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Characterisation of the Splicing Factor, Proline- and Glutamine-Rich (SFPQ)-RNA interactome in melanoma and how it impacts the cancer phenotype

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Abstract

Title: Characterisation of the Splicing Factor, Proline- and Glutamine-Rich (SFPQ)-RNA interactome in melanoma and how it impacts the cancer phenotype

Key words: SFPQ, Malignant melanoma (MM), long non-coding RNA (lncRNA)

There is emerging evidence linking aberrant expression of some long non-coding RNAs (lncRNAs) to cancer aetiology. Malignant melanoma (MM) is a lethal skin neoplasm, with the fastest rising incidence of all tumours and 5-year survival rates below 10%. Recently researchers have identified a role for the RNA-binding protein, Splicing Factor, Proline- and Glutamine-Rich (SFPQ) in several cancers, often via modulation of and interaction with long non-coding RNAs. The aim of this doctoral research project was to investigate (i) differentially expressed lncRNAs in melanoma and PMs (ii) identify novel SFPQ-lncRNA interactors via RIP-sequencing (seq) and determine the functional significance of these transcripts in melanoma (iii) establish if SFPQ contributes to the cancer phenotype in melanoma. RIP-seq uncovered a multitude of transcripts, including lncRNA, which specifically interacted with SFPQ in melanoma versus primary melanocytes (PM). Knockdown of these novel transcripts (*LINC01234* and *LINC00511*) led to a decrease in cell migration and proliferation in melanoma cells. Additionally, depletion of SFPQ in melanoma cells, resulted in a reduction in cell migration, proliferation, metabolism, and an increase in apoptosis. Furthermore, clinical data suggest a direct link between increased expression of SFPQ and poor patient survival, suggesting that SFPQ may have some utility as a prognostic biomarker in melanoma.

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Abbreviations

Acral lentiginous melanoma (ALM)

Adenosine De-Aminase RNA-specific (ADAR)

Anaplastic large-cell lymphomas (ALCLs)

Androgen receptor (AR)

Antisense of the *INK4* locus (*ANRIL*)

Brain-specific homeobox protein 3a (Brn3a)

Castration-resistant prostate cancer (CRPC)

Circular RNAs (circRNAs)

C-Jun N-terminal kinase (JNK)

Colorectal cancer (CRC)

C-reactive protein (CRP)

C-Terminal domain (CTD)

Cyclobutane pyrimidine dimers (CPDs)

Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4)

Death Effector Domain (DED)

Dihydroxyphenylalanine (DOPA)

DNA- and RNA-binding proteins (DRBP)

DOPAchrome tautomerase (DCT)

Double-strand breaks (DSBs)

Drosophila behaviour splicing (DBHS)

Electrochemotherapy (ECT)

Embryonic stem cells (ESCs)

Enhancer RNAs (eRNAs)

Extracellular signal–regulated kinases 1/2 (ERK1/2)

Fas-associated death domain (FADD)

Fibroblast activation protein-*α* (FAP-*α*)

Fibroblast growth factor (FBF)

Glucocorticoid receptor (GR),

Glycogen synthase kinase 3 (GSK3)

Growth differentiation factor 15 (*GDF15*)

Growth hormones (GHs)

Hepatitis *delta* virus (HDV)

Hepatocellular carcinoma (HCC),

Imprinting control regions (ICR)

Insulin-like growth factor 2 (*IGF2*)

Insulin-like growth factor-I (IGF-I)

Internal ribosome entry site (IRES)

Intraluminal vesicles (ILVs)

KIT proto-oncogene receptor tyrosine kinase (*KIT*)

Lactate dehydrogenase (LDH)

lentigo maligna melanoma (LMM)

Long non-coding RNAs (lncRNAs)

Lysine (K)-specific demethylase 1A (LSD1)

Malignant Melanoma (MM)

Melanocortin 1 receptor (MC1R)

Messenger RNAs (mRNA)

Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1)*

Micro RNAs (miRNAs)

Microphthalmia-associated transcription factor (MITF)

Mitogen activated protein kinase (MAPK)

Neural Crest (NC)

Nodular melanoma (NMM)

NONA/Paraspeckle (NOPS)

Non-coding RNA (ncRNAs)

Non-homologous end joining (NHEJ)

Nuclear enriched abundant transcript 1 (*NEAT1*)

Nucleotide excision repair (NER)

Oculocutaneous albinism (OCA)

Oncogene-induced senescence (OIS)

Open reading frames (ORFs)

Oral squamous cell carcinoma (OSCC)

Oaired box gene 3 (PAX3)

Peroxisome proliferator-activated receptors (PPARs)

Phosphatase and tensin homolog (*PTEN*)

Piwi-interacting RNA (piRNAs)

Polycomb-repressive complex 2 (PRC2)

Post-translational modifications (PTMs)

Programmed cell death (PD)-1 protein and one of its ligands, PD-L1

Prostate Cancer gene 3 (*PCA3*)

Prostate Specific Antigen (PSA)

PSPC1 (paraspeckle protein component 1)

PTB-associated splicing factor (PSF)

Red hair colour (RHC)

Replicative lifespan telomerase reverse transcriptase (*TERT*)

Retinoblastoma protein (RB)

Ribonucleoprotein (RNP)

RNA component of telomerase (*TERC*)

RNA-recognition motifs (RRM1 and RRM2)

SCF (stem cell factor)

Signal recognition particle (*SRP*) RNA

Small cajal body-specific RNA (scaRNA)

Small interfering RNA (siRNA)

Small nucleolar RNA (snoRNA)

Splicing factor proline-glutamine rich (SFPQ)

Splicing small nuclear RNA (snRNA)

Sprouty4-intronic transcript 1 (*SPRY4 IT1*)

SRA-like non-coding RNA1 (*SLNCR1*)

SRY (sex-determining region Y)

Superficial spreading melanoma (SSM)

The American Joint Committee on Cancer (AJCC)

The androgen-responsive lncRNA *CTBP1*-AS

The maternally expressed 3 lncRNA (*MEG3*)

The α-Melanocyte-stimulating hormone α-MSH receptor 1 gene (*MC1R*),

The *X-chromosome inactivation* (*XIST)*

TNF receptor-associated death domain (TRADD)

Transcriptional start sites (TSS)

Transfer RNAs (tRNAs)

Transforming growth factor β (TGFβ)

Translation ribosomal RNA (rRNA)

tumour necrosis factor (TNF)

Tumour protein p53 (*TP53*)

Tumour suppressor (TS)

Tyrosinase (TYR)

Ultraviolet radiation (UVR)

Chapter 1: Introduction

1.1 Background

Malignant Melanoma (MM) is one of the most aggressive, complicated, and heterogeneous cancers (Andor et al., 2016), with the fastest rising incidence of all tumours and 5-year survival rates below ten percent (Weyers, 2012). MM arises due to transformation of melanocytes via distinct aberrations arising in multiple signalling and cellular processes, such as cell cycle regulation, cell signalling, cell adhesion, differentiation and apoptosis (Hill, Gartner, Samuels, & Goldstein, 2013). Such heterogeneity suggests that multiple mechanisms participate in disease aetiology and this is reflected in the contribution of both different mutations and differential gene and protein expression associated with MM development and progression (Wangari-Talbot & Chen, 2013). Treatment of melanoma at early stages is important and usually includes surgical excision followed by adjuvant chemotherapy, however, some patients relapse and develop resistance. Although positive response rates have been observed with targeted immunotherapy, unfortunately, some individuals develop resistance (Flaherty & McArthur, 2010; Ribas et al., 2016; Ugurel et al., 2016; Wagle et al., 2011). Thus, there remains an urgent need to better our understanding of the underlying molecular mechanisms of specific genes contributing to the development and progression of the disease (Schmitt & Chang, 2016).

There is emerging evidence linking aberrant expression of some long non-coding RNAs (lncRNAs) to cancer. (Balas & Johnson, 2018; M.-C. Jiang, Ni, Cui, Wang, & Zhuo, 2019; Schmitt & Chang, 2016). Specifically in melanoma, various lncRNA have been reported to be deregulated and implicated in pathogenesis (Hulstaert, Brochez, Volders, Vandesompele, & Mestdagh, 2017; Ledong Sun et al., 2019). These lncRNA exert their function via different pathways, where they interact with various molecular targets and promote MM progression (Hulstaert et al., 2017). The RNA-binding

protein, Splicing Factor, Proline- and Glutamine-Rich (SFPQ) is also involved in the development of various cancers (I. W. Chang, Huang, & Sung, 2009; Knott, Bond, & Fox, 2016; K.-I. Takayama et al., 2017a). Interestingly, SFPQ often appears to mediate is function via interaction with lncRNAs (de Silva, Lin, Phillips, Martin, & Baxter, 2019a; Imamura et al., 2014; Q. Ji et al., 2014). This includes MM, where researchers reported that the SFPQ-binding lncRNA, *LLME23*, which is exclusively expressed in melanoma cells, modulates the ability of SFPQ to repress proto-oncogene expression (C. F. Wu, G. H. Tan, C. C. Ma, & L. Li, 2013). The aim of my doctoral research was to investigate the role of SFPQ binding to lncRNA in MM with a view to better understanding how this contributes to MM phenotype.

1.1.2 Melanocyte lineage

The Neural Crest (NC) was discovered by Wilhelm His during the 19th Century, characterised as a band of migratory cells arising between the neural tube and ectoderm (X. Huang & Saint-Jeannet, 2004). Unique to vertebrates, the NC is a transient population of cells that arise from the dorsal neural tube early during embryonic development. NC cells migrate to specific sites in the embryo and differentiate to form bone, adipose tissues, endocrine cells, and various types of neurones and pigment cells (Le Douarin and Kalcheim, 1999).

Melanoblasts are undifferentiated, unpigmented cells which migrate from the NC to the dermis and hair follicles, where they differentiate and synthesise melanin (Tolleson, 2005). Melanogenesis is a multistep process which occurs in membrane bound organelles called melanosomes (J. Y. Lin & Fisher, 2007). Melanosomes are transferred via dendrites to their neighbouring keratinocytes. This process is initiated and controlled by keratinocyte-derived factors (Hirobe, 2005; Lo Cicero et al., 2015). Mature melanocytes interact with microtubules which in turn undergo bi-directional

actin-dependent transport from the perinuclear area towards dendrites (T. Kondo & Hearing, 2011; Park, Kosmadaki, Yaar, & Gilchrest, 2009). While the exact delivery mechanism remains to be elucidated, various mechanisms have been proposed, such as heterophagocytosis of melanocyte dendrites by keratinocytes, exocytosis of the melanin core with subsequent endocytosis by keratinocytes, transfer by nanotubes or by melanocyte filopodia, and direct fusion with the keratinocyte membrane (S. K. Singh et al., 2008; Tarafder et al., 2014; Van Den Bossche, Naeyaert, & Lambert, 2006). Differences in pigmentation arise due to variations in size, number, and distribution of melanosomes. Maturation of melanosomes occurs in four stages as shown in Figure 1.1. Pre-melanosomes resemble the vacuolar domains of early endosomes, with intraluminal vesicles (ILVs) containing fibrils of the pigment cell-specific protein PMEL17, which is responsible for melanin polymerisation (Fowler et al. 2006) and the shape of the melanosome (Theos et al. 2006; Leonhardt et al. 2011). During stage II, melanosomes develop an organised, structured fibrillar matrix formed by glycoproteins such as PMEL17 and MART-1, alongside Tyrosinase and other enzymes of melanogenesis. Melanin production takes place during Stage III, where PMEL17 forms a fibrillar matrix before melanin pigments are deposited, as they are synthesized during later stages of melanosome development (Berson, Harper, Tenza, Raposo, & Marks, 2001; Theos, Truschel, Raposo, & Marks, 2005). During the last step, the melanosomes are filled with melanin so that the internal structure is masked (stage IV melanosomes), giving an intense dark colour (Leonhardt et al. 2011).

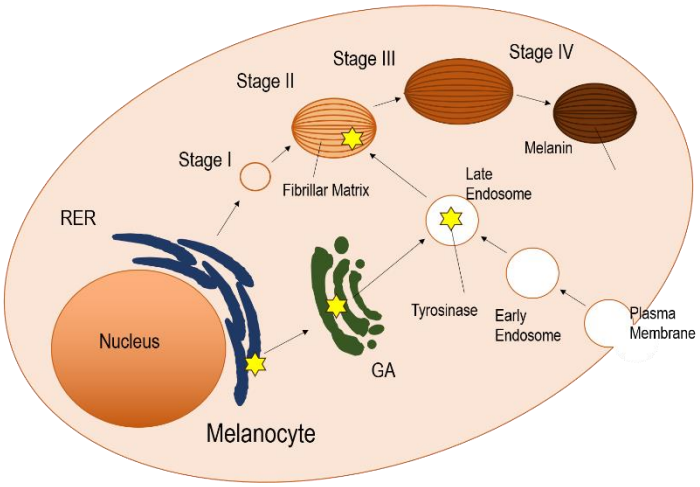


Figure 1.1 Developmental stages of melanosomes during melanin synthesis.

1.1.3 Melanogenesis

The first step in melanin biosynthesis is catalysed by the enzyme tyrosinase (TYR), which converts tyrosine to dihydroxyphenylalanine (DOPA), which is consequently is converted to DOPAquinone. In the presence of cysteine, stoichiometrically reaction occurs with nascent DOPAquinone to yield 3- or 5-cysteinyl DOPAs, which is then oxidised and polymerised, producing a yellow-red soluble melanin known as pheomelanin, endowed with poor photoprotective properties (Hennessy et al., 2005). As cysteine is depleted, surplus DOPAquinone spontaneously cyclises to synthesise an orange intermediate known as DOPAchrome. As a result, the carboxylic acid of DOPAchrome is lost, generating 5,6-dihydroxyindole (DHI), which oxidises and polymerises to produce the dark brown/black melanin which displays photoprotective features. Additionally, if DOPAchrome tautomerase (DCT) is available, DOPAchrome will tautomerise without losing its carboxylic acid group to form DHI-2-carboxylic, which can oxidise and polymerise to make a third type of melanin, known as DHICA-melanin, which is a lighter brown colour (Ito & Ifpcs, 2003).

Human skin comprises a mixtures of all three types of melanin, the ratio of melanosomes determines visible phenotype (Wakamatsu et al., 2006). During embryogenesis, melanocyte migration and survival is dependent on a large number of genes (>25), including pathways such as Wingless signalling (Wnt)/-catenin, the endothelin B receptor and its ligand endothelin-3, the receptor tyrosine kinase KIT and its ligand KIT-ligand/SCF (stem cell factor), NOTCH (Osawa et al., 2005; Schouwey et al., 2007) and transcription factor's activity, such as paired box gene 3 (PAX3), SRY (sex-determining region Y)-box10 (SOX10), hairy/enhancer of split (HES1), and microphthalmia-associated transcription factor (*MITF*) (Aoki et al., 2003; Lang et al., 2005; Shibahara et al., 2000). In humans, mutations in the genes KIT, PAX3, SOX10, and MITF lead to pigmentary diseases, such as Piebaldism, Waardenburg, or Tietz syndromes, characterized by patchy depigmentation. Patients suffering from the Waardenburg or Tietz syndromes are also defined by profound deafness (J. Y. Lin & Fisher, 2007; Wakamatsu et al., 2006). The receptor tyrosine kinase KIT is required for the development and survival of melanocytes in vertebrates via the PI3K/AKT and the RAS/RAF/MEK/ERK pathways (Wehrle-Haller, 2003). Mutations in the KIT gene have been found in ~30% of mucosal, 20% of acral, and 20% of melanomas arising in chronically sun-damaged skin (Beadling et al., 2008; Curtin, Busam, Pinkel, & Bastian, 2006; Handolias et al., 2010). Furthermore, *SOX10* is an important transcription factor during the embryonic development of melanocytes. *SOX10* controls multipotency, survival, and proliferation of neural crest cells, and differentiation into peripheral glial cells and pigment cells (Harris, Baxter, Loftus, & Pavan, 2010). In mice models, homozygous knockdown of SOX10 resulted in mice embryonic lethality and SOX10 haploinsuffiency resulted in pigmentation defects (Herbarth et al., 1998). Upon differentiation into the melanocyte lineage, *SOX10* stimulates dopachrome

tautomerase and tyrosinase and *MITF*. *SOX10* is vital for melanoma cell survival and proliferation as it regulates *MITF*, which is responsible for melanocytes development (L. A. Garraway et al., 2005). *SOX10's* regulation of *MITF* has consequences for melanomagenesis, as *MITF* mutations have been identified in a subset of melanomas (Cronin et al., 2009).

1.1.4 Melanoma

MM is a severely aggressive type of skin cancer due to its rapid progression and capacity to metastasize to distant organs and regional lymph nodes. Melanoma contributes to around four percent of all skin cancers, yet it is the major cause of mortality in eighty percent of skin cancer patients (Miller and Mihm 2006; MacKie et al. 2009), with a 5-year survival rate of ten to fifteen percent once the tumour has disseminated to distant tissues (Siegel, Miller, & Jemal, 2016). Over the last few decades, the incidence of melanoma has increased rapidly worldwide within Caucasian populations, almost tripling within the last twenty years; mainly effecting fair skinned individuals who are excessively exposed to sunlight and frequent sun beds users (Colantonio, Bracken, & Beecker, 2014; Gallagher, Spinelli, & Lee, 2005; Suppa & Gandini, 2019). Melanoma shows distinct geographic distribution, with an increase in melanoma incidence from fifteen to twenty-two people per 100,000 population per year. (Cormier et al., 2006; Siegel, Miller, & Jemal, 2018). Melanoma occurs frequently in Australia as the third predominant cancer in men and women and cause of mortality in young adults. Furthermore, males are at a greater risk compared to females, except for women over thirty-nine years who appear to be at higher risk than young males (Indini et al., 2018; Reed et al., 2012; Robsahm, Bergva, Hestvik, & Moller, 2013). Indeed, women appear to have a survival advantage over males, possibly due to more robust UV protection practices and a higher likelihood of visiting doctors, however, this

does not fully explain why sex remains an independent prognostic factor for the disease (Balch et al., 2009).The pathogenesis of MM is a complex interaction between genetic, environmental (UV radiation exposure) and phenotypic factors (fair phototypes, multiple nevi, positive family history for melanoma). Many cases of MM are diagnosed at an early stage and are curable via surgical excision, with an overall five year survival rate of ninety-two percent (Drzewiecki, Ladefoged, & Christensen, 1980; Torre et al., 2015). Treatment during the early stages is crucial due to poor prognosis in late stage patients. Blocking the interaction between the programmed cell death (PD)-1 protein and one of its ligands, PD-L1, has demonstrated a high rate of anti-melanoma response and has shown exceptional clinical benefits. Pembrolizumab and nivolumab were the first anti-PD-1 checkpoint inhibitors to gain approval from FDA. Nivolumab has been linked with increased overall survival compared with dacarbazine in patients (C. Robert, Long, et al., 2015; Topalian et al., 2014; Wolchok et al., 2013). Despite recent advances, there remains an urgent need for novel, effective therapeutic treatments, due to patients developing resistance. To this end, understanding the factors and molecular mechanisms involved in the invasive phenotype and disease progression remains an important area of melanoma research.

1.1.5 Classification of melanoma

Melanoma is generally classified based on the relationship between the level of sun exposure and the primary tumour site, or an evaluation of the tumour growth pattern (Curtin et al. 2005; Balch et al. 2009). According to the second criterion, four histological types have been outlined: superficial spreading melanoma (SSM), nodular melanoma (NMM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM) (Figure 1.2) (Balch et al. 2009).

SSM is the most common type of melanoma and accounts for seventy percent of cases. SSM is related to sporadic sun exposure and typically found around the back of the legs of women and on the backs of men. arising *de novo* or in association with a nevus (Markovic et al. 2007). Clinically, SSM are presented by various colours including tan, brown, grey, black, violaceus, pink and rarely blue or white. The lesion outline is sharp and marginated, with one or more irregular peninsula-like protrusions. The surface may have a palpable papule or a nodule that extends several millimetres above the skin surface.

Around five percent of melanomas are NMM, which develop on the trunk and limbs during the fifth or sixth decade of life, largely present in males compared to females. NMM are ulcerated and lack a radial growth phase (Markovic et al. 2007). Clinically, NMM appears as a smoothly surfaced nodule, an ulcerated polyp or as an elevated plaque with irregular outlines, usually brown, black, or blue-black in colour. In around fifty percent of cases, NMM is achromic and associated with intermittent exposure to the UV. Furthermore, NMM has been shown to invade the dermal layer, with small aggregates of cancer cells forming the overall tumour nodule (Duncan 2009).

LMM represents around four to fifteen percent of melanomas, which appear around the neck and head. While both NMM and SSM are associated with long-term UV exposure and old age, LMM is not. LMM can emerge for decades before invading into the papillary dermis (Viros et al. 2008). Furthermore, LMM are characterised by many colours such as black, brown or brown on a tan background, with irregular outlines and are classed as invasive due to papule appearance regardless of tumour size. Finally, the least prevalent type of melanoma is ALM, which comprises just five percent of melanomas in Caucasians. However, ALM is more common among Asians, accounting for fifty-eight percent of cases, and around sixty to seventy percent of

cases in African patients (J. W. Chang et al., 2004; Hudson & Krige, 1995). ALM is generally localised on glabrous skin and adjacent skin of digits, palms and soles (Markovic et al., 2007).

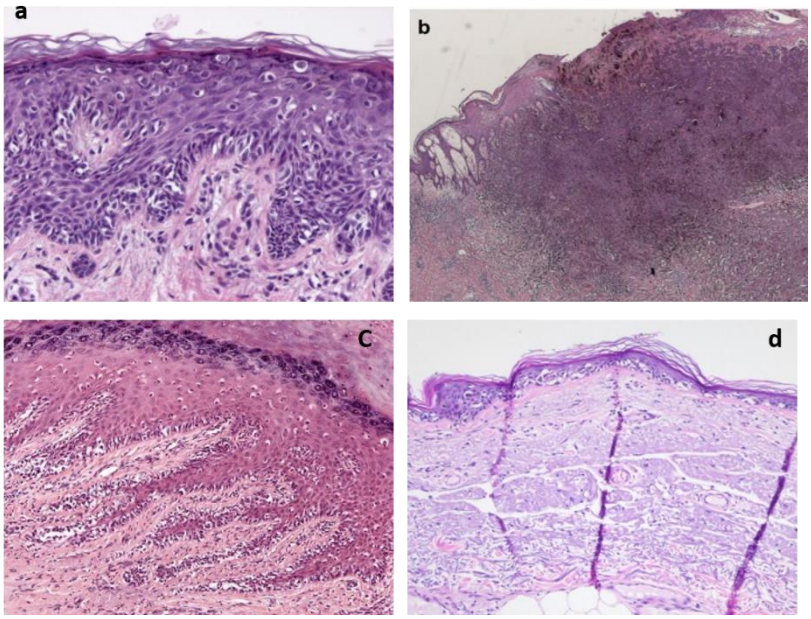


Figure 1.2 Classification of melanoma histology. (a) SSM, (b) NMM, (c) LMM and (d) ALM (Scolyer, Long, & Thompson, 2011).

1.2 Environmental Risk factors contributing to melanoma

Epidemiologic studies have found around sixty to seventy percent of melanomas are caused by the transformation of melanocytes, a result of excessive sunlight exposure and sunburn during childhood and early adolescence years (Arnold et al., 2018; Cust et al., 2011; Parkin, Mesher, & Sasieni, 2011). Ultraviolet radiation (UVR) is divided into 3 bands: UVA (wavelengths 320-400nm), UVB (wavelengths 290-320nm) and UVC (200-280nm). UVA is much more abundant than UVB in sunlight and accounts for around ninety five percent of solar UV radiation (van Weelden, de Gruijl, van der Putte, Toonstra, & van der Leun, 1988). UVA is predominately utilised in tanning beds,

and tanning beds can reach UVA doses twelve times that of the Sun. While UVA penetrates more deeply into the dermis than UVB, it is less genotoxic (Bruls, Slaper, van der Leun, & Berrens, 1984; de Gruijl, 2002; van Weelden et al., 1988). This is because UVB damages DNA directly in the form of photoproducts, such as cyclobutane pyrimidine dimers (CPDs) and 6−4 photoproducts (6-4PPs) (Shah & He, 2015). The nucleotide excision repair (NER) pathway recognises and repairs CPDs and 6-4PPs and contains proteins which recognise DNA damage, such as XPC, DDB1, DDB2, and XPA, which bind DNA damage sites and stimulate the repair response (Shah & He, 2015). Aberrations in NER pathways are linked to skin carcinogenesis and result in Xeroderma pigmentosum, a condition which increases the risk of skin cancer by 1000-fold (Bradford et al., 2011). Newer strategies are now available that aim to help repair damaged DNA thereby reducing the incidence of melanoma. In 2015, the Nobel Prize in chemistry was awarded to Aziz Sancar (University of North Carolina) for his work in determining the mechanisms of photolyase in DNA damage repair (Sancar, 2016). Photolyase belongs to a class of flavoproteins, repairing DNA photoproducts as a result of UVB exposure (Sancar, 2003; Todo et al., 1993). There are two types of photolyases; CPD photolyase and (6–4) photolyase, based on the class of photoproducts they repair (Sancar, 2003). Despite similarity in their structure, both have specialised functions against one or the other type of photoproducts they repair (Z. Liu, Wang, & Zhong, 2015). Humans don't naturally produce photolyase, they have the NER repair pathway, which is inefficient in repairing CPDs, with decreased activity with age (Bohr, Smith, Okumoto, & Hanawalt, 1985; Hoeijmakers, 2001; Petruseva, Evdokimov, & Lavrik, 2014). Therefore, it is interesting to incorporate photolyase which can repair UV-induced DNA

damage into a sunscreen, adding an exciting new facet to the strategic approach in protecting against UV damage.

Both UVB and UVA have been shown to stimulate melanoma growth in mice. UVB radiation promotes MM, while perinatal UVA exposure was not enough to trigger melanoma progression (De Fabo, Noonan, Fears, & Merlino, 2004). UVR promotes MM by directly altering DNA, promoting the cellular constituents of the skin to increase growth factors, reducing the skins immune response and triggers reactive oxygen species production (Meyskens, Farmer, & Anton-Culver, 2004). An exclusive feature of the melanocyte is the production of melanin, a molecule with a range of complicated redox radical free properties and the ability to interact with oxygen. Data suggests that during melanoma pathogenesis, melanin acts as an antioxidant via oxidation of reactive oxygen species generated by UVA, normal metabolic processes, or inflammatory responses, and the pro-oxidant quinone-imine content is increased (Sarna, Duleba, Korytowski, & Swartz, 1980). High levels of oxidative stress lead to reduced regulation of melanosomes (Rhodes, Seki, Fitzpatrick, & Stern, 1988), which in turn alters melanocyte biology to favour cell growth and migration in addition to negatively regulating apoptosis (Satyamoorthy & Herlyn, 2002).

Interestingly, data from a recent study which deeply profiled the UVR induced melanoma proteome found that UVB activates melanocytes via various growth factors, such as stem cell factor (SCF), fibroblast growth factor (FBF) and increased expression of transforming growth factor β (TGFβ). Resulting in activation of the Ras/RAF/MEK/ERK pathway and transcription of an array of genes implicated in cell proliferation and migration (Berking et al., 2004; Konstantakou et al., 2018) . Similarly, in normal human melanocytes UVR initiates TGF-*β*1 production, followed by high

levels of fibroblast activation protein-*α* (FAP-*α*), which is a serine protease expressed on the surface of activated fibroblasts. FAP-*α* has both collagenase and protease activity and occupies a critical role in the modification and degradation of the extracellular matrix (ECM) (Huber et al., 2003; Scanlan et al., 1994). One study found a link between UVR and FAP-α expression in fibroblasts, melanocytes and melanoma cells, suggesting that UVR of MM changes fibroblasts into FAP-α expressing and ECM degrading fibroblasts thereby facilitating invasion and migration (Wäster, Rosdahl, Gilmore, & Seifert, 2011). Furthermore, in zebrafish embryos, the team observed that FAP-*α* degraded the ECM thus, promoting migration and invasion (Waster et al., 2017).

Both studies highlight UVR as an initiation factor in promoting migration and invasion in melanoma, thus emphasising the importance of UVR protection to prevent the dissemination and invasion of melanoma. ECM degrading enzymes, including matrix metalloproteinases and cathepsins have received substantial attention in terms of therapy. Unfortunately, these have proven difficult due to their widespread expression in tumour as well as normal tissues (Matarrese et al., 2010; M. Yin et al., 2012). In contrast, FAP-α may serve as an attractive target due to its tumour-specific expression (Waster et al., 2017).

While UVR is a major driver of MM, the disease is also characterised by a diverse range of genetic aberrations. These include oncogenic change: (i) genetic aberrations (such as gene mutations, deletions, amplifications, or translocations) (Bauer et al., 2011; Fargnoli, Gandini, Peris, Maisonneuve, & Raimondi, 2010; Gerstenblith, Goldstein, Fargnoli, Peris, & Landi, 2007; Kennedy et al., 2001; Pasquali et al., 2015; van der Rhee et al., 2011) and (ii) epigenetic changes (DNA methylation or chromatin

defects) (L. Gao et al., 2013; Hodis et al., 2012; Moran, Silva, Perry, & Gallagher, 2018; Sarkar, Leung, Baguley, Finlay, & Askarian-Amiri, 2015; Spugnardi, Tommasi, Dammann, Pfeifer, & Hoon, 2003). A better understanding of the underlying genetic factors predisposing to melanoma progression is required to develop targeted therapeutics.

1.3 Genetic components of Melanoma

1.3.1 Genes with High Penetrance

At the molecular level, melanoma comprises a heterogeneous group of disorders. Genetic, phenotypic, and environmental risk factors all contribute to melanoma predisposition. The main changes underlying the genetic basis of melanoma occur as spontaneous acquired mutations within melanocytes.

Aberrations in driver genes disrupt vital cellular processes, such as cell signalling, cell cycle regulation, apoptosis, cell adhesion and cell differentiation (Lomas, Martin-Duque, Pons, & Quintanilla, 2008). A stepwise progression of mutations transforms a normal melanocyte into a primary and metastatic melanoma (Bennett, 2003; A. J. Miller & Mihm, 2006). This transition is a result of activated/modified oncogenes and inactivation of tumour-suppressor genes (TSG) (Ko & Fisher, 2011). Identifying cancer predisposing genes is vital to provide early diagnosis for patients. Genes that predispose melanoma are grouped into high and low penetrance genes (see table 1.1). The genes CDKN2A and CDK4 are involved in cell cycle regulation and senescence, thus controlling cell proliferation and tumour suppression (Jones et al., 2007; Serrano, Hannon, & Beach, 1993). Familial melanoma is prevalent within a family which have several atypical nevi. In these families, the pattern of heritability is

consistent with an autosomal dominant inheritance with incomplete penetrance. The genes CDKN2A and CDK4 have been implicated in familial melanoma and account for about eight to 12 percent of all cutaneous melanoma cases (Gandini et al., 2005; Hemminki, Zhang, & Czene, 2003; M. Rossi et al., 2019; Tsao, Chin, Garraway, & Fisher, 2012).

Table 1.1 Melanoma predisposition high and low penetrance genes

Gene Penetrance	Gene	Encoded Protein	Function	Prevalence of mutation	Reference
High-Penetrance	*CDKN2A*	p16INK4a	Regulates cell cycle	~20%-40% of Families	(Goldstein et al., 2006; Hussussian et al., 1994; Kamb et al., 1994)
		p14ARF	Regulates cell cycle	~1% of families	(Bahuau et al., 1998; Pedace et al., 2011; C. Pellegrini et al., 2017)
	CDK4	CDK4C	Regulates cell cycle	17 families	(Fargnoli et al., 2010; Goldstein et al., 2007)
	POT1	POT1	Maintaining Telomere	14 families	(Robles-Espinoza et al., 2014; J. Shi et al., 2014)
	TERT	Catalytic subunit of telomerase	Telomere lengthening	2 families	(Harland et al., 2016; Horn et al., 2013)
Low-penetrance	*MC1R*	MC1R	Melanogenesis and melanocyte proliferation	N/A	(Cordoba-Lanus et al., 2014; Fargnoli et al., 2010)
	MITF	MITF	Development and differentiation of melanocytes	N/A	(Bertolotto et al., 2011; Yokoyama et al., 2011)

CDKN2A is an important driver in melanoma; it is the major high-penetrance susceptibility gene, with mutations arising in around twenty to forty percent of melanoma suffering families around the world (Goldstein et al. 2006; Goldstein et al. 2007; Maubec et al. 2012; (Hayward, 2003). The frequency varies across continents and is inversely related to melanoma incidence (Goldstein et al., 2006; Goldstein et al., 2007). For example, Australia and UK share common mutations including, M53I, IVS2-105A/G, R24P and L32P (Goldstein et al., 2006). These mutational differences in penetrance between countries are due to the interplay between genetics and environment, where family members are exposed to the same level of UVR, as well as various other heritable genetic modifiers (Goldstein et al., 2007). Furthermore, variants of the master regulator of pigmentation melanocortin 1 receptor (MC1R) have been shown to increase penetrance of CDKN2A (Goldstein et al. 2005). Moreover, various independent factors have been correlated with mutations in CDKN2A gene, such as early onset of cutaneous melanoma, multiple primary melanomas and a Breslow thickness greater than 0.4 mm (Goldstein et al. 2006; Pedace et al. 2011; van der Rhee et al. 2011).

The CDKN2A gene is located on chromosome 9p21 and is composed of four exons that code for two unrelated proteins. The first of these, P16 inhibitor of cyclin dependent kinase 4 (p16INK4A), is transcribed from exons 1α, 2 and 3. Conversely, the alternate reading frame (p14ARF) is transcribed from exons 1β, 2 and 3 and is a tumour suppressor that functions to inhibit cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), thus maintaining tumour suppressor retinoblastoma protein (RB) in a hypo-phosphorylated state and stalling cell cycle S phase entry Figure 1.3 (Goldstein et al. 2006; Goldstein et al. 2007).

Mutations that affect $p16^{INK4a}$ and $p16^{ARF}$ result in disruption of specific interactions with CDK4 and CDK6, which obstructs their association with the cyclin D complex (Goldstein et al., 2007). $P16^{INK4a}$ loss activates CDK4 and CDK6, which results in hyper-phosphorylation of retinoblastoma protein *(*pRB), yielding an overall effect of increased cell division and proliferation. RB can activate transcription factor E2F1, responsible for controlling the transcription of genes involved in S phase and cell cycle progression (Lilischkis, Sarcevic, Kennedy, Warlters, & Sutherland, 1996). This increases cell proliferation and initiation of melanoma progression. Moreover, inactivation of $P16^{INK4a}$ is an early event in melanoma and occurs during the transition from *in situ* to invasive. Thus, the $p16^{INK4A}$ cyclin D-CK4/6-RB cascade is pivotal in melanomagenesis and may serve as a potential therapeutic target (B. Lee, Sandhu, & McArthur, 2015; Sheppard & McArthur, 2013).

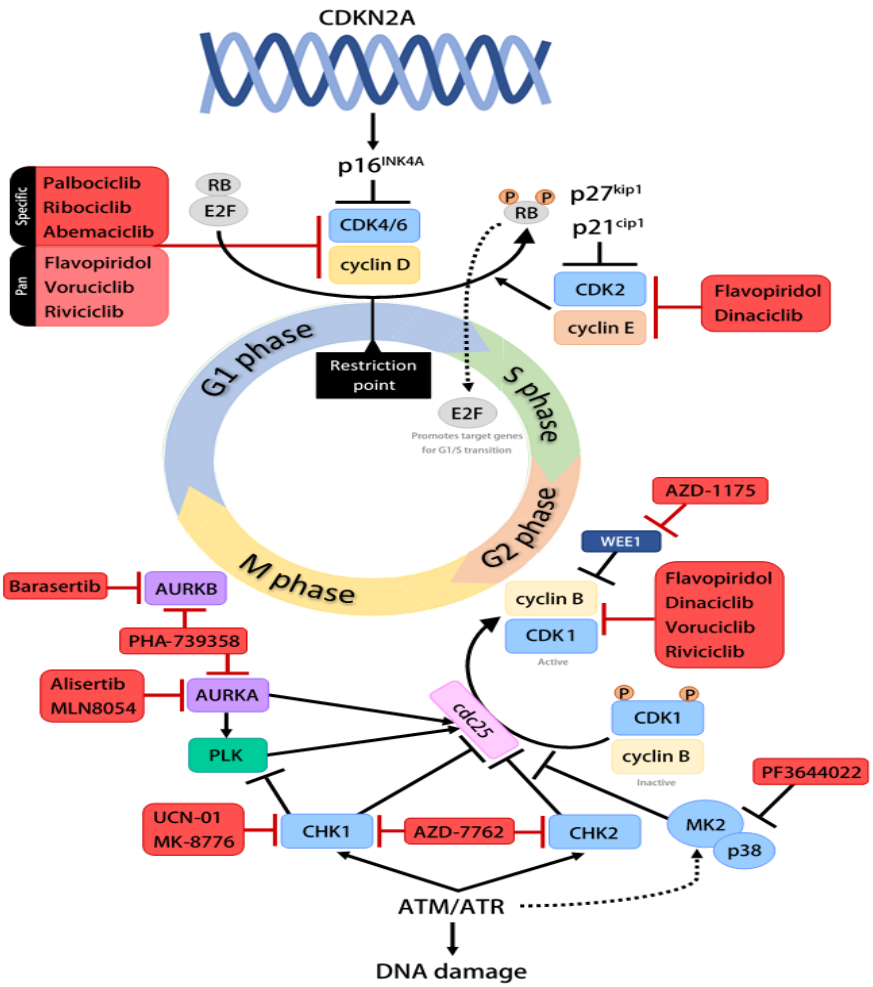


Figure 1.3 Cell cycle regulation is highly controlled at specific checkpoints due to the dynamic interactions of cyclins and their partners CDKs. CDK4/6 interact and form active complex with cyclin D to phosphorylate RB. As a result, E2F transcription factors separate from RB and enter the nucleus to stimulate target genes to start the G1 to S phase transformation. CDKN2A gene encodes the tumour suppressor p16^{INK4A} which binds to CDK4/6 and abrogates its association with cyclin D. Likewise, p27kip1 and p21cip1 block the interaction between CDK2 and cyclin E. Entry into M phase is controlled by the cyclin B/CDK1 complex, which is activated via dephosphorylation by CDC25. Upon DNA damage ATR-CHK1 and ATR-CHK 2 kinase signalling pathways which inhibit CDC25 are activated, preventing entry from the G2 to M phase via inactivating CDK1. The p38/MK2 pathway is parallel to the CHK 1 and 2 pathways and suppresses CDC25. WEE1 blocks M phase entry via direct phosphorylation of CDK1. As presented in red are various checkpoint inhibitors which are currently in clinical development.

1.3.2 Low penetrance genes

Low penetrance genes are defined as genes with low sequence variants or polymorphisms, where the majority of individuals carrying these genes will not develop the disease (Houlston & Peto, 2004; Shawky, 2014).

1.3.2.1 MITF

Minor familial melanoma loci are commonly found in the population and confer a small to moderate increased relative risk of developing melanoma (Bertolotto et al., 2011; Yokoyama et al., 2011). *MITF*, a member of the MYC supergene family, encodes a transcription factor crucial for the development of melanocytes (Steingrimsson, Copeland, & Jenkins, 2004). Previously, *MITF* was considered a regulator of melanocyte differentiation. However, recent research suggests a role in controlling cell proliferation, survival, and in the development of melanoma, leading to the moniker of 'master transcriptional regulator' of melanocyte lineage (Cheli, Ohanna, Ballotti, & Bertolotto, 2010; Yasumoto, Yokoyama, Shibata, Tomita, & Shibahara, 1994). Genomic amplification of *MITF* is present in around ten percent of primary melanoma and twenty percent of MM and correlates with a poor survival outcome (Levi A. Garraway et al., 2005; Ugurel et al., 2007). *MITF* promotes melanogenesis by activating pigmentation genes such as *TYR*, *TYRP1, DCT, PMEL*, and *MLANA* (Bertolotto et al., 1998; Du et al., 2003; Yasumoto et al., 1994) and regulates specific ubiquitously expressed genes that are critical for melanocyte survival (e.g, *BCL2*) and proliferation (e.g., *CDK2*) (Du et al., 2004; McGill et al., 2002). *MITF* transcription is activated via cAMP-CREB, the canonical Wnt signalling pathways, PAX3, SOX10, and ONECUT2, and transcriptional activity is supressed by ALX3, FOXD3, POU3F2, TGF-*β*, TNF-*α*, and hypoxia (Feige et al., 2011). Research suggests aberrations in certain

signalling pathways may contribute to dysregulation of *MITF*. Various groups have studied the germline MITFE318K mutation (Bertolotto et al., 2011; Yokoyama et al., 2011), demonstrating that MITFE318K protein has a higher transcriptional regulatory activity compared with *MITF* Wild Type (WT). This is due to the mutation occurring at a consensus sequence, which is important for SUMOylation that normally represses WT transcriptional function (A. J. Miller, Levy, Davis, Razin, & Fisher, 2005). The MITFE318K mutant thus results in a gain-of-function activity, which is evident by the increased non-blue eye colour phenotype among patients harbouring this germline allele (Yokoyama et al., 2011).

Furthermore, a team of scientists utilised RT-qPCR to determine the expression levels of *MITF* in melanocytic nevi, primary tumours, and metastatic tumours. Their findings reveal *MITF* amplification is predominately expressed in metastatic melanoma tumours and correlates with a decreased five-year survival rate. Thus, these observations unveil *MITF* amplification importance in the development and severity of a subset of human melanomas. They hypothesised that genetic amplification of *MITF* could promote tumour growth and survival when CDK inhibitors such as p16 or p21 are derailed and the MAP kinase pathway has increased activation. To address this, *MITF* was overexpressed in primary melanocytes and it was discovered that vital pathways such as p53 and p16/CDK4/RB were derailed. These findings demonstrated a markedly increased proliferative capacity of melanocytes. Moreover, the team demonstrated that ectopic expression of *MITF* combined with the $BRAF^{V600E}$ mutation transformed primary human melanocytes, and thus *MITF* can function as a melanoma oncogene. (L. A. Garraway et al., 2005). Additionally, bioinformaticians have revealed differential expression of *MITF* in melanoma. They found *MITF*-low melanomas are metastatic, while *MITF*-high melanomas are less invasive (Hoek et al., 2006). High

MITF stimulates the transcriptional activity of cell cycle and differentiation genes, causing melanomas to become more proliferative and differentiated. Meanwhile, the underlying mechanisms explaining why *MITF*-low melanomas are invasive are yet to be elucidated.

1.3.2.2 MC1R

The α-Melanocyte-stimulating hormone α-MSH receptor 1 gene (*MC1R*), located on chromosome 16q24, plays an important role regulating pigmentation in humans (Mountjoy, Robbins, Mortrud, & Cone, 1992). *MCR1* is a transmembrane G-protein coupled receptor, present on the cell surface of epidermal melanocytes (Chhajlani & Wikberg, 1992; Mountjoy et al., 1992). Functional *MC1R* occupies a pivotal role to protect melanocytes against UVR induced damage via three mechanisms: (1) stimulation of DNA repair pathways NER and base excision repair; (2) inhibition of reactive oxygen species production, activation of antioxidant enzymes, and increased expression of antioxidant genes; (3) increased eumelanin biosynthesis (Kadekaro et al. 2005; Kokot et al. 2009; Song et al. 2009; Kadekaro et al. 2010; Swope et al. 2014). Upon stimulation via melanocyte-stimulating α-MSH and adrenocorticotropic hormone, MC1R activates the cAMP/PKA/CREB cascade which results in increased protein levels of tyrosinase, tyrosinase-related protein (TRP) -1 and -2, and subsequently, stimulates biosynthesis of eumelanin pigments (Abdel-Malek et al. 1993; Abdel-Malek et al. 1995; Slominski et al. 2004).

Eumelanin reduces the accumulation of UV induced photoproducts, while pheomelanin contributes to UV damage by triggering formation of free radicals (Kadekaro et al., 2010). Epidemiology studies have demonstrated that *MC1R* is extremely polymorphic and differences in human pigmentation are often due to allele

variation in this gene (Gerstenblith et al., 2007). In African countries, the polymorphism M*C1R* variant is predominantly expressed, which encodes a dark skin photoprotective phenotype to protect against the deleterious effects of UVR (Harding et al., 2000). This therefore suggests a selective pressure that abrogates development of variants which decrease eumelanin synthesis (Harding et al., 2000; Rana et al., 1999). In comparison, *MC1R* is highly polymorphic in Northern European populations, with individuals harbouring variants such as R151C, R160W, and D294H and exhibit the red hair colour (RHC), light freckled skin phenotype, with deregulated or absent tanning response to UVR. These variants, specifically the RHC alleles, have been linked to an increased risk of developing melanoma (Cordoba-Lanus et al., 2014; Davies et al., 2012; Rees, 2000; Sturm, 2002). Furthermore, RHC alleles result in the loss of function in *MC1R* due to minor conformational changes in the receptor, which subsequently inhibits signals transduced via α-MSH-bound receptor. Consequently, this leads to an increased risk of melanocytes to acquire UVR signatures, all of which contribute to MM (Kadekaro et al., 2010; M. C. Scott et al., 2002).

Moreover, the presence of an *MC1R* variant coupled with *CDKN2A* mutation significantly increases the melanoma disease penetrance to 84%, with a mean age at onset of 37.8 years compared with patients carrying a CDKN2A mutation alone (N. F. Box et al., 2001; Neil F. Box et al., 2001; van der Velden et al., 2001). Finally, individuals carrying *MC1R* variants R151C, R160W, and D294H have a 5 to 15-fold increased risk of undergoing BRAF mutation regardless of signs of excessive UV exposure (Fargnoli et al., 2008; Landi et al., 2006).

Oculocutaneous albinism (OCA) is a group of conditions that affect pigmentation of the skin, hair, and eyes. OCA arises as a result of decreased melanin production, due to mutations occurring in several pigmentation genes such as *TYR, OCA2, TYR1* and

SLC45A2. Cutaneous hypopigmentation demonstrates a light hair and sun-sensitive skin phenotype (R. A. King, Willaert, et al., 2003). The two major mutations are (*OCA1*); as a consequence of mutations in the tyrosinase gene on chromosome 11p, and (*OCA2*); due to mutations in the P gene on chromosome 15q (R. A. King, Pietsch, et al., 2003; R. A. King, Willaert, et al., 2003). Mutations in these genes disrupt the cell's ability to synthesize melanin and decrease pigmentation in the skin, hair, and eyes. OCA display a range of phenotypes, from white hair and skin to various shades of light brown or light-to-dark-blond hair. Furthermore, alterations in MC1R can alter the appearance in individuals with oculocutaneous albinism type 2 (*OCA2* mutations). These individuals display light-coloured eyes and vision problems, however they display the red hair instead of the usual yellow, blond, or light brown hair phenotype (Branicki, Brudnik, & Wojas-Pelc, 2009; R. A. King, Willaert, et al., 2003).

1.3.4 Somatic mutations

Somatic genetic alterations can also promote melanoma development. These mutations arise in chronic or intermittent sun-exposed skin melanomas, disrupting vital genes such as *BRAF*, *NRAS*, growth and metabolism phosphatase and tensin homolog (*PTEN*), *KIT* proto-oncogene receptor tyrosine kinase (*KIT*), tumour protein p53 (*TP53*) and replicative lifespan telomerase reverse transcriptase (*TERT*). These genes normally control central processes such as proliferation and resistance to apoptosis.(Hodis et al., 2012; Krauthammer et al., 2012).These genetic changes typically lead to the aberrant stimulation of two important signalling pathways in melanoma, namely the RAS/RAF/MEK/ERK signalling cascade and the phosphoinositol-3-kinase (PI3K)/AKT pathway (Chappell et al., 2011).

A major pathway implicated in MM is the RAS/RAF/MEK/ERK cascade. This pathway is controlled by receptor tyrosine kinases, cytokines, and heterotrimeric G-protein-coupled receptors (Goodall et al., 2004). The small G protein RAS (HRAS, KRAS, and NRAS in humans) are located on the plasma membrane and stimulate the downstream factor RAF (ARAF, BRAF and CRAF in humans), subsequently activating MEK and ERK, finally transducing the signal to regulate transcription in the nucleus (V. Gray-Schopfer, Wellbrock, & Marais, 2007). This pathway is constitutively stimulated by several growth factors including stem cell factor (SCF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and glial-cell-derived neurotrophic factor (GDNF) (Bohm et al., 1995; Narita et al., 2009).

BRAF is a serine–threonine protein kinase that is part of the RAF/MEK/ERK cascade and regulates numerous cellular processes, such as growth, survival and differentiation (Claudia Wellbrock & Hurlstone, 2010). Activating mutations of the BRAF oncogene are present in more than seventy percent of cutaneous melanomas, 90% of which are $BRAF^{V600E}$ mutations (Helen Davies et al., 2002). Oncogenic mutations generally occur in the kinase domain, the common $BRAF^{V600E}$ mutations being the substitution of valine at the 600 position for glutamic acid (V600E), or more rarely an arginine (V600K). These mutations result in the constitutive activation of the mitogen activated protein kinase (MAPK) pathway, in turn activating cell survival and proliferation via upregulation of MITF expression (Davies et al. 2002; (C. Wellbrock et al., 2008).

BRAF mutations are rarely initiated at later stages of disease, emphasising their importance in tumour initiation and advancement (Hoeflich et al., 2006). This notion is supported by studies in mice, where melanocytes incorporating the $BRAF^{V600E}$ have

been shown to promote the formation of melanocytic nevi and melanoma growth, followed by loss of proliferative activity and stabilization of size (Dhomen et al., 2009). A similar phenotype occurs following sustained $BRAF^{V600E}$ expression in human melanocytes, where growth arrest is followed by activation of $p16^{INK4a}$ and expression of the senescence marker, acidic β-galactosidase (V. C. Gray-Schopfer et al., 2006; Michaloglou, Vredeveld, Mooi, & Peeper, 2008). These data illustrate that melanocytic nevi are benign clonal tumours which temporarily undergo proliferation upon stimulation via oncogenic *BRAF* signalling, prior to growth arrest due to oncogene-induced senescence (OIS) (Dhomen et al., 2009; Michaloglou et al., 2005). Therefore, *BRAF* gene mutations alone are not enough to drive oncogenesis in nevi and other factors must be required during this transformation. $P16^{INK4a}$ plays a critical role in replicative oncogene-induced senescence (Wajapeyee et al. 2008). Moreover, $P16^{INK4a}$ expression and growth inhibition could be stimulated by oncogenic BRAF in melanocytes *in vitro* (V. C. Gray-Schopfer et al., 2006) and $p16^{INK4a}$ deficiency is essential for Ras-induced melanoma in mice (Chin et al., 1999). Together, these data propose an interesting model in which $p16^{INK4a}$ may serve as a brake to oncogenic *BRAF* stimulated melanocyte transformation. In this model, oncogenic *BRAF* stimulates hyperproliferation and $p16^{INK4a}$ mediated senescence, which abrogates OIS, thus generating naevi that contain clones of senescent melanocytes. The role of p53 in melanoma has been nicely highlighted using zebrafish. p53-depleted fish models demonstrated that activated BRAF induced formation of melanocyte lesions which developed into aggressive and invasive melanomas, which resembled human melanoma (E. E. Patton et al., 2005). Given this fact, it is possible for benign nevi with mutated BRAF to evade the OIS and become melanomas, thus supporting the high

percentage of this mutation in sporadic disease (Wellbrock et al., 2004b; Dhomen et al., 2009).

