Investigating the role of RAMP1 on Oncogene Induced Senescence in Lung Cancer driven by the MAPK Signalling Pathway

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Abstract

Worldwide, lung cancer remains the leading cause of cancer death. Gene mutations accumulate as a natural part of ageing but can also be acquired by other means of DNA damage. Aberrant activation of the RAS-RAF-MEK-ERK (MAPK) signalling cascade is found in 75% of lung adenocarcinomas, the most common subtype of Non-Small Cell Lung Cancer (NSCLC). Oncogene Induced Senescence (OIS) is a protective mechanism where cells are unable to re-enter the cell cycle, protecting the organism from further damage and uncontrolled proliferation. Interestingly, MAPK pathway mutations in upstream activators RAS and RAF were found to stimulate induction of OIS, and by inactivating senescence regulators, this mechanism may be bypassed.

A shRNA-based lentiviral genetic screen was conducted to identify novel OIS regulators. This screen identified Islet Amyloid Polypeptide (IAPP or Amylin), a small peptide hormone. Loss of IAPP or its co-receptor Receptor-Activity Modifying protein (RAMP3), allowed bypass of BRAF-induced senescence in immortalized fibroblasts. There are three different RAMP proteins which interact with Calcitonin receptor (CALCR) to form a heterodimeric IAPP receptor complex, this work focuses on RAMP1. To determine if RAMP1 has a role in regulation of OIS, several knockdown experiments were conducted providing results that suggest RAMP1 works in tandem with the other members of the RAMP family to compensate for functional loss of one.

In this thesis, a genetically engineered mouse model (GEMM) is used to model BrafV600E activation in the lung followed by loss of p53. Cre-LoxP and Flp-FRT technologies are widely used in cancer research and are useful in studies which investigate tumor development and the progression of lung cancer. This specific model permits the expression of mutant Braf in

the lung via recombinase-mediated recombination. Flippase recombinase (Flp) is introduced by nasal instillation of an adenoviral vector expressing Flp. At a later timepoint, Cre activity is activated using a ubiquitously expressed Cre:ER transgene. This Cre:ER allele is comprised of Cre fused to a modified Estrogen Receptor which is activated by tamoxifen, an estrogen analog. Cre activation results in Cre-recombinase-mediated recombination and the tumor suppressor gene p53, is ablated. Using this dual-recombinase system, at an early timepoint of 12 weeks after Flp adenoviral infection to initiate mutant Braf, tamoxifen injections are administered resulting in loss of p53. This two-step model mimics the process of cancer development in humans where mutations are spatially and temporally separated. In comparison to p53 loss at later timepoints, prior research has demonstrated early p53 loss results in tumors larger in size, higher in burden, and higher pathological grade. To investigate the genetic differences that permit size, burden, and grade progression, lungs were harvested, and tumors were isolated for downstream analysis.

Resumé

Mondialement, le cancer du poumon reste la cause de mort de cancer. Les mutations de gènes accumulent en tant du process naturel du vieillissement mais peut aussi être obtenu par autres moyens de dommages à l'ADN. L'activation aberrante dans la protéine kinase activée par des mitogènes cascade de signalisation (MAPK) est trouvé dans 75% de cas d'adénocarcinome pulmonaire, le sous-type du cancer du poumon non à petites cellules (CPNPC) le plus commun. La sénescence induite par oncogène (OIS) est un mécanisme de protection contre des dommages supplémentaires et de la prolifération prolongée. Intéressement, les mutations de la voie de la protéine kinase activée par des mitogènes dans des activateurs en amont RAS et RAF ont été trouvé a stimulé l'induction de OIS, et par inactiver les régulateurs de sénescence, ce mécanisme peut être contourné.

Un crible génétique lentiviral à base de shRNA a été mené pour identifier des nouveaux régulateurs OIS. Le crible à identifié le polypeptide amyloïde des îlots (IAPP ou Amyline), une petite hormone peptidique. La perte de IAPP ou de son co-récepteur protéine de modification de l'activité des récepteurs (RAMP3), contournement autorisé de la sénescence induite par BRAF dans des fibroblastes immortalisés. Il y a trois RAMP protéines différentes qui interagi avec le récepteur de la calcitonine (CALCR) pour former un complexe récepteur IAPP hétérodimérique, ce travail se concentre sur le RAMP1. Pour déterminer si le RAMP1 a un rôle dans la régulation de l'OIS, plusieurs expérimentations d'inactivation ont été mené qui ont fourni des résultats qui suggère que RAMP1 fonctionne en tandem avec les autres membres de la famille RAMP pour compenser pour la perte fonctionnelle d'un autre RAMP.

Dans cette thèse, un modèle de souris conçue est utilisé pour modeler l'activation BrafV600E dans le poumon suivi par la perte de p53. Les technologies Cre-LoxP et Flp-FRT

sont largement utilisés dans la recherche de cancer induit par les oncogènes et ils sont utiles dans les études qui étudient le développement de la tumeur et la progression du cancer du poumon. Ce modèle spécifique permet l'expression du mutant Braf dans le poumon via la recombinaison médiée par la recombinase. La recombinase flippasse (FLP) est introduit par l'instillation nasale d'un vecteur adénoviral qui exprime Flp. À un moment plus tard, l'activité CRE est activée en utilisant un transgène Cre:ER exprimé d'une manière omniprésente. Cet allèle Cre:ER est composé de Cre fusé a un récepteur d'œstrogène qui est activé par Tamoxifène, un analogue d'œstrogène. L'activation Cre résulte en recombinaison médiée par la Cre-recombinase et le gène suppresseur de tumeur p53, est ablaté. En utilisant ce système a double recombinase, à un stade précoce de 12 semaines après l'infection adénovirale Flp pour initier le mutant Braf, des injections de Tamoxifène sont administrés résultant en une perte de p53. Ce modèle à deux étapes imite le process du développement de cancer dans les humains ou les mutations sont séparés spatialement et temporellement. En comparaison a la perte de p53 en des moments plus tard, des recherches précédentes ont démontrés des pertes précoces de p53 résultant en tumeurs de grande taille, de charge plus élevée, et de grade pathologique supérieur. Pour investiguer les différences génétiques qui permettent la taille, la charge, et la progression de grade, des poumons ont été récolté, et des tumeurs ont été isolé pour l'analyse en aval.

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Authors Contributions

Unless otherwise stated, all experiments and analysis were performed by Hannah Buchanan. Experimental designs were a collaboration between H. Buchanan and D. Dankort.

The shRNA library genetic screen, IAPP knockdown, and RAMP3 knockdown experiments were performed by S. Garnett (**Figure 3-1, 3-2**).

D. Dankort provided the HF-E1T cell line. BRER cells were created and characterized by S. Garnett and R. Shu.

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List of Abbreviations

4OHT	4-hydroxytamoxifen
AdFlp	Flp-recombinase adenovirus
AT-I	Alveolar type I cell
AT-II	Alveolar type II cell
BRAF	Human BRAF gene
Braf	Mouse gene
BRafFA	Mouse Flp-activated Braf gene
BRAFV600E	Oncogenic Braf mutant
BRER	HF-E1T cells with ΔBRAF-ER
CALCR	Calcitonin receptor
CDK	cyclin-dependent kinase
Cre	Cre recombinase
DDR	DNA damage response
Dox	Doxycycline
E2F	TFs able to bind to E2 gene promotor of adenovirus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EtOH	Ethanol
GDP	Guanosine diphosphate
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
hTERT	Human telomerase reverse transcription
IAPP	Islet amyloid polypeptide
IL-1	Interleukin-1

IL-6	Interleukin-6
IL-8	Interleukin-8
LCC	Large cell carcinoma
LuAD	Lung adenocarcinoma
MAPK	Mitogen-activated protein kinase
MCP2	Membrane cofactor protein-2
MCP4	Membrane cofactor protein-4
MEK	Mitogen-activated protein kinase kinase
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-3α	Macrophage inflammatory protein-3 alpha
MMP	Matrix metalloproteinase
NF1	Neurofibromatosis 1gene
NSCLC	Non-small cell lung cancer
OIS	Oncogene induced senescence
ORR	Objective response rate
p15INK4b	Cyclin-dependent kinase 4 inhibitor B
p16INK4a	Cyclin-dependent kinase inhibitor 2A
p21CIP1/WAF1	Cyclin-dependent kinase inhibitor 1
p53	Tumor protein 53 protein
p53flox/flox	p53 Cre-conditionally null mouse
PCR	Polymerase chain reaction
PDGF-α	platelet derived growth factor alpha
RAF	Human rapidly accelerated fibrosarcoma
Raf	Mouse rapidly accelerated fibrosarcoma
RAMP1	Receptor activity modifying protein 1
RAMP2	Receptor activity modifying protein 2
RAMP3	Receptor activity modifying protein 3

Rb	Retinoblastoma
RT-qPCR	Real time quantitative PCR
RTK	Receptor tyrosine kinase
rtTA	Reverse tetracycline transactivator
SA-β-gal	Senescence-associated-β-galactosidase
SASP	Senescence-associated secretory phenotype
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SOS	Son of sevenless
Tet	Tetracycline
tetO	Tetracycline operator
TetR	Tetracycline repressor
TF	Transcription factor
TGF-β	Transforming growth factor beta
TIS	Therapy-induced senescence
TNF-α	Tumor necrosis factor alpha
TP53	Tumor protein p53 gene
TRE	Tetracycline responsive element
TSG	Tumor suppressor gene
tTA	Tetracycline transactivator
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction and Literature Review

1.1 Introduction

Cancer affects one in two Canadians in their lifetime, developing as a result of accumulated DNA damage at the cellular level, which leads to uncontrollable growth [1]. Mitogen-Activated Protein Kinase (MAPK) pathways are often associated with cancer as many mutations in these pathways affect the control of cellular proliferation, differentiation, migration, apoptosis, and stress responses [2, 3]. Mutations in these pathways can drive malignancy through promotion of proliferation and survival despite many safeguards such as apoptosis, induction of cell death, and senescence. Senescence, as an intrinsic mechanism, is one of two main ways we are protected from proliferation of abnormal cells. Senescence halts the cell cycle while still allowing for metabolic activity, while apoptosis, a form of programmed cell death will eliminate cells by protein cleavage, both providing a line of defense against aberrant proliferation.

This thesis aims to analyze the mechanism of Oncogene Induced Senescence (OIS) within the context of developing Non-Small Cell Lung Cancer (NSCLC). Acting as an anticarcinogenic mechanism, OIS prevents damaged cells with abnormal oncogenic signalling from dividing, thus preventing further expansion of cells harboring harmful mutations. Some mutations permit bypass of the OIS mechanism, where senescence is not induced and cells continue to proliferate, only adding to the complexity of the disease by promoting malignancies. Understanding the processes involved in OIS induction and the underlying mechanisms permissive of bypass will provide further insight of what drives cancer development. Many new studies are emerging with results leading to new questions regarding their downstream therapeutic potential.

1.2 Cancer

Overview

Characterized by the uncontrollable growth of cells, cancer is an umbrella term that covers an incredibly diverse assortment of diseases. Cancerous tumors are categorized as one of two types, solid and liquid. Solid tumors consist of solid masses of cells that can occur nearly anywhere in the human body e.g., breast, colon, or lung cancer. Liquid tumors however are typically restricted to blood and bone marrow. Agents that result in mutation (i.e., chemical, radiation) or errors in DNA replication or repair mechanisms can lead unrestricted growth of abnormal cells. Termed in 2000 as the "Hallmarks of Cancer", there are acquired characteristics of cells suggested to be common across all cancer types, acting as drivers of tumor development [4]. Moreover, developing cancer occurs in a stepwise fashion where the accumulation of mutations underlying six features: sustaining proliferative signalling; evading growth suppressors; activating invasion and metastasis; enabling replicative immortality; inducing angiogenesis; and resisting cell death, occurs over time and together drive malignancy [4]. Additional Hallmarks of Cancer have emerged: deregulating cellular energetics and avoiding immune destruction, and genome instability and mutation, and tumor-promoting inflammation [5]. Over the course of one's lifetime, many mutations occur, and most do not end up causing cancer. DNA damage checkpoints are in place, damaged cells can be cleared by the immune system, and other safeguards such as programmed cell death and senescence exist.

1.3 Lung Cancer

Leading in both incidence (13%) and mortality (25%), it is projected that an average of 87 Canadians will be diagnosed with lung cancer each day in 2022 [6]. Although not all cancer

results in death, with a low 5-year survival rate of approximately 22% most patients will die as a result of lung cancer [1, 6].

Overview of The Lung

The lungs are a vital component of the respiratory system (**Figure 1-1**), ultimately facilitating intake of fresh oxygen and removal of waste gases (i.e., carbon dioxide). Comprised of five lobes, the left lung has two lobes while the right lobe has three. Air is inhaled and moves through the trachea, eventually reaching the lungs where it moves through the bronchial tubes (primary, secondary, and tertiary bronchi) which branch out into increasingly smaller bronchioles. At the ends of the bronchioles are the alveoli, small air sacs surrounded by capillary networks, which facilitate the exchange of oxygen into the blood and carbon dioxide out. As lung cancer develops, it can cause blockages of the lung and disrupt healthy oxygenation of the blood. Complications of these can lead to pneumonia while other symptoms affect the blood vessels, causing hemorrhaging or pleural effusion, which may cause dyspnea [7].

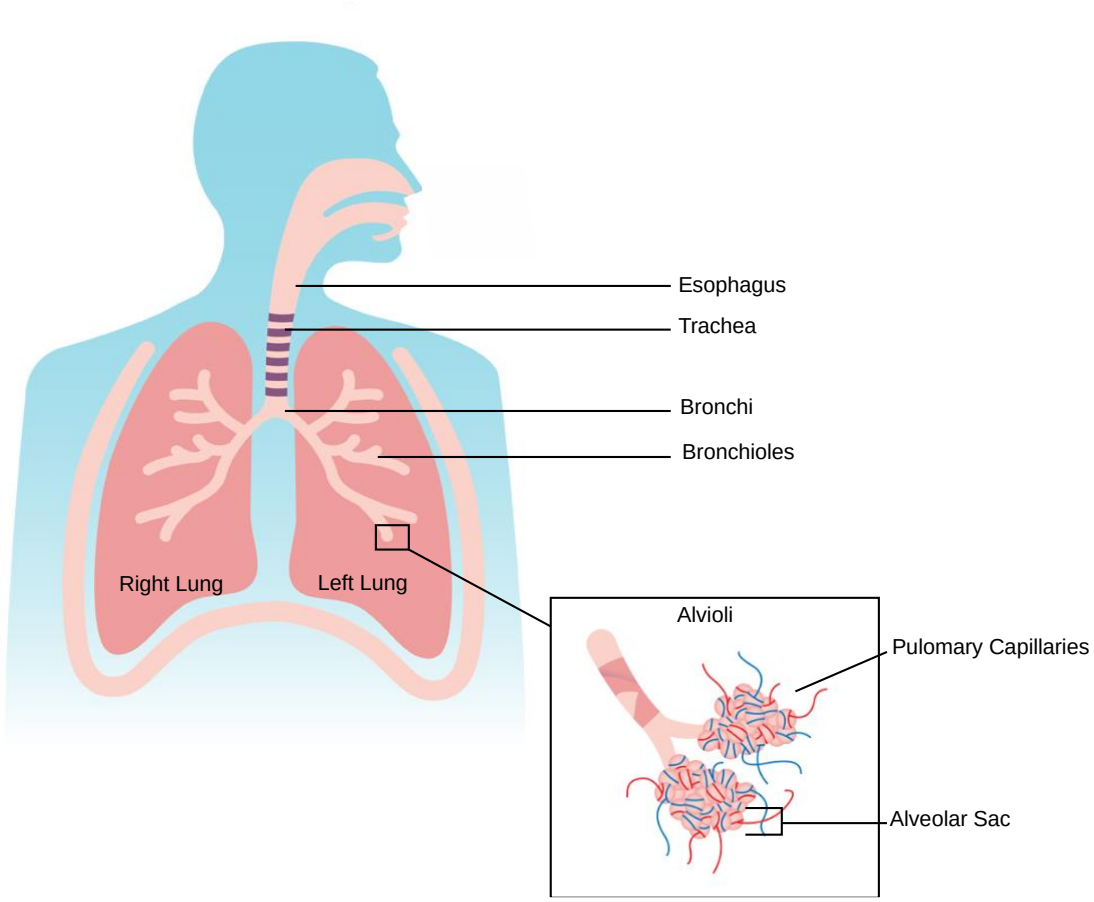


Figure 1-1. Human Lung Anatomy

Schematic displaying major parts of the respiratory system. As air is inhaled, it moves through the esophagus and trachea until it reaches the primary bronchi. From the primary bronchi the air moves through the increasingly smaller secondary and tertiary bronchi, finally reaching the bronchioles. Here, where the capillary-covered alveoli are located is where gas exchange occurs. [Figure adapted from Canadian Lung Association, 2016].

