and cell shape. It is therefore not surprising that Figure 1 shows there to be large changes in abundance of genes associated with cellular process when the different age and diets are compared. For example if we are to assume that at 24 months many more of the mouse cells has become senescent then we would expect to see a change in many of the genes which control cell morphology given that senescent cells become larger and flattened

(Campisi 2005) and these changes would be shown under Biological and cellular processes. It should also be considered that genes associated with Biological and Cellular processes are the most altered in transcript abundance when an age matched diet comparison is made.

There are various tools available for high-throughput analysis including PANTHER, KEGG and omicsNET. PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system classifies proteins and their genes to facilitate high-throughput analysis (Haider et al., 2013). While panther does use the gene ontology data base to classify gene and show which pathways are enriched there is also the possibility of KEGG analysis (http://www.genome.jp/kegg/pathway.html) which uses manually drawn pathways to represent knowledge on molecular interactions and reaction networks (Haider et al., 2013). The gaps between PANTHER and KEGG can be filled using omicsNET (Haider et al., 2013). OmicsNET analyses protein interaction networks. This is a good method for analysing large scale data sets and would have been best practice to show this analysis here. It is a limitation of this study that this is not included. However, the PANTHER analysis does give the large overview of the gene set however more could be gained and understood from carrying out further analysis.

In almost the opposite approach to the broad overview of DESeq analysis shown in Figure 1, tables 1 and 2 highlight a specific subset of genes from the analysis. These tables bring focus to genes showing the largest changes in transcript abundance when the same comparisons were made between age and diet type as seen in Figure 1. The genes shown in tables 1 and 2 have been highlighted because of the $\log_2$ fold change in the comparison-this is a measure of the change in transcript abundance normalised by logarithms. When the $\log_2$ fold change shows a negative value, this indicates a decrease in mRNA abundance while a positive value indicates an increase in abundance. There is only one clear pattern in the genes which show the largest expression changes and this is the down regulation of MUPs (Major Urinary Proteins). This gene family is discussed and investigated further in chapter 3. The other genes shown in tables 1 and 2 vary greatly in function and it would likely be more beneficial to focus on what may be mediating these changes in gene expression. As such the project will now continue to investigate the theory that subtle changes to the expression of RNA binding proteins can affect expression of a variety of genes and gain a greater understanding of these changes. This is continued in chapter 4. RNA binding proteins would not likely show large changes in gene expression and therefore would not be highlighted in such an analysis as is shown in tables 1 and 2 but very small changes would produce large

changes in expression given that RNA binding proteins often have a broad range of targets in order to allow for the complexity of RNA biology (Glisovic et al. 2008a).

Chapter 3: Major Urinary Proteins (MUPs)

3.1: Major Urinary Proteins-Background and Introduction

3.1.1: MUP structure and function

The analysis shown in Chapter 2 produced some unexpected results and highlighted that many genes knows as Major Urinary proteins (MUPs) undergo large changes in transcript abundance during ageing. MUPs have previously been established as providing a mode of transporting pheromones and ensuring their slow release into the environment, made possible by the hydrophobic binding pocket or central cavity of the molecule (Logan, Marton, and Stowers 2008). The main function of the MUP family is to bind pheromones present in mouse urine and allow the slow release of these compounds into the environment, most likely employed by mice as a method to avoid in-breeding (Logan, Marton, and Stowers 2008; Cheetham et al. 2007). The pheromones bound by MUP genes are known to accelerate puberty within young mice and even induce changes within the brain (Xia et al. 2006). There is some evidence to suggest that MUPs may also act as volatile pheromones without a ligand present (Chamero et al. 2007), though this is a controversial idea made more complicated by the fact that little is known regarding mammalian pheromones (Logan, Marton, and Stowers 2008). It is suggested that for the MUPs to act a pheromones they should form a hexapeptide sequence, however when the coding potential of MUP genes was analysed by Logan et al they found little to suggest that MUPS are able to form such a sequence (Logan, Marton, and Stowers 2008). Additionally MUP4 is not present in urine but is expressed in the nasal mucus and the vomeronasal organ and is thought to play a role in ligand transport in this organ (Phelan et al. 2014). Major urinary proteins may be also be involved in response to changes in diet such as calorie restriction. Several studies have linked major urinary proteins to metabolism as these proteins show changes in expression due to altered calorie or fat intake (Giller et al. 2013; Hui et al. 2009; Zhou, Jiang, and Rui 2009).

MUP genes are part of the lipocalin family and there are various gene expansions including α_{2U}-globulins in rats (Logan, Marton, and Stowers 2008; Phelan et al. 2014). It has been suggested that the increasing complexity of the gene families similar to MUPs is a mechanism employed in the evolution of many species to inform of species specificity when breeding (Logan, Marton, and Stowers 2008; Grzyb, Latowski, and Strzałka 2006). As part of the lipocalin gene family MUP genes have a tertiary structure consisting of 8 beta barrels,

open at one end to form a hydrophobic binding pocket in the centre of the molecule (Grzyb, Latowski, and Strzałka 2006; Phelan et al. 2014). This structure allows pheromone binding and is stable to allow MUPs to slowly release pheromones into the environment (Cho et al. 2011; Grzyb, Latowski, and Strzałka 2006; Cheetham et al. 2009). Lipocalins are a widely diverse family with many physiological functions however the main role of lipocalins is the binding of lipophilic molecules though other member of the lipocalin family play important roles in development and reactions to stress factors (Grzyb, Latowski, and Strzałka 2006). While the lipocalins show a conserved secondary and tertiary structure it is not uncommon for the amino acid sequence of two members of the lipocalin family to show only a 30% sequence similarity (Grzyb et al., 2006). In mice lipocalins are clustered on chromosome 4 while humans show a single lipocalin cluster on chromosome 9. There is a building body of evidence that like other members of the lipocalin gene family, MUPs play a role in lipid metabolism (Phelan et al. 2014).

The MUP family consists of 21 genes and 21 respective pseudogenes although there may be more genes not yet annotated (Logan, Marton, and Stowers 2008; Phelan et al. 2014). This extensive repertoire of MUPs means that the combination of these proteins expressed by the mouse is also able to act as a recognisable pattern within mouse urine preventing in-breeding (Knopf, Gallagher, and Held 1983; Cheetham et al. 2009; Cheetham et al. 2007). These genes have been classified into a class A which is older and more divergent and class B which is formed by a recent duplication of gene/pseudogene pairs (Logan, Marton, and Stowers 2008). Genes in class B are arranged in a head to head manner where each gene is paired with a forwards facing pseudogene(Logan, Marton, and Stowers 2008). Analysis carried out by Logan et al has discovered that the intergenic region between MUP1 and MUP2 from Class A has high similarity to MUP genes within class B providing evidence that Class A MUPs are the ancestral genes (Logan, Marton, and Stowers 2008). The class B genes arising from this duplication may also be classed as ‘circulatory’ MUPs whereas class A may be described as ‘peripheral’ MUPs. Peripheral MUPs (MUP 3,4,5,6,20,21) show mutations which result in the narrowing of the central cavity of the molecule suggesting that these MUPs have a more specific binding site (Phelan et al. 2014).

3.1.2: An alternative role for MUPS

The pheromone carrying capacity of MUPs which allows for the chemical communication between mice likely explains why these proteins account for over 90% of the protein in adult male mouse urine (Zhou, Jiang, and Rui 2009). Studies have also previously shown that MUP expression is altered due to calorie restriction regimes (Hui et al. 2009; Giller et al. 2013). One possible explanation for this is that to reduce energy expenditure, and defer sexual reproduction during times of low food availability MUP expression is greatly lowered. Once food becomes more readily available it is possible that MUP expression increases and the energy expended in doing so will aid reproductive processes by attracting a suitable mate in order to produce offspring which are more likely to survive (Giller et al. 2013). There is also evidence of overcompensation in the expression of MUP levels upon refeeding after calorie restriction perhaps allowing the mice to very quickly take advantage of newly replenished food supplies and reproduce before food again becomes scarce (Giller et al., 2013). While this is likely true there is some evidence to suggest MUPs play a role in lipid metabolism (Giller et al. 2013; Hui et al. 2009; Zhou, Jiang, and Rui 2009) and the alteration in MUP expression in response to changes in diet could be indicative of MUPs playing a role in this metabolic process. Hui et al also suggest that although liver tissue show the highest expression levels of MUPs the metabolic target of these proteins may in fact be skeletal muscle (Hui et al., 2009).

The link between MUPS, metabolism and CR is then further complicated by the relationship between MUPs and Akt. Some urinary pheromones have been shown promote the phosphorylation of Akt/ERK kinase and the survival of vomeronasal neurons (Xia et al. 2006), it may be possible that MUPs are also able to mediate this effect if the ability of these proteins to act as pheromones is confirmed. Hui et al show that when recombinant MUP1 is administered via an osmotic pump mice shown an increase in insulin evoked-Akt signalling in skeletal muscle (Hui et al. 2009). This could further link MUPs to the molecular mechanisms altered during a CR diet as Akt has been shown to be involved in metabolism changes seen in CR(Fontana and Klein 2007; Schleit et al. 2013).

3.2: MUPs-Results

Table 3: Changes in transcript abundance of MUP in mouse liver tissue genes during ageing and CR Gene short names are shown. Log 2-Fold change is the logarithm (base 2) of the change between the two values when the first is divided by the second. For example, when abundance at 3 months is divided by abundance at 24 months. Table headings show how each fold change was calculated. A positive log 2-fold change shows in increase in transcript abundance in the comparison whereas a negative log 2-fold change indicates a decrease in abundance. Table heading 3AL/24AL indicates genes which have changed in transcript abundance in the 24 month aged mice fed an AL diet when compared with the 3 month mice. Table heading 3AL/24CR indicates genes which have changed in transcript abundance in the 24 month aged mice fed a CR diet when compared with the 3 month mice. Table heading 24AL/24CR indicated genes which have changed in transcript abundance in the 24 month aged mice fed a CR diet when compared with the 24 month mice fed an AL diet.

3 AL/24 AL			3 AL/24 CR			24CR/24AL		
Gene Name	Log 2-Fold Change	P Value	Gene Name	Log 2-Fold Change	P Value	Gene Name	Log 2-Fold Change	P Value
Mup1	-3.71	1.91E-57	***Mup1***	-10.06	5.48E-175	***Mup1***	-5.95	1.76E-52
Mup2	-2.69	1.52E-40	***Mup2***	-4.92	1.30E-14	***Mup2***	-2.95	4.77E-11
Mup3	-2.25	5.20E-24	***Mup3***	-4.37	7.03E-41	***Mup3***	-2.06	9.90E-08
Mup6	-1.74	0.0196777	***Mup5***	-2.51	0.0041495	***Mup8***	-4.87	1.48E-22
Mup7	-5.29	2.95E-107	***Mup6***	-2.63	0.0024454	***Mup11***	-1.64	0.0345345
Mup8	-3.13	6.18E-47	***Mup7***	-6.32	5.66E-18	***Mup12***	-4.48	1.31E-19
Mup9	-3.09	3.34E-54	***Mup8***	-8.50	1.63E-69	***Mup13***	-2.06	0.0046099
Mup10	-2.37	9.27E-29	***Mup9***	-1.86	0.0363221	***Mup14***	-2.19	0.0020951
Mup11	-3.46	1.59E-80	***Mup10***	-2.77	0.0013096	***Mup15***	-6.31	1.59E-43
Mup12	-4.06	1.23E-19	***Mup11***	-3.82	2.51E-06	***Mup16***	-3.14	9.28E-12
Mup13	-3.96	1.60E-121	***Mup12***	-9.56	1.59E-118	***Mup17***	-4.52	7.38E-20
Mup14	-4.70	1.11E-144	***Mup13***	-4.99	2.57E-11	***Mup18***	-1.65	0.0335426
Mup15	-4.46	9.00E-83	***Mup14***	-5.60	2.07E-14	***Mup19***	-5.14	2.18E-31
Mup16	-3.95	2.33E-100	***Mup15***	-11.31	1.80E-146	***Mup20***	-2.32	5.04E-05
Mup17	-6.51	8.35E-231	***Mup16***	-6.15	3.60E-23	***Mup21***	-1.87	1.92E-06
Mup18	-3.25	1.91E-57	***Mup17***	-11.33	1.08E-164	***Mup-ps12***	-1.78	0.0178122
Mup19	-3.37	7.83E-81	***Mup18***	-4.23	6.65E-08	***Mup-ps13***	-2.08	0.0014303
Mup20	-2.49	9.15E-35	***Mup19***	-8.92	6.72E-111	***Mup-ps16***	-2.92	1.14E-05
Mup21	-3.01	1.10E-43	***Mup20***	-4.62	1.30E-14	***Mup-ps19***	-1.92	0.0037841
Mup-ps1	-1.47	0.0089966	***Mup21***	-4.38	1.33E-14			
Mup-ps6	-2.21	0.000484	***Mup-ps1***	-3.03	1.23E-05			
Mup-ps7	-1.77	0.0042741	***Mup-ps2***	-2.59	0.002935			
Mup-ps8	-2.20	0.000335	***Mup-ps6***	-3.95	3.95E-07			
Mup-ps12	-2.66	2.93E-10	***Mup-ps7***	-4.28	1.55E-08			
Mup-ps13	-1.11	0.0027606	***Mup-ps8***	-4.08	1.18E-07			
Mup-ps14	-1.78	0.003039	***Mup-ps10***	-2.76	0.0013558			
Mup-ps15	-1.63	0.0289385	***Mup-ps12***	-5.55	2.27E-16			
Mup-ps17	-5.06	3.55E-50	***Mup-ps13***	-2.65	0.0013135			
Mup-ps18	-1.96	0.00847	***Mup-ps14***	-4.49	1.61E-10			
Mup-ps19	-2.18	5.52E-30	***Mup-ps16***	-4.25	2.28E-08			
Mup-ps20	-3.03	1.45E-05	***Mup-ps17***	-7.21	2.04E-33			
Mup-ps22	-4.36	2.42E-13	***Mup-ps18***	-2.43	0.0058358			
			Mup-ps19	-3.92	2.46E-09			
			Mup-ps20	-3.17	0.0001452			