Over-expression of $BRAF^{V600E}$ has been implicated in various mechanisms to transform normal melanocytes to MM. $BRAF^{V600E}$ activates the downstream MEK/ERK pathway, escaping cell senescence and apoptosis. This leads to increased replication and angiogenesis via MEK-dependent activation of HIF-1α and VEGF. Melanoma cells invade and metastasize via upregulating numerous proteins involved in migration, such as integrin signalling, cell contractility, tumour- and microenvironment-derived interleukin-8. Finally melanoma cells evade the immune response (Balmanno & Cook, 2009; H. Davies et al., 2002; V. C. Gray-Schopfer et al., 2006; Lehmann et al., 2000; S. Liang, Sharma, Peng, Robertson, & Dong, 2007; Palanisamy et al., 2010; Pritchard et al., 2004; Sewing, Wiseman, Lloyd, & Land, 1997). As a result of this, cell proliferation is increased followed by induction of senescence.

1.4 Melanoma metastasis

Metastasis is a cascade of intricate biological processes, which involves dissemination of neoplastic cells to different anatomic sites and adaptation to foreign tissue microenvironments (G. P. Gupta & Massague, 2006; Talmadge & Fidler, 2010). This process involves interplay between metastatic tumour cells, numerous host factors and homoeostatic mechanisms (Beans, 2018; Chambers & Werb, 2015; Fidler, Schackert, Zhang, Radinsky, & Fujimaki, 1999; Leiter, Meier, Schittek, & Garbe, 2004; Seyfried & Huysentruyt, 2013). Furthermore, metastasis entails a sequence of complex events, which requires local invasion of primary tumour cells into surrounding tissues; intravasation of these cells into the circulatory system, immune system evasion, lymphangiogenesis, arrest and extravasation through vascular walls into the

parenchyma of distant tissues. These tumour cells form micro-metastatic colonies and subsequently proliferate, finally forming metastatic lesions (Fidler et al., 1999; Leiter et al., 2004; Nguyen & Massague, 2007).

MM can metastasise via the blood and the lymphatic system. There are three major metastatic pathways involved in the progression of primary cutaneous melanoma (Meier et al., 2002; Mervic, 2012). Melanoma can metastasise as satellite or in-transit metastases, as lymph node metastases or as distant metastases (Leiter et al., 2004; Meier et al., 2002; Weide et al., 2013; Zbytek et al., 2008). Satellite metastasis is the development of metastatic nodules within two centimetres of the primary tumour. In-transit metastasis is represented by developing metastasis within the dermal and subdermal lymphatics in the drainage area (Bann, Chaikhoutdinov, Zhu, & Andrews, 2019; Leiter et al., 2004; Meier et al., 2002). Previously, satellite and in transit metastases were categorised as separate entities. However, in 2002 The American Joint Committee on Cancer (AJCC) classified both types as Intralymphatic metastasis, which has been linked to poor prognosis ("Melanoma of the Skin," 2002). This is due to satellite metastases residing within centimetres of the primary tumour and in transit metastases in between the primary site and regional lymph node basin (Grotz et al., 2011). This led to the hypothesis that both develop from tumour cell emboli entrapped in dermal lymphatic vessels between the primary tumour and regional lymph node basin (Borgstein, Meijer, & van Diest, 1999; Oashi et al., 2013). All three types of metastasis represent loco-regional metastasis, which occurs distally to the primary tumour in the limbs. Conversely, distant metastasis is characterised as metastasis beyond regional lymph nodes and readily involves visceral sites such as lung, liver, intestines and brain (Balch et al., 2009).

There are three major models used to explain the progression of primary melanoma (Pizarro, 2015). The stepwise spread model postulates that melanoma metastasises initially through lymphatic system into the regional lymph nodes, thus resulting in systemic dissemination (Mervic, 2012; Tejera-Vaquerizo et al., 2007). The second model suggests that primary melanomas metastasise simultaneously via haematogenous and lymphatic pathways (Pizarro, 2015). Finally, the third model describes the patterns of progression of melanoma (Tejera-Vaquerizo et al., 2007). This model suggests that there are various independent migration pathways; some melanomas do not have the biological potential to metastasise at all, yet others are able to metastasise only to regional lymph nodes. Others are able to metastasise only haematogenously, and some can metastasise both haematogenously and through the lymph system (W. H. Clark, 1991; Mervic, 2012). Tumour metastasis is extremely difficult to treat with conventional surgery or radiotherapy due to their ability to disseminate to distant organs, but also mainly due to developing resistance to cytotoxic agents. However, recent advances in understanding the molecular mechanisms has led to the development of effective therapeutics which include immune checkpoint drugs.

Downstream ERK regulates differentiation and proliferation of melanocytes in response to UV radiation. Subsequently, this pathway determines the survival of melanocytes via the *MITF* transcription factor, which strongly advocates its importance in the biology of a melanocytic cell and development of melanoma (Claudia Wellbrock & Hurlstone, 2010)**.** More interestingly, activation of NRAS/BRAF mediates an epithelial-to-mesenchymal transition (EMT) switch at a later stage in melanoma and is dependent on TWIST1; ZEB1, loss in E-cadherin results in invasion. This EMT switch

is an independent factor of poor prognosis in melanoma patients (Caramel et al., 2013).

Until recently, therapeutic options for disease, particularly late stage, were limited and largely ineffective. However, recent progress in the treatment of advanced melanoma has led to unprecedented improvements in overall survival. Researchers have focused on critical mechanisms involved in melanoma pathology and targeted these for treatment and immunotherapy. Given that melanoma remains a deadly type of cancer, particularly at an advanced stage, it is pivotal to elucidate melanoma biology and evolution, which may improve patient care and prognosis. Below I will discuss some of the current treatments in melanoma.

1.4 Melanoma therapeutics

1.4.1 Traditional therapeutics

Surgery remains the mainstay to treat primary melanoma, with a five year survival rate of ninety-two percent (Batus et al., 2013; Torre et al., 2015; van Zeijl, van den Eertwegh, Haanen, & Wouters, 2017). Surgical excision procedures vary depending on the clinical pathological features of the tumour. For *in situ* melanomas, excision includes safety margins of 0.5 cm-1 cm for tumours < 2 mm thickness and 2 cm for tumours thicker than 2 mm (van Zeijl et al., 2017). Complete lymphadenectomy is required for patients with metastasis to regional nodes (Garbe et al., 2010). For most patient's surgery, removal can be combined with chemotherapy, radiotherapy, biological therapy, or targeted therapy (V. Gray-Schopfer et al., 2007). While local excision of in-transit metastasis is often successful in patients with melanoma, this approach is not amenable to metastatic malignant melanoma. Unfortunately, MM is

predominately refractory to most therapeutics, with poor prognosis and a five year survival rate of less than five percent (Cummins et al., 2006). Therefore, diagnosing melanoma prior to the advanced metastatic stage is crucial in terms of positive treatment outcomes, but this has proven difficult due to lesions remaining asymptomatic for prolonged periods (Soengas & Lowe, 2003).

1.4.1.2 Chemotherapy

Cytotoxic chemotherapy has been used to treat metastatic melanoma for over four decades. Chemotherapy combinations improved clinical response, however, showed no changes in overall survival (Legha, 1989). Chemotherapy has been found to become less effective as tumour cells developed resistance to apoptosis (Soengas & Lowe, 2003). Although new effective therapies are available, chemotherapy remains vital in the palliative treatment of refractory, progressive, and relapsed melanomas (Legha, 1989).

In 1974, Dacarbazine received FDA approval as a standard chemotherapy medication for treating MM. Traditional chemotherapy for melanoma abrogates DNA replication during cell division (Legha, 1989). Dacarbazine is a pro-drug that requires conversion in the liver to 5-(3-methyl-1-triazeno) imidazole-4-carboxamide (MTIC), the active compound. Studies reported the response rate in ten percent of the cases, these showed improved overall survival and twenty five percent of patients remained alive at twelve months (Erdei & Torres, 2010; Middleton et al., 2000). Furthermore, agents used in combinations such as vinblastine, cisplatin/carboplatin, taxanes and dacarbazine showed an overall survival rate of forty percent in a phase 2 trial. Although cytotoxic agents when combined provide high response rates, they were also linked with greater toxicity (Bhatia, Tykodi, & Thompson, 2009; Legha et al., 1989)

Electrochemotherapy (ECT) requires the combination of cytotoxic drugs, such as bleomycin and cisplatin, with high-intensity electric pulses which promote successful delivery of drugs into the cells (Miklavčič et al., 2012; Testori, Ribero, & Bataille, 2017). ECT has gained success in treating cutaneous and subcutaneous nodules of melanoma (Marty et al., 2006; Matthiessen et al., 2011). Furthermore, the European Standard Operating Procedures of Electrochemotherapy carried out a study in which they show an overall response of eighty five percent and no major negative adverse effects (Marty et al., 2006)

1.4.2 Targeted therapy

To improve overall survival in melanoma patients, targeted therapy and immunotherapy are recommended. Around seventy percent of patients with cutaneous melanoma harbour mutations in critical signalling pathways (de Snoo & Hayward, 2005; Goel, Lazar, Warneke, Redston, & Haluska, 2006; Saldanha, Potter, Daforno, & Pringle, 2006). These oncogenic mutations are associated with melanoma cell proliferation, invasion and migration (Flaherty, 2012; Flaherty & McArthur, 2010). Targeted therapy utilises small molecule inhibitors or antibodies that affect these mutated proteins, which are required for the progression of melanoma.

As BRAF mutations are present in early-stage melanoma, *BRAF* is an appealing therapeutic target. Prior to 2011, only dacarbazine and high-dose interleukin-2, (HD IL-2), were used to treat metastatic melanoma (Jarkowski & Khushalani, 2014). However, recent efforts have led to the development of vemurafenib and dabrafenib. Vemurafenib is a specific and highly potent *BRAF* inhibitor. Dabrafenib, a potent inhibitor of $BRAF^{V600E}$, was approved by the FDA in 2013; it responded with favourable results during Phase I/II trials, with a response rate of fifty percent when compared to

dacarbazine with six percent (Hauschild et al., 2012). However, despite the exceptionally high response rate, a large proportion of the patients treated with these drugs went on to develop chemo-resistance. To explain this relapse, several mechanisms have been suggested, the prevailing hypothesis being reactivation of the derailed MAPK-signalling (Flaherty & McArthur, 2010) . Evidence comes from *in vitro* and *in vivo* studies which utilised melanoma cell lines and tumour biopsies with acquired resistance to *BRAF*^{V600E} (Greger et al., 2012; Nazarian et al., 2010; Villanueva et al., 2010). They monitored the activation of MEK and ERK, which showed an increase in downstream phosphorylation in *BRAF* inhibition, implying an alternative pathway activates MAPK (Nazarian et al., 2010). One mechanism suggested is the elevated expression of the kinases CRAF, COT1, or mutant *BRAF* (Johannessen et al., 2010; Villanueva et al., 2010). Another mechanism of resistance is the alternative splicing of *BRAF* (*p61 BRAF*), leading to the dimerisation of RAF kinase, downstream ERK phosphorylation and activated mutations in *N-RAS*, *MEK1*, or *AKT1* (Poulikakos et al., 2011). Furthermore, increased expression of receptor tyrosine kinases, such as platelet-derived growth factor receptor-β and insulin-like growth factor-1 receptor, confirm MAPK-independent resistance (Nazarian et al., 2010; Villanueva et al., 2010). Consequently, the mechanisms of *BRAF* acquired inhibition resistance are complicated, involving several critical pathways to evade inhibition and allow disease progression post first treatment. Thus, understanding the key mechanisms of resistance is vital in order to combine treatment which may increase median progression-free survival and improve response rates.

Combining BRAF and MEK inhibitors has been proposed to be more effective for BRAF^{V600} positive melanomas (D. B. Johnson et al., 2014). Trametinib was approved by FDA in 2013 as a MEK1/2 to treat MM harbouring *BRAF* mutations (Ballantyne &

Garnock-Jones, 2013; Livingstone, Zimmer, Vaubel, & Schadendorf, 2014). Trametinib inhibits growth factor-mediated cell signalling to decrease proliferation of tumour cells (Flaherty et al., 2012). In *in vitro* studies on melanoma cell lines resistant to dabrafenib, monotherapy showed restoration of inhibition when both drugs dabrafenib and trametinib were given in combination (Greger et al., 2012). These positive preclinical results led to rationale for a Phase I trial and a randomized Phase II clinical trial (Flaherty et al., 2012). Combination therapy showed a positive response rate of seventy six percent compared with fifty four percent for dabrafenib monotherapy (Flaherty et al., 2012). However, this treatment regimen is also prone to chemo-resistance, which may be due to melanocytes activating their MAPK signalling pathway. Similar mechanisms of resistance have been described in patients on *BRAF* inhibitor monotherapy, emphasising the role of *MEK2* Q60P mutations, *BRAF*-splice mutants and *BRAF* amplification. Additionally, a previously unreported *MEK2* mutation was identified in resistant tumours. Inhibition of MEK1/2 results in the abrogation of growth factor-mediated cell signalling and decreased proliferation of tumour cells (Fedorenko, Gibney, Sondak, & Smalley, 2015; Greger et al., 2012; Palmieri et al., 2015; Ribas et al., 2014; Strickland, Pal, Elmets, & Afaq, 2015). Clearly, there remains an urgent need to better our current knowledge of how chemo-resistance arises in BRAF positive tumours that are treated with BRAF inhibitors.

Furthermore, around two percent of melanomas are associated with germline mutations in *CDK4,* which is a key gene controlling cell proliferation (Soura, Eliades, Shannon, Stratigos, & Tsao, 2016). CDK4/6 inhibitors such as Aibociclib, Abemaciclib, and palbociclib have been used to treat MM and have shown positive results (O'Leary, Finn, & Turner, 2016). Moreover, Abemaciclib has demonstrated decreased tumour

growth in vemurafenib resistant melanoma models, in which elevated levels of cyclin D1 expression and MAPK-pathway reactivation were present (Yadav et al., 2014).

During the nineteenth century, it was proposed that cancer and the immune system are linked. This was due to the persistent appearance of tumours at sites of chronic inflammation and the presence of immune cells in the tumour (Balkwill & Mantovani, 2001). T-cells play an important role in recognising tumour-specific antigens where upon activation, they proliferate and differentiate, acquiring the ability to destroy cells that express tumour-specific antigens. A hallmark of cancer cells is evading T-cell detection, as they do not express B7 molecules (Sharma & Allison, 2015).

In recent years, immunotherapeutic strategies against melanoma have garnered immense excitement following studies demonstrating that checkpoint inhibitor drugs led to improvement in overall patient survival over conventional chemotherapy (Wilden, Lang, Mohr, & Grabbe, 2016). Interferon (IFN) α-2b are cytokines which are secreted via leukocytes. These cytokines function by disrupting viral replication and are important in immunomodulatory, antiangiogenic, antiproliferative, and antitumor activities (Rafique, Kirkwood, & Tarhini, 2015; Sanlorenzo et al., 2017). IFNs stimulate various immune cells such as such as T-cells, B lymphocytes, natural killer cells, and dendritic cells. In 1995, high-dose IFN α-2b was approved by the FDA as an adjuvant therapy to treat resected advanced stage melanoma (Kirkwood et al., 2001; Rafique et al., 2015). In MM, IFN-α stimulates an immunomodulatory antitumor effect, activating major histocompatibility complex class I expression in melanoma and immune cells, consequently decreasing the proliferation of melanoma cells (Roh et al., 2013). Data from a meta-analysis study reported that adjuvant IFN-α significantly decreased the risk of recurrence and resulted in a positive survival outcome in melanoma patients (Ives et al., 2017). Furthermore, IL-2 was the second cytokine to

receive FDA approval in 1998 to treat MM. IL-2 has anti-tumour activity, with a complete response rate of four percent, partial response of twelve percent, and overall response of nineteen percent (Bright, Coventry, Eardley-Harris, & Briggs, 2017).

To elicit an immune response, precise coordination of several signals is required. An intricate system of checks are vital to ensure an appropriate response to antigenic stimuli (Y. Zhu, Yao, & Chen, 2011). Derailment of these signals results in autoimmunity and causes damage to healthy normal tissue (Das et al., 2015). Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) belongs to the immunoglobulin superfamily; it is one of the best studied regulatory molecules and is expressed on the surface of activated T cells (Brunet et al., 1987; Waterhouse et al., 1995). Anti-CTLA-4 antibodies are antagonists, which have received great attention in the last decade. These monoclonal antibodies work by evading the inhibitory effect, enhancing production of pro-inflammatory T-cell cytokines, and increasing clonal T-cell expansion and infiltration in tumours (Ribas, 2007; Ribas et al., 2009). Ipilimumab and tremelimumab are human immunoglobulins of the IgG1 and IgG2 isotypes which target CTLA-4. In a controlled phase III clinical trial, Ipilimumab showed positive improvement in overall survival of ten months in MM patients (Hodi et al., 2010). In a second randomized phase III trial, Ipilimumab in combination with dacarbazine demonstrated significantly longer survival of 11.2 months versus 9.1 months for dacarbazine plus placebo (Caroline Robert et al., 2011). In a third randomized phase III trial of tremelimumab versus dacarbazine or temozolomide, no significant change in the overall survival was observed (Ribas et al., 2013). Due to the great survival success of Ipilimumab as a single therapy, this drug progressed to gain approval by the FDA to treat MM in 2011. Despite the positive survival outcome, there were adverse effects linked to ipilimumab such as dermatitis, colitis, drug-related hepatitis

and endocrinopathies. However, most were reversible when treated with corticosteroids and intense immunosuppressive medication (Batus et al., 2013).

As mentioned previously, there are various checkpoint pathways active to prevent immune cells from destroying normal host cells. Programmed death-1 (PD-1) is a type I transmembrane glycoprotein consisting of an immunoglobulin (Ig) V-type extracellular domain (Ishida, Agata, Shibahara, & Honjo, 1992). This is involved in the interaction of PD-1 and programmed death ligand-1 (PD-L1) (H. Dong, Zhu, Tamada, & Chen, 1999; Francisco, Sage, & Sharpe, 2010). PD-1 is located on various immune cells which include: T cells, B cells, natural killer cells and surface of regulatory T cells (S. Y. Tseng et al., 2001). PD-1 receptor binds to PD-L1 and PD-L2, inhibiting T-cell signalling and subsequently resulting in T-cell apoptosis (Chemnitz, Parry, Nichols, June, & Riley, 2004). Shortly following the discovery of Anti-CTLA-4 antibodies, novel therapeutic Nivolumab was reported, a high-affinity anti-PD-1 monoclonal antibody which supresses the association between the PD-1 receptors PD-L1 and PD-L2 (Melero, Grimaldi, Perez-Gracia, & Ascierto, 2013). Nivolumab received approval by the FDA in 2014 to treat MM (Raedler, 2015). Nivolumab inhibits the interaction between PD-1 and its ligands, which results in immune responses and has shown antitumor activity by causing T-cell apoptosis, reducing T-cell expansion and by blocking production of IL-2 and interferon-γ (H. Dong et al., 2002; Francisco et al., 2010). In clinical trials, Nivolumab displayed a greater progression free survival of 6 months in MM patients compared to monotherapies with ipilimumab (2.9 months) and chemotherapy (2.2 months) (Gomes, Serra-Bellver, & Lorigan, 2018; Douglas B. Johnson, Peng, & Sosman, 2015; Nakamura et al., 2016). Interestingly, treating patients with Nivolumab and ipilimumab combined demonstrated an overall survival at five years of fifty four percent 54% compared to single treatment with ipilimumab at

twenty six percent and forty four percent in the nivolumab group. (Gomes et al., 2018; Hodi et al., 2016; J. Larkin et al., 2019).

In 2014, Pembrolizumab received FDA approval, having a six month response rate that was approximately twice that of chemotherapy in patients which failed to respond to Ipilimumab (C. Robert, Schachter, et al., 2015). Nevertheless, as with kinase inhibitors, the limitations of immune checkpoint inhibitors were quickly exposed due to a large population of patients not responding to therapy, although no biomarker could be identified for patients who received long-term benefits.

Whilst individual therapies were successful, the next rational step was to combine CTLA-4 and PD-1 checkpoint inhibitors. Both CTLA-4 and PD-1 have separate regulatory roles during various stages of T-cell activation. Targeting both checkpoints stimulates non-reductant alterations in gene expressions and shows a synergistic interaction when combined (Curran, Montalvo, Yagita, & Allison, 2010; Das et al., 2015) . The combination of Nivolumab and Ipilimumab has been shown to provide a longer progression free survival benefit of 11.5 months overall, with 11.7 months in BRAF-mutant melanoma patients. However, Nivolumab or Ipilimumab alone demonstrate a shorter progression free survival of 6.9 and 2.9 months, respectively when compared to combined therapy (James Larkin et al., 2015). This finding was compared to combination therapy of dabrafenib and trametinib in melanoma patients positive with BRAF mutations with 9.3–11.4 months progression free survival benefit (C. Robert, Karaszewska, et al., 2015). Furthermore, a similar longer progression free survival benefit was observed with Nivolumab or Nivolumab in combination with Ipilimumab in patients who expressed PD-L1(Hodi et al., 2016).

1.5 Non-Coding RNA

Over the past two decades, improvements in next generation sequencing has expanded our understanding of genomics. The surprising discovery that the human genome encodes ~20,000 protein-coding genes, representing less than two percent of the entire genome sequence, was ground breaking in modern biology (Lander et al., 2001; Venter et al., 2001). Perhaps more shocking was the subsequent realisation that more than 90% of genomic nucleotides are transcribed, as demonstrated through cDNA sequencing and interrogation of the whole chromosome tiling arrays (Okazaki et al., 2002). This suggests that the cell places great importance on the regulatory function of ncRNAs, which comprise a large percentage of these pervasive transcripts (M. B. Clark et al., 2011).

1.5.1 Classification of ncRNAs

Functionally, ncRNAs can be categorised into two broad classes: housekeeping ncRNAs, and regulatory ncRNAs. Housekeeping ncRNAs are predominantly fundamental components of cellular mechanisms governing RNA processing, such as ribosomal RNA (rRNA), tRNA, splicing small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA). In contrast, regulatory ncRNAs are subdivided based on transcript length, with short noncoding transcripts comprising < 200 nucleotides and long noncoding RNAs comprising transcripts > 200 nucleotides. Short ncRNAs include miRNA, small interfering RNA (siRNA), piRNA, transcription initiation RNA, and small cajal body-specific RNA (scaRNA) (Kutter & Svoboda, 2008; L. Li & Liu, 2011).

1.5.2 LncRNAs

LncRNAs are >200 nucleotides in length and lack significant open-reading frames (Gibb, Brown, & Lam, 2011). The majority of lncRNAs are transcribed by RNA

polymerase II and are divided into subclasses of polyadenylated and non-polyadenylated transcripts (Mercer, Dinger, & Mattick, 2009; Shen, Qi, & Du, 2015). Initially, scientists argued that lncRNAs were unimportant due to their low level expression and lack of species conservation (T. Derrien, R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, G. Guernec, D. Martin, A. Merkel, D. G. Knowles, J. Lagarde, L. Veeravalli, X. Ruan, Y. Ruan, T. Lassmann, P. Carninci, J. B. Brown, L. Lipovich, J. M. Gonzalez, M. Thomas, C. A. Davis, R. Shiekhattar, T. R. Gingeras, T. J. Hubbard, C. Notredame, J. Harrow, & R. Guigo, 2012). However, *in situ* hybridization and gene expression profiling studies have shown lncRNA expression is regulated at developmental stages and is cell type and tissue specific, suggesting that they contribute to functional processes (T. Derrien, R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, G. Guernec, D. Martin, A. Merkel, D. G. Knowles, J. Lagarde, L. Veeravalli, X. Ruan, Y. Ruan, T. Lassmann, P. Carninci, J. B. Brown, L. Lipovich, J. M. Gonzalez, M. Thomas, C. A. Davis, R. Shiekhattar, T. R. Gingeras, T. J. Hubbard, C. Notredame, J. Harrow, & R. Guigo, 2012). LncRNAs are predominantly localised in the nucleus, with well-studied examples including *Xist*, *HOTAIR and NEAT1* (Brown et al., 1992; Hutchinson et al., 2007). Yet some are known to localise to both the nucleus and the cytoplasm, such as *MALAT1* (Wilusz, Freier, & Spector, 2008).

LncRNA are further sub-divided depending on their genomic derivation, which include sense, antisense, bidirectional, intronic and intergenic transcripts. Sense lncRNA transcripts overlap with the sense strand of a coding gene. Antisense lncRNA transcripts overlap one or more exons of another transcript on the opposite strand and may or may not be complementary to protein coding sequences in the sense-strand. Bidirectional lncRNA transcripts are expressed within 1kb of a coding transcript of the opposite strand. Intronic lncRNAs are derived from the introns of protein-coding genes,

and intergenic lncRNA (also known as lincRNA) are situated within the interval between two genes (Gibb et al., 2011; Hauptman & Glavac, 2013; Lizio et al., 2015; Orom et al., 2010).

1.5.3 LncRNA function

Through genomic initiatives such as ENCODE, FANTOM, GTEx, and GENCODE, >60,000 lncRNAs have been identified, several of which have been found modified in certain diseases, emphasising the significance of these transcripts ("The ENCODE (ENCyclopedia Of DNA Elements) Project," 2004; Frankish et al., 2019; "The Genotype-Tissue Expression (GTEx) project," 2013; Harrow et al., 2012). Nevertheless, to date, only a minute percentage of these lncRNAs have been described in the literature, with an even smaller number given a specific mechanistic function. Like proteins, lncRNAs utilise more than one mode of action (Kopp & Mendell, 2018), and despite studies being in their infancy, they have been implicated in a highly diverse range of biological functions that coalesce around the control of gene expression (Bernard et al., 2010; T. Derrien, R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, G. Guernec, D. Martin, A. Merkel, D. G. Knowles, J. Lagarde, L. Veeravalli, X. Ruan, Y. Ruan, T. Lassmann, P. Carninci, J. B. Brown, L. Lipovich, J. M. Gonzalez, M. Thomas, C. A. Davis, R. Shiekhattar, T. R. Gingeras, T. J. Hubbard, C. Notredame, J. Harrow, & R. Guigó, 2012; Dhir, Dhir, Proudfoot, & Jopling, 2015; Gong et al., 2015; Hawkins & Morris, 2010; Pandey et al., 2008; S. C. Wu, Kallin, & Zhang, 2010; Kyoko L. Yap et al., 2010). This functional plasticity is achieved in part via their complex and versatile secondary and tertiary structure, which enables binding to RNA, DNA and protein partners (L. Yang, J. E. Froberg, & J. T. Lee, 2014).

LncRNA can function as decoys, reducing the availability of specific regulatory factors by serving as a molecular sink. Decoy lncRNAs regulate gene expression by sequestering RNA-binding proteins, transcription factors, microRNAs, catalytic proteins, and subunits of larger modifying complexes (Wang and Chang 2011; Kung et al. 2013; Ma et al. 2013; Chen 2016). This class of lncRNA negatively regulates effector factors by titrating factors away from their targets. For example, during DNA damage, lncRNA *PANDA* interacts with transcription factor NF-YA to disrupt p53 induced apoptosis. NF-YA stimulates expression of numerous key genes for apoptosis and cell senescence. However, *PANDA* -NF-YA in complex titrates NF-YA away from target gene chromatin, consequently reducing transcription expression of genes responsible for apoptosis and senescence (Hung et al. 2011; Baldassarre and Masotti 2012; Peng et al. 2017).

Another example of decoy lncRNAs is *H19,* which reduces the availability of regulatory factors, and controls transcription via sequestering regulatory factors, transcription factors, chromatin modifying complexes and miRNAs (Kallen et al., 2013). LncRNAs can also serve as scaffolds by assembling multiple-component complexes (Lin Yang, John E. Froberg, & Jeannie T. Lee, 2014). RNA transcripts interact with proteins to form ribonucleoprotein particles (RNPs).The transcriptional activity of the ribonucleoprotein (RNP) complex once it has completely assembled is controlled by different RNAs and proteins which are available (Aguilo, Zhou, & Walsh, 2011; Kotake et al., 2011). A small number of lncRNA-protein interactions have been defined, however the lncRNA interaction is likely to be more complicated than one lncRNA interacting with one protein. The majority of these lncRNA are implicated in chromatin-dependent processes, these include: *HOTAIR, KCNQ1OT1* and *ANRIL,* which act as scaffolds and control the activities of histone-modifying complexes (Aguilo et al., 2011;

Ribeiro et al., 2018; Rinn et al., 2007; Thakur et al., 2004). As an example, lncRNA *HOTAIR* functions as modular scaffold linking a histone methylase and a demethylase. The 5′ domain of *HOTAIR* binds Polycomb Repressive Complex 2 (PRC2), whilst the 3′ domain of *HOTAIR* associates with the LSD1/CoREST/REST complex. The ability to link two different complexes allows the assembly of PRC2 and LSD1, and coordinates targeting of PRC2 and LSD1 to chromatin for coupled histone H3 lysine 27 methylation and lysine 4 demethylation. This results in the repression of the *HOXD* locus. Another example comes from the highly abundant Nuclear Paraspeckle Assembly Transcript 1 (*NEAT1*), required to form paraspeckles. *In vitro* analysis revealed that *NEAT1* RNA binds with paraspeckle associated proteins PSP1 and p54 and is required for their localization to paraspeckles (Christine M. Clemson et al., 2009). There are also examples of ncRNAs such as the RNA component of telomerase (*TERC*) and signal recognition particle (*SRP*) RNA, which function as scaffolds at telomeres and on translating ribosomes during protein targeting to the endoplasmic reticulum, respectively (Halic et al., 2004; Zappulla & Cech, 2006).

Guide lncRNAs can control changes in gene expression, either *in cis* or *in trans*, in a manner that is not easily predicted based on lncRNA sequence. LncRNA guides are required for the precise localisation and organization of factors at specific genomic loci to regulate the genome. These lncRNA interact with transcription factors and chromatin modifiers, directing them to precise locations in the genome at either *in cis* or *in trans* sites from their locus of transcription (Khalil et al. 2009; Maenner et al. 2010; Wang and Chang 2011; Ma et al. 2013). The maternally expressed 3 lncRNA (*MEG3*) resides on human chromosome 14q32.3 and is a tumour suppressor gene (Miyoshi et al., 2000). Mondal *et al* explored the mechanisms by which *MEG3* targets chromatin. They demonstrated that *MEG3* and *EZH2* share similar target genes, including the

TGF-β pathway genes. Additionally, genome-wide mapping analyses of *MEG3* binding sites unveil that *MEG3* regulates the activity of TGF-β genes via binding to distal regulatory elements. The GA-rich sequences present in *MEG3* binding sites guide *MEG3* to the chromatin through RNA–DNA triplex formation. Furthermore, they show that RNA–DNA triplex structures are widespread and found over the *MEG3* binding sites linked with the TGF-β pathway genes (T. Mondal et al., 2015).

Additionally, enhancer RNAs (eRNAs) are derived from enhancer regions and function by affecting the 3-dimensional (3D) organization of DNA during transcription. One proposed model suggests that eRNAs may serve as tethers and do not disassociate from the enhancer, thereby tethering their interacting binding proteins to enhancer regions (Grote et al., 2013; X. Li, Wu, Fu, & Han, 2014). *HOTTIP* is an enhancer lncRNA shown to associate with the WDR5 protein, forming a vital part of the MLL-Trx complex that catalyses the activating H3K4me3 mark. *HOTTIP* is encoded on the distal 5′ end of the *HOXA* gene, and chromosomal looping of the 5′ end of the *HOXA* occurs in an enhancer like manner, moving *HOTTIP* into spatial proximity with multiple *HOXA* genes, implementing the gene activation and maintenance of H3K4me3 (Schuettengruber, Chourrout, Vervoort, Leblanc, & Cavalli, 2007). Interesting, *in vivo HOTTIP* loss of function experiments via gene silencing of *HOXA* expression lead to changes in limb morphology in mice, similar with its role in switching on *HOXA* genes. This study highlights the importance of lncRNAs interacting with chromatin modifying machinery, leading to enhancer-based gene activation (K. C. Wang et al., 2011). Some lncRNAs have also been implicated in cellular behaviour and regulation of the cell-cycle, cell growth, cell death, migration and apoptosis. Others are involved in metabolism, development, stress-response and

embryonic stem-cell pluripotency (L. L. Chen & Carmichael, 2010; Gibb et al., 2011; Mercer et al., 2009).

Consistent with their roles in differentiation and development, multiple functional studies have characterized lncRNA in controlling multi-step gene expression pathways via epigenetic modifications, transcriptional and post-transcriptional processing, and translational regulation (Batista & Chang, 2013; Berghoff et al., 2013; A. M. Bond et al., 2009; Kretz et al., 2013; N. Lin et al., 2014; Matkovich, Edwards, Grossenheider, de Guzman Strong, & Dorn, 2014; Wapinski & Chang, 2011; Whitehead, Pandey, & Kanduri, 2009). For instance, lncRNA *MALAT1* regulates post-transcriptional gene transcripts by controlling multiple processes, such as RNA maturation, and by modulating alternative splicing (Cooper et al., 2014; Tripathi et al., 2010). This latter action is achieved by interaction with splicing factors or by binding the 5' (GU - exon-intron boundary) and 3' (AG – intron-exon boundary) splice sites in pre-mRNA. LncRNAs bound to mRNA form double-stranded structures, promoting exosome-mediated RNA degradation, or alternatively, positively or negatively regulating their translation efficiency (K. C. Wang & Chang, 2011). Gene silencing can also be achieved via regulating chromatin structure and interaction with different RNA sub-classes (Bernstein & Allis, 2005; Whitehead et al., 2009).

Given the mounting evidence in terms of cellular function, it is not surprising that dysregulation of lncRNA expression has also been reported in a plethora of human disease (Betts et al., 2017; R. Jiang et al., 2017; Pu et al., 2015; X. Wang et al., 2016; Y. Wang et al., 2015; Wapinski & Chang, 2011; G. Yang, Lu, & Yuan, 2014; S. X. Yuan et al., 2016; S. Zhang, Zhang, & Liu, 2016; P. Zhu et al., 2016). This is particularly true in neurodegenerative disorders and to an even greater extend in cancer, where

lncRNA dysregulation is emerging as a key event in some neoplasms (Briggs, Wolvetang, Mattick, Rinn, & Barry, 2015; Riva, Ratti, & Venturin, 2016; A. Wang, Wang, Liu, & Zhou, 2017). Below, I will discuss some of the lncRNA mechanisms ascribed to disease progression in cancer.

1.5.4 Oncogenic lncRNA

Epigenetics is the study of gene expression without altering the DNA sequence. Epigenetic changes, such as DNA methylation, histone modification, nucleosome remodelling, RNA-mediated targeting and genomic imprinting regulate critical biological processes. Dysregulation of these mechanisms are considered as key drivers in neoplasm growth (Dawson & Kouzarides, 2012) . An explosion of evidence has emerged from numerous studies, identifying multiple lncRNAs which affect cellular transformation and cancer progression (see Table 1.2), predominantly by influencing gene transcription via cross talk with the epigenetic machinery (Prensner & Chinnaiyan, 2011).

Table 1. 2: Oncogenic LncRNA

LncRNA	Cancer type	Reference
aHIF	Overexpressed in renal, breast cancers	(Cayre, Rossignol, Clottes, & Penault-Llorca, 2003)
H19	Highly expressed in HCC. Correlates with c-Myc in primary breast and lung carcinomas	(Barsyte-Lovejoy et al., 2006; Matouk et al., 2007)
HULC	Increased in HCC	(Y. Liu et al., 2012)
KCNQ1OT1	Increased expression in colorectal cancer	(Nakano et al., 2006)
DD3 (PCA3)	Increased in prostate cancer patients	(Crawford et al., 2012)
LSINCT5	Overexpressed in breast and ovarian cancer	(Silva, Boczek, Berres, Ma, & Smith, 2011)
PVT1	Colorectal	(Y. Y. Tseng et al., 2014)
PCAT-1	Prostate	(Eeles et al., 2008; Prensner et al., 2011)
PTCSC3	Thyroid	(Jendrzejewski et al., 2012)
TERC	Oral cavity	(Dorji et al., 2015)

Numerous lncRNAs despite their low numbers, are evolutionary conserved and have shown distinct conservation patterns compared to mRNAs of protein-coding genes, this might be due to similarities in their regulatory promoter elements (Thomas Derrien et al., 2012; Guttman et al., 2009). Furthermore, in a study which compared lncRNAs in mammalian transcripts with lncRNAs found in zebrafish found only a few significant examples of sequence conservation, which were mainly restricted to short sequence stretches (Ulitsky, Shkumatava, Jan, Sive, & Bartel, 2011).

MALAT-1 is one of the most abundant lncRNA present in normal tissues and is highly conserved within mammalian species (Hutchinson et al., 2007). RNA polymerase II transcribes *MALAT1* from the human chromosome 11q13, giving rise to a ~6.5 kb transcript (Wilusz et al., 2008). At the molecular level, *MALAT-1* is recruited to nuclear speckles and is reported to regulate alternative splicing by interacting with the

serine/arginine-rich (SR) family of nuclear phosphoproteins (Bernard et al. 2010; Tripathi et al. 2010). Specifically, *MALAT-1* appears to regulate the distribution of pre-mRNA splicing factors to nuclear speckles, which in turn affects the phosphorylation state of SR proteins (Tripathi et al. 2010). Indeed, regulation of SR protein concentration and phosphorylation state are crucial because SR proteins affect the alternative splicing patterns of many pre-mRNAs. Even minute changes can disrupt the balance that controls mRNA variability between different cells and tissue types (Long and Caceres 2009). Thus, *MALAT-1* functions as a fine-tuning mechanism to regulate SR protein activity. For example, in *MALAT-1* depleted cells, high levels of mis-localised and hypo-phosphorylated SR proteins are evident, resulting in a higher frequency of exon inclusion (Tripathi et al. 2010). Neurons are highly enriched in *MALAT-1,* where it plays a key role in the control of synaptogenesis (Bernard et al. 2010) via modulation of neuronal SR splicing factors activity, thereby regulating the transcription of genes involved in synapse formation, density and maturation (Bernard et al. 2010). Consequently, *MALAT-1* functions within a post-transcriptional gene-regulatory mechanism by coordinating specific patterns of mRNA expression in different cell types.

Whilst many lncRNA have been shown to act *in cis* with regards to genetic imprinting, the lncRNA, HOX transcript antisense RNA (*HOTAIR*) has been shown to regulate gene expression *in trans*. *HOTAIR* is transcribed from the *HOX-C* gene cluster and physically interacts with polycomb-repressive complex 2 (PRC2) and lysine (K) specific demethylase 1A (LSD1). This brings them in contact with the *HOX-D* cluster DNA motif (Chu, Qu, Zhong, Artandi, & Chang, 2011), promoting the transcriptional repression of this 40 kb region (L. Li et al., 2013; Rinn et al., 2007; Tsai et al., 2010). Furthermore, *HOTAIR* regulates gene expression at numerous other

genomic locations by interacting with the LSD1/CoREST/REST complex, which then recruits PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and K4 demethylation (Tsai et al., 2010). Clearly, *HOTAIR* occupies a crucial role in epigenetic modification and transcriptional activity, so it is perhaps unsurprising that dysregulated expression of *HOTAIR* has been reported in numerous cancers, including breast, lung, colorectal and pancreatic. (C. Ding et al., 2014; X. H. Liu et al., 2013; Niinuma et al., 2012; Qiu et al., 2014; Sorensen et al., 2013; Z. H. Wu et al., 2014). The specifics of how increased *HOTAIR* expression impacts on cancer is still being delineated, however in breast cancer, elevated levels of *HOTAIR* have been shown to alter the epigenome towards a metastatic state and correlate with poor prognosis (R. A. Gupta et al., 2010).

The lncRNA, antisense of the *INK4* locus (*ANRIL*) comprises a region of 30–40 kb antisense to the *INK4b* locus, which encodes the p15 (INK4b) and p16 (INK4a) tumour suppressor genes (Pasmant, Sabbagh, Vidaud, & Bieche, 2011) . *ANRIL* modulates *INK4a* transcriptional repression *in cis* by interacting with a member of the polycomb repressive complex 1 (PRC1) Pc/Chromobox 7 (CBX7) to remodel the chromatin landscape, thereby contributing to development of cancer (K. L. Yap et al., 2010). In prostate cancer tissue, high levels of CBX7 and *ANRIL* have been detected, which correlate with low levels of INK4a. Furthermore, there is evidence that point mutations in CBX7 abrogate RNA binding to ablate the ability of PRC1 to repress the *INK4b*/*ARF*/*INK4a* locus in order to restrain cell senescence (K. L. Yap et al., 2010). This highlights *ANRILs* importance in the abnormal silencing of *INK4b*/*ARF*/*INK4a* locus and demonstrates that it serves as an initiation factor in prostate cancer development.

Further examples of lncRNA-mediated chromatin modification include the *X-chromosome inactivation* (*XIST)* and *H19* genes, which have been implicated in X-chromosome inactivation (XCi) and imprinting, respectively. In XCi, a large region of one X-chromosome is inactivated to ensure correct gene dosage levels in female somatic cells (J. T. Lee & Bartolomei, 2013). The lncRNA *H19* is an evolutionary conserved lncRNA, abundantly expressed in foetal tissue and placenta, and is dysregulated in majority of adult tissues (Gabory, Jammes, & Dandolo, 2010). A few lncRNAs use multiple strategies that, in combination, might be essential for their biological function. For example, the action of *H19* on gene expression reveals the complexity of the combinatorial mechanisms of regulation determined by one lncRNA. *H19* is expressed from the maternally-inherited chromosome, and insulin-like growth factor 2 (*IGF2*) is expressed from the paternal chromosome (Gabory et al., 2010; Zemel, Bartolomei, & Tilghman, 1992). On the maternal allele, methylation sensitive CCCTC (CTCF) binds to the imprinting control regions (ICR), silencing *IGF2* expression and stimulating the transcription of *H19*. On the paternal allele, the ICR undergoes DNA methylation in the male germline, disrupting the interaction with CTCF. As a result, on the paternal chromosome, *IGF2* associates with the enhancers from this chromosome (Court et al., 2011). DNA methylation on the paternal chromosome results in secondary methylation of the *H19* promoter via a mechanism which remains unknown, and consequently *H19* is silenced on the paternal chromosome (Court et al., 2011; Murrell, Heeson, & Reik, 2004). *IGF2* is critical in prenatal growth, for instance the placenta specific *IGF2* transcripts regulate the growth of the placenta and supply of maternal nutrients to the developing foetus (Constância et al., 2002). Moreover, the *H19* gene encodes an untranslated RNA which serves as a trans-regulator of the imprinted gene network, coordinating

embryonic growth in mice (Gabory et al., 2009). Research using mice models demonstrates that loss of *H19* results in maternal expression of *IGF2*, leading to an overgrowth phenotype (Gabory et al., 2009; Ripoche, Kress, Poirier, & Dandolo, 1997). Mechanistically, the control region between *H19* and *IGF2* comprises a differentially-methylated chromatin control region. *H19* interacts with methyl CpG binding domain protein 1 (MBD1), subsequently interacting with histone lysine methyltransferases, resulting in H3K9 methylation, repressing transcription (Monnier et al., 2013).

Additionally, lncRNAs transcribed from enhancer regions in the genome (eRNAs) function to stabilize and maintain chromatin loops (Lam, Li, Rosenfeld, & Glass, 2014; W. Li et al., 2013; Orom & Shiekhattar, 2013), allowing distally-located enhancers to associate with their target gene promoters (G. Li et al., 2012; Sanyal, Lajoie, Jain, & Dekker, 2012; Visel, Rubin, & Pennacchio, 2009). For example, studies have shown a correlation between the levels of eRNAs produced by upstream enhancers of the *PSA* gene and the normal levels of *PSA* gene expression, suggesting eRNA and chromatin interaction (Hsieh et al., 2014). Furthermore, the presence of eRNAs that bind the transcription factor p53 have been reported. Interestingly, knockdown of these eRNAs resulted in reduced transcription at neighbouring genes and reduced p53-dependent cell cycle arrest (C. A. Melo et al., 2013).

1.5.5. Tumour-suppressor lncRNA

In contrast to the oncogenic lncRNA discussed above, there are also reports of lncRNAs that have been shown to suppress tumour growth, however their expression has been dysregulated in various cancers.

MEG3, is found on the human chromosome 14q32.3 within the DLK1-MEG3 locus (Wylie, Murphy, Orton, & Jirtle, 2000; Y. Zhou, Zhang, & Klibanski, 2012). *MEG3* is a maternally imprinted gene, an mRNA-like RNA with ten exons that encodes a 1.6 kb non-coding RNA (X. Zhang et al., 2010). *MEG3* produces numerous transcripts as a result of alternative splicing and containing a few small open reading frames (ORFs) (Croteau, Charron, Latham, & Naumova, 2003; Schuster-Gossler, Bilinski, Sado, Ferguson-Smith, & Gossler, 1998). lncRNA *MEG3* resides in the nucleus and cytoplasm and is expressed in numerous human tissues, with increased expression predominately in the brain and pituitary gland (X. Zhang et al., 2003). Failure to regulate imprinting at the locus may result in developmental disorders (Benetatos, Vartholomatos, & Hatzimichael, 2011). Various studies have shown *MEG3* to function as a tumour suppressor, and its expression has been derailed in numerous human cancers which include Breast Cancer, Liver Cancer, Colorectal Cancer, Glioma, Cervical Cancer, Gastric Cancer, Ovarian Cancer, Lung Cancer and Osteosarcoma (Bando et al., 1999; Braconi et al., 2011; R. Cheng, Lo, Huang, & Tsao, 1997; Greife, Knievel, Ribarska, Niegisch, & Schulz, 2014; Jiao & Zhang, 2019; J. Liu et al., 2015; J. Wang, Xu, He, Xia, & Liu, 2018; Y. Wang & Kong, 2018; J. L. Wu, Meng, & Li, 2018; D. D. Yin et al., 2015). P53 is known as the guardian of the genome, controlling cellular growth by maintaining DNA integrity from cellular stress (Zilfou and Lowe 2009). The p53 protein is normally expressed at low levels due to MDM2 (Murine/human double minute 2), which regulates ubiquitin degradation of p53 (Manfredi 2010). Various stimuli trigger the activation of p53, such as DNA damage, telomere erosion, hypoxia, metabolic deprivation, and oncogenic stress (Yee and Vousden 2005; Riley et al. 2008). p53 activation subsequently activates several molecular pathways, including cell cycle arrest, DNA repair, senescence and apoptosis (Green and Kroemer 2009;

Zilfou and Lowe 2009). Many cancers are due to mutations arising in p53, thus p53 is one of the most extensively studied tumour suppressors (Haupt and Haupt 2017). Numerous groups have shown that overexpression of *MEG3* and its isoforms significantly increased p53 proteins levels and resulted in p53-dependent transcription via a p53-responsive promoter. The authors utilised functional assays to demonstrate the importance of the folding of *MEG3* RNA being necessary for its function. Additionally, *MEG3* activates expression of the growth differentiation factor 15 (*GDF15*) via enhancing the interaction of p53 to the *GDF15* gene promoter (Y. Zhou et al., 2007). However, *MEG3* does not switch on the transcription of $p21^{CIP1}$, therefore implying that *MEG3* regulates the specificity of p53 transcriptional activation. Furthermore, MDM2 levels were reduced in cells over expressed with *MEG3* construct, indicating that MDM2 inhibition is partly due to p53 accumulation stimulated via *MEG3*. Finally, they demonstrate that *MEG3* supresses cell proliferation in the absence of p53. Thus, *MEG3* functions as tumour suppressor, whose action is controlled by both p53-dependent and p53-independent pathways (X. Zhang et al., 2010; Y. Zhou et al., 2007). Moreover, knockdown of *MEG3* stimulates cell proliferation in multiple cancers via both p53-dependent and p53-independent pathways (Huarte et al., 2010; X. Zhang et al., 2003). For example, Zhao *et al* observed that cyclic AMP (cAMP) stimulates *MEG3* expression, and both the p53 and Rb pathways that regulate cellular proliferation (J. Zhao, Zhang, Zhou, Ansell, & Klibanski, 2006).

Interestingly, *MEG3* has an implicated a role in MM. Evidence from several reports demonstrate that *MEG3* expression is downregulated in melanoma (P. Li et al., 2018; J. Long & X. Pi, 2018). Until recently, the biological role of *MEG3* remained unknown, however Li and associates focused their efforts on the biological mechanisms by which

MEG3 contributes to melanoma development. Initially, they determined expression levels of *MEG3* in various melanoma cell lines, such as A375, SK-MEL-1, B16, and A2058, and compared expression to primary melanocytes. Data shows *MEG3* expression was significantly downregulated in melanoma cell lines versus primary melanocytes. Over expression of *MEG3* in melanoma cells inhibited cell proliferation, colony formation and induced apoptosis. Importantly, they investigated the role of the Wnt signalling pathway, which is involved in the progression of numerous cancers (Stewart, 2014; Suzuki et al., 2004; Van Scoyk et al., 2008; Weeraratna, 2005; T. Zhan, Rindtorff, & Boutros, 2017). The authors observed Wnt signalling in melanoma cells was highly activated, with increased β-catenin expression and decreased GSK-3β expression. *In vitro* over expression of *MEG3* resulted in inactivation of Wnt signalling pathway by decreasing β-catenin and Cyclin D1 and increasing GSK-3β levels. Together, this study highlights the importance of *MEG3* as a tumour suppressor and its biological function in melanoma development via suppression of the Wnt signalling pathway (Peng Li et al., 2018).

Furthermore, another group investigated the role of *MEG3* in inhibiting cell proliferation and invasion in melanoma via controlling the expression of CYLD mediated by sponging miR-499-5p. The *CYLD* gene is predominately mutated in familial cylindromatosis (Blake & Toro, 2009; Poblete Gutiérrez et al., 2002). Various studies have shown a tumour protective role for CYLD in numerous malignant tumours, by controlling critical signalling pathways such as Wnt/*β*-catenin, nuclear factor-*κ*B , and transforming growth factor-*β* (Lim et al., 2012; Tauriello et al., 2010; Urbanik et al., 2014). CYLD expression is decreased in melanoma tissues and cell lines, inhibiting cell proliferation and metastasis by obstructing the JNK/AP-1 and β1-integrin signalling pathways (H. Ke et al., 2013). Furthermore, miR-499-5p supressed

tumour growth and invasion in lung cancer via regulating the expression of VAV3 (M. Li et al., 2016). Evidence also suggests that miR-499-5p may stimulate proliferation of colorectal cancer cells by supressing the expression of *FOXO4* and PDCD4 (X. Liu et al., 2011). In this study, the authors established the expression levels of *MEG3* in melanoma tissues and cell lines, which were significantly decreased with *MEG3* and associated with poor prognosis. Next, they observed a reduction in CYLD and an increased expression of miR-499-5p in melanoma tissues and cell lines. Importantly, *MEG3* could interact with miR-499-5p and CYLD mRNA occupied at a binding site of miR-499-5p. *In vitro* Luciferase reporter assay and western blot confirmed that *MEG3* controlled the expression of CYLD via sponging miR-499-5p. Furthermore, functional analysis revealed that increased expression of *MEG3* supressed cell proliferation, invasion and migration, and stimulated apoptosis. Additionally, *MEG3* halted the melanoma cell cycle, by mediating the expression of E-cadherin, N-cadherin, and cyclin D1, by sponging miR 499 5p to control expression of CYLD. Overall, this research highlights the significance of *MEG3* in the suppression of melanoma, with potential roles as a novel therapeutic (Jianwen Long & Xianming Pi, 2018).

