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Cooperative regulation of autophagy by oncogenic PI3-kinase and NRF2 signaling pathways

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COOPERATIVE REGULATION OF AUTOPHAGY BY ONCOGENIC PI3-KINASE AND NRF2 SIGNALING PATHWAYS

by

CAITLIN MARGARET GUTHLEIN

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Approved by

First Reader _____

Anurag Singh, Ph.D.
Assistant Professor of Pharmacology and Medicine

Second Reader _____

Neil Ganem, Ph.D.
Associate Professor of Pharmacology and Medicine

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COOPERATIVE REGULATION OF AUTOPHAGY BY ONCOGENIC PI3-KINASE AND NRF2 SIGNALING PATHWAYS

CAITLIN GUTHLEIN

ABSTRACT

Lung cancer is the leading cause of cancer death worldwide with 2.2 million new cases diagnosed and 1.8 million deaths per year. Lung squamous cell carcinoma (LSCC) is an aggressive histological subtype of non-small cell lung cancers (NSCLC), which is strongly associated with cigarette smoking and exposure to environmental pollutants. In collaboration with the Computational Biomedicine group at Boston University, we identified several putative cancer driver mutations in benign premalignant lung tumors, extracted from upper bronchial airway epithelium. The gene mutations from premalignant tumors are thought to initiate neoplasia but cannot promote malignancy independently. It is hypothesized that additional cooperating mutations will have a compounding effect on tumorigenesis if co-expressed in the same tumor cell. We used cancer genomics data from LSCC primary tumors in the Cancer Genome Atlas (TCGA) database to identify lung pre-malignancy associated genes that are significantly co-mutated. Two of the identified mutant genes, *PIK3CA* and *NFE2L2,* were shown to co-occur at a statistically significant rate in LSCC primary tumors.

The *PIK3CA* gene encodes the PI3K lipid kinase, which regulates the AKT and mTOR kinase signaling pathways, thus promoting cell proliferation and survival. NRF2,

the product of *NFE2L2* gene, is a transcription factor that regulates the antioxidant response, playing a protective role against oxidizing cellular damage. NRF2 promotes the transcription of key proteins in the antioxidant response such as glutathione S transferase and NADPH oxidase. NRF2 is normally subject to ubiquitin-mediated degradation, which is regulated by the KEAP1 protein. Loss of function KEAP1 gene mutations are common in lung cancer. When cells are exposed to oxidizing agents, KEAP1 is modified by these agents, resulting in release and stabilization of NRF2, and the subsequent transcription of antioxidant response genes.

Studies of PI3K and NRF2, and their downstream effectors have shown that both the PI3K/AKT/mTOR and NRF2/KEAP1 signaling pathways control autophagy, which is a catabolic process that regulates the recycling of macromolecules under conditions of nutrient deprivation. PI3K and NRF2 both control the activity of the SQSTM1/p62 protein, which plays a major role in autophagic degradation of cargo proteins. Autophagy has been implicated as a tumor suppressive mechanism. Both PI3K and NRF2 are known to inhibit autophagy in lung cancer cells. Based on the significant frequency of co-occurrence of *PIK3CA* and *NFE2L2* gene mutations in pre-malignant LSCC lesions, we hypothesize that PI3K and NRF2 cooperate to inhibit autophagy to promote LSCC progression.

To test our hypothesis, we co-expressed mutant forms of *PIK3CA (E545K)* and *NFE2L2 (T80K)* into a non-transformed Human Bronchial Epithelial Cell line (HBEC-3KT). We performed a series of Western Blots to verify PI3K and NRF2 protein

expression as well as downstream AKT activation and markers of autophagy pathway activation. mTORC1 is an effector of PI3K and plays a central role in the inhibition of autophagy through the PI3K/AKT/mTOR signaling network. Therefore, we performed Western Blot analysis of samples treated with the mTORC1 inhibitor Everolimus to compare the effects of mTORC1 inhibition on autophagy activation in control, single *PIK3CA*, *NFE2L2* and double mutant HBEC3-KT cells. We observed significant suppression of autophagy in the PI3K/NRF2 double mutant cells. Moreover, the studies also showed that the double mutant cells are more sensitive to anti-proliferative effects of Everolimus compared to control and single mutant cells.

Taken together, our studies show that *PIK3CA* and *NFE2L2* mutations cooperate to hyperactivate the AKT kinase and to suppress autophagy pathway activation. This represents a key mechanism of the malignant transformation of benign premalignant LSCC lesions. This warrants further research into the cooperation between PI3K and NRF2 in lung cancer pathogenesis. Our results have important implications both for diagnosis and treatment of LSCC. Though many important advances in the treatment of lung cancer have been made over the past few decades including the use of tyrosine kinase inhibitors (TKIs) such as Erlotinib, there is still much to understand about the biology and mechanisms of the disease.[1] Blockers of the T-cell checkpoint, such as anti-PD-1 drugs are currently FDA-approved first lines of therapy for NSCLC. In addition, immunotherapy has shown some efficacy in lung cancer patients.[2] Our studies provide rationale for the development of therapeutics that suppress NRF2 and PI3K activity in the

treatment of LSCC.[3] Since mTORC1 inhibitors cause robust inhibition of *PIK3CA/NFE2L2* double mutant cell proliferation, future studies will be aimed at testing combinations of mTORC1, PI3K and NRF2 pathway inhibitors to treat LSCC.

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LIST OF ABBREVIATIONS

4EBP.....eIF4e Binding Protein

ARE.....Antioxidant Response Element

AMPK.....AMP dependent kinase

EGFR..... Epidermal Growth Factor Receptor

HBEC.....Human Bronchial Epithelial Cells

HIF1..... Hypoxia Inducible Factor 1

KEAP1.....Kelch-like ECH-associated protein 1

LC3..... Microtubule Protein Light Chain 3

LSCC..... Lung Squamous Cell Carcinoma

mTOR..... Mechanistic Target of Rapamycin

NRF2.....Nuclear factor erythroid 2-related factor

NSCLC.....Non-Small Cell Lung Cancer

OIS.....Oncogene-induced Senescence

PH.....Pleckstrin Homology

PIP3..... Phosphatidylinositol Trisphosphate

PI3K..... Phosphoinositide 3-kinase

ROS..... Reactive Oxygen Species

S6K.....p70 Ribosomal Protein S6 Kinase

SREBPs..... Sterol Response Element Binding Proteins

TKIs.....Tyrosine Kinase Inhibitors

ULK1 ..... Unc-51 Like Autophagy Activating Kinase

INTRODUCTION

Overview of Non-Small Cell Lung Cancer (NSCLC)

The lungs are a pair of spongy, air-filled organs on either side of the chest. Air is initially inhaled into the trachea which conducts air into the lungs via the bronchi. The bronchi divide into smaller bronchioles and eventually end in single cell sacs called alveoli. The lung's main function is to oxygenate red blood cells in the blood. Lungs also maintain blood pH in the body by increasing or decreasing the amount of carbon dioxide exhaled, and filter out small gas bubbles in the bloodstream.

Lung cancer is the leading worldwide cause of cancer death with only a 16% five year survival rate.[4] According to the World Health Organization, in 2018 lung cancer was responsible for 2.1 million deaths and is projected to increase to 3.3 million deaths per year by 2040.[5] Lung cancer has a much lower survival rate than most other epithelial malignancies. One reason for this is that the lung tissue is uniquely susceptible to tumorigenesis as it is constantly exposed to environmental toxins and airborne carcinogens.[6] Though the most common aetiology for lung cancers is smoking tobacco, the prevalence of lung cancers in non-smokers is on the rise due to exposure to carcinogens in pollution. Pollutants containing polycyclic aromatic hydrocarbon compounds are known to cause oxidative stress and inflammation.[4] As the concern with environmental pollution grows, lung cancer is not only a risk for current and former smokers, but for the population as a whole.

The histological subtype, non-small cell lung cancers, accounts for 85% of lung cancer diagnoses. This includes squamous cell carcinomas, adenocarcinomas and large cell carcinomas.[6] Therefore finding potential therapeutics that target NSCLC is of primary importance to reducing the cancer burden. Though many important advancements in the treatment of NSCLC have been made over the past few decades including the use of tyrosine kinase inhibitors (TKIs), there is still much to understand about the biology and mechanisms of the disease.

This thesis explores the role of two oncogenes commonly found in human pre-neoplastic NSCLC squamous tumors, *PIK3CA* and *NFE2L2*, in the transition pre-malignant human bronchial epithelial cells (HBEC) into a transformed and malignant state. *PIK3CA* encodes the PI3K alpha protein, which promotes mitogenic and cell survival signaling pathways. *NFE2L2* encodes the NRF2 transcription factor, which regulates expression of anti-oxidant response genes. Using genomics data from the Cancer Genome Atlas (TCGA/cBioportal), we determined that *PIK3CA* and *NFE2L2* gene mutations are found to be mutually co-occurring in squamous lung cancers, at a high level of statistical significance (log2 Odds Ratio = 1.648; $p < 0.001$). Based on this observation, our central hypothesis is that *PIK3CA* and *NFE2L2* activating gene mutations act cooperatively to promote cancer malignancy. We further hypothesize that the mutant forms of PI3Kα and NRF2 cooperate to cause increased inhibition of the autophagy pathway. Illuminating the mechanisms of autophagy pathway regulation by

PI3Kα and NRF2 is of vital importance to directing the search for potential cancer therapeutics to treat lung squamous cell carcinoma (LSCC).

The PI3K/AKT/mTORC1 Signaling Pathway

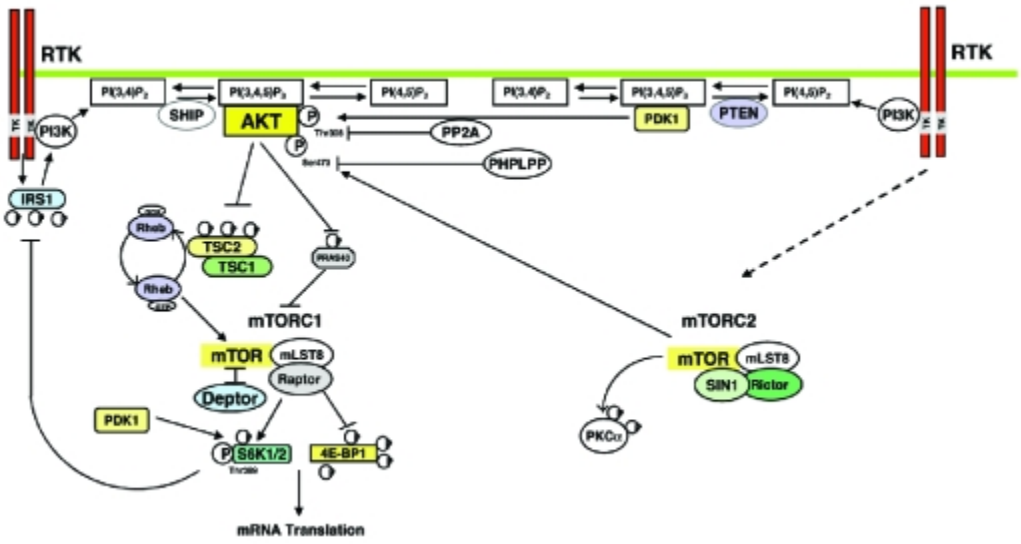


Figure 1: Schematic representation of the PI3K/AKT/mTOR Signaling Network. PI3K is recruited to the plasma membrane by RTKs and phosphorylates PIP3 which recruits PDK1 to phosphorylate AKT. Phosphorylated AKT inhibits TSC2 via phosphorylation preventing the inactivation of the small G protein, Rheb and allowing it to accumulate in its GTP-bound state. Rheb-GTP indirectly activates mTORC1.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase composed of a catalytic subunit (p110) and regulatory subunit (p85). PI3K initiates a signal transduction cascade that enhances cell growth, survival and metabolism by enhancing cellular replication and reducing growth inhibition and apoptosis.[7] Oncogenic PIK3CA mutations, including E545K and H1074R, cause a constitutive activation of PI3K by disrupting the attachment

of its regulatory and catalytic subunits leading to enhanced signaling and cellular transformation.[8,9]

The PI3K complex is activated and recruited to the plasma membrane by receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR).[10] At the plasma membrane it mediates the phosphorylation of phospholipid phosphatidylinositol 4,5 bisphosphate at the 3 position of the inositol ring and converts it to phosphatidylinositol trisphosphate (PIP3). PIP3 then functions as a second messenger and controls processes related to cell survival, growth, morphology, and motility by recruiting proteins with pleckstrin homology (PH) domains such as PDK1 and serine/threonine kinase AKT to the plasma membrane. There PDK1 activates AKT, also known as protein kinase B, by phosphorylation. (Figure 1)[7]

AKT is an important downstream effector of PI3K signaling. It is fully activated by mTOR-RICTOR kinase complex (mTORC2) via insulin/IGF Receptor or RTK stimulation. Activated AKT promotes MDM2 dependent p53 proteolysis and the phosphorylation of BAD which leads to the inhibition of apoptosis. AKT also suppresses apoptosis by activating the mTOR-RAPTOR kinase complex (mTORC1). The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that regulates eukaryotic cell growth and metabolism by controlling fundamental cellular processes such as protein translation/synthesis, lipid synthesis, and autophagy. The mTOR kinase can function in two distinct complexes, mTORC1 and mTORC2. The mTORC1 complex is inhibited by rapamycin and is activated by nutrients such as amino acids while the

mTORC2 complex is unresponsive to rapamycin and nutrients. AKT indirectly activates mTORC1 by associating with TSC1 in the TSC1/TSC2 complex to inhibit the GTPase Rheb. The accumulation of Rheb-GTP activates mTORC1 by inhibiting FKBP38. mTORC1 then phosphorylates p70 S6 ribosomal kinase which has a role in cell proliferation and positively regulating protein synthesis by increasing mRNA translation and thereby increasing protein synthesis. (Figure 1)[7]

PI3K is inactivated when PIP3 is hydrolyzed to PIP2 by PTEN, a lipid phosphatase and the enzymatic antagonist of PI3K. PTEN is inactive in many lung cancers. However, loss of PTEN function does not universally correlate with enhanced PI3K signaling as the cellular changes in tumors in response to diminished PTEN function are very different from those seen with gain of PI3K and have different drug sensitivities.[11]

Downstream Targets: The Importance of mTORC1

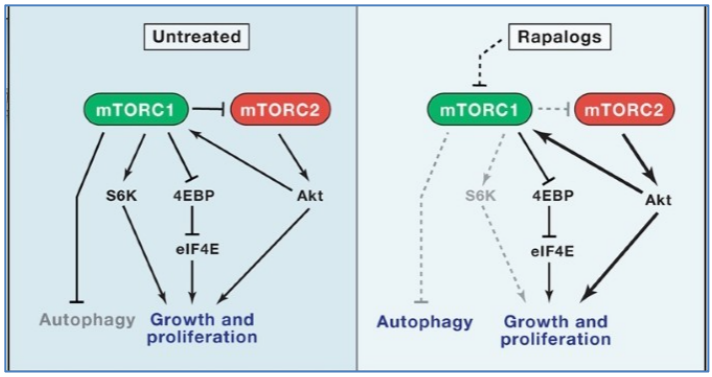


Figure 2: Overview of the function of mTOR Inhibitors. Left panel: Untreated mTORC1 inhibits autophagy, and promotes growth and proliferation by activating S6K and inhibiting 4EBP. mTORC1 also inhibits mTORC2 preventing activation of AKT. Right panel: mTORC1 rapalogs such as rapamycin

prevent mTORC1 from inhibiting autophagy and promoting growth and proliferation via S6K activation. It also prevents inhibition of mTORC2 by mTORC1 allowing AKT activation. Growth and proliferation can still be promoted by mTORC1 by inhibition of 4EBP.

