SGK-1 AS A REGULATOR OF METABOLISM AND SURVIVAL OF ECM-DETACHED CELLS

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Successful metastasis requires cancer cells to overcome both anoikis - caspase-dependent cell death triggered by extracellular matrix (ECM) detachment - and ECM-detachment-induced metabolic defects (namely loss of glucose uptake and ATP generation) that compromise cell survival. However, the precise signaling pathways utilized by cancer cells to overcome anoikis and promote ATP generation remain poorly understood. One way cancer cells can overcome anoikis and promote productive metabolism is through activating oncogenes, such as Ras. We discovered that oncogenic Ras uses divergent downstream effectors to overcome anoikis (through PHLPP1/p38 signaling) and promote glucose-mediated ATP generation (via PI(3)K/SGK-1) to facilitate survival during ECM-detachment. Given the novelty of discovery of SGK-1-mediated ATP generation (through promoting glucose uptake) specifically during detachment, as well as the ever-increasing evidence for the importance of SGK-1 in promoting tumorigenesis, we have expanded these studies and found that SGK-1 signaling is required in a variety of cell types and oncogenic backgrounds for ATP generation and survival during detachment.
When examining the mechanism by which SGK-1 promotes ATP generation, we surprisingly found that treatment with the mitochondrial uncoupler CCCP did not impact the ability of SGK-1 to promote ATP generation suggesting that the TCA cycle is not required for SGK-1-mediated ATP generation. Intriguingly, ATP generation instead requires flux through the pentose phosphate pathway (PPP) and consequent production of glyceraldehyde-3-phosphate (G3P). PPP-derived G3P is then shuttled back to glycolysis where ATP production can robustly occur. This metabolic pathway appears to be critical for the anchorage-independent growth of cancer cells as genetic or pharmacological disruption of glucose flux through the PPP significantly abrogates colony formation in a variety of distinct cancer cell lines. Overall, our data suggest that oncogenic insults like Ras can utilize multiple downstream effectors to overcome anoikis activation, stimulate ATP generation, and promote survival during ECM-detachment. Further, our data suggest that SGK-1 may act as a master regulator of glucose metabolism and energy production during ECM-detachment that may be amenable to novel targeted therapies aimed at eliminating ECM-detached cancer cells through disruption of metabolism.
For Marcie, Talan, Elijah, and Clara.

“[Love] bears all things, believes all things, hopes all things, endures all things.”

1 Corinthians 13:7
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CHAPTER 1:
INTRODUCTION

1.1 Cell survival during ECM-detachment

Many cells require attachment to the extracellular matrix (ECM) to regulate cell growth, differentiation, and survival (Buchheit et al., 2012; Buchheit et al., 2014b; Meredith et al., 1993; Nelson and Bissell, 2006; Schafer et al., 2009). In order for cancer cells to survive during metastasis, they must be able to overcome anoikis, or caspase-mediated apoptotic cell death caused by loss of matrix attachment (Debnath et al., 2002; Frisch and Francis, 1994; Schafer et al., 2009). Anoikis is also induced when cells attach to a foreign or abnormal ECM (Frisch and Screaton, 2001). Thus, in order for cancer cells to successfully metastasize, they must resist anoikis at every step of the metastatic cascade, from localized invasion to survival during circulation and embedding into a secondary site.

While there have been many studies and advances in understanding how cancer cells overcome anoikis – primarily in mammary epithelial cells using the 3-dimensional cell culture system where a hollow lumen is generated by ECM-detachment-induced cell death signals - it has become abundantly clear that inhibition of apoptosis is not sufficient to promote survival of ECM-detached cells in the luminal space (Debnath and Brugge, 2005; Debnath et al., 2002; Humphreys et al., 1996; Mailleux et al., 2007). Furthermore,
ECM-detached cells undergo many changes that lead to cell death independently of anoikis, such as metabolic deficiencies that are mediated by ECM-detachment (Buchheit et al., 2012; Buchheit et al., 2014b; Davison et al., 2013; Mason et al., 2016; Mason and Schafer, 2016; Schafer et al., 2009). These metabolic alterations include deficiencies in ATP production, glucose uptake, fatty acid oxidation (FAO), glutamine uptake, enhanced levels of reactive oxygen species (ROS), and autophagy induction (Avivar-Valderas et al., 2011; Buchheit et al., 2012; Davison et al., 2013; Fung et al., 2008; Grassian et al., 2011b; Mason et al., 2016; Mason and Schafer, 2016; Schafer et al., 2009). Recently, there have begun to be studies aimed at understanding how ECM-detached cancer cells overcome ECM-detachment-induced metabolic defects. (Davison et al., 2013; Mason et al., 2016; Schafer et al., 2009). Overall, these data suggest that ECM-detached cancer cells must overcome anoikis induction and rectify ECM-detachment-induced metabolic deficiencies to survive during metastasis. However, the precise signaling mechanisms that allow ECM-detached cancer cells to overcome anoikis and rectify metabolic deficiencies remain poorly understood.

1.1.1 Anoikis regulation

Upon loss of integrin attachment to the ECM, normal cells undergo caspase-dependent cell death termed anoikis (Greek for homelessness) (Frisch and Francis, 1994). This suggests that anoikis is a crucial barrier for cancer cells to overcome during disease progression. In line with this, while anoikis is triggered in normal cells, cancer cells require the ability to overcome anoikis to survive during ECM detachment, which mimics an
environment faced during each step of the metastatic cascade (Buchheit et al., 2014b). Further, numerous studies suggest that sensitization of cancer cells to anoikis induction leads to decreases in survival during detachment (Buchheit et al., 2012; Buchheit et al., 2014b; Mason et al., 2016; Rayavarapu et al., 2015; Schafer et al., 2009; Weigel et al., 2014).

In order to overcome anoikis, cancer cells use a myriad of distinct signaling pathways, such as signaling through receptor tyrosine kinases (RTKs) and other kinases, small GTPases, and undergoing an epithelial to mesenchymal transition (EMT). While these signaling pathways have numerous downstream effectors responsible for their effect, each of them converge on triggering the release of cytochrome c from the mitochondria, which triggers caspase activity and cell death (Figure 1.1).
Figure 1.1 Overview of anoikis induction.
Adapted from (Buchheit et al., 2014b).
1.2 Metabolic regulation of ECM-detached cell survival

While numerous studies have begun to unravel the molecular mechanisms underlying anoikis evasion in cancer cells, studies using the 3-dimensional model of mammary morphogenesis suggest that inhibition of anoikis alone is insufficient to promote long-term survival of ECM-detached cells (Debnath et al., 2002). Further, these centrally located cells in the luminal space experience large amount of metabolic stress that must be overcome in order to promote luminal filling (Schafer et al., 2009). Thus, ECM-detachment-induced deleterious metabolic deficiencies, namely the ability to produce ATP, are a critical barrier that cancer cells must overcome to promote survival. While few studies have been aimed directly at elucidating how ECM-detached cells overcome metabolic deficiencies, many studies highlight potential metabolic signaling networks that may be essential for promoting ATP generation and facilitating ECM-detached cell survival (Figure 1.2).
Figure 1.2 Overview of ECM-detachment-induced metabolic changes.
Adapted from (Mason et al.).
1.2.1 Metabolic regulation in cancer cells