In Hepatocellular carcinoma (HCC), the novel lncRNA located on chromosome 8p12, known as TSLNC8, functions to suppress transcription via inactivation of STAT3 and transkelotase (J. Zhang, Z. Li, et al., 2018). Moreover, the growth-arrest-specific 5 (*Gas5*) lncRNA regulates apoptosis by controlling glucocorticoid activity in response to nutrient starvation (Kino, Hurt, Ichijo, Nader, & Chrousos, 2010). Under normal cellular conditions, glucocorticoid receptor (GR) signalling regulates anti-apoptotic genes, including cellular inhibitor of apoptosis 2 (cIAP2), and inhibits caspases 3, 7, and 9 (Webster et al., 2002). However, during growth arrest, *Gas5* is expressed and interacts with the DNA-binding domain (DBD) of glucocorticoid receptor (GR), serving

as a decoy and obstructing GR association with its cognate glucocorticoid response elements (GRE). This serves to derail GR binding ability to the cIAP2 GRE, reducing cIAP2 expression levels and consequently deregulating its suppressive effect on caspases and promoting apoptosis (Kino et al., 2010). *Gas5* tumour suppressor function appears to be clinically relevant in breast cancer, as low levels of *Gas5* are observed in tumour cells compared with normal breast epithelia (Huarte et al., 2010) .

Interactions between lncRNA and key regulatory proteins, such as *Gas5* and GR in the example above, represent an important and emerging mechanism for lncRNA function that remains poorly understood due to the technical challenges associated with identifying RNA-protein interaction partners (McHugh & Guttman, 2018). RNA-binding proteins (RBPs) are obvious and widely reported interactors with lncRNA (Barrandon, Spiluttini, & Bensaude, 2008; Bierhoff, 2018; J. Kim et al., 2016; Mohamadkhani, 2014). Components of the paraspeckle (SFPQ/p54/NONO) appear to be particularly promiscuous in their interactions with lncRNA. Of these proteins, SFPQ-lncRNA interactions have been reported in several cancers (Z. Gao et al., 2019; J. Huang et al., 2014; Q. Ji et al., 2014; J. Lu, Shu, & Zhu, 2018; Munschauer et al., 2018; P. Yang et al., 2016; C. Zeng et al., 2018), however a global analysis of SFPQ-lncRNA interactions in melanoma has yet to be carried out.

1.5.6 Dysregulated LncRNA in melanoma

There is emerging evidence linking dysregulated expression of specific lncRNA in melanoma, as shown in Table 1.3.

Table 1.3 Dysregulated LncRNA in melanoma

LncRNA	Expression	Reference
HOTAIR	Overexpressed	(M. Cantile et al., 2017; Lihua Tang, Wei Zhang, Bing Su, & Bo Yu, 2013)
BANCR	Overexpressed	(Flockhart et al., 2012; R. Li et al., 2014)
ANRIL	Overexpressed	(E. Pasmant et al., 2007; S. Xu et al., 2016)
MALAT1	Overexpressed	(Lei Sun, Sun, Zhou, Gao, & Han, 2016; Tian, Zhang, Hao, Fang, & He, 2014)
LLME23	Overexpressed	(C. F. Wu et al., 2013)
SPRY-IT1	Overexpressed	(Khaitan et al., 2011; Y. Shi et al., 2015; Tan et al., 2017; Xia Zhang, Chi, & Zhao, 2017)
UCA1	Overexpressed	(Tian et al., 2014; Wei et al., 2016)
SAMMSON	Overexpressed	(Eleonora Leucci et al., 2016)
SLNCR1	Overexpressed	(Karyn Schmidt et al., 2016)

As mentioned previously, *HOTAIR* occupies an important role in epigenetic modification and transcription. Therefore, it is not shocking that *HOTAIR* expression is upregulated in various cancers, including breast, lung, colorectal, pancreatic, ovarian, hepatocellular and gastric (C. Ding et al., 2014; Geng, Xie, Li, Ma, & Wang, 2011; K. Kim et al., 2013; Kogo et al., 2011; X. H. Liu et al., 2013; Niinuma et al., 2012; Qiu et al., 2014; Sorensen et al., 2013; Z. H. Wu et al., 2014; L. Zhan, Li, & Wei, 2018). In melanoma, *HOTAIR* is overexpressed in lymph-node metastasis ~100-fold in comparison with primary lesions (L. Tang, W. Zhang, B. Su, & B. Yu, 2013). Furthermore, increased expression of *HOTAIR* is a risk factor promoting metastasis in MM, as shown by functional studies that demonstrated knockdown of *HOTAIR* decreased the migratory potential and invasion of melanoma cells *in vitro*. A second oncogenic lncRNA implicated in melanoma is *MALAT1*, with increased levels reported in MM compared to normal tissues. Moreover, *in vitro* silencing of *MALAT1* reduced the migration potential of melanoma cells (Tian et al., 2014).

In 2011, the first lncRNA described in melanoma was derived from an intron of the *SPRY4* gene Sprouty4-intronic transcript 1 (*SPRY4 IT1*). Unlike *HOTAIR*, very little is known about the cellular role of *SPRY4 IT1*. *SPRY4* belongs to the Ras/Erk inhibitor encoding Sprouty family of genes, located on chromosomal region 5q31.3 and encodes a protein of 322 amino acids. Moreover, *SPRY4* inhibits the EGF-receptor transduced MAP kinase signalling pathway but fails to supress MAP kinase activation by constitutively activting V12 Ras. Additionally, it functions upstream of RAS activation and impairs GAP-assisted GTP → GDP hydrolysis. (Khaitan et al., 2011; Leeksma et al., 2002; Tennis et al., 2010). Clearly, the tumour suppressor role of *SPRY4* is abrogated in MM. *SPRY4-IT1* is upregulated in melanoma and silencing of this lncRNA decreased cell proliferation and promoted apoptosis in melanoma cells (Khaitan et al., 2011). Additionally, *SPRY4-IT1*-depleted melanoma cells exhibited a significant decrease in both cell migration and invasion as opposed to the control (Mazar et al., 2014).

Furthermore, to establish if the stability of *SPRY4-IT1* and *SPRY4* were independently regulated, melanoma cell line A375 were treated with polymerase II transcriptional inhibitor α-amanitin and mRNA expression was quantified via RT-PCR. The authors show that *SPRY4* RNA decayed quicker compared to *SPRY4-IT1* in both the nucleus and cytoplasm, highlighting the functional independence of *SPRY4-IT1* and *SPRY4*. Furthermore, transcript-specific siRNAs were utilised to silence *SPRY4* and *SPRY4-IT1* in melanoma cell line A375. Their data shows in A375 cells, invasion was supressed by fifty percent by silencing *SPRY4-IT1* but was unaffected by *SPRY4* knock-down. Additionally, knockdown of *SPRY*4-IT1 stimulated apoptosis in melanoma cells more effectively compared to *SPRY4* knockdown. Together, this

study highlights the functional and transcriptional independence of *SPRY4-IT1* and its gene *SPRY4* (Khaitan et al., 2011).

Schmidt and colleagues profiled lncRNAs expressed in patient-derived melanomas. They discovered SRA-like non-coding RNA1 (*SLNCR1*), a novel lncRNA with sequence similarity to the lncRNA steroid receptor RNA activator. They found that increased expression of *SLNCR1* correlates with decreased survival in melanoma patients. Furthermore, functional and mechanistic characterisation showed that the brain-specific homeobox protein 3a (Brn3a) and the androgen receptor (AR) form complex with *SLNCR1's* conserved sequence and an adjacent sequence. Importantly, a previous report demonstrates that Brn3a is involved in melanoma cell cycle progression (Hohenauer et al., 2013). The role of *SLNCR1* in melanoma invasion was determined using melanoma cell line A375, which expresses low levels of *SLNCR1* versus melanoma patients. In the over expressed *SLNCR1* melanoma cell line A375 an increased invasion phenotype was observed compared to over-expression of a *SLNCR1* mutant without the highly conserved sequence, which did not result in increased invasion. Clearly, these data highlight that the conserved region is essential and enough for *SLNCR1*-mediated melanoma invasion.

Furthermore, the team next sought to investigate genes which regulate increased melanoma invasion. Utilising melanoma cell line A375, they performed RNA-seq. Two transcripts were significantly upregulated by *SLNCR1's* conserved sequence: RARRES2P8, a pseudogene of the retinoic acid receptor responder, and MMP9. Previous studies have demonstrated MMP9 involvement in early melanoma invasion via remodelling of the extracellular matrix (Hofmann, Houben, Bröcker, & Becker, 2005; MacDougall, Bani, Lin, Muschel, & Kerbel, 1999; MacDougall, Bani, Lin, Rak, &

Kerbel, 1995). In this study, the authors observed that *SLNCR1* promotes invasion in melanoma via transcriptionally upregulating the gene encoding the gelatinase, MMP9. Also, *SLNCR1* binds to AR, involving a hormone-responsive transcription factor in melanoma invasion. Hence, these findings may indicate why males have higher incidence of melanoma dissemination compared to females (Aubuchon et al., 2017; K. Schmidt et al., 2016).

More recently, Leucci and colleagues described the lncRNA *SAMMSON*, situated on chromosome 3p13p14, which also contains the melanoma-specific oncogene *MITF*. This study showed *SAMMSON* is co-amplified with *MITF,* and its expression is lineage specific. Functional data revealed exogenous *SAMMSON* increased the proliferation of melanoma cells and silencing *SAMMSON* rapidly decreased melanoma cell viability (E. Leucci et al., 2016). Mechanistically, the lncRNA *SAMMSON* binds to p32 and increases its mitochondrial localisation. Silencing *SAMMSON* decreased oxidative phosphorylation, mitochondrial ribosome biogenesis, and respiratory chain complex activity in a cancer-cell-specific manner. Thus, this data advocates that silencing *SAMMSON* may prove to be an effective melanoma therapy (E. Leucci et al., 2016).

As discussed above, aberrations of the Ras/ERK MAPK signalling pathway are common in MM, with more than fifty percent of tumours harbouring mutations in *BRAF* and crucially, over ninety percent of these cases occupying the same $BRAF^{V600E}$ substitution (H. Davies et al., 2002; Flaherty & McArthur, 2010). This prompted the development of $BRAF^{V600E}$-specific inhibitors, which work well for some patients, but unfortunately ~fifty percent of those treated with these drugs relapse and became chemo-resistant ultimately leading to fatality (Pérez-Lorenzo & Zheng, 2012; Torres-Collado, Knott, & Jazirehi, 2018; Tsao et al., 2012). Clearly, there is an urgent

need to gain a better understanding of how *BRAF* associates with the molecular machinery in the cancer cell to impact disease (Bamford et al., 2004; Sullivan & Flaherty, 2013).

With this goal in mind, Flockhart *et al* in his recent report utilised RNA-sequencing of primary melanocytes transduced with lentivirus expressing $BRAF^{V600E}$ in order to determine lncRNA expression induced by the *BRAF* mutant (Flockhart et al., 2012). The authors discovered a novel lncRNA, termed *BRAF*-activated non-coding RNA (*BANCR*), a 4-exon transcript of 693 bp situated on chromosome 9 (Flockhart et al., 2012; McCarthy, 2012). *BANCR* has been implicated in numerous human malignancies, including colorectal cancer, melanoma, gastric cancer, lung carcinoma, and bladder cancer. Interestingly, *BANCR* is overexpressed in $BRAF^{V600E}$ positive melanocytes and melanoma. To gain a better understanding on the functional role of *BANCR*, siRNA mediated *BANCR*-depletion was carried out, which resulted in decreased melanoma cell motility. Further functional studies revealed that the chemokine CXCL11 is positively regulated by *BANCR* and rescued the reduced cell motility phenotype observed in *BANCR*-depleted melanoma cells (Flockhart et al., 2012). This study highlights the pivotal role of the $BRAF^{V600E}$ mutation to stimulate overexpression of *BANCR*, resulting in the increase in the expression of CXCL11 to promote cell motility. Another study demonstrates that *BANCR* expression increased during later stages in melanoma (R. Li et al., 2014). Silencing *BANCR* expression substantially decreased the cell viability of melanoma cells via inactivation of the extracellular signal–regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) components of the MAPK pathway (R. Li et al., 2014). Clinically, patients with high *BANCR* expression in melanoma tissues suffered a poorer prognosis and lower survival rate (Ruiya Li et al., 2014).

1.5.7 Melanoma Biomarkers

As described earlier, treating melanoma during the earlier stages is linked to better survival outcome. Thus, the primary goal in the field of melanoma research is focused around developing novel therapeutics. New improved targeted and immune therapies have reshaped the care of patients with melanoma. The ability to predict melanoma growth and monitor or predict response to therapy has become the golden focus of research into biomarkers in melanoma. Biomarkers are characterised as measurable changes in biological substances that are linked with normal or abnormal conditions (Strimbu & Tavel, 2010). Cancer patients contain a myriad of tumour-derived genetic, proteomic and cellular components which are frequently released into circulation. Clearly, there is an urge to utilise these circulating biomarkers in the clinic to facilitate personalised therapy in melanoma.

In melanoma, various prognostic and predictive biomarkers have been used clinically, which make up the updated version of the 8th edition of AJCC melanoma staging system (Balch et al., 2009). This staging system is focused on the histological features of melanoma, such as tumour thickness, ulceration, number of naevi and mitotic rate. To date, the only circulating protein biomarker with significant prognostic value in the AJCC staging system is lactate dehydrogenase (LDH) (Balch et al., 2009). LDH is a cytoplasmic enzyme required for the conversion of pyruvate to lactate. Cancer cells that proliferate utilise anaerobic or glycolytic mechanisms to create a survival advantage (Agarwala et al., 2009; Koukourakis et al., 2003). Increased levels of LDH correlates with poor survival in stage IV melanoma and is an independent predictor of poor outcome in individuals treated with combination dabrafenib and trametinib (Agarwala et al., 2009; Long et al., 2016). Conversely, a significant decrease in LDH is linked with a positive response to immunotherapy (Diem et al., 2015). Other

serological biomarkers include S100B; an immunohistochemical biomarker that can indicate an advanced stage melanoma. Increased S100B levels are linked to metastasis, poor treatment response, relapse, and overall survival (Kaskel et al., 1999; Kruijff et al., 2009; Schultz, Diepgen, & Von Den Driesch, 1998). Additionally, the C-reactive protein (CRP) belongs to the pentraxin protein family (Thompson, Pepys, & Wood, 1999) and is a nonspecific marker of inflammation, infection, and tissue injury that is triggered via hepatocytes due to circulating cytokines, such as IL-6 (Moshage et al., 1988; Pepys & Hirschfield, 2003). In melanoma, elevated levels of IL-6 correlate with melanoma late stages. Furthermore, elevated CRP is associated with melanoma progression from stage I, II, or III to stage IV melanoma (Deichmann, Kahle, Moser, Wacker, & Wüst, 2004). Thus, implicating IL-6 and CRP with disease progression (Deichmann et al., 2000; Mouawad et al., 1996). Another increased protein found in melanoma is an autocrine growth factor termed Melanoma-inhibiting activity (MIA) (Bogdahn et al., 1989). In mouse models, it was shown that melanoma cells transfected with recombinant human MIA cDNA lead to increased invasiveness and migration of melanoma cells (Guba et al., 2000). Serum levels of MIA were found to correlate with both disease stage and progression, and in most cases responsiveness to therapy (Bosserhoff et al., 1997; Cao et al., 2007; Deichmann et al., 2001; Stahlecker et al., 2000).

Human serum and plasma contains circulating RNAs which include protein-coding messenger RNAs (mRNA), micro RNAs (miRNAs), piwi-interacting RNA (piRNAs) and transfer RNAs (tRNAs) (X. Chen et al., 2008; Danielson, Rubio, Abderazzaq, Das, & Wang, 2017; Freedman et al., 2016; Hornick et al., 2015; Inns & James, 2015; K. M. Kim, Abdelmohsen, Mustapic, Kapogiannis, & Gorospe, 2017; T. Yuan et al., 2016). Various types of RNAs have been utilised as biomarkers in cancer such as mRNA,

piRNA, small nucleolar RNA (snoRNA), miRNAs and long non-coding RNAs (lncRNAs). In cancer, circular RNAs (circRNAs) have been shown to regulate the function of other molecules and are directly involved in tumorigenesis (Z. Xu et al., 2018). CircRNAs are abnormally expressed in cancer compared to normal tissues and therefore, have received immense attention as potential biomarkers to characterise tumour state and progression (Freedman et al., 2016; Inns & James, 2015; K. M. Kim et al., 2017; Meng et al., 2017; Mitchell et al., 2008).

MiRNAs are small conserved non-coding RNAs, twenty-two nucleotide in length, and regulate a myriad of processes. These include: proliferation, differentiation, senescence, autophagy, migration, survival and alter cellular metabolism and genome stability (Ambros, 2004; Bartel, 2004; Kozomara, Birgaoanu, & Griffiths-Jones, 2019; Umu & Gardner, 2017; Wahid, Shehzad, Khan, & Kim, 2010). They can function as both oncogenes and tumour suppressors (Penna et al., 2011; B. Zhang, Pan, Cobb, & Anderson, 2007). Therefore, small changes in miRNA levels can result in various human diseases and cancers, including melanoma (Bell et al., 2014; Iorio & Croce, 2012; Noguchi et al., 2014; Sandoval et al., 2015; V. Sun, Zhou, Majid, Kashani-Sabet, & Dar, 2014; Hong Xie et al., 2014). Various studies have reported miRNAs as major players in melanoma progression (Hartman & Czyz, 2015; Seton-Rogers, 2015). Derailed expression of miRNAs have been shown in metastatic melanoma cells compared with melanocytes as a result of chromosomal abnormalities, epigenetic regulation, and disruption of miRNA biogenesis processes (Caramuta et al., 2010; J.-Y. Li, Zheng, Wang, & Hu, 2016; Mannavola, Tucci, Felici, Stucci, & Silvestris, 2016; S. A. Melo & Esteller, 2011; Mione & Bosserhoff, 2015; Mueller, Rehli, & Bosserhoff, 2009; Wozniak, Mielczarek, & Czyz, 2016). Moreover, the MAPK signalling pathway controls a network of 420 miRNAs and is over-expressed in melanoma (Couts,

Anderson, Gross, Sullivan, & Ahn, 2013). Muller et al. demonstrated that *miR-let-7a* was reduced significantly in metastatic melanoma cell lines versus melanocytes, and *miR-let-7a* decreased N-ras oncogene expression in melanoma cells (Mueller et al., 2009). Moreover, miRNAs are dysregulated at various stages of melanoma development, including intravasation into vessels, survival in lymphatic systems, extravasation, and formation of tumour in distant organs. Therefore, miRNAs serve as biomarkers with potential diagnostic and prognostic value (Latchana et al., 2017; Mirzaei et al., 2016; Mo, Chen, Fu, Wang, & Fu, 2012; Varamo, Occelli, Vivenza, Merlano, & Lo Nigro, 2017; Wozniak et al., 2016). Numerous miRNAs have been determined from preclinical and clinical trials which may be associated with melanoma (Fleming et al., 2015; Friedman et al., 2012; Douglas B. Johnson & Sullivan, 2014; Kanemaru et al., 2011). For example, *miR-221* was reported by Kanemaru and colleagues to be over expressed in MM. Serum levels of *miR-221* were statically higher in individuals with stage III–IV melanoma compared to early stage melanoma (Kanemaru et al., 2011). Additionally, upon surgical resection, serum levels of *miR-221* decreased, thus implying its potential as a novel biomarker for differentiating MM from stages I–IV, for identifying tumour progression, and for monitoring patients progression post treatment (Kanemaru et al., 2011).

lncRNA are another class of non-coding RNA (ncRNAs) with dysregulated expression profiles in various cancer types, where numerous evidence suggests that progression and pathogenesis of these cancers is directly associated with lncRNAs (J.-H. Chen et al., 2017; H. Li et al., 2014; W. Li, Xie, & Ruan, 2016; F. Ma et al., 2016; Pang, Yang, Fu, & Liu, 2015; S. H. Wang et al., 2016; B. Yang et al., 2017; B. Zhao et al., 2018). One major advantage of lncRNAs as potential diagnostic and prognostic biomarkers is their high stability whilst circulating in body fluids, specifically in exosomes and

apoptotic bodies (Akers, Gonda, Kim, Carter, & Chen, 2013). Evidence suggests that lncRNAs are able to resist degradation via ribonucleases, despite high quantities of ribonucleases being present in different body fluids (T. Shi, Gao, & Cao, 2016).

Moreover, derailed expression of lncRNAs is evident in tumour tissues which is mirrored in bodily fluids, such as whole blood, plasma, urine, saliva, and gastric juice (Reis & Verjovski-Almeida, 2012; Sartori & Chan, 2014; Shao et al., 2014; H. Tang, Wu, Zhang, & Su, 2013). LncRNAs therefore provide an opportunity to develop effective lncRNA-based biomarkers with minimal invasiveness compared to conventional biopsies, therefore being better tolerated by patients. Numerous lncRNAs have demonstrated potential value as biomarkers. For example, lncRNA Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1)* is an effective prognostic parameter used to determine patient survival in stage I non-small cell lung cancer (P. Ji et al., 2003). Furthermore, *HOTAIR* is over expressed in saliva samples in patients with oral squamous cell carcinoma (OSCC). Increased expression was observed in metastatic patients, thus *HOTAIR* implicates its role as a candidate for metastatic oral cancer diagnosis (H. Tang et al., 2013). Additionally, the role of *HOTAIR* as a prognostic marker was determined in colorectal cancer. Data showed increased blood levels of *HOTAIR* were linked to poor prognosis and high death rate. Additionally, expressional levels of *HOTAIR* could also predict the survival time of patients (Svoboda et al., 2014). In gastric cancer patients, plasma samples have demonstrated increased expression of lncRNA *H19*. This lncRNA has received great attention as a potential biomarker due it its high diagnostic value for the detection of gastric cancer, with a sensitivity of eighty-two percent and specificity of seventy-nine percent. Interestingly, *H19* is effective at detecting the cancer at an earlier stage compared to conventional biomarkers, such as CEA and CA199 (X. Zhou, Yin, Dang,

Ye, & Zhang, 2015). lncRNA Prostate Cancer gene 3 (*PCA3*) is the first lncRNA to gain approval by the FDA as a urine biomarker for prostate cancer (Bussemakers et al., 1999; Fradet et al., 2004). *PCA3* is more specific and sensitive compared to the Prostate Specific Antigen (PSA) blood test, due to its significantly increased expression in prostate cancer patients (Hessels et al., 2003; G. L. Lee, Dobi, & Srivastava, 2011; Scott B. Shappell, 2008; S. B. Shappell et al., 2009; Tinzl, Marberger, Horvath, & Chypre, 2004; Xue, Ying, Jiang, & Xu, 2014).

In melanoma, several lncRNA are reported to be over-expressed, such as *MALAT-1, SPRY4-IT1, BANCR, HOTAIR* and *UCA1* (Luan et al., 2016; Mazar et al., 2014; L. Tang et al., 2013; Tian et al., 2014). Data is currently limited for lncRNA as potential biomarkers in melanoma. Given the importance in expression level of lncRNA in various cancers, deregulated lncRNAs may serve as useful prognostic and predictive liquid biomarkers in melanoma. Moreover, there is emerging evidence linking ncRNA to melanoma development and metastasis. In particular, lncRNAs are gaining traction as key drivers of cancer development and metastasis (for review see (Akhbari, Whitehouse, & Boyne, 2014; Hulstaert et al., 2017; J. Li, Meng, Bai, & Wang, 2016; Slack & Chinnaiyan, 2019). However, our current understanding of how lncRNAs contribute to the genetic milieu of melanoma remains incomplete.

1.6 SFPQ

The *Drosophila* behaviour splicing (DBHS) family are a multifunctional family of proteins involved in regulating diverse cellular processes. One important member of this family is the human protein PTB-associated splicing factor (PSF), also known as splicing factor proline-glutamine rich (SFPQ). (C. S. Bond & Fox, 2009; J. G. Patton, Porro, Galceran, Tempst, & Nadal-Ginard, 1993). DBHS proteins (other notable

members include p54nrb (NONO) and PSPC1 (paraspeckle protein component 1) are found within the nucleoplasm, nucleolar caps and paraspeckles (C. S. Bond & Fox, 2009; Fox & Lamond, 2010; Shav-Tal & Zipori, 2002). The proteins are defined by their highly conserved core domain of ~300aa and differ in their N and C terminal regions. The core domain comprises of two RNA-recognition motifs (RRM1 and RRM2), a ~100 amino acid coiled-coil domain, and a conserved intervening 52aa sequence known as a NONA/Paraspeckle (NOPS) domain (B. Dong, Horowitz, Kobayashi, & Krainer, 1993). DBHS proteins form homo and hetero dimers via their conserved core domains (Fox, Bond, & Lamond, 2005; Passon et al., 2012), whereas RNA interaction is believed to occur via the RRM motifs (Fox et al., 2005). Different DBHS family members can be differentiated by the size and sequence complexity that exists outside of the conserved core region (M. Lee et al., 2015).

Initially, SFPQ was identified via an interaction with polypyrimidine tract-binding protein (PTB) (J. G. Patton et al., 1993). Early work demonstrated that PTB formed a complex with an uncharacterised splicing factor, which was necessary for the splicing of the α-tropomyosin pre-mRNA in nuclear extracts (J. G. Patton, Mayer, Tempst, & Nadal-Ginard, 1991). However, subsequent research has revealed that SFPQ regulates numerous pathways in mammals, including circadian rhythms (C. S. Bond & Fox, 2009), pluripotency in embryonic stem cells (ESCs), cell growth and development, tumour suppression (Passon et al., 2012; G. Wang, Cui, Zhang, Garen, & Song, 2009), DNA repair, transcriptional regulation, splicing and RNA transport (Shav-Tal & Zipori, 2002). How SFPQ regulates such diverse functions is discussed in more detail below, starting with the different structural regions of SFPQ.

1.6.1 SFPQ protein structure

In this section I will briefly discuss each of the domains present in SFPQ and the roles attributed to each domain. Figure 1.4 below represents the different regions of the SFPQ protein.

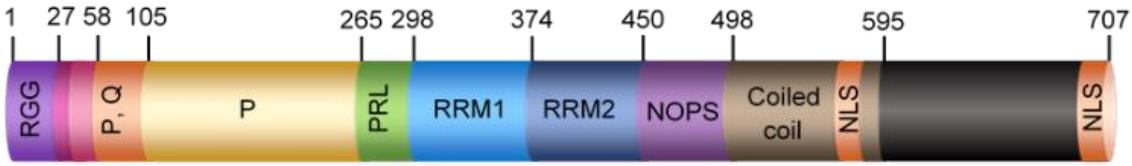


Figure 1.4 Domain structure of SFPQ. The region of SFPQ that comprises the DBHS core domain. RGG box, glutamine-rich subdomain (P, Q), proline domain (P), proline- rich linker (PRL), nuclear-localisation sequence (NLS), RNA-recognition motif (RRM1,RRM2), NONA/paraspeckle (NOPS) and coiled-coil motif *(Yarosh, Iacona, Lutz, & Lynch, 2015)*.

The N-terminal 27 amino acids of SFPQ are rich in arginine and glycine, containing numerous trimeric RGG repeats, known as an RGG box. RGG domains were initially identified in nucleolar proteins such as NSR1, GAR1, nucleolin, and fibrillian, as well as in the non-nucleolar heterogeneous nuclear ribonucleoprotein particle (hnRNP) A1/A2 (Nagai, 1996). These motifs are rare, present in about 100 human proteins and facilitate high RNA-binding affinity via interaction with G-quartet nucleic acid structures (Kiledjian & Dreyfuss, 1992), which are formed by guanine tetrads arranged in a planar conformation (Hanakahi, Sun, & Maizels, 1999). The protein arginine methyltransferases (PRMTs) target RGG motifs as substrates, resulting in the methylation of the RGG box and interruption of their association with other proteins and RNA (Thandapani, O'Connor, Bailey, & Richard, 2013). Interestingly, several sites of arginine methylation have been identified in SFPQ. Arginine residues within the RRG motif are asymmetrically methylated, an event that has been shown to be carried

out by PRMT1 *in* vitro, and is antagonised by citrullination of SFPQ (Snijders et al., 2015).

Following the RRG box motif, the next ~200 amino acids contain numerous proline and glutamine rich residues. The proline-rich region in SFPQ functions predominantly in protein-protein interactions, with non-DBHS proteins. Experimental data supports SFPQ interaction with the proline-binding SH3 domain of the T-cell signalling molecule Nck. Nck connects receptor and non-receptor tyrosine kinases to components of the actin cytoskeleton to regulate activation-dependent processes during cell polarisation and migration (Badour, Zhang, & Siminovitch, 2004; Lettau et al., 2010; Lettau, Pieper, & Janssen, 2009). Finally, one report has suggested the proline-rich domain of SFPQ binds to RAD51, a eukaryotic homologue of bacterial RecA. RAD51 functions as an important DNA recombinase, with roles in both meiotic and mitotic homologous recombination, thus suggesting SFPQ ability to bind DNA (Morozumi, Takizawa, Takaku, & Kurumizaka, 2009).

RNA-recognition motifs (RRMs) were first characterized in 1990 and function in RNA recognition and binding (Kenan, Query, & Keene, 1991; Query, Bentley, & Keene, 1989). SFPQ contains two tandem RRMs (RRM1 and RRM2), each spanning ~70–80 amino acids, located towards the centre of the protein, proximated by a seven amino acid, alanine-rich linker (Shav-Tal & Zipori, 2002). The crystal structure of RRMs in SFPQ remains to be established. However, the structure of RRMs of the other human DBHS proteins have been determined via x-ray crystallography, from a co-crystal of p54nrb/NONO and PSPC1, containing both RRMs from each protein plus additional sequences (NOPS and coiled-coil), which together make up the core structure of the DBHS protein (Passon et al., 2012). In this complex, the RRM uses a

canonical fold; the two RRMs of each monomer form a rigid extended conformation, interacting with the NOPS domain of one monomer and RRM2 domain of the other. The crystallized interaction of NOPS–RRM2 results in the RNA-binding face of RRM2 becoming accessible, enabling RRM2 to bind RNA. Furthermore, due to high similarity in homology between p54/NONO, PSPC1, and the general structural similarity of all RRMs, PSPC1 and p54nrb/NONO structures are likely to be representative of the RRM regions of SFPQ.

The common mode of association between RNA and RRM motifs involves the stacking of aromatic residues on β-strand 1 (RNP2) and 3 (RNP1), which are required to bind RNA. Additionally, these residues are important in stacking interactions with RNA bases or are inserted between two sugar rings (Daubner, Cléry, & Allain, 2013). Interestingly, RRMs can interact with RNA via specific interactions of RNA with loops at the base of the β-sheet and/or with the α-helices (Dominguez, Fisette, Chabot, & Allain, 2010; Hardin, Hu, & McKay, 2010; M. Singh et al., 2012).

Data from preliminary studies suggest that the SFPQ C-terminal RRM2 has RNA binding function despite the low levels of aromatic residues present on the β-sheet. Therefore, it is predicted that RRM2 of SFPQ interacts with RNA via a non-canonical mode. In contrast, RRM1 has low RNA-binding activity and is more likely to function in a canonical manner, with conserved abundant aromatic and charged residues (Ha, Takeda, & Dynan, 2011; Melton, Jackson, Wang, & Lynch, 2007; Shav-Tal & Zipori, 2002). Thus far, there is clear evidence that SFPQ binds to pyrimidine-rich RNAs, GA-rich sequences and GU-rich sequences (Cho et al., 2014; Melton et al., 2007; J. G. Patton et al., 1993; R. Peng et al., 2002; D. Ray et al., 2013). Additionally, the hepatitis *delta* virus (HDV) relies on host proteins for replication, and SFPQ binds the

terminal stem–loop domains of HDV RNA, suggesting potential SFPQ importance in the survival of HDV (Greco-Stewart, Thibault, & Pelchat, 2006). Interestingly and as discussed above, SFPQ has been shown to bind various lncRNAs, however, the precise regions of SFPQ that are mediating these interactions remain mostly uncharacterised.

In addition to mediating protein-RNA interactions, several studies have reported that RRMs also function in protein-protein interactions (Clery et al., 2013; Maris, Dominguez, & Allain, 2005). Indeed, the multiplicity and complexity in function of the two RRMs of SFPQ means that they are not interchangeable, as evidenced by studies showing that RRM1 is required for binding to VP16-activation domains, whereas RRM2 mediates RNA Pol II C-terminal domain interaction (Emili et al., 2002; Rosonina et al., 2005; Ryan, Murthy, Kaneko, & Manley, 2002). Furthermore, RRM2 deletion results in reduced levels of nuclear SFPQ, suggesting that RRM2 is integral for subnuclear localization of SFPQ to speckles (Dye & Patton, 2001).

The NOPS domain spans 52-aa between the RRM2 and the coiled-coil domain. Previously, the NOPS domain was believed to be an extension of RRM2 (Rosonina et al., 2005). However, recent studies disagree, and NOPS is now considered a distinct domain. Researchers have found in PSPC1/NONO, the NOPS domain binds to the opposite side of the RRM's β-sheet to the canonical RNA-binding surface, thus allowing further interactions. Additionally, the NOPS domain is also important in forming hydrophobic interactions with both RRMs and the distal region of the coil-coiled domain. However, mutations in the NOPS domain abrogate the association of PSPC1 and DBHS proteins, and the localisation of SFPQ to paraspeckles in cells, implying that the major function of the NOPS domain is to mediate protein–protein

interactions, with a vital role in the formation of functional dimers in the cell (Passon et al., 2012). Given the structure of PSPC1, this study provides a framework for the interpretation of other DBHS family members, including SFPQ and NONO.

The coiled-coil interaction motif of SFPQ spans 528-555aa, forming an extended structure via an anti-parallel coiled-coil, resulting in a linear polymer of SFPQ dimers within crystals (Passon et al., 2012). This motif associates with neighbouring dimers in the cell in a homotypic anti-parallel left-handed coiled-coil. This polymerization is required for the formation of paraspeckles, localisation of SFPQ to paraspeckles, binding of SFPQ to DNA and associated transcriptional regulation (M. Lee et al., 2015). It is evident that polymerization is reversible in solution, and thus is likely important in cellular functions (M. Lee et al., 2015).

The C-terminal region of SFPQ is important in mediating protein localization, an interaction between a part of the coiled-coil region and the final seven amino acids of SFPQ have been shown to mediate nuclear localization (Passon et al., 2012). SFPQ contains two weak nuclear localization signals (NLS) that are both essential for nuclear transport. The first is a C-terminal sequence similar to the classical NLS of the SV40 large T antigen and the second is sequence overlapping bipartite NLSs, where both NLSs are important for protein localisation in the nucleus (Dye & Patton, 2001).

Indeed, the C-terminus is essential for the interaction of SFPQ with other molecules, and there is flexibility within this domain. The C-terminus also contains PTM sites, where data from studies suggest phosphorylation of T687 is required for SFPQ to interact with the regulatory partner TRAP150, thus altering protein function (Heyd & Lynch, 2010). Interestingly, deletion of the final C-terminal amino acids demonstrates

that TRAP150 does not bind directly with T687, but phosphorylation of this site functions as a regulatory switch to enable interaction (Heyd & Lynch, 2010).

1.6.2 Post-Translational Modifications of SFPQ

All cellular proteins are regulated by post-translational modifications (PTMs). In recent years, it has become evident that in addition to phosphate groups, methyl groups are also important in terms of controlling elements in protein function (Clarke 1993). The RGG motif of SFPQ can be mono or di-methylated (Ong, Mittler, & Mann, 2004; Snijders, Hung, Wilson, & Dickman, 2010), which promotes mRNA binding via an unknown mechanism (Snijders et al., 2015). Interestingly, *in vitro* deamination of SFPQ prevented RGG methylation and reduced interaction with mRNA, emphasising a dynamic control of SFPQ functionality regulated by methylation (Snijders et al., 2015).

Despite this key role for protein methylation in SFPQ function, unsurprisingly phosphorylation is the most widely described modification of SFPQ. Emerging evidence from proteomic studies have revealed numerous phosphorylation sites within the RGG motif of SFPQ (Hornbeck et al., 2015). One example of SFPQ regulation via phosphorylation is phosphorylation of serine 8 and 283 by the MAP kinase interacting kinase MNK (Buxade, Morrice, Krebs, & Proud, 2008). Both these sites can be found at a location within or adjacent to the domains associated with binding of nucleic acids: S8 (within RGG) and S283 (within PR linker) (Buxade et al., 2008). Data shows that MNK phosphorylation increases SFPQ binding ability to the the 3′UTR of TNFα (tumor necrosis factor α), suggesting that MNK acts as novel substrate of SFPQ and modulates the binding of SFPQ to specific mRNAs (Buxade et al., 2008).

Signal-induced alternative splicing is an important, yet poorly understood mechanism for mediating protein isoform expression due changes in cellular surroundings (Lynch, 2007; Shin & Manley, 2004). In humans, signal-induced splicing regulation is required for neuronal depolarization, insulin signalling and T cell activation (An & Grabowski, 2007; Chalfant et al., 1995; J. A. Lee et al., 2007). A well characterised example of signal-induced splicing regulation is CD45, a transmembrane tyrosine phosphatase which encodes around five isoforms as a result of highly regulated alternative splicing (Hermiston, Xu, Majeti, & Weiss, 2002). For the immune system to function properly, signal-induced alternative splicing of the *CD45* gene in human T cells is required. Data shows that in resting T cells SFPQ is phosphorylated via glycogen synthase kinase 3 (GSK3), thereby aiding the interaction of SFPQ with TRAP150, which in turn supresses SFPQ from binding to CD45 pre-mRNA (Heyd & Lynch, 2010). Once T cells are activated, a decrease in GSK3 results in reduced SFPQ phosphorylation, thus relieving SFPQ from TRAP150 and allowing it to bind CD45 splicing regulatory elements and inhibit exon inclusion. Overall, this study demonstrates signalling pathways which link T cell receptor involvement with SFPQ-regulated exclusion of alternatively spliced CD45 exons and have identified GSK3 and TRAP150 as two vital mediators of this pathway (Heyd & Lynch, 2010).

Moreover, the oncogenic fusion tyrosine kinase nucleophosmin/anaplastic lymphoma kinase (NPM/ALK) stimulates cellular change in anaplastic large-cell lymphomas (ALCLs), occupying the t (2;5) chromosomal translocation (Bonvini, Gastaldi, Falini, & Rosolen, 2002; Coluccia et al., 2005; Pulford, Morris, & Turturro, 2004). In NPM/ALK, protein-protein interactions are required to turn on downstream signalling pathways (Coluccia et al., 2005; Ouyang et al., 2003). In this study, the authors revealed novel NPM/ALK-associated proteins which promote oncogenic transformation. They utilised

proteomic approaches and identified numerous RNA/DNA-binding proteins which coimmunoprecipitated with NPM/ALK, including SFPQ. The association between NPM/ALK and SFPQ was dependent on the activated ALK kinase domain and SFPQ was shown to be tyrosine-phosphorylated in NPM/ALK-expressing cell lines (Galietta et al., 2007). *In vitro* experiments reveal SFPQ is phosphorylated at tyrosine 293 within the PR linker by kinase domain from ALK, resulting in SFPQ localization to the cytoplasm. Consequently, mis-localisation of SFPQ might alter SFPQ nuclear-associated functions, and it is likely that SFPQ may be implicated in the oncogenic activity of NPM/ALK. Conversely, overexpression of SFPQ supressed cell proliferation and increased apoptosis in cells expressing NPM/ALK. This study highlights the significance of SFPQ as a novel NPM/ALK-binding protein and it is plausible SFPQ function may be abrogated in NPM/ALK-transformed cells (Galietta et al., 2007).

1.7 SFPQ cellular functions

The multi-domain structure of SFPQ enables the protein to regulate a plethora of cellular mechanisms. Intriguingly, in many cases the role of SFPQ in these processes can be modulated via binding of lncRNAs.

1.7.1 RNA processing

Extensive proteomic studies have shown that SFPQ is a key component of pre-catalytic spliceosome complexes (Ajuh et al., 2000; Jurica & Moore, 2003; Makarov et al., 2002). Moreover, biochemical analysis suggests a role for SFPQ in the second catalytic step of splicing of some, (Gozani, Patton, & Reed, 1994) but not all, (Lindsey, Crow, & Garcia-Blanco, 1995) pre-mRNA substrates. Furthermore, it appears that SFPQ also regulates alternative splicing in a variety of different cell backgrounds. This includes cluster of differentiation 45 (CD45) and Tau transcripts, where in the case of

Tau, SFPQ directly binds at the exon–intron boundary downstream of exon 10, resulting in the exclusion of this exon from the final mRNA (P. Ray et al., 2011). Examples of SFPQ promoting exon inclusion have also been reported. For example, SFPQ stimulates the neural-specific inclusion of the N30 exon of non-muscle myosin heavy-chain II-B, through mediating the binding of the splicing regulator RNA-binding protein, fox-1 homolog 3 (Rbfox3) to the substrate pre-mRNA via protein–protein interactions (K. K. Kim, Kim, Adelstein, & Kawamoto, 2011). Finally, SFPQ has been implicated in development of human disease via aberrant-splicing of SMN2 (survival of motor neuron 2) in neuroblastomas cells, which results in neurologic pathology (Cartegni & Krainer, 2002; Z. Jiang, Cote, Kwon, Goate, & Wu, 2000).

mRNA biogenesis in eukaryotic cells is a coordinated multi-step process, which includes the addition of a 5′-methyl cap, and the splicing and polyadenylation of the 3′-end (Colgan & Manley, 1997; Wahle & Keller, 1996). SFPQ has been implicated in 3'-end processing and forms part of the SF-A complex that also contains the U1A protein (Lutz, Cooke, O'Connor, Kobayashi, & Alwine, 1998; O'Connor, Alwine, & Lutz, 1997). Specifically, SFPQ has been implicated in alternative polyadenylation of the cyclooxygenase (COX) enzyme, COX2, via regulation of upstream auxiliary upstream sequence elements (USEs) integral for polyadenylation (Hall-Pogar, Liang, Hague, & Lutz, 2007). How exactly SFPQ is driving these mechanisms remains unclear, with current models favouring stabilisation or recruitment of the basal polyadenylation machinery to pre-mRNA transcripts.

The mammalian nucleus is highly organized in terms of structure. Individual chromosomes reside in discrete territories, and nucleic acids and specific proteins are enriched in subnuclear structures including nucleoli, Cajal bodies, nuclear speckles

and paraspeckles (Cremer & Cremer, 2010; Cremer et al., 2006; Platani & Lamond, 2004). The organization of the nucleus is important in maintaining the genome and controlling gene expression, and thus impacts on development, growth and cellular proliferation. By extension, aberrations in nuclear organization are associated with diseases, for example, in acute promyelocytic leukaemia (PML), loss of subnuclear promyelocytic leukaemia bodies is observed (Weis et al., 1994). Paraspeckles are dynamic structures, they are not present in human embryonic stem cells and only form upon differentiation (L. L. Chen & Carmichael, 2009). They are 0.5–1.0µm in diameter and vary in number, depending on cell type (Cardinale et al., 2007; C. M. Clemson et al., 2009; Fox et al., 2002). Paraspeckles are comprised of a small number of proteins, including PSPC1, P54NRB/NONO and SFPQ, with the highly expressed P54NRB/NONO and SFPQ being important for paraspeckle integrity (Sasaki, Ideue, Sano, Mituyama, & Hirose, 2009).

In addition to the proteins above, paraspeckle formation is dependent on the lncRNA, *NEAT1*. The *NEAT1* gene has two isoforms: *NEAT1v1* (3.7 kb) and *NEAT1v2* (23 kb) (Hutchinson et al., 2007). *NEAT1v2* appears to exert a greater effect in formation of paraspeckles, compared to *NEAT1v1* (Sasaki et al., 2009), via interaction with a SFPQ/NONO heterodimer (R. Peng et al., 2002; Sasaki et al., 2009). Several biological functions have been linked to paraspeckles, including cellular differentiation, RNA metabolism, gene regulation, miRNA processing, stress response via nuclear retention of mRNAs, developmental processes and disease (Carter et al., 1993; Cornelis, Souquere, Vernochet, Heidmann, & Pierron, 2016; Fox et al., 2002; Fujimoto et al., 2016; L. Jiang et al., 2017; Nakagawa, Naganuma, Shioi, & Hirose, 2011; Nakagawa et al., 2014; Standaert et al., 2014). Nuclear retention occurs due to adenosine (A) to inosine (I) editing, a reaction catalysed via the RNA editing enzyme,

ADAR (Adenosine De-Aminase RNA-specific), which binds specifically to double stranded RNA (Hundley & Bass, 2010). The deamination A-to-I results in hyper inosine-containing-RNA that bind to SFPQ with high affinity, obstructing edited-mRNA export to the cytoplasm (L. L. Chen & Carmichael, 2009). The essential role of SFPQ in this process is evident, as paraspeckle-disruption via depletion of *NEAT1* also causes mislocalisation of SFPQ and increased export of mRNAs (L. L. Chen & Carmichael, 2009).

SFPQ also functions during cytoplasmic internal ribosome entry site (IRES) mediated translation (H. A. King et al., 2014; Sharathchandra, Lal, Khan, & Das, 2012). IRESs are complex secondary or tertiary RNA structures that enable ribosome assembly in the absence of a 5'methyl cap. SFPQ is involved in IRES-mediated translation of apoptosis-related transcripts during TRAIL (TNF-related apoptosis-inducing ligand) induced apoptosis. Specifically, redistribution of SFPQ to the cytoplasm in response to TRAIL-induced apoptosis positively correlates with IRES-activity of apoptotic-regulated genes. This suggests that cytoplasmic localisation of SFPQ may have a role in the cellular response to damage, stress, and signals induced by TRAIL mediated apoptosis (H. A. King et al., 2014) .

1.7.2 Transcriptional regulation

Whilst SFPQ was identified as an RNA-binding protein and as discussed above, functions in a range of RNA processing mechanisms, it is also able to bind DNA. Such DNA- and RNA-binding proteins (DRBP) are rare, with around ~60 proteins falling into this category (Binns et al., 2009). SFPQ is known to act both as a positive and negative regulator of transcription. For example, SFPQ associates with a type of distant enhancer termed a tandem sequence motif and positively regulates expression of

ribosomal protein genes, including 60S ribosomal protein L18 (RPL18) (Roepcke et al., 2011). Furthermore, SFPQ and the p54nrb/NONO complex regulate transcription by bridging interaction between RNA Pol II and other nuclear proteins. Specifically, SFPQ and p54nrb/NONO interact with the RNA Pol II C-Terminal domain (CTD) and facilitate recruitment of other splicing and/or polyadenylation factors, such as SR family proteins, snRNP components and U2AF65 to transcriptional start sites (TSS) (Emili et al., 2002; Rosonina et al., 2005). Conversely, SFPQ in its homodimer or heterodimer form has also been reported to function as a transcriptional repressor. Emerging evidence from various studies demonstrate that SFPQ directly interacts with promoters of target genes and facilitates the recruitment of epigenetic silencers, such as Sin3A and HDAC (X. Dong, Shylnova, Challis, & Lye, 2005; X. Dong, Sweet, Challis, Brown, & Lye, 2007; X. Dong et al., 2009). Upon recruitment of epigenetic regulators, SFPQ binds to various hormone receptors, such as thyroid and retinoid X receptors (Mathur, Tucker, & Samuels, 2001), or forms complexes with steroidogenic factor 1 (SF-1), repressing the human *CYP17* gene and genes involved in circadian rhythms (Duong, Robles, Knutti, & Weitz, 2011; Sewer et al., 2002; Sewer & Waterman, 2002a, 2002b).

1.7.3 Apoptosis

Apoptosis is important for all multi-cellular organisms during development to remove damaged cells, control cell proliferation and maintain tissue homeostasis (Kerr, Wyllie, & Currie, 1972). There are two molecular pathways that govern apoptosis: the extrinsic pathway and the intrinsic pathway (Cryns & Yuan, 1998).

Furthermore, peroxisome proliferator-activated receptors (PPARs), specifically PPARγ, and their ligands have shown to demonstrate activity in both apoptotic

pathways. Interestingly, SFPQ is able to influence apoptosis via an interaction with PPARγ, a nuclear receptor involved in the regulation of cell proliferation and apoptosis. The RB activates PPARγ, resulting in the arrest of cells at the G1 phase of the cell cycle. Furthermore, PPARγ-mediated control of the cell cycle and apoptosis is dependent on histone deacetylase 3 (HDAC3) acting on PPARγ. Thus, PPARγ controls cell proliferation and apoptosis in an RB dependant manner (Fajas et al., 2003). Studies working with CRC cells lines (HT-29) which express high-levels of PPARγ demonstrated that knock-down of SFPQ leads to the loss of the autophagy marker, LC3B (microtubule-associated proteins 1A/1B light chain 3B), and an increase in apoptosis activity via caspase-3. This appears to be dependent on the expression of PPARγ, as the same effect was not observed in DLD-1 cells, which express low levels of this protein. Indeed, data from these studies provide evidence that SFPQ may function in regulating cell death in some colon cancer cells and is depended on the levels of PPARγ (T. Tsukahara, H. Haniu, & Y. Matsuda, 2013; Tsukahara, Matsuda, & Haniu, 2013)**.**

During apoptosis, the changes in sub-cellular localisation of SFPQ have been described in various studies. Interestingly, during apoptosis there is a reduction in nuclear SFPQ due to hyperphosphorylation at the N-terminus on serine and threonine residues, which results in an altered protein interaction profile that includes binding to U1-70K and SR proteins (serine/arginine-rich splicing factor) due to epitope masking (Shav-Tal et al., 2001). How these interactions contribute to apoptosis (if at all) is not currently understood (Shav-Tal et al., 2001).

LncRNA expression has been found to be derailed in various cancers. Some lncRNA have been reported as negative regulators of apoptosis in different tumours. For example, the *HOXA-AS2* gene resides between *HOXA3* and *HOXA4* genes on the

antisense strand (Rinn et al., 2007; X. Zhang et al., 2009). Its transcript is expressed in neutrophils and in human promyelocytic leukaemia cell lines (NB4). Zhao and associates showed the lncRNA *HOXA-AS2* utilises both the intrinsic and extrinsic pathway to suppress apoptosis in promyelocytic leukaemia (Hang Zhao, Zhang, Frazão, Condino-Neto, & Newburger, 2013). They observed NB4 cells treated with *trans* retinoic acid (ATRA) stimulate apoptosis by activating caspases. Silencing of *HOXA-AS2* resulted in ATRA-induced apoptosis. Additionally, increased expression of BAX confirmed the involvement of the intrinsic apoptotic pathway. Interestingly, another study noted an increase in TRAIL in *HOXA-AS2* knockdown cells treated with ATRA. This therefore shows the involvement of the extrinsic apoptotic pathway, as *HOXA-AS2* negatively regulates ATRA-induced TRAIL production, making it plausible that *HOXA-AS2* may directly affect the transcription of the TRAIL gene (Altucci et al., 2001). Another example comes from the lncRNA AFAP1-AS1, transcribed from the antisense strand of the *AFAP1* coding gene locus, found to be upregulated in esophageal adenocarcinoma tissues and cell lines (W. Wu et al., 2013). Knockdown of *AFAP1-AS1* stimulated apoptosis in esophageal adenocarcinoma. Moreover, cell cycle analysis demonstrated that knockdown of *AFAP1-AS1* promotes G2/M-phase arrest. Together, this study reveals *AFAP1-AS1* is able to mediate both apoptosis and proliferation in esophageal cancer cells (W. Wu et al., 2013). Furthermore, the melanoma associated lncRNA *SPRY4-IT1* has demonstrated anti-apoptotic effect. Khaitan and colleagues showed silencing of *SPRY4-IT1* in melanoma cell line WM1552C, lead to increased annexin V positive cells. However, no changes were observed in propidium iodide-positive cells, suggesting that silencing *SPRY4-IT1* stimulates cell death via apoptosis and not necrosis. Remarkably, the authors noted

SPRY4-IT1 predominately localised to the cytoplasm and suggest that *SPRY4-IT1* acts as a sponge for proteins or RNAs (Khaitan et al., 2011).