	Mup-ps22	-4.85	2.49E-11	

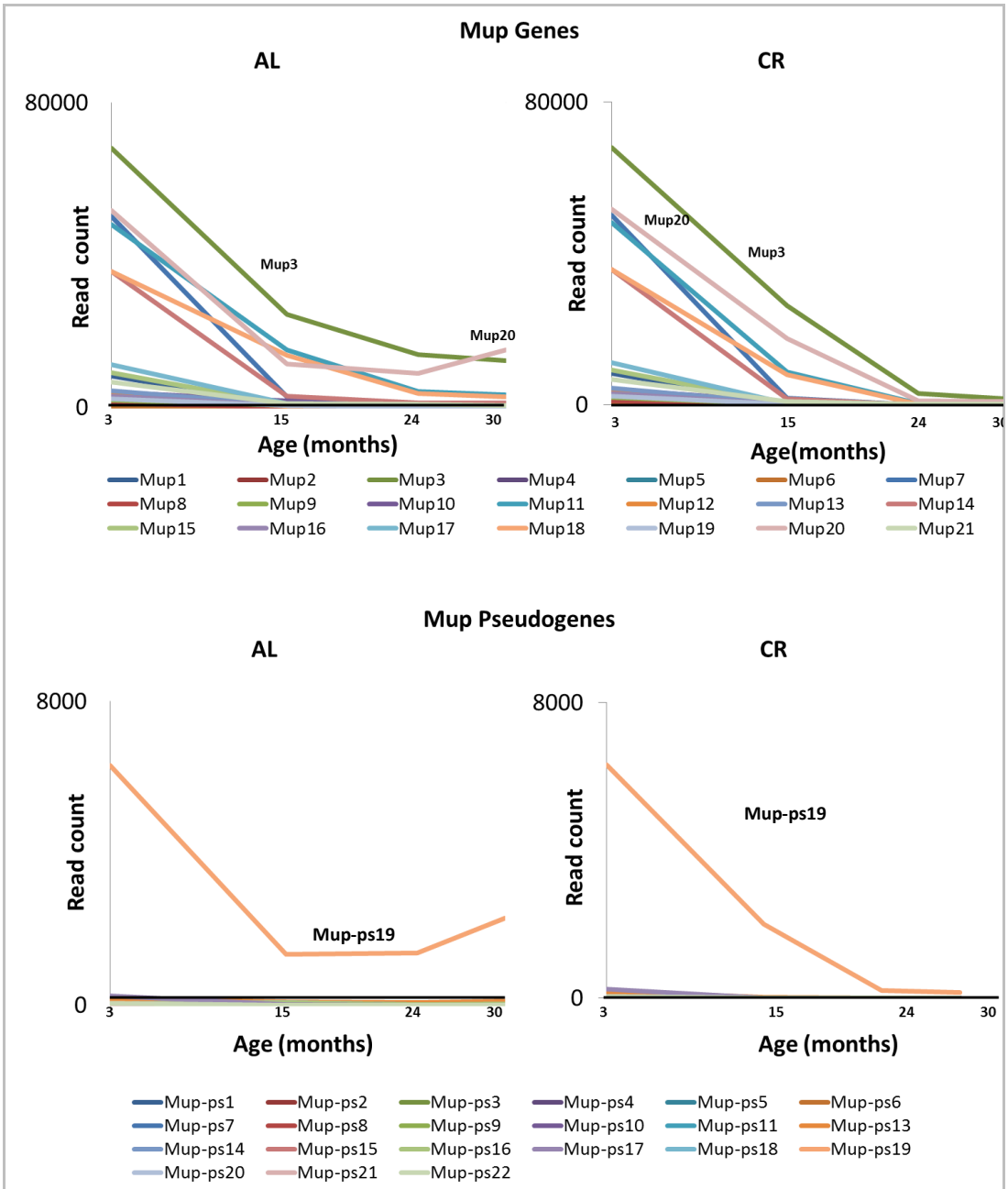


Figure 2: Transcript abundance of MUPs in mouse liver tissue during ageing and CR. The MUP gene and MUP pseudo genes have been separated. All graphs show the average gene count value for the age group. Genes which show a particularly noteworthy expression pattern have been annotated directly on the graph.

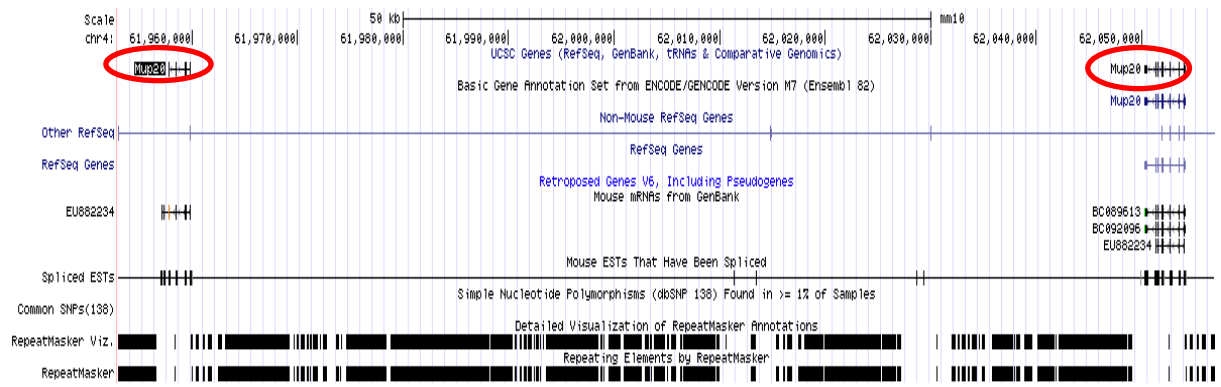


Figure 3: MUP20 gene annotation in mouse Shows an image taken from the UCSC Genome Browser Website (https://genome.ucsc.edu/) Which highlights the difficulty in accurately studying the MUP genes as they are incorrectly annotated. Red circles have been used to highlight two separate genes which have been labelled in this genome browser as MUP20, however the gene shown on the left of the image is at position chr4:61,957,790-61,959,911 is MUP-PS19 according to the ensemble (http://www.ensembl.org/) while the gene shown to the right side of the image is MUP20.

3.3 MUPs- Discussion

The information presented in Figure 2 demonstrates the severity of the decrease in MUP expression throughout the ageing process and between AL and CR diets in mouse liver tissue. Whether the mice have been fed an AL or CR diet the abundance of the MUP transcripts shows a very severe decrease during ageing, table 3 highlights which of these changes are significantly different using data from the DESeq analysis. Figure 2 also highlights how the transcript abundance of MUP3 and MUP20 appears to outlie the pattern of expression seen in the other MUP genes. MUP3 and MUP20 transcripts present at 24 months and these genes show a less severe reduction in expression, even increasing in expression levels at 30 months in the AL mice (though they still show a large decrease from young). Table 3 shows that these changes are still significant from expression levels in the 3 month mice. Both of these MUP genes have been classified as 'peripheral' MUP genes likely possessing a more specific binding cavity which could suggest the protein products of these transcripts carry out a more specific function other than the reproductive role originally thought and may not be readily reduced to such low levels as other MUP genes (Phelan et al. 2014).

In general, the MUP pseudogenes show very low transcript abundance though Figure 2 shows that MUP ps-19 is also an outlier in expression levels when compared to other MUP pseudogenes. While the pseudogenes in general are expressed at very low levels (many genes showing gene counts of 0) MUP ps-19 shows comparatively very high expression in the young mouse group and the trend in the abundance of this transcript follows a very similar pattern to that of the MUP non-pseudogenes in particular MUP20. Figure 3 in fact highlights an incorrect annotation of MUP-ps19 in the UCSC genome browser (https://genome.ucsc.edu/) where MUP-PS19 has been annotated as MUP20. This incidence highlights a key problem in effectively studying MUPs as these genes are poorly annotated. This is also coupled with the high sequence similarity of the genes meaning that primer design specific to a certain MUP gene is extremely difficult given that specific primer design relies heavily on data bases such as UCSC and unique sequences in genes. On further investigation, it would appear that primers used in the literature to target genes such as MUP1 may not be specific-however to know this for certain would rely on accurate annotations in gene databases.

There is much to consider when investigating the information currently provided regarding the effect of diet on the expression levels of major urinary proteins. There are some differing results from experiments to determine the effect of MUPs on glucose and lipid metabolism. Two studies investigate the effect of MUP1 on diabetic and obese mice, both suggesting that MUP1 levels are markedly lowered in diabetic mice and that replenishment of MUP1 increases insulin sensitivity (Zhou, Jiang, and Rui 2009; Hui et al. 2009). Zhou et al incubated primary hepatocytes from male mice in a medium containing MUP1 adenovirus for viral infection and injected diabetic mice with MUP1 adenovirus. (β-gal used as a control) (Zhou, Jiang, and Rui 2009). Therefore Zhou et al suggest that MUPs are able regulate glucose metabolism and that the addition of MUP1 to a primary hepatocyte culture inhibited gluconeogenic gene expression and well as inhibiting gluconeogenic program in diabetic mice (Zhou, Jiang, and Rui 2009). Hui et al also suggest MUP1 is a novel metabolic regulator and deliver MUP1 to diabetic mice using an osmotic pump leading to the conclusion that although MUP1 is produced in the liver the metabolic target is skeletal muscle where MUP1 increases mitochondrial biogenesis and decreases lipid accumulation (Hui et al. 2009). There are therefore two separate studies at whole organism level and cell culture level which suggest that the delivery of MUP1 reduces the symptoms of diabetes. This is of course contradictory to the results presented in Figure 2 which suggest that a calorie restricted diet results in a decrease in the expression of MUPs and there is a huge body of evidence informing us that a CR regime decreases risk of diabetes. For example, calorie restriction has been shown in many studies to increase mitochondrial biogenesis, locomotor activity and insulin sensitivity in a range of model organisms (Fontana and Klein 2007; Willcox et al. 2007), all changes seen in diabetic mice when MUP1 levels were increased (Zhou, Jiang, and Rui 2009)

The argument is then further complicated by other data shown by Hui et al which also suggests that overnight fasting induces a 50% reduction in the expression of MUP1 which is restored an hour after refeeding (Hui et al. 2009).Further evidence that a CR regime reduced the expression of MUP genes is presented by Giller et al, which reports that during short-term calorie restriction MUP 5, alongside other MUPs shows significantly decreased expression levels (Giller et al., 2013). This down regulation is then reversed upon refeeding where Giller et al observed an overcompensation in MUP5 levels (Giller et al. 2013). Yet, the addition of major urinary proteins to mice via an osmotic pump induces many of the physiological changes observed in mice on a CR diet (Zhou, Jiang, and Rui 2009; Giller et al. 2013; Fontana and Klein 2007). This is then further complicated by a separate study where

Resveratrol (a CR mimetic) was administered to mice consuming a high fat diet and expression levels of MUP1 and MUP3 in liver were significantly increased (Baur et al. 2006). Resveratrol has been considered by many as a CR mimetic as it has been suggested to promote the longevity increase and reduction in age associated disease mediated by CR although this topic is still controversial (Miller et al. 2011; Timmers et al. 2011; Baur et al. 2006). It is therefore interesting that when Resveratrol was administered to mice MUP expression increased whereas when mice are fasted overnight or fed a CR diet MUP levels are markedly decreased (Hui et al. 2009; Giller et al. 2013). All of the above evidence has been collected using liver samples however a study focusing on feeding induced changes in the hypothalamus transcriptome showed a low fat diet induced the expression of MUP 1 and 3 (De Giorgio, Yoshioka, and St-Amand 2009). We can explore the above arguments in various ways and though we can see that Zhou et al focus more closely on MUP1 and Giller et al on MUP5 the data in Figure 2 provides evidence that the pattern of downregulation during a CR diet is seen across the MUP gene family.