Lung Cancer

Cancer that originates in the lungs generally falls under two subtypes, small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). While less common, some lung cancers fall outside of these two categories such as a hybrid of NSCLC and SCLC (adenosquamous carcinoma). While SCLC is the more aggressive of the two, NSCLC accounts for the vast majority of cases, approximately 85-88% of cases [1]. Lung cancer develops due to a

wide variety of mutations in genes considered to fall within two main categories, oncogenes, and tumor suppressor genes (TSGs). These mutations occur at random or can be caused by environmental or behavioural risk factors. Smoking tobacco is the number one risk factor, directly causes up to 80% of Canadian lung cancer cases. Along with other risk factors such as hazardous exposure to carcinogenic materials, radiation exposure, breathing polluted air etc., through minimization or altogether avoiding exposure, lung cancer is largely preventable [8]. As a preventative measure, lung cancer screening is not typically performed, due to this, many patients are unaware of their lung cancer until they have reached late stage. At this stage the cancer will have metastasized, spreading to other areas of the body. In lung cancer especially it is common to see patients presenting only after advanced-stage symptoms appear, such as coughing up blood or experiencing significant breathing problems.

In total, 49% of all lung cancer diagnoses are caught at stage 4. In patients with NSCLC, 32% of squamous cell carcinoma, and 51% of large cell carcinoma subtype cases are caught at stage 4 [1]. As lung cancer is rarely caught early enough when intervention and treatment are most effective, treatments available to those with late stage have a lower likelihood of success and require more aggressive treatment plans. With a 5-year survival of 19% for all lung cancers and for those diagnosed with late stage NSCLC adenocarcinoma 0-10%, it is imperative to understand the disease in order to develop more effective and improve upon current treatments [1].

Non-Small Cell Lung Cancer

The three main subtypes of NSCLC are lung adenocarcinoma (LuAD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). These three histologic subtypes are grouped under NSCLC based on similar prognoses despite originating in different cell types. Originating

in epithelial tissues, carcinomas proliferate unchecked and typically form solid tumors. The most common NSCLC subtype is LuAD which accounting for 88%, appears to form from secretory cells called type II pneumocytes [1]. Adenocarcinomas are a type of cancer in glandular tissues in the lining of organs, thought to originate from Alveolar type II, Club (Clara; bronchiolar exocrine cells), or basal cells [9]. Adenocarcinomas are typically seen in the breast, colon, lungs, and pancreas [10]. LuADs are commonly associated with cigarette smoking but are also the most common form of lung cancer seen in non-smoking patients with mutations observed in many genes including *EGFR*, *KRAS*, *BRAF*, *TP53* [11, 12].

While lung cancers are grouped into subtypes, it is critical to highlight that no two lung cancers are the same. Each cancer is influenced by individual mutation patterns and environmental factors, heterogeneity exists between patients, between a single patients' tumors, and between cell populations that exist within the same tumor [13]. Excluding single gene diseases like cystic fibrosis (*CTFR*) or retinoblastoma (*RB*), no two patients have the same disease due to individual gene interactions paired with a variety of other genes carried. This is one of many reasons why cancer treatments may fail, they may work initially but the cancer returns, or the treatment does not have the same benefits for one patient as it does another [14].

1.4 MAPK Signalling Pathway

MAPK Signalling Pathway Overview

Mutations in the RAS-activated RAF–MEK–ERK mitogen-activated protein kinase (MAPK) signaling pathway (**Figure 1-2**) have been found in a variety of cancers, typically playing a role in cancer development, tumor formation, and drug resistance. Indeed, MAPK pathway mutations are particularly abundant in lung adenocarcinomas with a prevalence that reaches 75%.

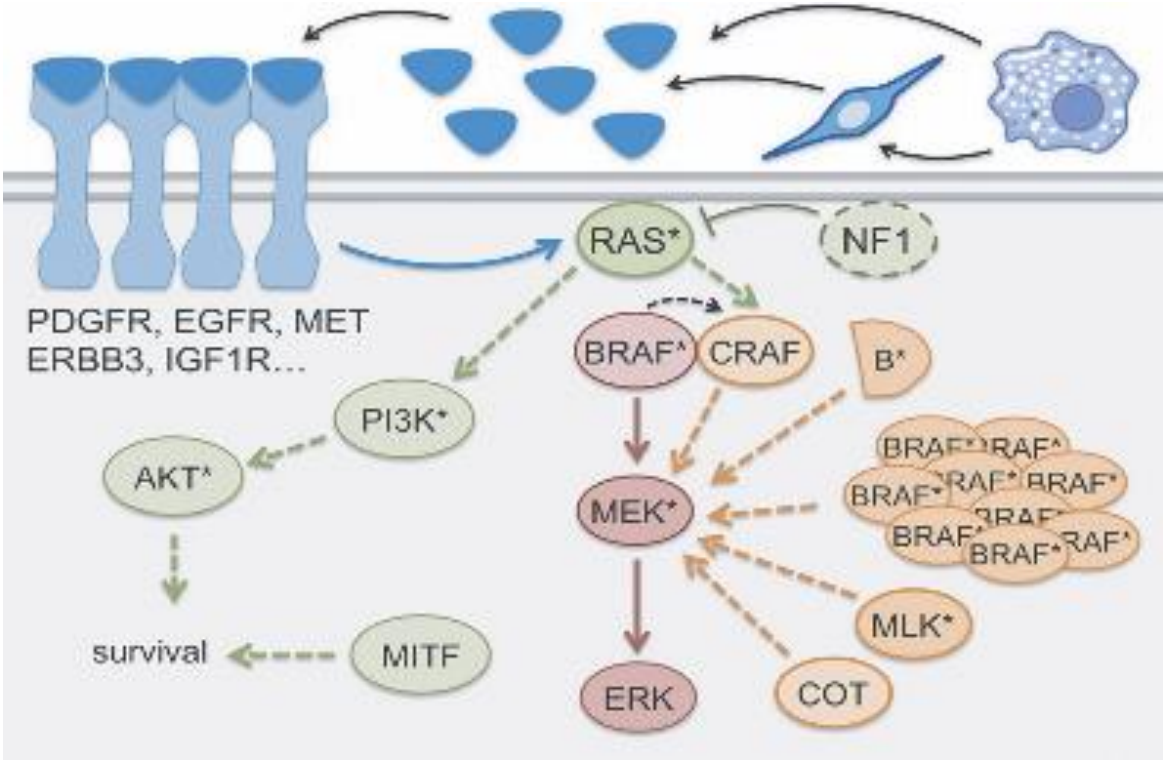


Figure 1-2. RAF-MEK-ERK Mitogen-Activated Protein Kinase (MAPK) Signalling Pathway

At the plasma membrane, growth factors such as EGF bind to their receptor EGFR (Receptor Tyrosine Kinase) which activates RAS. Active RAS recruits BRAF thereby activating the downstream effectors ERK1 and ERK2. When ERK is activated, it translocates to the nucleus. Here ERK acts as a regulator of transcription factors and gene expression. [Figure taken from Azozarena, 2017]

These mutations commonly occur in Receptor Tyrosine Kinases (RTKs) (24%), *KRAS* (32%), *BRAF* (8%) and loss of the negative Ras regulator *NF1* (11%) [15-17]. In this ERK-MAPK pathway, at the plasma membrane, GFs (Growth Factors) bind to RTKs (Receptor Tyrosine Kinase) and initiates the MAPK signal transduction cascade. Reaching downstream effector proteins and finally to the nucleus, a variety of transcription factors are activated which regulate cell growth, proliferation, and senescence [11, 18]. Because this pathway plays an

important role in cell survival, studying its targets, and potential druggable factors are of particular interest in research.

The MAPK signalling pathway (**Figure 1-2**) is initiated through EGF (Epidermal Growth Factor) ligand binding (EGF-related peptide growth factor family) to RTK's in the Epidermal Growth Factor Receptor (EGFR) family. Phosphorylation of EGFR creates Grb2 (adaptor protein) and Shc2 (Src homology 2) binding sites which through SOS (son of sevenless), stimulates the guanine nucleotide exchange of GDP to GTP, thereby activating RAS [19-21]. In its GTP-bound active complex, the RAS effector domain interacts with both the Ras-binding and cytosine-rich domain to activate RAF, which phosphorylates and activates the downstream effectors ERK 1 and ERK2 [22]. In the cytoplasm, activated ERK translocates to the nucleus. Here, ERK can regulate transcription factors and gene expression, affecting various cellular processes, including proliferation [23].

1.5 Oncogenes and Tumor Suppressor Genes

Oncogenes

Proto-oncogenes are genes when mutated or aberrantly expressed, have the potential to play a causal role in tumour formation through deregulation of a variety of signalling pathways involved in cell growth i.e., proliferation, differentiation, and cell death. In many cases, proto-oncogenes regulate proliferation and, when mutations occur, these can become oncogenes as the ability to induce cancer is acquired [24]. In the MAPK pathway, genes such as *RAS* and *BRAF* are considered oncogenes and are often seen mutated in human cancers. In NSCLC, mutations of *RAS* are observed in approximately 30-32% of cases, and *RAF* are present in about 7-8%. Interestingly, *RAS* and *RAF* mutations seem to be mutationally exclusive as dual mutations are observed in only a handful of cases [16, 17, 25-28].

Tumor Suppressor Genes

On the other hand, tumor suppressor genes (TSGs) encode proteins which act as a barrier to excessive proliferation. Inactivating mutations are seen in TSGs such as *TP53*, *PTEN*, *INK4*, and *RB1* that often cause loss of their suppressive roles [29]. The extensively studied *TP53* regulates approximately 500 genes and is found mutated in approximately half of all cancer cases [30]. This TSG encodes a protein (p53) that is normally found in low levels in the cell as it is quickly degraded. MDM2 regulates p53 levels through a negative feedback loop where p53 induces expression of the ubiquitin ligase, mouse double minute 2 (MDM2/HDM2), and MDM2 targets p53 for degradation [31-33]. In response to stressors, including oncogene activation, p53 levels increase as MDM2 is inhibited. When p53 binds directly to DNA, the *CDK1A* (Cyclin-dependent Kinase Inhibitor 1A) gene is transactivated, and stimulates production of its encoded protein, p21. As a key player in cell cycle progression, p21 regulates different cyclin-dependent kinases (CDKs) across the different phases of the cell cycle. Functioning as a promotor of CDK4/CDK6 kinase activity, the cell can progress through the G1 phase. As an inhibitor of CDK2, p21 leaves the kinase unable to activate the RB (retinoblastoma) TSG. Inactive, unphosphorylated RB remains bound to the family of E2F transcription factors and prevents transcriptional activation of E2F targets. These targets include genes involved in DNA replication, cell cycle progression, and exit. When E2F activity is repressed, the cell is unable to progress through G1 to S phase or exhibits senescence [34-39].

RAS

RAS proteins are a family of four small GTPases (guanine triphosphatases), encoded by *H- N-*, *K-RAS* that respond to signals by cycling between inactive (GDP-bound) and active (GTP-bound) states. Most often, mutations occur in *KRAS* codons 12, 13, or 61 in any of the 3

isoforms [40]. However, while mutations in other codons do occur, they are rarely observed [41]. Mutated *RAS* often works alongside other mutations, particularly tumor suppressor p53 or p16 loss, to drive tumorigenesis [42]. In NSCLC, *KRAS* (Kirsten rat sarcoma 2 viral oncogene homolog) is the most commonly mutated oncogene across all cancers, and 20-25% of LuADs harbour a mutation in this gene [41, 43-46]. Prognosis for the patient is associated with more or less favourable outcomes depending on the specific mutation which underlies their disease. For example, the KRAS-G12C mutation is observed in 39% of overall NSCLC cases, this transversion results in the GTP-bound state (active) of KRAS being more favourable. With increased levels of activated KRAS, multiple pathways including the PI3K/AKT/mTOR (phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin) MAPK pathways [47-49]. When overactivated KRAS recruits RAF and activates the protein kinase, this creates a surge in MAPK signalling and causes cells to proliferate excessively and increases their survival [50-52]. KRAS-G12C is observed in many patients who are heavy tobacco smokers, and this mutation is currently considered a hallmark of tobacco smoke exposure. Resulting in hyperactivation of KRAS, the KRAS-G12C mutation is most often associated with poor outcomes as patients typically exhibit a complex disease where both high mutation loads and high rates of concurrent p53 mutations are observed [44]. Prior decades of research emphasized the difficulties in targeting KRAS, citing issues in overcoming the high affinity for GTP, scarcity of clear druggable pockets due to the smooth surface shape, and that current drugs could not provide a strong enough inhibition provide. Consequently, KRAS was deemed “undruggable” until the early 2010’s, and other avenues of indirect inhibition were explored (i.e., inhibiting downstream effectors rather than KRAS itself) [41, 50, 53-55]. Advances include the discovery of a new allosteric target site and the subsequent development of new inhibitor drugs sotorasib and

adagrasib to specifically target the KRAS-G12C mutant protein. Both sotorasib and adagrasib work by binding to a small pocket that is only present in the inactive conformation of the mutated protein. The G12C mutation results in hyperactivation of KRAS as this glycine to cysteine substitution disrupts GTPase activity of the protein, trapping it in the GTP-bound (active) state. Sotorasib and adagrasib both target KRAS-G12C and create a covalent bond with cystine 12, inhibiting KRAS hyperactivation by preventing the protein from switching to its active state [50, 51, 56]. Both inhibitor drugs aim to decrease downstream signalling, and thus far the clinical results have been encouraging although acquired drug resistance remains a major problem. Further investigation of the mechanisms underlying *KRAS* driven cancer and inhibitor resistance are necessary to ensure future development of effective therapeutic drugs [57, 58].