1.7.4 DNA damage repair

Among DNA damage events, double-strand breaks (DSBs) represent the most deleterious lesions. The most common route by which DSBs are repaired is non-homologous end joining (NHEJ), which is an efficient but error-prone process that requires DNA-dependent protein kinase (PK) and DNA ligase in complex with XRCC4 to repair the breaks. An alternative repair route exists that utilises homologous recombination (HR) and involves the RAD51 complex, which enables strand invasion of the homologous sister chromosome and subsequent template-mediated repair (Shrivastav, De Haro, & Nickoloff, 2008; Sonoda, Hochegger, Saberi, Taniguchi, & Takeda, 2006). Intriguingly, several studies have shown a direct role for SFPQ in the recognition and repair of DNA DSBs (Ha et al., 2011; Morozumi et al., 2009; Rajesh, Baker, Pierce, & Pittman, 2011; Salton, Lerenthal, Wang, Chen, & Shiloh, 2010). The process involves direct binding of SFPQ to DSBs via the RGG box and proline-rich domains present in the N-terminal region of the protein (Ha et al., 2011; Morozumi et al., 2009; Salton et al., 2010). Moreover, the N-terminal region of SFPQ is crucial for association with and activation of the recombinase RAD51D (DNA repair protein RAD51 homolog 4) (Morozumi et al., 2009; Rajesh et al., 2011), which triggers HR by initiating strand invasion (Morozumi et al., 2009). SFPQ has also been shown to function in NHEJ via recruitment of p54nrb/NONO to DSB, which results in the assembly of additional machinery involved in NHEJ. These data are supported by studies demonstrating that loss of SFPQ results in delayed DSB repair, increased frequency of sister chromatid cohesion defects and the accumulation of cells in S

phase that exhibit chromosomal instability and sensitivity to DNA-damaging agents (Rajesh et al., 2011; Salton et al., 2010).

1.7.5 SFPQ as a tumour suppressor

Given its role in DSB repair and evidence that SFPQ binds and represses the transcriptional activity of proto-oncogenes, SFPQ has previously been described as a tumour suppressor (TS) protein (Gozani et al., 1994; J. G. Patton et al., 1993).

The Insulin-like growth factor-I (IGF-I) is a trophic factor found at high levels in the bloodstream and regulates many of the effects of growth hormones (GHs) in the body (Adashi, 1992; Laron, 2001). IGF-I is derived from granulosa cells, and has been assigned various functions, such as activation of steroidogenesis, and increased immunoprecipitable P-450 cholesterol side-chain cleavage (P450scc) protein levels and mRNA concentrations (Hammond, Mondschein, Samaras, & Canning, 1991; Urban, Garmey, Shupnik, & Veldhuis, 1990; Veldhuis, Rodgers, Dee, & Simpson, 1986). The rate limiting enzyme in the steroidogenic pathway, P450scc, is responsible for conversion of the C_{20}–C_{22} bond that releases the C_{22}–C_{27} side chain of cholesterol (W. L. Miller, 1988). P450scc contains an insulin-like growth factor response element, IGFRE (~30-bp), which regulates IGF-1 induced gene expression and forms a complex with ubiquitous transcription factor Sp1. In this study, the authors utilised micro sequence analysis and sequence-specific DNA affinity chromatography on HeLa cell nuclear extract protein and found SFPQ to bind P450scc IGFRE. Next, they determined the functional significance of SFPQ binding to the IGFRE using expression vectors in porcine granulosa cells in transfection experiments. They found that Sp1 induces, and SFPQ suppresses transcriptional activity of the porcine P450scc IGFRE. However, due to the complicated interactions of SFPQ and Sp1 in the porcine P450scc

IGFRE, the authors were not able to determine the mechanism (Urban, Bodenburg, Kurosky, Wood, & Gasic, 2000).

In melanoma, SFPQ's tumour suppressor role was determined using mouse models. Some elegant work by *Wang* and team demonstrated that binding of the mouse retrotransposon *VL30–1* lncRNA to SFPQ disrupts SFPQ mediated suppression of proto-oncogene *rab23* via its DNA-binding domain. A reduction in *VL30–1* lncRNA resulted in supressed proliferation and the converse was observed for high levels of mouse *VL30–1* RNA, which favoured tumorigenesis and proliferation both *in vitro* and *in vivo*, suggesting a possible role for SFPQ in the aetiology of MM (Song et al., 2002; G. Wang et al., 2009).

The absence of a human homologue of the mouse VL30-1 lncRNA prompted the search of a similar mechanism in humans. Research by *Wu et al* utilised an RNA-SELEX approach to enrich human RNAs interacting with SFPQ. Through subsequent cDNA library construction, gel electrophoresis and molecular assays, they identified a 1,600nt lncRNA termed *LLME23*. lncRNA *LLME23* is a SFPQ-binding lncRNA exclusively found in human melanoma cell lines, compared with other cancer cell lines, suggesting it may be important for melanoma pathology. (C. F. Wu et al., 2013). Functional experiments showed that *LLME23* expression reversed SFPQ-mediated suppression of *RAB23,* suggesting that *LLME23,* like *VL30-1,* derails SFPQ tumour-suppressive function (G. Wang et al., 2009). Indeed, increased expression of *LLME23* upregulated *RAB23* expression, whereas *LLME23* depletion led to down-regulated *RAB23,* implicating *LLME23* in melanoma progression (C. F. Wu et al., 2013). However, the authors were unable to identify a TSS for *LLME23* and it remains poorly annotated and absent from many public NGS datasets, raising doubt over the expression of this transcript *in vivo*. A similar lncRNA-mediated mechanism operates

in CRC, where *MALAT1* binds to SFPQ, disrupting its association with PTBP2 and relieving transcriptional repression of PTBP2 from the SFPQ/PTBP2 complex, consequently resulting in metastasis of CRC (Q. Ji et al., 2014).

1.7.6 SFPQ as an oncogene

Chromosomal translocations of SFPQ have been reported that lead to oncogenic fusion proteins, as seen in renal cell carcinoma and acute lymphoid leukaemia, where SFPQ is fused to the transcription factor TFE3 (Kuroda et al., 2012) or ABL kinase (Hidalgo-Curtis et al., 2008), respectively. It has been suggested that the dimerization and oligomerization properties of SFPQ are controlled by the kinase fusion to promote constitutive oncogenic kinase activity. Recently experimental data has found SFPQ on cell membranes in malignant cell lines of leukaemia patients, but the functional implications remain undefined (Ren et al., 2014).

Takayama et al demonstrates SFPQ contribution in the progression of prostate cancer. SFPQ may function as an oncogene due to increased SFPQ expression in prostate cancer cell lines and tumour samples compared with low expression in normal prostate cells. Evidence suggests that SFPQ may serve as potential target in treating CRPC (K.-I. Takayama et al., 2017a). *In vitro* silencing of SFPQ supressed cell growth in prostate cancer cells and *in vivo* silencing of SFPQ in mice castrated after tumour development resulted in reduced tumour growth. Additionally, decreased expression of SFPQ was shown to induce p53 and p21 proteins, where previous reports have shown SFPQ inhibited these cell cycle genes (K. Takayama et al., 2013). Furthermore, the authors identified global RNAs bound to SFPQ, such as lncRNA *CTBP1-AS*, and a prostate cancer-associated lncRNA *(SchLAP1),* which were positively mediated by SFPQ in prostate cancer cell lines. Interestingly, SFPQ is important in maintaining

AR mRNA stability and splicing, as CLIP signals were detected in intron and 3′-UTR regions of *AR* gene (K.-I. Takayama et al., 2017a). Various spliceosome genes were highly expressed in metastatic CRPC tissues and were identified as primary targets of SFPQ, suggesting this gene cluster is specifically mediated by SFPQ as an RNA-binding protein. Intriguingly, SFPQ may regulate the activation of various oncogenic pathways via controlling expression of spliceosome genes and interacting with these factors at protein level. Together, this study highlights SFPQ importance in the regulation of splicing machinery in prostate cancer (K.-I. Takayama et al., 2017a).

1.8 SFPQ: a promiscuous partner for lncRNAs

There is now emerging evidence that many SFPQ-mediated cellular functions are regulated in part by interactions with a diverse number of lncRNA that appear to be tissue and cell type specific. The full extent of the SFPQ-lncRNA interactome in these various cell types is currently unknown. Below, I discuss some of the reported SFPQ-lncRNA interactions and how these impact on cellular functions and contribute to disease phenotype.

MALAT expression is aberrantly increased in colorectal cancer (CRC), bladder cancer, non-small cell lung and hepatocellular carcinoma, compared with control tissue, suggesting a role in cancer development (L. Chen, Yao, Wang, & Liu, 2017; Fan et al., 2014; L. H. Schmidt et al., 2011; Haibiao Xie et al., 2017). In CRC, *MALAT1* promotes tumour progression and metastasis by competitively binding to the RNA binding domain of SFPQ, disrupting the interaction of SFPQ/*PTBP2* complex at the promoter region, leading to increased cell proliferation and migration. Furthermore, this study highlights SFPQ's critical role in mediating the effect of *MALAT1* on SFPQ/PTBP2 complex and the downstream cell proliferation and migration. As

silencing of *MALAT1* had a minimal effect on the SFPQ/PTBP2 complex and the amount of SFPQ-detached from PTBP2, thus, no increase in cell proliferation and migration was observed in CRC cell lines (Q. Ji et al., 2014).

A second example of SFPQ-lncRNA interaction driving cancer phenotype involves the lncRNA, *GAPLINC*. Human CRC cell lines HCT116, HT29, SW480, DLD-1, and SW620 abundantly expressed *GAPLINC* and CRC patients reported a positive correlation between increased expression of *GAPLINC* with tumour size, advanced tumour stage, advanced node stage increased mortality and shorter survival. Specifically, it appears that *GAPLINC* promotes CRC invasion by binding to SFPQ/NONO and partly inducing the expression of SNAI2, a *GAPLINC* associated gene known to play a role in epithelial to mesenchymal transition (EMT). Further research is required to determine the mechanisms by which SFPQ/NONO binds to *GAPLINC* and activates SNAI2 to promote invasion in CRC (P. Yang et al., 2016).

Interestingly, various studies have described involvement of the nuclear enriched abundant transcript 1 (*NEAT1*) lncRNA in regulating SFPQ multifunctional activities. *NEAT1* is necessary for nuclear body paraspeckles formation and is bound directly by SFPQ and p54nrb/NONO. *NEAT1* expression is stimulated through cellular stresses, including viral infection proteasome inhibition (Imamura et al., 2014; Song, Sui, & Garen, 2004). Consequently, SFPQ disassociates from target gene promoters, leading to abnormal upregulation of hundreds of SFPQ-dependent transcription targets (Hirose et al., 2014; Imamura et al., 2014). In response to influenza virus infection, *NEAT1* relocates SFPQ from the *IL8* promoter to the paraspeckles, leading to transcriptional activation of *IL8*, which is normally transcriptionally repressed by SFPQ (Imamura et al., 2014). Similarly, the mouse retrotransposon *VL30* RNA binds

and sequesters SFPQ, relieving SFPQ-dependent gene repression in mice (Song, Sun, & Garen, 2005; G. Wang et al., 2009).

Moreover, *NEAT1* is essential in the formation of paraspeckles, contributing in paraspeckle enlargement in various conditions, including viral infection and myotube differentiation. Luciferase reporter assays and immunofluorescent electron microscopy have revealed that paraspeckle elongation is due to up-regulation in expression of *NEAT1* and proteasome inhibition, rather than build up of degraded paraspeckle proteins. Furthermore, it was hypothesized that ADARB2 RNA-specific adenosine deaminase B2 expression is controlled by *NEAT1*-dependent sequestration of SFPQ. Microarrays in *NEAT1*-knock-down cells reveal *NEAT1* represses transcription of various genes, including *ADARB2*, and SFPQ is required for transcription of ADARB2. Consistent with these results, the authors also described expression of ADARB2, which is strongly reduced upon increased SFPQ sequestration by proteasome inhibition, due to concomitant reduction in SFPQ binding to the ADARB2 promoter. The findings of this study were able to confirm that paraspeckles are stress-responsive nuclear bodies and provide a model in which induced *NEAT1* controls target gene transcription by protein sequestration into paraspeckles (Hirose et al., 2014).

The androgen-responsive lncRNA *CTBP1*-AS is located within the AS region of the transcriptional corepressor gene, *CTBP1*. Androgen and its partner, the androgen receptor (AR), are key players in progression and proliferation of prostate cancer. Functional analysis studies revealed a novel sense–antisense mechanism for *CTBP1* repression by *CTBP1-AS*. *CTBP1* is directly repressed by *CTBP1-AS*, which interacts with SFPQ and histone deacetylases to repress transcription at the *CTBP1* promoter

(K. Takayama et al., 2013). Furthermore, *CTBP1-AS* possesses global androgen-dependent functions by inhibiting tumour-suppressor genes via this SFPQ-dependent mechanism, consequently promoting cell cycle progression. This suggests that lncRNAs are directly contributing to prostate cancer progression and it is tempting to suggest that similar SFPQ-lncRNA interactions may also be important for disease progression in other cancers (K.-I. Takayama et al., 2017a; K. Takayama et al., 2013).

A second study carried out global systematic analysis to determine the transcriptional targets and SFPQ interactors using castration-resistant prostate cancer (CRPC) model cells. Additionally, they investigated the mechanisms by which RNAs bind to SFPQ. The AR is also an important target of SFPQ in CRPC, where it has been shown to regulate numerous genes affecting the prostate cancer cells (C. D. Chen et al., 2004; Q. Wang et al., 2009). AR operates in ligand-dependent manner thus, androgen inhibition therapy is effective in decreasing tumour growth (Yuan, Cai, Chen, Yu, & Balk, 2014). Unfortunately, this approach does not always work, as patients develop resistance resulting in CRPC progression (C. D. Chen et al., 2004; Q. Wang et al., 2009). The variant AR-V7 is responsible for regulating distinct and androgen-independent activation of its downstream signals, which promotes the progression of CRPC (S. Sun et al., 2010). Therefore, AR and variant AR-V7 are suitable potential targets to treat CRPC. Utilising a deep-sequence–based approach, clinicopathological analysis and public database analysis to determine mechanistic insight, Prensner *et al* showed that the global RNAs bound with SFPQ. They discovered various miRNAs and lncRNAs which are targets of SFPQ. For example, *CTBP1-AS* and *SchLAP1* were upregulated in an SFPQ-dependent manner in prostate cancer cells. This mirrors a previous study that reported dysregulated expression of *SchLAP1* in metastatic prostate tumours (Prensner et al., 2013). Remarkably, this

study demonstrated that spliceosome genes are almost uniformly upregulated in metastatic CRPC tissues and showed that SFPQ interacts with other splicing factors and NONO in the intronic region of *AR* transcripts in order to control AR splicing, stimulating production of AR and its variants at the mRNA level (K.-I. Takayama et al., 2017a).

1.8 Aims

The underlying hypothesis of this PhD thesis is that SFPQ-RNA interaction may contribute to the development of MM by impacting metastatic phenotype. In this regard, the project aims are summarised as follows:

1. Characterise the oncogenic lncRNA signature in melanoma cells. This will be achieved by investigating lncRNA expression via RT-qPCR in a range of melanoma cell lines and primary melanocytes. Expression level will then be correlated with migration potential of each cell line to establish if a relationship exists that might be utilised for prognostic analysis.
2. Determine the SFPQ-RNA interactome in a melanoma cell background compared with primary human melanocytes via RIP-seq and analyse these data to establish the functional nature of SFPQ interactors via gene ontology analysis.
3. Investigate the functional role of SFPQ and novel melanoma cell-specific SFPQ-lncRNA interactors in melanoma cell growth via functional knockdown and cancer phenotypic assays.

Chapter 2: Materials and Methods

2.0 Materials and methods

2.1 Cell culture

All cell lines, unless stated otherwise, were obtained from European Collection of Authenticated Cell Cultures and certified mycoplasma-free. Immortalised cell lines were maintained in either Roswell Park Memorial Institute 1640 Medium (Sigma-Aldrich, UK), Dulbecco's Modified Eagle Medium (DMEM) or MEM α (Minimum Essential Medium) (Sigma-Aldrich, UK), as indicated in Table 2.1 Growth media were supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher, UK) and 100 U/ml of penicillin and 100 µg/ml of streptomycin (Life Technologies, UK). Primary melanocytes (CELLnTEC, Switzerland) were grown in a mixture (2:1) of keratinocyte serum free media (K-SFM) and Eagle's minimal essential medium (EMEM) (Sigma-Aldrich, UK). K-SFM was supplemented with 25 µg/ml bovine pituitary extract (BPE), 0.2 ng/ml rEGF, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine (Sigma-Aldrich, UK). EMEM was supplemented with 2% FBS, 1× nonessential amino acids, Primocin (100 U/ml)/streptomycin (100 µg/ml) (InvivoGen, UK), 2 mM l-glutamine, 5 ng/ml basic fibroblast growth factor (Tebu-bio, Europe) and 5 ng/ml endothelin-1 (Sigma, Dorset, UK). All Cell lines were incubated at 37°C in a 5% CO_2 atmosphere and routinely passaged at 70-80% confluency.

Table 2.1 Cell lines used in this study.

Cell line	Growth media
M14	RPMI
A2058	DMEM
UACC-62	MEM
A375	MEM
Primary Melanocytes	K-SFM and EMEM (2:1)
SKMEL-28	RPMI

2.2 Oligonucleotides

All primers were supplied as desalted 0.25 µM (SIGMA-ALDRICH, UK) (Table 2.2). Primer pairs were prepared at a stock concentration of 10 µM for use in qRT-PCR reactions. Primers in Table 2.2 were optimised by setting up numerous serial dilutions across a temperature gradient which determined optimum annealing temperatures, melt curves were used to confirm specificity. Furthermore, qPCR efficiency was obtained by generating standard curves.

Table 2.2 Oligonucleotides used during this study

LncRNA	Sense	Antisense
GAPLINC	ACACACAGCAGCCTGGTTTC	ATGGCACAATCAGGGCTCTT
CTBP-As	AACCTGGCAGCACGGAAGT	GAGCACAACCACCACCTCATC
SPRY4-IT1	GCTGAGCTGGTGGTTGAAAGGAATC	GCTTGGCCCACGATGACTTG
BANCR	ACAGGACTCCATGGCAAACG	ATGAAGAAAGCCTGGTGCAGT
MALAT1	GGATTCCAGGAAGGAGCGAG	ATTGCCGACCTCACGGATTT
ANRIL	CAACATCCACCACTGGATCTTAACA	AGCTTCGTATCCCCAATGAGATACA
RMEL3	ATGTGCTCCAAGAAAACCAGAG	CTTTGTCACAGGAATACCCAAC
SLNCR1	GAAAGAGGATGGGAAGGACTG	ATCAAATCCAGAGCTCCTGC
SAMMSON	TTCCTCAACTATGCAACTCAA	TAGACTACGGGCTCATGACTT
HOTAIR	CAGTGGGGAACTCTGACTCG	GTGCCTGGTGCTCTCTTACC
UCA1	CATGCTTGACACTTGGTGCC	GGTCGCAGGTGGATCTCTTC
LLME23	TTTGACACGAGTGACTGTATTTTGAA	ATTTATGAATTGTCACAGGACCTCT
R18s	TGAGAAACGGCTACCACATC	TTACAGGGCCTCGAAAGAGT
RPS13	GGTTGAAGTTGACATCTG	ATCTGTGAAGGAGTAAGG
GAPDH	CGGAGTCAACGGATTTGGTC	GGATCTCGCTCCTGGAAGATG
EMX2OS	AGTGTATAGAACGGCTTA	ATGATAGTGAGTGAGTGA
FENDRR	GACTGCGAATATCTGTTG	GATAGGAAGGAGAGTTGAT
EGFR-AS1	GCTTGCTTACCTTGTTAT	TCTGATGTCTCTGTTCTTA
RMDN2	TCATTTCGTGTCATTCTT	ATGGTTCTGTGTAATCTC
VCAN-AS1	CTGACATACATCCATCTG	TGCTTCACATAGTAGGTT
LINC00602	TTTCCTCTTCCCATTCATA	CATCCATCCATTCATTGC
LINC00511	TATAATGCCTAACACAAC	TGGATATAAATATACATAGTCA
TMEM51-AS1	ATATCGTTAGTCTGATTATTCCT	CACACTTCCTCAACTCTC
LINC01291	TTCCGCATTCATCACCTT	GATTCTTCATAGAGTCCAGACA
LINC02616	ATTCAGCCTAATACATCACT	TTGCCAGTTAGTTCTTGA
DUXAP8	CCTCATCAATACCTTCACTCA	CTGGATTCTGGACTCTTCTG
FOXD2-AS1	CTCACCCTACTCCCATTC	AAAGCAAACATAAGCGAAGA
SFPQ	TGGACAACAGAGCGAGAC	AACAGAAGTAGCACAAGGAGAT
NEAT1	AAGTTCTTAGCCTGATGA	TTAGCACAACACAATGAC

MEG3	TGGCATAGAGGAGGTGAT	AGACAAGTAAGACAAGCAAGA
LOC105378644	GGCTAACATAAGAGACCAAT	CCAGATGCTTGAACTTGT
LINC00689	TTCAGAAGATGCCAGTTACTAC	TAAGGTCTCCAGCGTTCA
HMMR-AS1	TTCAGAAGATGCCAGTTACTAC	TAAGGTCTCCAGCGTTCA
GRPEL2-AS1	GAGGTGGCACATAATGAA	ATGGTAAGGACTTCGTAAG
LINC00702	GTCACCTCTAACCTTCCA	AATCATCTTAGCAGTCATTGT
MTOR-AS1	GCTTGAGAGGAGGTATTATC	ACTTGGTCTTTCTGGTAAC
LINC00622	TCTGAGTGGAACCGATAA	CAAGCGAATGTTATGTCAAT
GATA2-AS1	TATGCCAAAGGTAGTATT	TAATCCATCTGAAGTTGA
LINC00200	ATGAATGACAGAATAAGAGTA	TACACAACACAGATATGC
SOX10	CAGTGGTATTTGAATAAAGTATG	CAGGAGACAGTAATGAGTT
AMIGO2	TTCTGGATTCTGAGTGGATTC	TGCTGGTGATGTTGTTATGA
MAGEA3	GCAGTCAGCATTCTTAGT	CTCATTCAACCATCCGTTA
R18s	TGAGAAACGGCTACCACATC	TTACAGGGCCTCGAAAGAGT
RPS13	GGTTGAAGTTGACATCTG	ATCTGTGAAGGAGTAAGG
GAPDH	CGGAGTCAACGGATTTGGTC	GGATCTCGCTCCTGGAAGATG

2.3 Transfections of immortalised melanoma cell lines

Cells were seeded into 24-well plates at a density of ~1.6×10^5 and cultured at 37˚C and 5% CO_2 until 70-90% confluence. Cells were transfected with LNA GapmeR antisense oligonucleotide (ASO) at a final concentration of 5 nM (QIAGEN, USA) using HiPerFect transfection reagent (QIAGEN, UK) (see Table 2.3). GapmeRs were diluted in 100 µl of serum free media (SFM) and 3 µl of HiPerFect, samples were mixed and incubated for 7 minutes at room temperature. Transfection reactions were added in a dropwise manner onto cells and plates were gently swirled to ensure uniform distribution prior to incubation for 24-72 hours, depending on experiment.

Antisense LNA GapmeRs oligonucleotides were designed by taking the primary transcript of each target of interest from ENSEMBL and then run with Qiagen online LNA GapmeR design tool. This service was provided by (Qiagen, USA).

Table 2.3 Specifically, designed GapmeRs sequence for each target

ASO GapmeRs	Primer Sequence
Antisense LNA GapmeR Control	LG00000002-DDA
MALAT1	339515 LG00000003-DDA
LINC01234	LG00232526-DDA LG00232527-DDA
LINC00511	LG00235729-DDA LG00235730-DDA
SFPQ	LG00232595-DDA LG00232596-DDA

2.4 SiRNA SFPQ knockdown

Cells (~1.6x10^5) were seeded onto a 24-well plate and incubated at 37˚C for 24 hours. The following day transfection mixes were prepared in 1.5 ml Eppendorf's, with either 37.5 ng of SFPQ SiRNA (FlexiTube siRNA, Qiagen, UK) or with MISSION® siRNA Universal Negative Control at final concentration 5 nM (ThermoFisher, UK). These siRNAs were mixed with 12 µl of HiPerFect diluted in 100 µl of SFM. Transfection mixes were gently vortexed and incubated at room temperature for 10 minutes. Next, transfection mixes were added in a dropwise manner were gently swirled and incubated for 24-72 hours, depending on experiment.

2.5 Wound Healing Assay

Cells were seeded into 6-well plates at a density of ~4x10^5 and incubated at 37˚C and 5% CO_2 until they reached 80-90% confluence. Cells were then serum starved and incubated for a further 24 hours. To generate the wound a 200 µl pipette tip was used

to create a vertical scratch through the cell monolayer. Growth media was then discarded, and cells carefully washed twice with 1 ml of PBS prior to addition of 2 ml of serum-free media. Several images of each wound were captured at 0h, 6h, 12h and 24h (EVOS XL Core Cell Imaging System, 4x objective). The area of wound closure was subsequently measured using the MRI wound healing tool and image J software. (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool).

2.6 Total RNA extraction

Total RNA was extracted from ~$1x10^6$ cells using the Aurum Total RNA Mini Kit (BIO-RAD, UK). Briefly, cells were washed in 1 ml of PBS and resuspended in 350 µl of lysis buffer by pipetting up and down several times, prior to the addition of 350 µl of 70% ethanol and further mixing by pipetting. Next, lysates were transferred to an RNA binding column placed inside a 2 ml collection tube and centrifuged at 12000g for 30 seconds. Flow through was discarded and 700 µl of low stringency solution was added to column and centrifuged at 12000g for 30 seconds. 40U of DNase I solution was added carefully to the centre of column and incubated at RT for 15 minutes. The column membrane was washed by consecutive addition of 700 µl of high stringency solution and centrifugation at 12000g followed by 700 µl of low stringency solution and centrifugation at 12000g for 30 seconds. The column was then centrifuged for an additional 60 seconds to remove residual wash solution and transferred to a nuclease free 1.5 ml microcentrifuge tube. RNA was eluted from the column by addition of 40 µl of elution buffer to the centre of column, followed by incubation at RT for 1 minute before centrifuging for 2 minutes at 12000g at RT. Isolated RNA was routinely stored at -80˚C.

2.7 Turbo DNA Free RNA

Ambion® TURBO DNA-free™ DNase Treatment kit (ThermoFisher, UK) was used to remove contaminating DNA from RNA samples according to the manufacture's guidelines. Briefly, 0.1 volume of 10x TURBO DNase Buffer and 1 µl of TURBO DNase was added to the RNA in a 1.5 ml RNA-free Eppendorf, samples were gently mixed and incubated at 37˚C for 30 minutes. DNase inactivation reagent 0.1 volume was added to samples which were mixed well and incubated for a further 5 minutes at room temperature. Finally, samples were centrifuged at 10,000g for 1.5 minutes, RNA was transferred to a fresh tube and stored immediately at -80˚C.

2.8 Quantification of RNA

RNA concentrations were measured using NanoPhotometer P330 (Implen GmbH, Munich, Germany). The instrument was programmed to measure the concentration at 0.2 mm path length using Lid 50, 1 µl of reference sample (elution buffer) was used as a blank. Concentration of RNA was measured and recorded as ng/µl. A260/280 was also recorded for each sample, as an indication of RNA purity.

2.9 First strand cDNA synthesis

cDNA was generated using iScript™ gDNA Clear cDNA Synthesis Kit (BIO-RAD, UK). Briefly, DNase master mix was prepared by combining 0.5 µl of iScript DNase and 1.5 µl of iScript DNase buffer, 500 ng of RNA and nuclease-free water up to 16 µl. Next, first strand synthesis reaction mix was prepared by adding 4 µl iScript Reverse Transcription Supermix to 16 µl of DNase-Treated RNA template. For no reverse transcriptase (NRT) negative control reactions, 1 µl of nuclease free water and iScript No-RT Control Supermix was used. Reactions were briefly mixed and incubated in a CFX Connect Real-Time PCR Detection System (BIO-RAD, UK) as follows:

DNase Reaction:

1. DNase Digestion at 25˚C for 5 minutes.
2. DNase Inactivation at 75˚C for 5 minutes.
3. Store at 4 ˚C or on ice until RT-Step.

cDNA synthesis

1. Priming at 25˚C for 5 minutes.
2. Reverse Transcription at 45˚C for 20 minutes.
3. Reverse Transcription inactivation at 95˚C for 1 minute.

All cDNA samples were diluted 5-fold prior to being stored at -20˚C.

2.9.1 First Strand synthesis for RNA-IP

cDNA was generated using iScript Select cDNA Synthesis Kit (BIO-RAD, UK). Briefly, first strand synthesis reactions were set up in nuclease-free PCR tubes using 4 µl of 5x iScript select reaction mix, 2 µl random primers or of Oligo(dT)20, 500 ng of total RNA,1 µl iScript reverse transcriptase and nuclease-free water made up to 20 µl. For no reverse transcriptase (NRT) negative control reactions, 1 µl of nuclease free water was used instead of reverse transcriptase. Reactions were incubated as follows, Oligo(dT)20 primers: 1. 42˚C for 30 minutes 2. 85˚C for 5 minutes. Finally, with random primers: 1. 25˚C for 5 minutes 2. 42˚C for 30 minutes 3. 85˚C for 5 minutes. All cDNA samples were stored at -20˚C.

2.10 Quantitative Real-Time PCR

Mastermix (MM) qPCR reactions were set up using 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD, UK), forward and reverse primers (300 nM each) (Table 2), 3.4 µl H_2O and 1 µl of cDNA. All reactions were carried out in triplicate in

CFX96 Real-Time system (Bio-Rad, UK). Optimal annealing temperatures for each primer pair were empirically determined, general qRT-PCR cycle conditions were:

- Initial denaturation and polymerase activation at 95°C for 30 seconds, cycle 1.
- Denaturation at 95ºC for 5 seconds, followed by annealing and plate read at 60ºC (or optimum temperature) for 30 seconds (40 amplification cycles).
- 65-95°C (+0.5°C increments, 5 seconds/increment) with plate read for melting curve.
- Data for qPCR was analysed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA). Gene expression was analysed using ΔΔCq (normalised expression) method (Livak & Schmittgen, 2001; Vandesompele et al., 2002).

2.11 Total protein isolation

Adherent cells were washed once in PBS prior to the addition of ice-cold Radioimmunoprecipitation assay (RIPA) buffer (SIGMA-ALDRICH, UK), supplemented with complete protease inhibitor cocktail (Roche) and incubated on ice for 30 minutes. Cell lysates were centrifuged at 12000g for 60 seconds to remove insoluble material, prior to the addition of Laemmli buffer (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.005% Bromophenol Blue, pH 6.8) and 1M Dithiothreitol (DTT) at 2x final concentration. Samples were then heated at 95°C for 5 minutes prior to being stored at -20°C.

2.11.1 Bradford Assay

10µL of Quick Start™ BSA Standards (BioRad) were used for standards in a 96 well plate and 10µL of samples were used in triplicate. 200µL of Quick Start TM BSA Dye Reagent (BioRad) was added to each well and incubated in darkness for 10 min.

Absorbance was read at 595nm using the Tecan Infinite F50 Robotic absorbance plate reader with Magellan Data Analysis software.

2.11.2 SDS-PAGE and western transfer of proteins

20 µg of total protein was loaded on an Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gel (BIO-RAD, UK) and electrophoresed in 1x Tris/glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SD, pH 8.3) at 120V for 1h, prior to being transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot® Turbo™ Transfer System (BIO-RAD). PVDF membranes were then incubated in 1xTBST (1x Tris-buffered saline 20 mM Tris and 150 mM NaCl) containing 5% non-fat milk for an hour at RT to block non-specific antibody binding before being incubated overnight with the primary antibody diluted in 1xTTBS containing 5% non-fat milk (Table 2.4) at 4°C on a shaking platform. Membranes were then washed with TBST for three times and incubated with the appropriate secondary HRP-conjugated IgG secondary antibody diluted in 1xTTBS containing 5% non-fat milk for 1 hour at RT, prior to 3x5 minute washes with TBST and incubation with 2ml of Clarity™ Western ECL Substrate (BIO-RAD) for 5 minutes and visualisation via Gel Doc XR+ imager system (BIO-RAD, UK).

Table 2.4 List of primary and secondary antibodies with dilution ratios

Antibody (supplier)	**Dilution ratio (v/v)**
Anti-SFPQ antibody (ab38148 ABCAM, UK)	1:1000
Mouse anti Human GAPDH antibody (Bio-Rad, UK)	1:5000
E-Cadherin (4A2) Mouse mAb (Cell Signaling, Danvers, Massachusetts)	1:1000
Vimentin (D21H3) XP® Rabbit mAb Cell Signaling, Danvers, Massachusetts)	1:1000
secondary HRP-conjugated IgG	1:2000
Goat anti Mouse IgG antibody	1:2000

2.12 RNA immunoprecipitation

RNA immunoprecipitations were performed using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore, USA) according to the manufacturer's recommendations. Briefly, ~1.0 x 10^6 cells were washed twice in 10 ml of ice cold PBS, scraped and collected via centrifugation at 300g for 5 minutes at 4°C. Cells were lysed in RIP Lysis Buffer by pipetting up and down until homogenous and incubated for 5 minutes on ice prior to storage at -80°C. Magnetic beads for immunoprecipitation were prepared by adding 5 µl of magnetic beads suspension into an RNAse-free micro centrifuge tube prior to adding 0.5 ml of RIP Wash Buffer. Tubes were placed on a magnetic separator to pellet beans and the supernatant was discarded, this wash was then repeated a further two times. Following the final wash, magnetic beads were resuspended in 100 µl of RIP wash buffer and 5 µg of SFPQ antibody or 5 µg of rabbit IgG for control was added to the beads incubated at RT with rotation for 30 minutes. Samples were pulse centrifuged and placed on a magnetic separator, supernatant was discarded, tubes removed from the magnetic and beads washed twice with 0.5 ml of RIP wash buffer, as described above. RIP Immunoprecipitation Buffer was prepared accordingly for each immunoprecipitate as follows: 35 µl of 0.5 M EDTA, and 5 µl RNase inhibitor to 860 µl of RIP wash buffer made to a final volume of 900 µl was added to the magnetic beads. RIP lysate stored at -80°C was thawed immediately on ice, centrifuged at 14,000 rpm for 10 minutes at 4°C and 100 µl added to each bead-antibody complex in RIP immunoprecipitation buffer made up to a final volume of 1.0 ml. 10 µl of the supernatant of RIP lysate was removed, placed into a tube labelled as 'input' and stored at -80°C, this served as a positive control for total RNA. An additional 10 µl of the RIP lysate was removed and Laemmli buffer added, followed by heating at 95°C and storage at -20°C, this served as a positive control for total protein. RIP

tubes were incubated overnight at 4°C with end-over-end mixing. The following morning, RIP samples were centrifuged briefly and washed six times in 0.5 ml of RIP Wash Buffer, as described above. On the final wash step 50 µl of the 500 µl beads suspension was removed and resuspended in Laemmli buffer followed by heating at 95°C prior to centrifugation and stored at -20°C, this served as a positive control for immunoprecipitation of SFPQ. 150 µl of Proteinase K buffer was prepared for each immunoprecipitated sample by adding 117 µl of RIP Wash Buffer, 15 µl of 10% SDS, 18 µl of 10 mg/ml proteinase K with a final volume of 150 µl and mixed with each immunoprecipitate. Proteinase K buffer containing 107 µl of RIP Wash Buffer, 15 µl of 10% SDS, and 18 µl of proteinase K was resuspended with the thawed 'input' sample giving a final volume of 150 µl, all samples re-suspended in proteinase K buffer were incubated at 55°C for 30 minutes with shaking to digest the protein. Following incubation tubes were centrifuged and placed on magnetic separator, supernatant was transferred into a new tube before adding 250 µl of RIP Wash Buffer to each tube. 400 µl of phenol: chloroform: isoamyl alcohol was added to each sample prior to mixing for 15 seconds, samples were centrifuged at 14000 rpm for 10 minutes at RT to separate the phases. 350 µl of the aqueous phase was carefully removed and placed in a new tube. 400 µl of chloroform was added and vortexed for 15 seconds and centrifuge at 14000 rpm for 10 minutes at RT. 300µl of the aqueous phase was removed placed it in a new tube. To each tube 50 µl of Salt Solution I, 15 µl of Salt Solution II, 5 µl of Precipitate Enhancer and 850 µl of 100% ethanol was added and mixed by inverting and stored at -80°C overnight to precipitate the RNA. The precipitated RNA tubes were centrifuge at 14,000 rpm for 30 minutes at 4°C supernatant carefully removed. 800 µl of 80% ethanol was used to wash pellet prior to centrifugation at 14,000 rpm for 15 minutes at 4°C. Supernatant was discarded and pellets air dried. Pellets were re-

suspended in 20 µl of RNase-free water. For first strand synthesis, cDNA was prepared using 5 µl of RNA using random primers as described above in section 2.9.1.

2.12.1 Analysis of RIP-IP Data

Enrichment over input was calculated using RIP-qPCR Data Analysis for % Input and Fold Enrichment. Calculations are briefly outlined below.

RIP-qPCR Data Analysis (ΔΔCt method)

Each RIP RNA fraction Ct value was normalised to the Input RNA fraction Ct value for the same qPCR Assay (ΔCt) to account for RNA sample preparation differences using the following formula:

- **ΔCt [normalized RIP] = (Ct [RIP] – (Ct [Input] – Log2 (Input Dilution Factor)**

Input dilution fraction was taken as 1%, which is a dilution factor of 100 or 6.644 cycles (i.e. log2 of 100). Thus, 6.644 was subtracted from the Ct value of the 1% Input sample. Average normalized RIP Ct values for replicate samples were calculated by the % Input for each RIP fraction (linear conversion of the normalized RIP ΔCt):

- **% Input = 2(-ΔCt [normalized RIP])**
    - The normalized RIP fraction Ct value for the normalized background [non-specific (NS) Ab] fraction Ct value (first ΔΔCt) was adjusted using the following formula.

- **(ΔΔCt [RIP/NS] = ΔCt [normalized RIP] – ΔCt [normalized NS]**
    - IP Fold Enrichment was calculated above the sample specific background using (linear conversion of the first ΔΔCt).
    - Fold Enrichment = 2 (-ΔΔCt [RIP/NS]

2.13 Transwell Migration Assays

In vitro cell migration was measured using 8 µm TC-inserts (Starstedt, Germany). Briefly, in a 24-well plate ~$1.6x10^5$ cells were transfected with the appropriate GapmeRs and incubated at 37°C for 24 hours. The following day transfected cells were serum starved for 24 hours prior to the assay. Cells were detached using 0.05% Trypsin-EDTA (incubated for 5 minutes at 37°C) and centrifuged at 1500rpm for 5 minutes and resuspended in serum free media to obtain $1x10^5$ cells per well. 1 ml serum free media and 1 ml of 2% serum DMEM was added to the lower chambers of a 24 well plate with 8 µm TC-inserts and 500 µl cell suspension was added into the insert. Cells were incubated for at 37°C for 6 hours, then fixed with ice cold 70% ethanol overnight. Insert membranes were stained in 0.2% crystal violet (Fisher Science, UK) for 10 minutes and mounted onto slides. 10 random high-power images were taken (EVOS XL Core Cell Imaging System, 100x objective) and an average of the number of cells moved through the pores for each image was calculated.

2.14 Proliferation/Viability Assay

Initially, Cells (~$1.6x10^5$) were transfected with the appropriate GapmeRs in a 24-well plate and incubated at 37°C for 24 hours. The following day transfected cells were detached using 0.05% Trypsin-EDTA. Next, $1x10^4$ cells/well were seeded in complete growth media into a (white) 96 well plate (ThermoFisher, UK) and incubated 37°C for a further 24 hours. Media was replaced with 100 µl of SFM and incubated at 37°C until day of assay. Cell viability was carried out using CellTiterGlo Luminescent Cell Viability Assay (G7570, Promega, UK) 100 µl of CellTiterGlo was added to cells which were then placed on an orbital shaker for 2 minutes and incubated at room temperature for 10 minutes. Luminescence was recorded using (Promega GloMax Explorer).

2.15 Cell Apoptosis Assay

Cells (~$1.0x10^6$) were transfected in a 6-well with SFPQ GapmeRs and incubated at 37°C for 48 hours. Cell death was determined in control GapmeR and SFPQ knock-down cells using the Annexin V-FITC Apoptosis Kit (BioLegend, UK) according to manufacturer's protocol. Briefly, cells were detached using 0.05% Trypsin-EDTA and centrifuged at 12,000rmp for 2 minutes, then washed twice with PBS and resuspended in 1x Binding buffer at a concentration of $1x10^6$ cells/ml. Next, 100 µl of $1x10^5$ cells were transferred to an eppendorf and 5µl of FITC Annexin V was added to each sample. For Propidium iodide (PI) 50 ug/ml was added. Samples were gently vortexed and incubated in the dark at room temperature for 15 minutes. Finally, 400 µl of 1× binding buffer was added to each tube and samples were analysed via flow cytometry (FACS) using the Guava EasyCyte.

2.16 Immunofluorescence

Glass coverslips 22x22x0.17mm (Thermo Fisher Scientific, UK) were placed into a 6-well plate and coated with poly-L-lysine (SIGMA-ALDRICH, UK) for 20 minutes. Next, poly-L-lysine was carefully removed and left to air dry for ~5 minutes prior to being washed twice with 1 ml of PBS. Cells were harvested (5 $x10^5$ cells/ml) and seeded on top of the cover slip prior to incubation at 37°C in a 5% CO_2 atmosphere. Transfection mix was prepared (as described above 2.3) complexes were added dropwise to the cells and incubated at 37°C for a further 48 hours. Transfected cells were then fixed using 4% formaldehyde in PBS for 15 minutes, washed with PBS and treated with 1% Triton-X 100 for 15 minutes. After a final PBS wash coverslip were mounted on glass slides in mounting medium for fluorescence with DAPI (Vector Laboratories, USA).

2.17 Seahorse

The Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were determined using a Mito Stress Test Kit and XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA) according to the manufacturer's protocol. Briefly, $1.6x10^5$ cells were transfected with GapmeRs in a 24-well plate and incubated at 37°C for 24 hours see section 2.3. A day prior to the assay, transfected cells were detached using 0.05% Trypsin-EDTA and re-seeded ($2x10^4$) per well into XFe 96 cell culture microplates in growth medium, 200 µl of sterile water was added to the moats around the cells and incubated at 37°C in a 5% CO_2 for 24 hours. Cartridges were hydrated by adding 200 µl of Seahorse XF Calibrant Solution (100840-000 Aligent, UK) to the wells and 400 µl around the moats and placed in a non-CO_2 incubator at 37°C overnight. The day of the measurement cells were washed twice in Seahorse XF DMEM pH 7.4 (103577-100 Aligent, UK) supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose and placed at 37°C in a non-CO_2 incubator for one hour prior to assay. Next, Oligomycin (1.5 µM), FCCP (1 µM), and rotenone/antimycin A (0.5 µM) were loaded onto the XFp sensor cartridges and these were injected at the indicated time points. The oxygen consumption rate was normalized by Sulforhodamine B assay (SRB).

2.17.1 SRB Assay

Briefly, cells were fixed with 100 µl of Trichloroacetic acid (TCA) Solution 10% (w/v) (SIGMA-ALDRICH, UK) and placed into the fridge for an hour. Fixed cells were carefully washed 4 times with deionised water and left to air dry for 2 days. Next, 100 µl of SRB (0.057% w/v) in 1% v/v acetic acid (SIGMA-ALDRICH, UK) was added to each well and left at room temperature for half an hour. Cells were then washed 1% acetic acid 4 times and left to dry at room temperature for 15 minutes. 200 µl of 10 mM

Tris base (unbuffered) was added to each well and placed on a rocker at 60/70rpm for 5 minutes and subsequently absorbance (510nm) was measured using a plate reader.

2.18 SFPQ mRNA stability

Cells (~$1.6x10^5$) were transfected with SFPQ GapmeRs in a 24 well plate and incubated at 37°C and 5% CO_2 for 48 hours. RNA was extracted (see section 2.6) from control GapmeR and SFPQ-knockdown transfected cells at t=0 for control. Next, Actinomycin D (1 µg/ml) (SIGMA-ALDRICH, UK) was diluted with 10% DMEM and added dropwise to the remaining wells containing control and SFPQ transfected cells, plates were swirled gently to ensure uniform distribution and incubated with RNA being extracted at 3h and 6h post-addition of the drug. cDNA was synthesised (see 2.9) and mRNA expression levels of SFPQ were assessed via qPCR.

2.19 RNA sequencing (RNA-Seq)

RNA-Seq was used to explore the RNA interactors of SFPQ in PMs and A2058 cells. RNA samples were prepared from SFPQ immunoprecipitations and corresponding IgG control as described in section 2.12. Single end and stranded Illumina NextSeq 500 sequencing was performed at the Next Generation Sequencing facility (Leeds Institute of Molecular Medicine, Saint James's University Hospital). Bioinformatics analysis was performed as described below by Dr Chinedu A. Anene at the CRUK Barts Centre, Queen Mary University of London.

2.19.1 RNA-Seq alignment and processing

Raw reads were processed for gene and transcript expression as previously described (Bolger, Lohse, & Usadel, 2014). Quality filtered, and adapter trimmed reads (Trimmomatic) were aligned to the GRCh38/hg38 assembly of the human genome using HISAT2 (V 2.1.0). StringTie (V 1.3.4) was used to assemble and quantify the

RNA abundance based on the GRCh38 annotation (GENCODE Release 32). The expression levels were normalised by the “transcripts per kilobase million” (TPM).

2.19.2 RIP-Seq differential enrichment

Peaks were called using MACS2 under the option of *-no model* with effective genome size and *-shift size* set to 3.0 x 10^8 and length of RNA fragments, respectively. To reduce the rate of false-positive enrichments, only uniquely mapped and non-duplicated reads were used for peak calling. Peaks were called against IgG control libraries with $p < 10^{-5}$ and genomic coordinates annotated using the R Bioconductor packages.

2.19.3 Cluster analysis

To compare the gene expression level in RIP with control library we extracted and applied unsupervised clustering on the differentially enriched genes in RIP versus Input, restricted to lncRNAs. To assess the robustness of the RNA enrichment results, we extracted and analysed the abundance of the enriched lncRNAs from a publicly available SFPQ-RIP in prostate cancer cells (K.-I. Takayama et al., 2017a).

2.19.4 Gene ontology analysis

To investigate the biological processes associated with the differentially enriched genes, we used the R ClusterProfiler and the human Bioconductor annotation database (org.Hs.eg.db) to compare the enriched biological processes between PM-enriched and A2058-enriched genes. All enrichment analyses were performed with a strict p-value and q-value < 0.01 cut-off with reduced redundancies by semantic similarity analysis (J. Z. Wang, Du, Payattakool, Yu, & Chen, 2007).

2.20 Production of RIP and control libraries

Three libraries were generated after antibody selection for each sample; a standard, a total RNA and a RIP library. These libraries were made using the same kit, with the total RNA samples entering at the standard start point and the antibody selected samples entering after the rRNA depletion step.

rRNA removal from control samples

Briefly, 100 ng of total RNA from each control sample were diluted to a final volume of 10 µl, to which 5 µl of rRNA Binding Buffer and 5 µl of Ribo zero gold, rRNA depletion probes were added prior to heating at 68˚C for 15 minutes and cooled down to room temperature. Once cooled, 35 µl of resuspended rRNA removal beads were added to the sample and incubated at room temperature for 1 minute. The sample was then pipetted up and down thoroughly, then placed on a magnetic stand for 1 minute. The supernatant was transferred to a new tube and placed on a magnetic stand to remove any remaining rRNA depletion probes bound to the magnetic beads.

RNA clean up (entry point for IP selected RNA samples)

99 µl of resuspended RNAClean XP beads were added to each sample from above or IP selected samples, which were mixed by pipetting and incubated at room temperature for 15 minutes. Next, the beads were pelleted by placing samples on a magnetic stand for around 5 minutes and discarding the supernatant. The beads were then washed in 200 µl of 70% ethanol for 30 seconds and pelleted using a magnetic stand. The supernatant was removed and the pellet air dried for 15 minutes. The pellet was then re-suspended in 11 µl of elution buffer and incubated for 2 minutes at room temperature. Beads were pelleted using a magnetic stand and 8.5 µl of the supernatant was removed into a new tube to which 8.5 µl of “Elute, Prime, Fragment

High Mix" was added. This was then mixed by pipetting followed by incubation at 94˚C for 8 minutes, prior to cooling to 4˚C.

First strand synthesis

8 µl of First Strand Synthesis Act D Mix (to which superscript II has been added) was added to each sample, mixed and incubated at 25˚C for 10 minutes, then 42˚C for 15 minutes followed by 70˚C for 15 minutes and finally cooled down to 4˚C.

Second strand synthesis

10 µl of Resuspension Buffer was added to each sample, followed by 20 µl of Second Strand Marking Master Mix, then incubated at 16˚C for 60 minutes prior to cooling sample down to room temperature (This step removes the RNA and creates a second strand in which dUTP is incorporated in the strand rather than dTTP).

Purification of cDNA

Each sample was mixed with 90 µl of AMPure XP beads and incubated at room temperature for 15 minutes, prior to pelleting on a magnetic stand for 5 minutes. The supernatant was discarded, and the pellets were washed twice in 200 µl of 80% ethanol. Next, the ethanol was removed and pellets air dried on the magnetic stand for 15 minutes at room temperature, prior to resuspending in 17.5 µl of resuspension Buffer. Following incubation for two minutes at room temperature, the beads were then pelleted using a magnetic stand and the supernatant removed into a new tube.

Adenylation of 3′ ends

5 µl of Resuspension Buffer and 12.5 µl A-Tailing Mix was added to each sample and incubated initially at 37˚C for 30 minutes, then at 70˚C for 5 minutes.

Addition of sequencing adaptors

2.5 µl of resuspension Buffer, 2.5 µl of Ligation Mix and 2.5 µl of the appropriate indexed RNA adaptors were added to each sample and incubated at 30˚C for 10 minutes. Samples were cooled down to 4˚C and 5 µl of the Stop Ligation Buffer was added to each sample. The libraries were cleaned by adding 42 µl of AMPure XP beads and incubated at room temperature for 15 minutes. The beads were then pelleted using a magnetic stand until the solution turned clear. The pelleted beads were washed twice with 200 µl of 80% ethanol and after the removal of the ethanol, the beads were air dried for 15 minutes at room temperature. The beads were re-suspended in 52.5 µl of resuspension buffer and incubated at room temperature for 2 minutes. Next, the beads were pelleted and 50 µl of the solution was moved into a fresh tube. A second round of bead clean-up was carried out on the samples as described above, but this time the samples were resuspended in 22.5 µl of resuspension buffer and 20 µl of the supernatant was removed to a fresh tube.

Enrich of DNA fragments

Samples were placed on ice and 5 µl of PCR Primer Cocktail and 25 µl of PCR Master Mix was added to each sample and PCR amplified as follows: Denatured at 98˚C for 30 seconds, 15 cycles at 98˚C for 10 seconds, 60˚C for 30 seconds and 72˚C for 5 minutes. After the final extension at 72˚C for 5 min, the samples were cooled to 4˚C. Next, the amplified DNA was cleaned by the addition of 47.5 µl of AMPure XP beads as described above and washed twice in 200 µl of 80% ethanol. The air-dried pellets were resuspended in 32.5 µl of resuspension buffer, then samples were pelleted and 30 µl of the supernatant was placed into a fresh tube.