The first observation highlighting cancer metabolism occurred in the 1920’s, when Otto Warburg observed that cancer cells consume immense amounts of glucose compared to normal cells, which is commonly known as the “Warburg effect” (Koppenol et al., 2011; Tennant et al., 2009; Warburg, 1956a, b; Warburg et al., 1924). Furthermore, Warburg noticed that these cancer cells undergo rapid glycolytic flux for lactate production, even in the presence of oxygen (Warburg, 1956b). While the Warburg effect was originally thought to occur due to mitochondrial oxidative metabolism impairments in cancer cells (Warburg, 1956a), it has since been shown that, even though there are many mitochondrial gene mutations in cancer cells, many cancer cells do not actually harbor oxidative metabolism defects (Dupuy et al., 2015; Moreno-Sanchez et al., 2007; Wallace, 2012).

While cancer cell metabolism is closely linked with acquiring the well-established hallmarks of cancer (Hanahan and Weinberg, 2011), the role of metabolism in cancer supports growth and proliferation (Kroemer and Pouyssegur, 2008), and many metabolic processes are negatively regulated by ECM-detachment (Buchheit et al., 2012; Buchheit et al., 2014b; Grassian et al., 2011a; Ward and Thompson, 2012). Thus, cancer cells must overcome ECM-detachment-induced metabolic deficiencies to promote successful metastasis.
1.2.2 Glucose metabolism

The most well-defined metabolic shift that occurs in cancer cells is the Warburg effect, where there is a stark increase in glucose and lactate metabolism even in the presence of oxygen (Warburg et al., 1924). This increase in glucose and lactate metabolism accounts for the high production of ATP (DeBerardinis et al., 2008), as even though the yield of ATP per glucose consumed is low, the high glycolytic flux is high enough that the percent of ATP produced from glycolysis exceeds that of the much slower oxidative phosphorylation (Guppy et al., 1993; Warburg, 1956b). Furthermore, high rates of glycolysis can minimize the production of free radicals from oxidative phosphorylation (Guppy et al., 1993). Therefore, the Warburg effect can promote high levels of ATP generation and decrease ROS levels; both of which are required for ECM-detached cancer cell survival (Buchheit et al., 2012; Buchheit et al., 2014b; Davison et al., 2013; Schafer et al., 2009).

1.2.2.1 Upstream regulators/promoters of glucose metabolism

Upon detachment from the ECM, epithelial cells rapidly lose their ability to maintain glucose uptake, and this must be overcome in order to promote ECM-detached cell survival (Buchheit et al., 2014b; Grassian et al., 2011b). In order to promote glucose uptake and cell viability, cancer cells utilize multiple mechanisms to promote a translocation of glucose transporters to the plasma membrane (Wellen and Thompson, 2012). ECM-detached cancer cells also upregulate receptor tyrosine kinases such as ErbB2 and epidermal growth factor receptor (EGFR) (Schafer et al., 2009; Tong et al., 2009;
Weihua et al., 2008) in order to promote survival. Interestingly, different receptors promote glucose uptake through different downstream effectors: ErbB2 through a phosphatidylinositol 3-kinase (PI3K)/Akt-dependent manner (Schafer et al., 2009) and EGFR through the sodium-glucose cotransporter molecule 1 (SGLT1) (Weihua et al., 2008). Furthermore, and independently of upstream tyrosine kinase upregulation, PI3K has been shown to promote glucose uptake (Plas and Thompson, 2005; Tong et al., 2009) and signaling through Akt to protect cells from starvation (Kalaany and Sabatini, 2009). Additionally, PI3K-mediated glucose uptake can be dependent upon the activation of serum and glucocorticoid-regulated kinase 1 (SGK-1), independently of Akt (Mason et al., 2016).

Apart from upregulating receptor tyrosine kinases and the well-established PI3K signaling network, ECM-detached cells also promote high rates of glycolysis through the induction of autophagy. In particular, the increased glycolytic flux that is observed in oncogenic Ras-mediated transformation is dependent upon autophagic flux (Lock et al., 2011). Furthermore, overcoming ECM-detachment-induced ATP deficiencies is accomplished at least partly through increasing the total level, and activity, of AMP-activated protein kinase (AMPK) (Davison et al., 2013). While it was not directly measured, these data suggest that AMPK is acting as a biological switch, its’ canonical role, for glucose uptake, FAO, and biosynthesis of glucose transporters and mitochondria during ECM detachment (Bergeron et al., 1999; Durante et al., 2002; Ojuka, 2004; Thomson et al., 2007). Overall, these data suggest that ECM-detached cells can use a multitude of effectors to promote glucose uptake and subsequent glycolytic flux.
1.2.2.2 Downstream effectors promoting glucose breakdown and ATP production

Undergoing high rates of glycolysis promotes survival advantages for cancer cells, such as high rates of ATP production and producing carbon units for macromolecule and nucleotide synthesis (DeBerardinis et al., 2008). While macromolecule and nucleotide synthesis are essential for growth and proliferation, ATP production is essential for ECM-detached cell survival (Buchheit et al., 2014b; Davison et al., 2013; Mason et al., 2016; Schafer et al., 2009). To promote the increase in glycolytic flux that is oftentimes seen in cancer cells - and is the predominate source of ATP generation - cancer cells oftentimes express the M2 splice isoform of pyruvate kinase (PKM2), which is not typically expressed in adult tissues and has little to no enzymatic activity (Bensinger and Christofk, 2012; Christofk et al., 2008; Hosios et al., 2015). As the final step in glycolysis, PKM2 does not allow efficient transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, which would typically yield one molecule of ATP and one molecule of pyruvate (2 molecules in total as 1 glucose molecule yields 2 molecules of PEP), which is then converted to lactate, acetyl-CoA, or alanine. While the decreased enzymatic activity of PKM2 promotes increases in macromolecule biosynthetic pathways in nutrient rich conditions (Macintyre and Rathmell, 2011; Vander Heiden et al., 2010), it is essential for promoting ATP generation in periods of nutrient stress or the absence of growth signals (Macintyre and Rathmell, 2011), suggesting that PKM2 may be required for ECM-detached cells to promote ATP generation. In support of this possibility, PKM2 has been shown to promote tumorigenesis in vivo (Vander Heiden et al., 2010), where cancer cells undergo many periods of ECM detachment.
While ECM detachment decreases carbon flux through many pathways, such as glycolysis, the TCA cycle, and the pentose phosphate pathway (PPP) (Buchheit et al., 2012; Grassian et al., 2011a; Schafer et al., 2009), there is a substantial drop in pyruvate flux through pyruvate dehydrogenase (PDH) (Grassian et al., 2011b). Lack of flux through PDH leads to a decrease in glucose-derived carbon units entering the TCA cycle, which is required for lipid and nucleotide synthesis (Grassian et al., 2011a). Furthermore, in ECM-detached cells, there is a drastic increase in glucose-derived carbons that are secreted from the cell as lactate, alanine, or pyruvate (Caneba et al., 2012; Grassian et al., 2011b). It is conceivable to think that cancer cells, which must rectify glucose uptake and ATP deficiencies caused by ECM detachment, may decrease PDH flux in order to promote ATP-dependent glycolytic flux for survival. In line with this, cancer cells oftentimes utilize glutamine metabolism for carbon sources that enter the TCA cycle as intermediates for macromolecule synthesis (DeBerardinis et al., 2007; Dupuy et al., 2015).