It would be interesting to investigate the mechanism by which MUP1 is able to regulate metabolism and this is likely through interaction with peroxisome proliferator-activated receptor gamma (PPARγ) (Zhou, Jiang, and Rui 2009). Zhou et al show that addition of MUP1 adenovirus significantly suppressed the expression of PPARγ and it is suggested that MUP1 inhibits hepatic lipogenesis by suppressing the expression of lipogenic genes such as PPARγ (Zhou, Jiang, and Rui 2009). Hui et al also suggest that diabetic mice show decreased levels of MUP1 as well as showing that the addition of PPARγ agonist rosiglitazone increases the expression of MUP1 (Hui et al. 2009). PPARγ is known to promote adipocyte differentiation, induce lipogenic enzymes and glucoregulatory proteins ensuring that cells maintain a normal level of glucose sensitivity by interacting with ligands (Olefsky 2000).

Currently the evidence presented leads us to no direct conclusion to the effect of MUPs on metabolic process as both high and low levels of MUPs appear to have similar physiological effects. However, from Figure 2 it is clear than during ageing in the AL group MUP levels show a large decrease in transcript abundance and this is further downregulated in the CR group. This may lead us to believe that MUPs are not involved in altering metabolic processes however, the administration of recombinant MUP1 via an osmotic pump clearly has an effect on metabolism (Zhou, Jiang, and Rui 2009; Hui et al. 2009). If these genes are to be studied and characterised more effectively there is a clear need for better annotation.

Phelan et al also suggest there is likely more MUP genes located on chromosome 4 and this will also require further analysis to fully annotate the MUP gene family (Phelan et al. 2014)

This may be interesting regarding metabolism and human ageing as humans also express genes which are members of the lipocalin gene family which have been associated with metabolic syndrome in humans. However, this link is tenuous and would require further investigations to establish a link between the two. This work was carried out to explore a possible lead for future work but does not establish a causal link or provide evidence to suggest lipocalins or major urinary proteins form the basis of the molecular mechanism underpinning caloric restriction. This lead was investigated based on the 20 gene most altered during the ageing process when dietary types are compared however looking more broadly than the top 20 genes would have given a broader overview.

Chapter 4: RNA Binding Proteins

4.1: RNA Binding Proteins-Introduction

4.1.1 RNA binding proteins

Tables 1 and 2 demonstrate that besides the large changes seen in the expression levels of MUP genes there is no overall pattern in genes which show the largest changes in transcript abundance during ageing or when age-matched dietary comparisons are made. In an attempt to understand the variety of RNA detected during the RNA sequencing experiment the transcript abundance of RNA binding proteins- the molecules which regulate the RNA found within the cell- was further analysed. The transcriptional profile of cells is essential in determining function and differentiation of the cell and it is therefore likely that there are consistent changes within the transcriptional profile of cell which occur during ageing and senescence (McCarroll et al. 2004). While many studies have detected the pathways directly regulated by ageing and CR and others have focused on the changes to the gene expression profile of the cell. As a result of these conditions, few have looked closely at the mechanism mediating these changes. In depth analysis of changes to the transcriptome during ageing and calorie restriction could be essential to determining the underlying cause of ageing and the downstream effects of nutrient sensing pathways.

RNA binding proteins (RBPs) are essential in modulating the transcriptome of the cell, and are involved in all elements of RNA biology including alternative splicing, mRNA export and mRNA localisation and may also act in combination with RNA form dynamic ribonucleoproteins. The processing of RNA is conserved throughout species and disruptions to these pathways within the cell results in disease (Glisovic et al. 2008b; Castello et al. 2012). The functions of RBPs are carried out via a limited repertoire of RNA binding motifs; the range of which is likely not yet full known (Castello et al. 2012). Specificity of an RBP is increased through multiple RNA binding motifs, auxiliary domains, post translational modifications and alternative splicing (Glisovic et al. 2008b). The differing combinations of these domains enables RNA binding proteins, one of the most abundant classes of human proteins, to meet the complexity of RNA (Mercer and Mattick 2013). It would seem logical that RBPs are essential for the cell to undergo the large changes in protein expression seen during cellular senescence. Studies have revealed that RNA binding proteins such as HuR, CUGBP1 and TIA are associated with ageing and senescence and likely regulate the expression of growth proteins (W. Wang 2012; Marasa, Martindale, and Halushka 2009).

Another aspect to consider is emerging evidence that many RBPs have a multitude of roles within the cell and are involved in the regulation of different cellular processes depending on cellular or environmental conditions (Naro et al. 2015; Twyffels, Gueydan, and Kruys 2011). For example the Serine-Arginine (SR) family of RBP were traditionally described as regulators of alternative splicing however under certain conditions members of this family undergo changes in phosphorylation status causing the protein to shuttle from the nucleus to the cytoplasm and play a role in translation (Twyffels, Gueydan, and Kruys 2011; Änkö 2014). The target RNA sequence of an RNA binding protein can be determined using bioinformatic tools and computational predictions but these are limited and further analysis such as CLIP-Seq should be used to fully determine the RNA binding specificity of a given RBP(Castello et al. 2012; Park 2009). Since each RNA binding protein is likely regulating more than one specific RNA molecule and RNA binding proteins are responsible for modulating the transcription, translation, alternative splicing and many other functions of the cell we have hypothesised that small changes to the expression of RNA binding proteins would produce wider changes to the expression of other proteins in the cell almost emulating a cascading effect.

Alternative splicing is a specific function largely mediated by RNA binding proteins and it is via alternative splicing that the cell is able to produce a diverse array of proteins generated from a relatively small genome (Stamm et al. 2005; Padgett 2012). It is predicted that 95-100% of all mRNA transcripts containing multiple exons are alternatively spliced to produce multiple isoforms and combined with non-sense mediated decay (NMD), alternative splicing may also regulate transcript abundance (Nilsen and Graveley 2012; Kelemen et al. 2013). There are two main types of alternative splicing; constitutive splicing in which introns are removed to produce a processed mRNA transcript and alternative splicing where introns may be retained or skipped, or alternative splice sites are used. Generally it seems that both constitutively and alternatively spliced exons share similar sequences however these may deviate more from the consensus sequence in alternatively spliced exons resulting in a weaker affinity for the spliceosome which may suggest why these spice sites are not consistently recognised by the spliceosome (Kelemen et al. 2013).

There are seven main type of alternative splicing carried out by the cell; skipped exon, alternative 3’ splice site, alternative 5’ splice site, retained intron, mutually exclusive exons, alternative promotors and alternative poly A sequences (Blencowe 2006). All splicing is carried out by the spliceosome, of which there are a major and minor; the major

spliceosome being more widely studied and therefore more greatly understood (Padgett 2012). The spliceosome is a dynamic multi-megadalton ribonucleoprotein complex containing within it 5 small nucleolar ribonucleoproteins (snRNP) as well as many other proteins which may be associated with the spliceosome at certain points as the network is continually formed and rearranged during the splicing process (Will and Lührmann 2011). The spliceosome recognises a specific, conserved sequence found at the 5' and 3' splice site which defines an intron however other cis-acting splicing factors recognise pre-mRNA elements known as exonic and intronic splicing enhancers (ESEs or ISEs) or silencers (ESS or ISS) (Will and Lührmann 2011). Serine-arginine (SR) proteins generally bind to ESEs enhancing the ability of the spliceosome to act on this exon while heteronuclear ribonucleoproteins (hnRNPs) bind to intronic and exonic splicing silencers decreasing the ability of the spliceosome to act on these genes in a position dependent, combinatorial or competition dependent manner (Änkö 2014; Will and Lührmann 2011). This chapter also seeks to begin investigating how alternative splicing may be altered during the ageing process and what the effects of this may be.

4.1.2 RNA Binding Proteins and Ageing

Little is known regarding core changes to alternative splicing throughout ageing due to the difficulties of such analysis. The misregulation of splicing has been linked to a number of human diseases such as Spinal Muscular Dystrophy (Padgett 2012). In fact, around 10% of human diseases can be attributed to a mutation in the consensus splice site sequences while as much as 25% of human genetic diseases are linked to a mutation in other splicing regulatory sequences (Padgett 2012; Will and Lührmann 2011). A genome wide analysis of peripheral blood leukocytes carried out by Harries et al (2011) finds that many aspects of mRNA metabolism show ageing-associated alterations including RNA binding and RNA Splicing (Harries et al. 2011). Further to this finding it has been reported that during ageing there is a general deregulation of splicing and a decrease in the expression of splicing factors, highlighted in a study by Holly et al which suggests that 38% of splicing factors are altered during ageing (Holly et al. 2013). More specifically an analysis focusing on alternative splicing within neuronal tissues during ageing and Alzheimer's disease showed significant splicing changes in 1174 exons most likely attributed to the splicing factor TAR DNA binding protein 43 (TARDBP) (Tollervey, Wang, and Hortobágyi 2011).

There are also many other factors which can affect the pattern of alternative splicing. For example, alternative splicing may also be effected by epigenetic mutations which result

in histone modifications and this can affect the rate of mRNA translation resulting in altered folding of the nascent chain differing the availability of alternative splice sites (Nilsen and Graveley 2012) as well as reversible phosphorylation which acts by altering the function of RNA binding proteins (Will and Lührmann 2011). Identifying the different global and tissue specific splicing patterns altered during disease or ageing may prove a daunting task as an analysis carried out by Yeo et al suggests that there is variation in the alternative splicing across human tissues (Yeo et al. 2004). Yeo et al highlight how different tissues show variation in the type of alternative splicing most commonly occurring and in the expression patterns of splicing factors, for example the human adult liver is a clear outlier in splicing factor expression when compared to other tissues (Yeo et al. 2004).

The prominent role of RNA splicing in the control of the expression profile of a cell means that splicing factors play an important role in the initiation and maintenance of DNA damage repair. RNA splicing and splicing factors mediate the changes in the expression profile of a cell undergoing the DNA damage response (DDR) (Naro et al. 2015). Therefore it is likely that splicing factors and RNA binding proteins are involved in the induction and maintenance of senescence in cells (Marasa, Martindale, and Halushka 2009). There is also evidence to suggest that splicing factors can aid the prevention and repair of DNA damage. One such example of this is the splicing factor Srsf1/Srp40- often referred to as the prototype of the serine-arginine rich family (SR family) of splicing factors which has been shown to prevent R-loop formation (Naro et al. 2015) and has also been shown to stabilise p53 in the induction of senescence (Fregoso et al. 2013).

4.1.3 SR proteins

This chapter will also seek to look more closely at the Serine-Arginine (SR) family of RNA binding proteins. SR proteins are named so as each protein has at least one domain which contains serine-arginine repeat (RS domain) (Twyffels, Gueydan, and Kruys 2011; Änkö 2014). To be classified as an SR protein specific sequence criteria are defined as: one or two conserved RNA binding domains as well as an RS domain located at the C-terminus. These domains may be extensively phosphorylated, a mechanism that is emerging as increasingly important in the regulation of RNA binding proteins (Will and Lührmann 2011). This results in a family of 12 SR proteins although SRSF8 is not expressed in mice (Twyffels, Gueydan, and Kruys 2011). SR domains are thought to stabilise the interactions of RNA with spliceosomal components via protein-protein interactions (Will and Lührmann 2011). Generally these proteins have been described as trans acting splicing factors known to act as exon splicing

enhancers (ESE) (Will and Lührmann 2011) however, recent evidence suggests that members of the SR protein family are able to translocate from the nucleus to the cytoplasm under certain conditions where they play a role in translation (Mo, Ji, and Fu 2014; Maslon et al. 2014; Twyffels, Gueydan, and Kruys 2011).

Transportin-SR is responsible for the nuclear export of SR proteins. This protein surveys the SR domain and will export the SR protein when the correct phosphorylation pattern is detected (Lai et al. 2000). These phosphorylation patterns are regulated by several protein kinase families such as the SRPK family (Twyffels, Gueydan, and Kruys 2011; Lai et al. 2000). However, the regulation of sub-cellular localisation of the SR proteins via the phosphorylation of the RS domain can also prove irrelevant when there is a nuclear retention signal present in the protein sequence. This is displayed in the SR protein SRSF2 which contains two long stretches of RS domains able to undergo phosphorylation but remains in the nucleus due to a nuclear localisation signal (Cazalla et al. 2002). SR proteins associate with various classes of RNA including non-coding RNA and intronless transcripts (Breig and Baklouti 2013) which first indicated that SR proteins have functions outside of alternative splicing.

The expression pattern of the SR proteins within the cell can alter the splicing landscape and these patterns can vary greatly between different cell types (Twyffels, Gueydan, and Kruys 2011). SRSF1 and SRSF2 are considered to be the prototype of the SR family (Twyffels, Gueydan, and Kruys 2011) and as such these proteins have been more extensively studied. A study in 2000 using *C.elegans* suggests that there is a degree of redundancy between the SR proteins in order to protect against deletions of one SR protein, the exception to this is SRSF1 which proved essential for the successful development of nematodes (Twyffels, Gueydan, and Kruys 2011). To understand more about how the emerging variety of roles played by SR proteins within the cell impacts on the ageing process the RNA-Seq analysis of mouse liver tissues was probed further to analyse how the expression of this family may change during the ageing process.