Quantification and pooling

1 µl of each library was analysed on a TapeStation (Agilent) using a standard sensitivity DNA tape reagent to check for a fragment size of ~260 bp and the absence of adaptor dimers. Each library was diluted to a 10nM solution and pooled to form an equimolar pool of all the indexed libraries, prior to being sequenced on 75 bp single end, NextSeq lane. The exported BCL formatted data base was called and demultiplexed using the BCL2FASTQ application to produce read 1 fastq.gz file for each sample.

2.21 SFPQ Survival curves

Transcriptomic data from primary melanomas of 703 patients (Illumina DASL array HT12.4, deposited at the European-Genome Phenome Archive, accession number EGAS00001002922, Nsengimana et al, PMID: 29664013; Thakur et al., PMID: 31515461) as well as clinical data were provided by collaborators from the Melanoma Research Group, Leeds Institute of Medical Research at Saint James, University of Leeds. This data is part of the Leeds Melanoma Cohort (LMC) a large population-based melanoma cohort which has a median follow up time of 8 years.

Dependence between tumour characteristics including Breslow thickness and mitotic rate and tumour expression of targets was carried out using Spearman correlation. These expressions were further tested for association with melanoma-specific survival (i.e. excluding deaths from other causes) after their split into quartiles by applying Cox proportional hazards regression and plotting Kaplan-Meier survival curves. Where appropriate, certain quartiles were combined to reduce the number of parameters. Both single gene models and bivariate models were applied with an interaction term to test the independence between genes. These analyses were conducted in STATA

v14 (StataCorp, Texas, USA) by Jeremie Nsengimana, Senior Statistician at the University of Leeds.

2.22 Statistical Analysis

Statistical analysis was done using GraphPad Prism 6 (GraphPad, San Diego, CA, USA). One-way ANOVA was used to determine if there were any statistically significant differences between two or more independent groups. Two-way ANOVA was used to compare the mean differences of two independent variables on a dependent variable (Fowler et al. 1998). Followed by Tukey's Honest Significant Difference test, a post-hoc test based on the studentised range distribution. This test is used to determine which specific group's means compared with each other are different. Thus, compares all possible pairs of means. T-test was used to compare the means to determine level of significance which is known as p-value. Significant results are denoted as $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ ns= not significant.

Chapter 3

3.1 Establishing a hierarchy of migration potential in melanoma cancer cell lines

There is emerging evidence linking lncRNAs as key drivers of cancer development and progression. Interestingly, numerous groups have demonstrated a role for lncRNAs in re-modelling the tumour microenvironment and tumour metastasis. Metastasis requires cancer cells to detach from the primary tumour, invade the basement membrane and nearby tissues, intravasate into blood vessels and eventually establish growth at distal organs. To achieve these individual steps, the precise coordination of cell movement and remodelling of the matrix is important (Lauffenburger & Horwitz, 1996). Migratory cancer cells undergo various molecular and cellular changes via remodelling their cell-cell interactions, cell-matrix adhesion and their actin cytoskeleton (Berx & Van Roy, 2001; Nieman, Prudoff, Johnson, & Wheelock, 1999; Palamidessi et al., 2008).

If lncRNAs are acting as metastatic drivers in melanoma, one hypothesis is that melanoma cell lines with high metastatic potential will express differentially increased levels of oncogenic lncRNA compared with less aggressive melanoma cell lines. To investigate this possibility, I initially set out to characterise the migration potential of several melanoma cells lines via wound healing assays. Melanoma cell lines were selectively chosen based on their reported ‘metastatic potential’, as previously reported in the literature. A study investigating the changes which occur in the cellular metabolome and lipidome via gas chromatography-mass spectrometry and direct infusion-mass spectrometry discovered aminomalonic acid as a novel potential biomarker which helped differentiate the different stages of melanoma metastasis. Their findings revealed increased levels of aminomalonic acid in high metastatic cells A2058 and SKMEL-28 cell lines compared with the low metastatic melanoma cell line,

A375 (H.-Y. Kim et al., 2017). Similarly, lipid analysis demonstrated phosphatidylinositol (PI) was abundant in A2058 as opposed to A375 (H.-Y. Kim et al., 2017). Consistent with these findings the metastatic potential in A2058 and A375 melanoma cells was confirmed via a Transwell migration assay (Q. Cheng et al., 2017; Giles, Brown, Epis, Kalinowski, & Leedman, 2013). Moreover, evidence of UACC-62 as a low metastatic melanoma cell line and M14 as highly metastatic was established in a study which looked at the gene expression signatures in melanoma (Jeffs et al., 2009). Based on these findings the reported high metastatic melanoma cell lines, M14, A2058, SKMEL-28 and low metastatic A375 and UACC-62 were selected for further analysis in this study.

To establish if migration potential resulted in the same reported hierarchy described above, cell line wounds were created for each melanoma cell line in triplicate on a monolayer of confluent cells and images captured at 0h, 6h, 12h and 24h time points. Images were then analysed using ImageJ and the MRI wound healing tool. For each cell line an average of the area was taken to determine percentage wound closure over 24 hours (Figure 3.1 A-F). As can be seen in Figure 3.1 F, significant differences in wound closure were observed between melanoma cell lines. Specifically, A375, UACC-62 and cell lines displayed a significantly lower level of wound closure at all time points, compared with A2058 and M14 Figure 3.1 A & B, in agreement with published convention. The SKMEL-28 cell line displayed an intermediate rate of wound closure that was significantly increased at 12h and 24h-post wound (Figure 3.1 C, compared with A375 and UACC-62. Together these data establish a hierarchy between the six melanoma cells lines analysed, in terms of migration potential (Table 3.1).

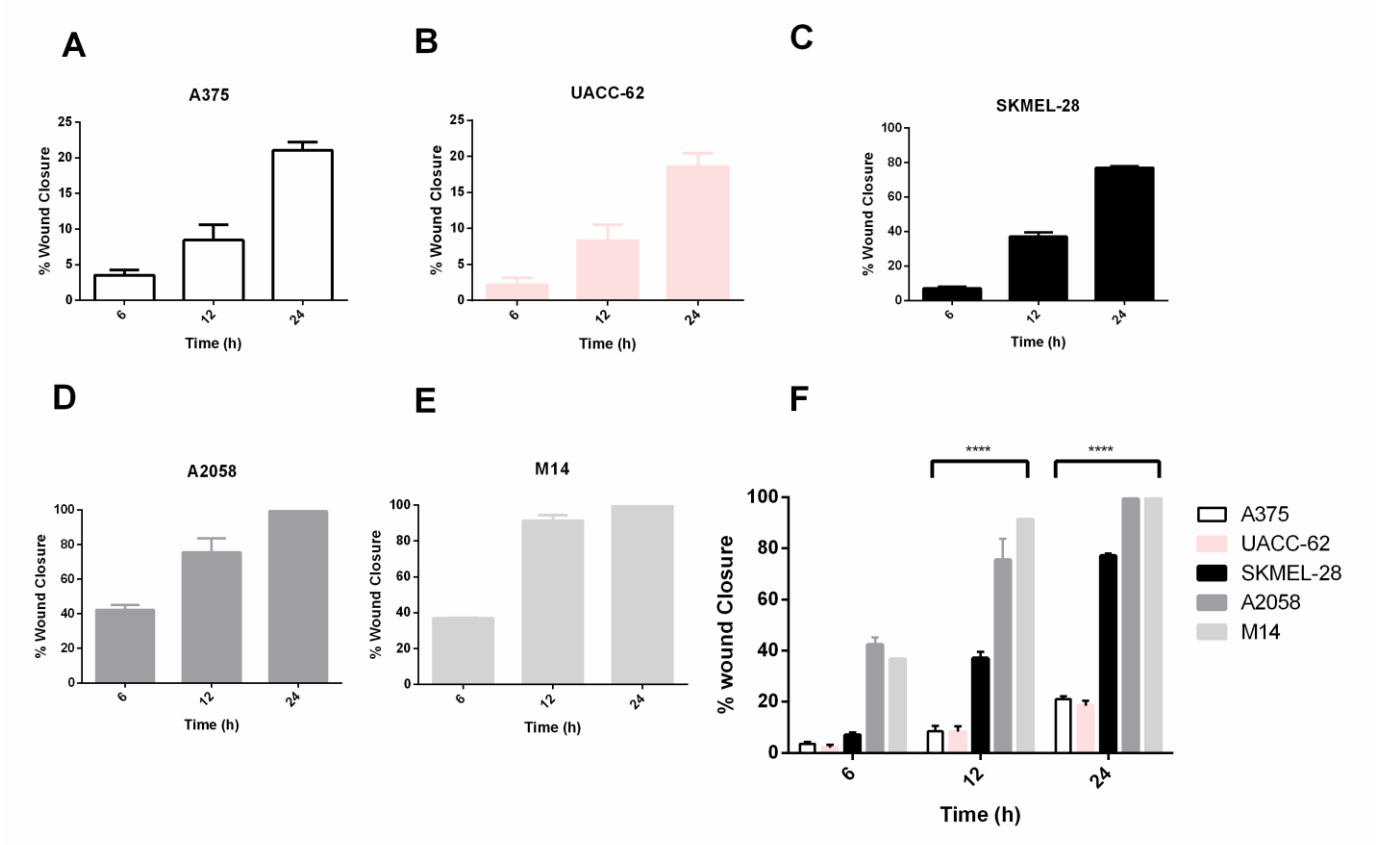


Figure 3.1. Melanoma cell lines possess significantly different migration potential, *In vitro*. Melanoma cell migration was assessed *In vitro* via wound healing assays. Following scratch wound, the percentage closure rate was determined for A375, UACC-62, SKMEL-28, A2058 and M14 cell lines over a 24h period (A-F). Direct statistical comparison of wound closure between panels A-F was carried out via Using 2-way Anova followed by Tukeys multiple comparisons test n=5 independent biological repeats. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.001, ns= no significance.

Table 3.1 Statistical analysis on melanoma cell migration. Data is presented as the mean of the percentage wound closure (n=5 replicates) ± ** denotes p <0.01, *** p<0.001, **** p<0.0001, ns (no significance) using two-way ANOVA test Using 2-way Anova followed by Tukey's multiple comparisons test.

Cell lines	Statistical significance 6h	Statistical significance 12h	Statistical significance 24h
A375 vs UACC-62	Ns	Ns	Ns
A375 vs SKMEL-28	Ns	****	****
A375 vs A2058	****	****	****
A375 vs M14	****	****	****
UACC-62 vs SKMEL-28	Ns	****	****
UACC-62 vs A2058	****	****	****
UACC-62 vs M14	****	****	****
SKMEL-28 vs A2058	****	****	****
SKMEL-28 vs M14	****	****	****
A2058 vs M14	Ns	***	Ns

3.2 Comparative analysis of oncogenic LncRNA

Having determined the migratory potential of several melanoma cell lines I next sought to test the hypothesis that these phenotypes may correlate with dysregulated lncRNA expression. To this end, published qPCR primers were utilised to amplify a panel of lncRNAs previously reported as melanoma-associated (Table 3.2). Each of the melanoma cell lines described in Figure 3.1 were cultured to confluency prior to total

Table 3.2 Melanoma associated oncogenic lncRNA.

LncRNA	Expression in Melanoma	Confirmed Effect	References
SPRY4-IT1	Upregulated	Stimulates melanoma cell proliferation, migration, invasion, and halts apoptosis.	(Khaitan et al., 2011; T. Liu et al., 2016; Mazar et al., 2014; W. Zhao et al., 2016)
MALAT1	Upregulated	Stimulates melanoma cell proliferation and migration	(Y. Sun et al., 2017; L. Tang et al., 2013; Tian et al., 2014)
HOTAIR	Upregulated	Stimulates melanoma cell proliferation, migration, invasion and degradation of extracellular matrix	(Monica Cantile et al., 2017; L. Tang et al., 2013; Tian et al., 2014)
BANCR	Upregulated	Stimulates melanoma cell proliferation and migration	(Flockhart et al., 2012; R. Li et al., 2014; McCarthy, 2012)
SLNCR1	Upregulated	Favours melanoma cell invasion	(K. Schmidt et al., 2016)
UCA1	Upregulated	Stimulates melanoma cell proliferation and migration	(Tian et al., 2014; Wei et al., 2016)
ANRIL	Upregulated	Stimulates melanoma cell proliferation and migration	(Eric Pasmant et al., 2007; Huaping Xie et al., 2016; Shiqiong Xu et al., 2016)
RMEL3	Upregulated	Stimulates melanoma cell proliferation and migration	(Goedert et al., 2016; Sousa et al., 2010)
SAMMSON	Upregulated	Stimulates melanoma cell migration	(E. Leucci et al., 2016)
LLME23	Upregulated	Promotes melanoma cell migration	(C.-F. Wu, G.-H. Tan, C.-C. Ma, & L. Li, 2013)

RNA isolation, quantification and subsequent first strand cDNA synthesis. Care was taken to ensure that all cDNA reactions were consistent in terms of amount of RNA reverse transcribed and qRT-PCR reactions were performed for each lncRNA target across all cell lines. Importantly, each target gene was cross referenced to two genes, to ensure robust normalisation across all cell lines (Kozera & Rapacz, 2013). As can be seen in Figure 3.2, *ANRIL*, *LLME23*, *BANCR* and *SAMMSON* exhibit expression levels between the melanoma cell lines that mirror migration potential (Figure 3.2A-

D). This was in contrast to *MALAT1*, *RMEL3* and *UCA1*, which to different degrees are enriched in the lower migratory cells (Figure 3.2 F-H). To investigate this further,

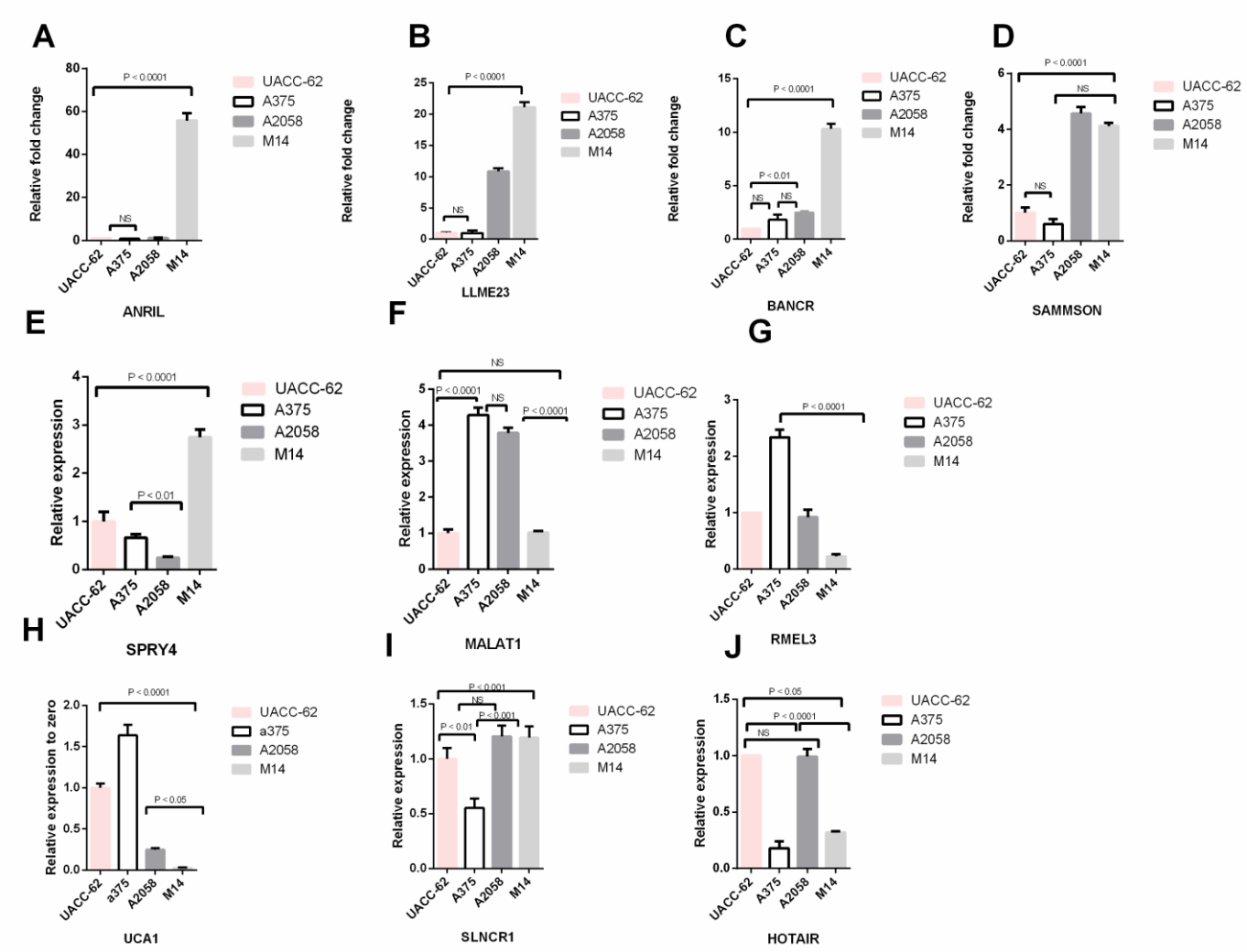


Figure 3.2. Melanoma-associated lncRNA are differentially expressed between melanoma cell lines. Melanoma-associated lncRNA are differentially expressed between melanoma cell lines. Gene expression of Melanoma-associated lncRNA were determined via qRT-PCR in UACC-62, A375, A2058 and M14 melanoma cell lines. Each target: *ANRIL, LLME23, BANCR, SAMMSON, SPRY4, MALAT1, RMEL3, UCA1, SLNCR1* and *HOTAIR* are presented as fold change of *UACC-62* (A-J). Statistical comparison of lncRNA expression levels in melanoma cells was carried out via 2-way Anova followed by Tukeys multiple comparisons test n=4 independent biological repeats. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.001, ns=no significance.

correlative analysis was carried out on the relative levels of migration for each cell line compared with lncRNA expression to generate a correlation co-efficient for each lncRNA target. As can be seen in Figure 3.2.1, *LLME23, SAMMSON, BANCR, HOTAIR, SLNCR1* and *SPRY4* all exhibit positive correlation scores that are >0.5, suggesting that increased expression of these lncRNA possess a positive, but not necessarily causal, relationship with melanoma cell migration potential. Interestingly, *MALAT1, RMEL3* and *UCA1* display an inverse correlation with migration potential. Together, these data identify a subset of lncRNA whose expression is positively correlated with migration potential in melanoma, *in vitro.*

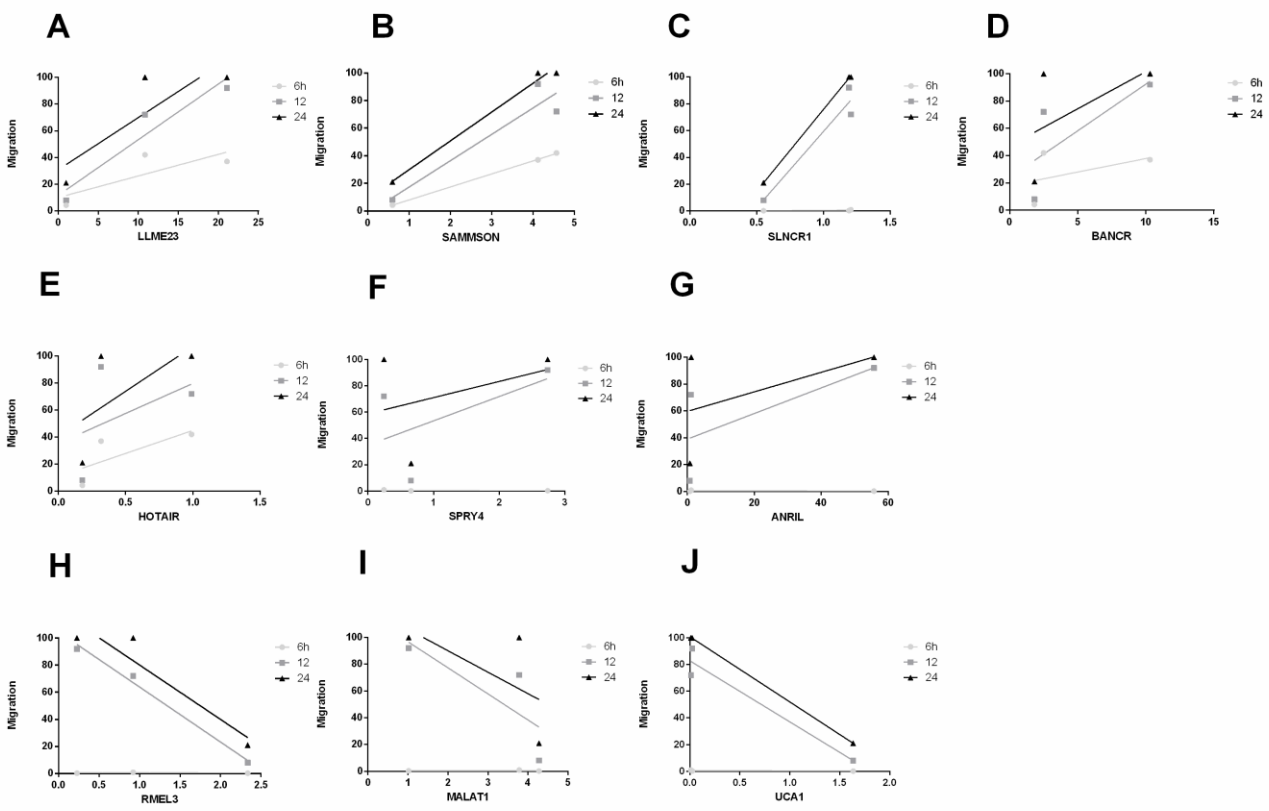


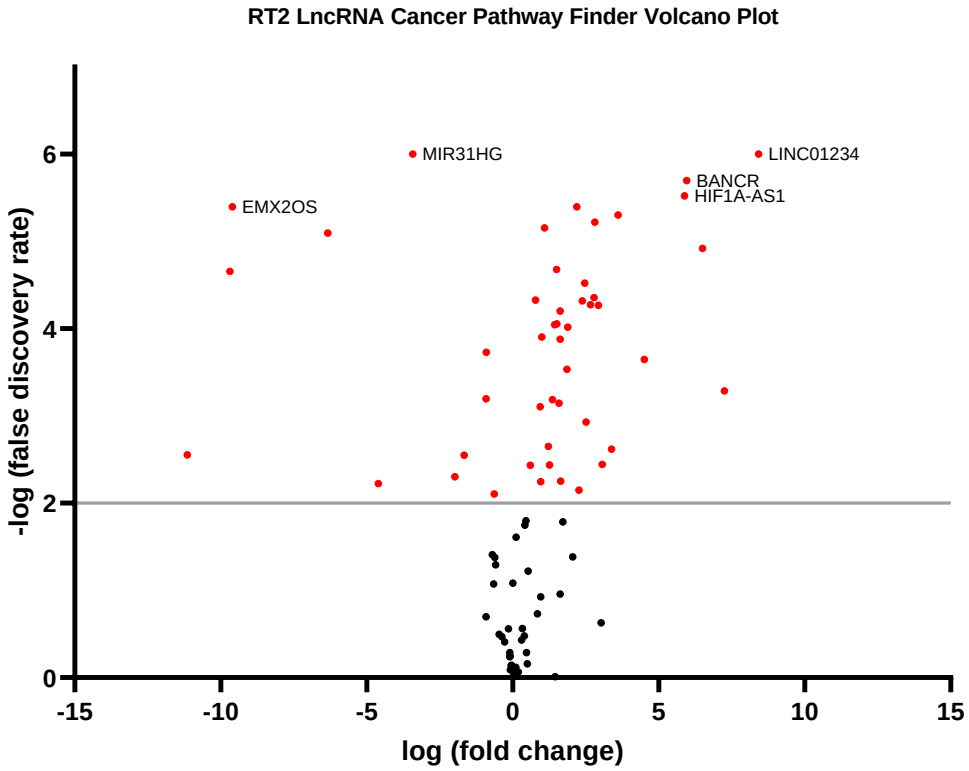
Figure 3.2.2. Melanoma associated- lncRNA expression correlates with metastatic melanoma migration potential. Pearson's analysis determined correlation between expression levels of lncRNA *LLME23, SAMMSON, SLNC1, BANCR, HOTAIR, SPRY4, ANRIL, RMEL3, MALAT1* and *UCA1* (x-axis) and migration potential (y-axis) in melanoma cell lines A375, A2058 and M14 over 24-hour period (A-J).

3.3 Differential lncRNA expression analysis between primary melanocytes and metastatic melanoma cell lines

The positive correlation described in section 3.2 between expression levels of several lncRNA with migration potential in melanoma cells suggests that specific lncRNAs may function as driver genes for cancer cell metastasis, in broad agreement of the published literature summarised in Table 3.2. While the association of lncRNA expression in melanoma cells has been extensively studied (Mestdagh et al., 2016), far less is known about changes in lncRNA expression that occur in primary melanocytes as a consequence of transformation. Therefore, it was of interest to investigate if lncRNA are also differentially expressed in PM compared with melanoma cells, *in vitro*.

To this end, we utilised qRT-PCR-based mini-arrays (RT² Profiler™ PCR Array Human Cancer PathwayFinder™), to analyse the expression of 83 cancer-associated lncRNA targets in PM compared with the melanoma cell line, A2058. The A2058 cell line was chosen as it represents an 'aggressive' melanoma cell line that is well characterised in the literature (H.-Y. Kim et al., 2017; Quiñones & Garcia-Castro, 2004). Total RNA was extracted from PM and A2058 cells, quantified and cDNA generated via first strand synthesis prior to use in these assays. The RT² Profiler™ arrays contain several control elements including, multiple reference genes and internal positive and negative controls to detect RNA sample quality, genomic DNA contamination and general PCR performance. Following analysis, RT² Profiler™ data revealed numerous lncRNA that were significantly differentially expressed in A2058 melanoma cells compared with PM (Figure 3.3). Interestingly, targets upregulated in A2058 cells included numerous poorly annotated lncRNA (*LINC01234, HIF1A-AS1* and *DLX6-AS*). Moreover, two lncRNAs were significantly downregulated in A2058 cells compared with PM and these

included the established tumour suppressor lncRNA, *MEG3* and the less well understood tumour suppressor *EMX2OS*.



Gene	Log2 (fold change)	-Log10 FDR
LINC01234	8.420579493	6
MIR31HG	-3.422699068	6
BANCR	5.955918932	5.698970004
HIF1A-AS1	5.88644176	5.522878745
EMX2OS	-9.59924871	5.397940009
NBR2	2.198729468	5.397940009
H19	3.605912896	5.301029996
CRNDE	2.817168436	5.22184875
HOTAIRM1	1.091555899	5.15490196
GAS6-AS1	-6.336719478	5.096910013
DLX6-AS1	6.498369078	4.920818754
TUG1	1.503995648	4.677780705
XIST	-9.683746575	4.657577319
SNHG16	2.469418326	4.522878745
CAHM	2.786113243	4.356547324
RMRP	0.785310902	4.327902142
MIR17HG	2.383920249	4.318758763
LINC00963	2.665296004	4.27572413

HOXA11-AS	2.931932502	4.26760624
GAS5	1.626593209	4.200659451
RN7SK	1.510991898	4.055517328
PVT1	1.433039344	4.045757491
HEIH	1.883069352	4.017728767
RPLP0	0.99447192	3.903089987
PRNCR1	1.627719709	3.879426069
TERC	-0.906890596	3.728158393
WT1-AS	4.504855762	3.647817482
DLEU2	1.85725279	3.53313238
BLACAT1	7.24863041	3.288192771
MIR155HG	-0.914883386	3.195860568
LINC00312	1.365464153	3.185752404
SNORA73A	1.584196373	3.144480844
CBR3-AS1	0.944674545	3.104577454
PTCSC3	2.514573173	2.928117993
SUMO1P3	1.22180006	2.650528201
PCAT1	3.388697107	2.619426997
MEG3	-11.14636411	2.552841969
MALAT1	-1.660640949	2.550213153
PCA3	3.066442143	2.442853858
ACTB	1.255472933	2.436163081
LUCAT1	0.60728517	2.433916216
CDKN2B-AS1	-1.983256736	2.30390584
HOXA-AS2	1.641015306	2.250882338
ZFAS1	0.962655456	2.246493543
HAND2-AS1	-4.606273007	2.222790742
KCNQ1OT1	2.266128977	2.147398031
HIF1A-AS2	-0.632359644	2.106349183

Figure 3.3. LncRNA display differential expression in A2058 vs PM. RT^2 profiler arrays were used to determine the expression levels of numerous lncRNA in A2058.The volcano plot displays statistical significance versus fold-change on the y- and x-axes, respectively. Table shows log fold change and -log FDR for each gene. The volcano plot combines a p-value statistical test with the fold regulation change.

To determine if similar differential expression was observed between PM and other melanoma cell lines, the top 10 lncRNA targets in terms of differential change and p-value were selected for analysis in the A375, UACC-62 and M14 cell lines. Total RNA was extracted from PM, A375, UACC-62 and M14 cells, quantified and quality assessed prior to the generation of cDNA via first strand synthesis. The expression of selected lncRNA transcripts was then determined via qRT-PCR with each target normalised against three independent reference genes, RPS3, R18S and GAPDH. As shown in Figure 3.3.1 (A-D) several lncRNA which were upregulated in A2058 melanoma cells, were also differentially upregulated in A375, UACC-62 and M14 versus PM. Specifically, *LINC01234*, *BLACAT1* and *LINC00963* were upregulated in M14 cells compared to PM. While, increased expression of *HIF1A-AS1* was observed in A375 Figure 3.3.1 A. Consistent with our previous finding demonstrating decreased expression of *MEG3* and *EMX2OS* in A2058 cells, expression of these genes was significantly downregulated in A375, UACC-62 and M14 melanoma cells, compared with PM (Figure 3.3.1). Interestingly, several of the targets that were dysregulated in A2058 compared to PM, exhibited differential expression levels between melanoma cell lines that appear to correlate with the migration potential of these cell lines, as described in 3.2.

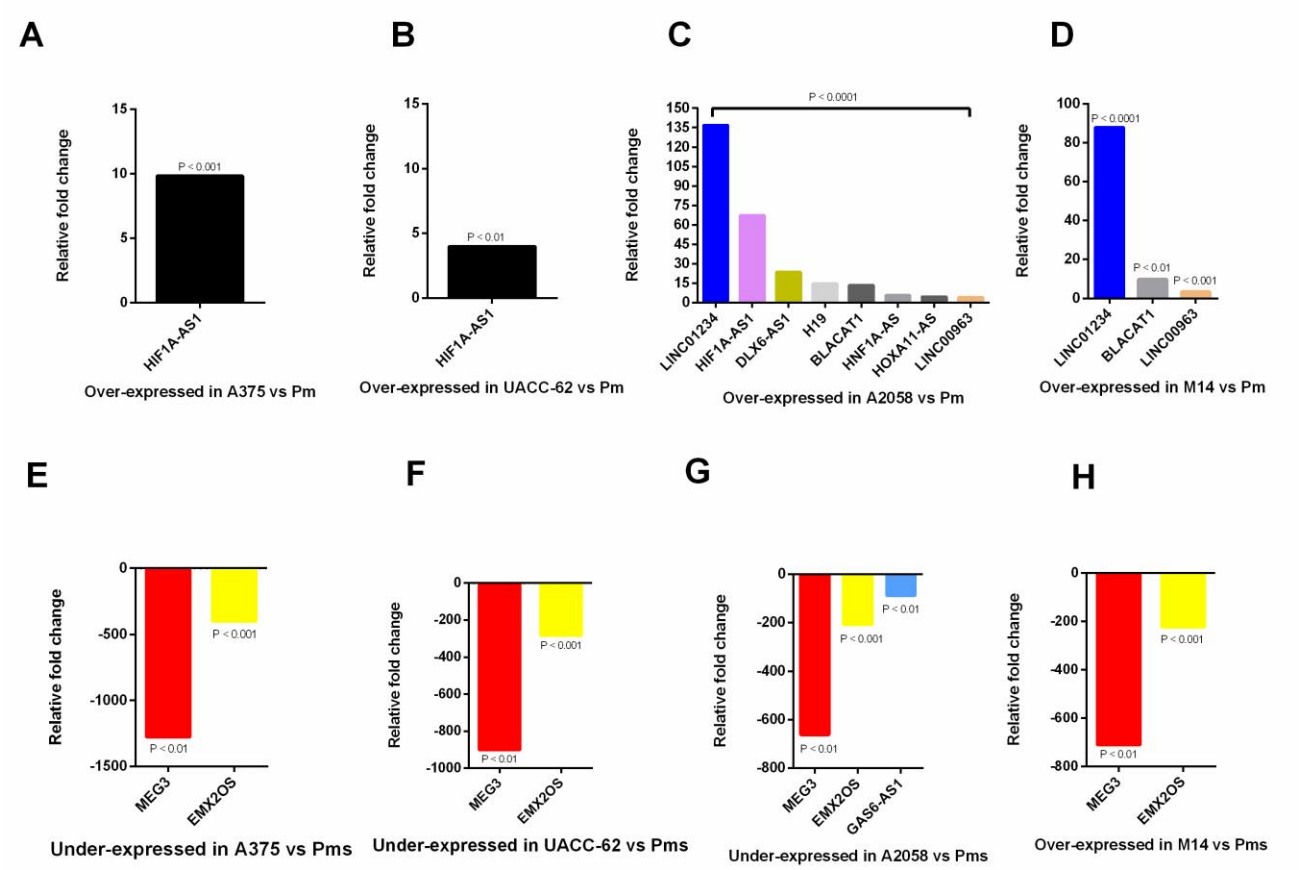


Figure 3.3.4. Dysregulated expression of lncRNA in melanoma cells vs PM. Expression of lncRNAs was accessed in melanoma cell lines and PM via RT-qPCR. Data was normalised to RPS3, R18S and GAPDH.

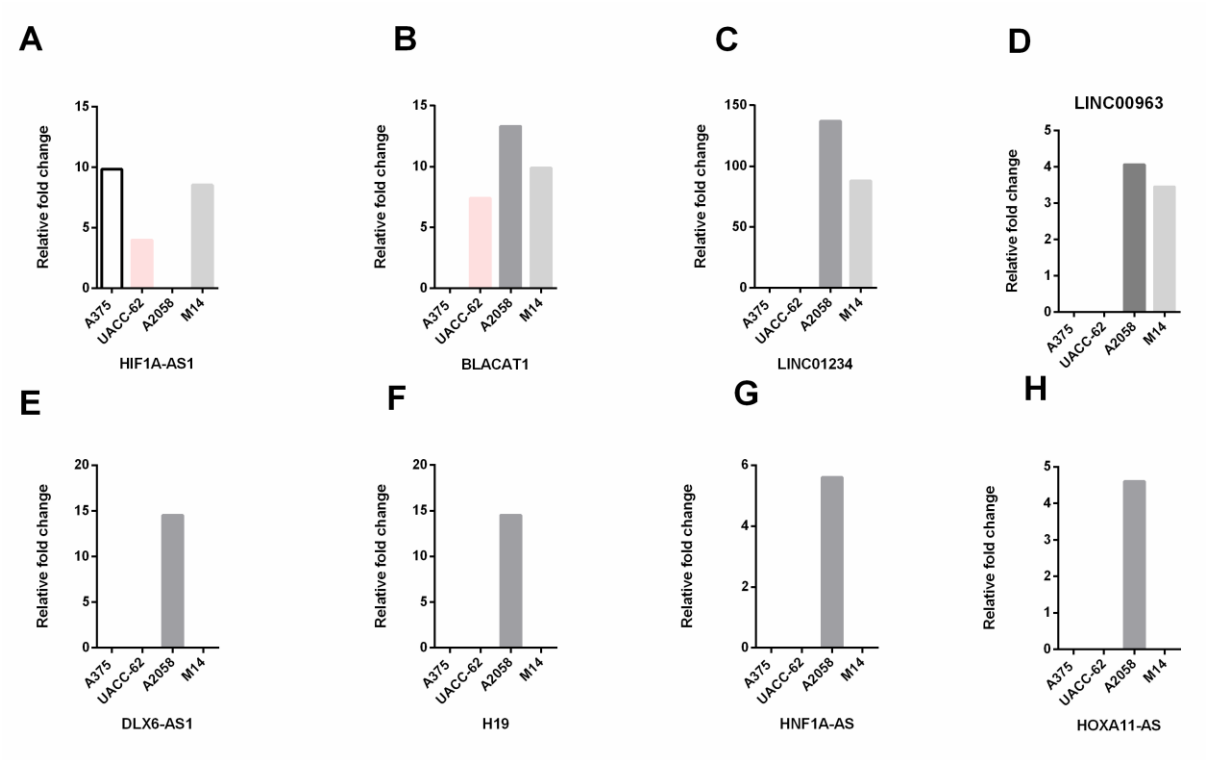


Figure 3.3.5. Previously unreported lncRNA are upregulated in metastatic melanoma cells. Top 8 lncRNA were selected and subjected to RT-qPCR, (A-H) shows differential expression of lncRNA in melanoma cell lines, A375, UACC-62, A2058 and M14, which mirrors migratory potential.

As shown in Figure (3.3.2) numerous lncRNA are upregulated in highly metastatic melanoma cells compared to PM. Next, it was interesting to determine if the melanoma- associated lncRNA Figure 3.1 display differences in expression between PM and melanoma cells. The relative gene expression of each lncRNA was determined via qRT-PCR with each target normalised against three independent reference genes, RPS3, R18S and GAPDH in PM and melanoma cells. Data below shows melanoma- associated lncRNA also demonstrate a differential expression in melanoma cells vs PM (Figure 3.3.3). Similarly, RT^2 targets were significantly upregulated in metastatic melanoma cells Figure 3.3.3 (F&H). Furthermore, the lack of expression of *BANCR* Figure 3.3.3 was unexpected and may reflect a lack of

sensitivity due to different primers being utilised to those immobilised on the RT2 profiler arrays Figure 3.2.

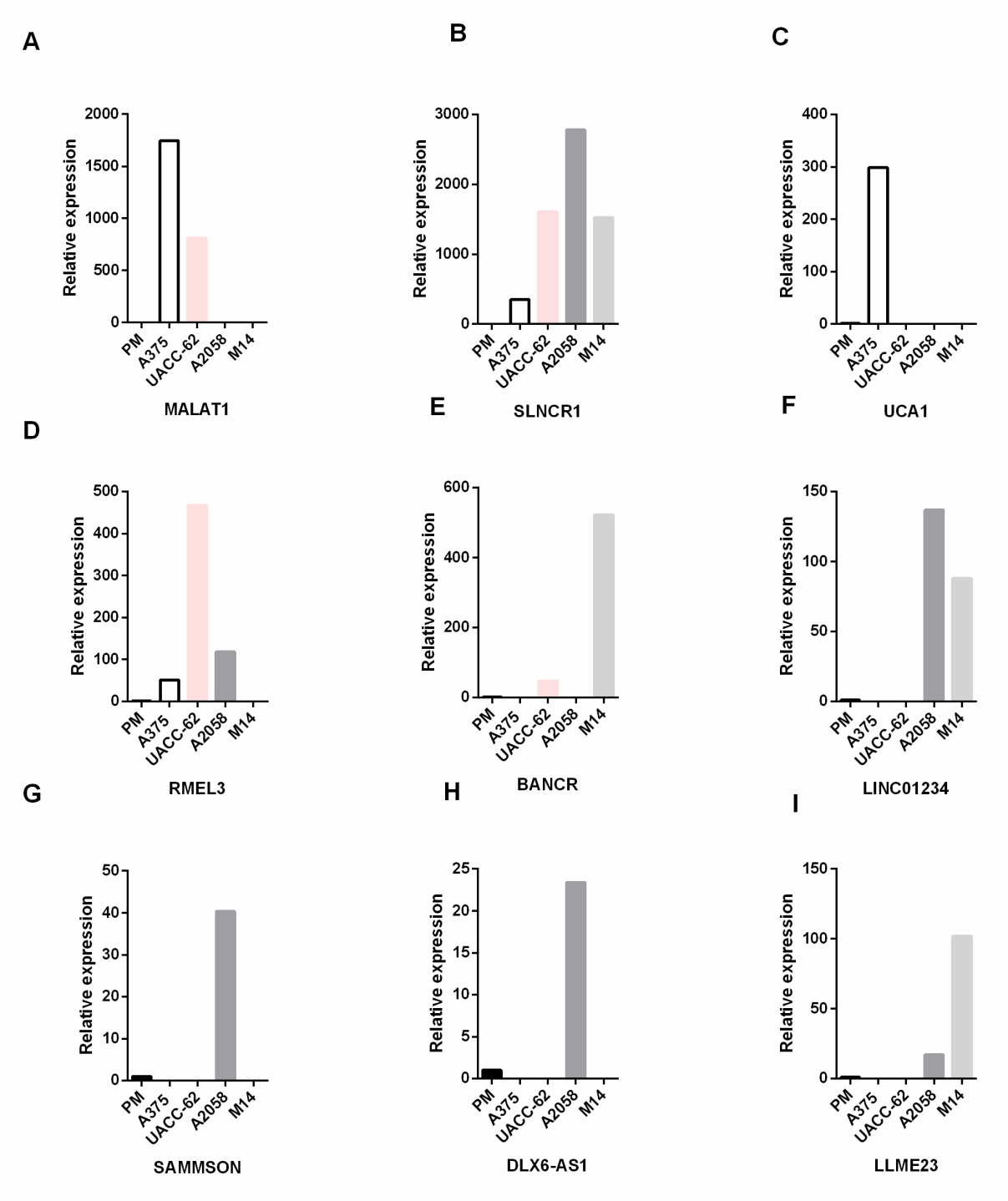


Figure 3.3.6. Melanoma cells display differential patterns of lncRNA expression. Melanoma-associated lncRNA and RT^2 profiler targets were selectively chosen and subjected to RT-qPCR.

3.4 SFPQ is over-expressed in melanoma cells

There is limited evidence to suggest that the RNA-binding protein, SFPQ, is a tumour suppressive lncRNA target in melanoma. Specifically, SFPQ is displaced from the promoter region of the *RAB23* proto-oncogene by binding of the poorly annotated lncRNA, *LLME23* (C.-F. Wu et al., 2013). Subsequent to and in contrast with this publication there have been a number of articles demonstrating a positive role for SFPQ in the aetiology of several other cancers (Q. Ji et al., 2014; Luisier et al., 2018; K. I. Takayama et al., 2017; Takeuchi et al., 2018; Tamotsu Tsukahara, Hisao Haniu, & Yoshikazu Matsuda, 2013). Indeed, while Wu *et al* investigated the role of *LLME23* in melanoma via knockdown, they did not directly investigate the function of SFPQ. To address this gap in the literature, I initially set out to determine the expression of SFPQ protein in PM compared with a range of melanoma cell lines. Total protein was isolated from melanoma cells and quantified via BCA assay, prior to loading and separation of proteins via SDS-PAGE and western transfer for subsequent SFPQ expression analysis Figure 3.4.

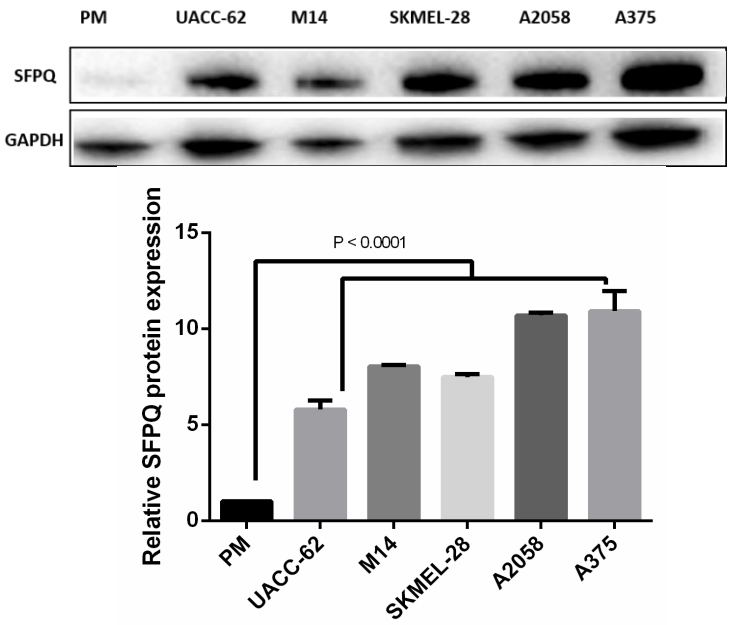


Figure 3.4. SFPQ is over-expressed in melanoma cell. (A) Western blot of total protein expression of SFPQ in PM and melanoma cell lines, UACC-62, M14, SKMEL-28, A2058 and A375. (B) densitometry analysis of SFPQ shows a significant increase in fold change between melanoma cells and PM. Statistical analysis was carried out via 2-way Anova followed by Tukeys multiple comparisons test n=3 independent biological repeats. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001, ns= no significance.

Given the proposed role of SFPQ as a tumour suppressor in melanoma, via the transcriptional repression of *RAB23* (C.-F. Wu et al., 2013) the observed dramatic increase in SFPQ expression between PM and the each melanoma cell line was unexpected and suggests that increased expression of SFPQ may be important for melanoma cell growth. As mentioned earlier, SFPQ contributes to the phenotype of several cancers via interaction with various lncRNAs. To determine if lncRNAs previously reported to bind SFPQ in other cancers, but not yet associated with melanoma growth might also correlate with melanoma migration potential, the lncRNA, *GAPLINC*, which binds SFPQ in colorectal cancer (P. Yang et al., 2016) was selected for further analysis via qRT-PCR. Interestingly, I observed a significant increase in

GAPLINC expression between PM and two metastatic melanoma cell lines that becomes striking in terms of difference for A2058 and M14 and is reflected via high correlation co-efficient between *GAPLINC* and migration potential in melanoma Figure 3.5. This raises the interesting possibility that SFPQ-interacting lncRNA might be of relevance to melanoma cell biology, a hypothesis that I go on to test in the following chapter.

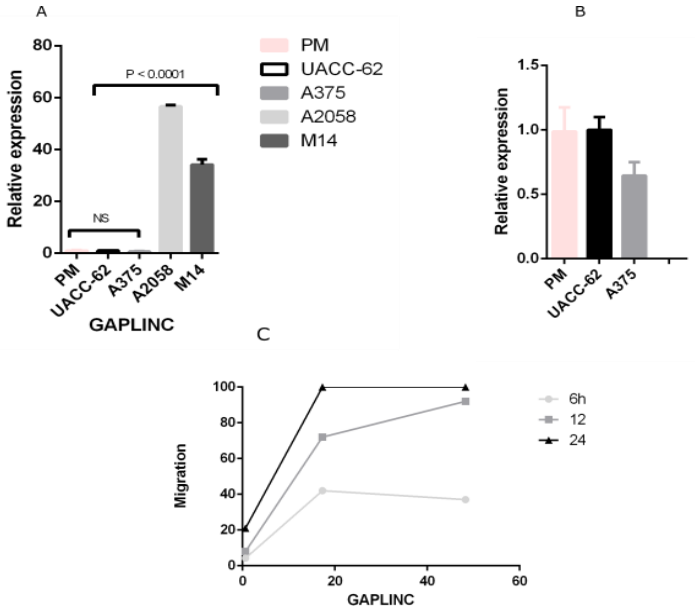


Figure 3.5. LncRNA *GAPLINC* is highly expressed in metastatic melanoma cells. GAPLINC expression levels were determined via RT-qPCR in PM, UACC-62, A375, A2058 and M14 melanoma cells. (B) Rescaled data for *GAPLINC* expression in PM compared with UACC-62 and A375 cell lines. (C) Pearson's analysis shows expression of *GAPLINC* mirrors migratory potential over 24 hours. Statistical analysis was carried out using 2-way Anova followed by Tukey's multiple comparisons test n=3 independent biological repeats. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.001, ns= no significance.

3.5 Discussion

Metastasis is the event that mostly significantly impacts the prognosis and overall survival of melanoma patients. There is mounting evidence linking the expression of numerous lncRNA to poor prognosis and metastasis in various cancers and more specifically in melanoma (Abbastabar, Sarfi, Golestani, & Khalili, 2018; D.-L. Chen et al., 2017; D. Chen et al., 2018; H. Lu et al., 2019; X. Luo et al., 2018; R. Tang et al., 2019; S. Wang et al., 2017; J. Yu, Yan, Hua, & Ming, 2019; X. Yu et al., 2018; Haiying Zhao et al., 2017). For example, dysregulated expression of lncRNA HOX transcript antisense intergenic RNA expression has been implicated as a potential biomarker for the prognosis of patients with carcinoma of digestive systems (G. Ma et al., 2015). Another example comes from the meta-analysis demonstrating the increased expression of the lncRNA, HOXA transcript at the distal tip (*HOTTIP*), which correlates positively with lymph node metastasis and poor survival outcome in various cancers, suggesting that *HOTTIP* may serve as a potential novel predictor of lymph node metastasis and survival in a pan-cancer manner (Zhicong Chen, He, Wang, Liu, & Huang, 2017) .

Numerous studies have demonstrated the involvement of numerous lncRNA in melanoma pathogenesis. The lncRNA *SPRY4-IT1* is abundantly found in the cytoplasm and stimulates proliferation, migration and invasion in melanoma cell lines, conversely low expression levels of *SPRY4-IT1* are associated with better patient outcomes (Khaitan et al., 2011; T. Liu et al., 2016; Mazar et al., 2014). Another study focused its efforts on identifying how the lncRNA *BANCR impacted on melanoma cell growth by silencing BANCR expression. They observed* decreased migration of melanoma cells, possibly due to the upregulation of the chemokine CXCL11 (Flockhart et al., 2012). Furthermore, it has been suggested that increase in melanoma

proliferation is stimulated by *BANCR*-mediated activation of the ERK1/2 and JNK MAPK pathways (Ruiya Li et al., 2014). The lncRNA *GAS5* has been reported as the only published tumour suppressor in melanoma, overexpression of *GAS5* in melanoma cell lines reduced expression of MMP2 resulting in collagen degradation and a reduction in cell migration (L. Chen et al., 2016). Numerous other lncRNA have been implicated in promoting melanoma growth such as, *HOTAIR, MALAT1, SAMMSON, SLNCR1*, and *ANRIL* (E. Leucci et al., 2016; K. Schmidt et al., 2016; L. Tang et al., 2013; Tian et al., 2014; Wei et al., 2016; Huaping Xie et al., 2016). Specifically, *SAMMSON* a melanoma specific lncRNA appears to serve as promising prognostic biomarker to detect melanoma disease progression at the early stage (E. Leucci et al., 2016).

Clearly, lncRNA occupy an important role in melanoma progression. Therefore, it was of interest to test the hypothesis that a panel of lncRNA may be present in melanoma that might correlate positively or inversely with metastatic potential. To investigate this, I opted to use cell migration as a proxy for metastatic potential. Cell migration is a key process during melanoma progression, which is controlled in various stages of tumour progression via cytokines and growth factors which enable cells to grow and metastasise to distant organs (Lázár-Molnár, Hegyesi, Tóth, & Falus, 2000). There are several methods that serve as relevant proxies for determining metastasis such as cell invasion assays, proliferation, expression of MMPs, colony formation assays, and perhaps most widely used, in *vitro* cell migration assays. I opted to utilise the classic wound-healing assay as a proxy for the metastatic potential of the melanoma cells in this study, for several reasons. Firstly, there is significant precedent for the use of this assay when investigating metastatic phenotype in melanoma cells. For example, Flockhart and associates determined *BANCR* as a novel BRAFV600E-induced

lncRNA which regulates melanoma cell migration *in vitro* via wound-healing assays (Flockhart et al., 2012). Moreover, work on *SOX4,* which is dysregulated in melanoma as well as other cancers (Q. Cheng et al., 2017; Y.-W. Huang et al., 2009; Jafarnejad, Wani, Martinka, & Li, 2010; R. Sun et al., 2015) used Transwell migration and wound-healing assays to assess the impact of siRNA-mediated knockdown and lentivirus-mediated overexpression of *SOX4* in melanoma cell lines A375 and A2058 (Q. Cheng et al., 2017). Furthermore, another study investigating the role of neurotrophin in melanoma cell migration utilised Transwell migration and wound-healing assays (Truzzi et al., 2008). Wound-healing assays also represent a cost-effective technique that was an important consideration in terms of budget management, given the significant costs associated with RNA-seq later in the project cycle.