Another mechanism that may be used by ECM-detached cells to promote survival is through the activation of large kinase B1 (LKB1), which is activated upon reduction in glucose uptake (Paoli et al., 2013). Once activated, LKB1 can activate AMPK, which acts as a sensor for the cellular ADP:ATP ratio (Hardie et al., 1998; Paoli et al., 2013). When AMPK senses a low level of ATP and an increase in ADP, which has been observed in ECM-detached cells (Schafer et al., 2009), AMPK inhibits anabolic processes in lieu of stimulating energy consuming, or catabolic, processes (Hardie et al., 1998). Therefore, upon detachment from the ECM, it is feasible that the LKB1/AMPK-signaling axis could be utilized to stimulate ATP production and cell viability. Overall, these data suggest that
ECM-detached cells can signal through a plethora of downstream effectors to promote ATP generation, and subsequent survival, following glucose uptake.

1.2.3 Fatty acid metabolism

While many studies have focused on the ability of cancer cells to promote glucose uptake and subsequent glycolytic flux for ATP production, there have been recent studies indicating alternate metabolic pathways, such as FAO, are utilized for ATP production and survival of ECM-detached cells (Buchheit et al., 2012; Buchheit et al., 2014b; Moreno-Sanchez et al., 2007; Tennant et al., 2009). While ECM-detached cells typically undergo drastic reductions in FAO, this can be rescued in MCF-10A cells by overexpressing ErbB2 (Schafer et al., 2009). In this study, it was shown that ATP production, and subsequent survival in ECM-detached MCF-10A cells overexpressing ErbB2, was dependent upon FAO (Schafer et al., 2009). In line with these data, cells experiencing glucose deprivation maintain their ATP production through increases in FAO (Buzzai et al., 2005; Zaugg et al., 2011). These data suggest that, in certain contexts, ECM-detached cells must promote FAO in order to maintain ATP production and viability.

Apart from oftentimes being deprived of glucose, cancer cells must also be able to survive during periods of low oxygen, or hypoxia (Brahimi-Horn et al., 2007; Buchheit et al., 2014b; Vaupel and Mayer, 2007). It has been shown that hypoxia can promote ECM-detached cell survival through promoting invasion and inhibiting anoikis (Chien et al., 2008; Whelan et al., 2010). Hypoxia also contributes to the metabolic rewiring of cancer cells, partially by filling energetic demands of cancer cells by promoting FAO for ATP
production (Verdegem et al., 2014; Zaugg et al., 2011). Overall, these data suggest that FAO may play a critical role in promoting ATP generation and ECM-detached cell survival during hypoxia.

1.2.4 Amino acid metabolism

For many decades, it was assumed that glucose uptake and the subsequent glycolytic flux was sufficient to fill the metabolic demands of highly proliferative cancer cells. However, due to the inherent inefficiency of the Warburg effect, many studies have examined alternative pathways utilized by cancer cells to promote productive metabolism and ATP production (Bender, 2012; Cairns et al., 2011; Hsu and Sabatini, 2008; Koppenol et al., 2011; Vander Heiden et al., 2009). Amino acid metabolism, particularly serine, glycine, and glutamine, may also have the potential to promoting productive metabolism and survival of ECM-detached cancer cells.

1.2.5 Serine and glycine metabolism

Two of the most well-studied amino acids in cancer metabolism are serine and glycine (Amelio et al., 2014; Locasale, 2013), particularly as a source of one-carbon units. Serine is a nonessential amino acid that is required for many processes that occur during metabolic reprogramming, including glycolysis (Kalhan and Hanson, 2012). Furthermore, serine and glycine metabolism can occur through both cytoplasmic and mitochondrial pathways, which are both upregulated in cancer (Jain et al., 2012; Labuschagne et al., 2014; Locasale et al., 2011; Possemato et al., 2011; Snell, 1985; Tibbetts and Appling, 2010; Zhang et al., 2012). Upon serine uptake, or following de novo synthesis through
glycolytic intermediates, serine is converted to glycine by serine hydroxymethyltransfrase (cytoplasmic, SHMT1; mitochondrial, SHMT2). This conversion of serine to glycine produces one-carbon units that enter the tetrahydrofolate (THF) cycle for nucleotide synthesis (Amelio et al., 2014; Labuschagne et al., 2014). Glycine can then be further cleaved by the mitochondrial glycine cleavage system, which has components that are amplified in some cancers (Zhang et al., 2012). Like serine cleavage, glycine cleavage produces one-carbon units that can then enter the THF cycle (Tibbetts and Appling, 2010). Thus, serine and glycine significantly contribute to nucleotide synthesis for proliferating cells.

Apart from the traditional role of serine and glycine contributing to one-carbon cycles for nucleotide synthesis, it has been established that the cytosolic synthesis of serine is actually in excess of what is needed for macromolecule synthesis (Possemato et al., 2011). These data indicate that serine and glycine metabolism is being used for more than just macromolecule synthesis. In line with this, serine and glycine metabolism have recently been shown to contribute to ATP production through one-carbon metabolism using methylenetetrahydrofolate dehydrogenase (MTHFD1, converts ADP to ATP in the cytosol; MTHFD2, converts ADP to ATP in the mitochondria) (Tedeschi et al., 2013). Serine is also an allosteric activator of PKM2, which leads to a buildup of glycolytic intermediates that provides de novo serine synthesis and minimizes carbon flux into the TCA cycle (Chaneton et al., 2012; Ye et al., 2014). Furthering these data, in breast cancers and melanomas there is an amplification of phosphoglycerate dehydrogenase (PHGDH), which is the rate-limiting enzyme for de novo serine biosynthesis (Locasale et al., 2011;
Possemato et al., 2011), suggesting a potential positive feedback loop for meeting the energetic demands of cancer cells. Finally, in the poorly vascularized tumor regions of gliomas, high SHMT2 activity and glycine cleavage is required for cancer cell survival (Kim et al., 2015). Overall, these data suggest that serine and glycine metabolism is not only required for nucleotide synthesis, and that during periods of stress, such as ECM detachment, serine and glycine metabolism may be crucial for promoting ATP generation and cancer cell survival.