RAF

ARAF, BRAF, and CRAF (Raf-1) are three *RAF* isoforms present in the human genome, encoding cytoplasmic serine/threonine kinases. *CRAF/RAF1* was the first isoform to be discovered, deriving its name from the murine retroviruses encoding the viral rapidly accelerated fibrosarcoma (V-RAF oncogene homolog B) [59]. Primarily observed in melanoma at a prevalence of 40-60% and half of papillary thyroid cancers, *BRAF* mutations are also seen in a wide variety of other cancers such as colorectal, ovarian, and several brain cancers [60-62]. Activating mutations are seldom observed in *ARAF* and *CRAF* whereas mutations of *BRAF* are present in about 7-8% of NSCLC. The lower occurrence of activating mutations in *ARAF* and *CRAF* is thought to be a result of the additional phosphorylation event within the N-terminal necessary for activation. Additionally, *BRAF* possesses higher basal kinase activity and is more easily activated by RAS [63, 64].

Mutations of *BRAF* can be divided into classes I, II, and III (**Figure 1-3**)[65]. Class I mutations refer to kinase-activating V600-monomers. These act independently from RAS, resulting in high *BRAF* kinase activity, and therefore MAPK activity. Class II consists of RAS-independent non-V600 mutations that have somewhat weaker (moderate to high), *BRAF* kinase activity, and these dimers are typically BRAF inhibitor (vemurafenib)-resistant. The "kinase-dead" heterodimers fall under class III, termed as such because they have impaired or lack of *BRAF* kinase activity. Preferentially binding to active RAS, class III heavily relies on upstream (RTK, RAS-activating, or TSG (*NF1*) deletion) mutations for increased proliferation [66-68]. Half of all *BRAF* mutations are caused by a class I BRAF-T1799A to BRAF-V600E point mutation ($BRAF^{V600E}$), a thymine to adenine transversion in exon 15 (nucleotide 1799) resulting in a valine to glutamic acid substitution at codon 600 [69]. Observed in a small percentage of NSCLC patients, the $BRAF^{V600E}$ mutation destabilizes its inactive form, and results in its active state being more favourable [67].

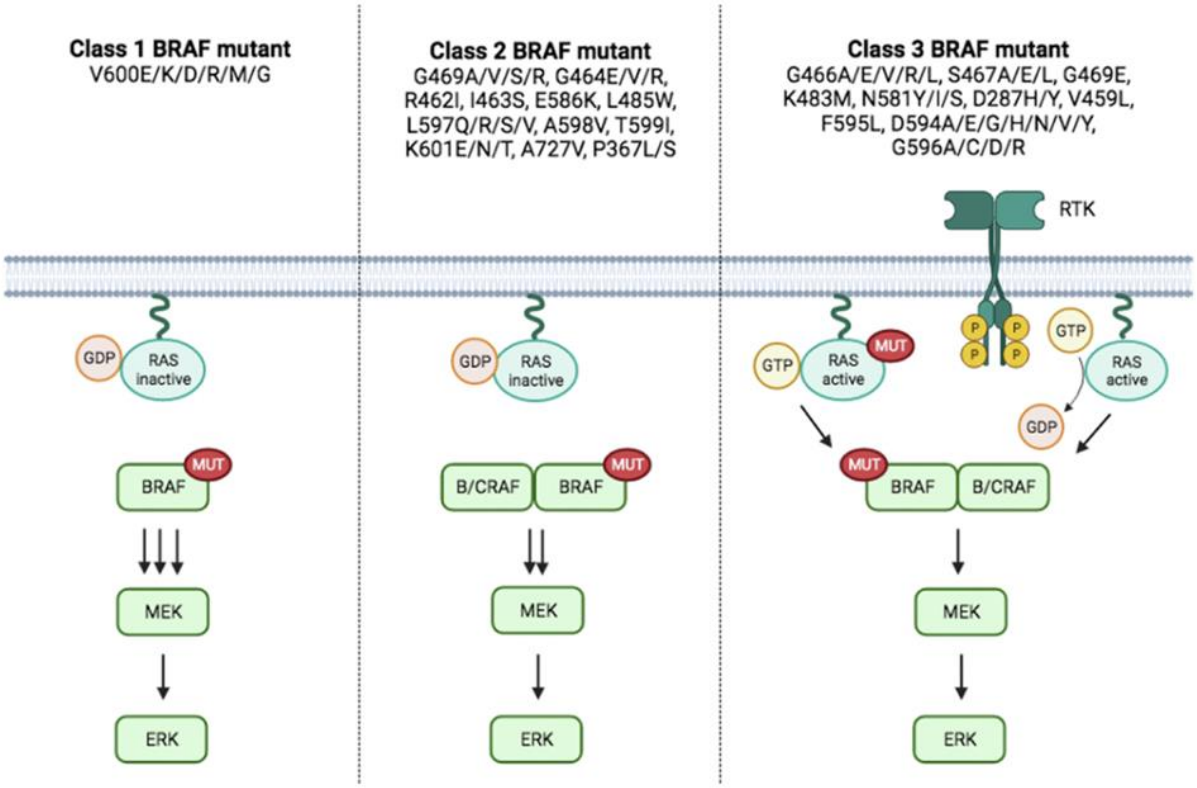


Figure 1-3. Class I, II, and III BRAF Mutation Classes.

BRAF mutations are characterized as class I, II, or III based on their Ras-dependency, dimerization status, and kinase activity levels. Class I mutations are Ras-independent, kinase-activating V600-monomers, resulting in high *BRAF* kinase activity, and therefore MAPK activity. Class II consists of RAS-independent non-V600 dimers that exhibit moderate to high *BRAF* kinase activity. Class III mutations are Ras-dependent heterodimers referred to as "kinase-dead" due to low or no *BRAF* kinase activity.[Figure taken from Tabbò, 2022]

Traditional Cancer Treatment

Out of common treatments available to Canadians, drug therapy, radiation therapy, and surgery are the most widely used. For all cancers, treatment plans differ based on many factors including the stage the disease is caught at, and physical condition of the individual. Relying on many factors, namely stage at clinical presentation, NSCLC patients have treatment options ranging from surgical removal to palliative care. In early stages of NSCLC, when the tumor is

locatable, surgery is by far the most successful treatment where it is possible for either part (wedge resection, segmentectomy, lobectomy) or the whole lung (pneumonectomy) to be removed. Through removal of the affected area, surgery dramatically increases 5-year survival rate up to 70% [18, 24]. In some cases, surgery is the only treatment necessary, while in other cases, drug therapy (chemotherapy) or radiation therapy will be used prior to or after surgery to shrink and inhibit tumor growth [70]. For patients who cannot have surgery, or the cancer has metastasized, radiation therapy and chemotherapy can be used alone or together as part of treatment.

Targeted Therapy

Targeted therapy is available to candidates harbouring specific mutations in genes such as *HER2, ALK, EGFR* or *BRAF*, which can be targeted with drugs. In the case of BRAF-mutated cancers, single or dual use of inhibitors to both BRAF (vemurafenib, dabrafenib, encorafenib) and its downstream effector, MEK (trametinib, cobimetinib, binimetinib) are used, sometimes in conjunction with more traditional lines of treatment (e.g. chemotherapy) [71-74]. Similarly, in EGFR-driven cancers, a handful of RTK inhibitors (gefitinib, erlotinib, afatinib, osimertinib) and monoclonal antibodies (mAbs) [75, 76].

Immune Therapy

More recently available in Canada, immunotherapy (immune therapy) is a type of treatment able to utilize the body's own immune system to target and eliminate cancer [77]. Typically used after other treatments have failed, the results of immunotherapy prove to be quite promising [78, 79]. There are many benefits of this type of therapy such as the lack of severe side effects seen in more traditional treatments (e.g., nausea, fatigue, alopecia) but patients run the risk of an inflammatory cytokine release syndrome which in some cases results in death, or

their immune system attacking healthy tissues [79-81]. There are various types of immunotherapies (e.g., immune checkpoint inhibitors, mAbs, cancer vaccines, adoptive cell therapies, etc.), and interestingly, the Chimeric Antigen Receptor T-Cell (CAR-T) adoptive cell therapy is among the most recently approved [82]. CAR-T therapy involves removal of T cells from the body, activation, reintroduction back into the body, and in preliminary research, has been used to successfully treat cancers. Through adoptive cell transfer, cells are transferred to the patient via infusion. After extraction, the T cells are genetically modified where CARs specific to cancerous cells is expressed [83-85]. The cells are then returned to the body and cancer cells will be targeted and cleared by the patient's own immune system [86]. In Canada, currently only patients with select blood cancers (i.e., leukemia and lymphoma) are candidates but face additional barriers as the treatment is both age-restricted and unavailable in most provinces [87]. CAR-T cell therapy is specific to each patient, typically only requiring one infusion and has proven to be enormously effective [88, 89]. It's a highly targeted method and as such, comes with an exorbitant price tag. Current work emphasizes the need for additional development of more CAR-T immunotherapies targeting more cancer types, focusing both on long-term patient outcomes and affordability of treatment [90-93]. Kymriah (tisagenecleulecel, Novartis Pharmaceuticals Canada Inc; CAR-T immunotherapy drug) was approved by Health Canada in 2019, and its single-infusion price sits at $475,000 USD with other drugs estimated to exceed $1 million USD.

Furthermore, if the patient is unable to withstand any of the aforementioned treatments, or the quality of life would be significantly affected, palliative care is another alternative that not only enhances the quality of life but extends survival as well [94, 95].

Targeting the MAPK Pathway

In the MAPK pathway, BRAF inhibitors have been developed to selectively inhibit the mutated oncoprotein, precisely targeting tumor cells with mutant BRAF, thus acting as a blockade to prevent aberrant downstream signalling [70]. The BRAF kinase inhibitor vemurafenib was developed for use as a monotherapy in treatment of late-stage metastatic melanoma. More recently it was administered to a cohort of *BRAF* V600E NSCLC patients; treatment resulted in tumor regression in most patients and the objective response rate (ORR), where tumors are eliminated or reduced, was 42% [96, 97]. In other clinical trials of BRAF V600E NSCLC, combined treatment with another BRAF kinase inhibitor, dabrafenib, resulted in an ORR of 33%, while treatment with a combination of dabrafenib and trametinib (a MEK1/2 inhibitor) resulted in an ORR of 64% [98-100]. These results demonstrate the utility of direct BRAF V600E inhibition in NSCLC and highlight the role of elevated BRAF activity in this disease.

Initial tumor regression followed by a secondary mutation, cancer recurrence, or drug resistance is common in drug targeted therapy and more work must be done to further understand these processes in BRAF-mutated NSCLC [101, 102]. While results of many clinical trials focusing on BRAF-targeting drug therapy are promising, due to complexity of the disease, resistance to BRAF inhibitors is common as observed in melanoma [103]. In future work there is a need to uncover more effective targets for a more comprehensive treatment strategy [27, 104, 105].

1.6 Senescence

How Senescence is Triggered

In 1961, Hayflick and Moorhead observed a finite proliferative capacity in human fibroblasts grown in cell culture, characterizing this novel phenomenon of irreversible growth arrest as senescence [106, 107]. Our current understanding of senescence is that it is triggered in response to damage and is important in wound healing, tissue remodelling, and is an important anticancer mechanism [108-112]. Senescence-inducers can be divided into two main categories, physical damage and developmentally programmed cues [111]. Physical damage can be induced by many things such as cellular aging (i.e., telomere shortening resulting from replicative stress), oxidative stress, UV light exposure, chromatin remodeling, exposure to drugs, oncogene-induced senescence (OIS), and therapy-induced senescence (TIS).

Role of Telomeres in Senescence

Human chromosomes consist of linear double stranded DNA located within the nucleus. The genetic material is protected by telomeres, chromatin structures that cap the ends of each chromosome with TTAGGG repeats, from degradation or events such as unintended activation of the DNA damage response (DDR) which recognizes the ends of chromosomes as DNA breaks. Normally as cells divide and chromosomes are replicated, the DNA strands become marginally shorter. As telomerase (hTERT) maintains telomere length, the DNA lost in the replication process is telomeric rather than genetic material. In stem cells and the germline, telomerase is responsible for maintenance of telomere length which allows for [113-115]. In somatic tissue, throughout many cycles of cell division, telomeres are eventually shortened. After approximately 40-60 divisions, without telomerase activity extending the telomeres, they will have atrophied to a critical length [107, 116-118]. The cell recognizes the short telomeres to

be double-stranded DNA breaks, which in turn triggers the DDR [119-121]. The DDR is a network of cellular pathways that recognize and respond to DNA lesions, the areas in DNA that harbors damage [122]. When damage is detected, the DDR signals for cell replication to be halted at G1 and for repair to be initiated. The streams of response include DNA reparation, senescence, or cell death [123]. Here, hTERT acts as a protective mechanism, a checkpoint of sorts to prevent proliferation of cells harbouring unstable chromosomes which would allow the cells to accumulate mutations that can drive cancer development [124-127]. In many forms of cancer transcriptional upregulation of hTERT is observed, allowing for evasion of the DDR, senescence, and allows proliferation past the normal finite limit [116, 125, 126, 128]. In other diseases related to telomerase activity, if hTERT expression is too low to maintain telomere length, the resulting premature telomeric atrophy is associated with Alzheimer's and Dyskeratosis congenita [129-132].

Other Functions of Senescence

More recent work has presented some evidence of an additional role in various processes including embryo development and adult regeneration, induced by developmentally programmed signals [133, 134]. Observed in human, mouse, chicken, and quail embryos, cellular senescence occurs in early developmental stages and is involved in patterning. In the absence of senescent cells during the process of embryogenesis, morphology appears altered [110, 111, 134].

In wound healing, in absence of senescent cells which secrete the platelet derived growth factor alpha (PDGF-α), wound closure is significantly delayed as PDGF-α is necessary for differentiation of fibroblasts into myofibroblasts (contractile fibroblasts). If cells are unable to differentiate, fibroblasts cannot contract granulation tissue resulting in delayed wound healing [135, 136]. In tissue remodelling, the premature elimination of senescent cells affects the

remodelling process additionally affecting the development of fibrosis [137]. As an anticancer mechanism, senescence prevents damaged cells from re-entering the cell cycle thereby locking the cells in a metabolically active state, without the ability to proliferate, thus acting as a checkpoint [138-141].

Some events causing DNA damage trigger cellular apoptosis or necrosis, whereby cells are eliminated through a form of death, however, senescence prevents re-entering of the cell cycle whilst the cells remain both viable and metabolically active. When DNA damage is sensed, p53 is recruited to the site and functions as a transcriptional activator of many genes involved in cell cycle progression, resulting in cell cycle arrest [121].

E2F/RB Pathway in Senescence

The E2F family of transcription factors regulates the cell cycle by activating genes responsible for both the G1 to S phase transition and DNA replication [142]. Regulated by RB, E2F binds to the unphosphorylated RB pocket protein and is unable to activate its target genes. When RB is phosphorylated, it will release E2F which can then bind to relevant gene promotors, initiating gene transcription and cell cycle can progress. p21 is a CDK activated by p53 and is a regulator of RB. When damage is sensed, p53 accumulates and p21is targeted for activation. p21 regulates RB by inhibition in the face of damage where RB cannot be phosphorylated and will remain inactive. p53 targets a CDK inhibitor, p21, that forces RB to remain inactive. p21inhibits RB phosphorylation, where it remains bound to the E2F family of transcription factors, which are then unable to regulate transcription of downstream targets. Cells cannot re-enter the cell cycle as it remains bound to the E2F family of transcription factors. As phosphorylation of RB permits E2F to regulate transcription of other genes involved in the G_1 to S phase transition, inhibition of RB traps the cells in G_1 phase of the cell cycle [121, 128, 133, 143]. When

establishment of this protective mechanism fails, or cells acquire the ability to evade senescence, this may drive tumor development [144, 145].