4.1.4 SRSF1

When studying the transcript abundance of the SR protein family there was no single SR protein which stood out from the family however as previously mentioned there is likely other regulatory processes occurring with alter the function of some SR proteins. In order to investigate this further the SR protein SRSF1 was used as case study and analysed more

closely. SRSF1 was chosen for a number of reasons namely because this protein is the most extensively studied of the SR proteins (Maslon et al. 2014) therefore more comparable data is available. SRSF1 also shows significant change in the transcript abundance seen during ageing in both diet types. Further evidence to look more closely at SRSF1 is provided by a study by Holly et al (2013) which finds the expression of SRSF1 to be significantly altered during ageing (Holly et al. 2013).

SRSF1 has been shown to translocate from the nucleus to the cytoplasm when the C-terminus of the protein has become phosphorylated. In the cytoplasm SRSF1 plays a role in translation (Twyffels, Gueydan, and Kruys 2011) and has been linked with the stabilisation of p53- a tumour supressing protein (Fregoso et al. 2013). SRSF1 is thought to interact with mcl-1 alongside SRSF5 (Gautrey and Tyson-Capper 2012). Considering how SR protein expression is regulated leads to further investigations into the splicing of SRSF1 as well as the localisation under these conditions. Expression of SR proteins within the cell are generally controlled by post-transcriptional mechanisms where the transcript undergoes alternative splicing, producing non-coding transcripts which are retained in the nucleus or targeted to the NMD pathway (Ni et al. 2007; Lareau and Brenner 2015). Ni et al (2007) suggest that the cell is able to control alternative splicing by sensing the splicing status of mRNA transcripts and uses NMD as a method to regulate the expression of the appropriate splicing factors (Ni et al. 2007).

4.2.1: RNA Binding Proteins-Results

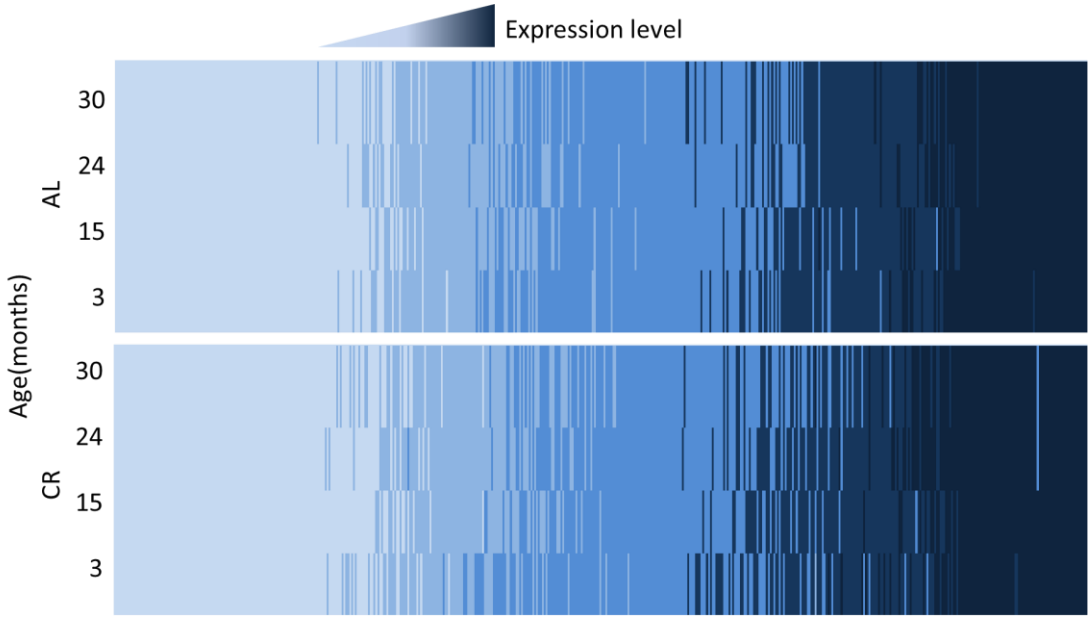


Figure 4: Heat map showing changes in transcript abundance of RNA binding proteins in mouse liver tissue during and ageing and CR. The data used to plot this was a normalised gene count file. The data was clustered according to expression level. The gene list of RNA binding proteins used was assembled using a combination of genes from Galante et al (Galante et al. 2009) and using genes assigned a the GO annotation 0003723 (RNA binding protein). As the scale bar shows the lighter colours represent lower gene read counts while darker colour represents high expression level.

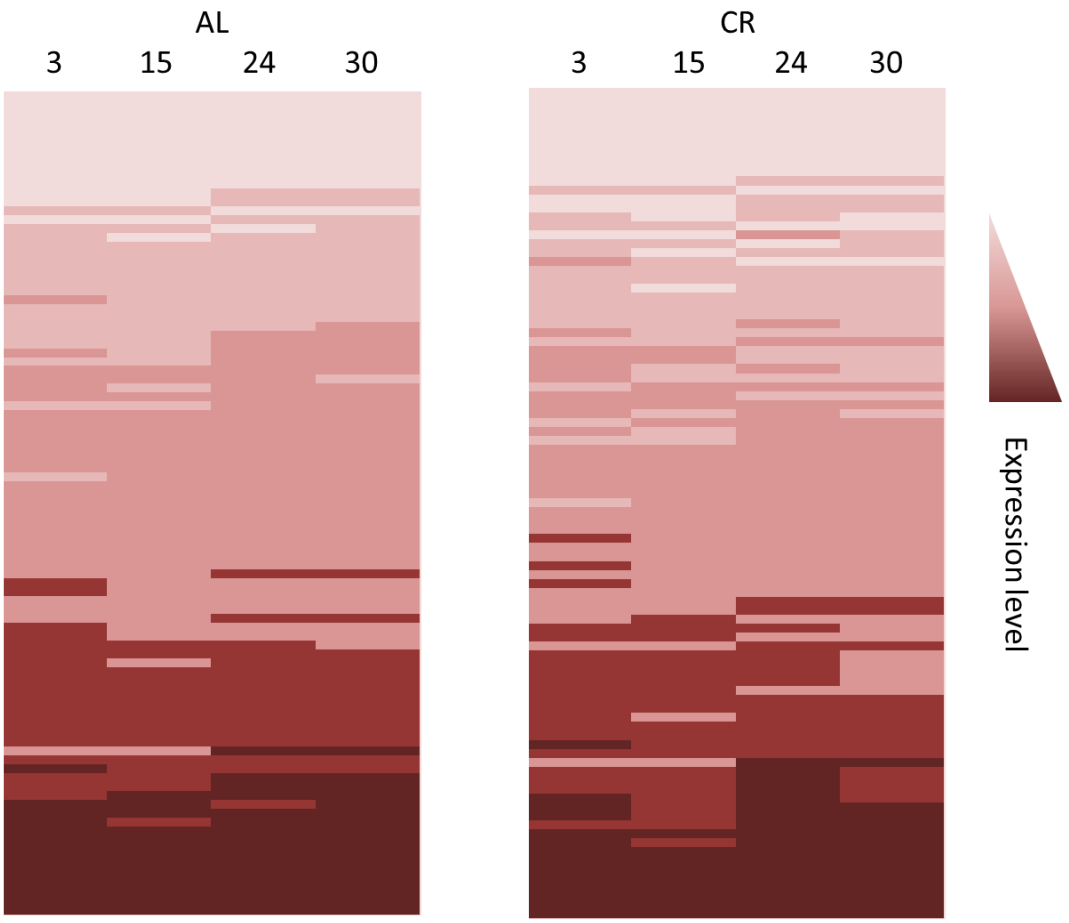


Figure 5: Heat map showing changes in transcript abundance of RNA splicing factors in mouse liver tissue during and ageing and CR. The data used to plot this was a normalised gene count file, and was clustered according to expression level. The gene list of splicing factors used was assembled by cross referencing a list of proteins assigned the Panther Protein Class of mRNA splicing as well as the GO term RNA splicing:0008380. As the scale bar shows the lighter colours represent lower gene read counts while darker colour represents high expression level.

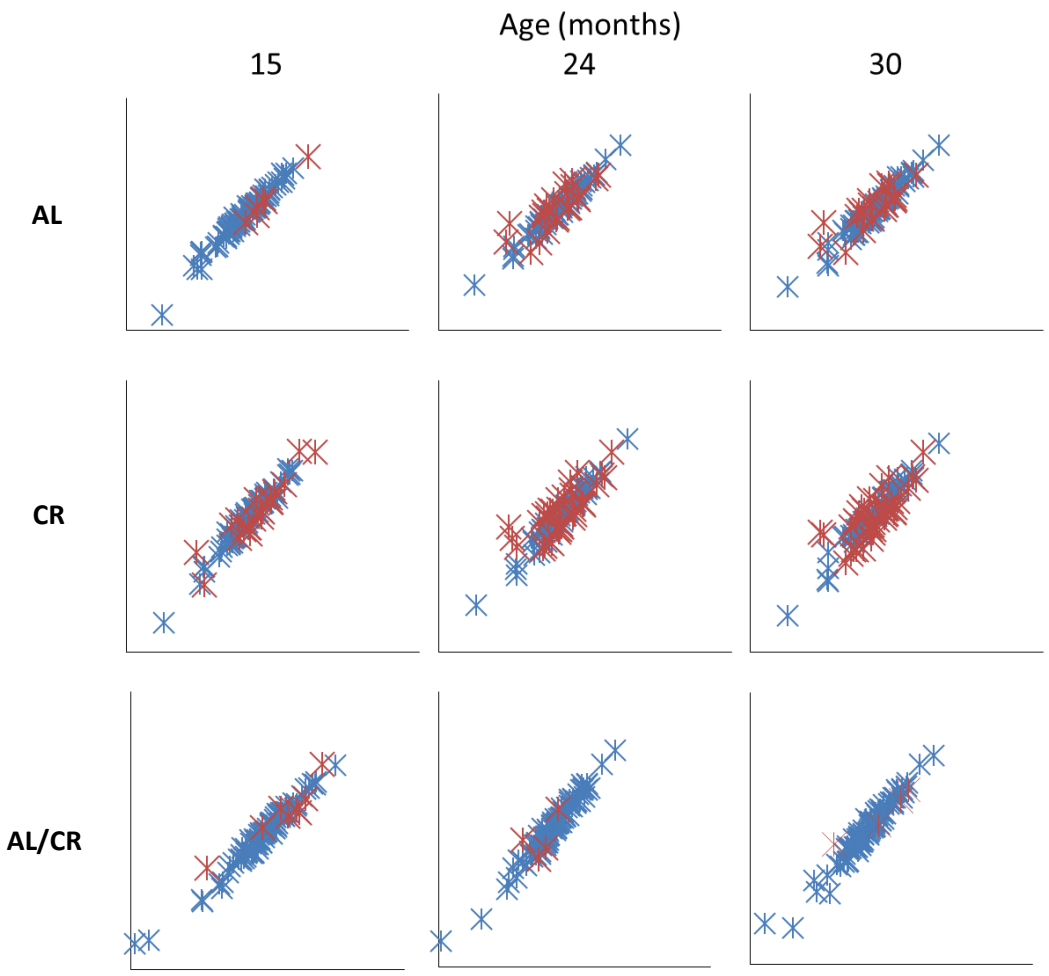


Figure 6: Scatter plot showing changes in transcript abundance of splicing factors during two way comparisons between ageing and diet type. The gene list of splicing factors used was assembled by cross referencing a list of proteins assigned the Panther Protein Class of mRNA splicing as well as the GO term RNA splicing:0008380. Data shown is normalised gene counts, values are log10 to make the data easier to interpret. Values deemed significant by DESeq analysis are shown those data points which are coloured red represent RNA binding proteins while blue data points represent all other genes with significant transcript abundance. All axis have been normalised so they have the same maximum value. Row 1 shows AL values for each age group compared to 3 month mice. Row 2 shows CR values compared to 3-month-old mice, 3 month values are always shown on the x-axis. Row 3 shows age matched comparisons between AL and CR diets with CR values always shown on the X-axis

Table 4: Significant changes in transcript abundance of SR genes during ageing and CR in mouse liver tissue. Gene short names are shown. Log 2-Fold change is the logarithm (base 2) of the change between the two values when the first is divided by the second. For example, when abundance at 3 months is divided by abundance at 24 months. Table headings show how each fold change was calculated. A positive log 2-fold change shows in increase in transcript abundance in the comparison whereas a negative log 2-fold change indicates a decrease in abundance. Table heading 3AL/24AL indicated genes which have increased in transcript abundance in the 24 month aged mice fed an AL diet when compared with the 3 month mice.