mTORC1 promotes cell growth and proliferation by increasing anabolic processes like nucleotide, protein, and lipid synthesis and suppressing catabolic processes such as autophagy. One target of mTORC1 related to protein synthesis is p70 ribosomal protein S6 kinase (S6K). S6K is phosphorylated by mTORC1 on its hydrophobic motif. S6K can then be activated by PDK1 via a second phosphorylation on its T-loop. S6K then goes on to activate its substrates via phosphorylation to promote mRNA translation. The first identified substrate of S6K was a component of the 40S ribosome, ribosomal protein S6. (Figure 2)[11] In addition, mTORC1 also promotes protein translation by phosphorylating eIF4e Binding Protein (4EBP) which normally inhibits the assembly of translational machinery by binding to eIF4e. Phosphorylation by mTORC1 changes the conformation of 4EBP, freeing eIF4e and allowing translation machinery to assemble. Both S6K1 and 4EBP bind raptor, a critical mTOR-interacting scaffold protein, and are regulated by amino acid levels. Depletion of amino acids, especially the branched chain amino acids leucine and isoleucine, leads to reduced protein synthesis due to dephosphorylation of S6K1 and 4EBP. This is rapidly revered by replenishing amino acid levels. In addition, S6K1 is also regulated by glycolysis, mitochondrial function, cell stress, and growth factors such as insulin.

mTORC1 induced proliferation requires increased lipid synthesis to meet the membrane assembly needs of dividing cells. The S6K1 pathway is also involved in lipid synthesis by activating transcription factors called sterol response element binding proteins (SREBPs).[11]

mTORC1 also plays an important role in promoting DNA replication by regulating nucleotide synthesis. Again, mTORC1 acts via S6K1 which activates carbamoyl-phosphate synthase, an enzyme involved in the de-novo pyrimidine synthesis pathway and urea cycle, via phosphorylation. mTORC1 activation of SREBPs also controls nucleotide synthesis. The activated SREBPs redirect more cellular resources to the pentose phosphate pathway generating more NADPH needed for lipid and nucleic biosynthesis. mTORC1 also increases nucleotide synthesis by upregulating bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase which donates carbons for purine synthesis in the mitochondrial-tetrahydrofolate cycle.[11]

Another important target of mTORC1 is hypoxia inducible factor 1 (HIF1), a transcription factor involved in the expression of survival genes. Translation of the alpha subunit of HIF1 is increased following mTORC1 activation leading to increased production of glycolytic enzymes. This marks a shift in glucose metabolism, leading to increased glycolysis and reduced oxidative phosphorylation. In addition, as discussed later, mTORC1 also promotes cell growth by inhibiting the key catabolic process of autophagy.[11]

PI3K in Cancer

The PI3K/AKT/mTORC1 signaling pathway controls cell growth, proliferation and survival by increasing anabolism, and suppressing catabolism, including autophagy, which is discussed later.[2] There are three classes of PI3K proteins with class IA having the strongest association with cancer.[7] *PIK3CA* encodes for the catalytic subunit of PI3K, p110a. It is mutated in approximately 3-12.7% of NSCLCs.[12] Studies of p110a inhibitors on leukemic cells have shown they inhibit growth, suggesting that PI3K is an oncoprotein. There are three common 'hotspot' mutations in PIK3CA that are commonly found in cancer; one in the kinase domain and two in the helical domain. All three mutations cause a gain of function of p110 and a significant upregulation of PI3K's lipid kinase activity. This increased oncogenic activity of PI3K is observable by elevated phosphorylation of AKT which can be shown on a Western Blot. It can also be shown in cell culture by induced anchorage independent growth.[10]

Overexpression of PI3K or its downstream effectors leads to constitutive mTORC1 activation and is pivotal to many LSCCs as it increases protein translation, proliferation and inhibition of the autophagy pathway through repression of ULK1 (Unc-51 Like Autophagy Activating Kinase 1) and the MIT/TFE family of transcription factors. Similarly, tumor suppressors TP53 and LKB1 function as negative regulators of mTORC1. TP53 and LKB1 mutations are common in cancers, conferring increased mTORC1 activity. In addition, mTORC2 has also been implicated as an oncogene through activation of AKT causing increased glucose uptake, glycolysis, and inhibition of apoptosis.

Cancer Therapeutics: mTORC1 inhibitors

To investigate PI3K-mTOR signaling in cell lines, we used mTORC1 kinase inhibitor Everolimus (see Materials and Methods). However, clinically mTOR inhibitors have shown limited therapeutic efficacy when administered as single agents. One reason for this is the complexity of the PI3K/AKT/mTORC1 axis. When mTORC1 alone is inhibited, there is an activation of the mTORC1 negative feedback loop. This results in the activation of AKT by S6K induced upregulation of the IRS-1 and TGFBR-1 pathways. Therefore, inhibition of mTORC1 alone is not sufficient to fully suppress PI3K-AKT signaling.[13] Another reason for the lack of efficacy of mTORC1 inhibitors is that they do not equally inhibit the phosphorylation of all mTORC1 substrates equally. Thereby some substrates such as S6K1 are considered rapamycin sensitive while others such as 4EBPs are considered rapamycin insensitive. Interestingly, mTORC1 inhibitors also activate autophagy. The role of autophagy in cancer is complex and often context dependent--in some cases activation of autophagy promotes cancer survival while in other cases, usually earlier stages, it prevents tumorigenesis. The activation of autophagy by mTOR inhibitors have been shown to help cancer cells survive nutrient deprived conditions by increased recycling of cellular materials, allowing the cell to become resistant to apoptotic cell death. Though currently available mTORC1 inhibitors have limited clinical efficacy, there remains a strong interest in mTORC1 and downstream effectors as therapeutic targets due to their central role in oncogenesis. For example,

mTORC1-actitvated S6K1 phosphorylates and activates oestrogen receptor α leading to breast cancer progression.[14]

Treatment of LSCC is most effective in patients with targetable oncogenes such as EGFR.[2] One of the first lines of treatment in LSCC are EGFR TKIs. However, over activation of the PI3K/AKT/mTORC1 axis is a common resistance mechanism to EGFR TKIs accounting for 14.9% of resistance events. This is of great concern as co-occurrence of EGFR activating mutations with mutations in the PI3K pathway predicts a worse prognosis for LSCC patients. Because of this, despite the limited efficacy of mTORC1 inhibitors including Everolimus, development of new inhibitors of the PI3K/AKT/mTORC1 axis still remains of great interest in the treatment of LSCC. Moreover, the Ras/Raf/MEK/ERK pathway inhibits the TSC1/TSC2 complex as ERK phosphorylates TSC2. This promotes Rheb-mediated activation of mTORC1 independent of the PI3K/AKT pathway. This complicates the potential use of PI3K/AKT/mTORC1 inhibitors as potential therapeutics due to parallel survival signaling mediated by the Ras pathway. Application of PI3K inhibition sensitizers, such as CDK4/6 inhibition, may enhance clinical response.[13,14] Recently, ATP-competitive catalytic inhibitors that inhibit both mTORC1 and mTORC2 have also shown promise.

NRF2 and the Antioxidant Response

Nuclear factor erythroid 2-related factor (NRF2) is a transcription factor encoded by *NFE2L2* that is part of an evolutionary conserved intracellular defense mechanism to overcome oxidative stress in the lung. NRF2 is part of the Cap "n" Collar family of

transcription factors and contains the highly conserved basic region-leucine zipper motif. NRF2 and its principle negative regulator, the E3 ligase adaptor Kelch-like ECH-associated protein 1 (KEAP1), reduce oxidative stress through regulation of glutathione and thioredoxin dependent systems and are critical for the maintenance of redox, metabolic and protein homeostasis and regulation of inflammation.[2] Reactive oxygen species (ROS) are generated in the body from external exposure or internally from metabolic processes. They include $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, $RO_2^{\bullet}$, $RO^{\bullet}$, $^1O_2$, and O_3 and are primarily produced by the mitochondria during aerobic respiration. Nearly all enzymes that utilize oxygen as a substrate are known to produce ROS.[15] Oxidants can act as signaling molecules for cell division, immune function, and autophagy. ROS present a big concern for the lung because even though it is an internal tissue it is continuously exposed to the outside environment during respiration. For that reason oxidative stress is not only implicated in the initiation of lung cancer, but also asthma and COPD. Consequently, NRF2 is a potential therapeutic target for many respiratory diseases.[16]

NRF2's Role as a Transcription Factor

Redox homeostasis is maintained through a counterbalance of ROS and antioxidant systems.[15] NRF2 is a potent transcriptional activator that recognizes and binds to a unique DNA sequence called the antioxidant response element (ARE). By regulating expression of ARE-containing genes, NRF2 can prevent oxidative stress-induced genetic damage by inducing antioxidant and radioprotective mechanisms and enhancing clearance of DNA damaging agents in non-malignant cells. When NRF2 is

allowed to accumulate by inactivation of the KEAP1. sequestering mechanisms there is a swift activation of the ARE-dependent cytoprotective genes.[3,15] Though ROS can cause damage to cells, recent studies have identified them as signaling molecules for many intracellular proteins. The NRF2 transcriptional response via the ARE-dependent genes reduces the levels of ROS below the threshold for these proteins. These proteins are often involved in tumorigenesis and include NF-κB, mitogen-activated kinases, PI3K, and hypoxia inducible factor 1.[2] NRF2 has long been thought of as a 'pro-survival' transcription factor that protects us from a variety of diseases. However, despite NRF's role in preventing tumorigenesis, NRF2's protective abilities are often used by cancer cells to create a pro-survival microenvironment to foster tumor growth and drug resistance.[15]

Downstream Targets of NRF2

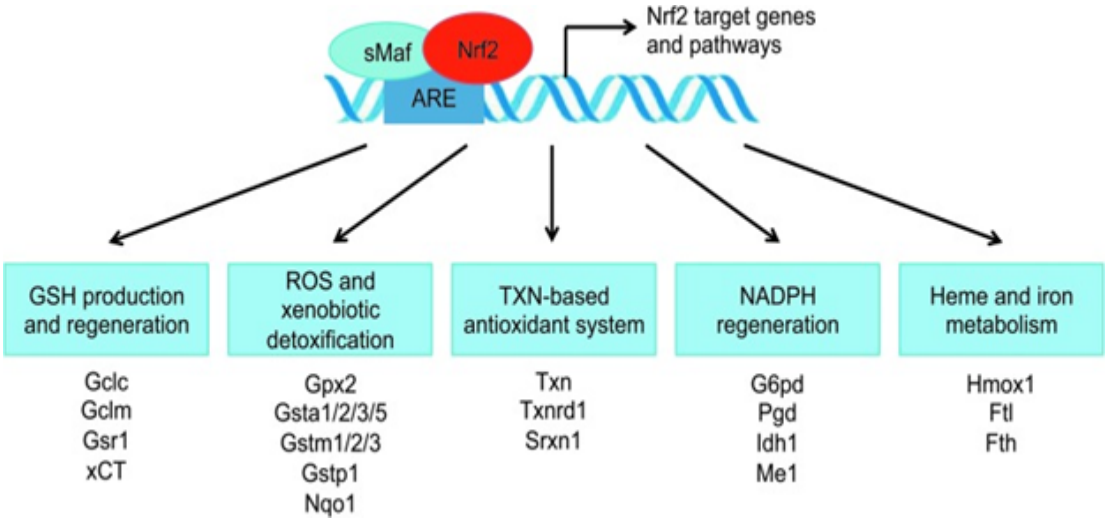


Figure 3: Transcriptional Targets of NRF2. NRF2 binds to antioxidant response elements to increase transcription of gene involved in glutathione production and regeneration, ROS and xenobiotic detoxification, the TXN-based antioxidant systems, NADPH regeneration, and heme and iron metabolism.

NRF2 and its downstream targets regulate antioxidant signaling and cell survival. The role of NRF2's downstream targets include antioxidants and detoxification enzymes but also expand to stress response genes, xenobiotic metabolizing genes, genes involved in the ubiquitin-mediated proteasomal degradation system, intracellular redox-regulating genes, genes controlling cell growth and genes encoding transporters. (Figure 3)[17] The many functions of NRF2 can be explained by the various antioxidant response element (ARE)-regulated genes it controls and it is clear that the NRF2-dependent response is involved in a cell's attempt to defend itself from harmful conditions.[15] As mentioned earlier NRF2 maintains redox homeostasis through a counterbalance of ROS and antioxidant systems. It does this through many mechanisms including by inducing the synthesis of reducing factors such as the antioxidant Glutathione and NADPH.

NRF2 also controls the basal and induced expression of a variety of drug metabolizing enzymes and has been linked to genes that play a role in drug resistance including transporters such as multidrug resistant associated protein 1-6. Initiation of drug metabolism through ARE likely developed as a strategy of detoxification of endogenous oxidants but evolved to include environmental toxins.[18]

As discussed in the autophagy section below NRF2 also regulates some oxidant signaling proteins such as p62.

Regulation by KEAP1

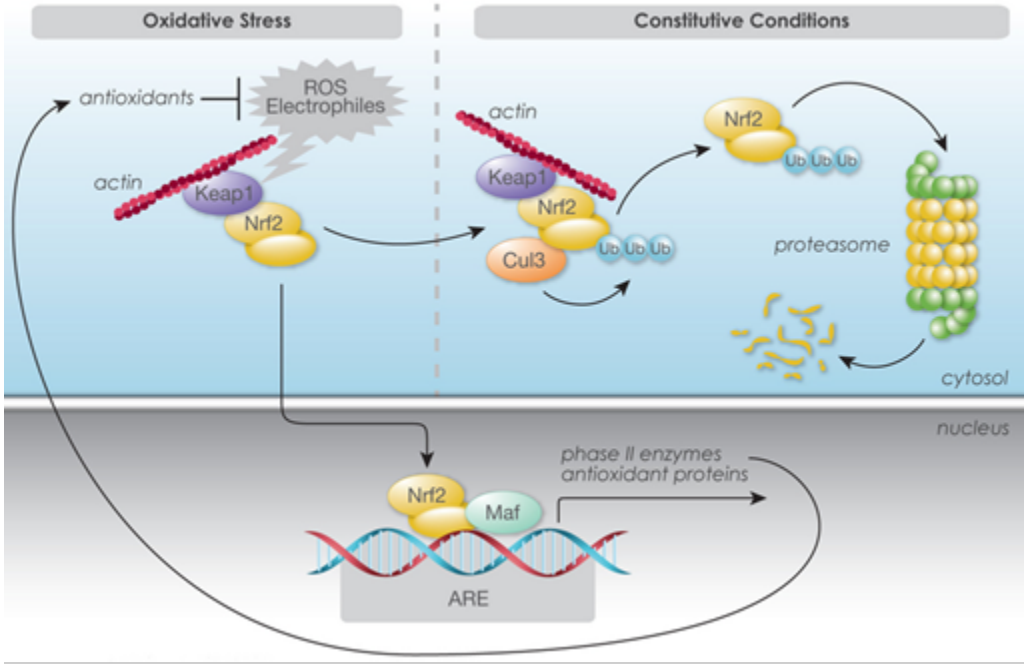


Figure 4: The NRF2/KEAP1 Pathway. Under constitutive conditions NRF2 is bound to KEAP1 and sequestered in the cytosol. Ultimately this complex is ubiquitinated and degraded by the proteasome. In the presence of ROS NRF2 is freed from KEAP1 and can travel to the nucleus where it promotes transcription of antioxidant proteins. Eventually these antioxidant proteins eliminate ROS species and NRF2 remains sequestered in the cytosol by KEAP1.