Senescence in Cell Culture

In cell culture, replicative senescent cells are morphologically distinct, observably enlarged and flattened. Moreover, these cells express specific markers such as senescence-associated-β-galactosidase (SA-β-gal), chromatin remodelling, metabolic alterations, as well as increased levels of cell cycle inhibitors p16INK4a, p15INK4b and p21CIP1 (**Figure 1-4**)[146-148]. *in vivo*, senescent cells do not adopt the altered morphology observed in cell culture, instead they are characterized by prolonged growth arrest coupled with a combination of 'senescent cell markers' including SA-β-gal, p16INK4a, p15INK4b, and other proteins [110, 121, 148-150]. Senescent cells are distinct from quiescent cells, as the growth arrest is permanent, and there is additional expression of a variety of secreted factors referred to as the senescence-associated secretory phenotype (SASP) [23, 151-157].

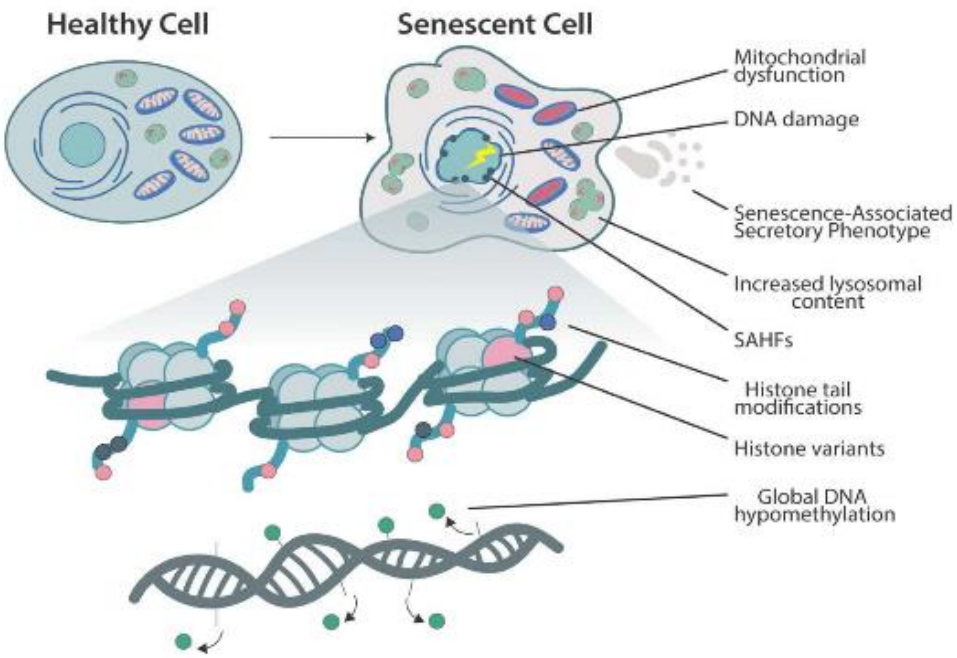


Figure 1-4. Hallmarks of a Senescent Cell: Morphological and Cellular Alteration

Once senescence is established the cells are visibly bloated and become flattened, expressing the Senescence-Associated Secretory Phenotype, along with other senescence biomarkers including senescence-associated heterochromatic foci (SAFH, condensed chromosomes)(Zhang, 2007), senescence-associated b-galactosidase(SA-β-Gal)/increased lysosomal content, increased expression of tumor suppressor genes (i.e., p53, p16INK4a, p15INK4b), [Taken from Crouch, 2022]

Oncogene-Induced Senescence

Contrary to what was expected, it was first discovered that high *HRAS* oncogenic signalling can trigger cellular senescence [42]. This has since been found to be true with high signalling of other oncogenes such as *RAF* or *MYC* and was thus termed Oncogene Induced Senescence (OIS) [42]. Furthermore, the growth arrest was found to be driven by upregulation of tumor suppressor networks involving genes such as p53, Rb and INK4a [5, 42, 112].

Some gene oncogene mutations including those in *RAF* affect MAPK pathway signalling, and have demonstrated the ability to bypass this anticancer defense [158]. Despite acting as a

barrier in tumor development, failure to establish senescence can result in cells harbouring unstable genomic programming gaining the ability to proliferate unhindered.

To model hormone-induced OIS in cell culture, ΔBRAF-ER cells (BRER) are used, these are human lung fibroblast TErt-expressing (HF-TE) immortalized cells. They express the BRAF kinase domain fused to a modified human estrogen receptor hormone binding domain (hER) (**Figure 1-5**). When BRER cells are treated with 4-hydroxytamoxifen (an estrogen analog), the fusion construct is stabilized thereby activating BRAF kinase activity [159, 160]. Because oncogenic signalling induces senescence, the BRER cells will exhibit growth arrest.

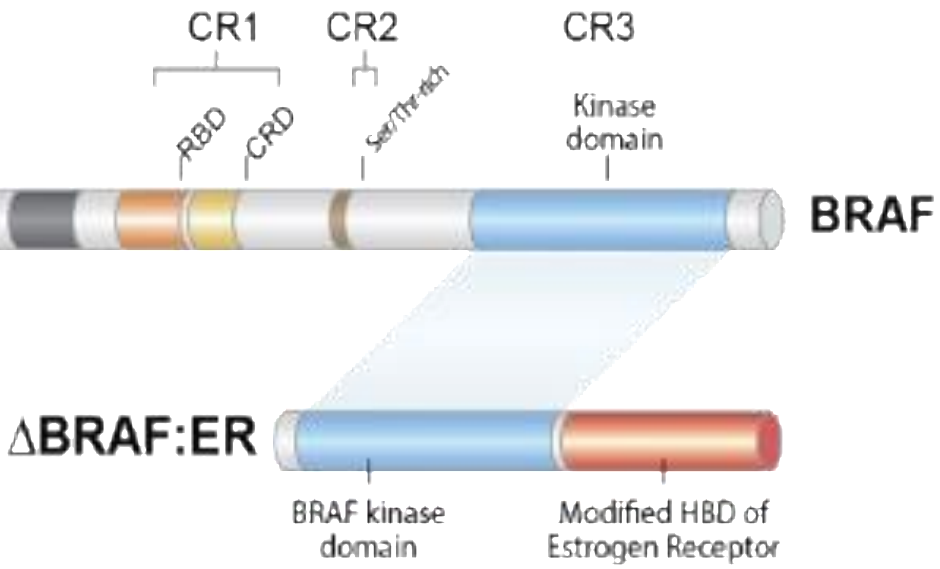


Figure 1-5. ΔBRAF:ER construct

Experimental DBRAF:ER cells carry the depicted fusion protein where the BRAF domain harbouring kinase activity is fused to the modified hormone binding domain of human estrogen receptor (hER). When DBRAF:ER (BRER) cells are treated with 4-hydroxytamoxifen (4OHT) media, the fusion construct will stabilize thereby activating BRAF kinase activity. [Taken from Garnett, 2019 unpublished, as modified from Lavoie and Therrien, 2015]

Senescence-Associated Secretory Phenotype

In cell culture, the distinct secretome expressed by senescent cells is referred to as the SASP [161]. This molecular profile includes soluble and insoluble factors such as cytokines, chemokines, growth factors, metalloproteinases (MMPs), ECM components, and others [162, 163]. Along with other markers of senescence (i.e. cell cycle arrest associated proteins, senescence-associated β-galactosidase), these factors may affect the tumor microenvironment in both pro-cancer or anti-cancer manners, as senescent populations can be capable of altering the behaviour of neighboring cells by inducing senescence or promoting proliferation [164-167].

When pro-inflammatory cytokines and chemokines are secreted (e.g. IL1, IL6, IL8, MCP2, MCP4, MIP-1α, MIP-3α, etc.), the adaptive immune system (i.e. T-cells, natural killer cells, macrophages) is recruited to the site and senescent cells may be eliminated in this way [168-170]. In immunodeficient mice, it was shown the animals lack the capability to clear oncogene-induced senescent cells from tumors [169]. In some cancers, senescence can be induced if the malignancy is driven by p53 loss. Because p53 is a regulator of senescence through the p53/RB pathway, if p53 can be rescued, and senescence induced it will lead to tumor regression [171, 172].

Two decades ago, SASP-expressing cells were found to be promotors of proliferation of both pre-malignment and malignment cells *in vitro*, it has since been discovered that through cell-non-autonomous action, proliferation can be both promoted and inhibited [144, 161, 170]. Some SASP factors plays a role various pathways involved in tumor development and metastases. The vascular endothelial growth factor (VEGF) promotes formation of new blood vessels, angiogenesis, which supplies the tumor with a constant blood supply and enables its growth and metastasis [173, 174]. Other factors such as IL-6 and IL-8 aid the tumor in

dissemination beyond its primary location through promotion of the epithelial-to-mesenchymal transition (EMT). This transition allows epithelial cells to adopt invasive and migratory properties [175, 176]. MMPs are other factors which have been shown to degrade the ECM, thus contributing to the invasiveness of the cancer [177].

A recent body of research has focused on senescence and methods of targeting and removing senescent cells using drugs (senolytics). This is of interest as removal of senescent cells has been demonstrated to relieve symptoms in various pathologies and improve overall health [133, 177-179]. More recent studies have provided evidence that suggests small subsets of therapy-induced senescent cells from non-small lung, colon, and breast cancer are capable of growth arrest escape at an extremely low frequency of 1 in 10^6 cells [180]. Despite these findings, this emergence (bypass) of senescence suggests that therapy induced senescent cells may play a role in cancer dormancy. Although this "escape" of senescence occurred in a small subset of tumor cells, it could be explained by genetic alterations they already carry, and thus can acquire the ability to proliferate once again [180, 181].

1.7 Cell Model

Hormone Inducible OIS Model

To model OIS in cultured cells, hormone inducible ΔBRAF-ER cells (BRER, **Figure 1-5**) are used. BRER cells were created from immortalized human lung fibroblast TErt-expressing (HF-TE) cells, these express the BRAF domain containing kinase activity fused to the modified hormone binding domain of human estrogen receptor (hER) expressed under the Chicken Actin Gene promotor (CAG) [182]. When BRER cells are treated in 4-hydroxytamoxifen (4OHT, an estrogen analog), the fusion construct stabilizes thereby activating BRAF kinase activity in a

concentration dependent fashion (K Dutchak, 2019 unpublished). As oncogenic signalling induces senescence, the BRER cells will exhibit growth arrest [160].

1.8 IAPP as a Requirement for RAF Oncogene Induced Senescence

Identification of IAPP

Using hormone-inducible BRER cells, a genetic screen was conducted by S. Garnett in attempt to discover novel regulators of OIS, to do so, BRER cells were infected with a pooled lentiviral shRNA library targeting approximately 5000 human genes. Following the stable integration of the shRNAs, cells were treated with 4OHT to activate BRAF activity and were cultured for an additional three weeks to allow the formation of colonies of proliferating cells. These colonies were expanded and for shRNA identification by PCR and sequencing. The strongest candidate was identified as islet amyloid polypeptide (*IAPP),* a small metabolic regulator, more often referred to as amylin [183]. Most research on IAPP has focused on the function in relation to diabetes whereas the role of IAPP in relation to OIS or the connection to the MAPK pathway are poorly understood.

IAPP

IAPP is a 37-residue hormone predominantly produced by pancreatic β-cells, which functions in regulation of glucose metabolism by promoting the breakdown of glycogen (into glucose-1-phosphate, glucose) in the liver. Released into the bloodstream along with insulin after meal consumption, those with Type-I and II diabetes are also IAPP-deficient. IAPP acts as a satiation factor in the area postrema region of the brain, here, when IAPP binds to its receptor hypophagia is experienced [184]. Other functions of IAPP are in gastric emptying regulation and glucagon secretion [185-187].

Most notably, these IAPP-knockdown cells continued to proliferate in culture, and additionally were found to have lower expression of senescence markers in comparison to the control which suggests IAPP is an important factor in establishment of OIS.

*IAPP g*ene knockdown was confirmed and BRER-shIAPP cells were treated with 4OHT in culture, the cells continued to proliferate and this additionally, decreased expression of senescence markers in comparison to the control cells which exhibited both OIS and senescence markers was observed [160]. Moreover, OIS bypass was also observed using CRISPR-mediated knockdown while treatment of BRER-shIAPP cells with exogenous amylin or expression of a non-targeted IAPP cDNA both restored a BRAF-induced growth arrest to the cells [41]. Again, demonstrating these results are true and loss of IAPP is permissive of OIS bypass.

1.9 Receptor-Activity Modifying Protein Family

RAMP Overview

The Receptor-Activity Modifying Protein (RAMP) family consists of three transmembrane proteins that interact with G-protein coupled receptors (GPCRs) resulting in different functions across the body [188, 189]. IAPP-specific coreceptors consist of the calcitonin receptor (CALCR) and either RAMP1 or RAMP3. One of the distinct functions of the RAMP family is that peptide binding is dependent on the specific RAMP protein that interacts with the GPCR to form a receptor. Different combinations of these RAMP/GPCR co-receptors can bind to a multitude of peptides including calcitonin, adrenomedullin, and IAPP. RAMPs control trafficking, signalling, and pharmacology changes as peptide binding and the resulting function of the receptor is dependent on the specific RAMP protein [190-192]. The coreceptor complex of RAMP3 and CALCR has the highest affinity for IAPP and as such, was chosen for further investigation [193, 194]. While calcitonin like receptor (CTLR) shares 55% of its amino

acid sequence with CALCR, the coreceptor complex CTLR/RAMP1 act as a GPCR receptor while CTLR/RAMP2 or 3 complexes act as receptors to adrenomedullin [189, 191, 195]. shRNA mediated knockdown of RAMP3 permitted bypass of BRAF induced OIS [160]. Combined with S. Garnett's IAPP knockdown and rescue experiments, the results suggest the role of IAPP in OIS is dependent on both its' coreceptor as well as an unspecified cellular role.

1.10 Mouse Model

Genetically Engineered Mouse Model Overview

Using Genetically Engineered Mouse Models (GEMMs) to study the MAPK pathway and OIS in vivo is invaluable in the study of cancer. Tumorigenesis in GEMMs closely imitates the human disease in terms of genetics, histology, and molecular qualities [196-198]. Furthermore, senescent cells do not exhibit the altered morphological features as observed in cultured senescent cell and tissue structure is inhibitive of many reliable senescence markers used in cell culture [199, 200]. Effects on the whole animal can be observed versus cells cultured in vitro, and biological processes can be better observed; beyond that, non-cell autonomous actions and the accelerated tumor development allow for a more comprehensive study of cancer [201]. Since the first transgenic (i.e. foreign DNA from another species is introduced into the host genome) mice were created in 1974, these models have since been designed to develop cancerous tumors, which have been analyzed in the study of lung cancer [202]. Early GEMMs were able to study effects of gene knockouts throughout the whole organism, but many of these experiments did not allow for development of viable animals, or the knockout was lethal [203]. In the study of oncogenic and tumor suppressive mutation effects, although gene knockouts throughout the whole organism are valuable in determining the many functions of each gene, as GEMMs have progressed, so has the target specificity. In modern models, the

problem of lethality can be avoided altogether through tighter control over the gene of interest, location, and timing of gene modifying events [204, 205].