24AL/Y

Gene Name	Log 2-Fold Change	P Value
Srsf4	0.99	2.09E-07

24CR/Y

Gene Name	Log 2-Fold Change	P Value
Srsf1	-0.78	0.000168
Srsf4	1.15	5.34E-07
Srsf9	-0.61	0.015713
Srsf10	-0.51	0.011236
Srsf11	0.56	0.015514

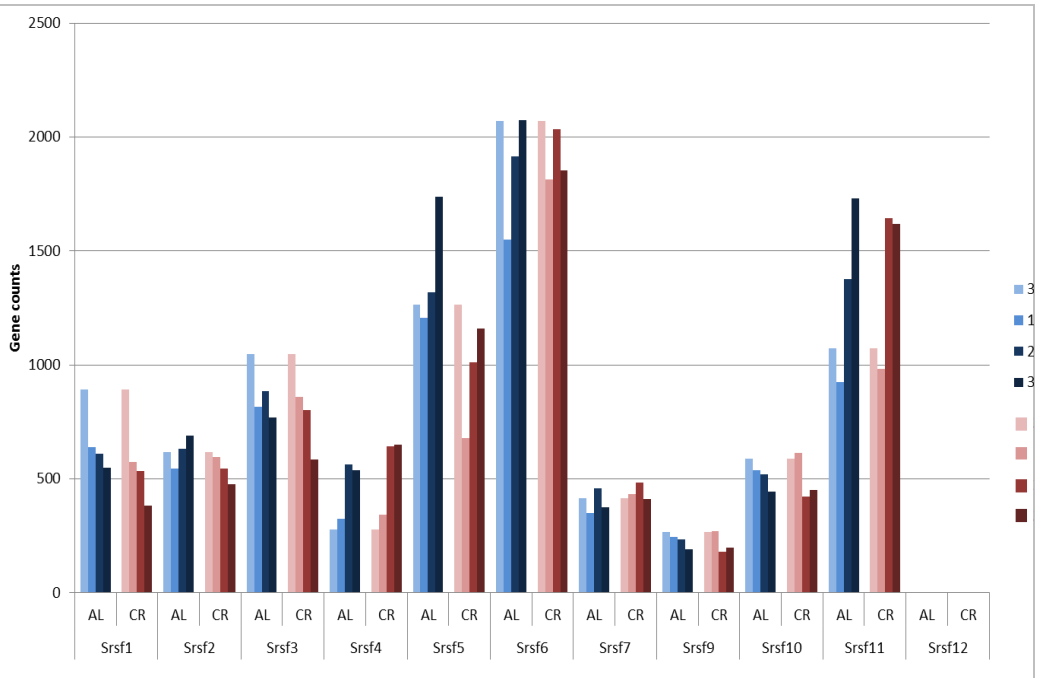
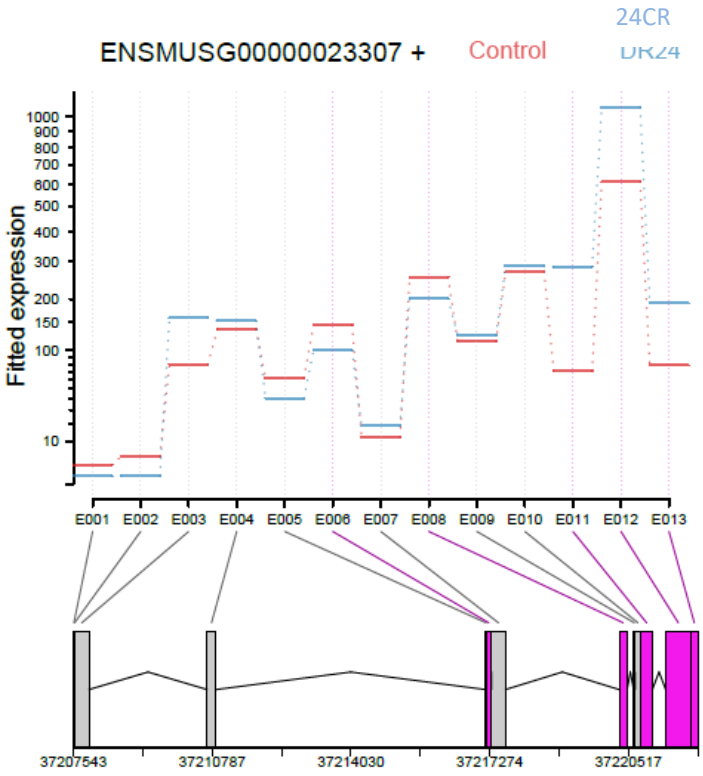


Figure 7: Gene counts aligned to SR proteins from mouse liver tissues across all ages and diet types. The Figure legend indicates the age in months of the mouse which the sample was taken from. AL mice are shown in varying shades of blue with CR mice are shown in varying shades of red. Srsf12 shows no data as no reads were aligned to this gene.

A



B

24CR/Y

Gene name	Exon ID	P Value	Log 2-fold Change	Location
Srsf1	E011	0.000244	4.34	Chr11: 88049216-88049411

Figure 8: DEXSeq analysis showing alternative slicing of SRSF1 gene in mouse liver tissue A) Image taken from DEXSeq analysis of RNA Sequencing showing possible alternative splicing patterns of SRSF1 in mouse liver **tissue**. Each possible event is assigned a number and the number of reads aligned to this part of the genome is shown as 'fitted expression'. Reads aligned to the 'control' or 3-month mouse are shown in red while reads aligned to the 24CR shown in blue. The alternative splicing event detailed in Figure 9 is shown here as E011. B) **DEXSeq data for SRSF 1 alternative splicing**. Log 2-fold change when the number of reads for E011 where compared between the control and the 24 CR mouse. Data also includes a p value for E011 and a chromosome location for the event.

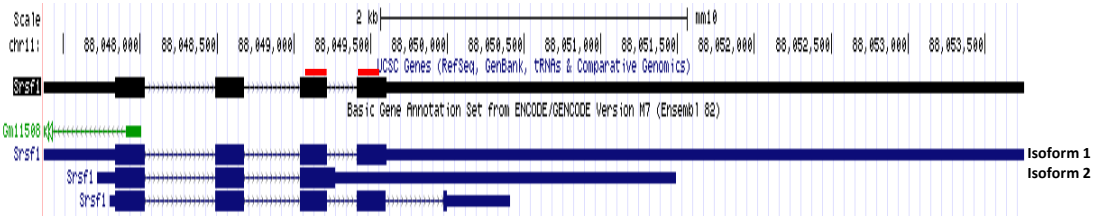


Figure 9: Structure of the SRSF1 gene in mouse Images taken from UCSC Genome Browser(https://genome.ucsc.edu/) sho the varying isoforms of the SRSF1 gene in Mouse. The primer alignments are shown as the red bars at the top of the arrang and two isoforms discussed in the chapter are highlighted. A list of primers is shown in the appendix

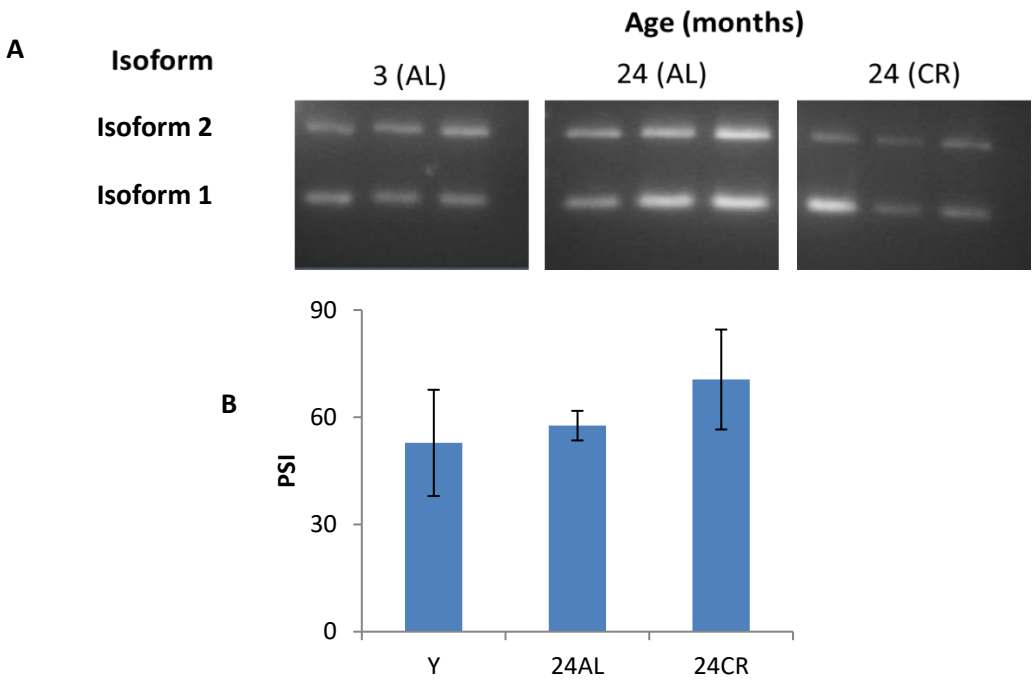


Figure 10: Alternative splicing of SRSF1 in mouse liver tissue A) photographs of a gel showing the products of a PCR using cDNA from mouse liver tissue (as described in methods) under UV light. Primers used are shown in Figure 9 And exact sequences of primers are shown in the appendix. B) shows the PSI (percentage of the functional transcript present) in mouse liver tissue and was calculated using image J the larger the value of the PSI the greater ratio of isoform 1 to isoform 2. A list of primers is shown in the appendix

4.3: RNA Binding Proteins-Discussion

Splicing factors are an interesting area of the RNA biology to investigate in such an analysis as there is very little known about how the splicing landscape changes during the ageing process and any changes in this would likely be attributed to changes in the expression of splicing factors. Splicing is employed by the cell to create the required complexity in protein function from a relatively small genome and is therefore an essential process. Our analysis has focused on changes to splicing factors as core components of the spliceosome are unlikely to change and the project was time limited. It was hypothesised here that changes in the expression of splicing factors is a method employed by the cell to undergo the large changes in many functions seen during the ageing process such as altered metabolism and cellular senescence. The available RNA Sequencing and DESeq data was analysed to determine the effects of ageing and diet on the transcript abundance of RNA binding proteins and splicing factors

Figure 4 and 5 do not shown any significantly large changes in the expression levels of RNA binding proteins during the ageing process. While Figure 4 illustrates the general trend in the overall expression of RNA binding proteins these are a broad array of proteins modulating a diverse range of functions within the RNA biology of the cell and no significant change is shown. Holly et al(Holly et al. 2013) report that a decrease in the transcript abundance of splicing factors during the ageing process However we could take into account that there may be variation between different tissue types as Holly et al study peripheral blood whereas this study focuses on liver tissue (Holly et al. 2013). This data becomes more difficult to interpret as many of the RNA binding proteins can carry out other functions within the cell, for example by translocating. This suggests that regardless of the expression level of the RNA binding protein RNA or splicing factor RNA this does not necessarily imply these proteins are carrying out the same functions at different ages or when mice are fed different diet types. This is a limitation of this analysis and in future we should seek to obtain further information about the proteins expressed within liver tissue including phosphorylation status. Figure 6 adds more detail to Figures 4 and 5 and represents changes to the transcript abundance of splicing factors according to the DESeq comparisons between differing age groups and dietary regimes, highlighting in red the number of statistically significant changes. This indicates that the greatest number of statistically significant changes are shown when the 30-month CR mice are compared to young. While there is a clear trend showing more significant changes during ageing there are comparatively few significant changes between

dietary types and these genes which do show significant changes will be interesting targets for further study.

The results shown in table 4 and Figure 7 then highlight how the RNA transcript abundance of SR proteins in particular change during the ageing process. Figure 7 represents the number of reads aligned to each of the SR proteins demonstrating that there is no single overall trend in how the transcript abundance of the SR proteins change during ageing. This is perhaps to be expected given that the liver shows the most diverse expression of SR proteins (Twyffels, Gueydan, and Kruys 2011) In general there appears to be lower transcript abundance in the aged mice however SRSF4 and SRSF11 show an increase in the aged mice in both diet types. SRSF2 and SRSF6 are interesting as these genes show an increase in the 24 and 30 month mice fed an AL diet and a decrease in abundance in the 24 and 30 month mice fed a CR diet. There are clearly some significant changes in the expression of SR proteins as shown in table 4 with the majority of significant changes shown between the young mice and the 24-month-old CR mice. However, there was no significant changes when aged matched 24 month mice were compared by diet type. This can be compared to the study carried out by Holly et al where it is reported that there is a significant decrease in the expression of SRSF1, SRSF2, SRSF3 and a significant increase in the expression of SRSF5 and SRSF 11 during the ageing process however Holly et al find that only SRSF1 and SRSF6 are significantly associated with ageing (Holly et al. 2013). This suggests that SRSF1 is likely an important splicing factor for this investigation to focus on more closely.

Again, using only transcript abundance data this is difficult to interpret given the alternative splicing of the SR proteins themselves as well as the ability of the SR proteins to change function depending on cellular localisation. As described in the introduction, on a post-translational level the serine amino acids found in the SR domain may also undergo extensive phosphorylation allowing control of the sub-cellular localisation of SR proteins (Twyffels, Gueydan, and Kruys 2011; Will and Lührmann 2011). Therefore, data describing the location and phosphorylation of the SR proteins would be needed to investigate this area completely. A link may then be made between the altered transcript abundance, location and phosphorylation status allowing further investigation of the role of SR proteins within the cell.