Initially, a panel of cell lines were assessed for migration potential to determine a hierarchy of low and high cell lines. Data shown in Figure 3.1 demonstrates the melanoma cell lines used in this study exhibit a range of migration potential, ranging from low (UACC-62) to high (M14). These phenotypic differences were consistently observed via scratch wound assays across five biological repeats, suggesting that the differences observed are due to cell line specific effects, rather than variation between assays. The observation that M14 melanoma cells are highly migratory is supported by several studies. Specifically, compared with other melanoma cell lines M14 has been shown to express high levels of C-X-C chemokine receptor type 7 (CXCR7) (Wen et al., 2017), which regulates cell migration, invasion, growth and interacts with extracellular matrix thereby promoting tumour development (Balkwill, 2004; D. Rossi & Zlotnik, 2000). Moreover, knockdown of CXCR7 significantly inhibits M14 cell migration and invasion, highlighting the importance of this receptor in melanoma cell migration (X. J. Li et al., 2017). CXCR7 is also expressed in A2058 cells (Shields et

al., 2006), which in my data show a similar migration profile to M14 and are significantly more migratory than A375, UACC-62 and SKMEL-28 cells. The low migration potential observed for these cell lines is also in broad agreement with the work of Kim *et al*, who in their work investigating the utility of aminomalonic acid as a potential melanoma biomarker characterised A375 cells as 'low metastatic potential', compared with the aggressive A2058 cell line (H.-Y. Kim et al., 2017). Together, this supports my data demonstrating that my chosen panel of melanoma cell lines represent a broad spectrum in terms of migration potential and that these differences can be exploited to determine if correlative patterns exists between melanoma cell migration and lncRNA expression.

Having established a panel of melanoma cell lines that exhibit significantly different migratory potential, I opted to carry out comparative analysis of transcript levels for several oncogenic lncRNA reported as melanoma-associated in the literature. In each case, the genes listed in Table 3.2 have been implicated in melanoma metastatic phenotype and except for *SLNCR1*, this also involves cell migration. One of the caveats of the many lncRNA studies that focus on the overexpression of a single lncRNA gene on cancer phenotype is a lack of understanding in terms of how changes in the expression of these genes relate to other dysregulated lncRNA in the cancer cell. As shown in Figure 3.2, all of the published melanoma-associated oncogenic lncRNA were readily detected by qRT-PCR in each of the four melanoma cell lines, however, several genes exhibited significant differential expression that correlated either positively or negatively with melanoma cell migration. Consistent with the literature, several of these lncRNA exhibited increased expression in the more aggressive melanoma cell lines, including *BANCR* (R. Li et al., 2014) and the recently identified melanoma-specific lncRNA, *SAMMSON* (E. Leucci et al., 2016).

To the best of our knowledge whilst no other study has attempted to correlate expression levels of lncRNA to a cancer phenotype in melanoma cells, there are datasets that include a comparative analysis of lncRNA expression in different melanoma cell lines. Most notable of these is a recent study that utilised the NCI60 cancer cell line panel (Shoemaker, 2006) to assess the expression of 1707 human lncRNAs via high-throughput nanowell RT-qPCR (Mestdagh et al., 2016). Curation and analysis of expression data for UACC-62 and M14, which are the two cell lines common to the work described in this chapter and the NCI60 datasets, reveals a list of lncRNA that are differentially expressed between these two cell lines. Unfortunately, it is not possible to cross-reference the genes that display the greatest differential expression and correlation to migration (*LLME23*, *GAPLINC* and *SAMMSON*), as the first two genes are not present on the array employed by this study - one of the drawbacks of biased array-based experiments, as opposed to non-biased RNA sequencing data-sets. Moreover, and somewhat concerning, is the lack of any *SAMMSON* expression for either cell line in the NCI60 lncRNA datasets, despite clear expression of *SAMMSON* in all of the cell lines investigated in this project (Figure 3.2 D) and others (E. Leucci et al., 2016).

In contrast to limited published data comparing lncRNA expression between melanoma cell lines, almost nothing is known regarding the differential expression of lncRNA between non-transformed PM and melanoma cells. I decided to investigate lncRNA expression between PM and the aggressive melanoma cell line, A2058 via a qRT-PCR array harbouring 84 lncRNA dysregulated in various cancers. Expression of each lncRNA was assessed initially in melanoma cell line A2058 and PM and then the top ten dysregulated targets were analysed across the additional melanoma cell lines, A375, UACC-62 and M14 (Figure 3.3.1).

Interestingly, several previously unreported lncRNA were upregulated in A2058, including *LINC01234*, which was significantly upregulated by ~135-fold in melanoma cell line and has not been previously associated with melanoma. However, increased expression of *LINC01234*, which is a highly conserved mammalian noncoding RNA situated at 12q24.13, has been observed in several other cancers (W. Guo et al., 2016). Initially, *LINC01234* gained interest as a promising prognostic marker to predict survival in breast cancer patients (W. Guo et al., 2016). *LINC01234* was subsequently also found to be significantly upregulated in non-small-cell lung cancers (NSCLC) and exerts tumour growth by promoting invasion and migration and is linked to poor patient survival (Z. Chen et al., 2020). In oesophageal cancer cells, *LINC01234* drives metastatic phenotypes such as invasion, proliferation and apoptosis (Ghaffar et al., 2018). Clearly, these studies suggest that *LINC01234* may serve as a promising biomarker for cancer and its role in melanoma cell growth warranted further investigation, which is presented in Chapter 5.

Another interesting target that is significantly over expressed in A2058 cells is HIF 1 alpha-antisense 1 (*HIF1A-AS*). A common feature of tumour growth is hypoxia (Erler et al., 2006; Pouysségur, Dayan, & Mazure, 2006; G. L. Wang, Jiang, Rue, & Semenza, 1995), which is regulated by Hypoxia- inducible factor (HIF)-1 and HIF-2, controlling gene expression for tumour angiogenesis, glucose metabolism, and resistance to oxidative stress (Bertout, Patel, & Simon, 2008; Dayan, Roux, Brahimi-Horn, Pouyssegur, & Mazure, 2006; Erler et al., 2006; Wouters & Koritzinsky, 2008). *HIF1A-AS* is over-expressed in in a number of renal cancers, (Bertozzi et al., 2011) and in heart disease, where interplay between *HIF1A-AS1* and apoptotic proteins promote proliferation and apoptosis of vascular smooth muscle cells (VSMCs), contributing to the development of thoracoabdominal aorta aneurysm (Y. Zhao, Feng,

Wang, Yue, & Zhao, 2014). Furthermore, *HIF1A-AS1* may serve as a potential biomarker to detect non-small cell lung cancer (NSCLC) as levels of *HIF1A-AS1* were significantly higher in tumour tissues or serum from NSCLC patients versus control group (Tantai, Hu, Yang, & Geng, 2015). The role of *HIF1A-AS1* in melanoma has not been investigated, however, recent data implicating this lncRNA in HIF-1α mediated autophagy suggests that it could be playing a similar role in melanoma (F et al., 2020).

Finally, distal-less homeobox 6 antisense 1 (*DLX6-AS1*) resides at the 7q21.3 chromosomal region and has been implicated in various cancers such as lung adenocarcinoma , renal cell carcinoma , and hepatocellular carcinoma and gastric cancer (Juan Li et al., 2015; X. Zeng et al., 2017; L. Zhang, He, Jin, Gang, & Jin, 2017). The clearest picture of *DLX6-AS1* function comes from knockdown experiments in gastric cancer, which revealed that silencing of *DLX6-AS1* significantly decreased cell proliferation, colony formation, cell cycle progression, migration and invasion, although a molecular mechanism for this was not put forward by the authors (Fu, Tian, Kuang, Wen, & Guo, 2019).

In addition to overexpressed lncRNA I also identified two lncRNA that were significantly down regulated in melanoma cell lines compared with PM. The first of these was the well-characterised maternally expressed gene 3 (*MEG3*), which is a tumour suppressor gene located in chromosome 14q32 and was downregulated by ~500 fold in A2058 cells (Tanmoy Mondal et al., 2015; J. V. Schmidt, Matteson, Jones, Guan, & Tilghman, 2000; Wylie et al., 2000). *MEG3* expression is derailed in several cancers such as cervical cancer, bladder cancer, colorectal cancer, glioma, endometrial carcinoma and breast cancer (Qin et al., 2013; W. Wang et al., 2019; Shoudan Zhang & Guo, 2019; W. Zhang et al., 2017). In melanoma, *MEG3* has

previously been shown to be downregulated in A375, SK-MEL-1, B16, and A2058 melanoma cell lines compared to PM (Peng Li et al., 2018; Jianwen Long & Xianming Pi, 2018), in board agreement with my data. A second downregulated lncRNA identified in my analysis was *EMX2OS,* a polyadenylated transcript which is expressed on the strand opposite to the *EMX2* gene that encodes the Homeobox transcription factor, Emx2 (Noonan, Goodfellow, Staloch, Mutch, & Simon, 2003). Emx2 is an important gene which regulates the development of the brain and urogenital system during embryonic development (Cecchi & Boncinelli, 2000; Miyamoto, Yoshida, Kuratani, Matsuo, & Aizawa, 1997; Noonan et al., 2003; M. Pellegrini, Pantano, Fumi, Lucchini, & Forabosco, 2001; Tole, Goudreau, Assimacopoulos, & Grove, 2000; T. Zhao et al., 2006). There is mounting of evidence to suggest that *EMX2OS* acts as a tumour suppressor in many cancers, including lung, gastric and glioma, presumably via downregulation of Emx2, which behaves as a negative regulator of the Wnt signalling pathway (Falcone, Daga, Leanza, & Mallamaci, 2016; J. Li et al., 2012; Okamoto et al., 2010; Yue et al., 2015). A report linking *EMX2OS* expression in papillary thyroid cancer to unfavourable Kaplan-Meier curves also demonstrated, via *in silico* analysis, that genes co-expressed with *EMX2* or *EMX2OS* were highly overlapped. Analysis of the KEGG pathway unveiled that these genes were abundant in targets associated with inhibitors of cancer initiation and development, such as genes regulating ECM-receptor interaction, focal adhesion, PI3K-Akt signalling, protein degradation and absorption and proteoglycans (Y. Gu, Feng, Liu, Zhang, & Yang, 2018). It will be of interest to establish if our observation of decreased *EMX2OS* expression in melanoma cell lines has a similar functional importance in melanoma (something beyond the scope of this project) and if decreased EMX2OS predicts poorer patient outcomes, which I investigate in chapter 5.

The increased expression of SFPQ observed in all the melanoma cell lines compared with PM was somewhat unexpected, given the proposed tumour suppressor role for SFPQ in melanoma (C. F. Wu et al., 2013). Given the plethora of functions attributed to SFPQ, it is difficult to speculate how exactly overexpression might impact on melanoma biology, if at all. However, there is compelling evidence in prostate cancer that increased SFPQ expression drives cancer phenotype via post-transcriptional regulation of RNA transcripts (K.-i. Takayama et al., 2017b). Here, in its capacity as a splicing factor, SFPQ is responsible for the expression of AR-responsive genes and other components of the splicing machinery and as such promotes a 'cancer transcriptomic state'. Intriguingly, the authors demonstrate that in addition to prostate cancer associated mRNA transcripts, SFPQ also regulates the expression of oncogenic lncRNA. This prompts the question is SFPQ performing a similar function in melanoma? I test this hypothesis in the next chapter, via comparative RIP-seq analysis of the SFPQ interactome in PM and A2058 melanoma cells.

Chapter 4: Characterisation of the SFPQ-RNA interactome in melanoma cells

4.1 RIP-pull down of SFPQ in A2058 cells

SFPQ has emerged as a key regulator of metastasis in several cancers including colorectal, prostate and melanoma. Importantly, where it has been shown to be implicated in cancer, a common mechanism involves interaction with lncRNAs (Q. Ji et al., 2014; K. Takayama et al., 2013; P. Yang et al., 2016). Thus far, the melanoma specific lncRNA *LLME23* has been reported to bind to SFPQ, derailing its role in transcriptional repression of *RAB23* and positioning SFPQ as a tumour suppressor with regards to melanoma progression (C. F. Wu et al., 2013). However, Wu *et al* were unable to characterise the full length *LLME23* transcript via RACE and the gene remains incompletely annotated. Moreover, work in prostate cancer suggest an oncogenic role for SFPQ, whereby the splicing accessory regulates expression of key metastatic drivers. Clearly, the functional role of SFPQ in cancer is complex and multifaceted and a broader appreciation of its role in melanoma remains unclear.

To this end, I sought to characterise the SFPQ-RNA interactome in melanoma via an unbiased approach utilising RIP-seq. Given the increased expression of SFPQ in melanoma cancer cell lines compared with PM (Figure 3.4). I was particularly interested in identifying melanoma-specific shifts in the SFPQ-RNA interactome. Initially, ~1.0 x 10^6 cells were lysed in RIP lysis buffer and incubated for 5 minutes on ice prior to storing at -80°C. Next, magnetic beads were loaded with SFPQ antibody or rabbit IgG for control and used to immunoprecipitations with RIP lysates. Following washes 10% of beads were removed and precipitation of the SFPQ protein confirmed via western blot. As can be seen in Figure 4.1, specific immunoprecipitation of SFPQ protein was observed for SFPQ IPs, compared with samples immunoprecipitated with IgG antibody control. RNA extraction was carried out for PM and A2058 RIP samples by re-suspending remaining beads in proteinase K buffer and subsequently isolating

RNA via phenol/chloroform and ethanol precipitation. RNA pellets were re-suspended in RNase-free water quality assessed via NanoDrop. RNA samples were then sent to the Next Generation Sequencing facility at The University of Leeds. Briefly, RIP and control libraries were generated, rRNA was removed from control samples, RNA was cleaned (entry point for IP selected RNA samples), first and second strands were synthesised, cDNA was purified and adenylation of 3′ ends performed followed by the addition of sequencing adaptors. Libraries were then quantified and pooled for sequencing on an Illumina NextSeq platform.

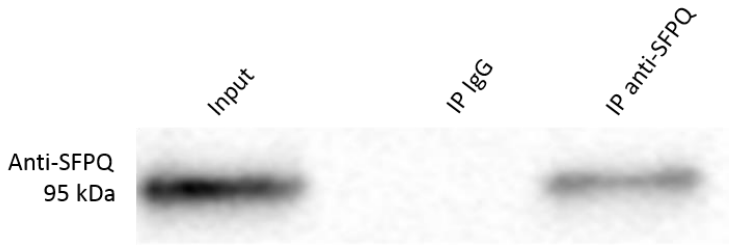


Figure 4.1 RNA immunoprecipitation of SFPQ. 10% input (total A2058 lysates) IgG and SFPQ were immunoprecipitated with anti-SFPQ, followed by detection via western blot. Lane 1 (10% Input), lane 2 (IP IgG), lane 3 (IP anti-SFPQ).

4.2 The SFPQ-RNA interactome is reprogrammed between PM and A2058 cells

Raw sequencing data were processed and analysed in collaboration with Dr A. Anene (Centre for Cancer Genomics and Computational Biology, Barts Cancer Institute, QMUL) and gene lists provided detailing differential enrichment of SFPQ-binders in each cell background. Raw sequencing has been uploaded to Array Express and will remain embargoed until publication (work described in this thesis is currently under review with *Molecular Cancer*).

Initially, I identified transcripts that were (i) significantly enriched with SFPQ in PM compared with A2058 cells; (ii) enriched with SFPQ in both PM and A2058 cell backgrounds; (iii) significantly enriched with SFPQ in A2058 cells compared with PM (Figure 4.2 A). Most SFPQ-enriched transcripts comprised mRNA, with 8124 unique peaks called, however, further analysis revealed 345 unique SFPQ-lncRNA interactions across both cell backgrounds (Figure 4.2 B). While most SFPQ-enriched transcripts were present in both PM and A2058 cells, we observed significant differential enrichment of both mRNA and lncRNA transcripts between PM and A2058 cells. To rule out the possibility that baseline expression of these genes in PM and A2058 transcriptomes was influencing SFPQ-enrichment, whole-cell RNA expression profiles were integrated with RIP-Seq data and peaks called with unsupervised clustering. As can be seen in Figure 4.2 C, I observed no association between whole-cell RNA transcript abundance and the specificity of the SFPQ-RNA interactions, suggesting that the differential SFPQ-RNA interactome identified in A2058 melanoma cells is due to reprogramming. To investigate if reprogramming of the SFPQ-RNA interactome might have any biological importance for melanoma, gene ontology analysis was carried out to identify enriched biological processes for PM-specific and A2058-specific SFPQ-mRNA interactors, respectively. As can be seen in Figure 4.3, specific GO terms were identified that are enriched for either PM or A2058-specific SFPQ-binding mRNAs.

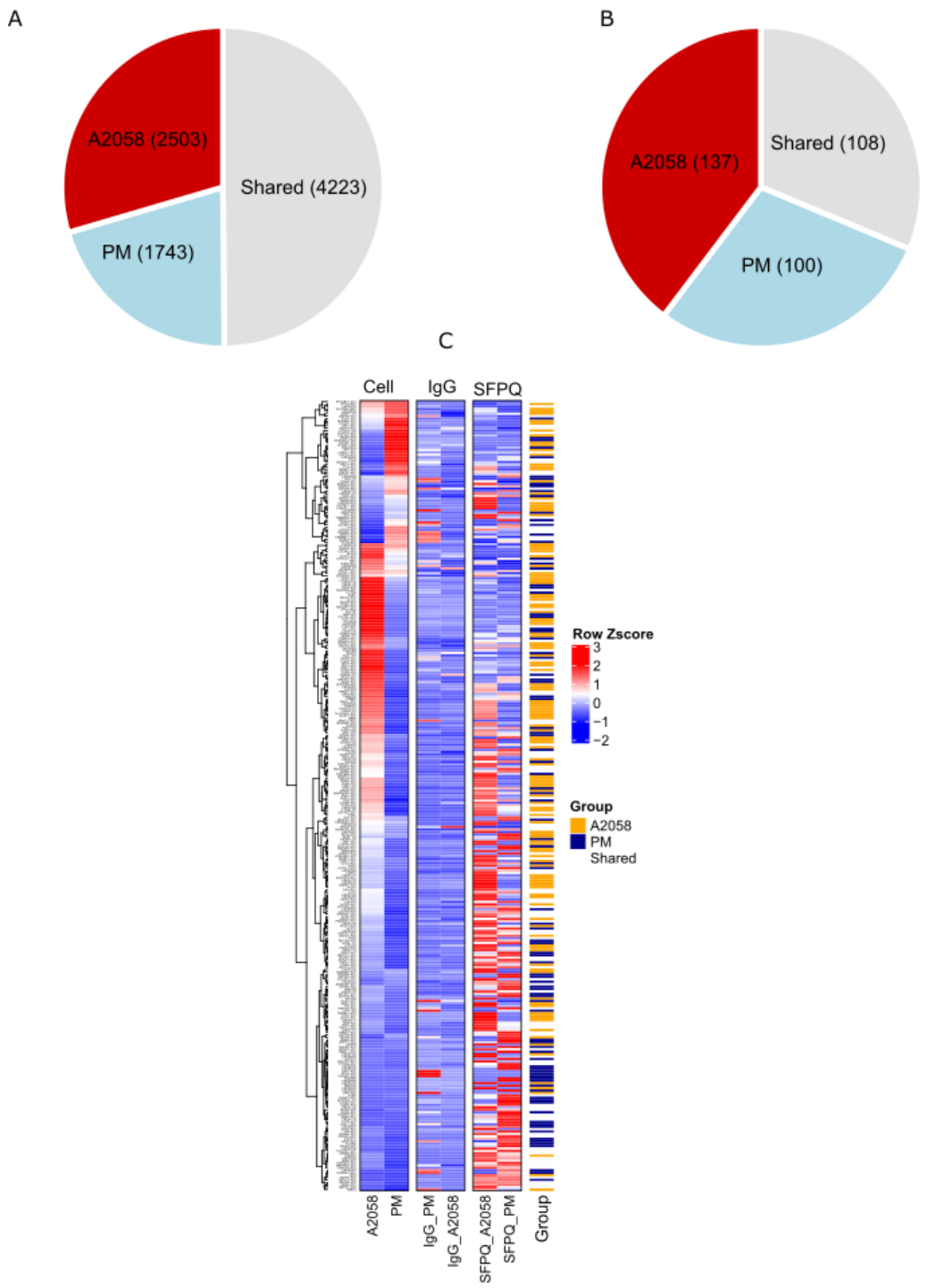


Figure 4.2. The SFPQ-RNA interactome is reprogrammed between PM and A2058 cells. (A) Pie chart depicts the proportion of all interaction RNA transcripts shared between PM and A2058, specific to PM or specific to A2058. (B) Pie chart depicts the proportion of lncRNA transcripts shared between PM and A2058, specific to PM or specific to A2058. (C) Heat map of relative expression profile of identified lncRNA interactors across whole cell RNA (A2058 and PM), IgG control and SFPQ-IP. Within the heatmap, red colour indicates high expression relative to whole cell RNA and blue colour indicates low expression.

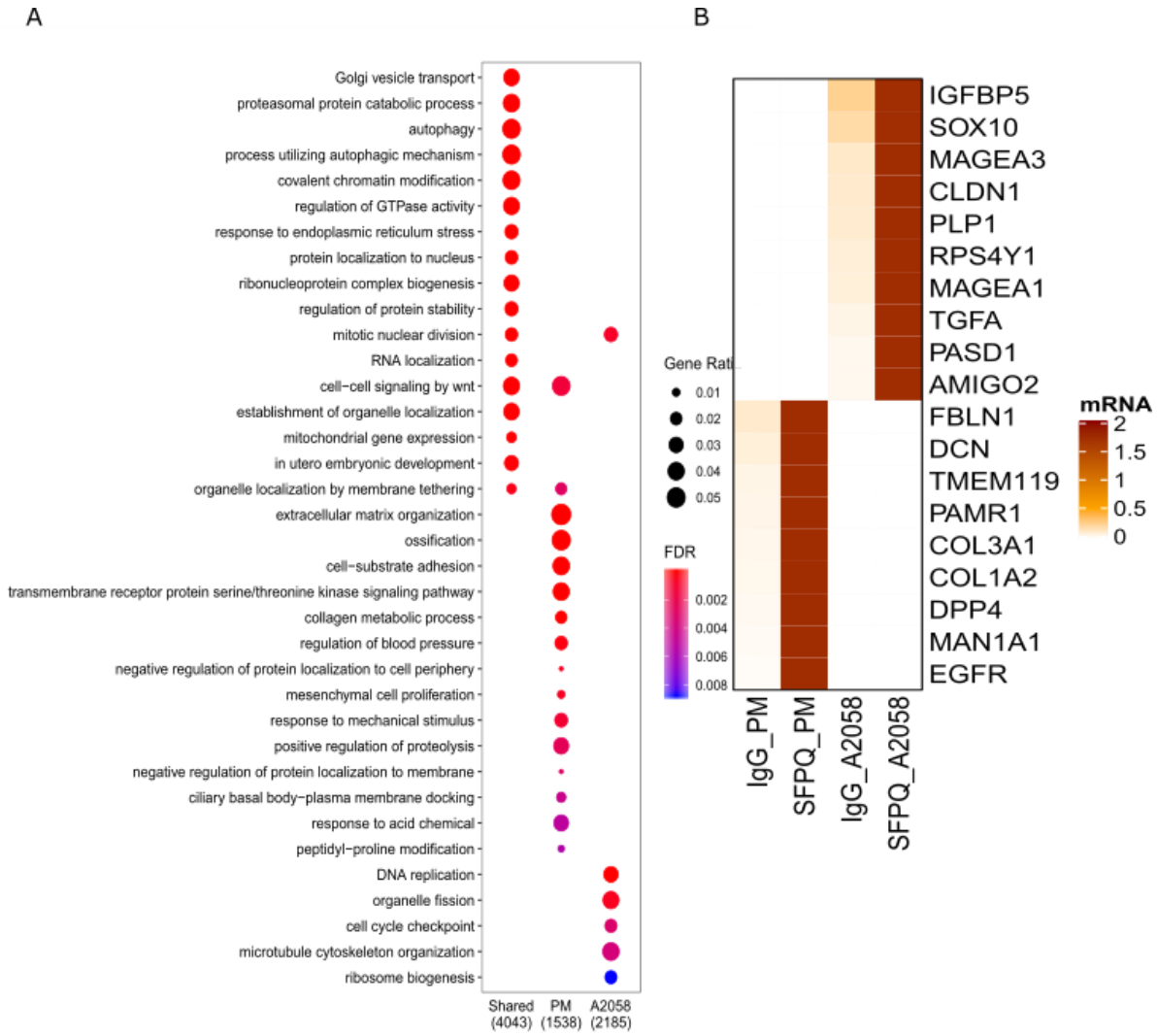


Figure 4.3. Cluster comparison plot of enriched biological processes for transcripts shared between PM and A2058, specific to PM or specific to A2058. Gene ratio = genes in ontology/total gene set and FDR = false discovery rate. (B) Heat map of relative expression profile of identified mRNA interactors (A2058 and PM), IgG control and SFPQ-IP.

Given the widely documented relationship between SFPQ and lncRNAs in several cancers, including melanoma, SFPQ-lncRNA interactors were also ranked according to their PM- and A2058-specificity (Figure 4.4 A). Discerning the functional importance of enriched lncRNA gene lists from RNA-seq studies such as the one reported here remains problematic, as the vast majority of lncRNAs remain unannotated. However, it is interesting to note that the most PM-specific SFPQ-lncRNA interactors include

genes associated with TS function, such as *EMX2OS* and *FENDRR*, whereas the most A2058-specific SFPQ-lncRNA interactors comprise genes widely reported as oncogenic. These observations are like those reported in prostate cancer, where SFPQ-RNA interactions have been shown to be reprogrammed in disease. To investigate if melanoma-specific SFPQ-RNA interactions were conserved in prostate cancer we integrated an existing SFPQ RIP-seq dataset. Data from three different prostate cancer cell lines LNCAP, LTAD, 22RV1 were analysed and data extracted for the top 10 SFPQ-enriched lncRNAs (Figure 4.4 B). Interestingly, the enrichment pattern across the prostate cancer cell lines broadly followed the same pattern observed in our study. Specifically, SFPQ-lncRNAs enriched in A2058 cells were similarly present in SFPQ RIP-seq data derived from prostate cancer cells. Moreover, PM specific lncRNAs demonstrated the inverse trend (Figure 4.4 B). Two PM-specific SFPQ-lncRNAs, *FENDRR* and *LINC00602*, were not enriched in any of the prostate cancer cells, underlying their cancer specific interaction with SFPQ.

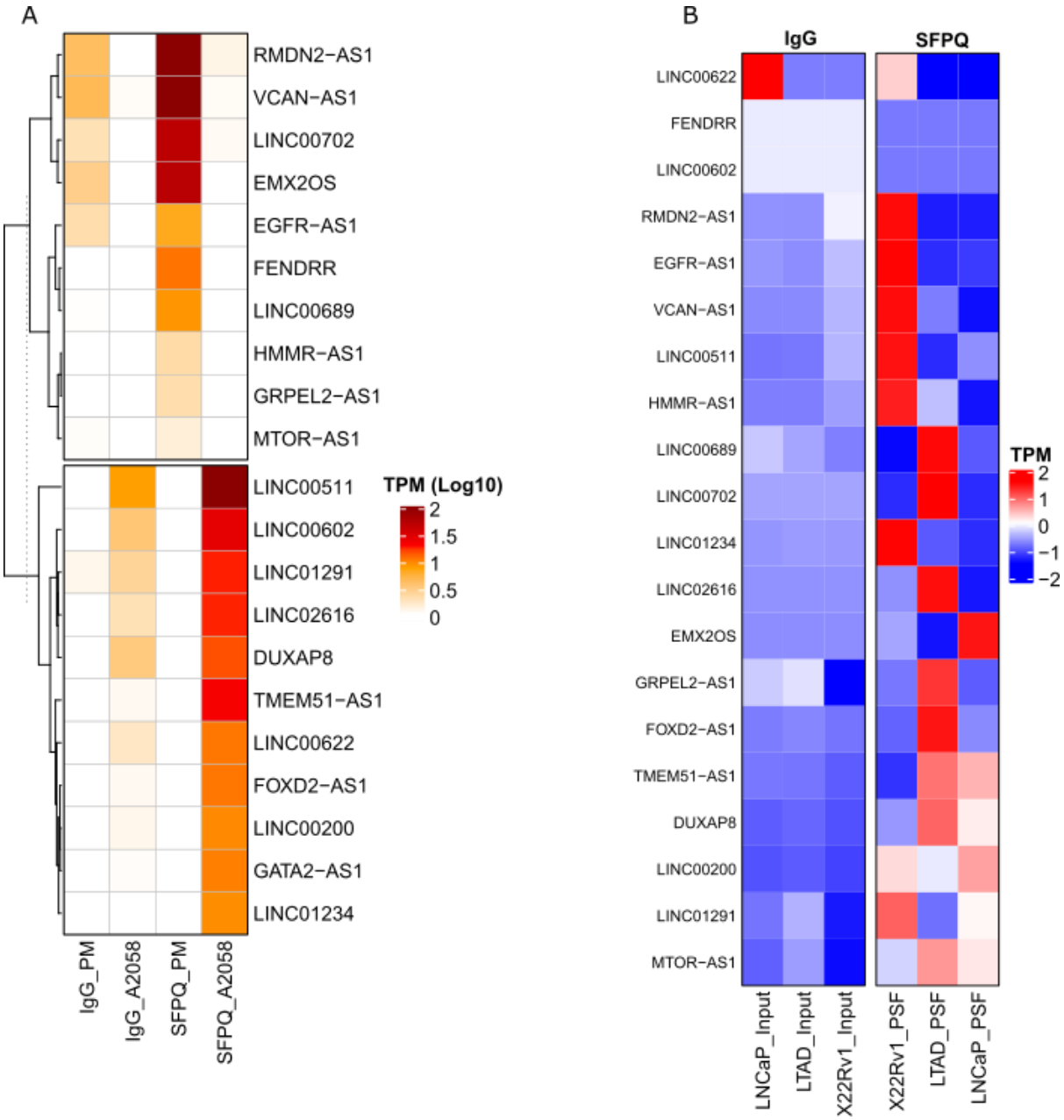


Figure 4.4. SFPQ-lncRNA interactors were ranked according to their PM- and A2058-specificity. A. Heat map of expression profile of top 10 cell specific lncRNA interactors across IgG control and SFPQ-IP. Within the heatmap, orange colour indicates enrichment in SFPQ-IP compared to IgG and white colour indicates no enrichment. B. Heat map of expression profile of top 10 cell specific lncRNA interactors across Input control and SFPQ-IP in prostate cancer cells. Within the heatmap, red colour indicates high expression relative to whole cell RNA and blue colour indicates low expression.

4.3 SFPQ binding profile suggests regulation of splicing

While peak calls in RIP-seq experiments lack the resolution offered by CLIP-seq, it is possible to obtain limited data relating to the location of protein binding on interacting transcripts via calling only those peaks that are uniquely mapped and non-duplicated against IgG control libraries and annotating the genomic coordinates of those with p-values of $<10^{-5}$ via the R Bioconductor packages. This analysis was carried out in collaboration with Dr Anthony Anene (CRUK Barts Centre, Queen Mary University of London) to gain insight into the binding location of SFPQ on a range of enriched transcripts. As can be seen in Figure 4.5, SFPQ appears to predominantly bind transcripts at either exon-intron boundaries or at the 3'UTR, which is consistent with the reported roles of SFPQ in pre-mRNA splicing and mRNA stabilisation (Cosker, Fenstermacher, Pazyra-Murphy, Elliott, & Segal, 2016; X. Dong et al., 2007; Knott et al., 2016; Landeras-Bueno, Jorba, Pérez-Cidoncha, & Ortín, 2011; Song et al., 2005; K.-i. Takayama et al., 2017b).

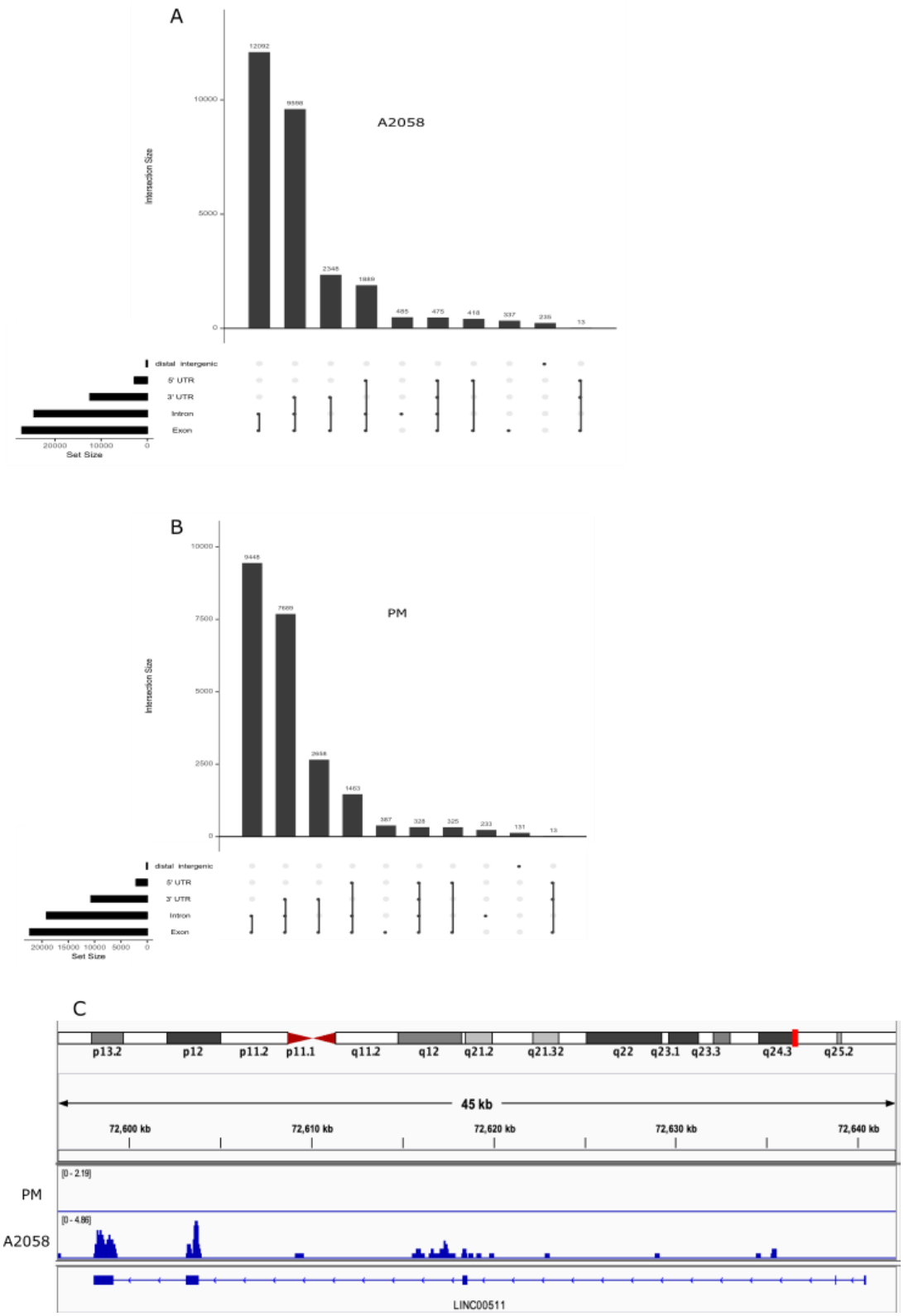


Figure 4.5. SFPQ binding profile suggests regulation of splicing. A. Structural features of SFPQ RNA binding in A2058. B. Structural features of SFPQ RNA binding in PM. E. IGV profile of SFPQ binding to *LINC00511* across PM and A2058.

4.4 SFPQ depletion effect on gene expression and stability

SFPQ has previously been shown to function as a transcriptional regulator in a variety of cell types including melanoma (K.-I. Takayama et al., 2017a). Moreover, it has also been shown to stabilise mRNA transcripts in prostate cancer. Therefore, it was interesting to determine if SFPQ was regulating, activating, or stabilising specific transcripts in melanoma. To this end, it was important to determine in the absence of SFPQ the expression of genes of interest, targets were selected based on top SFPQ-lncRNA binders in A2058 (*TMEM51-AS1, LINC00511*, *LINC01234*, *TMEM51-AS1)* and mRNA transcripts which include *SOX10*, *AMIGO2* and *MAGE3*, *SAMMSON* was selected as a control this target is not an SFPQ interactor. Initially SFPQ was silenced via GapmeRs for 48 hours, prior to treatment with ActinomycinD, RNA was extracted at 0, 3 and 6 time points for control and SFPQ-knockdown cells. Next, cDNA was synthesised, and gene expression was assessed via RT-qPCR. Each target was normailsed to three house-keeping genes GAPDH, RPS13 and TBP (Vandesompele et al., 2002). As shown in Figure 4.6 gene expression of *LINC00511*, *LINC01234*, *TMEM51-AS1, SOX10* and *MAGEA3* were significantly reduced in the absence of SFPQ. Furthermore, RNA stability of several transcripts was determined, as can be seen in Figure 4.7 stability of *SOX10* and *TMEM51-AS1* decreased in SFPQ-knockdown cells.

Collectively, these data show that SFPQ-RNA interactions in melanoma are reprogrammed compared with PM and skewed towards oncogenic coding and non-coding transcripts. Moreover, SFPQ appears to regulate the expression of a subset of enriched transcripts, possibly via stabilisation of the transcripts mediated by interaction with the 3’UTR.

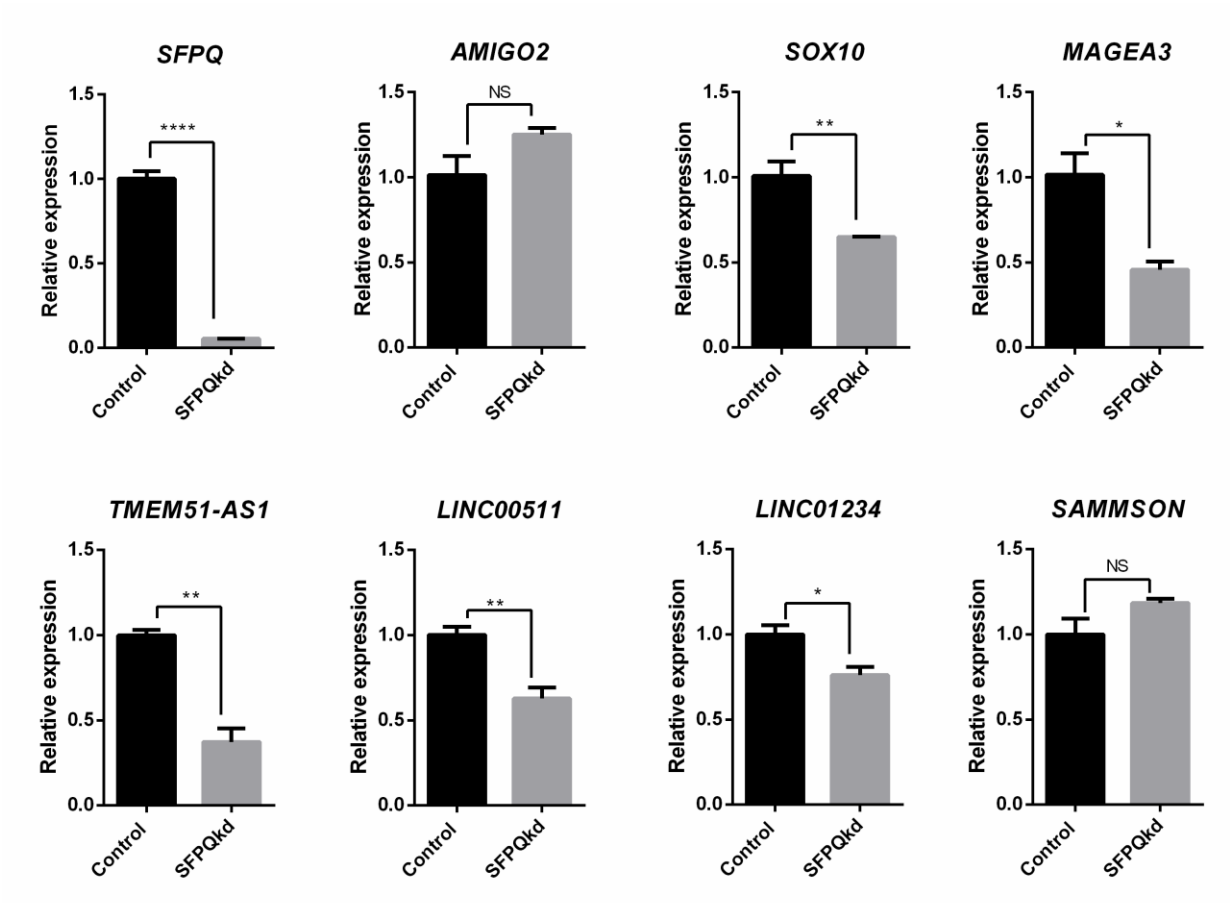


Figure 4.6. Expression of gene transcripts in the absence of SFPQ. GapmeRs were transfected into A2058 cells, 48 hours post transfection cells were treated with actinomyinD. RNA was extracted at 0, 3 and 6 hours, cDNA synthesised, and gene expression was determined via RT-qPCR. Each target was normalised to three house-keeping genes GAPDH, RPS13 and TBP. N=3, Statistical analysis was carried out using t-test, *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001 ns (no significance).

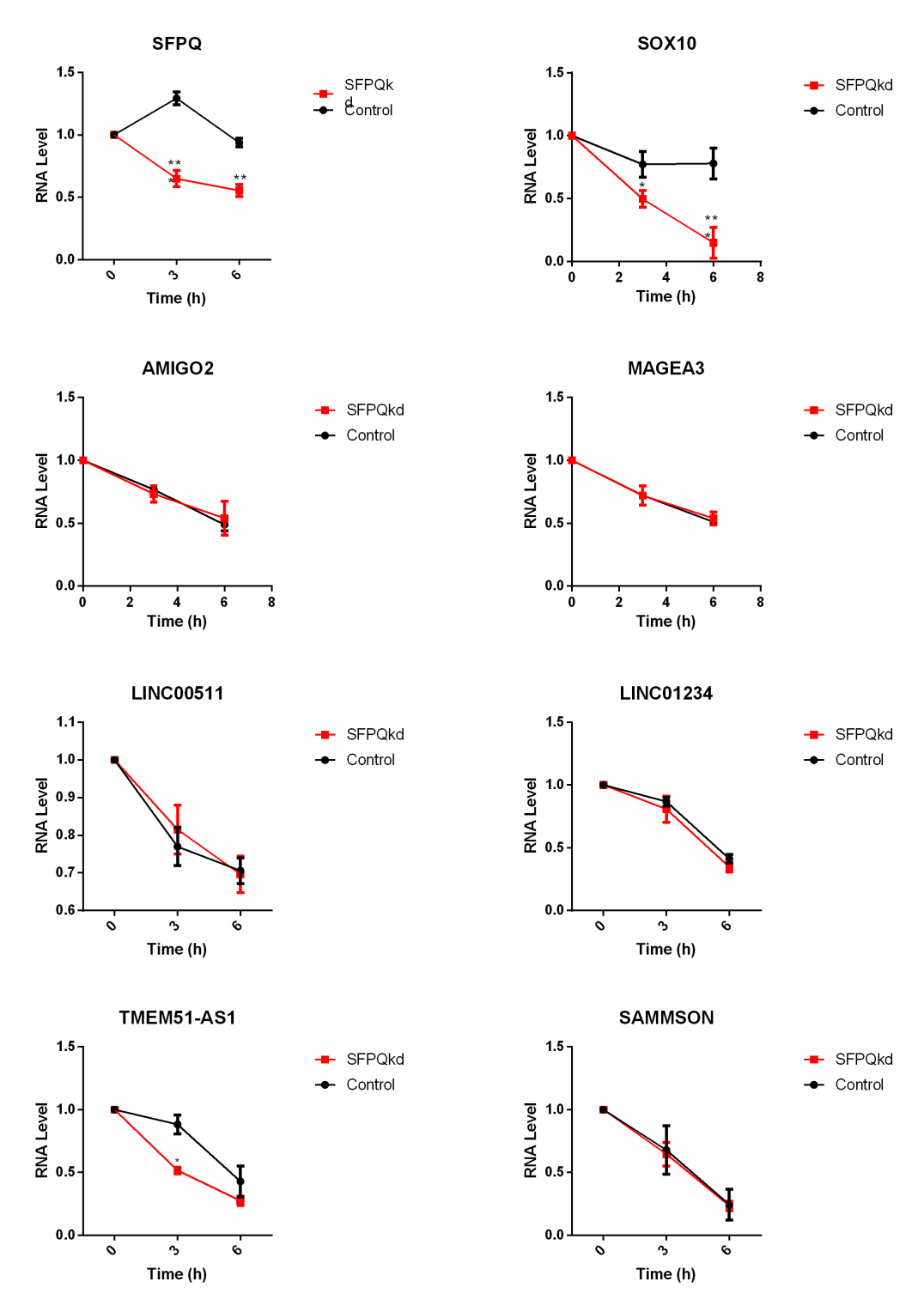


Figure 4.7. RNA stability of transcripts in the absence of SFPQ. GapmeRs were transfected into A2058 cells, 48 hours post transfection cells were treated with actinomyinD. RNA was extracted at 0, 3 and 6 hours, cDNA synthesised, RNA stability was determined via RT-qPCR. Each target was normalised to three house-keeping genes GAPDH, RPS13 and TBP. N=3. Statistical analysis was carried out using t-test, *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001 ns (no significance).

4.6 Discussion

This aim of the work presented in this chapter was to characterise the SFPQ-lncRNA interactome and to determine its reprogramming in melanoma. Bioinformatics analysis of RNA-Seq datasets revealed a multitude of transcripts (mRNA, miRNA and lncRNA) associated with SFPQ in both primary melanocytes and melanoma cells (Figure 4.2 A). By combining peak calls with transcript abundance analysis, we revealed numerous SFPQ-lncRNA interactors specific to A2058 and PMs. Further, we integrated publicly available RNA expression profiles from whole-cell lysate of PMs and A2058 data sets from ENCODE and GEO database to investigate the effect of baseline expression levels on these cell-type specific interactions. Hierarchical clustering of the data suggests there is no association between whole-cell RNA level and the specificity of the SFPQ- lncRNA interaction. Confirming that high expression of transcript in PMs or A2058 does not influence the observed interaction specificity, implying a functional regulation of these SFPQ-lncRNA interactions (Figure 4.2 C).

We next used the ratio of SFPQ-IP to IgG-IP read counts to select top 10 specifically enriched lncRNAs in PMs and A2058 (Figure 4.4 A). An interesting target specifically enriched in PM is *FENDRR*, a gene 3099nt in length, found on chr3q13.31 and comprised of four exons. *FENDRR* is essential for the development of heart and body wall development in mice (Grote et al., 2013) and binds to both PRC2 and Trithorax group/MLL protein complexes (TrxG/MLL), which have critical roles in regulating structure of chromatin and gene activity (Khalil et al., 2009; Schuettengruber et al., 2007). Beyond this, *FENDRR* appears to function as a tumour suppressor gene in gastric cancer, where its expression is reduced in metastatic cells compared to normal gastric epithelial cells (T. P. Xu et al., 2014). Moreover, reduced expression of *FENDRR* was correlated with deeper tumour invasion, higher tumour stage and

lymphatic metastasis (T. P. Xu et al., 2014). Interestingly, a recently, a study showed that *FENDRR* supresses invasion and migration of metastatic melanoma cells (X.-E. Chen et al., 2020). The authors observed decreased expression of *FENDRR* in metastatic melanoma cells leading to significant increases in cell proliferation, migration and invasion. Conversely, over-expression of *FENDRR* in melanoma cells decreased the cancer phenotype (X.-E. Chen et al., 2020). These data suggest *FENDRR* may play a tumour suppressor role by inhibiting development of metastatic phenotype in MM. Our observation that SFPQ interacts with *FENDRR* in PM but not in A2058 suggests that SFPQ may be involved in the regulation of *FENDRR* expression and that this role is reprogrammed in melanoma (Figure 4.4 A). A second PM-specific SFPQ-enriched lncRNA is *EMX2OS*. Data shown in chapter 3 demonstrate that *EMX2OS* is predominantly expressed in PM (Figure 3.3), however, SFPQ-interaction appears to be also be PM specific, which raises the possibility that SFPQ may be reprogrammed in melanoma and thus contributes in some way to reduced expression of *EMX2OS*. Little is currently known in terms of *EMX2OS* function and it has been proposed to function as an oncogene in ovarian cancer (M, M, C, H, & M, 2020) and a tumour suppressor in thyroid cancer (Y, C, T, B, & L, 2018), suggesting that function may be tissue specific.

The top SFPQ-enriched transcript in melanoma cells was *LINC00511*, which has been widely reported to function an oncogene in various cancers, including breast, renal, gastric, lung and bladder (Z. Chen, Wu, Zhang, Li, & Liu, 2019; Deng et al., 2019; L. Jiang, Xie, Ding, Mei, & Bi, 2019; J. Li, Li, Meng, Fu, & Kong, 2018; G. Lu et al., 2018). However, to date no research has been carried out investigating the role of *LINC00511* in melanoma. In breast cancer, expression was linked with chemoresistance, as the therapeutic efficacy of paclitaxel-induced chemotherapy was increased following

depletion of *LINC00511* (H. Zhang, Zhao, Wang, Zhang, & Yu, 2019). Furthermore, *LINC00511* is upregulated in liver hepatocellular carcinoma (LIHC) tissues and cell lines and positively correlates to TNM staging, lymphatic metastasis and predicated a poor prognosis in patients (Hu et al., 2019). Furthermore, *in vitro* silencing of *LINC00511* reduced migration and proliferation of Huh7 and Hep3B cells. A similar functional role has been ascribed in ovarian cancer and NSCLC (C. C. Sun et al., 2016; J. Zhang et al., 2019). This suggests that *LINC00511* functions as an important oncogene and I investigate if this gene is also contributing to the cancer phenotype in melanoma in the chapter 5, alongside two other lncRNAs associated with SFPQ and melanoma, *LINC01234* and *GAPLINC*.

Functional assessment of multiple SFPQ-enriched lncRNA, while desirable, is problematic. While there are numerous databases which provide important information regarding lncRNAs structure, expression and interaction, such as NONCODE, LncRNAdb, ChIPBase, lncRNome, LncRNADisease (Bhartiya et al., 2013; G. Chen et al., 2013; Quek et al., 2015; Y. Zhao et al., 2016), far fewer databases exist for the functional annotation for lncRNAs. Ultimately, the main method of identifying lncRNA function (and thus annotating the gene) remains empirical biological experiments, however, these are expensive and time consuming. A few computational methods have been reported (J. Zhang, Zhang, Wang, Liu, & Deng, 2018). *Guo et al* developed a network-based approach, lnc-GFP. In lnc-GFP, first a bi-coloured biological network is formed according to co-expression and protein interaction data, then lncRNAs are annotated by running a global propagation algorithm on the bi-coloured network (X. Guo et al., 2013). Despite great efforts, lncRNA annotation remains an enigma, due to their sophisticated molecular regulatory mechanisms.