Inducible Gene Expression Systems

Many gene knockout and knockin systems are available for use in murine models, and the TetON/OFF, Cre-LoxP, and Flp-FRT recombinase systems are among the most used. Tetracycline systems control activation and suppression of the gene of interest through presence or absence of tetracyclines and their analogs. The Tet-Off model is tissue specific and uses a tetracycline-controlled transactivator protein (tTA). rTA is a fusion of the transcription activation domain (AD) from the herpes simplex virusVP16 protein and Tet repressor DNA binding protein (TetR), under the control of a tetracycline-responsive promotor element (TRE) containing several Tet Operator (tetO) recognition sites. In absence of doxycycline (dox; a tetracycline analog), rTA will bind to tetO sites in the TRE and transcription of the target gene is activated. In the presence of dox, a conformational change is induced whereby gene expression is inactivated. In this state, tTA is prevented from binding to the tetO sites in the TRE. In the tetracycline-on (Tet-On) model, the reverse tetracycline transactivator (rtTA) promotor is a similar fusion of the transcription (AD) from the VP16 protein and TetR. There is an additional modification which permits rtTA to act opposite of tTA where dox induces a conformational change allowing activation of the target transgene rather than preventing it. In this Tet-On system, he presence of dox is required for rtTA-tetO binding. The rtTA is only active in the presence of dox so if the treatment is withdrawn and dox is metabolized, rtTA cannot bind to tetO recognition sites and the transgene will not be activated [205-209]. The Tet-On and Tet-Off models are especially useful as the transgene activation or suppression can be reversed upon dox withdrawal.

Cre-LoxP and FLP-FRT Systems

Two other widely used systems are the Cre-LoxP system (Cre enzyme derived from the P1 bacteriophage-locus of X-ing over), and Flp-FRT system (flippase enzyme derived from saccharomyces cerevisiae-flippase recognition target) [201, 210]. Both Cre and Flp enzymes mediate site-specific recombination when active. Used to perform both gene knockouts and knockins, *in vivo*, Cre recombinase recognizes and binds to 34 base pair LoxP target sites and when inserted so that the sites face the same direction flanking the region of interest (floxxed), the DNA segment between the LoxP sites are excised. [198]. A method of initiating Cre-mediated recombination is through infection using adenovirus that expresses Cre recombinase (AdCre). Using adenoviral or lentiviral vectors as delivery vehicles, the enzyme can regulate gene expression of the target of interest after AdCre infection. Alternatively, a second approach is breeding transgenic mice where the Cre-recombinase gene is placed under a specific promotor to facilitate targeted expression. For gene knockouts, by floxxing the target gene, Cre-mediated recombination will result in an irreversible ablation of the gene. For gene knockins, through insertion of a stop codon between the promotor and target gene, when the stop codon is floxxed, Cre activity will excise this region, allowing the downstream target gene to be activated.

The Flp-FRT system functions in a similar manner where Flippase recombinase enzyme targets short, 34 base pair FRT sites [211, 212]. When FRT sites flank the target DNA segment in the same direction, when there is Flp activity, this region will be excised by Flp-mediated recombination.

Oncogene-Driven Models

NSCLC models of KRAS-driven LuAds are common, and more specifically, the KRAS-G12D variant is of interest as it appears most frequently in human cancers [204, 213]. A tet

GEMM that uses a conditional Kras-G12D mutation was used to investigate the effects of activated Kras on both initiation and maintenance of LuADs [214]. Developed that expression of rtTA protein is restricted to type II Alveolar cells, dox treatment allows mutant Kras-G12D expression, but is restricted to the lungs. It was found that mutant Kras allows for development of LuADs and following withdrawal of dox, tumors would regress. Additionally, mutant Kras activation paired with simultaneous loss of p53 also demonstrated that withdrawal of dox resulted in tumor regression. These findings concluded that Kras is indeed necessary, regardless of p53 status, for both tumor initiation and maintenance [214]. Another Kras LuAD GEMM uses two conditional alleles of KrasLSL-G12D (knock in) and p53 floxxed (knockout), where both are controlled by lentiviral Cre-recombinase (lenti-Cre). Experimental mice carry a LoxP-flanked stop codon followed by the Kras-G12D mutant, as well as floxxed p53 alleles. Upon intratracheal lenti-Cre administration, recombination excises the stop codon (LSL-cassette) along with p53, resulting in Kras knockin and p53 knockout. These mice model rapid Kras-driven tumorigenesis which is especially suitable for investigating effects of Nkz2-1 (NK2-related homeobox transcription factor), a potential tumor suppressor [201, 215].

Dual Recombination System

In this work, the LuAD model used was created to model both Braf activation and p53 loss under spatio-temporal control [216]. Experimental mice harbour two distinct site-specific recombinase mutant alleles, a Flp-activated BRafV600E (BrafFA) and a Cre-conditional p53 null (p53flox) allele (**Figure1-6)**. Because the BRAF-activating mutation is temporally separate from p53 loss, it more closely models how cancer develops in humans [211].

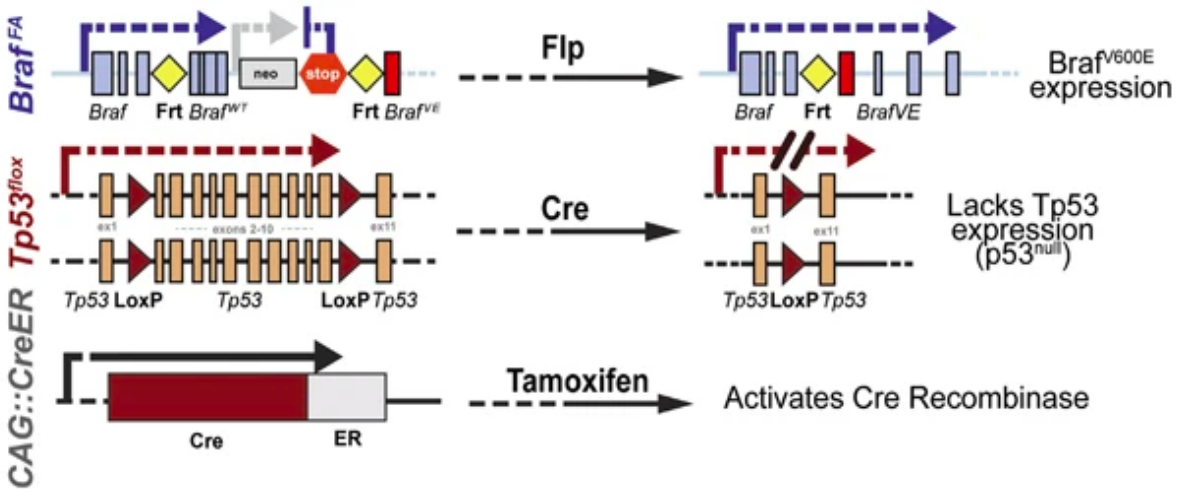


Figure 1-6. Schematic of the BrafFA and p53null alleles before and after recombinase-mediated recombination

Tamoxifen-inducible CreER allele is also depicted where tamoxifen treatment results in Cre-recombinase activity. Both alleles Braf and p53, exhibit wild-type expression until respective Flp and Cre activity allow for mutant expression of V600E and extirpation of the majority of the p53 locus. [Taken from Dankort, unpublished]

Wildtype Braf is expressed until infection with AdFlp via intranasal instillation, which restricts site-specific combination to the lungs. After AdFlp infection, Flp targets the FRT sites flanking region containing wildtype Braf (exons 15-18 and a stop codon) and Flp-mediated recombination excises Braf wildtype exons 15-18, the stop codon, and selection cassette. Removal of wildtype Braf permits mutant exon 15 (BrafV600E) expression which drives tumor development, where the mice form neoplastic growths that develop into adenomas. Cre activity is regulated through use of the CAG::CreER transgene, a fusion of a modified ligand binding domain of mouse estrogen receptor to Cre under the chicken actin gene (CAG; CMV-IE enhancer/chicken β-actin promotor) [182]. When tamoxifen is injected and metabolized into 4OHT, because p53 exons 2-10 are floxxed by LoxP sites, Cre is activated and results in p53 loss. Recombination disrupts the p53 and deletes the majority of the locus, rendering it null

[217]. The spatial separation of mutant expression allows the precise targeting of lung tissue while temporal separation of BrafV600E and p53 loss allows the effects of timing of these mutations to be explored.

1.11 Research Objectives and Rationale

The rationale behind this thesis is to determine 1) if RAMP1 plays a role in OIS establishment and 2) investigate tumors formed with early p53 loss. For the first objective, I used a lentiviral delivery system to introduce shRNAs to RAMP1 into BRER cells to determine whether this co-receptor too was required for OIS bypass. Results of S. Garnett's RAMP3 knockdown experiments demonstrated a partial bypass. As the IAPP co-receptors with high binding affinity is a complex of both CALCR and either RAMP3 or RAMP1, the next appropriate target was RAMP1. Through RAMP1 knockdown, assessment of OIS establishment and bypass are observable, and my work has focused on determining to what extent OIS is affected.

Additionally, for my second objective, my work has concentrated on tumor development using an inducible dual-recombination system of Brafv600E activation followed by p53 loss. The experiments were designed using GEMMs to investigate effects of the timing of mutations on tumor development and progression. Using of the Cre-LoxP and Ad-Flp site-specific recombination systems, when BrafV600E activation and p53 loss are induced separately, it more closely simulates the process of mutation accumulation in the normal ageing process seen in humans. In experiments performed by S. Garnett, it was discovered that when p53 loss occurred early (12 weeks post Brafv600E activation), tumors that formed were both larger, did not senescence, and were of a higher pathological grade. Through collection and isolation of these

tumors, they can be analyzed and the differences between early and late p53 loss can be determined.

Chapter 2: Materials and Methods

Cell Culture

Human Fibroblasts (HFTE) [160], and Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. All cells were cultured at 37°C and 21% O2, 5% CO2. 4-hydroxytamoxifen (4OHT, Sigma, H6278) was dissolved in EtOH. Media was changed every 3 days.

Plasmids

Five shRNA clones to RAMP1 were purchased from McGill High Throughput Screening Facility, sequences are listed in **Table A-1**. DH5α E. coli chemically competent cells were produced using the Z-Competent™ E. coli Transformation Kit (Zymo Research, T3001). Bacteria were transformed with plasmid DNA and subsequent mini and maxi preps were performed using standard protocol. Identity of plasmids were confirmed by Sanger sequencing performed by the McGill Genome Centre and restriction enzyme digest.

Lentivirus Production

HEK 293T cells were seeded at $5x10^6$ per100mm plate on day 1. Day 2 cells were transfected with 8µg of lentiviral vector plasmid DNA, 5.2µg PAX2 viral packaging vector, 2.8µg pCI VSVG viral envelope, 550µL Opti-MEM, and 42.6µL 1mgmL-1 polyethylenimine (PEI). Day 3 media was removed and replaced with full media. On day 4 virus was harvested by collecting media (viral supernatant) from plates and filtering through 0.22 µm filter. Virus was aliquoted and stored at -80°C.

Viral Infection

BRER cells were seeded at 50,000 cells per well into 6-well plates on day 1. At the end of day 1, once cells were adhered to the plate, media was changed to 1:1000 polybrene and 200μL of lentivirus encoding shRNA was added. Day 2, media was changed to fresh. Day 3 cells were replated in 100mm plates and selected for with 4mg/μL puromycin media.

Cell Proliferation Assay

Cells were counted and plated in triplicate in 12-well dishes at a density of $1.5x10^4$ cells per well. Day of plating was considered day -1. Cells were trypsinized and counted using a hemocytometer on days 0, 1, 3, 5, and 7. When drugs (EtOH/4OHT) were used, they were added to media on day 0. For crystal violet staining, cells were fixed using 0.5mL formalin (Sigma, Z2902) per well and left overnight. Plates were rinsed in water and left to air dry until all plates could be stained at the same time. Fixed plates were stained simultaneously with 0.5mL 0.1% crystal violet (Sigma, C0775) per well for 30 minutes, excess stain was rinsed off in water, and left to air dry.

IncuCyte™ Cell Proliferation Assay

Cells were manually counted with a hemocytometer and plated in triplicate in 96-well dishes at a density of 500 cells per well unless otherwise indicated. Day of plating was considered day -1. On day 0, the indicated 4OHT was added to reach final concentration of 100nM or an equivalent volume of ethanol, and the cells were incubated in a CO_2 Incubator housing the IncuCyte™ Zoom Live-Cell Analysis System (Essen™) for 7 days with 4 randomized locations per well captured 6 times per day. Image analysis was performed using the confluence mask in the IncuZyte™ ZOOM program.

Colony Forming Assay

BRER cells were plated in triplicate at a low density of $2.5x10^4$ cells per plate. Cells were incubated in presence of 100 nM 4OHT for approximately 3 weeks, fixed with methanol and then stained with 1:20 filtered Giemsa reagent (Sigma, GS-10). Stain was removed and plates were rinsed in water, air dried, and scanned.

Senescence-Associated β-Galactosidase Assay

BRER cells were plated in triplicate in 6-well dishes and treated with EtOH or 100nM 4OHT for 9 days then re-plated in 6-well dishes to be sub-confluent. On day 10, cells were fixed (2% formaldehyde, 0.2% glutaraldehyde PBS) and stained overnight at 37°C in absence of CO2 in ß-Gal staining buffer (40mM citric acid/Na phosphate buffer, 5mM K4[Fe(CN)6] 3H2O, 5mM K3[Fe(CN)6], 150 mM NaCl, 2mM MgCl2 and 1mgmL-1 X-gal in distilled water), briefly washed in 100% methanol, rinsed with water, and air dried before scanning.

RNA Isolation

Media was removed from cells and 1mL of TRIzol® reagent (ThermoFisher Scientific, 15596018) was added to lyse cells while maintaining the integrity of RNA for isolation. Cells in TRIzol were transferred to microcentrifuge tubes and homogenized. Total RNA was isolated following the manufacturer's instructions and stored at -80°C. Integrity was confirmed by presence of two bands at approximately 4.8kb and 2.0kb, representing 28S and 18S rRNA bands using 1% bleach 1% agarose gel electrophoresis [218]. To determine the quality and concentration of RNA, the samples were thawed on ice and measured using NanoDrop spectrophotometer.

cDNA Synthesis

After determining RNA concentration, reverse transcription was performed using the iScript™ Revserse Transcription Supermix cDNA synthesis kit (BIORAD, 1708841), with 4µg of starting RNA template following manufacturer's instructions and the conditions of incubation were 1x 5min 25°C, 1x 20min 46°C, 1x 1min 95°C.

RT-qPCR with SYBR® Green

cDNA was diluted to 1:1000 using sterile, RNase-free water. qPCR was performed in triplicate with a total reaction volume of 10µL 4µL cDNA, 5.7µL SYBR® Green Master Mix (ThermoFisher Scientific, 4364344), and 0.3µL 20mM primers (**Table A-2**) following manufacturer's instructions. qPCR was performed using the CFX96™ Real-Time PCR Detection System on the C1000™ Thermal Cycler (BIORAD). Analysis was ran performed using the complimentary CFX Maestro software.