Kelch-like ECH-associated protein (KEAP1) serves as an adaptor for the Cullin 3 based ubiquitin E3 ligase for NRF2. NRF2 is targeted for ubiquitin-mediated degradation by KEAP1 until the cell encounters oxidative stress/ oxygen free radicals. KEAP1 is rich in thiols and contains many reactive cysteine residues. Consequently, oxidative stress induces a conformational change when electrophiles directly modify the cysteine residues

in KEAP1. This inactivates KEAP1 and releases NRF2. NRF2 is then free to move to the nucleus, bind to the antioxidant response element (ARE) and enhance the production of antioxidants to eliminate the free radicals. (Figure 4)[16]

Protein turnover is the major mechanism for NRF2 regulation as Keap1 is a ubiquitin ligase that marks NRF2 for degradation. This keeps the concentration of free NRF2 low and henceforth also keeps the expression of NRF2 target genes low. When exposed to oxidative stress the cysteine thiols of KEAP1 and NRF2 are modified, inhibiting the ubiquitination of NRF2 and altering NRF2/Keap1 complex. The KEAP1 is therefore trapped in the complex and newly synthesized NRF2 can bypass KEAP1 and enter the nucleus. The level of NRF2 is increased and ARE-mediated transcription is upregulated. When oxidant or toxin exposure has ceased, KEAP1 translocates to the nucleus during the post induction period and removes NRF2 from the ARE regulatory sequences. NRF2 is shuttled back to the cytoplasm where it is degraded to turn off the NRF2-dependent response.[15]

NRF2 and Cancer

Mutations in the NRF2/KEAP1 stress response pathway are major drivers of cancer and are found in approximately 33% of LSCC according to the Cancer Genome Atlas.[19] Cancers with elevated NRF2 levels are associated with reduced survival due to chemoresistance and high proliferation.[2] However, NRF2 has a complex role in lung cancer as it acts as both a tumor suppressor and an oncogene. Its role as a TSG relates to its role in protecting cells from carcinogens and in doing so blocks tumor initiation and

cancer metastasis.[16] Loss of *NFE2L2* in coordination with other common mutations has been shown to lead to early tumor growth, indicating oxidative stress as a driver for the beginning stages of tumorigenesis. However, in transgenic mouse models loss of *NFE2L2* is also associated with increased survival by promoting oncocytomas. Relatedly, in lung diseases such as emphysema and COPD NRF2 deficiency or downregulation is linked to disease etiology.[16,20] Despite NRF2's role in clearing ROS, mutations such as T80K in the ETGE motif of the Neh2 domain interrupt KEAP1-NRF2 binding and lead to constitutively active NRF2 have been found in many pre-malignant LSCC lesions. High nuclear levels of NRF2 seem to promote cancer cell proliferation by creating an environment conducive to growth and survival despite detrimental conditions.[21,22] While reduced NRF2 levels can also cause cancer because of the lack of protection from carcinogens, elevated NRF2 levels can cause anabolic pathways to become activated and stimulate cell proliferation leading to cancer cell transformation. This thesis will focus on the oncogenic role of NRF2 but it's important to recognize the dual role of NRF2 as a tumor suppressor.[2] Furthermore, in some cancer cells the antioxidant abilities of NRF2 are hijacked to increase resistance to oxidants in cancer therapeutics such as cisplatin, doxorubicin and etoposide, creating chemoresistant tumors. In mice with KRAS-induced lung tumors drug sensitivity can be imparted to chemoresistant cancer cells with the use of the NRF2 inhibitor brusatol.[22] In addition, research by Wang & Sun showed that both introduction of NRF2-small interfering RNAs and overexpression of KEAP1 using lentiviruses caused increased drug sensitivity in chemoresistant LSCC.[15] Other potential

inhibitors of NRF2 that have been explored as therapeutic agents are agonists of the glucocorticoid receptor such as dexamethasone. Following ligand mediated activation and binding to the glucocorticoid response element, dexamethasone has been shown to inhibit transcriptional activity of NRF2.[3]

Also of note, loss of function mutations in KEAP1 also have a similar effect to NRF2 activating mutations as they keep NRF2 constitutively active and are common in cancers including LSCC where environmental factors are important etiological components.[16] In LSCC loss of heterozygosity at the genomic location of KEAP1, 19p13.2, is common. In addition, mutations in the Kelch and linker domains of KEAP1 that prevent NRF2 binding are also common. It is suggested that NRF2 inhibition during chemotherapy may be more effective when KEAP1 is non-functional or under expressed.[15] However, it unclear whether single mutations in either KEAP1 or NFE2L2 can initiate malignant transformation. Initiation and progression of malignant transformation requires cooperativity between two or more oncogenic driver mutations. For example, experiments done by Best & Souza showed that mice with elevated NRF2 due to a knockdown of Keap1 do not spontaneously develop tumors. However simultaneous deletion of KEAP1 and PTEN, a negative regulator of PI3K, promotes adenocarcinoma formation in the murine lung.[2] Co-occurrence of NRF2 mutations with alterations in the PI3K pathway have the strongest association with malignant transformation and is the foundation of this thesis.

Overall elucidating the mechanism through which NRF2 modulates the progression of pulmonary diseases is important for the development of therapeutics to target this pathway. Interestingly, NRF2 deletion in NSCLC cells has been shown to completely suppress tumor formation in athymic mice suggesting that NRF2 inhibitors or CRISPR-Cas9 NRF2 genes editing could be used to treat NSCLC. Discovery of NRF2 targeted agents could be a game changer not only for lung cancer but a variety of respiratory diseases.[16]

Autophagy

Overview:

Autophagy is a highly evolutionary conserved process that promotes recycling of macromolecules in response to a variety of cellular stresses such as nutrient deprivation, hypoxia, abnormal protein aggregation, and organelle damage. It serves a role in cell death and tumor suppression as well as maintaining metabolism and homeostasis. There are three major types of autophagy: macroautophagy (the focus of this thesis), microautophagy and chaperone-mediated autophagy. When cells are deprived of nutrients, autophagy is upregulated to maintain a source of proteins and nutrients essential to energy production. It also plays a housekeeping role to prevent cellular damage and serves to clear damaged organelles, including the mitochondria and peroxisomes, removes misfolded proteins, eliminates intracellular pathogens and maintains genomic integrity.[23] An important mechanism of autophagy is the intracellular degradation of proteins and organelles by the autophagosome, a double-membrane

organelle. The autophagosome expands by engulfing intracellular cargo and then reaches maturity when it fuses with the lysosome and becomes the autolysosome. (Figure 5)[14] The autolysosomal contents are then degraded by lysosomal proteases and exported back into the cytoplasm for re-use for energy or anabolism of macromolecules.[14]

Autophagy's role in cancer is complex and context dependent-- In mouse models of NSCLC autophagy ablation can initiate early tumor growth. However, in later stages, loss of autophagy dampens tumor growth. The heterozygous loss of the autophagy gene *Becn1* in mice causes tumorigenesis and deletion of *Atg5* or *Atg7* leads to the development of hepatomas. These findings suggest that autophagy plays a role in tumor suppression. On the other hand, in some cases autophagy is localized to the hypoxic and metabolically stressed areas of the tumor, enabling tumor cell survival. Conversely, the absence of autophagy may inhibit tumor growth due to the accumulation of defective mitochondria and reduced cellular respiration, which can impair cell survival and cause diminished stress tolerance. Tumors that are dependent on autophagy are labelled "autophagy-addicted" and autophagy inhibitors are of particular interest for treatment of these tumors.[20,24]

Both oncogenes, *PIK3CA* and *NFE2L2*, investigated in this thesis play a role in the autophagy pathway as elaborated on below. My central hypothesis is that *PIK3CA* and *NFE2L2* mutations promote cancer progression via enhanced cooperative inhibition

of the autophagy pathway.

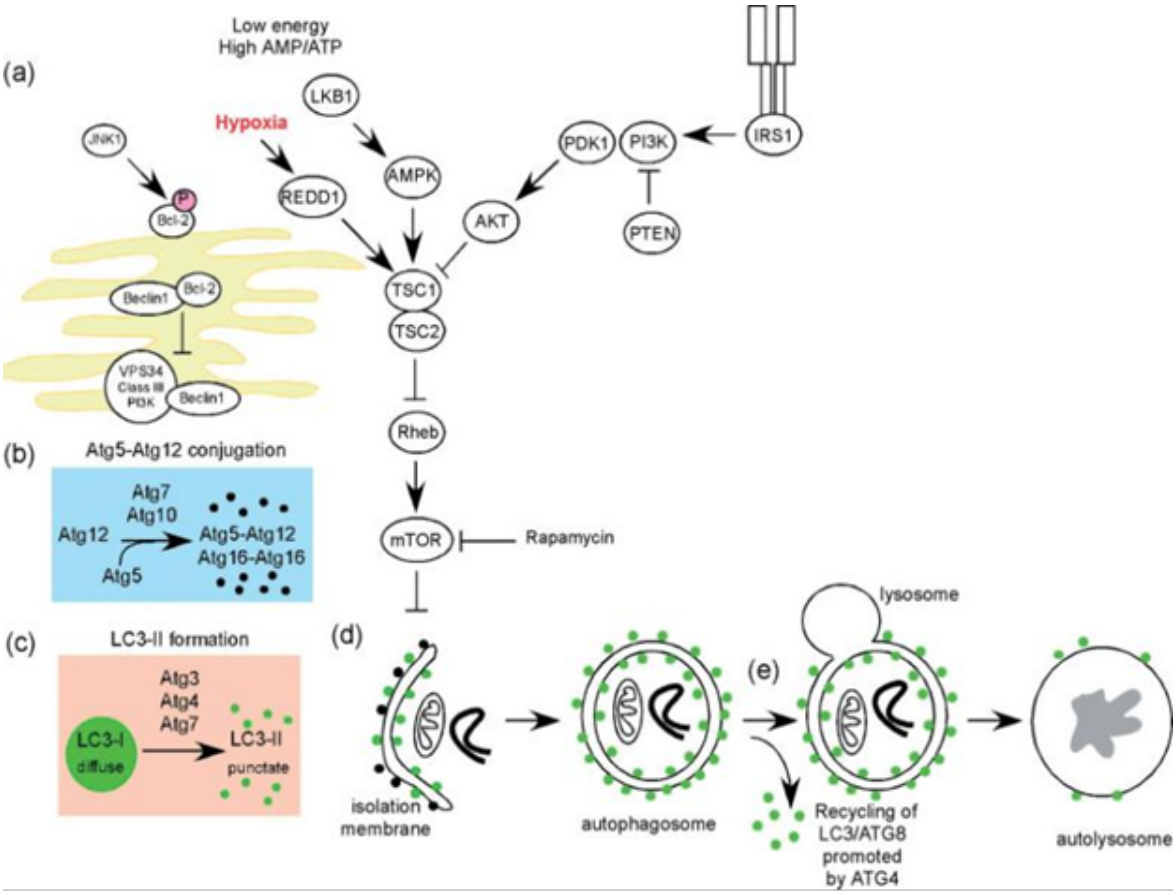


Figure 5: Signaling Pathways Regulating Autophagy. Autophagy is a multistep self-degradative process with several key steps: (a) the phagophore is formed from the ER membrane. Its formation is controlled by Beclin-1 in response to stress. (b) At the phagophore ATG5 and ATG12 conjugate. (c) LC3 is processed and inserted into the membrane of the extending phagophore. (d) Selective targets are captured for degradation. (e) The autophagosome fuses with a lysosome to form an autolysosome.

Assays to Study Autophagy

Accumulation of select autophagy-related proteins can be measured by Western blotting or by microscopy. The roles of these autophagy-related proteins are discussed

below. When autophagy is inhibited these proteins are unable to be broken down and they therefore accumulate within the cell. Methods to detect levels of these proteins can be used to measure autophagy inhibition.

LC3

There are two types of macroautophagy, selective and non-selective. Selective autophagy relies on adaptor proteins to identify contents to be degraded. Microtubule protein light chain 3 (LC3-II) is a cytosolic adaptor protein that is cleaved and then recruited and integrated into the growing autophagosome on both the internal and external surfaces. LC3 integration is dependent on the ATG5-ATG12 complex. LC3-II is a good indicator of autophagy inside cells. When autophagy is active LC3-II synthesis and processing is increased but it is degraded in the autolysosome making detectable levels of LC3-II very low. When autophagy is inhibited LC3-II remains undegraded and accumulates to high levels inside the cell.[14]

p62/ sequestosome 1

p62 is a multifunctional adaptor protein that interacts with LC3-II on the autolysosome. Like LC3-II it is also degraded within the autolysosome when autophagy is active and accumulates when autophagy is inactive. p62 facilitates selective autophagy by binding ubiquitinated protein aggregates and delivering them to the autophagosome. NF-κB signalling is also regulated by p62, with elevated p62 levels inducing tumorigenesis by deregulating NF-κB signaling. As discussed below, p62 plays a role in activating NRF2.

ATG5

An initial step in the autophagy pathway is the conjugation of ATG5 with ATG12. ATG16 complexes with ATG5-ATG12 to promote expansion of the nascent phagophore. Recruitment of LC3 is dependent on the presence of ATG5-ATG12 on the phagophore membrane. However, once the phagophore is completely formed ATG5-ATG12 dissociates.

ATG7

Atg7 is involved in the initiation of autophagosome synthesis. It is an E1 ubiquitin-activating enzyme that serves in the elongation step of the phagophore by activating ATG12 and LC3-I mediated conjugation systems.[25]

Regulation of Autophagy by the PI3K/mTOR Pathway

The autophagic mechanism is regulated by signaling pathways involving coordination of many proteins, including mTORC1 and mTORC2, which are involved in nutrient sensing and regulation of cell growth. When nutrients are plentiful mTOR inhibits autophagy and promotes cell growth by inducing ribosomal protein expression. As discussed earlier the PI3K protein and its corresponding gene PIK3CA are investigated throughout the laboratory work of this thesis. mTORC1 is activated downstream of PI3K. Therefore upregulation or downregulation of PI3K will have a corresponding effect on mTORC1 and on autophagy. Autophagy is initiated by AMP dependent kinase (AMPK) activation of ULK, a serine/threonine kinase. Activated mTORC1 inhibits autophagy by phosphorylating ULK1. This prevents the initiation of

autophagy as phosphorylated ULK1 cannot be activated by AMPK, thereby inhibiting the ATG1/ULK-1/-2 complex at the early stage of phagophore formation from the lipid bilayer. mTORC1 can also be negatively regulated by AMP-activated protein kinase (AMPK). When mTORC1 is suppressed, as is the case in the event of hypoxia or low cytosolic ATP levels, there is an increase in AMP which induces autophagy by activating AMPK.[14] The MiT/TFE family of transcription factors promote expression of genes needed for autophagy. The regulation of the MiT/TFE family of transcription factors is another mechanism by which mTORC1 suppresses autophagy. By phosphorylating TFEB, a member of the MiT/TFE family, and preventing its translocation to the nucleus and mTORC1 prevents expression of lysosomal hydrolases needed for formation of the autolysosome.

In cancer cells, autophagy inhibits cancer cell survival and facilitates cell death thereby promoting tumorigenesis. The mTOR complex is a major target for current and potential cancer therapeutics. As mentioned earlier, reduced mTOR activity induces autophagy and causes increased catabolism and reduced cell growth. Therefore rapamycin, which targets mTOR and reduces its activity, is used in cancer therapy as it blocks protein translation, inhibiting cell growth and also promotes autophagy.[14] In lung cancers, the autophagy pathway's role in protecting cells from environmental toxins is of particular interest as the bronchial epithelial cells are in direct contact with the outside world via the air inhaled. In addition, mitophagy, the autophagy-dependent degradation of mitochondria, is of special interest in regards to lung cancers as it limits the production

of carcinogenic ROS.[23] The interruption of the autophagy pathway could be a key initial step that makes HBEC more susceptible to mutations caused by environmental toxins such as those found in cigarette smoke or pollutants and ultimately lead to the progression into lung cancer.[14]

NRF2 and autophagy

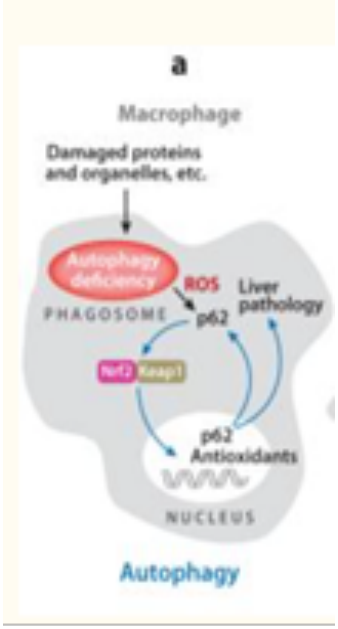


Figure 6: Schematic representation of the positive feedback loop between p62, NRF2, in autophagy. The presence of ROS from damaged proteins and organelles free NRF2 from being sequestered in the cytoplasm by KEAP1. This allows NRF2 to travel to the nucleus and promote the transcription of p62. After translation p62 binds to KEAP1 and prevents it from sequestering NRF2.