GO terms are built predominately around protein coding genes. In this study, bioinformatic analysis revealed numerous SFPQ-mRNA interactions that displayed cell type specific enrichment. GO analysis of all shared, PM and A2058-specific SFPQ-enriched mRNA revealed several interesting findings (Figure 4.3). PM specific GO terms broadly associated with extracellular matrix organisation, whereas A2058-specific SFPQ-interactors were significantly associated with GO terms relating to the positive regulation of the cell cycle. Indeed, as shown in Figure 4.3 B, several of the genes that ranked highest in our analysis of A2058-specific SFPQ-mRNA interactors have been previously implicated in melanoma progression, including the transcription factor, *SOX10* (JC et al., 2018) and the transmembrane protein, *AMIGO2* (B et al., 2017). In contrast, PM-specific SFPQ-bound mRNA transcripts were significantly enriched for components of the ECM, including *COL1A2*, *COL3A1* and *FBLN1*, which have been previously reported to function as melanoma TS genes (BJ et al., 2014; VF et al., 2011).

The top SFPQ-enriched mRNA in A2058 cells was the *SOX10* transcript, cytogenetically located at 22q13.1 (Bowles, Schepers, & Koopman, 2000). *SOX10* is an important nuclear factor which plays a critical role in the formation tissues and organs during embryo development (Bowles et al., 2000; Britsch et al., 2001). Importantly, in melanoma *SOX10* is required for melanocytic cell differentiation by activating *MITF* and melanogenic enzymes dopachrome tautomerase and tyrosinase (Bondurand et al., 2000; Dutton et al., 2001; Ludwig, Rehberg, & Wegner, 2004). *Graf et al* observed *SOX10* mRNA expression varied between melanoma cells and did not correlate with disease progression (Graf, Busch, Bosserhoff, Besch, & Berking, 2014). However *Cook et al* reported reduced *SOX10* expression in melanocytes compared with melanoma cells (Cook, Smith, Smit, Leonard, & Sturm, 2005). Moreover, *SOX10*

protein was highly expressed in metastatic melanoma cells suggesting association with a more invasive metastatic phenotype. Moreover, depletion of *SOX10* in several melanoma cell lines significantly reduced cell death and invasion (Graf et al., 2014). Mechanistically, the melanoma inhibitory activity (MIA), an important protein for melanoma cell invasion and migration was found to be a direct transcriptional target of *SOX10* (Graf et al., 2014). Correlation between expression levels of *SOX10* and MIA has been previously reported in melanoma cell lines and silencing of *SOX10* decreases MIA expression and promoter activity. Also, over-expression of MIA in *SOX10*-depleted melanoma cells promoted the invasion capacity, suggesting MIA is required for *SOX10*-regulated melanoma cell invasion (Graf et al., 2014). Another interesting target specifically enriched in melanoma cells is *AMIGO2* belonging to the Amphoterin-induced gene and ORF family (Kuja-Panula, Kiiltomäki, Yamashiro, Rouhiainen, & Rauvala, 2003). This gene comprises six LRRs flanked by cysteine-rich domains and an Ig-like domain (Rabenau et al., 2004). Initially, *AMIGO* proteins were identified in a screen for genes that regulate adhesion processes required for axon extension and fasciculation (Kuja-Panula et al., 2003). *AMIGO2* was independently discovered as pro-survival factor of cerebellar granule neurons upon depolarisation (Ono, Sekino-Suzuki, Kikkawa, Yonekawa, & Kawashima, 2003). Recent studies show *AMIGO2* as a pro-survival factor in endothelial cells with hypoxia, suggesting an important function in the vasculature (Ono et al., 2003). Bromodomain and extraterminal domain inhibitors (BETi) have gained great attention as promising therapeutics for MM. *Fontanals-Cirera et al* shows *AMIGO2*, as a BET target gene required for melanoma cell survival. Moreover, their findings show *AMIGO2* is upregulated in metastatic melanoma cells and tissues compared to normal human melanocytes, and that depleting *AMIGO2* in melanoma cells results in G1/S arrest

followed by increased apoptosis (Fontanals-Cirera et al., 2017). Interestingly, the pseudokinase, PTK7, interacts with *AMIGO2* to mediate survival of melanoma cells independently of PI3K/AKT and MAPK pathways (Fontanals-Cirera et al., 2017). *AMIGO2*-PTK7 has received attention as potential therapeutic target. So far, antibody-drugs against PTK7 have gained success in phase I clinical trials for solid tumours (Damelin et al., 2017). Leading the way to the development of high affinity monoclonal antibodies which inhibit the interaction of *AMIGO2* and PTK7 and may have potential therapeutic efficacy in melanoma. Our findings show SFPQ-specific enrichment of *AMIGO2* mRNA in A2058 melanoma cells, and it would be interesting in the future to determine the functional importance of this interaction.

In contrast to melanoma cells, the most PM-specific SFPQ-enriched mRNAs were collagen family members *COL1A2* and *COL3A1*. Type I collagen, is predominately found in connective and embryonic tissue (Cole, 1994) and is a vital structural component of the extracellular matrix. Type I collagen is composed of a heterotrimer of two α1 (*COL1A1*) and one α2 (*COL1A2*) chains (Exposito, Valcourt, Cluzel, & Lethias, 2010). Dysregulated expression of *COL1A1* and *COL1A2* has been reported in renal cancer, hepatocellular carcinoma and melanoma, similarly other family members expression has been implicated in carcinogenesis (Bonazzi et al., 2011; Ibanez de Caceres et al., 2006; Kita et al., 2009; Ramaswamy, Ross, Lander, & Golub, 2003; Y. H. Wu, Chang, Huang, Huang, & Chou, 2014). Epigenetic regulation of tumour suppressor genes (TSGs) have occupied an important role in melanoma development. During melanomagenesis, TSGs, such as *PTEN*, *CDKN2A/p16INK4A* and *RASSF1A* expression has been decreased by CpG island methylation (Richards & Medrano, 2009; Rothhammer & Bosserhoff, 2007). A study conducted by *Bonazzi et al* focused its efforts identifying methylation

status of the most promising genes using highly sensitive Sequenom EpiTYPER assays in a large panel of melanoma cell lines and resected melanomas, and melanocytes. Their findings show transcript levels *COL1A2* were inversely correlated with promoter methylation (Bonazzi et al., 2011). More than fifty percent of melanoma cell lines had no *COL1A2* mRNA expression, which correlated with an increase of *COL1A2* promoter methylation in sixty-seven percent of this subset. Moreover, they show *COL1A2* promoters were more than ten–fold more methylated in melanoma cell lines versus melanocytes (Bonazzi et al., 2011). These finding are in line with previous studies which confirmed *COL1A2* as methylated in thirty-five to eighty-nine percent of melanoma tumour samples (Koga et al., 2009; Muthusamy et al., 2006). Indeed, *COL1A2* gene is critical in melanocytic neoplasia, our data show that SFPQ is enriched with TS transcripts in PMs and suggest SFPQ regulation may be important for the expression of these transcripts in PMs.

SFPQ interacts with PTB/hnRNPI which in turn associates with the polypyrimidine tract of intronic mRNA, a region important in the 3′-splice site in mammalian cells (J. G. Patton et al., 1993). Several studies have demonstrated SFPQ in splicing for example, in order to elicit an immune response, *TRAP150* associates with the SFPQ RRM2 domain and inhibits splicing of numerous SFPQ-target genes in T Cells (Christopher A. Yarosh et al., 2015). Recently *Takayama et al*, discovered several spliceosome genes were upregulated in metastatic CRPC tissues. Using cell-based models they found SFPQ regulates the expression of these spliceosome genes. The most important targets identified for SFPQ was AR, because AR and AR-V7 stimulate the hormone-refractory state (Y. Li et al., 2011; L. L. Liu et al., 2014). RIP-seq analysis revealed increased association of AR transcripts with SFPQ in CRPC models cells compared with hormone-dependent cancer cells (K.-I. Takayama et al., 2017a).

Moreover, silencing of SFPQ in 22Rv1 cells supressed full-length AR and AR-V7 mRNA and protein, however, silencing of SFPQ in LNCaP cells did not impact AR mRNA and proteins levels. Thus, upregulation of SFPQ is responsible for increased expression of AR and AR-V7 by aberrant splicing activity for CRPC development. These findings imply that SFPQ may function as a commander of splicing machinery for prostate cancer development and the AR (L. L. Liu et al., 2014; K.-i. Takayama et al., 2017b).

This hypothesis gains further traction from studies of AR and SFPQ in Alzheimer's disease (AD), which have been shown to regulate the expression of the amyloid precursor protein (APP), a gene closely related to AD (K. I. Takayama, Fujiwara, & Inoue, 2019). Here androgen increases the production of APP at both mRNA and protein levels and is enhanced by AR overexpression, conversely knockdown of AR supresses APP production (K. I. Takayama et al., 2019). Data obtained from public transcriptomes of brain tissue in mice suggest that APP is tightly controlled via SFPQ post-transcriptionally, as silencing of SFPQ resulted in reduced expression of AAP due to destabilisation of APP mRNA (K. I. Takayama et al., 2019). Together these studies highlight the importance of SFPQ in regulating alternative splicing and transcript stability in a range of diseases.

In this study a similar approach was adopted to determine if SFPQ is affecting the expression and stability of interacting transcripts. As shown in Figure 4.6, silencing of SFPQ expression in melanoma cells led to a significant decrease in the expression of *SOX10, TMEM-AS1, LINC00511, LINC01234, AMIGO2* and *MAGE3*. Not all SFPQ-enriched transcripts were decreased, suggesting that SFPQ may not contribute directly to the expression of every RNA it binds and/or that any enhancement of expression was beyond the limit of detection using this assay. Interestingly, when I

investigated if the observed decease in expression was due to diminished mRNA stability, significant reductions in half-life were only observed for *SOX10* and the lncRNA, *TMEM-AS1*, suggesting that SFPQ may stabilise these transcripts. Aforementioned, *SOX10* occupies an important role in melanocyte development, my findings suggest SFPQ is having an upstream effect on *SOX10* as 50% of transcript was destabilised and SFPQ-*SOX10* interaction is important in driving melanoma. A recent study revealed *TMEM51-AS1* functions as ceRNAs promoting laryngeal squamous cell carcinoma (Hui, Wang, Zhang, & Long, 2019). However, no data has been reported for *TMEM-AS1* involvement in melanoma. Although, I was unable to look at splicing of these transcripts, it would be interesting to determine this in the future for some of the targets which showed a decrease in transcript expression and stability. Particularly, *SOX10* as this transcript has 7 splice variants and our data shows a significant decrease in stability and expression of this target.

Chapter 5: Determining how SFPQ impacts on the melanoma cancer phenotype

5. Functional role of SFPQ and SFPQ-interacting lncRNA in melanoma cells

In chapter three a significant increase in the expression of SFPQ and several oncogenic lncRNA was observed in melanoma cells and in the previous chapter several lncRNA were shown to interact with SFPQ in a melanoma-specific manner. These data prompt the hypothesis that melanoma cell growth is somehow supported by the increased expression of SFPQ and possibly by genes that it post-transcriptionally regulates. In this chapter I describe ASO-mediated knockdown of SFPQ and several of the SFPQ-enriched lncRNAs targets described in the previous chapters and report how these impact on cancer phenotype in melanoma cells.

5.1 Silencing of SFPQ

SFPQ is over expressed and directly important for cell growth in prostate cancer and CRC (K. I. Takayama et al., 2017; P. Yang et al., 2016). In chapter 3, SFPQ was shown to be significantly over expressed in a range of melanoma cell lines, compared with PM (Figure 3.4). However, to date no one has investigated if SFPQ expression is required for melanoma cell growth. To address this question, I opted to utilise the A2058 melanoma cell line, as this is reported to display a potent metastatic phenotype. Initially, short interfering RNAs (siRNAs) were obtained specific to the major SFPQ transcript (NM_005066.3) and transfected into A2058 cells prior to culturing for 24 and 48h. Following isolation of total RNA and first strand synthesis, SFPQ transcript levels were assessed via RT-qPCR, however, no significant knockdown was observed across several independent experiments (Figure 5.1 A). Therefore, an alternative approach was adopted using GapmeR® antisense oligonucleotides (ASOs).

Two independent SFPQ-specific GapmeRs® were obtained and transfected into A2058 cells, which were then cultured for 24, 48 and 72 hours, prior to RNA extraction

and cDNA synthesis. SFPQ transcript levels were then assessed via qRT-PCR. Data in Figure 5.1 B-D show that SFPQ was significantly depleted with either individual GapmeR and that when both GapmeRs® were combined depletion was enhanced. To confirm that GapmeR-mediated depletion of the SFPQ transcript also led to a significant reduction in SFPQ protein, total protein was isolated from GapmeR-transfected A2058 melanoma cells and quantified via BCA assay, prior to loading and separation of proteins via SDS-PAGE and western transfer for subsequent SFPQ expression analysis (Figure 5.1 E-F). All subsequent SFPQ knockdown experiments utilised the GapmeR® agents in combination.

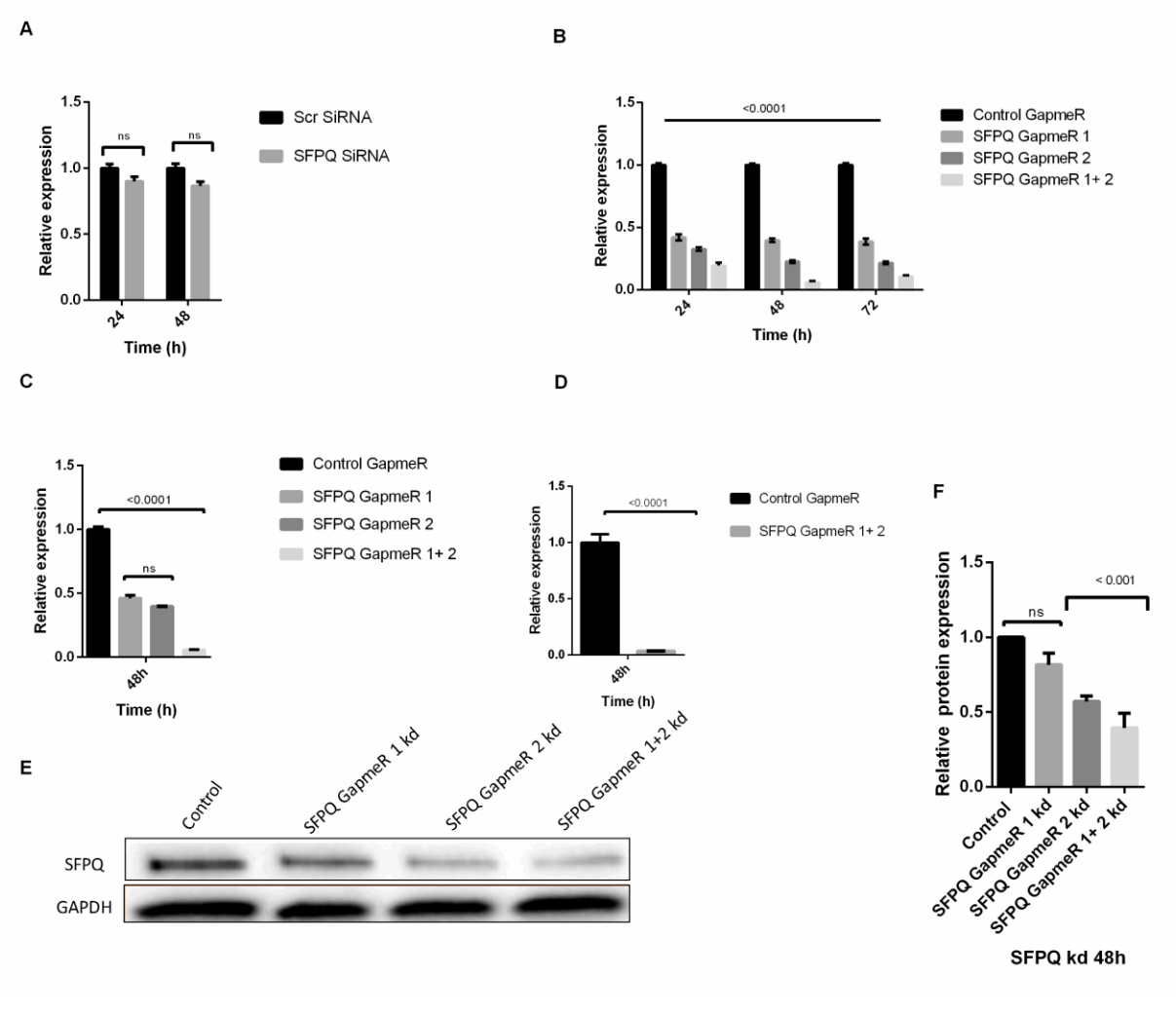


Figure 5.1. Knock-down of SFPQ in A2058 using siRNA and GapmeRs®. (A) A2058 cells were transfected with SFPQ siRNA (5nM), for comparison control cells were transfected with target-less scrambled (Scr) siRNA for 24 and 48 hours. SFPQ transcript levels were assessed via RT- qPCR. GAPDH and R18s served as reference genes. (B) A2058 cells were transfected with Control GapmeR®, SFPQ GapmeR® 1, 2 and combined (5nM), for 24, 48 and 72 hours. (C) Control GapmeR® and SFPQ GapmeR® 1, 2 and combined (5nM) 48 hours post transfection. (D) Control GapmeR and SFPQ GapmeRs combined (5nM) 48 hours post transfection. SFPQ transcript levels were determined via RT- qPCR, data was normalised to GAPDH, RPS13 and TBP reference genes. (E) Total protein was extracted 48 hours post transfection; protein lysates were subjected to western blot analysis for SFPQ and normalised to GAPDH. (F) Densitometry analysis of SFPQ-knockdown. Data represent mean ±S.D, N = 3 independent experiments, *=p<0.05, **=p<0.01, ***=p<0.001, ****= p<0.0001. Data analysed using 2-way Anova followed by Tukeys multiple comparisons test.

5.2 SFPQ functional impact on melanoma cells

To address the question if SFPQ knock-down affects melanoma phenotype, various phenotypic assays were utilised such as migration, proliferation, cell death and cell metabolism.

5.2.1 Knock-down of SFPQ reduced *in vitro* cell migration

To determine is SFPQ expression contributes to melanoma cell migrations, a combination of wound healing assays and transwell migration assays were used. A2058 cells were transfected with SFPQ and Control GapmeRs® and the following day scratch wounds were created in triplicate on a monolayer and images were taken at 0h,6h,12h and 24h time points. Images were then analysed using ImageJ and the MRI wound healing tool. For each GapmeR® an average of the area was taken to determine percentage wound closure over 24 hours. As can be seen in Figure 5.2 A silencing of SFPQ significantly decreased migration of A2058 cells at 12 and 24 hours compared to control cells. Next, transwell migration assays were carried out to validate these data via a second independent assay. Briefly, A2058 cells were transfected with SFPQ-specific and Control GapmeRs® cultured for 24h and on the day of assay cells were added to the upper chamber of the transwell insert and either SFM or complete growth media containing 10% FBS was added to the lower chamber, as a chemoattractant. Cells were cultured for a further 24h prior to fixing in ethanol and then stained with crystal violent. As shown in Figure 5.2 B, SFPQ significantly decreased both chemokinesis and chemotaxis of A2058 cells, supporting the observation made with wound healing assays in Figure 5.2 A.

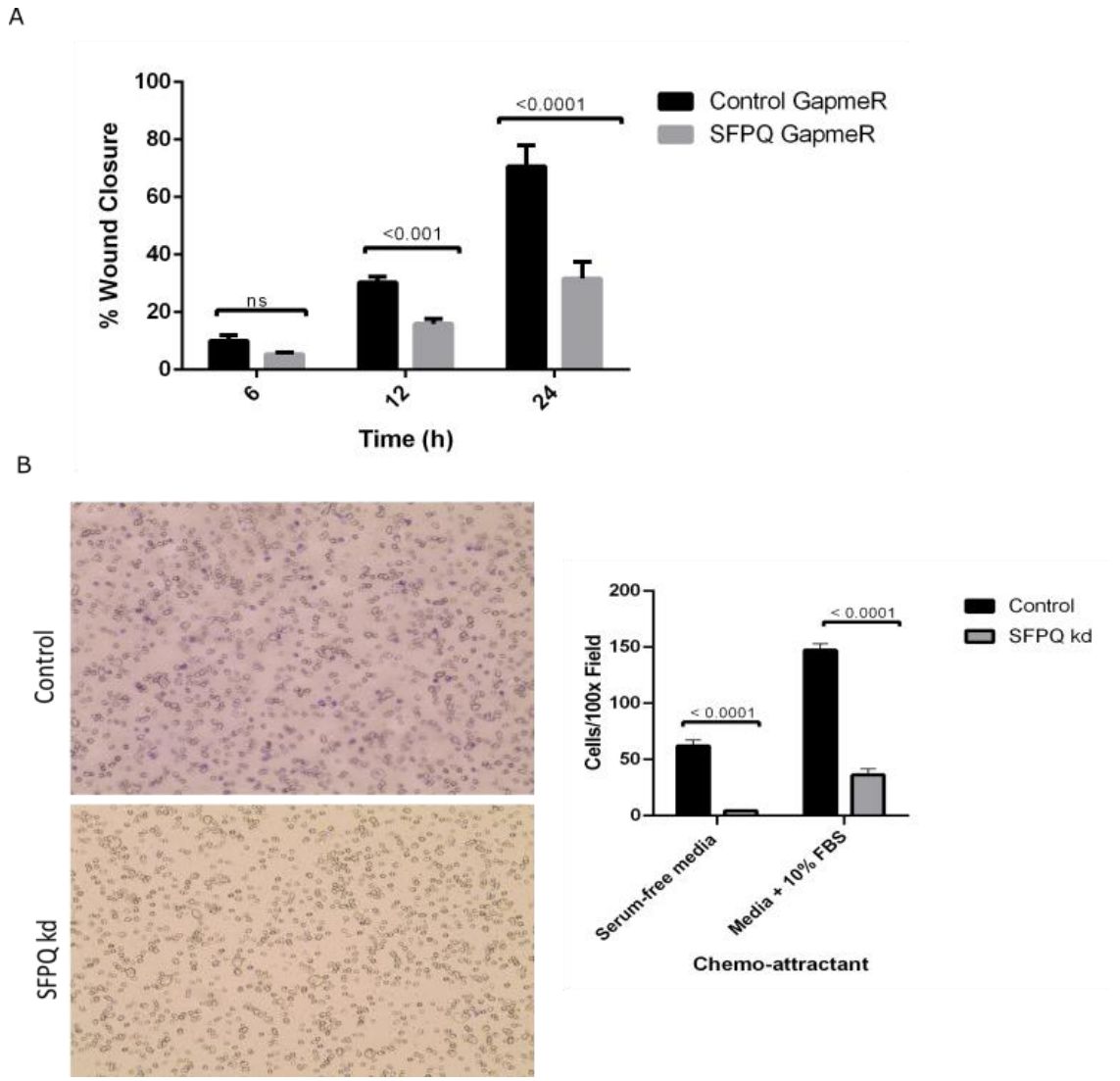


Figure 5.2. Silencing of SFPQ significantly decreased migration in A2058 melanoma cells. (A) Transfections were carried out in A2058 and in vitro cell migration was assessed via wound closure assays. Following scratch wound the percentage closure rate was determined for SFPQ and Control cells. (B) Cell migration was measured in vitro by Transwell migration assays, random images were taken for the number of cells migrated and an average was calculated for SFPQ and Control cells. Representative images are of four independent biological repeats. Direct statistical comparison of wound closure and number of cells migrated between control and SFPQ and was carried out via using 2-way Anova followed by Tukeys multiple comparisons test n=4 independent biological repeats, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns (no significance).

5.2.2 SFPQ knockdown decreased viable cell growth in melanoma A2058 cells

To determine if depleting SFPQ also affects cell growth, A2058 cells were transfected with SFPQ GapmeRs® and incubated for 24 hours prior to seeding in a 96 well plate and culturing over a 72h period. The number of cells per well were determined daily via addition of CellTiter-Glo® and incubation at room temperature for 10 minutes, prior to a luminescence reading being taken. As can be seen in Figure 5.3, silencing of SFPQ transcript significantly reduced cellular proliferation of melanoma A2058 cells compared to control.

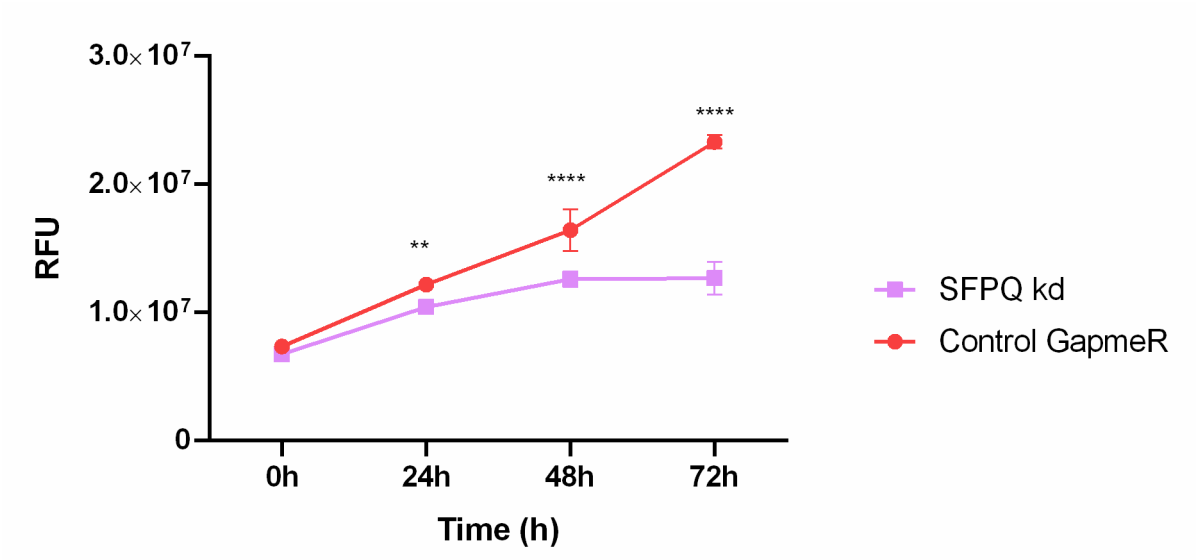


Figure 5.3. SFPQ-knockdown significantly reduced melanoma cell viability. A2058 cells were transfected, cell viability was measure via CellTiterGlo assay for control and SFPQ-depleted cells over a 72-hour period. Data is representative of n=3 biological repeats, statistical analysis was carried out using t-test. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001 ns (no significance).

5.2.3 SFPQ-Knockdown induced cell death in melanoma cells

The decrease in viable cell growth observed in SFPQ-depleted A2058 cells compared to control could indicate decreased proliferation and/or increased apoptosis. To test if SFPQ knockdown was increasing the amount of apoptosis occurring in cultured A2058 cells, SFPQ-specific GapmeRs® were transfected into A2058 cells and incubated for 48 hours. On the day of the assay, cells were detached, centrifuged, and resuspended in binding buffer, prior to the addition of FITC-Annexin V and PI to control and SFPQ-depleted cells and incubation in the dark at room temperature for 15 minutes. Additional binding buffer was then added, and samples were analysed via flow cytometry. As can be seen in Figure 5.4, I observed a nominal but significant increase in the percentage of SFPQ-depleted cells stained with AV and PI, which is indicative of cell in late apoptosis. This suggests that the reduction in viable cell growth is, in part, due to increased levels of apoptosis in SFPQ-depleted melanoma cells.

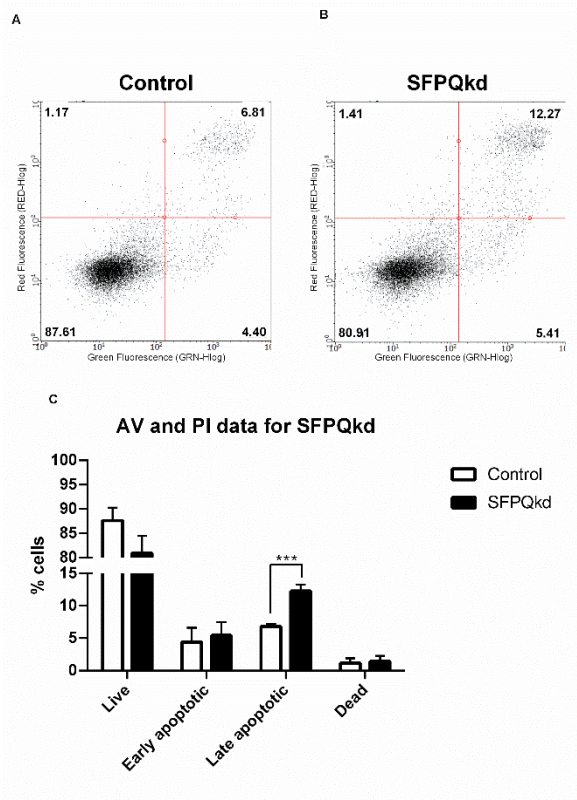


Figure 5.4. SFPQ silencing increased apoptosis. SFPQ and control GapmeRs were transfected into A2058 cells, 48-hour post transfection AV and PI were added to each sample and readings were taken. A-B representative dot blots for Control and SFPQkd during late apoptosis. C representative histogram for live, early apoptotic, late apoptotic and dead cells for control and SFPQ-kd. Data is representative of n=3, statistical analysis was carried out using multiple t-test. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001 ns (no significance).

5.2.4 SFPQ-Knock-down reduces melanoma cells metabolism

Ground breaking research by Warburg and Cori in 1920s demonstrated that cancer cells exhibit enhanced glycolysis and reduced oxidative phosphorylation (OXPHOS) (Warburg, Wind, & Negelein, 1927). Initially, it was thought that cancer cells were dependant on glycolysis due to derailed OXPHOS mechanisms. However, recent advances suggest that OXPHOS pathways are operating in majority of cancer cells and the high glycolysis consumption is due to the precise interplay between oncogenes and the tumour microenvironment (Zheng, 2012; Zu & Guppy, 2004). The

first major pathway in glucose catabolism is glycolysis which allows the retention of glucose within the cell when hexokinase phosphorylates glucose to glucose-6-phosphate. The ensuing steps of glycolysis then promote anaerobic synthesis of NADH, ATP, and pyruvate. Although, the production of lactate from glucose is less energy counter-productive, cancer cells primarily breakdown glucose to lactate regardless in the presence of oxygen. This phenomenon of aerobic glycolysis is called the 'Warburg effect' (W. H. Clark, 1991; Vander Heiden, Cantley, & Thompson, 2009). Increased glycolysis aids the survival and promotes tumour progression and metastasis of cancer cells. Glycolysis provides various macromolecules that are used by cancer cells to synthesise of vital cell components including proteins, nucleotides and lipids, that stimulates rapid proliferation of cancer cells (Lunt & Vander Heiden, 2011). Many cancer cells, including melanoma cells mainly rely on aerobic glycolysis (D. A. Scott et al., 2011). There are several reports demonstrating the role of lncRNA in cell metabolism disorders. For example, when stressed the lncRNA (NBR2) is stimulates AMPK via direct binding. Silencing of NBR2 resulted in metabolism disorders and subsequently favoured cell proliferation (Liu et al. 2016). Furthermore, lncRNA *SAMMSON* has received a lot of attention as it is bound to the major mitochondrial regulator p32 protein and is implicated in the progression and development of melanoma (Leucci et al. 2016).

To test whether SFPQ expression levels drive the metabolic program of melanoma cells, we compared key cellular metabolic and bioenergetic profiles in control and SFPQ-depleted A2058 melanoma cells. The Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were determined using a Mito Stress Test Kit. Briefly, SFPQ-specific GapmeRs® were transfected into A2058 cells, which were then cultured for 24 hours, prior to analysis via SeaHorse XF analyser. Briefly, the oxidative

phosphorylation inhibitor (Oligomycin), reversible inhibitor of oxidative phosphorylation (FCCP), and the mitochondrial complex I inhibitor rotenone plus the mitochondrial complex III inhibitor antimycin A (rotenone/antimycin A) were sequentially injected into each well at specific time points. Knockdown of SFPQ led to a significant decrease in basal respiration, maximal respiration, ATP production and spare respiration capacity. Intriguingly, proton leakage and the respiratory control ratio were increased, suggesting exacerbated oxidative phosphorylation (OXPHOS) dysregulation in cells with decreased expression of SFPQ (Figure 5.5). Together, these data demonstrate that ASO-mediated knockdown of SFPQ in melanoma cells results in a wide-ranging deceleration of the cancer phenotype, strongly suggesting that the principal functional output of increased SFPQ expression in melanoma cells is oncogenic

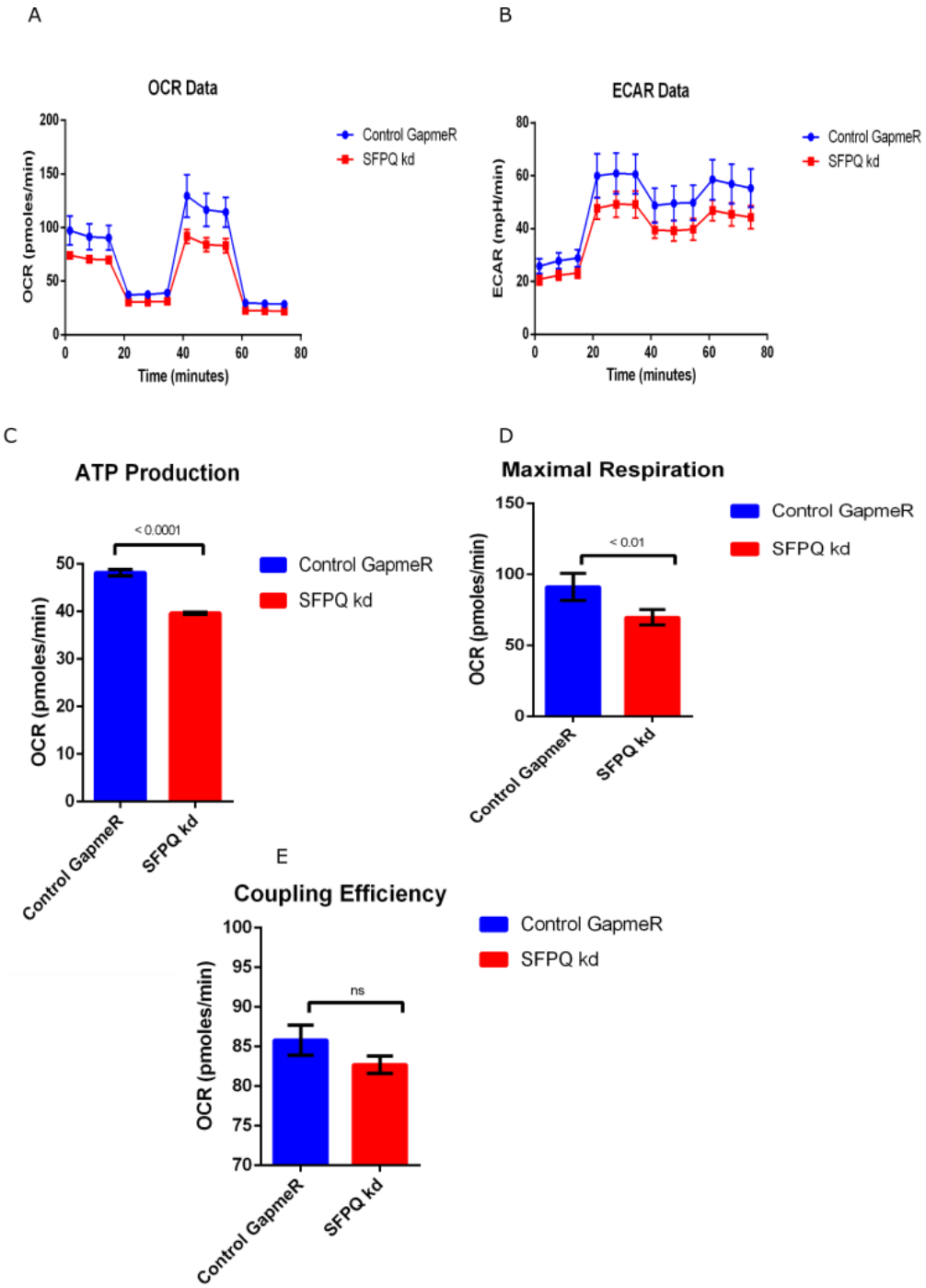


Figure 5.5. Silencing of SFPQ decreased melanoma cell metabolism. A2058 cells were transfected with SFPQ GapmeRs®, cell Mito stress kit was used (Oligomycin 1.5 µM and rotenone/antimycin A 0.5 µM) and the oxygen consumption rate was analysed using the Seahorse XFe analyser. (A) OCR, (B) EACR, (C) ATP production, (D) Maximal respiration (E) coupling efficiency. Data was normalised by SRB assay, Statistical analysis was carried out using t-test n= 3 independent repeats, *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001, ns (no significance).

5.3. SFPQ-lncRNA interactors

Numerous lncRNA are dysregulated in various cancers including melanoma. In chapter three I identified several melanoma-associated lncRNAs where expression positively correlates with melanoma cell migration potential. In chapter 4 I focused on finding novel SFPQ-lncRNA binders via RIP-sequencing which are specific in A2058 melanoma cells, compared with PM. Below, I assess several of these lncRNA to determine if ASO-mediated knockdown has any impact on the melanoma cancer phenotype.

5.3.1 Optimisation of ASSO-mediated knockdown of *GAPLINC, LINC00511* and *LINC01234*.

I decided to prioritise three lncRNA for further study, based upon either their previous association with a non-melanoma cancer phenotype and SFPQ (*GAPLINC)* or their specific enrichment with SFPQ in a melanoma cell background (*LINC00511* and *LINC01234*) not all GapmeRs were used in the experiments below due to time constraints . To this end, *GAPLINC*, *LINC00511* and *LINC01234* were depleted using specific GapmeRs, as described for SFPQ in section 5.1. As shown in Figure 5.6 robust and significant knock-down efficiency was observed for all lncRNA target 24 hours post-transfection.

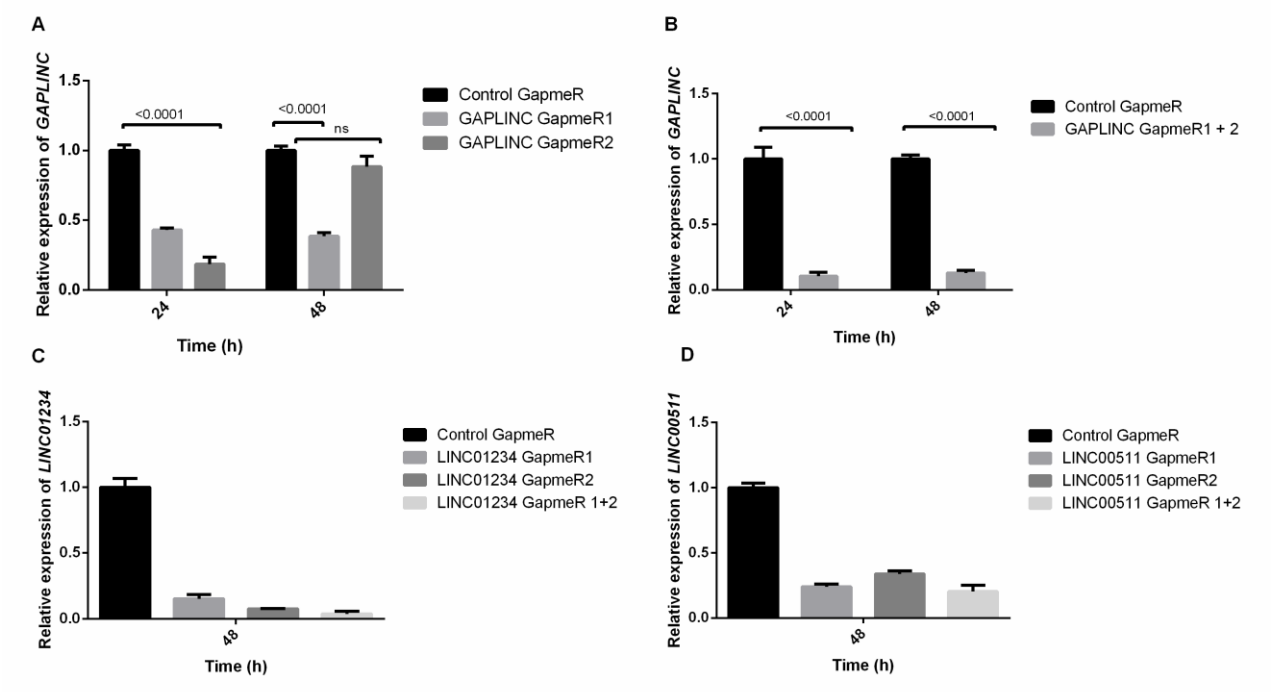


Figure 5.6. Significant knock-down of *GAPLINC*, *LINC01234* and *LINC00511* using GapmeRs in A2058 cells 24-hour post transfection. (A) Transfection of Control GapmeR® and GAPLINC GapmeR® 1 and 2 (5nM) in A2058 cells for 24 and 48 hours. (B) Control GapmeR® and GAPLINC GapmeRs® 1 and 2 combined (5nM) were transfected for 24 hours. (C-D) Knockdown of *LINC01234* and *LINC00511* in A2058 (5nM) at 24 hours. Data represented as mean±S.D, n=3, using 2-way Anova (Sidak's multiple comparisons test) *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns (no significance).

5.4 Knockdown of *GAPLINC*, *LINC01234* and *LINC00511* expression significantly suppresses A2058 cell migration

Having demonstrated successful depletion of *GAPLINC, LINC01234* and *LINC00511* I next sought to investigate the functional importance of these lncRNA in regulating melanoma cell growth. Initially, cell migration was assessed via wound healing assay. A2058 cells were transfected with the indicated GapmeRs® and the following day scratch wounds were created in triplicate on monolayers and images taken at 0h, 6h,12h and 24h time points. Images were then analysed using ImageJ and the MRI wound healing tool. For each GapmeR® an average of the area was taken to determine

percentage wound closure over 24 hours. As shown in Figure 5.7 A and B knock-down of *GAPLINC* and *LINC01234* significantly reduced migration compared to control cells. To confirm these data via a second approach, transwell migration assays were then performed. This time the proposed oncogenic lncRNA, *LINC00511* was included in the experiment, alongside *MALAT-1*, which has previously been reported to promote melanoma cell migration (Tian et al., 2014). A2058 cells were transfected with the respective GapmeRs®, as described above, and on the day of the assay cells were added to the upper chamber of the transwell insert and SFM or complete media added to the lower chamber. Cell were cultured for 24h prior to fixing in ethanol and then stained with crystal violent. As shown in 5.8 A-C knockdown of *GAPLINC*, *LINC01234*, *LINC00511* and *MALAT1* significantly reduced migration compared with control. Together, this suggests that each oncogenic lncRNA has a role in promoting migration of melanoma, *in vitro*.

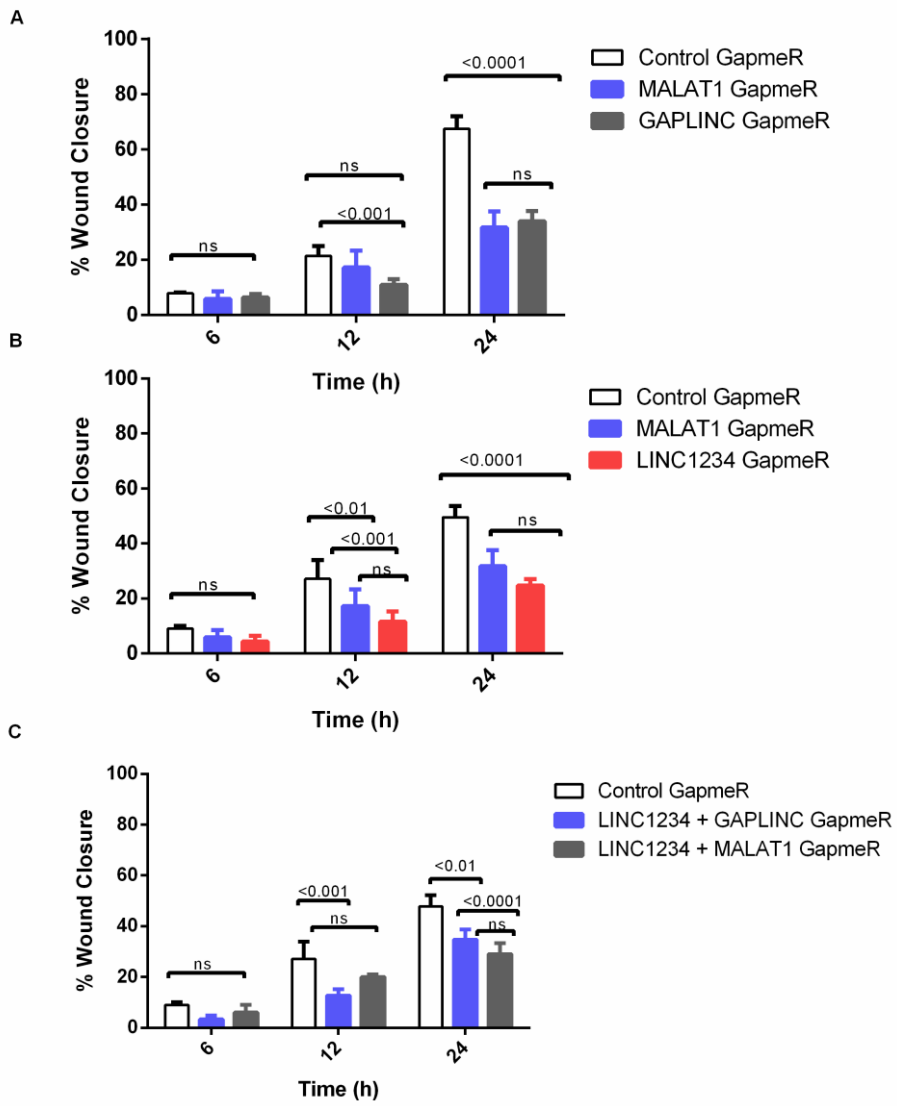


Figure 5.7. Silencing lncRNA *MALAT1, GAPLINC, LINC01234* reduced cell migration of A2058 melanoma cells. Transfections were carried out in A2058 and *In vitro* cell migration was assessed via wound closure assay. Following scratch wound the percentage closure rate was determined for *MALAT1, GAPLINC, LINC01234, LINC01234 + GAPLINC, LINC01234 + MALAT1* over a 24-hour period (A-C). Direct statistical comparison of wound closure between panels A-C was carried out via Using 2-way Anova followed by Tukeys multiple comparisons test n=5 independent biological repeats. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns (no significance).

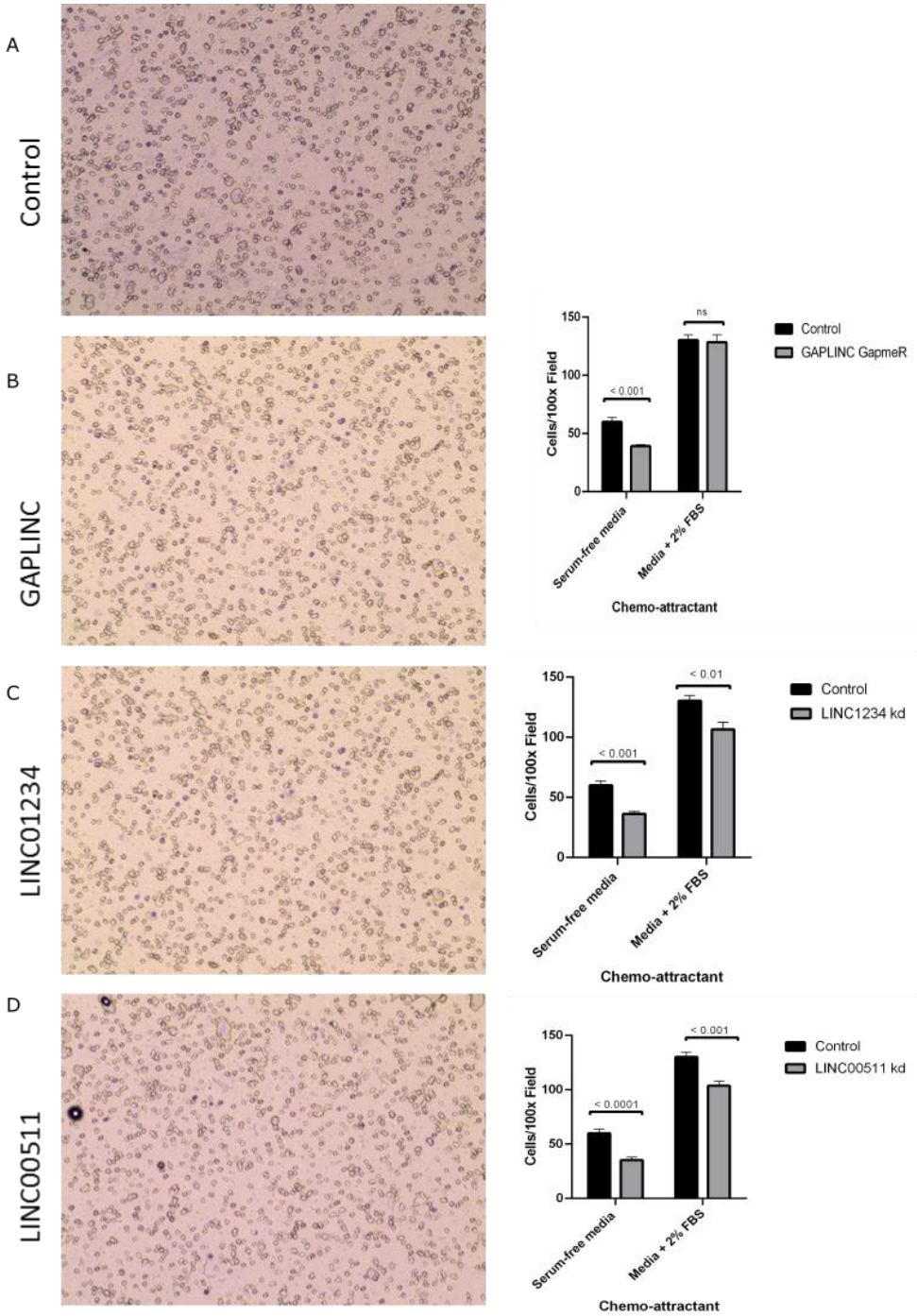


Figure 5.8 Knock-down of *GAPLINC, LINC01234 and LINC00511* significantly decreased migration *In Vitro* via Transwell migration assay. Transfections were carried out in A2058 cells for 24 hours. Cell migration was assessed *in vitro* by Transwell migration assays. For each lncRNA *GAPLINC, LINC01234 and LINC00511* random images were taken for the number of cells migrated and an average was calculated (A-D).Statistical comparison of number of cells migrated between each target an control were carried out via using 2-way Anova followed by Tukeys multiple comparisons test n=4 independent biological repeats. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.001, ****=p<0.0001, ns (no significance).

5.5 Knock-down of *LINC00511*, *GAPLINC* and *LINC01234* reduced viable cell growth in melanoma cells.

As part of the functional assessment, it was interesting to determine if silencing of *GAPLINC*, *LINC01234*, and *LINC00511* impacts cell proliferation in melanoma. In this regard, A2058 cells were transfected with GapmeRs® and incubated for 24 hours prior to monitoring growth over a 72h time course via CellTiter-Glo®. Analysis of luminescence revealed that knock-down of *LINC00511* and *GAPLINC* resulted in a marked and significant decrease in viable cell growth and to a lesser, but still significant level, the same was observed in *LINC01234* depleted cells (Figure 5.9 A-D).