Data describing the alternative splicing of transcripts was also available for this study so using SRSF1 as a case study the alternative splicing of SRSF1 was investigated. The results shown in Figure 8 show an image and data taken directly from a DESEQ analysis and

demonstrate a significant difference in the expression of a particular exon when RNA from a 3-month-old mouse was compared to that of a 24-month mouse consuming a CR diet. This was then investigated further using PCR and Figures 9 shows clearly the exon which was investigated. While the results of the PCR (shown in Figure 10) where analysed and do not show a significant difference this is only a semi-quantitative method and it is clear that the trend seen reiterates the results from the DEXSeq analysis which is a far more sensitive technique. It is suggested here that there is some alternative splicing of SRSF1 occurring in 3 month and 24-month-old mouse liver tissue and in all instances both isoforms are expressed. Figures 9 shows two isoforms, termed isoform 1 and isoform 2, and this relates directly to the post transcriptional controls first mentioned in the introduction this chapter (Ni et al. 2007; Lareau and Brenner 2015). Ni et al describe the inclusion of introns such as that seen in isoform 2 of SRSF1 as a method of targeting that isoform for degradation by the NMD pathway (Ni et al. 2007). The inclusion of cassette exons which are termed 'poisonous' exons as seen here in Figure 9 results in premature stop codons and is an emerging method for the control transcript abundance by the cell (Grellscheid et al. 2011). This could confirm the decrease in transcript abundance of SRSF1 seen in the 24-month CR mice as there is a greater abundance of isoform 2 and therefore more of the transcript is being destroyed via the NMD pathway. However a paper by Sun et al suggest that the inclusion of this intron (as in isoform 2) by the autoregulation of SRSF1 may not result in degradation via NMSD but results in the retention of SRSF1 in the nucleus and the protein product of this isoform is not able to shuttle to the cytoplasm (Shuying Sun, Zuo Zhang, Rahul Sinha, Rotem Karni 2010). We can see from Figure 9 that isoform 2 would result in a truncated version of SRSF1 and upon further investigation using ensemble (ww.ensemble.org) this showed that the truncated version would be missing part of the RS domains from the C terminus which allows phosphorylation and so shuttling out of the nucleus.

In summary while there is an overall decrease in the expression of RBP and an overall increase in the expression of splicing factors it becomes difficult to take real meaning from this given the diverse range of functions of these genes. There are different roles of splicing factors to consider, for example which are splicing silencers or enhancers and perhaps a more in depth analysis which divides the genes according to functions such as this would elude a clearer result or pattern. When looking more closely at the SR family of genes our analysis concludes that SRSF1 may be associated with ageing, reiterating a similar statement from holly et al (Holly et al. 2013). We can see that these changes may be associated with a change in the splicing of SRSF1 which also appears to be associated with

ageing. The result of this alternative splicing requires further investigation. Obtaining further information such as phosphorylation status and the location of the protein within the cell would further inform these investigations and contribute to gaining a greater understanding of how splicing is altered during the ageing process.

Chapter 5: Human Dermal Fibroblasts (HDF) as model for studying SRSF1 in senescence

5.1 HDF as a Model for Studying Senescence-Introduction

Human dermal fibroblasts (HDF) cells were grown in culture until replicative senescence to investigate further the role of SRSF1 in senescence (as described in materials and methods). The HDF cells used were first described in a by Pekovic et al (2001), referred to as DD1 cells throughout this research. These cells were taken from a biopsy of the foreskin of an adolescent male. Primary HDF cells in culture undergo only a limited number of population doublings before replicative senescence is reached making them a suitable model for studying cellular senescence *in vitro* (Cristofalo et al., 2004). Cellular senescence is accompanied by changes in protein expression know as senescence associated secretory phenotype (SASP) and altered cell morphology (Nelson et al. 2012) including the production of senescence associated beta galactosidase (sen- β-gal). A sen-β-gal assay was set up, as described in materials and methods to initially detect if cells were senescent after the time between population doublings began to increase. This assay is not a robust marker for cellular senescence alone as cells shown to express sen-β-gal may only be pre-senescent and are therefore able to revert back to a proliferative state (Greider 2001). To provide effective evidence that these cells have become senescent a fluorescence microscopy was used to detect the presence of phosphorylated γH2Ax foci (Nelson et al. 2012). γH2Ax is a DNA damage response protein and forms foci around damaged areas of DNA where there has been a double strand break, these foci are more frequent and persistent in the nuclei of senescent cells(Nelson et al. 2012).

Chapter 4 looked more closely at how the transcript abundance and alternative splicing of SRSF1 changes throughout the ageing process and sought to link the two together. However, one of the conclusions from this chapter was that investigating how the location of SRSF1 changes during ageing would be important in drawing an evidence based conclusion into the changing function of SRSF1 during the ageing process (if any). Therefore, senescent HDF cells were used as a model to investigate this.

5.2 HDF as a Model for Studying Senescence-Results

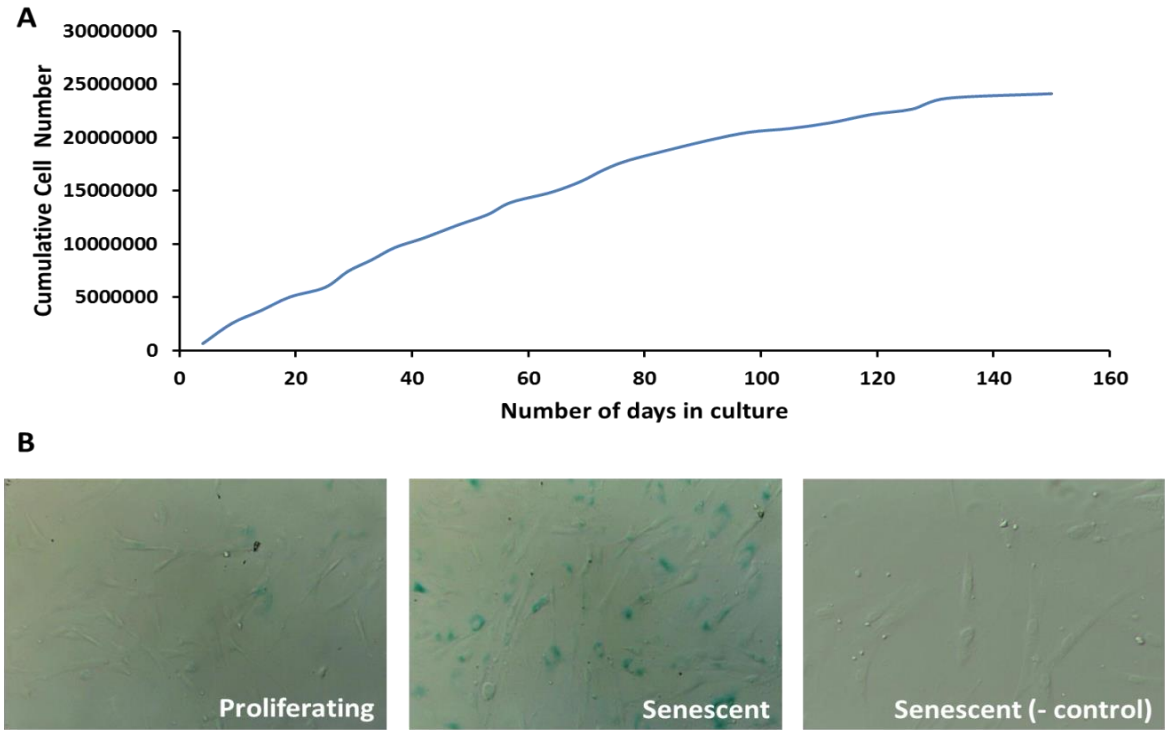


Figure 11: Evidence for senescence in HDF cells: growth curve and sen-β-gal staining. A) Population growth curve. Cells were grown in culture as described in materials and methods. Cumulative cell number was calculated after counting cells and plotted against the number of days the cells had been grown in culture. B) Shows the results of a sen-β-gal assay. Images were taken using a bright field microscope, methods were as described in the materials and methods section

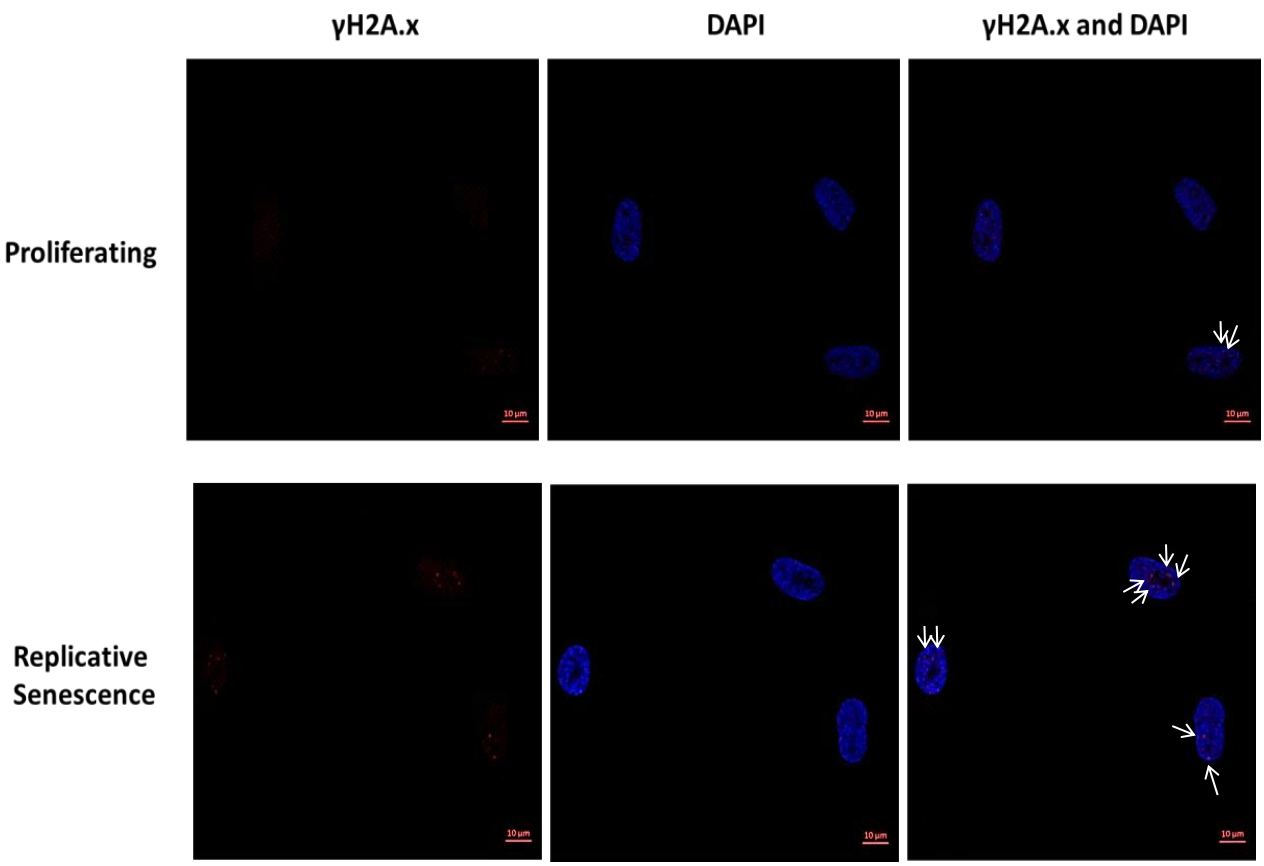


Figure 12: Evidence for senescence in HDF cells: False colour fluorescent image of HDF cells showing DNA damage foci. γH2A.x foci (red) and nuclei (blue) (as stated in methods section). Cells were taken at the following days: Proliferating-day 33, Senescent- day 132. Scale bars show 10µm. A list of primary antibodies is shown in the appendix

Table 5: Evidence for senescence in HDF cells: Quantitative analysis. Percentage of sen-β-gal positive cells and the number of cells with DNA damage foci

Percentage of Sen-β-gal positive cells		Number of cells with DNA damage foci	
Proliferating	Senescent	Proliferating	Senescent
10%	53%	1	3

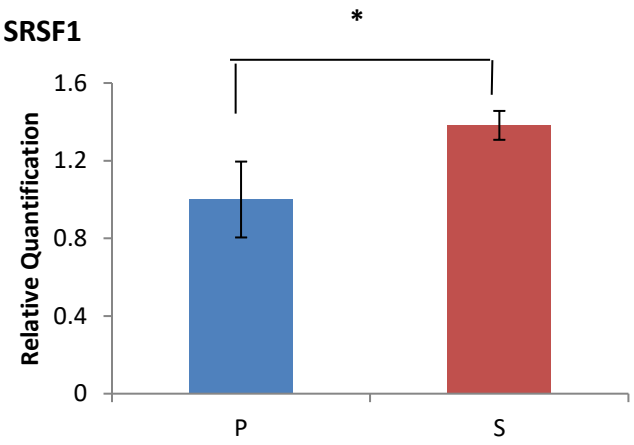


Figure 13: Transcript abundance of SRSF1 in HDF cells Relative Quantification levels of SRSF1 mRNA transcript present in HDFs as determined by qPCR when compared to a constitutively expressed gene, calculated using standard delta CT methods (see appendix for primers uses and calculation method followed). Asterisk symbol shows results which are significant different. Error bars shown indicate standard deviation. A list of primers is shown in the appendix