A key link between NRF2 and the autophagy pathway is the selective autophagic cargo receptor protein, p62. p62 removes KEAP1 suppression of NRF2 even in the absence of electrophiles leading to activation of NRF2 and increased ARE-dependent gene expression. *SQSTM1,* the gene that encodes for p62, is an ARE-dependent gene.

Therefore accumulation of NRF2 leads to increased p62 expression. (Figure 5)[18] In addition, p62 also positively regulates NRF2 transcription creating a positive feedback loop and when the autophagy pathway is inactivated there is a large accumulation of p62 levels leading to increased levels of NRF2.[26,27] This is supported by the experiments of Komatsu that found an overexpression of NRF2 in autophagy defective mice.[28] KEAP1 binds to the KEAP1-interacting region domain on p62 which is similar to the KEAP1-interacting ETGE motif on the Neh2 domain of NRF2 allowing p62 to sequester KEAP1 in the autophagosome. Thus, p62 plays a crucial role in regulating KEAP1 turnover and it has been shown that elevated levels of p62 reduces the half life of KEAP1. Without the presence of KEAP1 in the cytosol, ubiquitination of NRF2 is impaired and NRF2 becomes active. This is considered the non-canonical activation pathway for NRF2 since it does not require ROS. While our experiment models NRF2 as an oncogene by inducing its overexpression, impaired formation of the autophagosome via ATG7 or p62 deficiencies may be a contributing factor to the high NRF2 levels that lead to tumorigenesis instead of mutations in *NFE2L2* or *KEAP1*.[25]

Much is still not known about p62 regulation of KEAP1. Some studies have suggested that p62 has increased affinity for KEAP1 when it is phosphorylated resulting in prolonged accumulation of NRF2 and transcriptional upregulation of its target genes. In addition, ULK-1 is also thought to interact with p62 and facilitate its phosphorylation leading to increased degradation of cargo proteins including KEAP1 and thereby increasing NRF2 levels. This is an important link to the PI3K pathway as mTORC1, the

downstream target of PI3K prevents the ATG1/ULK-1/-2 complex formation at the early stage of phagophore formation releasing ULK-1 to associate with and phosphorylate p62, leading to NRF2 protein stabilization. By this mechanism PI3K indirectly leads to increased levels of NRF2.[25]

The role LC3 plays in the link between the NRF2 pathway and autophagy is not completely understood. The KEAP1-interacting region domain and the LC3-interacting region domain are very close to each other on p62. Some studies have suggested this adjacency leads to competitive bind, therefore limiting the KEAP1 sequestering capabilities of p62. However, other studies suggest that the formation of the KEAP1-p62-LC3 complex are necessary for the clearance of ubiquitin aggregates in response to oxidative stress.[25]

Recent studies of autophagy deficient mice have shown prolonged NRF2 activation leads to cancer. When autophagy is ablated by deletion of genes encoding for critical autophagy proteins, p62-KEAP1 aggregates accumulate in the cytosol resulting in prolonged NRF2 activation. *ATG7* deficient mice provide an important model for the connection between NRF2 and autophagy. When *ATG7* is deleted in mouse models, phagophore elongation is blocked leading to impaired formation of the autophagosome. KEAP1-p62 accumulation and constitutive NRF2 activation were observed in ATG*7*-deficient mice.[25] Similarly, these same KEAP1-p62 aggregates with NRF2 pathway activation were found in human hepatocellular carcinomas with *ATG7* deficiencies. When p62 was deleted in *ATG-7*-deficient cell lines, no KEAP1-p62 aggregates were

produced and the NRF2 pathway was blocked. In addition, anchorage independent growth was also suppressed in the absence of p62.[22] Much is still not known about the role of the interplay between the NRF2 and PI3K/mTOR-autophagy pathways in cancer initiation and progression. Reciprocal control of NRF2 and autophagy may be context dependent and a comprehensive understanding of the mechanism is needed before further research into therapeutic modulation of NRF2 and autophagy can be achieved.[25]

METHODS

Clonogenic Assay

The cell lines were plated at a density of 100cells/mL in a six well plate. Two wells were left as controls and only contained media. The cells were allowed to grow for 6 days in the incubator at 37°C and 5% CO2. They were then fixed with 10% buffered formalin, stained with 0.5% Crystal Violet Dye, and air dried overnight. The 6-well plate was then imaged with Bio-Rad Molecular Imager ChemiDoc™ XRS+ with Image Lab™ Software.

Initial Cell Lines

Frozen aliquots of HBEC3-KT cells transfected with the genes *HcRed*, E545K mutant *PIK3CA*, and T80K mutant *NFE2L2* were thawed and plated. HBEC3-KT are a line of cells manufactured by American Type Culture Collection (ATCC) that are non-tumorigenic and immortalized by the introduction of CDK4 and hTERT.[29,30] The transfected cell lines were allowed to grow to confluence in an incubator at 37°C and 5% CO2. Airway Epithelial Basal media manufactured by the American Type Culture Collection (ATCC) was used to house the cell lines. It was supplemented with the Bronchial Epithelial Cell Growth Kit (also manufactured by ATCC) and 1X Penstrep.

Lentiviral Transduction

The HBEC-*NFE2L2* line was plated in a 6-well dish. Lentiviruses diluted at a 1:1000 containing *HcRED* or *PIK3CA* were transduced using spin infection at 1,200 x g for 1 hour. Protamine sulfate was also added with the virus at a concentration of 6mg/mL

to help the virus bind to the plasma membrane more efficiently. This process created our HBEC- *NFE2L2* + *HcRED* and HBEC-*NFE2L2* + *PIK3CA* cell lines which we expanded into 100mm dishes. Later, we repeated this process with Lentiviruses containing an LC3 fusion protein with dual fluorescence of mCherry and GFP with the four parental cell lines in a six well plate. This lentivirus also contained a puromycin resistance gene which allowed us to select for the transfected cells by adding puromycin at a 1:500 concentration to each well.

Drug Dilution with Everolimus and Nutrient Deprivation

The four cell lines were plated at a density of 50,000 cells/mL in 96 well plates and allowed to grow to confluence. Once they reached confluence, Everolimus, a mTORC1 inhibitor, was added in concentrations varying from 0.125mM to 1mM. The drug concentrations were obtained using serial dilutions in DMSO and RPMI + Glutamax™ (1X) serum free media manufactured by Gibco. Three wells per cell line served as a control and were treated with only DMSO in serum free media. The cells were then incubated for 48 hours.

Nutrient deprivation was also performed on these 96 well plates. The media was aspirated off wells and 100µL of minimal media, Earle's Balanced Salt Solution manufactured by Gibco, was added. 6 wells per cell line were kept as a control. The cells were then incubated for 1 hour.

The drug treated, nutrient deprived, and control wells were then fixed and stained with lysotracker (1:7500) and Hoechst dye (1:2500) in formaldehyde and PBS and

incubated for another hour. They were then placed in the fridge until they were imaged by the BioRad cytation imaging reader.

Live Cell Imaging with Everolimus and Chloroquine

Each cell line was plated at a density of 65,000 cells/mL into three wells of a twelve well plate. The cells were incubated at 37°C and 5% CO2 for 48 hours. The cell lines were treated with 100µL of 10mM Everolimus, 50mM chloroquine, or DMSO diluted in RPMI in each of the three wells. The plate was then placed in the BioRad cytation imaging reader with a 10X objective lens at the same incubation settings. Nine fields per well were selected and images were taken at the initiation of the experiment and then at a time point of one hour for 18 hours using laser autofocusing. Light-emitting diode filter cubes were used to image GFP and mCherry fluorescence. Using Gen5 imaging software, the green and red LC3 puncta were quantitated by setting fluorescence intensity thresholds to count green/red puncta and red puncta alone.[31]

Western Blots for NRF2, PI3K, Downstream Targets and Autophagy Markers

Cells were plated in 100mm plates and allowed to grow to confluence. After splitting cells for maintenance, 900 µL of the 1mL cell suspension was pelleted. Cells were lysed with 100µL of 8M Urea Lysis Buffer and then sonicated at 20% amplitude for 15 seconds. The Pierce BCA protein Assay by ThermoFischer Scientific was used to normalize the samples. The normalized samples were boiled with SDS for 6 minutes then loaded into either 12% Poly-Acrylamide Gel or a Mini-Protean™ pre-cast gel by BioRAD depending on the protein being probed for. The samples were then run using

SDS-PAGE at 100V for 1 hour and the proteins were transferred to a polyvinylidene (PVDF) membrane by running the gel and membrane at 100V for 1 hour.

The membrane was blocked in TBS-T-BSA for 30 minutes. The primary antibodies were then added at the appropriate dilutions and the membrane was incubated overnight. The primary antibodies used were: NRF2 (Cell signaling), NRF2 (Santa Cruz Biotechnologies), PI3K (4294), PI3K (4253), AKT, phospho-AKT, ATG6, LC3, p62, GAPDH. GAPDH was used as a loading control. GAPDH is a housekeeping gene that is highly and constitutively expressed in many cells and tissues. The following day the secondary antibody diluted 1:5000 in TBS-T-BSA blocking solution was added and the membrane was incubated for 1 hour. The secondary antibodies were either anti-mouse or anti-rabbit HRP-conjugated antibodies depending on the primary antibody used. An enhanced chemiluminescent mixture was added and the membrane was imaged using the Bio-Rad Molecular Imager ChemiDoc™ XRS+ with Image Lab™ Software.

Western Blots for Everolimus treated Cells

This Western blot procedure was repeated with Everolimus treated cell lines. The cell lines were plated in two six-well plates at a density of 25,000 cells/mL and allowed to grow to confluence in an incubator at 37°C and 5% CO2. Everolimus at concentrations of both 1mM or 2mM was then added to each cell line. DMSO was also added to each cell line to establish controls.

Three samples per cell line, DMSO treated (control), 1mM Everolimus treated, and 2mM Everolimus treated, were collected 48 hours post treatment and a Western blot

was run according to the procedure above. Primary antibodies for LC3, p62, pS6, total S6, pS6K, and total S6K were used.

RESULTS

Co-occurrence of Commonly Mutated Genes in Squamous Cell Lung Cancer

A	B	Neither	A Not B	B Not A	Both	p-Value	Tendency
NFE2L2	PIK3CA	231	29	150	59	**<0.001**	**Co-occurrence**
PIK3CA	TP53	50	15	210	194	**<0.001**	**Co-occurrence**
FAT1	EP300	357	87	17	8	0.110	Co-occurrence
NFE2L2	TP53	55	10	326	78	0.287	Co-occurrence
NOTCH1	TP53	61	4	367	37	0.300	Co-occurrence
PIK3CA	FAT1	205	169	55	40	0.337	Mutual exclusivity
NOTCH1	EP300	404	40	24	1	0.337	Mutual exclusivity
NFE2L2	FAT1	305	69	76	19	0.414	Co-occurrence
NOTCH1	FAT1	342	32	86	9	0.455	Co-occurrence
PIK3CA	NOTCH1	238	190	22	19	0.468	Co-occurrence
NFE2L2	EP300	360	84	21	4	0.480	Mutual exclusivity
NFE2L2	NOTCH1	347	81	34	7	0.482	Mutual exclusivity
EP300	TP53	62	3	382	22	0.535	Co-occurrence
FAT1	TP53	52	13	322	82	0.554	Co-occurrence
PIK3CA	EP300	246	198	14	11	0.562	Mutual exclusivity

Table 1: Co-occurrence of Commonly Mutated Genes in LSCC. Using cBioPortal 469 patient samples from the Pan-Cancer Atlas were queried for co-occurrence of six commonly mutated genes in benign premalignant LSCC lesions: *FAT1, EP300, NFE2L2, NOTCH1, PIK3CA,* and *TP53.*

The general aim of our research was to investigate which genes are mutated in premalignant tumors that cause the transformation to malignancy in LSCC. Six

commonly mutated genes in benign premalignant LSCC lesions were investigated. These genes, *FAT1, EP300, NFE2L2, NOTCH1, PIK3CA,* and *TP53*, when mutated are all considered "first hits". Mutations of these genes are not sufficient to cause tumorigenesis on their own. Our research seeks to answer if two of these common mutations are combined in HBECs, is the "second hit" enough to initiate increased cell proliferation, attachment independent growth, inhibition of autophagy, and other hallmarks of tumorigenesis? Using the computational bioinformatics web site, cBioPortal, we discovered which of the fifteen pairs of genes co-occurred in human LSCC by querying a data set of 469 patient samples from the Pan-Cancer Atlas (TCGA). The TCGA is the product of a collaboration between the National Cancer Institute and the National Human Genome Research Institute. By analyzing over 11,000 tumors from common types of cancer the TCGA seeks to further explain the molecular etiology of tumor formation. As shown in Table 1, of the 15 pairs of genes we queried, only two gene pairs displayed statistically significant co-occurrence, as defined by a Fisher exact test with p-value ≤ 0.05[32] (*NFE2L2/PIK3CA* and *PIK3CA/TP53*). We chose to follow up on PI3KCA/NFE2L2 mutations because both are oncogenic and have been implicated in the control of the autophagy pathway. Out of the samples queried, 88% of the *NFE2L2* mutations and 89% of the *PIK3CA* mutations are activating. The remainder are unknown but are predicted to also be activating.[32] As discussed earlier autophagy has been shown to play a tumor suppressive role at the premalignant stages of tumorigenesis. When autophagy is suppressed by oncogenes such as PI3K, DNA damage-associated mutations

may accumulate leading to initiation of tumor growth. The nature of autophagy in cancer is bipolar and in later stages of tumor growth autophagy is often upregulated past basal levels to promote cell proliferation and the epithelial-mesenchymal transition. Our research focuses on determining how *NFE2L2* and *PIK3CA* mutations interact to inhibit autophagy, which will shed light on mechanisms by which these oncogenes promote tumor initiation.[33] In addition, abnormalities in tumor suppressor genes are not yet clinically applicable in treatment of LSCC and we sought to keep our research clinically relevant, we decided to focus on oncogenes only.[2] This eliminated *TP53-PIK3CA* from consideration for our research.

To test the effect of the co-occurrence of *PIK3CA* and *NFE2L2* we transduced a constitutively active *PIK3CA (E545K)* mutant gene into a cell line previously transduced with a constitutively active mutant form of *NFE2L2 (T80K).* The oncogenic variants of these genes we used for transduction are the same as those found in the TCGA database. In addition, *HcRED* was also transfected into the *NFE2L2* cell line as a positive control. An HBEC line containing just *HcRED* was used as the negative control. Another cell line containing *PIK3CA* alone was used to compare the effects of *PIK3CA* alone with the effects of *PIK3CA + NFE2L2.* Proteins from all four cell lines were harvested for Western blot analysis. A figure comparing the concentration of various proteins between the cell lines is shown below.