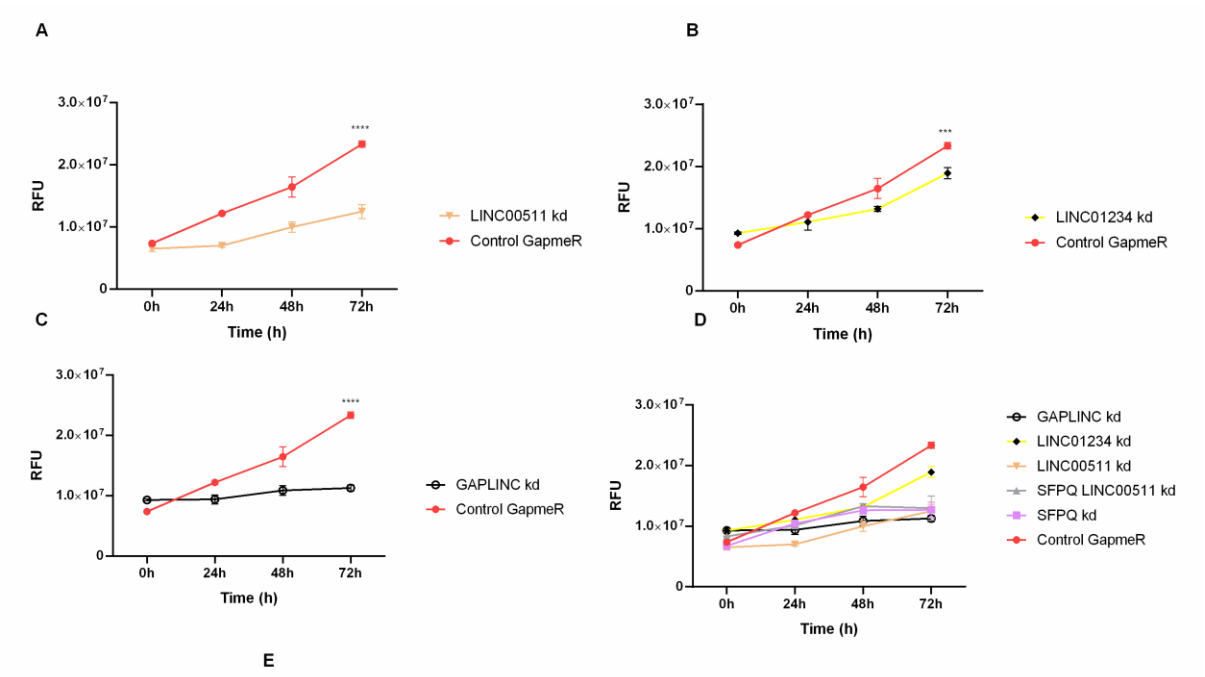


Figure 5.9. Depletion of *LINC00511, LINC01234 and GAPLINC* significantly reduced cell proliferation (A-D). A2058 melanoma cells were transfected with appropriate GapmeRs for 24 hours. CellTiterGlo was used to measure cell viability for Control, *LINC00511+ SFPQ, LINC00511, LINC01234 and GAPLINC* depleted cells over a 72-hour period. Data is representative of n=3, statistical analysis was carried out using t-test, *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001 ns (no significance).

5.6 Caspase-3 activity increased *in GAPLINC-kd* and *LINC01234kd* melanoma cells

To establish if silencing SFPQ-enriched lncRNAs was also having an impact on apoptosis in melanoma cells the ApoLive-Glo™ Multiplex Assay was utilised. Caspases are key regulators of apoptosis, with caspase-3 serving as an executioner protease, catalysing the specific cleavage of many key cellular proteins (Polverino & Patterson, 1997; Walsh et al., 2008). Scramble control, *GAPLINC*- and *LINC01234*-specific GapmeRs were transfected into A2058 melanoma cells, which were then cultured for 24 hours prior to viability reagent being added and readings taken, followed by addition of Caspase-Glo® 3/7 reagent and analysis of luminescence. Data was normalised to viable number of cells. As shown in Figure 5.10, Caspase-3 activity significantly increased for *GAPLINC* and *GAPLINC+LINC01234.*

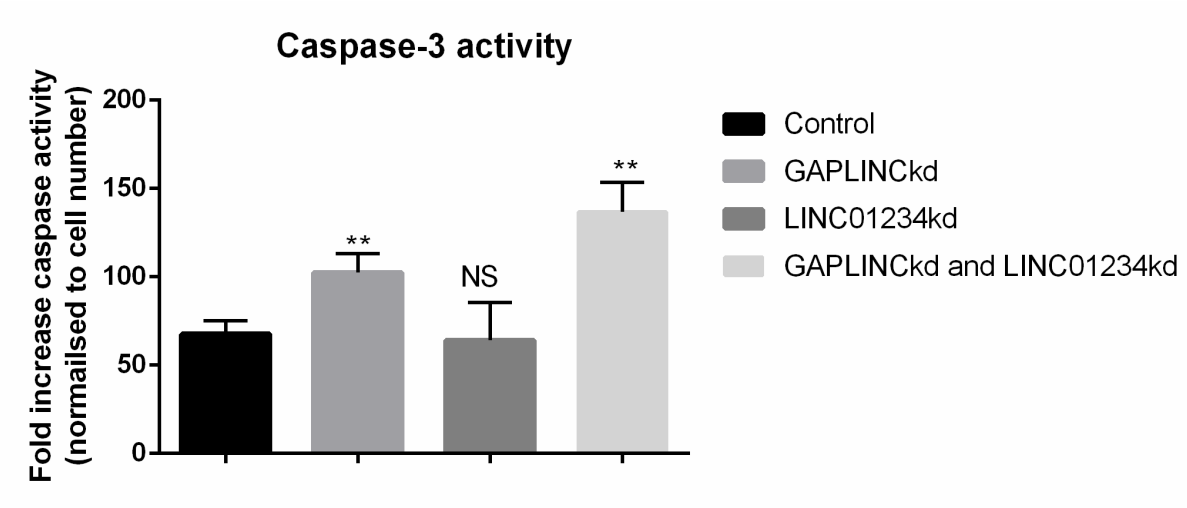


Figure 5.10. knockdown of *GAPLINC* and *LINC01234* and *GAPLINC+LINC01234* increased apoptosis via Caspase 3. Melanoma cells were transfected with GapmeRs, 24h post transfection Caspase-Glo® 3/7 reagent was added, and luminescence reading was taken. Data is representative of n=3, statistical analysis was carried out using t-test. *=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001 ns (no significance).

5.7 Knock-down of SFPQ interactor *LINC00511* reduced cell metabolism in A2058 melanoma cells.

I was keen to investigate if any SFPQ-enriched lncRNA were involved in metabolism, given the observation that SFPQ knockdown impaired this process and the reported role of another lncRNA, *SAMMSON*, in melanoma metabolism. Unfortunately, I was unable to test all three of the lncRNAs described above, due to budget constraints and therefore I opted to investigate *LINC00511*, which is ranked highest for melanoma-specific enrichment with SFPQ in my RIP-seq dataset. Briefly, *LINC00511* transcript was silenced by specific GapmeRs in A2058 cells and 24h post-transfection the OCR and ECAR levels were determined via cell Mito stress kit, Oligomycin, FCCP and rotenone/antimycin A were consecutively injected into each well at specific time points. As shown in Figure 5.11, depletion of *LINC00511* decreased OCR and ECAR levels compared to control cells.

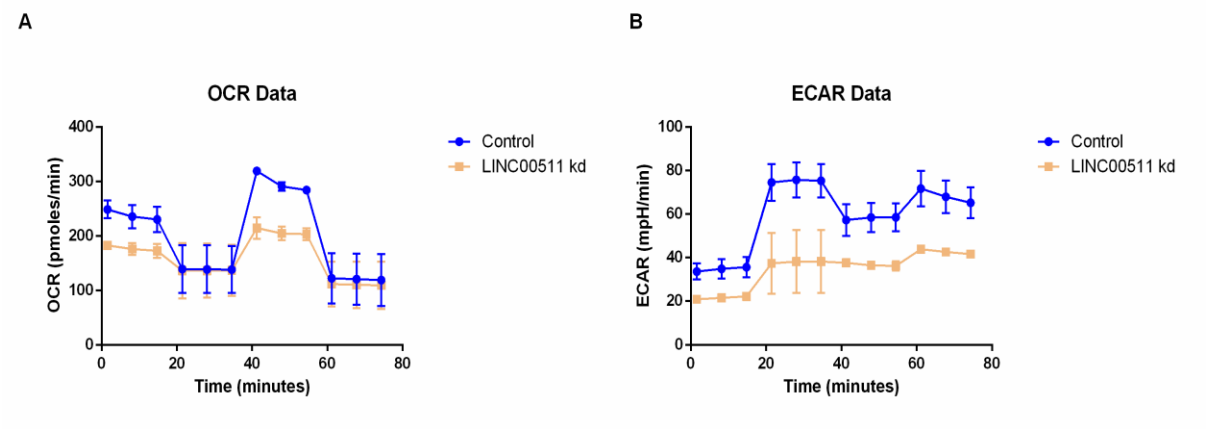


Figure 5.11. Knock-down of SFPQ interactor *LINC00511* reduced cell metabolism in melanoma. A2058 cells were transfected with specific GapmeRs, cell Mito stress kit was used (Oligomycin 1.5 µM and rotenone/antimycin A 0.5 µM) and the oxygen consumption rate was analysed using the Seahorse XFe analyser. (A) OCR, (B) EACR. N=3.

5.8 Significance of SFPQ expression in melanoma patients

The survival rate in melanoma patients has greatly improved within the last few years, due to the development of novel and more effective therapeutics (Bhatia et al., 2009; Domingues, Lopes, Soares, & Pópulo, 2018). Key prognostic factors are used to determine melanoma disease stage such as: breslow thickness, ulceration, lymph node involvement, mitotic rate and distant metastasis (Balch et al., 2009). The findings of this study and *Takayama et al* work observed increased SFPQ protein expression in melanoma (Figure 3.4) and prostate cancer cells, respectively. In turn, various functional analysis revealed that knock-down of SFPQ reduced metastatic melanoma phenotype *in vitro*. Therefore, it was interesting to determine if increased expression of SFPQ was observed in melanoma patients and if this might associate with poorer outcome or other markers of disease progression. Transcriptomic data from primary melanomas of 703 patients (Illumina DASL array HT12.4, deposited at the European-Genome Phenome Archive, accession number EGAS00001002922, Nsengimana et al, PMID: 29664013; Thakur et al., PMID: 31515461) as well as clinical data were provided by our collaborators from the Melanoma Research Group, Leeds Institute of Medical Research at Saint James, University of Leeds. These data form part of the Leeds Melanoma Cohort (LMC) a large population-based melanoma cohort which has a median follow up time of 8 years (see Table 5.1).

Table 5. 1 Leeds Melanoma transcriptomic cohort description (primary disease, n=703)

Characteristic	Number and percentage or median and range *
Male sex	310 (45.1%)
Age at diagnosis (years)	58.4 (18.3, 81.2)
Tumour site	
Limbs	288 (42.0%)
Trunk	230 (33.5%)
Head/neck	80 (11.7%)
Rare[&]	88 (12.8%)
Died from melanoma	202 (29.4 %)
Follow up time (years)	7.6 (1, 14.5)
AJCC stage v8	
I	236 (34.7%)
II	336 (49.3%)
III	109 (16.0%)
Mitotic rate (count per mm^2)	3 (0, 83)
TILs	
Brisk	61 (16.5%)
Non-brisk	383 (68.0%)
Absent	76 (15.5%)

* Missing data excluded in percentage calculation

[&] Rare site=non-exposed to the sun such as anal, perineal, penile, vulvar, oropharyngeal.

Dependencies between tumour characteristics including Breslow thickness and mitotic rate (which predict worse prognosis) and tumour expression of SFPQ were assessed using Spearman correlation. These expressions were further tested for association with melanoma-specific survival (i.e. excluding deaths from other causes) after their split into quartiles by applying Cox proportional hazards regression and plotting Kaplan-Meier survival curves. Where appropriate, certain quartiles were combined to reduce the number of parameters. Both single gene models and bivariate models were fitted with an interaction term to test the independence between genes. These analyses were conducted in STATA v14 (StataCorp, Texas, USA).

The data showed that tumour expression of SFPQ increased slightly with tumour thickness and mitotic rate, with correlation coefficients of 0.14 and 0.10 respectively (Figure 5.12 A-B). Note that these clinical variables were on a logarithmic scale to remove skew. Interestingly, increased SFPQ expression in tumours predicted poor patient survival (Figure 5.12 C). Although there is no evidence of a significant difference between the top 3 quartiles (q2, q3 and q4), they all have a significantly reduced survival compared to the low quartile (q1), with an overall p value of 0.004.

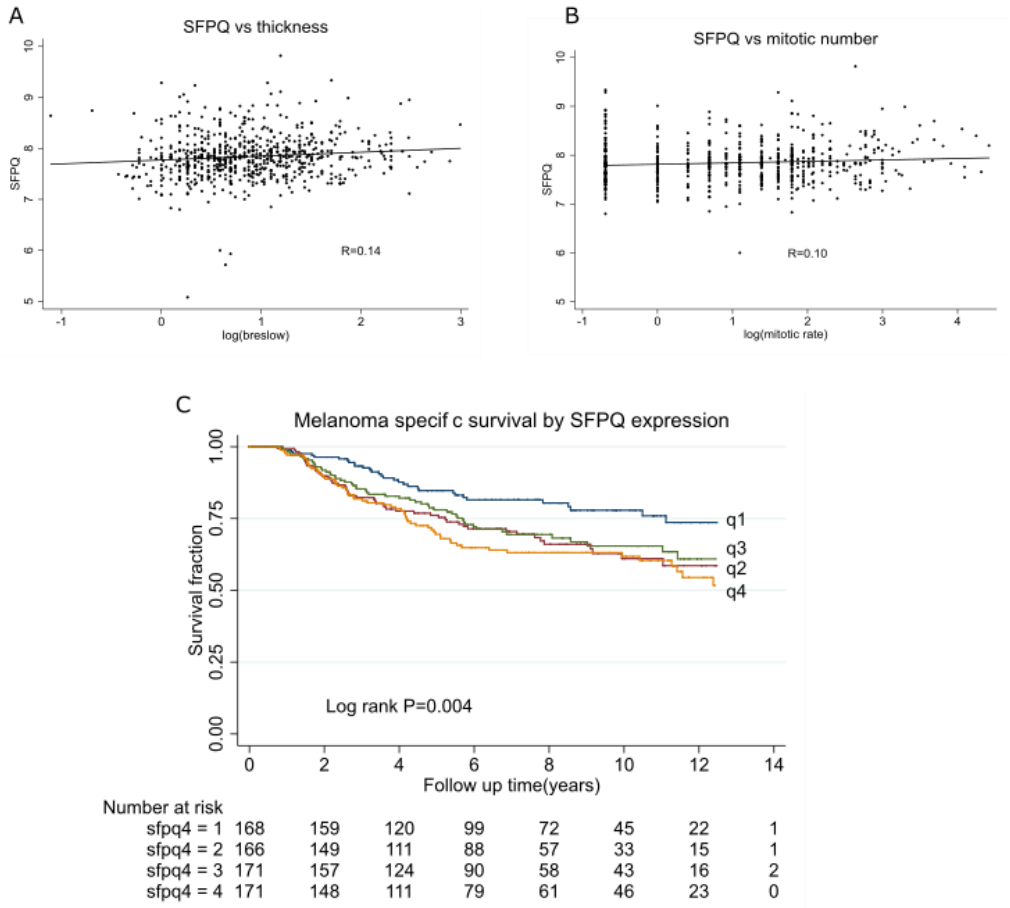


Figure 5.12. Association between tumour expression of SFPQ and log-transformed Breslow thickness (A), log-transformed mitotic rate (B) and melanoma-specific survival (C). For survival, the expression was split into quartiles labelled q1 (bottom 25%) to q4 (top 25%).

An obvious follow up to this analysis was to determine if any SFPQ-enriched lncRNAs were possessed prognostic power, however, unfortunately the HT12.4 array utilised in these studies is an early generation chip that has very limited ncRNA content and none of the oncogenic specific SFQP-enriched lncRNA identified in my RIP-seq analysis were present on the device. However, I was able to assess the expression of two lncRNAs which were SFPQ-enriched in PMs and hugely down-regulated in melanoma cell lines, *MEG3* and *EMX2OS*. *MEG3* is widely reported as tumour

suppressor and has been implicated in various cancers such as bladder, cervical, hepatocellular, prostate, CRC, gastric and melanoma (Braconi et al., 2011; J. Long & X. Pi, 2018; Luo et al., 2015; W. Peng et al., 2015; L. Wu et al., 2020; D. D. Yin et al., 2015; Ying et al., 2013; J. Zhang et al., 2016). While, little is known about the functional role of *EMX2OS*, it was initially identified as a CNS transcript then implicated in glioma (Falcone et al., 2016), however, to date it has not been associated with melanoma.

Remarkably, in melanoma tumour samples, expression of these lncRNAs is highly correlated with a correlation coefficient of 0.45 (Figure 5.13 H). Moreover, expression levels display an inverse relationship with tumour thickness and mitotic rate (Figure 5.13 A-D). Increased expression of both genes also predicted survival although for *MEG3* it was the bottom quartile that was significantly different from the other 3 quartiles combined, whereas for *EMX2OS* it was possible to split these three ways (Figure 5.13 E-F). Intriguingly, there appeared to be a significant synergy between the expression of these two lncRNA, as high expression of one without the other did not predict for improved survival (Figure 5.13 G). However, it should be noted that high expression of *MEG3* is observed in 3/4 of patients while that of *EMX2OS* is only seen in 1/4 of patients in this population-based cohort (i.e. with a fair representation of primary melanoma population as a whole). Together, these clinical data confirm that increased expression of SFPQ in melanoma is associated with more aggressive disease and poorer patient outcome.

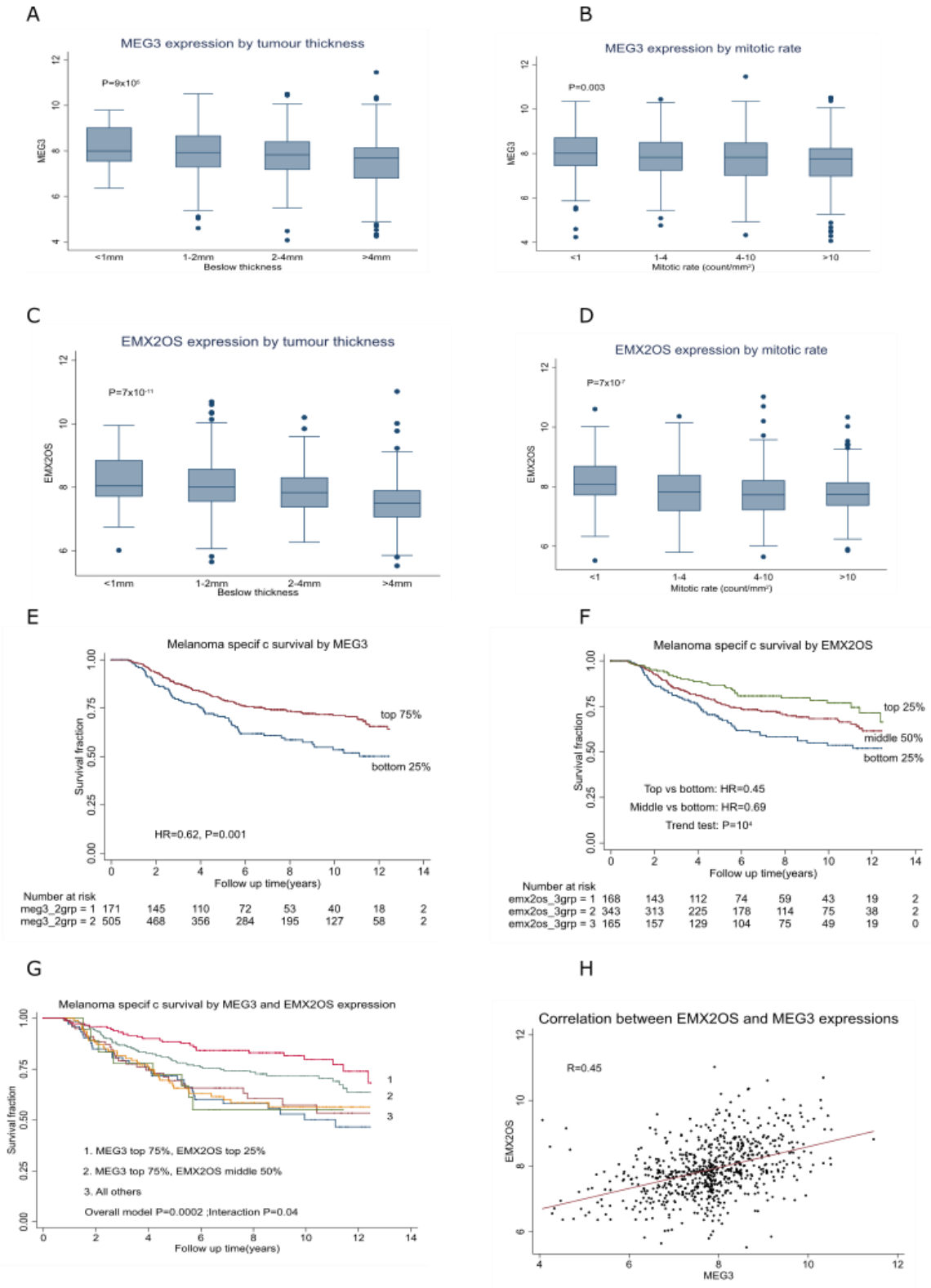


Figure 5.13. Association between *MEG3* and *EMX2OS* tumour expression, Breslow thickness, mitotic rate (A-D) and melanoma specific survival (E-F for single gene, G for joint effects).

5.9 Discussion

SFPQ is a multifunctional protein that regulates a myriad of biological functions, which include mRNA splicing, transcriptional regulation, mRNA processing, DNA damage and DNA repair (Knott et al., 2016; C. A. Yarosh et al., 2015). Various studies have reported its involvement in a number of cancers and neurodegenerative disorders (Y. D. Ke et al., 2012; K.-I. Takayama et al., 2017a; Takeuchi et al., 2018; C.-F. Wu et al., 2013; P. Yang et al., 2016). Despite this, little is known about the role of SFPQ in melanoma. Data presented in this chapter revealed that SFPQ positively contributes to an array of cancer phenotypes. Initially, an siRNA approach was adopted for knockdown of SFPQ expression, however, the level of depletion, while reasonable at the transcript level (~50%) was negligible at the protein level. Therefore, I opted to use an alternative technology, ASOs GapmeRs®, to knockdown SFPQ transcript and subsequently used this same technology successfully against SFPQ-enriched lncRNA transcripts. GapmeRs® function by degrading the target RNA via RNase H activity (Castanotto et al., 2015). Importantly, ASOs are effective and preferable to siRNA, as RNase H is found in the nucleus and cytoplasm, while siRNAs utilise the multiprotein RNAi-induced silencing complex (RISC) which functions in the cytoplasm, something that is particular important when depleting lncRNAs, which are often exclusively nuclear. Furthermore, LNA oligonucleotides have increased binding affinity for their complementary strand, compared with traditional DNA or RNA oligonucleotides, due to incorporation of locked nucleic acids (LNA). The extreme antisense-target duplex stability allows degradation of secondary structures of RNA targets. This results in unprecedented sensitivity and specificity (Grünweller & Hartmann, 2007). Using GapmeRs®, I was able to knockdown SFPQ protein expression by ~60% 48h post-transfection, this is comparable to the knockdown achieved in published work that

subsequently went on to identify phenotypic functions of SFPQ (de Silva, Lin, Phillips, Martin, & Baxter, 2019b; I et al., 2020; Rhee et al., 2017) and thus this was deemed sufficient to proceed with downstream assays.

The functional role of SFPQ in melanoma is unclear, beyond a solitary article that suggests SFPQ functions as a TSG via repression of *RAB23 (C. F. Wu et al., 2013)*. There are a number of issues with this observation, for example as discussed in chapter 3 the lncRNA described by the authors, *LLME23*, does not appear in any melanoma RNA-seq data sets and has yet to be formally annotated, in terms of its TSS. However, the major issue with the ascribed role of SFPQ as a TSG is that this assumption was made based upon SFPQ binding to *Rab23* in mouse and this observation was not confirmed via ChIP in human melanoma cells. Given the cell type specific nature of SFPQ interactions described in this thesis (Chapter 4) and elsewhere by several other groups (Luisier et al., 2018; K.-I. Takayama et al., 2017a; Christopher A. Yarosh et al., 2015) it seems probable that SFPQ interactions might differ between mouse and human. Indeed, I did not detect any enrichment of *LLME23* with SFPQ in A2058 cells. Therefore, to investigate the role of SFPQ in melanoma more thoroughly, I decided to carry out a functional analysis of several cancer phenotypes in A2058 melanoma cells depleted of SFPQ.

Initially, I investigated cell migration and found SFPQ silencing reduces migration *in vitro* (Figure 5.2). This is consistent with data from a study which looked at the link between SFPQ and LSD1 a key regulator shown to play a critical role during cerebral cortex development (Saud et al., 2017). Silencing of SFPQ via in utero electroporation of a shRNA resulted in decreased migration of new-born pyramidal neurons. Silencing SFPQ also reduced proliferation of progenitor cells. This study highlights SFPQ role in regulating proliferation and migration during the development of the cerebral cortex

(Saud et al., 2017). The phenotypic role of SFPQ in melanoma has not been investigated, our findings show a reduction in cell viability and migration in SFPQ-knockdown melanoma cells, thus implying increased expression of SFPQ promotes melanoma progression.

There are two types of programmed cell death, apoptotic cell death and autophagic cell death, both are distinguished via cell morphology (Y. Kondo & Kondo, 2006). PPARγ is a nuclear receptor which is important in cell proliferation, apoptosis, and inflammation. Various studies have reported PPARγ is overexpressed in many cancers, such as stomach, colon, breast, and lung cancer, thus indicating regulation of PPARγ is important in the development of cancer (Allred & Kilgore, 2005; J. Zhou et al., 2009). *Tsukahara et al* utilised proteomic assays and demonstrated SFPQ-PPARγ as a novel interacting partner which is required during the vital steps of proliferation in colon cancer cells. Knockdown of SFPQ induced apoptosis by increasing caspase-3 activity (Tamotsu Tsukahara et al., 2013). Silencing of SFPQ in colon cells decreased expression of the autophagic molecule LC3B and induced apoptosis. Suggesting, SFPQ-regulated reduction in LC3B plays a novel role in the mediating cell proliferation and apoptosis, which may serve as potential therapeutic strategy for colon cancer (T. Tsukahara, Y. Matsuda, et al., 2013). Furthermore, *Saud et al* demonstrated SFPQ- knockdown stimulated neuronal apoptosis in developing mouse brains (Saud et al., 2017). In line with our observations, silencing SFPQ may abrogate its role in repairing DNA double strand breaks (Jaafar, Li, Li, & Dynan, 2017) leading to increased apoptosis in melanoma cells.

Interestingly, herein I demonstrate silencing of SFPQ in melanoma cells led to a decrease in OXPHOS (Figure 5.5). Similarly, a recent study investigated SFPQ role in skeletal muscle, using SFPQ knock-out mice they observed a reduction in OXPHOS

complex I (35%), complex II (18%), and complex IV (35%) compared to control mice (Hosokawa et al., 2019). This study indicates SFPQ importance in energy metabolism, as aberration in skeletal muscles are a consequence of impaired postnatal skeletal muscle development and whole-body growth (Hosokawa et al., 2019). Furthermore, SFPQ regulates muscle-specific target genes required for the energy metabolism and to maintain muscle mass. Similarly, SFPQ may regulate specific energy metabolism genes in melanoma, however currently this remains unknown and future work is required.

Several lines of evidence support the notion that dysregulated expression of lncRNA are associated with human disease (Q. Ji et al., 2014; C.-F. Wu et al., 2013; P. Yang et al., 2016). Therefore, it was interesting to establish if silencing of melanoma and SFPQ associated oncogenic lncRNA identified in chapters 3 and 4 were having an impact on melanoma phenotype. I opted to focus on *GAPLINC,* as its expression has significant positive correlation with migration, in addition to *LINC01234* and *LINC00511,* which were both enriched with SFPQ in a melanoma-specific manner. Remarkably, my data show silencing *GAPLINC, LINC01234* and *LINC00511* significantly decreases migration and cell viability in melanoma cells. In terms of *GAPLINC* this is in broad agreement with published literature for CRC, as *GAPLINC* is over-expressed in CRC compared to non-cancerous tissue and knock-down led to decreased cell migration (Y. Luo et al., 2018), while the converse was observed for *GAPLINC* over-expression (Y. Luo et al., 2018). Various studies have shown certain lncRNAs act as competitive endogenous RNAs for miRNAs to exert oncogenic effects (X. Wu et al., 2016; F. Yu et al., 2015). Interestingly, the authors identified miR-34a as a downstream molecule of *GAPLINC*, miR-34a was shown to negatively regulate the migration and invasion of colorectal cells (Y. Luo et al., 2018). Additionally, our findings

show reduction in *GAPLINC* expression stimulated apoptosis by increasing caspase 3 activity in melanoma cells compared to control cells. A similar report has been made in non-small cell lung cancer (NSCLC) depletion of *GAPLINC* increased apoptosis and supressed cell viability in non-small cell lung cancer (NSCLC) (H. Gu, Chen, Song, & Shao, 2018). To date no reports have been made regarding *GAPLINC* role in melanoma, however, this study suggests increased expression of *GAPLINC* is promoting an aggressive phenotype in melanoma. Evidence from CRC studies supports a similar oncogenic role for *LINC01234,* as over-expression increased apoptosis *in vitro* in gastric cancer cells (X. Chen et al., 2018). My data supports an oncogenic role for *LINC01234* in melanoma, as silencing *LINC01234* significantly decreased viable cell growth kinetics and increased caspase-3 activity, however, this was not as prominent compared to the other targets. Interestingly, when *LINC01234* was co-depleted with either *GAPLINC* or *MALAT1* no additional decrease in phenotype was observed, suggesting that these lncRNAs may operate via a common mechanism to drive cancer phenotype. However, an alternative explanation might be that co-depletion impacts on the expression of either target, as gene expression was not investigated for these double knockdown conditions.

LINC00511 is gathering increasing attention in the literature as an oncogenic lncRNA and has been reported to be dysregulated in breast, cervical and gastric cancer (L. Liu, Zhu, Liu, Feng, & Chen, 2019; Mao, Xu, Zhong, Ding, & Meng, 2019; D. Wang, Liu, & Chen, 2020). *Wang et al* showed *LINC00511* is over expressed in gastric cancer cells and tissues compared to non-cancerous samples (D. Wang et al., 2020). Phenotypic analysis revealed silencing *LINC00511* decreased cell migration, proliferation and increased apoptosis in gastric cancer cells (D. Wang et al., 2020). Moreover, the authors report *LINC00511* behaves as a molecular sponge, regulating

miR-515-5p expression in gastric cancer, thereby promoting metastatic phenotype (D. Wang et al., 2020). These observations are consistent with our findings implying increased *LINC00511* expression favours melanoma metastasis. An intriguing observation was that knockdown of SFPQ appears to reduce the impact of *LINC00511* knockdown on viable cell growth (Fig. 5.9D). The mechanism for this is unclear, however, I observed that SFPQ depletion impacted on the expression of numerous associated transcripts, that may include regulators of *LINC00511* expression and in the future, it would be interesting to investigate the global effect of SFPQ knockdown on RNA transcript expression in melanoma via RNA-seq.

Whilst I was not able to look at *LINC00511*-miRNA interaction experimentally. *In silico* analysis using ENCORI data base revealed numerous *LINC00511*-miRNA binders (J. H. Li, Liu, Zhou, Qu, & Yang, 2014). An interesting target selected was miR-345-5p which functions as TS in pancreatic cancer by targeting CCL8 (Mou et al., 2019). RT-PCR confirmed miR-345-5p expression is reduced in pancreatic ductal adenocarcinoma cells (PDAC). Moreover, increased expression of miR-345-5p expression supressed proliferation and migration of PDAC cells (Mou et al., 2019). Furthermore, in prostate cancer miR-345-5p is upregulated and favoured metastatic phenotype (Tinay et al., 2018). In contrast, another study demonstrated miR-345-5p expression was reduced in gastric cancer and miR-345-5p supressed EMT to prevent the proliferation and migration of gastric cancer cells (Feng, Yuan, & Li, 2017). These study's highlight miR-345-5p plays different roles in human cancer. The functional role of miR-345-5p remains to be elucidated in melanoma, however, one study integrated mRNA and miRNA transcriptome sequencing data from PMs and melanoma cell lines to determine genes involved in the process of tumour EMT, they identified 11 miRNAs which includes miR-345-5p (D. Wang et al., 2015). Therefore, it would be interesting

to determine experimentally if *LINC00511* functions by sponging miR-345-5p and if this interaction is important in promoting cell migration, cell viability and metabolism in melanoma. LncRNAs are emerging as important regulators in cancer metabolism. *SAMMSON*, is specifically upregulated in melanoma and is essential for melanoma survival and growth (E. Leucci et al., 2016). While, in our RIP-seq analysis we were unable to detect an interaction between *SAMMSON*-SFPQ, *SAMMSON* has been shown to interact with p32 and regulates the expression of mitochondrial-encoded proteins, maintenance of mitochondrial membrane potential and oxidative phosphorylation (E. Leucci et al., 2016). Aberrations in mitochondrial function have been associated with various metabolic disorders and cancer. *SAMMSON* and *LINC00511* may be targeting miRNA's which regulate metabolic pathways in melanoma, additional functional work is required to determine this.

Over the recent year's melanoma incidence has increased dramatically worldwide (Garbe et al., 2016). The AJCC staging system, groups melanoma patients into three categories: localised stage I–II, regional stage III (disseminated to regional lymph nodes) and distant stage IV (metastatic sites) (Balch et al., 2009). Survival in melanoma patients is dependent on cancer stage, with a five-year survival rate of ninety-eight percent for localised, sixty two percent (regional) and nineteen percent for distant melanoma (SEER Cancer Statistics Factsheets, 2017). Melanoma diagnosis is based on histopathologic criteria; however, this is often insufficient at differentiating melanoma from certain types of benign nevi. As for prognosis, Breslow thickness, mitotic rate, and ulceration have been considered the most crucial prognostic factors (Abbas, Miller, & Bhawan, 2014). To date, increased circulating LDH levels are the only validated independent prognostic markers with high specificity during late stages of melanoma (Kelderman et al., 2014; Palmer, Erickson, Ichetovkin, Knauer, &

Markovic, 2011). Circulating biomarkers represent an interesting area of research in melanoma. In this study, patient melanoma data was analysed and showed SFPQ expression increased with tumour thickness. Moreover, high expression of SFPQ expression in tumours predicted a poor survival in melanoma patients. This is in line with the *Takayama et al* findings which showed SFPQ predicts worse prognosis of prostate cancer patients (K.-I. Takayama et al., 2017a). SFPQ might serve as useful as a prognostic circulating biomarker during Stage I/II melanoma. Furthermore, TS *MEG3* and *EMX2OS* are down regulated in various cancers, thus have potential as circulating biomarkers. This is an interesting area and work is currently being undertaken in collaboration with Prof Julia Newton-Bishop from the University of Leeds using a recently obtained and currently unpublished transcriptomics dataset from the Leeds melanoma cohort.

Chapter 6: Discussion

There is mounting of evidence linking dysregulated expression of numerous lncRNAs to various cancers such as: renal, breast, CRC, prostate, lung carcinomas and melanoma (Chakravarty et al., 2014; Hulstaert et al., 2017; Yongzhen Li et al., 2019; W.-C. Liang et al., 2015; Jianguo Shi, Zhang, Zhong, & Zhang, 2019; R.-X. Tang et al., 2017). Where lncRNAs have been shown to promote tumour growth, this is normally due to increased expression, however, for the vast majority the mechanisms by which lncRNAs exert this effect remains to be elucidated. Interestingly, the multi-functional protein SFPQ has been associated with a myriad of cellular processes such as splicing, transcriptional regulation, mRNA stability, DNA damage, innate immune response and miRNA synthesis (Knott et al., 2016; C. A. Yarosh et al., 2015). Several studies have reported an SFPQ-lncRNA axis in cancers such as CRC, prostate and melanoma (Q. Ji et al., 2014; K.-i. Takayama et al., 2017b; K. Takayama et al., 2013; C. F. Wu et al., 2013).

In this study I demonstrate several previously unreported lncRNAs are upregulated in melanoma, compared with PMs. Often where lncRNAs are upregulated they are affecting cancer phenotype (Z. Chen et al., 2016; Y. Tang, Xiao, Chen, & Deng, 2018; W.-T. Wang et al., 2016; F. Xu & Zhang, 2017). In this regard, lncRNA function was assessed in melanoma cells, my findings reveal silencing of *GAPLINC, LINC01234* and *LINC00511* decreased migration, cell viability and increased apoptosis *in vitro*, thus implicating their importance in the aetiology of melanoma. Similarly, the functional impact of SFPQ in melanoma cells was assessed, reduction in SFPQ promoted metastasis in melanoma. Interestingly, I also report that SFPQ binds to the *SOX10* transcripts and that this binding appears to regulate stability of the mRNA and by extension expression SOX10 expression.

Clearly, SFPQ-RNA appear to be important drivers of metastatic phenotype. Further functional work would help strengthen data obtained in this study. Specifically, further experiments assessing cell migration, such as Matrigel invasion assays and analysis of MMP expression would provide additional insight into how these gene regulate invasion of the basement membrane. Moreover, analysis of EMT marker expression in melanoma cell lines and how these genes impact on these will be of great interest. While I show that SFPQ knockdown and depletion of key lncRNA lead to increased cell death and caspase activity, additional functional studies will strengthen the role of these genes in modulating apoptosis. For example, TUNEL assays and biochemical analysis of apoptosis markers (cleaved PARP and □H2AX) would more clearly define a role for these genes in apoptosis. Also, given the reported role of SFPQ in DSB repair, Comet assays could be used to assess in increased DSBs are present following knockdown of SFPQ.

The significance of the interactions observed between SFPQ and the many lncRNA identified in chapter 4 remains unclear. For example, we do not know if SFPQ-enrichment of these genes reflects a role for SFPQ in post-transcriptional processing of the lncRNA transcript or if the lncRNA are binding to SFPQ as mature lncRNA to exert function. However, experiments to determine this are technically challenging and made more complex given recent reports that lncRNA themselves may regulate splicing (He, Luo, & Mo, 2019). Post-transcriptional lncRNA interactions with SFPQ, such as those reported to relieve transcriptional repression, represent attractive therapeutic targets and as such it would also be of great interest in the future to Inhibit specific SFPQ-lncRNA interactions, by generating dominate negative SFPQ mutants to determine the importance of SFPQ-lncRNA interaction in melanoma cancer phenotype. One way of blocking these interactions would be to drug these lncRNA.

The biological role and cellular regulation of RNAs are largely dependent on their secondary and tertiary structure (Y. Ding et al., 2014; Morris & Mattick, 2014). Guanine-rich RNA sequences can fold into four-stranded structures namely G-quadruplexes (G4-RNAs) (Leppek, Das, & Barna, 2018; Wan, Kertesz, Spitale, Segal, & Chang, 2011). One study reports G4-RNAs can be ligand-induced, G4-stabilising ligands such as BRACO-19 and RHPS4 can alter the G4 transcriptomic landscape (S. Y. Yang et al., 2018) and successfully destabilise *MALAT-1*. The higher order structures of most lncRNA remain unsolved and while modelling is improving and new techniques are coming online, such as SHAPE-seq, detailed information remains limited. If, for example, *LINC00511* was shown to also form G4-structures it would be interesting to take a similar approach and block the interactions of *LINC00511*-SFPQ to determine any impact this might have on melanoma cell growth. Moreover, *Amodio et al* successfully drugged *MALAT1* using ASOs specifically targeting regions in the lncRNA which bind to proteasome subunits NRF1 and NRF2, this inhibition promoted anti-multiple myeloma activity (Amodio et al., 2018). Detailed mapping on SFPQ-lncRNA interaction domains via oligo-mediated RNA pulldown might open options with regards to taking this same approach with SFPQ-specific binders in melanoma and other cancers.

The tumour suppressor *MEG3* has been studied extensively and is downregulated in numerous cancers, while little is known regarding *EMX2OS* (Braconi et al., 2011; Cecchi & Boncinelli, 2000; J. Zhang et al., 2016; X. Zhang et al., 2003; Y. Zhou et al., 2012). In this study, I observed decreased expression of *MEG3* and *EMX2OS* in melanoma cells compared to PMs, suggesting their tumour suppressive role in melanoma has been abrogated. Unfortunately, I was not able to over express *MEG3* and *EMX2OS,* due to time and budget constraints linked to this project. However,

several other groups have over-expressed *MEG3* and shown anti-tumour effects. For example, over-expression of *MEG3* inhibited tumorigenesis in breast cancer, supressed development of diabetic retinopathy via regulating VEGF and TGF-3 β, decreased proliferation of glioma cells and reduced metastasis in prostate cancer(Luo et al., 2015; P. Wang, Ren, & Sun, 2012; D. Zhang et al., 2018; M. Zhu et al., 2019). Far little is known for *EMX2OS,* data shows over-expression of *EMX2OS* increased proliferation and invasion in ovarian cancer cells, while the converse was observed for knockdown (Duan, Fang, Wang, Wang, & Li, 2020). Therefore, it would be interesting to over-express these transcripts in melanoma cells using expression construct such as pcDNA3.1, or via CRISPRa, which has the advantage of being able to regulate multiple genes in multiple pathways within a single experiment (La Russa & Qi, 2015).

The study reported here has parallels with a studies in prostate cancer and Alzheimer's disease, which show that SFPQ is able to stabilise mRNA transcripts and thus impact on disease-relevant gene expression (K. I. Takayama et al., 2019; K. I. Takayama et al., 2017). Herein, I demonstrate stability was reduced for several melanoma transcripts in the absence of SFPQ, including the transcription factor Sox10. It would be of great interest to expand my limited RNA stability assays and investigate how SFPQ impacts on RNA stability globally via RNA-seq analysis of SFPQ knockdown cells compared with control over an Actinomycin D time course. This would not only enable analysis of stability, but also any effect on global gene expression levels that arise due to SFPQ depletion by analysis of the t=0 timepoint.

Furthermore, numerous lncRNAs have been annotated in eukaryotic genomes, many of which are localised within the cytoplasm, where they could be involved in miRNA-mediated interactions with other transcripts (van Heesch et al., 2014). Experimental and computational evidence support the targeting of lncRNAs via miRNAs

(Paraskevopoulou et al., 2013) and systematic analyses of lncRNA-ceRNA network have been shown in breast cancer, glioblastoma and gastric cancer (Paci, Colombo, & Farina, 2014; Xia et al., 2014; X. Zhou, Liu, & Wang, 2014). Therefore, it would be interesting to carry out *in silico* analysis of lncRNA-miRNA interactions, with a view to identifying potential lncRNA-miRNA-mRNA networks. These analyses could then be supported and validated via additional sequencing experiments such as SFPQ knockdown and miRNA-seq will help better our understanding on affected RNA networks in melanoma.

Finally, I show compelling evidence that SFPQ expression in tumour positively correlates with tumour thickness, mitotic rate, and poor survival in a cohort of 703 melanoma patients. There is emerging evidence suggesting lncRNA may serve as suitable cancer diagnostic and prognostic biomarkers due their high stability whilst circulating in body fluids (Akers et al., 2013). To date no reports have been made regarding an FDA approved lncRNA biomarker in melanoma, however, *MALAT1* has been shown to serve as an effective prognostic marker for patient survival during early stage non-small cell lung cancer (P. Ji et al., 2003). Moreover, *PCA3* in patient urine samples has provided more sensitive and specific diagnosis of prostate cancer compared to PSA serum levels (Fradet et al., 2004; Scott B. Shappell, 2008; Tinzl et al., 2004). In this regard, this work might lead to patient benefit were we able identify novel prognostic lncRNA biomarkers in melanoma, particularly if these were present at elevated levels in blood. To this end, and in collaboration with Prof Newton-Bishop (University of Leeds) and Dr Jeremie Nsengimana (University of Newcastle), we are currently investigating the expression levels of SFPQ and numerous lncRNA in a recently obtained transcriptomic data set from over one thousand melanoma patients. Importantly, these data were obtained via next-generation Affymetrix chips that include

20,000 lncRNA transcripts and in addition to tumour sample we also have full transcriptomics data from both lymph node metastasis and blood. It will be extremely exciting to determine if SFPQ or any of its melanoma-associated RNA prove to have prognostic value as biomarkers, that are clinically relevant and may result in direct patient benefit in the future.

Appendix

mRNA		lncRNA	
PM	A2058	PM	A2058
COL1A2	AMIGO2	EMX2OS	LINC00602
COL3A1	RPS4Y1	FENDRR	LINC00511
MAN1A1	MAGEA3	EGFR-AS1	TMEM51-AS1
DPP4	IGFBP5	RMDN2-AS1	LINC01291
PAMR1	CLDN1	VCAN-AS1	LINC02616
DCN	TGFA	LINC00689	DUXAP8
TMEM119	PASD1	HMMR-AS1	FOXD2-AS1
FBLN1	MAGEA1	LINC00702	LINC00622
EGFR	MAGEA4	CCND2-AS1	GATA2-AS1
CA12	SPTBN2	CYTOR	LINC00200
CLDN11	MAGEA6	LOXL1-AS1	LINC01234
VGLL3	EYA1	LRP1-AS	LURAP1L-AS1
BCAT1	MAPK4	APCDD1L-DT	LINC02199
ABCC9	PDK4	LMO7-AS1	DIRC3-AS1
SPATA18	ATP6V0A4	MIR31HG	LINC01446
GREM2	NTRK1	MBNL1-AS1	LINC00518
CDH11	PIK3CG	ZNF433-AS1	PCAT7
LOXL1	LRAT	LINC00942	LINC00111
COL6A2	ARAP2	LINC00607	RAMP2-AS1
THY1	RXRG	LINC00839	TNRC6C-AS1
SLIT2	SLAIN1	KCNMA1-AS1	EWSAT1
MKX	LIMCH1	CYP51A1-AS1	FOXD3-AS1
MXRA5	MAGEB2	SEC23A-AS1	MYHAS
FGF5	MAGEA12	LNCOC1	UBA6-AS1
EML1	SOX8	ZNF436-AS1	C22orf34
COL1A1	LEF1	SUCLG2-AS1	MELTF-AS1
C14orf132	DDX3Y	CYP1B1-AS1	ALKBH3-AS1
GJA1	NEDD9	LINC01184	DLX6-AS1
TRPS1	PDE10A	OTUD6B-AS1	FLVCR1-DT
OLFML3	UGT8	MIR646HG	ZNF460-AS1
PBX1	GCNT2	PDZRN3-AS1	NBR2
TCF4	GNG4	LINC00565	FAM222A-AS1
LAYN	CDH19	DPYD-AS1	NALCN-AS1
NTNG1	CADM1	LINC00654	TENM3-AS1
PRKG2	CSAG1	VIM-AS1	LINC02241
ADGRL4	F11R	NDUFV2-AS1	KTN1-AS1
MASP1	PIEZO2	LINC01572	NNT-AS1
STEAP4	ZFY	SLC8A1-AS1	ADGRL3-AS1
THBD	GRAMD1B	P4HA2-AS1	LINC01271
KCNMA1	PDE4D	SNAI3-AS1	LINC01138
XG	ZNF704	GPRC5D-AS1	LINC01270
PLA2R1	C10orf90	LINC02035	MKNK1-AS1
CAVIN2	KDM5D	WDFY3-AS2	CKMT2-AS1
IRAK3	AIF1L	LIMD1-AS1	PROSER2-AS1
ITGBL1	NEDD4L	SRD5A3-AS1	CNTN4-AS1

CACNA2D1	LONRF2	KCTD21-AS1	SERTAD4-AS1
BNC1	KIF21B	ABALON	PTCHD1-AS
FBLN2	SCARA5	BAIAP2-DT	PRR7-AS1
SHC3	LPL	IL21R-AS1	TBX2-AS1
FGF14	FOXR2	SGMS1-AS1	SLCO4A1-AS1
OSR1	ADGRG6	NORAD	STAU2-AS1
LRATD1	SORCS1	BEAN1-AS1	SEMA6A-AS1
GALNT15	KRT80	WAC-AS1	SMG7-AS1
CPE	EXTL1	HCG11	ZFPM2-AS1
TNFRSF10D	PCDH1	PSMG3-AS1	ATP6V0E2-AS1
OLFML2B	CACNA2D4	NIPBL-DT	MIR600HG
CCBE1	ONECUT1	PINK1-AS	HAGLR
MMP3	POU3F2	TPT1-AS1	VLDLR-AS1
ADH1B	ACP7	ADD3-AS1	LINC02055
BMPER	ATP10B	SPRY4-AS1	LINC00997
TRIM22	PDE1C	MID1IP1-AS1	SLC7A11-AS1
VAT1L	ADGRL3	ATP2B1-AS1	COL4A2-AS2
NFASC	TUBB2B	DGCR11	EML2-AS1
TMEM47	ITGB4	SNHG26	PKP4-AS1
RGS4	RHPN1	FAM225A	CTBP1-DT
TNFRSF11B	LONRF3	PXN-AS1	HOXD-AS2
HS3ST3B1	CSMD1	LINC00667	SP2-AS1
CFH	RAB39A	IQCH-AS1	NRAV
GUCY1B1	SLC24A5	FER1L6-AS2	KIAA1614-AS1
HS3ST3A1	CDK18	USP2-AS1	LINC00205
SIM1	L3MBTL4	LINC01852	RARA-AS1
TNS1	CCNJL	IPO9-AS1	FLG-AS1
SH2D4A	SORBS2	PSMB1	GSEC
PTPRQ	LCP1	SNAP25-AS1	SLC25A25-AS1
HEPH	FOXD3	MIR4713HG	THRIL
ADAMTS2	SCG2	NAALADL2-AS3	SLFNL1-AS1
EDA2R	AFAP1L2	STEAP2-AS1	DLEU1
S1PR3	PRXL2A	RB1-DT	DCST1-AS1
CPXM2	FAM178B	LNX1-AS1	DNAJC3-DT
SLC16A2	HES6	GPC6-AS2	ITGA9-AS1
ISLR	SLC27A2	VCAN-AS1	TTN-AS1
RNF152	MYO5C	MIR31HG	POLR2J4
TSHZ3	MGAT5B	VPS33B-DT	NUP50-DT
AKR1C1	ELOVL2	ZNF426-DT	LINC01963
MYO1D	TENM1	BCRP3	LRRC8C-DT
PRTFDC1	TSPAN33	GNG12-AS1	LINC00963
NPR3	ADM2	GCC2-AS1	LINC01719
AKR1C3	UTY	TRAM2-AS1	ASH1L-AS1
VSTM4	PLA2G7	MAPKAPK5-AS1	HOXA-AS3
GPX7	EPB41L4B	LINC01182	TSPOAP1-AS1
SHISAL1	HOXD13	TBC1D8-AS1	LINC00539
TSPYL5	SLC45A3	MSC-AS1	STARD7-AS1

CCDC149	DMTN	FLNC-AS1	PTOV1-AS1
CCDC69	CLIC6	FAM198B-AS1	DIRC3
NUDT11	SNX10	ARRDC3-AS1	RNF219-AS1
GPR68	GPR37	IQCH-AS1	IL10RB-DT
ZNF385D	SLC35F1	ROR1-AS1	GMDS-DT
ARHGAP24	FAM163A	KCTD21-AS1	PHKA2-AS1
ZBTB16	EN2	RORA-AS1	BCDIN3D-AS1
APCDD1L	GRIP1	LINC01239	TMPO-AS1

Appendix 1: Top 100 mRNA and LncRNA specifically enriched with the SFPQ in PM and A2058 cell.

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