1.2.6 Glutamine metabolism

Much like serine and glycine, research examining glutamine, a nonessential amino acid, metabolism in cancer has recently intensified (DeBerardinis and Cheng, 2010; Hensley et al., 2013; Wise and Thompson, 2010). Glutamine is the most abundant amino acid found in the plasma (Bergstrom et al., 1974; DeBerardinis and Cheng, 2010), and similarly to glucose, many different signaling cascades regulate glutamine uptake (Tong et al., 2009). Furthermore, even with the ability of glutamine to be synthesized from glucose, some cancer cells can become addicted to exogenous glutamine (Eagle, 1955; Yuneva et al., 2007).

Following uptake or synthesis, glutamine undergoes a deamination reaction (via glutaminase) that yields glutamate and ammonia (Eng and Abraham, 2010; Hensley et al., 2013). Glutamate then undergoes another deamination reaction to the TCA intermediate α-ketoglutarate by glutamate dehydrogenase (mitochondrial, yields a second molecule of ammonia) or transaminases (cytosolic or mitochondrial) that produce nonessential amino
acids (Eng and Abraham, 2010; Hensley et al., 2013). In glucose rich conditions, the transamination process of glutamate to nonessential amino acids predominates (Yang et al., 2009). In contrast, upon glucose deprivation, which occurs during ECM detachment, cell survival depends upon an increase in glutamate dehydrogenase activity to supply TCA intermediates, predominately α-ketoglutarate, for macromolecule and nucleotide synthesis (Choo et al., 2010; Yang et al., 2009). However, the high rate of glutamine uptake does not seem to be solely for its role in macromolecule and nucleotide synthesis (Wise and Thompson, 2010), suggesting alternate roles for glutamine metabolism.

Indeed, apart from providing TCA intermediates for macromolecule and nucleotide synthesis, glutamine-derived α-ketoglutarate is a major source of energy by providing reducing equivalents for the electron transport chain (ETC) and oxidative phosphorylation (Reitzer et al., 1979). Additionally, glutamine metabolism yields glutathione and nicotinamide adenine dinucleotide phosphate (NAPDH), each of which reduce ROS levels (Amores-Sanchez and Medina, 1999; Chang et al., 2002; DeBerardinis et al., 2008; DeBerardinis et al., 2007), and neutralization of ROS is required to promote ATP generation and survival in ECM-detached cells (Schafer et al., 2009). Taken together, these data suggest that, apart from macromolecule and nucleotide synthesis, ECM-detached cells may utilize glutamine metabolism for producing reducing equivalents to promote ATP generation and cell survival.
1.2.7 Neutralization of ROS

Whenever the Warburg effect was defined, the belief at the time was that cancer cells must have mitochondrial deficiencies that inhibit oxidative phosphorylation (Warburg, 1956a). However, it has since been shown that the majority of cancer cells actually do not harbor oxidative metabolism defects (Dupuy et al., 2015; Moreno-Sanchez et al., 2007; Wallace, 2012). Thus, the Warburg effect is occurring because of alternate reasons than the inability of cancer cells to undergo oxidative phosphorylation. One of the advantages conferred by the Warburg effect would be the high rate of glycolysis that leads to an increase in net ATP production over oxidative phosphorylation (described above). Another survival advantage of the Warburg effect would be the decrease in damaging free radicals that are produced as a by-product of oxidative phosphorylation (Guppy et al., 1993).

Free radical levels act as a rheostat for cancer cell survival; low to moderate levels play important roles in signaling cascades to promote cell survival (Azad et al., 2009; Clerkin et al., 2008; Trachootham et al., 2008), whereas high levels of ROS promote cancer cell death (Benhar et al., 2002; Buchheit et al., 2014b; Trachootham et al., 2009). ROS levels have also been implicated to play a major role in inhibiting energetically favorable metabolism. In particular, high levels of ROS are required to be alleviated in order to promote ATP production from FAO in ECM-detached cells (Schafer et al., 2009). One possible explanation for this phenomenon is that fatty acyl CoA produces hydrogen peroxide as a by-product, which acts as a negative feedback loop to suppress its’ own activity (Hashimoto and Hayashi, 1990). Also, high ROS levels directly inhibit PKM2
activity, which is critical for cancer metabolism and tumorigenesis (Anastasiou et al., 2011; Christofk et al., 2008). ECM-detached cancer cells may utilize many signaling pathways to diminish high levels of ROS and promote ATP generation and cell survival.

1.2.7.1 Pentose phosphate pathway and NADPH production

In order for cancer cells to proliferate, they alter their metabolism for energy production, redox homeostasis, and macromolecule precursors generation (Cantor and Sabatini, 2012; Schulze and Harris, 2012; Ward and Thompson, 2012). Additionally, if a cancer cell is unable to establish redox homeostasis, it results in a decrease in energy production and cell death (Benhar et al., 2002; Buchheit et al., 2014b; Trachootham et al., 2009). Thus, cancer cells must be able to sufficiently produce reducing equivalents to maintain redox homeostasis, energy production, and cell survival.

The oxidative branch of the PPP, which is crucial for driving nucleotide biosynthesis (Cairns et al., 2011), is a major source of reducing power for protecting cancer cells from cell death (Buchakjian and Kornbluth, 2010; Schafer et al., 2009). Through a series of steps, oxidative PPP converts D-Glucose-6-phosphate to D-Ribulose-5-phosphate, producing large amounts of the reducing molecule NADPH that is used for reducing glutathione, lowering ROS levels, and promoting ECM-detached cell survival (Bensaad et al., 2006; Herrero-Mendez et al., 2009; Pandolfi et al., 1995; Schafer et al., 2009). Furthermore, since high levels of ROS completely inhibit PKM2 activity (Anastasiou et al., 2011), this leads to an increase in PEP that inhibits trioso-phosphate isomerase. This leads to an inhibition of glycolytic flux and a bottle neck effect where glucose-6-phosphate
is shuttled through the PPP for NADPH production, thus lowering ROS in order to promote glycolytic flux (Gruning et al., 2011).

Apart from oxidative PPP, cancer cells can utilize a variety of different mechanisms to produce reducing molecules that would maintain ATP production. During energetic stress, such as during migration, cancer cells can utilize FAO for NADPH production (Jeon et al., 2012). When AMPK is upregulated, it phosphorylates and inhibits acetyl-CoA carboxylase (ACC1, 2), which lowers the NADPH consumption in fatty acid synthesis. As a result, there is a net increase in NADPH production through FAO (Jeon et al., 2012). Serine and glycine can also generate reducing equivalents through methylenetetrahydrofolate dehydrogenase 2-like (MTHFD2L) in the mitochondria during the folate one-carbon cycle (Tedeschi et al., 2013) or the generation of glutathione (Locasale, 2013). SHMT2 (which cleaves serine to glycine in the mitochondria) is also induced during hypoxia to promote mitochondrial redox balance through NADPH generation (Ye et al., 2014). Overall, these data suggest that during periods of stress, such as hypoxia and ECM detachment, cancer cells may utilize a variety of mechanisms to promote reducing equivalents to diminish high ROS levels; leading to an increase in ATP production and cell viability.