Western Blots

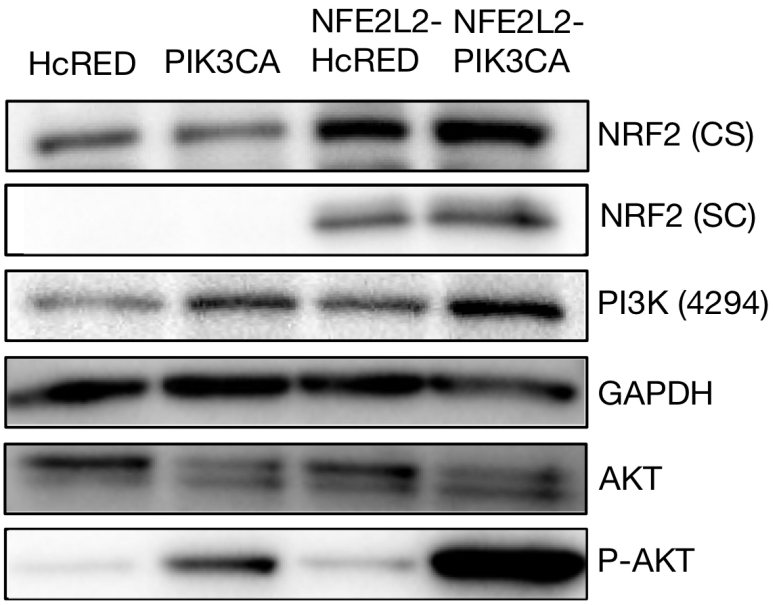


Figure 7: Western Blot analysis showing indicated protein levels following transduction of *HcRED* and *PIK3CA* lentiviral vectors into existing HBEC-*NFE2L2* cell lines. This Western blot shows the difference in protein levels between the four cell lines for the protein products of the genes being investigated (NRF2 and PI3K) as well as AKT, a downstream target of PI3K. Two antibodies with different molecular weights for NRF2 were used.

To begin to investigate the possible cooperation between oncogenic *NFE2L2* and *PIK3CA* mutations suggested by the results in Table 1, we performed a preliminary Western Blot to in order to compare protein levels between the four cell lines. The protein products, NRF2 and PI3K, of the genes being investigated were probed for to see if their expression increased in the presence of the second oncogene. In addition, AKT and P-AKT were also probed for. AKT is a downstream target of PI3K. When PI3K is constitutively activated levels of P-AKT is elevated. By probing for AKT and phospho-

AKT we sought to answer whether the mutant *NFE2L2* gene would augment phospho-AKT levels in the dual mutant.

Two NRF2 antibodies were used: one high molecular weight made by Cell Signaling (CS) and one low molecular weight made by Santa Cruz Biotechnology (SC). The high molecular weight NRF2 antibody showed the presence of basal NRF2 expression in the HBEC-*HcRED* and HBEC-*PIK3CA* lines. Both antibodies showed increased expression of NRF2 in the *NFE2L2* transduced lines. The high molecular weight NRF2 antibody showed slightly increased expression of NRF2 in the HBEC-*NFE2L2*+*PIK3CA*. Probing with the PI3K antibody showed elevated levels of PI3K expression in the HBEC-*PIK3CA* and HBEC-*NFE2L2*+*PIK3CA* above the basal PI3K expression shown in HBEC-*HcRED*. Equal amounts of total AKT, which includes both the phosphorylated and non-phosphorylated forms of AKT, were found in each cell line. The GAPDH antibody was used as a loading control and showed that the total protein in each sample loaded into the gel was relatively equal. (Figure 7)

This experiment confirmed that the oncogenic *NFE2L2* and *PIK3CA* cooperate. However, it does not answer how they cooperate. We hypothesized that one way they might cooperate is through the process of autophagy. In the Western Blot below in Figure 8 we investigated the cooperation of *NFE2L2* and *PIK3CA* on reducing autophagy by probing for three key autophagy components.

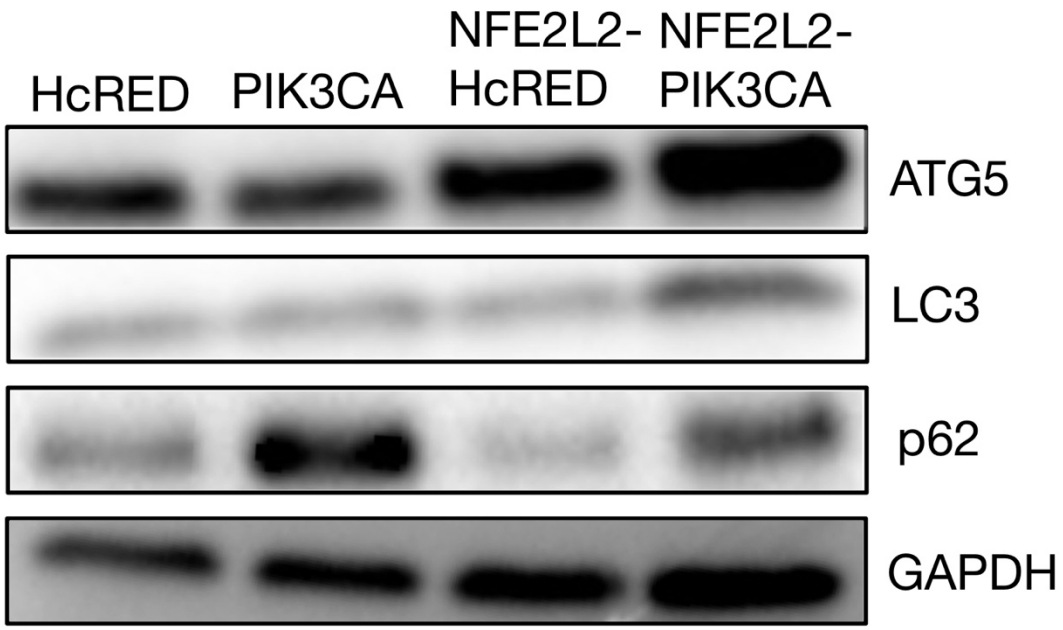


Figure 8: Western Blot Analysis of Autophagy Markers. Western blots showing the protein levels in each cell line for autophagy pathway components: ATG5, LC3, and p62. As these proteins are degraded in the autolysosome when autophagy is activated, higher levels of these proteins indicates inhibition of autophagy.

Autophagy is one known cellular process that both the PI3K/AKT/mTORC1 and NRF2/KEAP1 signaling pathways are known to converge on. Reduction in autophagy is thought to be a key step for the malignant transformation of pre-cancerous LSCC lesions. We performed this second western blot to investigate if autophagy may be a process in which NRF2 and PI3K act synergistically to inhibit autophagy and push pre-cancerous LSCC to metastasis. Three key markers of autophagy were probed for: LC3, p62, and ATG5. When autophagy is active these markers are degraded along with the rest of the

contents of the autolysosome, keeping their concentration at a relatively low level. When autophagy is inhibited these markers accumulate to high levels in cells.

The highest levels of ATG5 and LC3 were found in the HBEC-*NFE2L2-PIK3CA* dual mutant. Levels of ATG5 were approximately equal in the HBEC-*HcRED* and HBEC-*PIK3CA* samples but elevated from basal levels in HBEC-*NFE2L2-HcRED* samples . Levels of LC3 were similar between HBEC-*HcRED,* HBEC-*PIK3CA,* and HBEC-*NFE2L2-HcRED* samples. Levels of p62 were highest in the HBEC-*PIK3CA* sample. Compared to basal p62 expression levels as shown by the HBEC-*HcRED,* p62 expression was elevated above basal levels in the HBEC-*NFE2L2-PIK3CA* sample but was below basal levels in the HBEC-*NFE2L2-HcRED* sample. GAPDH was used as a loading control and showed the amount of protein loaded in each sample to be similar. (Figure 8)

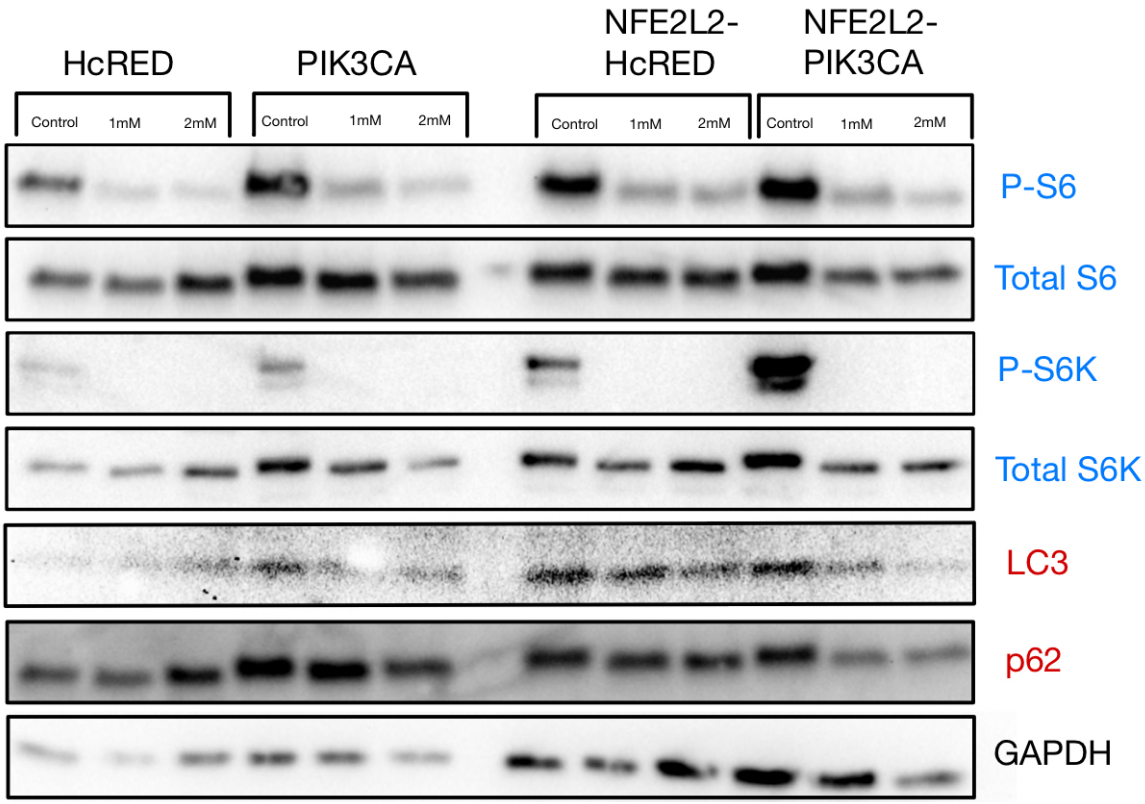


Figure 9: Western Blot Analysis of Cell Lines Treated with Everolimus. Blue text: Western Blot analysis of the effects of mTORC1 inhibition on expression levels of downstream mTORC1 targets across the four cell lines. Red text: Western Blot analysis of the effects of mTORC1 inhibition on select autophagy pathway components.

Autophagy is controlled my mTOR. After we observed a significant reduction of autophagy in the dual mutant HBEC line in Figure 8, we wanted to know if the cooperation in reducing autophagy between oncogenic *NFE2L2* and *PIK3CA* mutants could be reversed. To test this we treated the cells with the mTORC1 inhibitor, Everolimus. Samples were harvested for a Western Blot 48 hours post treatment. If the pathways did cooperate as we observed and hypothesized we would expect to see a near

complete restoration of autophagy in the dual mutant HBEC line, despite mTORC1 inhibitors being only know to interrupt constitutive PI3K activation and not NRF2 activation. In order to compare mTOR regulation across the four cell lines we needed to first confirm mTORC1 inhibition by establishing controls. To verify mTORC1 inhibition by Everolimus, antibodies for total and P-S6K and antibodies for the total and phosphorylated form of P-S6K's main substrate S6, a component of the 40S ribosome, were used. S6K is a major target of mTORC1 and therefore levels of P-S6K are a key indicator of mTORC1 activity. In addition, rapamycin, another mTORC1 inhibitor, has been shown to inhibit mitogen induced activation of S6K by preventing phosphorylation of Thr389 on S6K. Both Rapamycin and Everolimus inhibit the raptor scaffold protein, the binding site of S6K to the mTOR complex. This prevents mTORC1 from binding substrates such as S6K and mediating phosphorylation of rapamycin-sensitive sites. The mTORC1-S6K axis plays an important role in ribosome biogenesis, adipocyte differentiation, GSK3 regulation, and cell survival & motility.[34]

For each sample levels of phosphorylated S6 (P-S6) protein were highest in the control samples not treated with Everolimus. Levels of P-S6 were all elevated from the baseline established by HBEC-*HcRED* in the control, 1mM and 2mM Everolimus treated samples for the *PIK3CA, NFE2L2,* and dual mutant cell lines. For the *PIK3CA* and dual mutant line the samples showed a clear dose dependency as the 2mM Everolimus treated cells had reduced P-S6 expression compared to the 1mM treated cells. Total S6 protein was probed for and gave validity to our results for P-S6 by establishing the samples were

equally loaded and contained equal amounts of S6 protein. Phosphorylated S6 Kinase (P-S6K) levels were highest in the dual mutant cell line and elevated from the baseline shown in *HcRED* in the *PIK3CA* and *NFE2L2* transfected lines. In each cell line P-S6K expression was only observed in the control samples and not in the samples treated with Everolimus suggesting that Everolimus at both 1mM and 2mM concentrations is sufficient to prevent phosphorylation of S6K. Probing for total S6K showed that the observed reduced P-S6K levels are due to the inability of mTORC1 to phosphorylate S6K and not lack of S6K protein as the levels in each sample are comparable. (Figure 9). With these results we confirmed Everolimus was an effective mTORC1 inhibitor in our HBEC lines. This allowed us to proceed to investigating the effects of Everolimus treatment on autophagy activation in the dual mutant by probing for LC3 and p62.

LC3 levels were low in the *HcRED* line and equal between the control, 1mM, and 2mM Everolimus treated line. The *PIK3CA* transfected cells exhibited increased LC3 levels in the control sample from baseline and also exhibited reduced expression in samples treated with Everolimus. However there does not appear to be a dose dependency as there is not a difference in expression in the *PIK3CA* line between the 1mM and 2mM Everolimus treated samples. Both the *NFE2L2* and dual mutant line had high LC3 expression in their control samples. Levels in the *NFE2L2* line did not exhibit a response to Everolimus as the LC3 level shown on the Western Blot was similar between the control, 1mM, and 2mM treated samples. The dual mutant showed a dose-dependent

response to Everolimus with the greatest reduction in LC3 expression in the 2mM treated line.

The *PIK3CA*, *NFE2L2*, and the dual mutant showed an increase in p62 levels compared to baseline in the control sample. In the *PIK3CA* transfected line reduced p62 levels are seen in the sample treated with 2mM Everolimus but not the sample treated with 1mM Everolimus. The *NFE2L2* transfected line showed equal p62 levels across the three samples. The dual mutant showed decreased levels of p62 following treatment with Everolimus but the level of p62 detected was equal between the 1mM and the 2mM Everolimus treated samples indicating that the response in this line is not dose dependent. Probing with GAPDH indicated the protein levels across all twelve samples were comparable. (Figure 9)

An increased in LC3 and p62 protein levels indicates an inhibition of autophagy at the stage of autophagosome-to-autolysosome maturation because both proteins are degraded in the autolysosome. The reduction of p62 and LC3 levels following treatment with Everolimus observed in Figure 9 indicates the restoration of the autophagy pathway.

IC50 of Everolimus

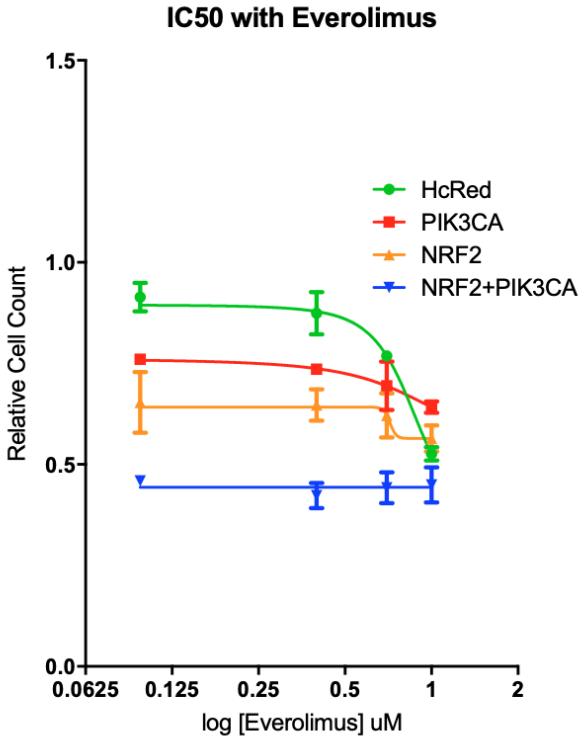


Figure 10: Normalized Cell Count vs. Concentration of Everolimus. IC50 of Everolimus is shown showing the differing sensitivities of each cell line to mTORC1 inhibition.