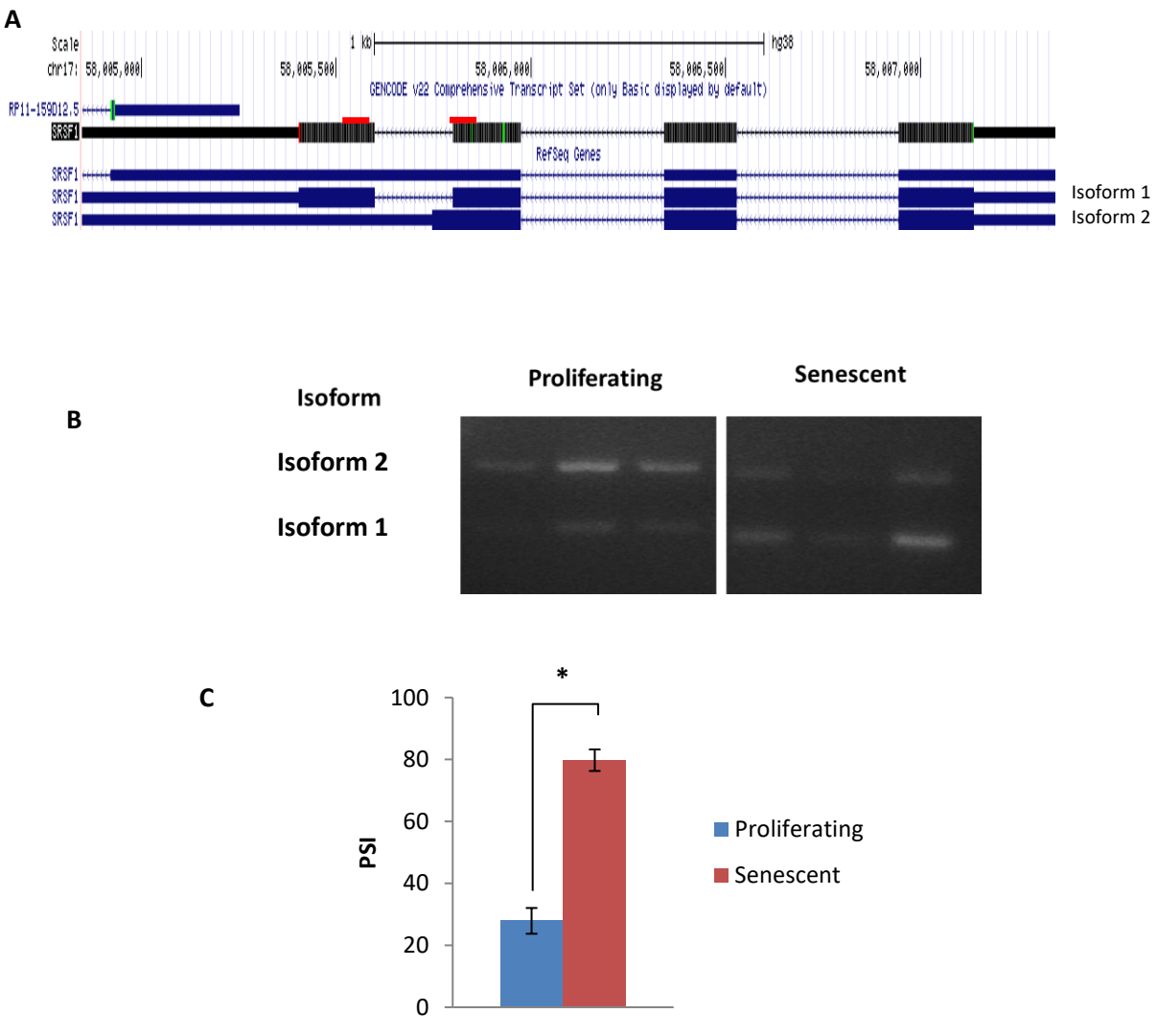


Figure 14: Alternative splicing of SRSF1 in HDF cells (A) Structure of the SRSF1 gene. Images taken from UCSC Genome Browser(https://genome.ucsc.edu/) showing the varying isoforms of the SRSF1 gene in Humans. The primer alignments are shown as the red bars at the top of the arrangement and two isoforms discussed in the chapter are highlighted (B) shows photographs of a gel showing the products of a PCR using HDF (as described in methods) under UV light using the primers shown in part A. (C) shows the PSI (percentage of the isoform 1 present) in HDF cells and was calculated using image J the larger the value of the PSI the greater ratio of isoform 1 to isoform 2. A list of primers is shown in the appendix

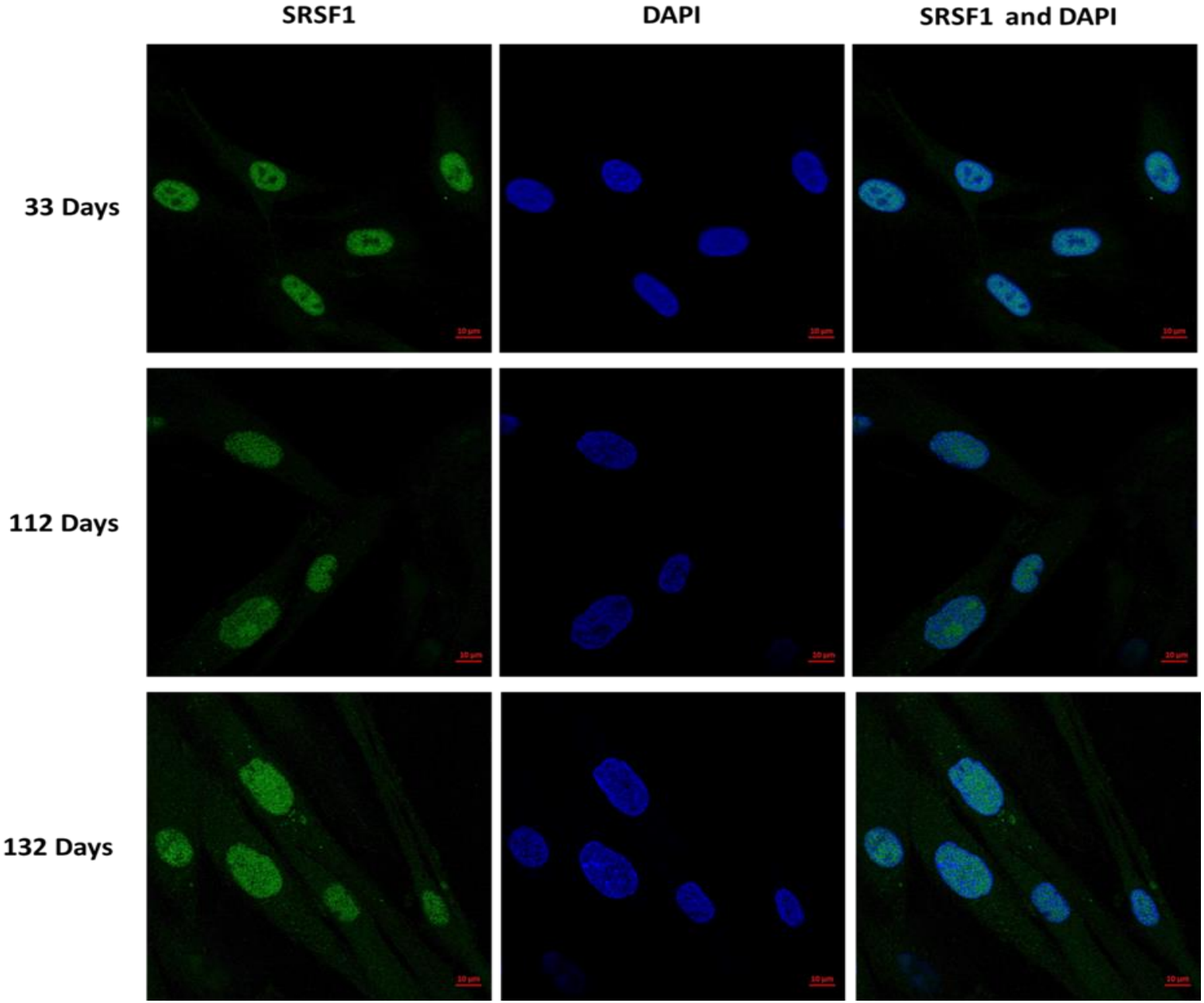


Figure 15: False colour fluorescent image of HDF cells showing location of SRSF1 during senescent and proliferating stages. SRSF1 (green) and nuclei (blue) (see methods section). Cells were fixed at the indicated time points and a population growth curve is shown in figure 11. A list of primary antibodies is shown in the appendix. No quantification was performed.

5.3 HDF as a Model for Studying Senescence- Discussion

There is a clear difference in the staining of the proliferating and senescent cells shown in Figure11 B. The Sen-β-gal assay produces a blue stain when the presence of senescence associated beta galactosidase is detected and there is clearly a larger amount of blue staining in the senescent cells both compared to the proliferating cells and the negative control. There are an increased number of γH2A.x foci seen in the cells fixed at 132 days providing further evidence that these cells may be senescent. However, Figure 11 A shows that these cells are slow growing and therefore this could have affected the results presented throughout this chapter. Normally HDF cells undergo between 3 and 4 population doublings per week and since these cells have been used successfully in previous studies (Pekovic et al. 2011) it is likely that the growth conditions for theses cells are questionable. This means that to confirm the results presented these experiements would need to be reproduced with a different set of HDF cells which demonstrate an expected growth rate.

The results show that there is increased expression of SRSF1 within HDF cells (Figure 13) which is accompanied by a change in alternative splicing (Figure 14) resulting in the exclusion of a retained intron. This is opposite to the data shown in Chapter 4 where there is a decrease in the expression of SRSF1 mRNA in 24 month mice (of both diet types) when compared to 3-month-old mice. As previously discussed the inclusion of cassette exons such as that shown in Figure 14 which are termed 'poisonous' exons results in premature stop codons and is an emerging method for the control transcript abundance by the cell (Grellscheid et al. 2011). DEXSeq data shown in Chapter 4 also suggests that in the 24-month CR mice there is a significant change in alternative splicing of SRSF1 which leads to inclusion of the retained intron whilst there was no significant change detected in the 24 month AL mice. However, the analysis shown in Figure 14 shows that there is an increase in the abundance of isoform 1 in HDF. These results while opposite in differing cell types do show consistency if we are to hypothesise that inclusion of this 'poisonous exon' in isoform 2 means there is a decrease in the transcript abundance of SRSF1 due to the degradation of SRSF1 via NMSD pathway (Grellscheid et al. 2011). The method employed to take some quantitative meaning from the gel images shown in Figure 14 must however be treated with caution. Gels are a semi-quantitative measure of transcript abundance and therefore it is not possible to take true quantitative meaning from this. It is clear from the gel images that there is a difference in the isoform expression between proliferating and senescent cells however to truly quantify this qPCR should be carried out.

Figure 15 demonstrates how the localisation of SRSF1 changes during senescence. During the proliferating state it appears that SRSF1 is located within the nucleus and at the senescent state (132 days) it appears SRSF1 may also be found in the cytoplasm. However, when the cells fixed at 112 days were stained there appear to be some localisation to certain areas of the nucleus, this may be nucleoli but further experiments using markers for the nucleoli would be needed to investigate this further. A search of the literature shows no evidence of SRSF1 previously being associated with the nucleolus. This appears to be a novel result and although this result needs to be replicated before it can be considered conclusive it is worth noting that this localisation appears to be possible and could perhaps be part of a pre-senescence mechanism. However these results are not conclusive as there has been no negative controls provided in the images above therefore this would also need to be provided as supporting evidence and is a limitation of the results presented. The possible translocation of SRSF1 from the nucleus to the cytoplasm during a senescence is shown in Figure 15. This is supported by the evidence presented in Figures 13 and 14 suggesting a mechanism whereby the increase in transcript abundance seen is linked to alternative splicing of the gene. This alternative splicing should allow a full protein product to be produced if the mRNA is to be translated by the cell and this fully functional protein product contains RS domains at the C terminus which may be able to undergo phosphorylation and translocate to the cytoplasm during senescence. To determine if this is the case, the SRSF1 proteins within the cells could be investigated further. Protein extractions were taken from the cells however it was beyond the time limits of this project to investigate this further. This would provide further supporting evidence to the data presented here. This can be used to inform our interpretation of the evidence presented by Sun et al (Shuying Sun, Zuo Zhang, Rahul Sinha, Rotem Karni 2010) which suggests that the inclusion of the 'poisonous exon' found in isoform 2 would result in retention of the gene product in the nucleus as we still do not know conclusively whether the isoform 2 is degraded by NMSD. While the evidence presented does not prove SRSF1 is found within the cytoplasm is senescent HDF cells there is evidence provided which could be investigated further. Why the pattern should be reversed in the aging mouse liver, which should contain senescent cells, is not known and again this would require further investigation into the localisation of SRSF1 in this liver tissue. Perhaps, since the liver expresses the most diverse pattern of SR proteins the role fulfilled by SRSF1 in HDF cells is made redundant by another SR protein in liver cells or in mouse cells. The use of differing cell types is a limitation of this experiment as is the slow growth rate of

the HDF cells presented here which could also suggest there was issues in the growth conditions of these cells making these results unreliable.