Cell Lysis for Protein Isolation

Media was removed from plates, and cells were washed in PBS before lysing in PLC buffer (PLC lysis buffer (50mM HEPES pH 7.5, 150mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1mM EGTA, 1.5mM MgCl2, 10mM NaF, and 10m Na4P2O7, Aprotinin, Leupeptin, and Pepstatin at 1µg/mL, 1mM PMSF, 1mM orthovanadate)) in a shaker at 4°C for 30 minutes. Lysates were clarified by centrifugation at 4°C for 30 minutes. Protein was quantified using the BCA Protein Assay Kit (Millipore,71285). Samples were diluted with ddH2O, 4X Laemmli buffer (Tris pH 6.8, glycerol, 2-Mercaptoethanol (β-mercaptoethanol), SDS, Bromophenol Blue) and boiled at 100°C prior to loading.

Western Blots

Protein samples were resolved on 4% stacking and 10% separating sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN system (Biorad, 1658026). Gels were semi-dry electrotransferred at 15V for 40 minutes using Trans-Blot® SD Cell (Biorad, 1703940) to polyvinylidene difluoride (PVDF) membranes. To improve sensitivity, membranes were blocked in 5% (w/v) non-fat milk (NFM) in TBS-T buffer for 1 hour and then probed with primary antibodies at indicated dilution (**Table A-3**) overnight at 4°C. Blots were washed 3x 10 minutes in TBS-T before conjugated secondary antibody was incubated at room temperature for 1 hour. Blots were washed 3x 10 minutes in TBS-T before signal development with HRP substrate (Millipore, RPN2209, or if the signal was weak, WBLUR0500).

Mouse Work

All experiments with mice (AUP 5819) were performed in accordance with the recommendations by the Canadian Council on Animal Care and conditions set by the McGill University Animal Care Committee.

Mouse Strains

Mice were maintained in a FVB background. Alleles used were the Flp-activated BrafV600E ($Braf^{FA}$)[219], conditionally null $p53^{flox}$ (Trp53tm1Brn/J), and CAG::CreER (Tg(CAG-cre/Esr1*)5Amc/J) [182, 216], both purchased from Jackson Laboratory. $Braf^{FA/+}$; 5^{LL}; CreER ($Braf^{BrafFlp\ Activated\ BrafV600E/+}$; $p53^{flox/flox}$; CAG::CreER) were bred, weaned at three weeks of age, and genotyped as described.

Genotyping

1-2mm tail clippings were taken at time of weaning. Genomic DNA was extracted from the tissue samples by boiling at 100°C for 20 minutes in an alkaline lysis reagent (25mM NaOH, 0.2mM disodium EDTA, pH 12.0) and neutralized in 40mM Tris-HCL, pH 5 reagent [220]. Mice were genotyped using 25µL PCR reaction volume (1µL gDNA, 17.65µL $_{dd}$H2O, 5µL 5X GOTaq PCR Buffer, 0.3µL 100mM dNTPs, .25µL 40mM Spermadine, 0.3µL 10mM primers (**Table A-4**), and 0.5µL Taq Polymerase) using primers listed in Table A-3. PCR was performed using Taq DNA Polymerase (homemade or Promega, M3001) 1x (2min 95°C), 6x (20sec 95°C, 30sec 66 to 69°C ↓0.5°C/cycle, 45sec 72°C), 30x (20sec 94°C, 30sec 60°C, 45sec 72°C), 1x (3min 72°C), 1x (10sec 18°C), 1x (0sec 4°C). Products were resolved by 2% (1:1 mixture low melt: regular melt agarose) gel at 220V for ~17 minutes or until resolution was satisfactory.

Adenoviral Infections

Adenovirus encoding Flp recombinase (Ad-Flp, FlpOE cloned into pAD/CMV/V5-DEST^{TM} (Invitrogen), made by Dr. Dankort) purchased from Viraquest. Virus diluted in A195 Buffer (10mM Tris, 0.1mM EDTA, 1mM MgCl2, 10mM Histidine, 75mM NaCl, 5% Sucrose, 0.02% PS-80, 0.5% EtOH, pH 7.40) to a concentration of $2.5x10^6$ plaque forming units (pfu) per uL was precipitated in EMEM with $CaCl_2$ for 30 minutes at room temperature prior to infection with a total volume of 45µL/infection. At 8 weeks of age, mice were anaesthetized with isoflurane, and adenovirus was administered via intranasal instillation as previously described [201, 221-223].

Tamoxifen Injections

12 weeks post infection (at 20 weeks of age), mice were weighed prior to tamoxifen injections. The animals were manually restricted and held in the supine position with the head lowered. Tamoxifen (Sigma T5648, dissolved in corn oil) injections were administered interperitoneally at 1mg/20g body weight once a day for 5 days.

Necropsy, Lung Extraction, Tumor Isolation, Whole and Partial-Lung Fixation

Mice were anaesthetized with avertin (2,2,2 tribromoethanol, Sigma, T48402) until unresponsive to multiple pedal withdrawal reflexes (toe pinches) by forceps. Once anaesthesia was confirmed, mice were secured to a dissecting board with pins in dorsal recumbent and sprayed down with 70% ethanol. Incisions are made to expose the abdominal and thoracic cavity. The heart was perfused by PBS through the right ventricle. Once the fluid draining out of the animal runs clear and is free of blood, the heart is removed, and the animal is sacrificed. Lungs are removed with the trachea attached and non-lung tissue is separated and removed. Through the trachea, lungs are perfused with PBS for inflation. Using a microscope (Olympus SZ2-ILST, LED Illuminator Stand), lungs were placed in PBS and inspected for visible solid tumors. If present, single tumors were isolated from either the left lung or one of the four right lobes and stored at -80°C. What remained of the lobe was isolated, trimmed, and embedded in optimal cutting temperature (O.T.C., Tissue-Tek, 4583) with a cryomold to be stored at -80°C. Leaving the bronchus and a small section of lung attached or if the lungs had no visible tumors, the lungs were perfused with PBS and then with 10% Zn^{2+} Formalin (Sigma) through the trachea to fix all tissue equally. Isolated lungs were rocked at 4°C overnight in Zn^{2+} formalin and dehydrated the next day by rocking in 30%, 50%, and 70% ethanol sequentially for 60 min each. Lungs were stored in 70% ethanol at 4°C.

Statistics

All experiments were carried out at least 3 times in triplicate and statistical analysis was performed using GraphPad Prism Software.

Chapter 3: Results

While RAMP1/CALCR work together as a co-receptor to IAPP, due to the reasoning that CALCR is not specific to a single RAMP, rather that its ligand specificity is controlled by which RAMP the GPCR interacts with (i.e. RAMP1-3/CALCR form heteromeric IAPP co-receptors whilst CALCR alone is a calcitonin receptor), we chose to investigate the effects of knockdown of the RAMP and not CALCR.

IAPP As a Novel Senescence Regulator

Using a cell model where the CR3 region containing the kinase domain of BRAF is fused to a modified estrogen receptor (hER), BRAF activity can be directly regulated by 4-hydroxytamoxifen (4OHT), a hormone antagonist. Upon exposure to media containing 4OHT, the effects of increased BRAF activity and furthermore OIS, on proliferation are observable.

To identify novel regulators of OIS, an shRNA-based was conducted (performed by S. Garnett) to select for regulators of OIS (**Figure 3-1**). BRER cells were infected with a lentiviral shRNA library containing shRNAs targeting approximately 5000 human genes, particularly those that had been deemed "Signalling Pathway Targets" (Human Mod 1: Signalling Pathway Targets, addgene #28285). By introduction of shRNAs into BRER cells, target genes were knocked down by RNA interference. Following the stable integration of the shRNAs, BRER cells were treated with 4OHT to induce BRAF-induced OIS. Individual colonies that formed after three weeks of continuous BRAF activation were hand-picked and cultured for DNA extraction used for identification via PCR and sequencing. The strongest novel OIS regulator candidate was discovered to be islet amyloid polypeptide (*IAPP)*. Greater presence was detected in more colonies in comparison to other targets, confirming that IAPP was of importance. In

addition, IAPP was found as the exclusive target in some colonies. This signified that IAPP as a candidate was capable of OIS without ambiguous cooperation of other knockdown targets.

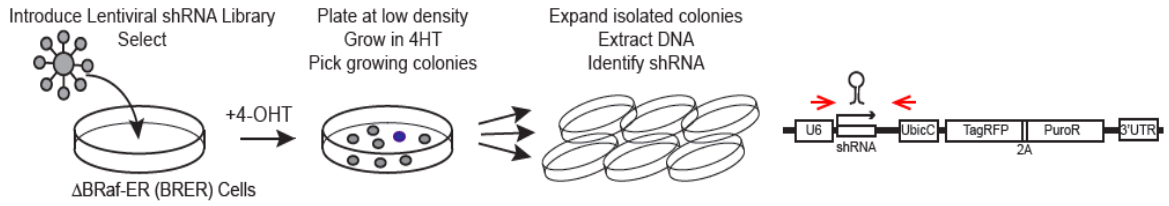


Figure 3-1. Lentiviral genetic screen identifies novel OIS regulators

Schematic diagram of lentiviral-based shRNA screen experiment. Once the shRNA library with targets to approximately 5000 genes was introduced into BRER cells, these cells were grown in 4OHT media and colonies were picked and identified. Following identification of the gene the shRNA targeted, BRER cells were re-infected with lentivirus to create stable cell lines for experimental use. [Taken from Garnett, 2021]

Knockdown was confirmed by a dual-luciferase reporter assay and in further experiments shIAPP-BRER cells were treated with 4OHT in culture, to investigate proliferation. The results from growth assays demonstrated that IAPP knockdown cells were in fact able to continue proliferating (**Figure 3-2 A-B**). Moreover, the shIAPP-BRER cells were further examined for expression of senescence markers versus the control cells; found to exhibit both OIS and senescence markers. These findings raised the question of whether the IAPP coreceptor had any functional role in establishment of OIS.

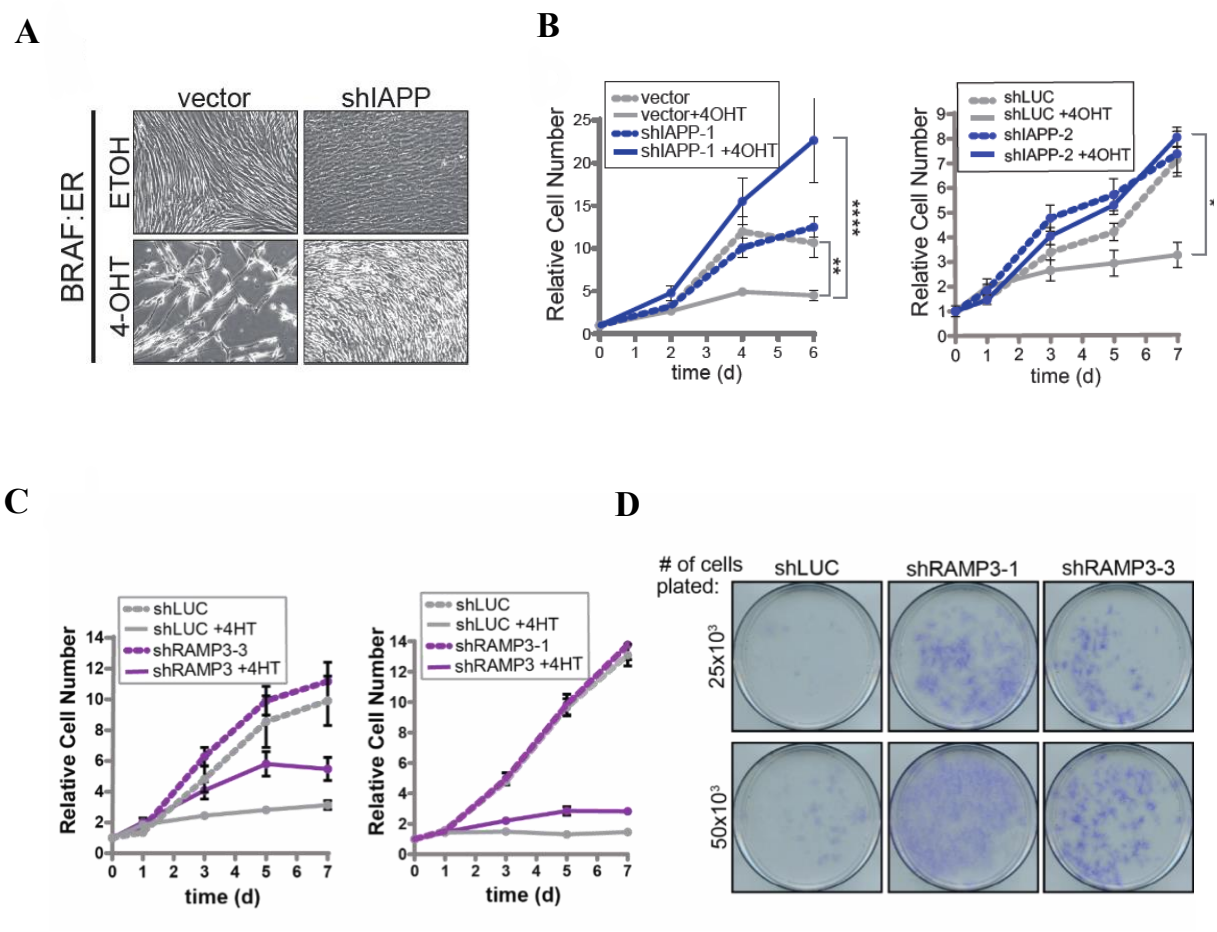


Figure 3-2. IAPP and RAMP3 knockdown allows bypass of OIS

(**A**) Cell images of BRER (empty vector BRAF:ER) and shIAPP after treatment with 4OHT for 7 days. (**B**) Growth curve results demonstrating that both shIAPP cell lines continued proliferation after 4OHT treatment. (**C**) Growth curve results showing that both shRAMP (shRAMP3-3 and shRAMP3-1) cell lines were able to continue proliferating in treatment of 4OHT. (**D**) Giemsa staining of colony formation assays plated at two densities, imaging showing in comparison to shLUC, both shRNA lines formed colonies. [Modified from Garnett, 2019, unpublished and Garnett, 2021]

Creation of RAMP1 Knockdown shRNA BRER Cell Lines

Five shRNA clones were obtained, and BRER cells provided by D. Dankort were transduced with each of the five shRNAs to create shRAMP1-1, shRAMP1-2, shRAMP1-3, shRAMP1-4, and shRAMP1-5 lines. To create a cell line using the BRER model, lentivirus for

RAMP1 was produced and harvested from HEK293T (human embryonic kidney) cells and Human Lung Fibroblasts (HF-E1T) harboring ΔBRAF-ER (BRER cells) were transduced. Successfully infected cells were selected for by puromycin resistance. To investigate bypass of OIS, the hormone-inducible BRER cells with shRNA to RAMP1 (shRAMP1 cells) were cultured in 4OHT media to mimic loss of RAMP1 concomitant with overactivation of BRAF.