In addition to measuring the effects of Everolimus on levels of key autophagy proteins using Western Blots, we also calculated the half maximal inhibitory content, IC50, of Everolimus for each cell line. IC50 indicates how much of a drug inhibitor is needed to reduce the targeted biological function by 50%. Therefore lower the IC50 confers greater sensitivity to a drug. It is important to investigate whether Everolimus had the greatest effect on the dual mutant HBEC line. This would support our hypothesis of cooperation between the oncogenic *NFE2L2* and *PIK3CA* mutations as well as have important implications for treatment of LSCC tumors with these mutations.

As hypothesized, Everolimus had the biggest effect as shown by the lowest IC50, on the HBEC-*NFE2L2-PIK3CA* line. Extrapolation from our results showed the IC50 of Everolimus for the HBEC-*NFE2L2-PIK3CA* line to be 0.46 µM. However these results are ambiguous as there is no intersection point for 50% as this cell line displayed maximal inhibition at 0.625µM, the lowest drug concentration we investigated. The IC50 for HBEC-*NFE2L2-HcRED* was extrapolated to be 5.21 µM but these results were also ambiguous. The IC50 for HBEC-*HcRED* and HBEC-*PIK3CA* were 6.77 µM and 6.07µM respectively. (Figure 10)

Fluorescence Assays

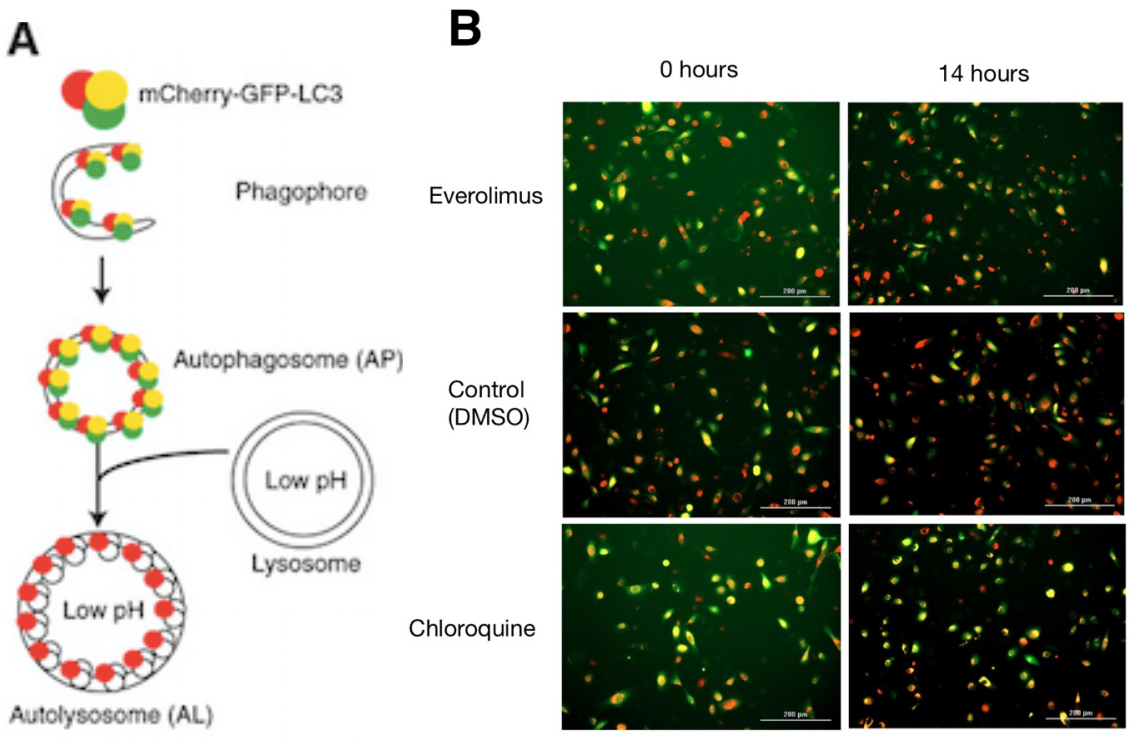


Figure 11: Fluorescent Assay for Autophagy. Panel A: Schematic representation of the indications of fluorescent colors. The engineered mCherry-GFP-LC3 appears yellow before fusion of the autophagosome and lysosome as the red and green fluorescence mix. After fusion GFP is degraded due to the high acidity of the autolysosome and only red fluorescence is detected. **Panel B: Representative data of Fluorescent assay for HBEC cells expressing GFP mCherry.** HBEC-*HcRED* cells were treated at indicated agents at indicated time points.

To assay autophagy, we transduced an engineered form of LC3 into the cell lines. The LC3 fusion protein we used contains both GFP and mCherry. Fluorescence from GFP and mCherry are able to be read using the BioRad cytation imaging reader. Since the fluorescence from GFP is quenched under acidic conditions, when only red fluorescence from mCherry is present it indicates the presence of an autolysosome and the activation of autophagy. (Figure 11A)[31] On the other hand, the presence of yellow fluorescence, the mixture of green and red fluorescent signals, indicates the autophagosome has not fused with the lysosome. Accumulation of the yellow fluorescent signal suggests there is a block in autophagy.[35]

As in previous experiments we then treated the cells with Everolimus and the control (DMSO). In addition, we also treated with chloroquine. Chloroquine is an autophagy inhibitor that acts by deacidifying the lysosome and preventing fusion with the autophagosome thereby preventing formation of autolysosomes.

Upon a 14 hour incubation with Everolimus, there is reduced green fluorescence in the *HcRED* line. The cells treated with DMSO showed a slight decrease in green

fluorescence after 14 hours. Compared to the control the cells treated with chloroquine have increased green fluorescence, even at time point zero. After the fourteen hour incubation, the cells treated with chloroquine have a slightly diminished green fluorescence from time point zero but it is still greatly elevated compared to the control treated cells. (Figure 11B)

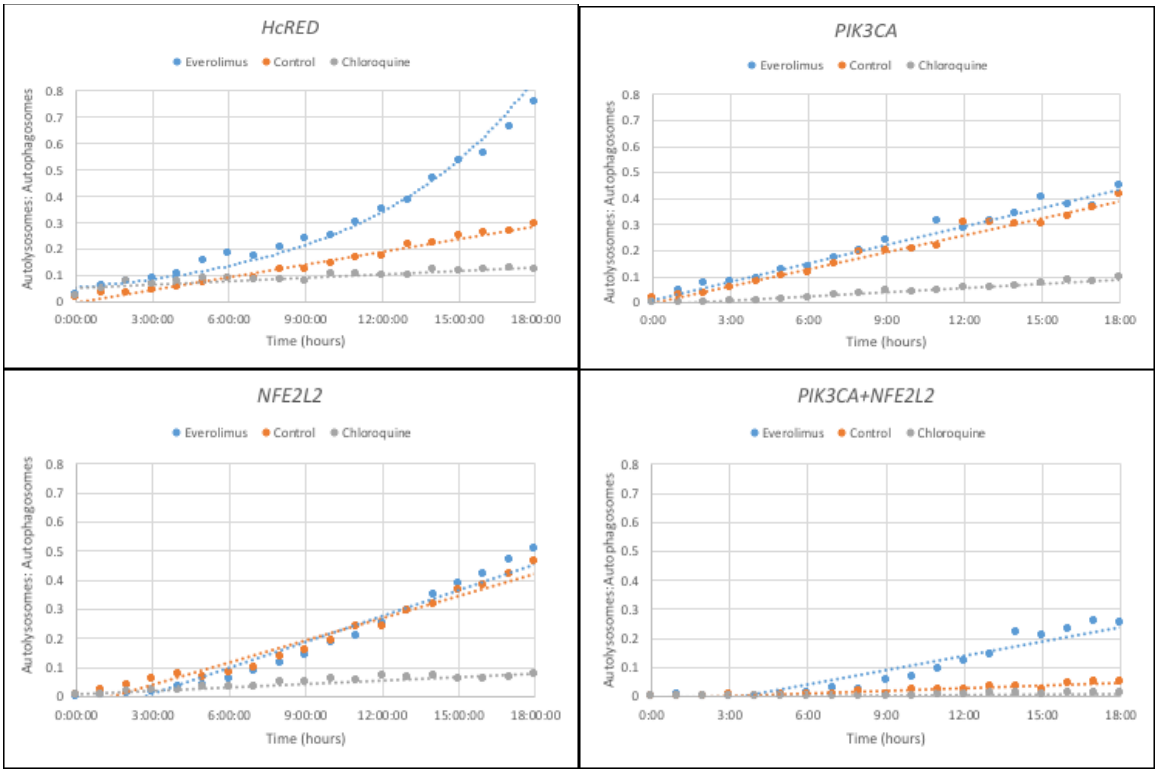


Figure 12: Autolysosome to Autophagosome Ratio in Everolimus, Control, and Chloroquine Treated HBEC Cells over time. Using live cell imaging fluorescence from the GFP-mCherry labeled LC3 was used to quantify the autolysosomes and autophagosomes and obtain the ratios used. From this conclusions about autophagy in the four cell lines could be made.

Using the same fluorescent technology as in Figure 11B, we assayed autophagy by counting the yellow (mixture of red and green puncta) and the red puncta alone. As discussed earlier presence of red puncta alone is indicative of activation of autophagy while yellow puncta is indicative of inhibition of autophagy. From these counts the autolysosome: autophagosome ratios were calculated. A greater ratio suggests a high level of autophagy while a ratio close to zero suggest autophagy inhibition.

In the *HcRED* cell line there was an exponential increase in the ratio of autolysosomes to autophagosomes upon treatment with Everolimus. There was also a notable linear increase in the ratio over time in the control treated cells and a very slight increase in the chloroquine treated cells. In the *PIK3CA* line there was only a slight difference in autolysosome:autophagosome ratio between the Everolimus and the control treated cells, though the ratio did increase over time in both treatments. The ratio in the chloroquine treated cells slightly increased over time. In the *NFE2L2* line there is again very little difference in autolysosome:autophagosome ratio between the Everolimus and the control treated cells and the ratio in the chloroquine treated cells slightly increased over time. In the dual mutant cell line there is a significant increase in the autolysosome:autophagosome ratio in the Everolimus treated cells over time. However there is only a slight increase in the ratio in the control treated cells. In the chloroquine treated cells there is little change over time and the ratio remains close to zero indicating that autophagosomes greatly outnumber autolysosomes. (Figure 12)

Clonogenic Proliferation Assay

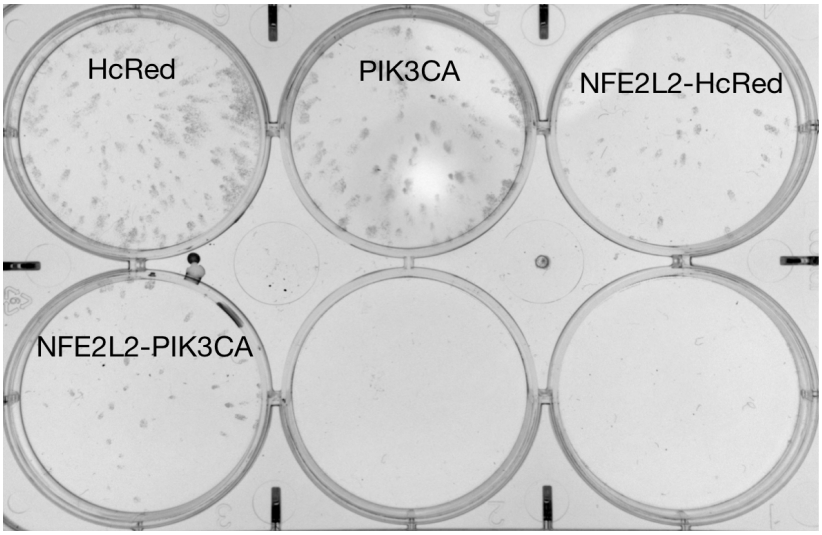


Figure 13: HBEC Clonogenic Proliferation Assay. Cells from the four lines were plated at 100 cells/mL and allowed to grow for 72 hours. The wells were then fixed and stained to observe colony growth. Proliferation in *NFE2L2-HcRED* and *NFE2L2-PIK3CA* lines were drastically reduced compared to the control, *HcRED,* and the *PIK3CA* line.

Early on in our investigation we performed a Clonogenic Proliferation Assay to determine the effect of *PIK3CA* and *NFE2L2* on proliferation. It is well known that the PI3K/AKT/mTOR signaling network is a major player in cell growth and proliferation and often involved in tumorigenesis. We hypothesized when *PIK3CA* was transduced into the existing HBEC-*NFE2L2* line, we would see increased cell prolifertation. However, our results were perplexing. (Figure 13) It has been well documented that the PI3K/AKT/mTORC1 axis increases cell proliferation. We expected the overexpression of NRF2 and PI3K would work synergistically to further increase cell proliferation considering both work to augment the pentose phosphate pathway which provides

necessary materials for cell growth and division as well as promote metabolic reprogramming to shunt glucose into anabolic pathways. In addition, research by Mitsuishi et al. have shown that in the presence of enhanced proliferation by active PI3K/AKT/mTORC1 signaling, NRF2 augments the expression of metabolic genes that lead to cell proliferation.[2] Confoundingly, our proliferation assays showed reduced proliferation in both *NFE2L2* transfected cell lines compared to HBEC-*HcRED* and HBEC-*PIK3CA*. Not only did the overexpression of NRF2 suppress proliferation in the HBEC-*NFE2L2-HcRED* line but it seemed to counteract the proliferative effects of PI3K overexpression in the HBEC-*NFE2L2-PIK3CA* line.

DISCUSSION

Mechanism of Synergy

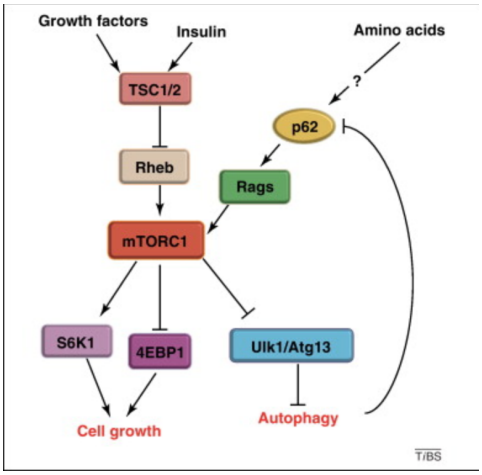


Figure 14: Schematic Representation of the Intersection of mTORC1 and p62. mTORC1 is a downstream target of the PI3K/AKT/mTOR signaling network while p62 transcription is controlled by NRF2. This schema shows a connection between the PI3K/AKT/mTOR and NRF2/KEAP1 pathways that could explain their observed cooperation.