Chapter 6: Conclusions

The data shown in the results sections demonstrates that there is a variety of genes which show the greatest alteration in transcript abundance during the ageing process when mice are fed an AL or CR diet. The only clear trend shown in Tables 1 and 2 is the downregulation in transcript abundance of the MUP gene family which is further investigated in chapter 3. There is a clear and significant trend in the transcript abundance profile of these genes during ageing when mice where fed an AL or CR diet, and the decrease in abundance of MUPs is more severe in the CR mice. This data, alongside that of previously published data suggest that while MUPs are clearly involved in the reproductive processes of mice there may also be a role for MUPs in the regulation of lipid metabolism throughout ageing and when a CR diet is initiated (Hui et al. 2009; Giller et al. 2013). This is an interesting development and would benefit from further investigations however in order for this investigation to be conclusive and accurate an improvement in the annotation of MUP genes in bioinformatics would be necessary. Alongside of this further investigation into the role of MUP-ps19 may also yield some interesting results.

This report also sought to gain some insight into the overall trends in the expression of RNA binding proteins and splicing factors during the ageing process and while the data presented in Chapter 4 does make some way to understanding this there is no clear trend overall and perhaps this aim was too ambitious considering the variety of function and complexity of RNA binding proteins and splicing factors. However, when the SR protein family was investigated more closely this suggested SRSF1 as an age associated gene as demonstrated in previous studies (Holly et al. 2013; Harries et al. 2011). Our investigations lead to a clear proposed pathway for the regulation and function of SRSF1 where the alternative splicing of SRSF1 results the inclusion of a 'poisonous exon' possibly resulting in the degradation of SRSF1 by the NMD pathway or in a protein product lacking a functional RS domain which is therefore unable to translocate to the cytoplasm.

For future directions there is a clear need to investigate proteomics data in the mouse liver and HDF cells more closely in order to ascertain whether the isoform, termed here as isoform 2 is degraded via NSMD. Obtaining this proteomics and phosphorylation data would also be useful in investigating the SR family more closely. Some fluorescence microscopy which focused on the localisation of SRSF1 in mouse liver tissue would also be useful to inform the hypothesis proposed here regarding the alternative splicing of SRSF1. It also becomes difficult to make conclusions regarding the function of SRSF1 given the there are two

variables to take into account; the different organism which the cells originate from as well as differing tissue types. It would therefore be recommended that for future investigations into the function of SRSF1 in senescence also carry out investigations in mouse dermal fibroblasts as well as human liver cells. This was unfortunately beyond the scope of this project due to time constraints and the availability of cells.

Chapter 7: Materials and methods

7.1: Mice

The RNA taken from C57Bl/6 male mice and used in experiments described in this thesis were kept as a long-established ageing colony at Newcastle. Male mice were housed of groups of 4–6 that did not change from weaning. Mice had AL access to water CR at 60% of AL intake was initiated at 3 months of age and lasted for either 5 or 12 months. Group sizes for lifespan experiments were (censored events in brackets): C57Bl/6 AL 310 (172), C57Bl/6 CR 241 (157) and ICRFa 2391 (1,061). This work is more fully described in previous work by Miwa et al (2014) and Cameron et al (2012) including full detail of how the mice were housed. All work complied with the guiding principles for the care and use of laboratory animals and was licensed by the UK Home Office (PPL60/3864). Live tissue was extracted and RNA extracted and treated with DNAse prior to this experiment being initiated.

7.2 Computational Biology

The computational data presented in this thesis is the result of analysis carried out by David Dolan and Albert Lahat who have provided the following as an overview of the analysis carried out: Strand specific paired end libraries for RNA-seq were generated from DNAse treated total RNA using ScriptSeq, then run with and illumina 2500 sequencer to obtain 100 base pair end reads. The sequenced fastq were filtered to remove low quality reads by kraken (Davis et al,. 2015) . Reads were aligned using tophat2 (Kim et al., 2013) without multi-mapping or novel junctions. Sequence Alignment files were sorted by name (Li et al., 2009) for HTSeq-count (Anders et al., 2013) to read gene counts. Gene coding counts were normalized and compared sample by sample using DeSeq2 (Love et al., 2013).

7.3 Cell culture and media

Human Dermal fibroblasts (HDF) were obtained from the lab group of Prof. Quinlan at Durham University and were first used by Pekocvnic et al (2011) and are referenced throughout this paper at DD1 cells (Pekovic et al. 2011) these cells were used as they have a finite replicative lifespan when cultivated and this can be used as a model for senescence. HDFs where obtained from the foreskin of an adolescent male. HDF cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Sigma-D5796) supplemented with 10% Foetal Bovine Serum (FBS)(Labtech.com-FCS-SA), 5ml of Penicillin-Streptomycin (Sigma-P4333-100ML) and 5ml of L-Glutamine (Sigma-G7513). Cells were cultured at 37°C in humidified

incubators containing 5% CO. Trypsin-EDTA solution (Sigma-T3924) was used to when passaging cells after they reached 80-100% confluency and after washing with phosphate buffered saline (PBS) (Sigma: D1408).

7.4: RNA extraction

Cells were seeded in 6 well dishes at 80,000 cells per well, media was aspirated and washed when cells reached 80% confluency. 200µl of TRI Reagent (Sigma-T9424-25ML) was added to each well and the dish was vortexed vigorously until cells were completely homogenised then incubated for 5 mins at room temp. The Homogenised cells and TRI Reagent were aspirated into a separate Eppendorf tube before adding 40µl of chloroform and vortexing vigorously for 15 seconds then the tube was centrifuged at 13,000 RPM for 15 mins. The upper aqueous phase was removed to a new tube. RNA was precipitated by adding 120µl of isopropyl alcohol and incubated at room temperature for 10 mins then centrifuged at 13,000 RPM for 10 mins. The supernatant was then removed and the pellet washing with 70% ethanol before centrifuging again at 13,000 RPM and allowing the pellet to air dry. The pellet was then dissolved in 20µ of RNAse and DNAse free H_2O and stored at -20˚C.

7.5: cDNA synthesis

cDNA was synthesised using qScript cDNA Synthesis Kit (Quanta Biosciences-95047-100). RNA concentration was determined using absorbance 280nm and the correct volume of RNA to give a concentration of 1µg was used in the reaction, RNAse free H_2O was then added to a final volume of 10µl before adding 4µl of qScript reaction mix and 1µl of qScript reverse transcriptase. The tubes were placed in the thermal cycler under the following cycle: 22˚C-5mins, 42˚C-30mins, 85˚C-5 mins, 4˚C-hold. Samples were then diluted to 200µl to give a final concentration of 3ng/µl.

7.6: Primer design

All primers were designed in house spanning an exon junction where possible to eliminate amplification of genomic DNA. DNA sequence was determined using Ensembl (http://www.ensembl.org/index.html), Primer 3 (http://primer3.ut.ee/) was then used to generate primers to give products of the appropriate length (80-120bp for qPCR). These primers where then checked using the UCSC in-silico PCR tool (https://genome.ucsc.edu/). The resulting primers were then ordered from Integrated DNA Technologies (IDT (https://www.idtdna.com/site)). Once primers arrived they were diluted to a concentration of 100nM and stored at -20˚C.

7.7: PCR

PCR was carried out in order to test newly ordered primers before carrying out qPCR or to look for some cases of alternative splicing. GAPDH was used as a house keeping primer. 2µl of cDNA was added to MyTaq Red DNA Polymerase and MyTaq red buffer (both Bioline:BIO-21108) the amount of primer added varied depending on the primer but was generally 1 µl, H_2O was then added to give a final volume of 23µl before adding 2µl of cDNA. Reactions were carried out using the following cycle:

Step	Temp (˚C)	Time (seconds)	Cycles
Initial denaturation	72˚C	60	
Denaturation	72˚C	15	
Annealing	53-57˚C	15	30
Extension	95˚C	20	
Hold	4˚C	-	

7.8: qPCR

At least two separate master mixes were set up; cDNA master mix and primer master mix. Housekeeping primers of HPRT were used in human cells and GAPDH was used for mouse liver tissue.10 µl of the cDNA master mix and 5µl of the primer master mix were added to the final reaction after mixing thoroughly and each reaction was set up in triplicate. Primer master mix-0.8 of each primer to give a final concentration of 400nM, 3.4µl of Nuclease free H_2O. cDNA master mix-6ng of cDNA (3µl), 10µl of SYBR No-ROX kit (Bioline: BIO-98005), 2µl of Nuclease free H_2O. A calibrator was also used which contained no cDNA. Rotor-gene Q was used to collect data and carry out analysis including determining ct values. Reactions were carried out using the following cycle:

Step	Temp (˚C)	Time (seconds)	Cycles
Polymerase activation	95	120	
Denaturation	95	5	
Annealing	65	10	40
Extension (acquisition)	72	20	
Melt curve	55-95	N/A	

7.9: Calculating relative quantification from qPCR results

Ct=calculated using Qiagen Rotor-Gene software *(curves were checked to ensure that this was accurate*)

Δ Ct= $Ct_{\text{gene tested}} - Ct_{\text{housekeeping gene}}$

$\Delta\Delta Ct = \Delta Ct_{\text{sample 1}} - \Delta Ct_{\text{calibrator}}$

Δ Ct SD= Standard deviation *(calculated using the delta Ct values of the triplicates-values were considered valid when Delt Ct SD was less than 0.25*)

RQ= Ralative quanitification= $2^{-\Delta\Delta Ct}$

7.10: Statistical Tests:

All statistically significant results presented in this thesis were calculated using standard devition determined by the following formula:

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{N}}$$

Where σ= the standard deviation, $\bar{x}$ = the sample mean, *n*=the number of scores in the sample, Σ=the sum of

7.11: Staining

Before staining could be carried out cells were seeded onto a cover slip at 18,000 per well in a 24 well dish. After 1 day cells were washed twice in PBS (Sigma: D1408) and fixed by incubating in 4% Paraformaldehyde (PFA) for 10 mins. Fixed cells were stored in PBS at 4˚C.

7.11.1: Senescence-associated β galactosidase (Sen-β-gal) assay

A staining solution was made using 8ml of Sen-β-gal solution (1.5mL 1M NaCl, 20µL 1M MgCl2, 800µL 0.5M citric acid, 1.2mL 0.1M sodium phosphate, 6.48mL distilled H2O-pH 6.0) 0.2ml of X-gal and 1.0ml of Iron bru solution (PBS-Mg solution containing 50nM Potassium Ferrocyanide, 50nM Ferricyanide). 1ml was added to each well and the cover slips were incubated in a dry incubator for 16 hours. Cover slips were mounted on a glass slide using a single drop of mounting media (Vectashield: H-100) and imaged using a bright field microscope.

7.8.2: Fluorescence microscopy

Cover slips were incubated in 0.5% Triton X-100 for 5 mins then washed twice in PBS before being incubated in blocking solution (1% new born calf serum in PBS). Cells were washed twice in PBS then incubated in 50µl of primary antibody for 1 hour at room temperature or overnight at 4˚C in a moist staining chamber. Cells were then washed 5 times in PBS before being incubated in 50µ of secondary antibody for 1 hour at room temperature, 50 minutes into this incubation 50µl of DAPI was added again carried out in a moist staining chamber. All antibody dilutions were made using blocking solution and at a concentration suggested by the supplier then adjusted according to image quality if necessary (see antibody table). Cover slips were then washed 5 times in PBS and mounted on a glass slide using a single drop of mounting media (Vectashield:H-100).

Chapter 8: Appendix

8.1 Primers used

Gene	Forwards	Reverse	Figure
Mouse Housekeeping (GAPDH)	ATGGTGAAGGTCGGTGTGAA	ATGAAGGGGTCGTTGATGGC	12
Human Housekeeping (HPRT)	AAGCTTGCTGGTGAAAAGGAC	AAGCTTGCTGGTGAAAAGGAC	9 and 10
Mouse SRSF1	CACTGGTGTCGTGGAGTTTG	GCCCATCAACTTTAACCCGG	9 and 10
Human SRSF1	CACTGGTGTCGTGGAGTTTG	GCCCATCAACTTTAACCCGG	12
Human SRSF1 (alt splicing)	CACTGGTGTCGTGGAGTTTG	GCCCATCAACTTTAACCCGG	13

8.2Primary Antibodies Used

Protein	Host	Supplier	Dilution
γH2A.x	Mouse	Millipore	1 in 200
SRSF1	Mouse	Thermo Fisher	1 in 50

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