RAMP1 Gene Knockdown in Cell Culture

An initial Colony Formation Assay was performed on 5 BRER cell lines transduced with shRNA targeting RAMP1 along with Luciferase (negative) and IAPP (positive) control lines to determine if RAMP1 knockdown cells would continue proliferation despite BRAF activation.

7-day cell proliferation assays were performed to yield quantifiable results using the Incucyte and manual counting. After comparing results between the three cell growth assays (**Figure 3-3 - 3-5**) and assessing which shRNA cell lines continued to proliferate in the presence of 4OHT, two shRNA cell lines, shRAMP1-1 and shRAMP1-5 were selected to move forward with for further experimentation.

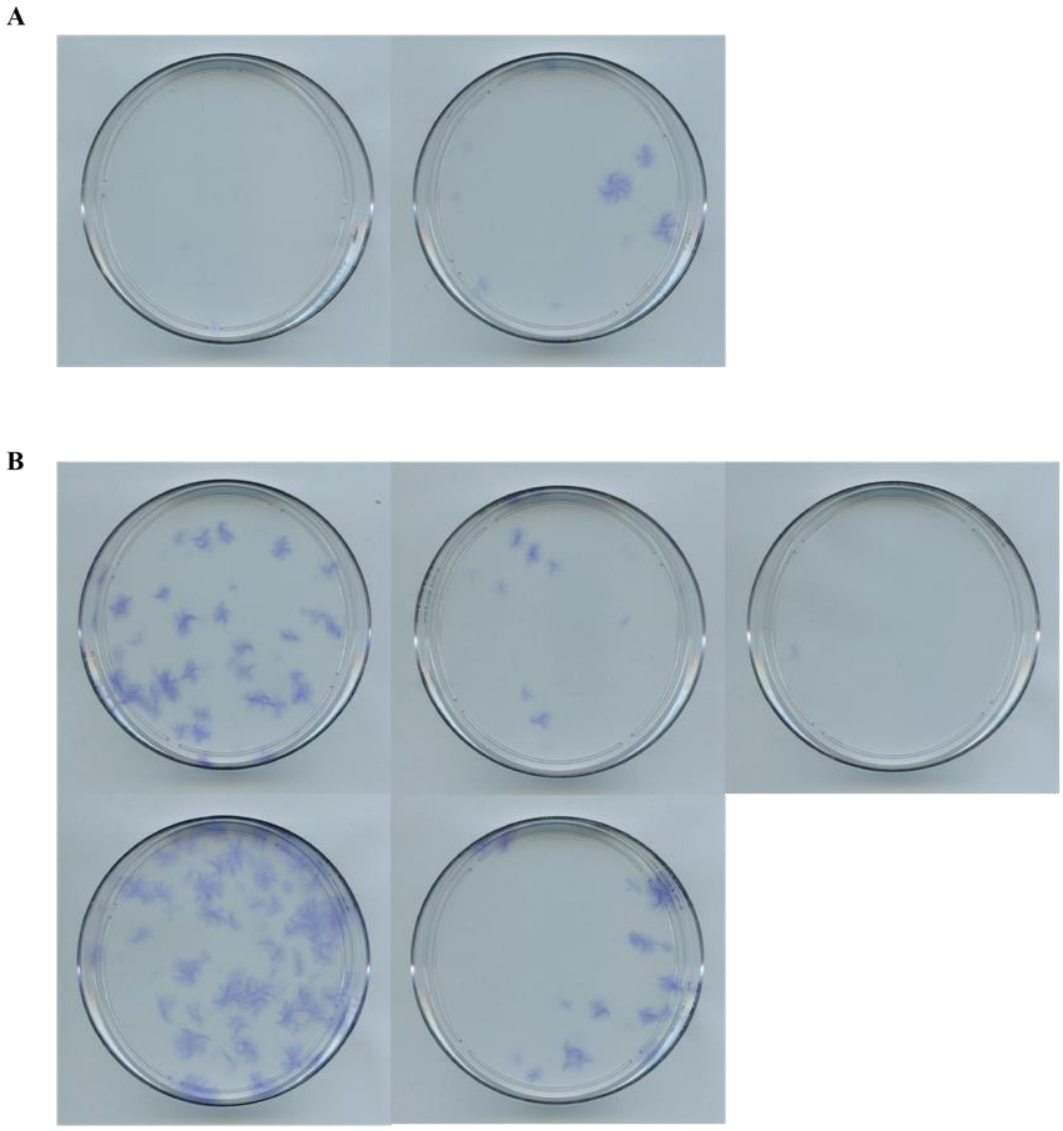
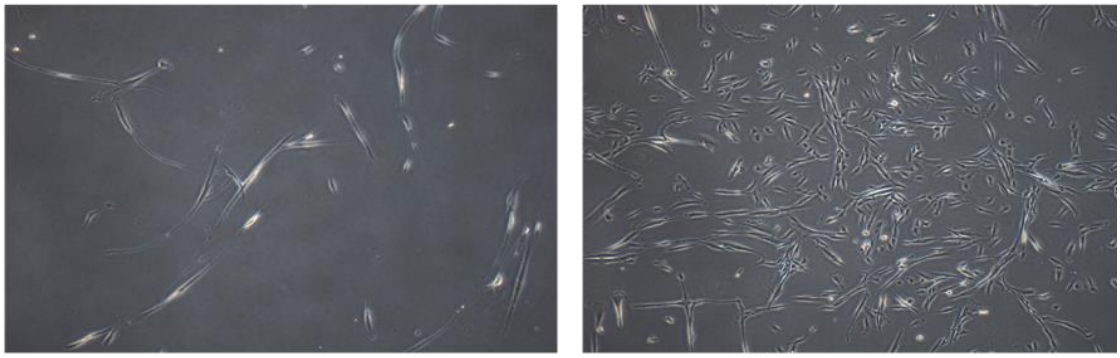


Figure 3-3. shRAMP1 BRER cells continue proliferating despite BRAF activation

Colony formation assay plated at a density of 25,000 cells per well, shRNA-infected BRER cultured in 4OHT media to allow for colony growth for 3 weeks before staining with Giemsa. (**A**) From left to right, shLUC (negative control), shIAPP (positive control). (**B**) Top row from left to right, shRAMP1-1, shRAMP1-2, shRAMP1-3. Bottom row from left to right, shRAMP1-4, shRAMP1-5. The positive control (shIAPP), and RAMP1 knockdown cells demonstrated colony growth visible to the naked eye after 3 weeks.

A



B

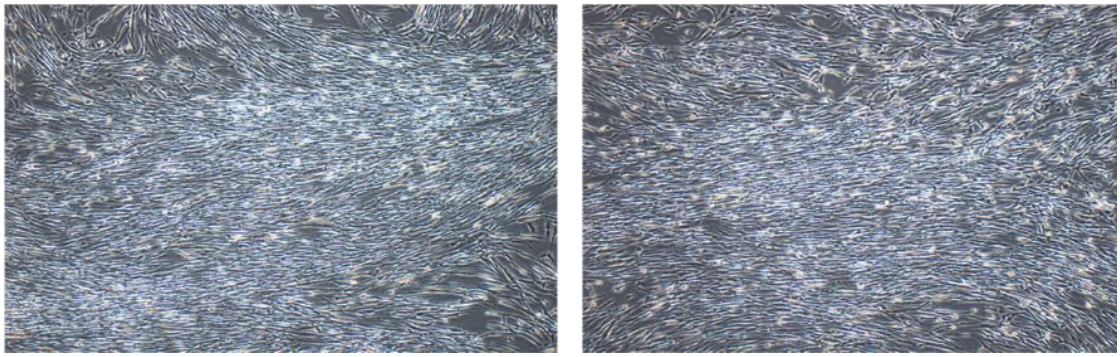


Figure 3-4 shRAMP1 BRER knockdown cells continue proliferating in 4OHT treatment

Representative images of BRER cell growth in 4OHT treatment, with BRAF activity (**A**) from left to right, shLUC, shIAPP (**B**) from left to right, shRAMP1-1, shRAMP1-5. Here the difference in morphology of cells undergoing OIS (shLUC) is visible in comparison to those which seemingly exhibit OIS bypass (shIAPP, shRAMP1-1, shRAMP1-5).

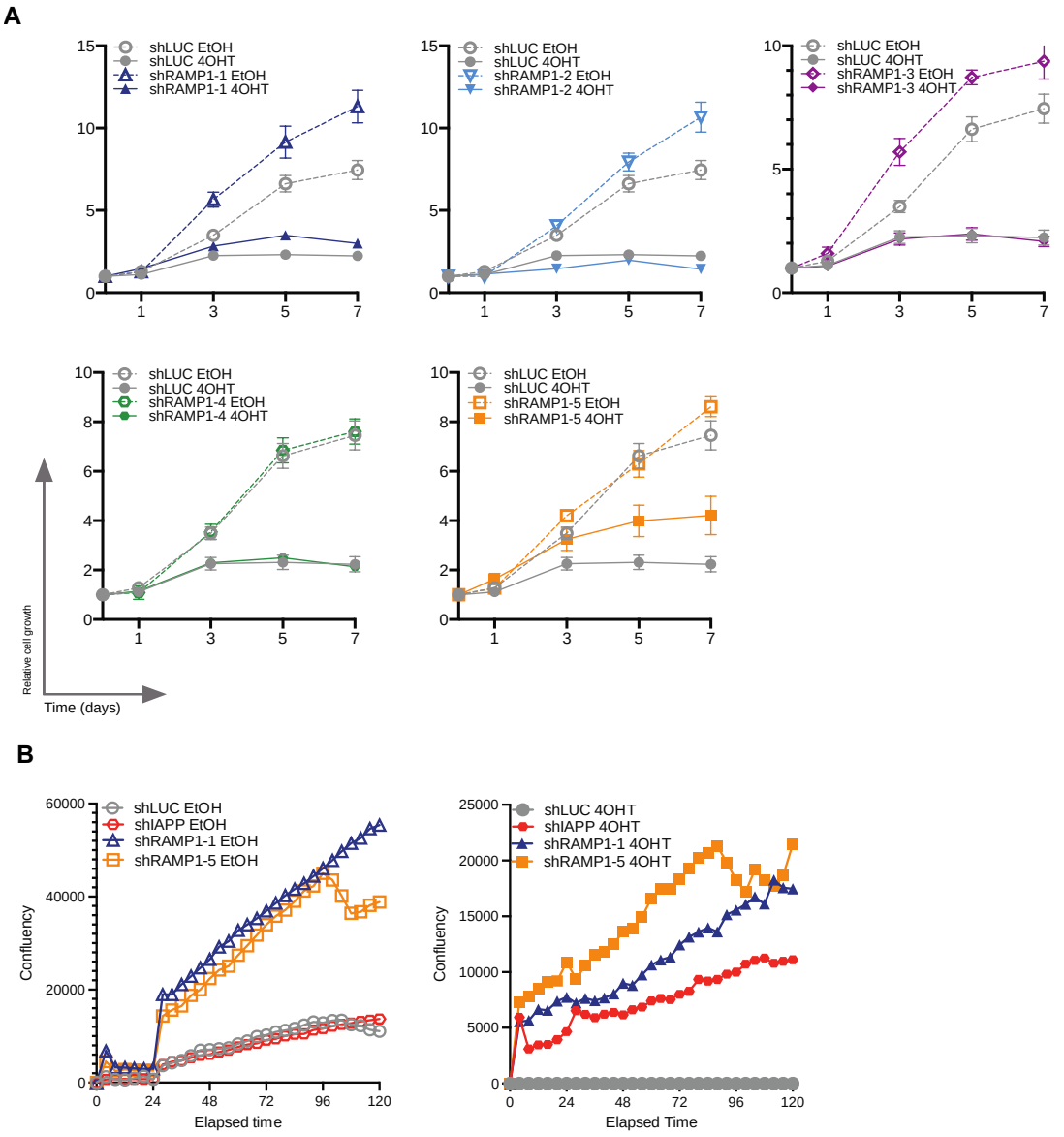


Figure 3-5. shRAMP1-1 and shRAMP1-5 BRER knockdown cells exhibit limited OIS bypass.

(**A**) Growth curve of five shRAMP1 BRER cell lines plated and grown in 100nm EtOH or 4OHT for 7 days. Showing that 4OHT treatment causes shLUC (control cells) to decrease proliferation while shRAMP1-1 and shRAMP1-5 BRER cells continue to proliferate. (**B**) Incucyte growth curves of shRAMP1-1 and shRAMP1-5 BRER cell lines plated at 1000cells/well and grown in 100nm EtOH or 4OHT showing continued proliferation after 4OHT-induced BRAF activation.

Additionally, protein was isolated from RAMP1-1 shRNA BRER cell lines to determine the extent of the knockdown effects on endogenous protein levels. Samples were treated with 4OHT and EtOH before performing immunoassay experiments but due to issues with antibody detection, no clear blots were produced (not pictured). α-tubulin was used as a control, and it was found that p-ERK was elevated in cells treated with 4OHT while tERK was level across both treatments but ER. Cell cycle inhibitors p15, p16INK4b, p21 could not be detected using this method, nor could PCNA, a marker of DNA synthesis.

Generation of Mice with Braf Activation and p53 Loss

Mice were bred to obtain genotype of BrafFA/+, p53flox/flox, CreER/- so that BRAF activation and p53 ablation could be induced separately by Ad-Flp infection and tamoxifen injections, respectively. BrafFA/+, p53flox/flox animals were also used where the lack of CreER served as a control and these animals would not experience p53 loss, remaining p53wildtype [224]. The animals were infected with AdFlp at a titre of $5x10^6$ pfu at approximately 8 weeks to induce Flp-mediated recombination resulting in mutant BrafV600E expression in the lungs. 12 weeks after BrafV600E activation, tamoxifen was administered for five days. Tamoxifen is metabolized into 4OHT and initiates Cre-mediated p53 ablation. 12 weeks after tamoxifen injections, the animals were sacrificed, and the lungs were harvested (**Figure 3-6**). The mouse colony was under continuous surveillance, and after tamoxifen administration not all animals survived to the 32-week timepoint but met clinical endpoint. Nevertheless, lungs were still harvested from those whose welfare was compromised, and early sacrifice was necessary.

Lung Sample Isolation and Collection

When animals reached clinical endpoint, whether at the 32-week mark or earlier due to welfare and health concerns, lungs were harvested and inspected to determine if individual tumors were visible to the naked eye. If tumors were present, the individual tumors were removed from the lobe with the most prominent tumor burden and isolated from the remaining lung tissue. Samples were obtained of both experimental (BrafFA/+; p53flox/flox; CreER/-) derived tumors and control (BrafFA/+; p53flox/flox) lungs are stored at -80°C for future use.