Our research sought to show that NRF2/KEAP1 and PI3K/AKT/mTORC1 pathways act synergistically to promote cancer malignancy mainly through their dual inhibition of autophagy. Studies into the carcinogenesis of HPV showed that the expression of mTOR was positively correlated with p62, a key autophagy protein. It is thought that increased p62 levels may play carcinogenic role in the PI3K/AKT/mTORC1 by enhancing the activation of mTOR leading to inhibition of autophagy, increased proliferation, and diminished antitumor ability.[36] p62 is thought to be selectively required for amino acid activation of mTORC1 by binding to Rag GTPase and raptor. As mentioned earlier, p62's expression is enhanced by elevated NRF2 levels via a positive

feedback loop. By this mechanism, NRF2 increases activation of the PI3K/AKT/mTORC1 axis and enhances the known carcinogenic properties associated with this axis. (Figure 14)[37]

Evidence that NRF2 enhances the PI3K/AKT/mTORC1 axis was also found in research on PTEN and KEAP1 deficient mouse liver cells. In PTEN and KEAP1 deficient mouse liver cells AKT and the downstream target of AKT, glycogen synthase kinase 3 (GSK3), were highly phosphorylated indicating robust augmentation of the PI3K/AKT/mTORC1 axis. Interestingly, when NRF2 function was eliminated in these mouse models by knocking out *NFE2L2,* normal phosphorylation patterns were completely restored. These results suggest NRF2 activation enhances the PI3K/AKT/mTORC1 axis. On the other hand, activation of the PI3K/AKT/mTORC1 axis is also thought to stabilize NRF2. Phosphorylation by AKT inactivates GSK3. However, active GSK3 promotes degradation of NRF2 in a KEAP1 independent manner. Therefore, conditions of prolonged PI3K/AKT/mTORC1 axis activation promotes the accumulation of NRF2.[24] It is likely that both pathways act in a positive feedback loop with increases in one leading to increases in another and so on causing a rapid transition to malignancy.

Effect of Synergy on Other Cellular Processes

In addition to autophagy, another cellular process in which NRF2/KEAP1 and the PI3K/AKT/mTORC1 axis have a synergistic effect is via the pentose phosphate pathway. The pentose phosphate pathway generates ribose-5-phosphate for nucleotide synthesis

and NADPH which is used in both nucleotide and lipid synthesis. Both ribose-5-phosphate and NADPH are critical for highly proliferative cancer cells. In addition to activating cytoprotective genes, NRF2 acts as a transcription factor for genes involved in the pentose phosphate pathway and has been shown to promote and increase the synthesis of purine nucleotides. NRF2 accumulation and the PI3K/AKT/mTORC1 axis work synergistically to promote purine synthesis. mTORC1 serves to increase glucose uptake and glycolytic activity thereby increasing the supply of glycolytic intermediates to enter the pentose phosphate pathway. In addition, the downstream target of mTORC1, SREBP, regulates the enzymes of the oxidative arm of the pentose phosphate pathway, while NRF2 upregulates the enzymes of the pentose phosphate pathway.[38]

In addition, NRF2 also augments metabolic reprogramming in cells by activating metabolic genes leading to the redirection of glucose and glutamine into anabolic pathways. The effect of NRF2 on gene expression and metabolic activities are most notable in the presence of active proliferative signals such as that of the PIK3/AKT/mTORC1 axis. The sustained activation of the PIK3/AKT/mTORC1 axis creates a highly proliferative environment in the cell. The functional expansion of NRF2 in these cells enhances anabolism and further promotes the activation of the PIK3/AKT/mTORC1 axis. This suggests the presence of positive feedback between NRF2 and the PIK3/AKT/mTORC1 axis in proliferating cells. However, it should be noted that upregulation of NRF2 does not initiate tumorigenesis. Rather it confers advantages to the cell in terms of proliferation and stress resistance once a cell acquires

uncontrolled proliferative properties.[38] We observed reduced cell proliferation in both *NFE2L2* transfected lines suggesting that in the early stages of tumorigenesis NRF2 may actually antagonize cell proliferation.

Summary of Other Research of NRF2 and PI3K Cooperation

Experiments done in mouse models suggest synergy between the NRF2/KEAP1 and PI3K/AKT/mTORC1 in cancer formation. It was shown that inactivation of KEAP1 and PTEN promotes adenocarcinoma formation in the lung. Though TSGs were not explored in our experiments, like *NFE2L2* overexpression, KEAP1 inactivation also leads to constitutive activation of NRF2. Likewise, PTEN inactivation also leads to constitutive activation of PI3K. The loss of *KEAP1* alone causes no morphological changes. Therefore cooperation with loss of PTEN or activation of the PI3K/AKT/mTORC1 axis is necessary for tumorigenesis.[2]

Perturbed PI3K signaling has also been shown to increase NRF2/KEAP1 pathway activation. NADPH Dehydrogenase (NQO1) is a key enzyme involved in the body's antioxidant response and its transcription is controlled by NRF2. Therefore NQO1 levels are used as biomarkers for NRF2 activation. In PTEN and KEAP1 deficient mice there was an synergistic increase in NQO1 compared to mice models with the loss of only one of these genes. This suggests that loss of *PTEN* increases NRF2 function in the lung by enhancing NRF2 stability independent of KEAP1.[38]

Interpretation of Results

Western Blots for NRF2, PI3K and Downstream Targets

The high molecular weight NRF2 antibody showed slightly increased expression of NRF2 in the HBEC-*NFE2L2+PIK3CA* line supporting our hypothesis that NRF2 and PI3K act synergistically. (Figure 7) In addition, probing with the PI3K antibody showed elevated levels of PI3K expression in the HBEC-*NFE2L2+PIK3CA* compared to HBEC-*PIK3CA* and basal PI3K expression. (Figure 7) This suggests that both pathways act upon each other to increase expression. The strongest evidence from this western blot to support the cooperation between NRF2 and PI3K, specifically the enhancement of the PI3K/AKT/mTORC1 axis by NRF2, is the expression of phospho-AKT (p-AKT). (Figure 7) AKT is a downstream target of PI3K and its phosphorylation is increased with elevated PI3K activity. Not only is p-AKT expression increased from basal levels in the HBEC-*PIK3CA* line but p-AKT is by far the highest in HBEC-*NFE2L2+PIK3CA*. This indicates that NRF2 via one of its effectors acts upstream of AKT to increase phosphorylation and therefore increase the detectable amount of p-AKT. Total AKT, which includes both the phosphorylated and non-phosphorylated forms of AKT, was also probed to verify that the concentration of total AKT was the same across the four cell lines and to give validity to our results for p-AKT. (Figure 7)

As discussed earlier the synergy between constitutively active NRF2 and the PI3K/AKT/mTORC1 axis can be explained by the enhanced transcription of p62 which activates mTORC1 via the same mechanism as nutrient sensing. However AKT is upstream of mTORC1 and its enhanced phosphorylation in the presence of constitutively active PI3K and NRF2 can not be explained by this pathway. Investigations exploring the

role of hepatomegaly, the abnormal enlargement of the liver, suggest NRF2's transcriptional control over EGFR ligands, as a possible explanation for the elevated p-AKT levels we observed.[39] Upon binding to their ligand EGFR dimerize and activate PI3K which leads to downstream phosphorylation of AKT. Increased transcription of EGFR ligands lead to increased EGFR dimerization and PI3K activation. Due to the compounding effect of signal transduction even a small increase in PI3K can lead to big increase in activation of downstream targets explaining the huge increase in p-AKT when constitutively active PI3K and NRF2 are combined. As mTORC1 is a downstream target of PI3K/AKT, increased transcription of EGFR ligands by constitutively active NRF2 is another mechanism by which NRF2 acts synergistically with PI3K to inhibit autophagy.

Western Blots for Autophagy Markers

As shown in Figure 8, ATG5 levels were not elevated from basal levels shown by HBEC-*HcRED* in the sample taken from HBEC-*PIK3CA* line but were elevated in the HBEC-*NFE2L2-HcRED* sample. ATG5 levels were the highest in HBEC-*NFE2L2-PIK3CA* indicating that NRF2 and PI3K act synergistically to inhibit autophagy as the increase in ATG5 levels cannot be explained by summing the increase in ATG5 levels in HBEC-*PIK3CA* and HBEC-*NFE2L2-HcRED* since the level of ATG5 observed in HBEC-*PIK3CA* is not increased from baseline. The unchanged levels of ATG5 in cells only transfected with *PIK3CA* does not reflect autophagy activation in this line because the PI3K/AKT/mTORC1 axis mainly inhibits autophagy via phosphorylation of ATG13 and ULK1. This prevents the initial phagophore formation before the conjugation of

ATG5-ATG12. The antibody for ATG5 is for the ATG5-ATG12 and therefore does not provide a good marker for autophagy in the *PIK3CA* line. The increased ATG5 levels in the HBEC-*NFE2L2+PIK3CA* but not HBEC-*PIK3CA* can be explained by the PI3K/AKT/mTORC1 axis enhancing NRF2 activation by phosphorylating GSK3 thus preventing NRF2 degradation.

Levels for LC3 in Figure 8 were only found to be elevated in the HBEC-*NFE2L2+PIK3CA* dual mutant line. LC3 transcription is enhanced by high ROS levels.[26] The LC3 antibody used was for the LC3B-II, the processed form of LC3. The processing of LC3 to LC3B-II is dependent on autophagy activation and the formation of the ATG5-ATG12 complex. Though autophagy may be inhibited from basal levels in the HBEC-*PIK3CA* line, like with our results for ATG5 it is not observed because the PI3K/AKT/mTORC1 inhibits autophagy pre-ATG5-ATG12 complex formation. Since constitutive activation of NRF2 drastically reduces ROS levels, LC3 transcription is reduced. This explains why LC3 levels in the HBEC-*NFE2L2-HcRED* samples are not elevated from baseline even though we expect autophagy to be inhibited in this line. LC3 levels are elevated in the HBEC-*NFE2L2+PIK3CA* dual mutant samples. This result indicates that the inhibition of autophagy when both NRF2 and PI3K are constitutively active is greater than when either mutant protein is present on its own thus supporting our hypothesis that NRF2 and PI3K act synergistically.

Our results for p62 in Figure 8 were unexpected. While p62 accumulation was observed in the lines transfected with *PIK3CA* indicating reduced autophagy, its expression was reduced in lines transfected with *NFE2L2*, with its expression being lower in the HBEC-*NFE2L2-HcRED* line than even the control, HBEC-*HcRED.* In addition, p62 levels in HBEC-*NFE2L2+PIK3CA* were lower than in HBEC-*PIK3CA* suggesting that NRF2 expression counteracts the effect of the PI3K/AKT/mTORC1 axis on p62 accumulation. As discussed in the introduction section, this is surprising as p62 is a transcriptional target of NRF2. Increased NRF2 levels should increase p62 expression. One possible explanation for our observations is the interaction between KEAP1 and p62. Studies by Tian and Liu have shown that NRF2 activity is positively correlated with KEAP1 mRNA expression in squamous cell carcinomas. By using NRF2 activating drugs tBHQ and dimethylformamide on NRF2 deficient human lung cell line H292, Tian and Liu showed that KEAP1 mRNA expression increased. They also showed that KEAP1 mRNA expression is downregulated when NRF2 addicted human lung carcinoma line A549 is treated with NRF2 siRNA. The proposed mechanism for NRF2-driven transcription of KEAP1 is an ARE-sequence in KEAP1's promoter region.[40,41] Since p62 binds to KEAP1 and leads to the degradation of the complex, this would lead to decreased p62 levels in the *NFE2L2* transfected lines due to the increase in KEAP1 for p62 to bind to.

Western Blot Analysis After Treatment with Everolimus

P-S6 is a downstream target of mTORC1 and as expected we observed decreased expression in the presence of a mTORC1 inhibitor. In Figure 9, we observed increased P-S6 expression in the control samples of the *PIK3CA, NFE2L2,* and dual mutant lines. Since both the *PIK3CA* and the dual mutant contain an oncogenic, constitutively active form of PI3K elevated P-S6 levels were expected. This also explains the dose dependency observed in these samples in Figure 9. With more mTORC1 activated by the mutant PI3K, more Everolimus is needed to inhibit the additional complexes and reduce P-S6 levels. However, the elevated P-S6 levels were not expected for the sample from the *NFE2L2* transfected line. This result can be explained by the cooperation of the NRF2/KEAP1 and PI3K/AKT/mTORC1 pathways via p62. Constitutively active NRF2 leads to increased transcription of p62. After translation, p62 can activate mTORC1 and lead to increased P-S6 levels.

Expression of P-S6K, another downstream target of mTORC1, is reduced beyond detectable levels in all Everolimus treated samples in Figure 9. We also observed increased P-S6K expression in the control samples of the *PIK3CA, NFE2L2,* and dual mutant lines in this figure. The highest P-S6K level was observed in the control sample of the dual mutant, supporting the proposed synergy between the NRF2/KEAP1 and PI3K/AKT/mTORC1 pathways. The elevated levels of P-S6K in the dual mutant and *NFE2L2* line can again be explained by the linkage of the two pathways through p62.

As expected, LC3 levels were low in the three *HcRED* samples in Figure 9. Autophagy is unaffected in this line therefore treatment with Everolimus cannot act to

restore it. In the *PIK3CA* sample LC3 levels are elevated in the control sample and reduced in the Everolimus treated samples. These results differ from our results from the Western Blot for autophagy markers (Figure 8) that did not show increased LC3 levels in the *PIK3CA* line. One possible explanation is that we performed the Everolimus Western Blot months after the one performed for the autophagy markers. Oncogenes such as *PIK3CA* are toxic to cells. Increased activity of PI3K or its downstream targets may activate pathways in the cell to counteract the upregulation in the PI3K/mTORC1/AKT axis. For example, increased levels of PIP3, the product of PI3K, recruits more PTEN to the plasma membrane causing downregulation of the PI3K/mTORC1/AKT axis.[42] In addition, studies have shown that PTEN not only prevents AKT from being phosphorylated but also participates in the complex that marks P-AKT for degradation when P-AKT levels are high. However, the protective capacity of these cellular feedback mechanisms are finite. Eventually the constitutive activation of the PI3K/AKT/mTORC1 pathway will exceed the buffering ability of the cell. Perhaps enhanced PI3K signaling was buffered in earlier passages and autophagy remained active, preventing the accumulation of LC3 as seen in the Western Blot for the autophagy markers. A Western Blot for LC3 should be repeated to confirm our results. LC3 levels are also elevated in *NFE2L2* transfected line and do not vary between the control, 1mM and 2mM Everolimus treated cells as shown in Figure 9. This shows that mTORC1 inhibition has no effect on mitigating autophagy inhibition in this line. Since NRF2 inhibits autophagy independently from mTORC1 by removing ROS, we expected Everolimus to have little

effect on the *NFE2L2* cell line. Interestingly, in the dual mutant line LC3 is elevated in the control, reduced in the 1mM Everolimus sample, and further reduced to near basal levels in the 2mM sample. The LC3 level in the dual mutant 2mM treated sample is lower than in *PIK3CA* suggesting that transfection with both *NFE2L2* and *PIK3CA* oncogenes makes the cell more sensitive to mTORC1 inhibitors which could have important clinical implications. The reason for this increased sensitivity could lie in the p62 linkage between the two pathways. p62 is a transcriptional target of NRF2 but p62 also enhances transcription of NRF2 and activates mTORC1. Upon treatment with Everolimus, mTORC1 inhibition of autophagy is removed. Without input from this major player, autophagy is at least partly restored and a considerable amount of p62 is degraded in the autolysosome. This has major effects on activating autophagy as not only is transcription of NRF2 is reduced but activation of mTORC1 by p62 is also reduced.

This explanation is supported by our Western Blot for p62 that shows reduced p62 levels in the 1mM and 2mM Everolimus treated samples. (Figure 9) The Western blot for p62 also shows the *NFE2L2* transfected line is resistant to mTORC1 as the p62 levels were elevated from baseline but unchanged across the three sample conditions. The *PIK3CA* transfected line showed reduced p62 levels in only the 2mM but not the 1mM Everolimus treated line suggesting dose dependence.

Everolimus IC50

By calculating the IC50 of Everolimus on each cell line, we determined that HBEC-*NFE2L2*-*PIK3CA* had the greatest sensitivity to mTORC1 inhibition while the

control HBEC-*HcRED* had the least. (Figure 10) From these results we can conclude that NRF2 and PI3K act synergistically on the cell cycle and proliferation since the Everolimus had by far the greatest effect on reducing proliferation in the HBEC-*NFE2L2-PIK3CA* line.