1.2.7.2 Antioxidant enzymes

Apart from generating reducing equivalents as described above, cancer cells oftentimes utilize antioxidant enzymes to reduce ROS levels. Antioxidant enzyme expression is regulated by the transcription factor Nrf2, which is normally bound by Keap-1 and sequestered into the cytosol. However, high levels of ROS oxidize Keap-1, thus
releasing Nrf2 to the nucleus and inducing the expression of many antioxidants, such as: oxygenase-1, peroxiredoxin-1, heavy and light chains of ferritin, glutathione peroxidase, thioredoxin, catalase, and superoxide dismutase (SOD) (Li et al., 2012).

The data above suggests that cancer cells may utilize antioxidant enzymes to promote survival during ECM detachment. In agreement, breast cancer cells have been shown to increase levels of the antioxidant SOD2 upon ECM-detachment (Kamarajugadda et al., 2013), and catalase is required for the survival of ECM-detached cancer cells in vivo in an AMPK-dependent mechanism (Davison et al., 2013). Furthermore, treatment of MCF-10A cells with Trolox or N-acetyl cysteine (NAC) promotes ATP generation in ECM-detached cells and anchorage independent growth potential (Schafer et al., 2009). Also, inhibiting the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) reversed paclitaxel resistance (Ramanathan et al., 2005), suggesting that high antioxidant enzyme activity promotes chemoresistance and tumorigenesis. Overall, these data suggest that cancer cells must overcome ECM-detachment-induced ROS levels to promote survival, and this can be attained through upregulating antioxidant enzymes.

1.2.8 Oncogenic signal transduction cascades that regulate ATP metabolism

Upon detachment from the extracellular matrix, there is a drastic reduction in the ability of cells to promote glucose and glutamine uptake, FAO, and ATP production, as well as an increase in ROS levels (Avivar-Valderas et al., 2011; Buchheit et al., 2012; Davison et al., 2013; Fung et al., 2008; Grassian et al., 2011b; Schafer et al., 2009). In order to promote survival during ECM-detachment, cancer cells must be able to overcome
these ECM-detachment-induced metabolic deficiencies (Buchheit et al., 2014b). One way to alleviate many ECM-detachment-induced metabolic deficiencies is through promoting oncogenic signaling cascades, which are crucial for the survival of many cancer cells (Buchheit et al., 2015; Buchheit et al., 2012; Buchheit et al., 2014b; Mason et al., 2016; Schafer et al., 2009). Thus, oncogenic insults and their downstream effectors may be used by ECM-detached cancer cells to promote productive ATP metabolism and survival.

1.2.8.1 ErbB2

One of the easiest ways for cells to utilize extracellular signals to promote intracellular responses is through the use of receptor tyrosine kinases (RTKs). The RTK ErbB2 is amplified in 20-30% of breast cancers (Hayes and Thor, 2002), and has been shown to promote cell viability in ECM detachment (Buchheit et al., 2012; Rayavarapu et al., 2015; Schafer et al., 2009). Overexpression of ErbB2 in ECM-detached cells is sufficient to rescue ECM-detachment-induced ATP deficiencies by restoring glucose uptake and signaling through PI(3)K/Akt (Schafer et al., 2009).

ErbB2 also promotes ECM-detached cell survival by lowering ECM-detachment-induced ROS levels, at least in part by signaling through the PPP for generation of NADPH (Schafer et al., 2009). Furthermore, ErbB2 induces lactate dehydrogenase, which rapidly converts pyruvate to lactate (Fantin et al., 2006; Zhao et al., 2009), thus preventing entry into the TCA cycle and minimizing free radical production. ErbB2 can also promote ECM-detached cell survival by stabilizing HIF1 levels and signaling through Erk and Akt (Whelan et al., 2013), as well as signaling through Erk/Sprouty2 to stabilize EGFR and increase β1
integrin levels (Grassian et al., 2011c). Overall, these data suggest that ErbB2 is sufficient to overcome ECM-detachment-induced metabolic defects in order to promote ATP generation and cell survival.

1.2.8.2 Ras

Mutations in the ras gene are very common, as they occur in approximately 30% of all cancers (most commonly in pancreatic (90%), colon (50%), and lung (30%)) as a result of constitutive activation via mutations in codon 12, 13, or 61 (Bos, 1989; Downward, 2003; Prior et al., 2012; Pylayeva-Gupta et al., 2011). There are 3 ras genes that encode 4 different isoforms, K- (4A/B), H-, and N-Ras, which share high sequence similarity but are not functionally redundant (Omerovic et al., 2007). Of the isoforms, K-Ras is the most abundantly mutated in cancers (Prior et al., 2012), with varying incidence rates of isoforms in different cancers and specific Ras mutations being associated with certain cancers; K- and H-Ras with most solid tumors and N-Ras with most hematopoietic cancers (Bos, 1989). Furthermore, oncogenic Ras plays a critical role promoting tumor progression, metastasis, and cell survival (Downward, 2003; Eckert et al., 2004; Webb et al., 1998).

One of the primary downstream Ras effectors is PI(3)K, which has been shown to signal through Akt and SGK-1 to promote glucose uptake, glycolytic flux, and energy production (Frauwirth et al., 2002; Mason and Schafer, 2016). During hypoxia, oncogenic H-Ras can also signal through PI(3)K to phosphorylate and stabilize HIF1, leading to HIF1 binding to the glucose transporter 1 (GLUT1) promoter and promoting GLUT1 mRNA
expression (Chen et al., 2001). Furthermore, Ras can stimulate glycolytic flux and survival through the promotion of autophagy (in ECM-detachment; (Lock et al., 2011)) or by enhancing the levels of PFKBP3, a form of the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate that is highly expressed in tumors (Atsumi et al., 2002; Telang et al., 2006). These data suggest that oncogenic Ras signaling is sufficient to meet the energy demands of cancer cells experiencing stress, such as ECM detachment.