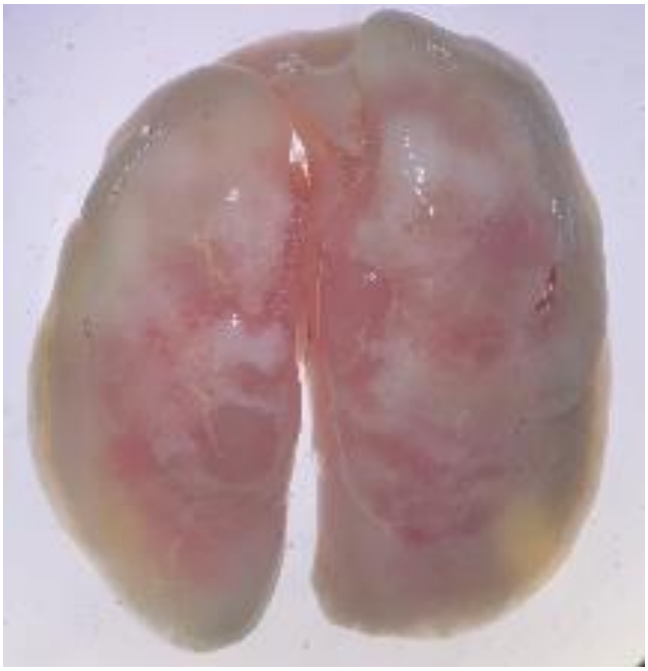


Figure 3-6. High tumor burden lungs harvested from BrafFA/+; p53flox/flox; CreER mutant mouse.

The animal was infected with adenovirus expressing Flp-recombinase, inducing transgene recombination. 12 weeks after infection the animal was injected with tamoxifen for 5 days, upon which, the majority of p53 was deleted. 12 weeks after tamoxifen injections (endpoint), the animal was sacrificed, lungs were perfused, and major visible tumors were isolated by microdissection and the remaining lobes were stored for future analysis.

Chapter 4: Discussion

Cellular Senescence and Disease

In the development of many diseases, senescence acts as a barrier against unchecked proliferation, and protects the organism from genomic instability [112]. Cellular senescence is driven by many stressors including tumor suppressor signalling, as seen in the p53/RB pathway, or can be triggered by oncogene activation such as MYC, RAS, and RAF hyperactivation, as seen in OIS. Here, the cellular senescence mechanism acts as a protective response to DNA damage. When senescence is induced, this mechanism traps these damaged cells in a metabolically active state of proliferative arrest. Morphology alterations and the distinct secretome of a senescent cell (SASP), are also observed. These secretome factors are capable of influencing the tumor microenvironment in various ways. Some factors may promote senescence induction, while others promote proliferation in the neighbouring population of cells [164, 165, 167].

In cancer, uncovering the underlying network behind senescence is imperative in the advancement of therapeutics. Originally discovered and defined as an irreversible growth arrest, additional morphological differences and a distinct secretome profile have since been uncovered. Further research has revealed additional involvement with other cellular processes including the DDR, wound healing, and regeneration [107, 121, 133, 146, 148]. Because senescence is involved with age-related disease and cancer, recent work has focused on the exploitation of senescent cells and how these findings can be used in efforts to treat human cancers and disease. For example, senescence induction initiated by rescuing p53 has demonstrated potential as a method of forcing tumor regression [171, 172]. Senolytics on the other hand, exploits senescence

by targeting the senescent population of cells for clearance, resulting in overall improved health and symptom relief in multiple pathologies [177-179].

Further Investigation of the Role RAMP1 Plays Within OIS

The RAMP family of three transmembrane proteins has been extensively studied in GPCR pharmacology. Prior research has confirmed that RAMPs control trafficking, signalling, and pharmacological changes because peptide specificity, and therefore function of the receptor, is reliant on which RAMP protein interacts with the GPCR to form a coreceptor [190-192]. Due to this, multiple subtypes of coreceptors exist, and each will bind to a different peptide namely, adrenomedullin, calcitonin, or IAPP. In the study of diabetes, IAPP has propelled RAMPs to the forefront of investigations as those with Type-I and II diabetes are IAPP-deficient and the IAPP coreceptors are comprised of RAMP1 or RAMP3 and the calcitonin receptor (CALCR) [184]. S. Garnett's research (2019, unpublished) on IAPP and RAMP3 revealed a connection to OIS while this work further explores the possibility that both RAMP3 and RAMP1 are involved in the process of cellular senescence.

Results of the initial BRER shRAMP1 colony formation assays (**Figure 3-3)** were promising and generally consistent between experiments, showing that upon BRAF activation, the shRAMP1 BRER cells were in fact able to form colonies visible to the naked eye, rather than senescing. In the later cell proliferation assays (**Figure 3-4, 3-5**), shRAMP1 cells exhibit a seemingly stronger evasion of OIS in comparison to shIAPP cells, yet these results were inconsistent between experiments where different shRAMP1 cell lines exhibited bypass to differing degrees in each round of experiments. Although interesting, these results may not be well grounded and future work should seek to clarify if a stronger bypass is indeed driven by the loss of RAMP1. Some of the discrepancies may possibly be due to issues with the cell lines

progressing to higher passages and acquiring additional mutations, or human error when manually counting and seeding the cells.

Taken together, these results suggest knockdown of RAMP1 does influence the establishment of OIS to some unknown degree, seemingly allowing for a partial bypass of BRAF-activated OIS. It was found that most of the experiments with samples consisting of older cells (approximately ten passages higher) yielded higher density colonies resulting in stronger Giemsa staining. The inconsistencies between cell count numbers may have been a result of mutations acquired through normal passaging of the cells and future work should consider the unintended effects of using higher passaged cells in experimental design.

Due to the nature of fibroblast cells, they exhibit low adherence and a major limitation of the cell culture experiments performed was difficulty in colony quantification after staining attempts at higher colony density. During the process of fixing and staining, the overlapping swirls of confluent cells exhibited extremely low plate attachment as they appeared to be growing on top of each other, forming clumps, and would lift off the plate. On successfully stained plates with high colony density, quantification was not accurate as the staining was quite intense and colony borders were poorly defined as there was significant overlap between them. Low plate attachment also resulted in unsuccessful attempts to stain for SA-βGal, where the cells would lift entirely off the plate during the staining process and the resulting scans were missing sections. Future cell culture experiments should determine if cell-plate adherence could be optimized by addition of an ECM gel coating or by using cultureware with a higher surface roughness to aid in cell attachment.

As shown in the results (**Figure 3-4 B**), when RAMP1 knockdown cells are treated with 4OHT, colonies continue to form and proliferate which suggests that RAMP1 functions in the establishment of OIS. shLUC cells enter OIS and halt proliferation whereas shRAMP1 cells continue proliferating, seeming to exhibit a limited bypass similar to observations from previous shRAMP3 experiments (**Figure 3-2 C**). In future work, colony growth and cell proliferation experiments should be performed within a defined passage age. Performing experiments within a set limit of passaging may account for additionally acquired mutations that result in possible genomic, transcriptional, and even functional alterations.

In S. Garnett's IAPP and RAMP3 experiments (2019, unpublished), it was thoroughly demonstrated that knockdown of IAPP using both CRISPR and shRNA methods resulted in OIS bypass, while knockdown of RAMP3 lead to a partial bypass [160]. Additionally, when Amylin was introduced to cell growth media following BRAF activation, OIS was partially rescued. Taking everything into consideration, these results suggested that in OIS induction, IAPP's function may rely in part on the interactions between it and the receptor (RAMP3). While the RAMP3 coreceptor has the highest binding affinity for IAPP, all three RAMPs are able to interact as CALCR/RAMP complexes to form an IAPP receptor; as the shRAMP1 BRER cells seemingly exhibited a partial bypass. These results tie into the previous RAMP3 studies and substantiate that a compensatory mechanism of RAMPs may exist, with a function in the induction of OIS.

Dual Recombinase GEMMs

Cre-LoxP and FLP-FRT inducible gene expression systems are widely used in cancer research and have been beneficial in the study of tumor progression, effects of oncogene activation, and TSG loss. Using Cre-LoxP and FLP-FRT systems together, recent mouse-based

experiments focused on creating a dual-recombinase GEMM. With both a Flp-activated Braf allele (BrafFA), and a conditional p53floxxed allele (p53flox), this model has been used in the study of BRAFV600E lung tumor initiation, progression, and therapy [216]. The BrafV600E-driven LuADs the animals produced were analyzed and tumor differences were observed and described by S. Garnett. In AdFlp infection at a high viral titre versus a low viral titre it was shown that low titre infection resulted in development of less tumors in comparison to high titre infections, but these tumors were also smaller in size, higher in grade, and proliferated at a more rapid rate. Other differences were also observed in tumor progression in animals whose p53 function was lost at an early timepoint in comparison to those who retained p53wildtype until later (Garnett, 2019, unpublished). In early timepoints it was discovered that p53 loss permits tumor progression to LuADs whereas in later p53 loss timepoints this is not true, and progression is inhibited by induction of OIS.

Tumor Sample Preparation

Based on prior findings linking Braf-driven LuADs, timing of p53 loss, and OIS, this work sought to generate additional samples that can be used in downstream experiments to explore the gene expression patterns, pathways involve, and underlying mechanisms that allow progression of benign hyperplasias into LuAD.

Experimental mice were heterozygous BrafFA/+, homozygous p53flox/flox, and hemizygous for CreER while the control group lacked CreER (**Figure 1-6**). Without CreER, p53 was not subject to Cre-mediated recombination and remained intact upon tamoxifen administration. Mice are first infected with AdFlp to facilitate the Flp-mediated recombination in the lungs, activating the mutant BrafV600E. At a later timepoint, tamoxifen is administered at the early timepoint which metabolizes into 4OHT, and experimental mice undergo Cre-mediated

recombination resulting in p53 ablation. Tumors that developed in experimental mice (**Figure 3-6**) progressed from neoplastic growths to adenomas and previously generated tumors stained with hematoxylin and eosin have been described as larger in size, of higher pathological grade, and higher burden in comparison to p53wildtype mice (Garnett, 2019, unpublished). The long-term goal of deriving these samples is for downstream RNA isolation and working out optimal conditions for reliable obtainment of RNA-sequencing (RNAseq) data.

Chapter 5: Conclusion and Future Directions

Cellular senescence is a protective mechanism that acts as a barrier between DNA damage and genomic integrity. Cells undergoing senescence become trapped in G1 phase and are unable to re-enter the cell cycle, yet they remain viable and metabolically active. This work has demonstrated the necessity of furthering our understanding of underlying cellular mechanisms of OIS in RAF-induced NSCLC. By examining potential regulators and investigating the underlying mechanism of cancer development, new therapeutic targets may be discovered. In an effort to more thoroughly define the mechanism of OIS and better characterize senescence regulators, this work has demonstrated that knockdown of RAMP1 has some effect on OIS, but more research is necessary to determine a definitive role and the exact mechanism by which this occurs.

Future experiments should aim to provide adequate confirmation of knockdown and investigate the effects of a dual knockdown. To further investigate effects of RAMP1 knockdown, immunoblotting should be re-examined to determine the presence or lack thereof, of cell cycle arrest markers and DNA synthesis markers. To investigate effects of a dual knockdown in cell culture, Gibson Cloning would be a practical technique. This DNA assembly method allows for the construction of plasmids containing multiple DNA fragments. By design, a plasmid carrying shRNAs to both RAMP1 and RAMP3 could be introduced into BRER cells, to allow for observation of effects resulting from a dual knockdown. This method would aid in determining if there is in fact, a compensatory mechanism that indeed restricts full OIS bypass, so long as one RAMP is functional. Furthermore, by designing plasmids with different combination of the three RAMPs it could be determined which specific RAMP's effects are most important for induction of senescence.

To better characterize the mechanism of action of RAMP1 in relation to OIS, although all three RAMPs have distinct functions, the results of prior studies are ambiguous and currently only a handful of defined functions the RAMPs exhibit have been described [190]. While RAMPs are an appealing drug target, past research has highlighted that they can function independently but have additional functions that overlap. Attempting to isolate the function of a single RAMP makes experimental design problematic, as difficulties arise when looking to study the effects of a single component involved in many distinct pathways [225].

GEM models have been widely used in oncogene-driven cancer development research where oncogene activation is induced by recombinase enzymes such as the Cre-LoxP or Flp-FRT systems. In this work, both these systems were utilized to temporally control mutation activation. BRAF activity is induced upon intranasal instillation of adenovirus expressing Flp-recombinase and 12 weeks later p53 ablation is induced by Cre recombination, generation of a combination of whole-, partial- lungs, and individual tumor samples were obtained from 64 mice and are available for future experiments. As lungs were harvested, those with visible tumors were micro dissected and isolated from the rest of the organ, these samples are frozen and available for further analysis. Future experiments should aim to focus on investigation of the transcriptome using next-generation sequencing (NGS). Examining the transcriptome will likely reveal valuable information (total mRNA, rRNA, tRNA) regarding genetic differences in adenoma to LuAD progression where these early p53 loss tumors are larger, of a higher pathological grade, and non-senescent. Despite limitations, this thesis highlights the importance of continued efforts to understand the connections and mechanisms behind cancer, tumor development and progression, and cellular senescence. Although further exploration of

RAMP1within the RAF-driven OIS mechanism is necessary, all of this work contributes to the advancement of cancer research and in the understanding of cancer development.

Appendix Tables

Table A- 1. RAMP1 shRNA clone sequences

Clone #	Clone ID	Target Name	Sequence
R-799-1	TRCN0000014208	shRAMP1-1	CCCTTCTTCCAGCCAAGAAGA
R-799-2	TRCN0000014209	shRAMP1-2	AGGTTCTTCCTGGCAGTGCAT
R-799-3	TRCN0000014210	shRAMP1-3	CCTCACCCAGTTCCAGGTAGA
R-799-4	TRCN0000014212	shRAMP1-4	CCAATGCAGAGGTGGACAGGT
R-799-5	TRCN0000014211	shRAMP1-5	CTCTGGCTGCTCCTGGCCCAT

Table A- 2. Primers used for qPCR

	Sequence	Direction
RAMP1 qPCR F1	TCCTGGCCCATCACCTCTT	3’ → 5’
RAMP1 qPCR R1	GTAGCTCCTGATGGTCCTGC	5’ → 3’
RAMP1 qPCR F2	CGGACTGCACTCGGCAC	3’ → 5’
RAMP1 qPCR R2	ATGAAGAGGTGATGGGCCAG	5’ → 3’
RAMP1 qPCR F3	GCTGCTCCTGGCCCATC	3’ → 5’
RAMP1 qPCR R3	CTACCTGGAACTGGGTGAGG	5’ → 3’

Table A- 3. Antibodies used for Western Blotting

Target	Supplier	Catalogue #	Dilution
α-tubulin	Sigma	T5168	1:10000
pERK	Cell Signalling Technology	9106s	1:1000
tERK	Cell Signalling Technology	4695s	1:1000
PCNA	Cell Signalling Technology	2586s	1:2000
p15 INK4b	abcam	Ab53034	1:500
p16 CDKN2A/P16INK4A	abcam	Ab108349	1:2000
p21	abcam	Ab107099	1:200
p21 Waf/Cip1	Santa Cruz	Sc-6246	1:200
p16 INK4A	Santa Cruz	Sc-1661	1:50
ERα	Santa Cruz	Sc-543	1:1000

* Diluted in 5% (v/w) NFM (Non-Fat Milk) in TBS-T

Table A- 4. Primers used for genotyping

Name	Sequence	Direction
$Braf^{FA}$	GGAAAGCCTGTCACGGGTC	3’ → 5’
	AGATTCGTATGTCCTCTGAAAGTC	5’ → 3’
p^{53}	AAGGGGTATGAGGGACAAGG	3’ → 5’
	GAAGACAGAAAAGGGGAGGG	5’ → 3’
CreER	GCCAGCTAAACATGCTTCATC	3’ → 5’
	ATTGCCCCTGTTTCACTATCC	5’ → 3’

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