Response of HBEC-*HcRED* cells to Everolimus, DMSO, and Chloroquine

Despite being unable to acquire clear images for the *PIK3CA, NFE2L2* or dual mutant lines due to oncogenic toxicity, the images acquired for *HcRED* exemplify the expected effect of these drugs on HBEC. In the Everolimus treated cells displayed in Figure 11, there is significant yellow fluorescence at time point zero. After a fourteen hour incubation, the green fluorescence is quenched and more cells appear red while less cells appear yellow. This indicates that Everolimus is activating autophagy as there are less autophagosomes present as shown by reduced yellow fluorescence. Presumably, more autophagosomes are fusing with lysosomes to become autolysosomes as there appears to be more cells that appear only red.

In the control (DMSO) treated cells there is a slight decrease in green fluorescence after a 14 hour incubation as compared to time point zero. (Figure 11) This is an unexpected result as we predicted there should be no change in autophagy in the control cells over time. The green fluorescence from GFP has been known to degrade over time and presents a confounding effect on interpreting the significance of our results. In addition, we did not change the media on the wells before beginning this

experiment. It is possible that overtime the cells became nutrient deprived and the reduction in green fluorescence is a result of the initiation of autophagy.

The chloroquine treated cells display increased yellow fluorescence even at time point zero in Figure 11. As expected, chloroquine potently inhibited autophagy as the accumulation of yellow fluorescence indicates the failure of the autophagosome and lysosome to fuse. However, after the fourteen hour treatment the yellow fluorescence is slightly decreased. Again, this is likely due to the degradation of the green fluorescent signal or an induction of autophagy due to unintentional nutrient deprivation.

Autolysosome to Autophagosome Ratio in Everolimus, Control, and Chloroquine Treated Cells

As shown in Figure 12, Everolimus treated cells in the *HcRED* line has an exponential increase in the autolysosome:autophagosome over time indicating that autophagy is overactivated. In the absence of an autophagy inhibiting oncogene, induction of autophagy by Everolimus has the greatest effect on the *HcRED* line as there is no counteraction by the products of mutant *PIK3CA* or *NFE2L2.* (Figure 12) In the *PIK3CA* and *NFE2L2* HBEC lines there is elevated autolysosome:autophagosome compared to the dual mutant. However, in the dual mutant the increase in the ratio in the Everolimus treated cells is significantly elevated about the increase in the ratio in the control cells while the increase in the ratio over time is almost equal between the Everolimus and control treated cells as shown in Figure 12. Though the dual mutant does not have a high baseline autolysosome:autophagosome compared to the *PIK3CA* and *NFE2L2* lines, the increase in the ratio in the Everolimus treated cells from the control

indicate these cells are more sensitive to mTORC1 inhibitors. It is also interesting that the increase in autophagy in these cells does not become quantifiable in Figure 12 until approximately 4-5 hours after treatment with Everolimus. This is consistent with our understanding that mTORC1 inhibitors change transcriptional activity in the cell and therefore their effects have a significant lag time post treatment.

The dual mutant has the greatest inhibition of autophagy under control conditions as shown by its low autolysosome:autophagosome in the DMSO treated cells which suggests an accumulation of autolysosomes. However, there is a slight increase in the ratio in the control treated cells of the dual mutant. In the other cell lines this increase is even more pronounced. (Figure 12) This is likely attributable to activation of autophagy caused by nutrient depletion. The increase in autophagy is more observable in the *HcRED, PIK3CA,* and the *NFE2L2* than the dual mutant because of confluence of the cell lines. Despite allowing the cells to incubate for 48 hours after plating, the dual mutant cells did not grow to confluence likely due to reduced proliferation from the combined oncogenic toxicity from *PIK3CA* and *NFE2L2.* Due to the differences in confluence the cell lines had different nutritional needs and autophagy was activated sooner in the control treated cells of the more confluent lines.

The autolysosome:autophagosome in chloroquine treated cells starts close to zero in all the cell lines indicating a stark inhibition of autophagy. However, in all the cell lines despite the dual mutant there is a gradual increase in autolysosome:autophagosome over time as evident in Figure 12. This suggests that the effect of chloroquine, even at a

high concentration, is fleeting and autophagy will begin to recover in the cells as soon as 3 hours post-treatment. In the dual mutant this is not observed, likely because the baseline ratio is already extremely low.

Clonogenic Proliferation Assay

One possible explanation for the perplexing results observed in Figure 13 is the increased clearance of ROS that accompany the overexpression of NRF2. ROS were once considered to only be harmful carcinogenic compounds that lead to cell damage and dysfunction. However, it is now thought that ROS have a physiological role as signal mediators involved in regulating important cellular functions such as cell proliferation and differentiation. For example, in murine models it has been shown that osteoclastogenesis is regulated by intracellular ROS, including H_2O_2. Elevated NRF2 levels in these models inhibit osteoclastogenesis by reducing ROS levels.[43] It is possible that a similar mechanism is at play in the *NFE2L2* and dual mutant lines.

Another explanation for the reduced growth in the mutant *NFE2L2* transduced lines is oncogene-induced senescence (OIS). Though oncogenes are thought to promote cell proliferation, hyperactivation of certain pathways can actually be toxic. OIS protects against this oncogenic stress by restricting proliferation of abnormal cells. However, OIS also has been shown to promote tumorigenesis by enhancing angiogenesis, metastasis, and promoting the formation of cancer stem cells.[44,45] OIS has also been linked to reducing the efficacy of cancer therapeutics.[46,47] Induction of OIS by oncogenic *NFE2L2* would explain the reduced proliferation observed.

Though we hypothesize that oncogenic driver mutations in *PIK3CA* and *NFE2L2* would act synergistically to transform pre-malignant NSCLC lesions, from this experiment it is evident that they do not do this by augmenting cell proliferation, at least not in the initial stages of tumorigenesis. This experiment led us to focus greater attention on other pathways of intersection between *PIK3CA* and *NFE2L2,* specifically autophagy.

Global Implications of Research and Next Steps

Understanding how early mutations in pre-malignant LSCC lesions act synergistically to promote metastasis has many potential benefits for enhancing early detection and improving cancer therapeutics. By identifying the proteins and cellular processes involved in this transformation by creating HBEC models we have established several targets for screening and drug research. Currently, over half of all lung cancers are detected in stage IV once clinical manifestations such as cough, dyspnea, and chest pain emerge.[48] This explains the mere 23.6% five year survival rate for LSCC.[49] Given this poor prognosis at late stage diagnosis there is strong interest in improving early detection of LSCC. Our results have identified autophagy as a pathway affected early in tumorigenesis as early oncogenic mutations in *NFE2L2* and *PIK3CA* work synergistically to inhibit autophagy. However, currently methods to quantitate autophagic flux in animals require tissue isolation. Our results support the need to develop non-invasive screening procedures to quantitate changes in autophagic flux by detecting autophagy biomarkers. This could be done by detecting circulating factors regulated by autophagy or by direct imaging of autophagic substrates. Positron Emission Tomography (PET) is

one potential approach to quantitating the abundance of target autophagy markers. PET is a nuclear imaging technology in which a small radioactive ligand is injected intravenously. The ligand then binds to the target molecule and the gamma rays released from the ligand-target complex is then read by the PET scanner. This gives a direct measurement of the target abundance.[49] From our results, LC3 would be a good target molecule for detection of autophagy by PET since it drastically accumulates in cells where autophagy is inhibited.

There are several experiments that could be performed to confirm our results and further support our hypothesis. One such experiment is a Western blot to probe for KEAP1 levels. As discussed, it has been demonstrated that elevated NRF2 levels create negative feedback loop by increasing transcription of KEAP1 mRNA and thereby promoting the creation of more KEAP1 to sequester NRF2 and inhibit its activity. This is a mechanism by which the constitutively active form of NRF2 mediates its oncogenic activity and delays tumorigenesis. It is important to verify whether this mechanism occurs in HBEC-*NFE2L2* transfected lines as it would explain the inhibition of proliferation observed in those lines. In addition, p62, the major link discussed between the NRF2-KEAP1 and PI3K/AKT/mTORC1 pathways, binds to KEAP1 and marks it for degradation. Elevated levels of KEAP1 would limit the levels free p62 available to activate mTORC1 thereby decreasing autophagy inhibition. This presents an argument against the cooperation of NRF2 and PI3K/AKT/mTORC1 pathway and further research should be pursued.

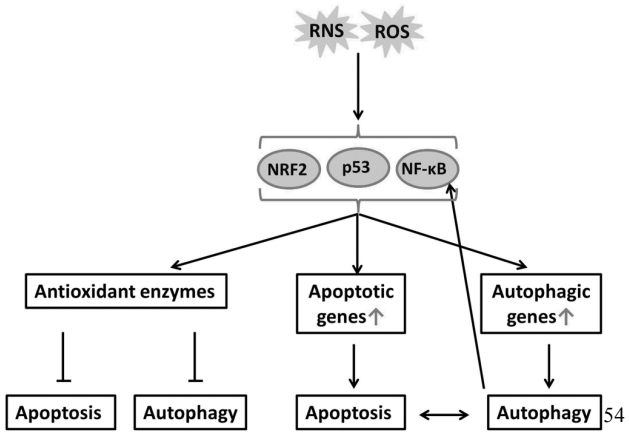
Relatedly, we proposed p62 as the major mechanism of synergy between NRF2/KEAP1 and PI3K/AKT/mTORC1 pathway to explain the increase in autophagy we observed. Further experiments are needed to verify p62's role in the cooperation of these pathways. Post transcriptional gene silencing using small interfering RNA (siRNA) is often used to research the roles of certain proteins on various cellular processes as it allows researchers to knock-out the expression of certain proteins without altering the genome of their cell lines. siRNA are double stranded RNA molecules that enter cells via transfection. They are then processed and the lagging strand is degraded inside the cell while the leading strand associates with the RNA-induced silencing complex (RISC). When this complex comes in contact with the mRNA of the target gene, base pairing between the leading strand and the mRNA occurs. The target mRNA is then cleaved and is rendered untranslatable thus silencing protein expression.[50] When silencing p62 expression in our cell lines we would expect autophagy inhibition to decrease as shown by decreased expression of ATG5 and LC3 on a western blot as well as decreased phosphorylation of mTORC1 targets as there would be decreased activation of mTORC1 by p62. Effectively silencing p62 should restore autophagy in the HBEC-*NFE2L2+PIK3CA* thereby preventing tumorigenesis. Regarding global implications, in recent years there has been much interest and research into the use of siRNA encapsulated in liposomes clinically in cancer treatment. If the expected results in this potential experiment were observed, this would support the use of p62 siRNA in the treatment of LSCC.

In this thesis, we explored cooperation of *PIK3CA* and *NFE2L2* in HBEC cell lines and there are many opportunities to further our research by exploring the cooperation of other oncogenes in LSCC. A logical next step after verifying our results, would be to investigate how additional mutations affect tumorigenesis when transfected in the *PIK3CA* and *NFE2L2* cell lines alone and when transfected into the *PIK3CA* and *NFE2L2* dual mutant cell line. The obvious selection for the third gene to study is *TP53* as it was found to co-occur with *PIK3CA* at a statistically significant rate. *TP53* is a tumor suppressor gene and is the most commonly mutated gene in human cancer. Out of our samples from the Pan Cancer Atlas data set 86% contained a mutation in *TP53*. *TP53* encodes the p53 protein, transcription factor, which activates autophagy by promoting the transcription of autophagy related genes such as damage-regulated autophagy modulator (*DRAM*), *ISG20L1,* and *ULK1*.[51] Downstream targets of p53 have been shown to interact with the PI3K/AKT/mTORC1 axis by inhibiting the mTOR complex.[33] By knocking out p53 expression, autophagy is expected to decrease. The question to be answered in future studies are whether oncogenic *PIK3CA* expression cooperates with the knock-out of *TP53* to inhibit autophagy and whether knocking out *TP53* in the *NFE2L2-PIK3CA* dual mutant lines has a greater effect on the inhibition of autophagy than would be expected.

Another possible third gene target is *KRAS. KRAS* is an oncogene that commonly arises in early pre-neoplastic adenocarcinoma lesions at a prevalence of 33% but was not identified as a common mutation in LSCC by our collaborators and does not co-occur

with *PIK3CA* and *NFE2L2* at a statistically significant rate.[32,52] Though it is apparent that *KRAS* mutations do not occur in LSCCs in vivo, creation of a *KRAS* + *PIK3CA* + *NFE2L2* mutant would provide important information about the role of autophagy in LSCC. *PIK3CA* and *NFE2L2* act synergistically to promote initiation of tumorigenesis by inhibiting autophagy. However, as the tumor develops autophagy must be promoted for further progression as the cells become more dependent on autophagy to sustain their metabolic needs due to changes in their metabolism. Being able to mark the transition from reduced autophagy to enhanced autophagy in a LSCCs has many clinical implications. For example drugs that may work in the early stages of LSCCs to restore the reduced autophagy, such as mTORC1, may actually promote tumorigenesis once the tumor switches to autophagy dependence. KRAS activation changes the metabolism of the cell by suppressing acetyl-CoA production thereby increasing the cell's dependence on autophagy. Inhibition of autophagy in *KRAS* mutant cells causes robust tumor suppression due reduced mitochondrial oxidative phosphorylation and lack of an energy source. As inhibition of autophagy causes damage to DNA and leads to additional mutations a possible experiment would be to transfect *KRAS* into the cell lines at different time points. At the initial time points it could be hypothesized that the addition of *KRAS* would inhibit tumorigenesis in the *PIK3CA+NFE2L2* line as it is autophagy addicted and the reduced autophagy in this line would send the cells into metabolic crisis. However over time as the *PIK3CA+NFE2L2* line acquired more mutations the transfection of *KRAS* might promote tumor growth.

Concluding Remarks



[54]

Figure 15: Schematic Representation of the effect of ROS on Autophagy. Presence of ROS increase the activity of several transcription factors including NRF2 that regulate autophagy and apoptosis. Upregulating antioxidant gene expression through activation of NRF2 leads to inhibition of autophagy.

Both the NRF2-KEAP1 pathway and autophagy are cellular stress responses. Both serve to reduce oxidative stress and ROS levels. NRF2 reduces ROS levels by increasing transcription of components of the glutathione and thioredoxin antioxidant systems while autophagy reduces ROS levels by digesting damaged mitochondria. (Figure 15)[53] Accumulation of NRF2 when autophagy is deficient causes disruptions in redox homeostasis.[17,25] Constitutive activation of NRF2 disrupts autophagy by eliminating ROS. Studies have shown that the presence of ROS initiate autophagosome formation and increase the rate of autophagic degradation by activating transcription factors that upregulate the transcription of p62 and LC3.[26,54] When NRF2 is upregulated

or constitutively active, ROS species are eliminated to levels below baseline and autophagy is inhibited.[53,55]

To proceed with our research it is imperative that we further understand the mechanisms by which free radicals activate autophagy. To further elucidate these mechanisms acetylcysteine, an antioxidant and precursor to reduced glutathione, could be used to treat the cells. This would quench the ROS thereby mimicking NRF2 activation. We would expect autophagy to see autophagy inhibition in the *HcRED* line and a further inhibition in the oncogenic lines. We could also add free radicals to the cell lines and see if we observed an increase in autophagy to confirm if ROS are truly an activator of autophagy as the scientific literature suggests.

Overall, the cooperation between NRF2 and PI3K/AKT/mTORC1 signaling in inducing the malignant evolution of cancers is not just of interest for modeling LSCC development. Understanding the synergy between these two pathways could provide great insight for development of therapeutics. An effective cancer therapy could be found by disrupting feedback activation--Inhibitors of PI3K/AKT/mTORC1 signaling could weaken NRF2 activity, while a therapeutic design to mimic KEAP1 and sequester NRF2 in the cytosol could antagonize PI3K/AKT/mTORC1 activity in cancer cells.[38]

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