As described above, cancer cells must be able to overcome ECM-detachment-induced increases in ROS levels, such as by increasing PPP flux or utilizing antioxidant enzymes, to promote survival (Buchheit et al., 2012; Buchheit et al., 2014b). However, oncogenic K-Ras does not seem to utilize the same mechanisms as ErbB2 to overcome ECM-detachment-induced increases in ROS levels, as it does not seem to rely on oxidative PPP flux to generate reducing equivalents to neutralize ROS levels (Vizan et al., 2005). Alternatively, oncogenic K-Ras signals through Nrf2 to promote tumorigenesis and cell survival, and loss of Nrf2 results in an increase in cell death even in the presence of oncogenic K-Ras (DeNicola et al., 2011). In agreement with these data suggesting oncogenic Ras utilizes antioxidant enzymes to promote cell survival, cells harboring oncogenic H-Ras mutations have increases in peroxiredoxin-1 and thioredoxin peroxidase (Buchheit et al., 2012; Paoli et al., 2013). Overall, these data suggest that oncogenic Ras can promote glycolytic flux to meet energy demands, as well as alleviate high levels of ROS through stimulating antioxidant enzyme levels and activity. Thus, oncogenic Ras has the potential to utilize multiple mechanisms to promote ECM-detached cell survival.
1.2.8.3 c-Myc

C-Myc (hereafter referred to as Myc) is a transcription factor that belongs to the family of myc genes and has approximately 10,000 binding sites in the human genome (Dang, 2013). Myc is also frequently mutated or amplified in many different cancers through a variety of mechanisms, with an estimated 70,000 deaths per year being associated to changes in Myc (Augenlicht et al., 1997; Cole, 1986; Dang, 1999, 2013; Little et al., 1983; Mariani-Costantini et al., 1988; Munzel et al., 1991). While Myc does play a critical role in many signal transduction cascades that promote nucleotide and macromolecule synthesis and cell proliferation, it is also essential for promoting glycolytic flux and bioenergetics (Dang, 1999; Miller et al., 2012; Osthus et al., 2000; Tong et al., 2009; Wang et al., 2011).

Upon activation (either through overexpression or mutation), Myc binds to the promoter regions and upregulates almost all of the glycolytic genes, including hexokinase II, phosphoglucone isomerase, phosphofructokinase 1, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, lactate dehydrogenase A and enolase (Dang et al., 2008; Dang et al., 1997; Kim et al., 2004; Osthus et al., 2000; Tennant et al., 2009). Therefore, an increase in Myc activation is sufficient to promote the Warburg effect. In line with this, Myc also promotes glucose uptake by upregulating GLUT1 (Dang et al., 2008; Osthus et al., 2000; Tong et al., 2009). Additionally, Myc further promotes glycolytic flux by inducing PKM2 expression (David et al., 2010), which can be phosphorylated by Erk, causing PKM2 to translocate to the nucleus where it phosphorylates histone 3 and promotes Myc transcription (Yang et al., 2012). Thus Myc-induced PKM2 expression sets
up a positive feedback loop for Myc, PKM2, and glycolytic flux to meet the energy demands of cancer cells. These data suggest that ECM-detached cancer cells may utilize Myc as a means of promoting vast amounts of glucose uptake, glycolytic flux, and ATP production to promote cell survival.

While Myc promotes high rates of glycolytic flux, it also plays critical roles for alleviating ROS. In melanoma cells, Myc upregulates glutathione that is later reduced to lower ROS levels and promote cell survival (Biroccio et al., 2002). Similarly, Myc upregulates the monocarboxylase transporter 1 (MCT1/SLC16A1) to transport lactate, the predominate by-product of glycolytic flux, out of the cells (Doherty et al., 2014). Upon inhibition of MCT1, intracellular lactate levels are significantly increased, which leads to a decrease in glucose uptake, ATP production, NADPH production, and glutathione reduction (Doherty et al., 2014). This loss of ATP and reduced glutathione leads to substantially increased levels of ROS and cell death (Doherty et al., 2014). Another mechanism used by Myc to support NAPDH production is via the upregulation of glutamine uptake and metabolism (Tong et al., 2009; Vander Heiden et al., 2009). Similarly to Ras, Myc can also signal through Nrf2 to increase antioxidant enzyme levels, which reduce ROS levels and promote tumorigenesis (DeNicola et al., 2011). Overall, these data suggest that Myc amplification or mutation may be sufficient for cancer cells to overcome ECM-detachment-induced ATP deficiencies and increases in ROS levels to promote survival.
1.2.8.4 HIF1

Cancer cell survival during tumor progression depends on their ability to adapt to many environments, such as hypoxia. Cancer cells oftentimes do this through hypoxia-inducible factor 1 (HIF1), a heterodimeric transcription factor consisting of the oxygen-regulated HIF1α subunit and the constitutively expressed HIF1β regulatory subunit (Semenza, 2000; Wang et al., 1995). Under normoxia, HIF1α is hydroxylated on proline 402 and/or proline 564, a process known as prolyl-hydroxylation (Kaelin and Ratcliffe, 2008; Semenza, 2010b). Upon prolyl-hydroxylation, HIF1α is then bound to the tumor suppressor von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to target HIF1α for proteasomal degradation (Masson et al., 2001; Semenza, 2010b). However, during hypoxia, the HIF1α subunit is stabilized and translocates from the cytoplasm to the nucleus, where it binds to the HIF1β subunit and forms a transcriptionally active HIF1 complex (Huang et al., 1996; Kallio et al., 1997; Ke and Costa, 2006). The HIF1 complex then binds to the hypoxia response element, 5′ – RCGTG – 3′ and activates transcription of many genes (Goldberg et al., 1988; Semenza et al., 1996). Apart from hypoxia, mutations in VHL can also stabilize HIF1α levels and the HIF1 complex transcriptional activity (Kim et al., 2006; Vogelstein and Kinzler, 2004). Furthermore, an increase in HIF1α stability and activity has been associated to promote mortality in many cancer types (Semenza, 2003).

The HIF1 complex plays a key role in reprogramming cancer metabolism, as it activates many genes that play crucial roles in promoting cancer progression, such as angiogenesis, glucose uptake, cell survival, and invasion (Semenza, 2003, 2010a). In
particular, HIF1 directly activates glycolytic flux from glucose uptake to the subsequent ATP production through increasing the expression of glucose transporters, hexokinase II, PDK1, lactate dehydrogenase A, and PKM2 (Brahimi-Horn et al., 2007; Chen et al., 2001; Kim et al., 2006; Yeung et al., 2008). Additionally, hypoxic stabilization of HIF1α promotes a positive feedback loop for the Warburg effect because PKM2 hydroxylation promotes nuclear translocation and binding to HIF1, which in turn promotes the transcription of the glycolytic enzymes mentioned above (Luo et al., 2011). While HIF1 sufficiently promotes ATP generation under periods of stress or following genetic mutations, it also acts to promote redox homeostasis and cell survival (Semenza, 2010b). PDK1 expression that is induced by HIF1 inhibits PDH activity and entry into the TCA cycle, therefore reducing free radical production (Kim et al., 2006). Also, HIF1 induces SHMT2 activity in to provide one-carbon units for the reduction of ROS levels (Ye et al., 2014). Overall, these data, as well as the indication that ECM detachment can induce HIF1α signaling (Buchheit et al., 2012), suggest that stabilization of HIF1α and the HIF1 complex may promote ECM-detached cell survival through supporting the bioenergetics needs and eliminating death-inducing ROS.
CHAPTER 2:
METHODS

2.1 ATP assays

To measure ATP levels in ECM-detached cells, 400,000 cells were plated in 6-well poly-(2-hydroxyethyl methacrylate) (poly-HEMA, Sigma-Aldrich, St. Louis, MO, USA)-coated plates for the amount of time indicated in the figure legends. The ATP Determination Kit (Invitrogen, Carlsbad, CA, USA) was used following normalization of total protein concentration according to manufacturer’s instructions. In LY294002 (Calbiochem, Billerica, MA, USA), EMD638683 (ApexBio, Houston, TX, USA), 2-DG (Sigma-Aldrich), CCCP (Sigma-Aldrich), 6-AN (Sigma-Aldrich), NMN (Sigma-Aldrich), methyl malate (Sigma-Aldrich), and methyl pyruvate (Sigma-Aldrich) experiments, the indicated concentration of inhibitor was added at the time of plating.

2.2 Caspase-3/7 assays

Cells were plated at a density of 13,000 cells per well on 96-well poly-HEMA-coated plates. Caspase activation was measured using the CaspaseGlo 3/7 Assay Kit (Promega, Madison, WI, USA) according to manufacturer’s instructions. In p38 inhibitor (SB203580, Cell Signaling Technologies, Danvers, MA, USA), SGK-1 kinase inhibitor
(EMD638683), pentose phosphate inhibitor (6-AN, Sigma-Aldrich), and staurosporine (STS; Sigma-Aldrich) experiments, the indicated concentration of inhibitor was added at the time of plating. For SB203580 experiments, inhibitor was added every 24 hours (12 hours for 10A HrasG12V PHLPP1) throughout the duration of the experiment.

2.3 Cell culture

MCF-10A cells (ATCC, Manassas, VA, USA) and derivatives were cultured in Dulbecco’s Modified Eagle Medium/F12 (Gibco, Waltham, MA, USA) supplemented with 5% horse serum (Invitrogen), 20ng/mL epidermal growth factor (EGF), 10µg/mL insulin, 500µg/mL hydrocortisone, 100ng/mL cholera toxin, and 1% penicillin/streptomycin. HCT116 cells (ATCC) and derivatives were cultured in McCoy’s media (Gibco) plus 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. 4T07 cells (ATCC) and derivatives were cultured in Dulbecco’s Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 1% non-essential amino acids (Thermo) and 1% penicillin/streptomycin. KPL4 cells (ATCC) and derivatives were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. KPL4 cells (ATCC) and derivatives were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. MDA-MB-468 (ATCC) cells were cultured in Dulbecco’s Modified Eagle’s Medium/F12 with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. ATPIF1 KO cells (gift from lab of Navdeep Chandel, Ph.D.) were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 1% HEPES (Thermo), 100µg/mL uridine (Sigma-Aldrich), 10mg/mL blasticidin (Invivogen, San Diego, CA, USA), 50mg/mL hygromycin (Thermo), 1mM sodium pyruvate (Thermo), and 1% penicillin/streptomycin. DN-POLG was induced
in ATPIF1 KO cells by the addition of 10ng/mL doxycycline (Sigma-Aldrich) for 9 days prior to experimentation.

2.4 CellTiter Glo assays

Cells were plated at a density of 13,000 cells per well on either a 96-well plate (attached), or a 96-well poly-HEMA-coated plate (detached), for the indicated time. ATP levels were measured using the CellTiter Glo Assay (Promega) according to manufacturer’s instructions.

2.5 Cell viability assays

Cells were plated at a density of 13,000 cells per well on 96-well poly-HEMA-coated plates. AlamarBlue reagent (Invitrogen) was added to each well according to manufacturer’s instructions. EMD638683, LY294002, and SB203580 were added at the indicated concentrations at the time of plating.

2.6 Glucose limiting assays

The indicated cells were plated at 400,000 cells per well in 6-well poly-HEMA-coated plates for 24 hours. MCF-10A derivatives were plated in standard media (described above) or glucose free Dulbecco’s Modified Eagle Medium/F12 (US Biologicals, Salem, MA, USA) supplemented with 5% horse serum (Invitrogen) 20ng/mL epidermal growth factor (EGF), 10µg/mL insulin, 500µg/mL hydrocortisone, 100ng/mL cholera toxin, 1% penicillin/streptomycin, and 0.4mM glucose solution (Gibco). HCT116 derivatives
were plated in either standard media (described above) or glucose free Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin, and 0.4mM glucose solution (Gibco).

2.7 Glucose uptake assays

Glucose uptake was measured in ECM-detached cells using the Amplex Red Glucose Assay Kit (Invitrogen) according to manufacturer’s instructions. Cells were plated at a density of 13,000 cells per well, and baseline glucose measurements were taken from a media only control plated at the same time as the cells. In EMD638683 (ApexBio) experiments, the indicated concentration of inhibitor was added at the time of plating.

2.8 G3P addition

Cells were plated in detachment (poly-HEMA-coated 6-well plate) at a density of 400,000 cells per well for the indicated time. The cells were then harvested and incubated with .01% Saponin (Sigma-Aldrich) or PBS control and placed on ice for 30 minutes. Cells were then spun down at 900 rpm for 3 min and washed once with ice cold PBS. Cells were then resuspended in complete medium with the presence, or absence, of 100µM glyceraldehyde 3-phosphate (G3P, Sigma-Aldrich) for 30 min on poly-HEMA-coated plates at 37 degrees C. Following incubation, cells underwent lysis (described below) and ATP measurements (described above).
2.9 Immunoblotting

ECM-detached cells were harvested, washed once with cold PBS, and lysed in 1% Nonidet P-40 supplemented with protease inhibitors leupeptin (5µg/mL), aprotinin (1µg/mL), and PMSF (1mM) and the Halt Phosphatase Inhibitor Mixture (Thermo Scientific, Waltham, MA, USA). Lysates were collected after spinning for 30 minutes at 4°C at 14,000 rpm and normalized by BCA Assay (Pierce Biotechnology, Waltham, MA, USA). Normalized lysates underwent SDS-PAGE and transfer/blotting was performed as previously described (Davison et al., 2013). The following antibodies were used for western blotting: IGF-1Rβ (Santa Cruz Biotechnology, #sc-81167), SGK-1 (EMD Millipore, #07-315), p-Akt (Cell Signaling Technology, #4060), GAPDH (Cell Signaling Technologies, #5174), Ras (Cell Signaling Technology, #3965), p-Sek1 (Abcam, ab39403), DYKDDDDK (FLAG) tag (Cell Signaling Technology, #2368), α-Tubulin (Cell Signaling Technology, #2144), β-tubulin (Cell Signaling Technology, #2146), β-Actin (Sigma-Aldrich, #A1978), PHLPP (Bethyl Laboratories, #A304-029A), p-Erk1/2 (Invitrogen, #368800), FKBP5 (Cell Signaling Technology, #12210), p-MAPKAPK2 (Cell Signaling Technology, #3007), p-NDRG1 (Cell Signaling Technologies, #5482), GLUT1 (Cell Signaling Technologies, #12939), G6PD (Cell Signaling Technologies, #8866), p-GSK3β (Cell Signaling Technologies, #9322), and mtCOXII (Invitrogen, #A6404).

2.10 Immunofluorescence

Cells were plated at a density of 50,000 cells per well in a 6-well plate (attached), or a 6-well poly-HEMA-coated plate (detached) for 24 hours. The cells were then
harvested, washed twice with cold PBS, and deposited onto slides with a Shandon Cytospin3 (Thermo Scientific) at 800 rpm for 5 minutes. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X 100 in PBS. Cells were washed with 100mM glycine in PBS three times and blocked with immunofluorescence (IF) buffer containing 130mM NaCl, 7mM Na$_2$HPO$_4$, 3.5mM NaH$_2$PO$_4$, 7.7mM NaH$_3$, 0.1% BSA (Millipore, Billerica, MA, USA), 1.2% Triton-X 100, 0.5% Tween-20), supplemented with 10% goat serum (Invitrogen). Slides were stained with SGK-1 (EMD Millipore, #07-315) at a concentration of 1:200 in IF buffer. For secondary visualization, AlexaFluor 568 (Invitrogen, #A11031) was used at 1:200 in IF buffer. Nuclei were stained with 5µg/mL 4,6-diamidino-2-phenylindone (DAPI, Invitrogen) and mounted with ProLong Gold Antifade Reagent (Life Technologies). Imaging of MCF-10A cells was completed using the Applied Precision DeltaVision OMX fluorescent microscope (Applied Precision, GE Healthcare, Issaquah, WA, USA). Images shown are representative images at 60X magnification. Imaging for 4T07 and HCT116 cells was completed using the Nikon A1R-MP microscope (Nikon, Melville, NY, USA). Images shown are representative images at 100X magnification. Images were quantified for fluorescence intensity using FIJI (National Institutes of Health, Bethesda, MD, USA). Percentage of GLUT1/4 at the cell membrane indicates cells per field with staining of GLUT1/4 at the membrane – those in which GLUT1/4 completely surrounds the nuclei and forms a morphologically identifiable membrane structure – divided by the total number of nuclei per field.
2.11 Retroviral generation of stable cell lines

The pBABE-Puro-based retroviral vectors encoding constitutively active H-Ras, constitutively active K-Ras, and PHLPP1, the pBABE-Neo-based retroviral vectors encoding constitutive active H-Ras and constitutive active K-Ras, the pLPCX-Puro-based retroviral vectors encoding constitutive active SGK-1 (S422D) and kinase dead SGK-1 (K127M), and the pLNCX-Neo-based retroviral vector encoding myristoylated Akt (Myr-Akt) were used to generate stable cell lines. HEK293T cells were transfected with 0.75µg target DNA along with the packaging vector pCLAmpho (0.75µg) with Lipofectamine 2000 (Life Technologies). Virus was collected at 48 and 72 hours post-transfection, filtered through a 0.45µm filter (EMD Millipore), and used for transduction of MCF-10A, HCT116, 4T07, and MDA-MB-468 cells in the presence of 8µg/mL polybrene. Stable populations of puromycin-resistant cells were obtained using 2µg/mL puromycin (Invivogen, San Diego, CA, USA). Stable populations of neomycin-based cells were obtained using 300µg/mL G418 (Nalgene, Waltham, MA, USA).

2.12 Short hairpin RNA generation of stable cell lines

MISSION short hairpin RNA (shRNA) constructs against SGK-1 (NM_005627; TRCN0000094957, TRCN0000040175) and PHLPP1 (NM_194449; TRCN0000082796) in the puromycin-resistant pLKO.4 vector along with an empty vector control were purchased from Sigma-Aldrich. HEK293T cells were transfected with 0.5µg target DNA along with the packaging vectors pCMV-D8.9 (0.5µg) and pCMV-VSV-G (60ng) using Lipofectamine 2000 and PLUS reagent (Life Technologies). Virus was collected 24 and 48
hours post-transfection and filtered through a 0.45µm filter (EMD Millipore), and used for transduction of MCF-10A, HCT116, and MDA-MB-468 cells in the presence of 8µg/mL polybrene. Stable populations of cells (MCF-10A and HCT116) were selected using 2µg/mL or 8µg/mL (4T07) puromycin (Invivogen).

2.13 Soft agar assays

Cells were plated at densities of 20,000 – 30,000 cells per well in 1.5mL of growth media plus 0.4% low-melt agarose (Sigma-Aldrich) and layered onto a 3mL bed of growth media with 0.5% low-melt agarose. Cells were fed daily with 1mL of growth media, plus the indicated concentration of z-VAD-fmk, 2-DG, EMD638683, 6-AN, or NMN. At the indicated time, growth media was removed and viable colonies were stained using INT-violet (Sigma-Aldrich). Colony number was determined using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.14 siRNA transfection

The indicated cells were transfected with siRNA SMARTpools (Dharmacon, Lafayette, CO, USA) against G6PD. Non-targeting siRNA (Dharmacon) was used as a control. For each transfection, 200nM of siRNA was transfected into cells using oligofectamine (Invitrogen) according to manufacturer’s protocol. For experiments involving siRNA in detached cells, cells were plated on poly-HEMA-coated plates 48h after siRNA transfection and assays were conducted 72h after siRNA transfection. Knockdown efficiency was examined after 72h by immunoblotting.
2.15 Statistics

Statistical significance was determined using a two-tailed t-test. Error bars represent S.D.

2.16 Transient transfection

The indicated cells were transfected with 1.5µg target DNA for the pcDNA3-based vectors including an empty vector control and PHLPP1 (Addgene, Cambridge, MA, USA; plasmid #22404) using Lipofectamine 2000 and PLUS reagent (Life Technologies) according to the manufacturer’s instructions. Cells were plated for soft agar assays and immunoblotting was completed 48 hours post-transfection.

2.17 3D cell culture

To generate acini, 3,000 – 4,000 cells were plated per well in an 8-well chamber slide on a Matrigel (Lot# 9044) bed and allowed to form acinar structures as previously described (Mailleux et al., 2007) (Debnath et al., 2003) and according to the protocol at http://brugge.med.harvard.edu/. Overlay media (Dulbecco’s Modified Eagle Medium/F12 supplemented with 2% horse serum, 5ng/mL epidermal growth factor (EGF), 10µg/mL insulin, 500µg/mL hydrocortisone, 100ng/mL cholera toxin, 2% Matrigel, and 1% penicillin/streptomycin) was added at day 0 and 4. Overlay media, consisting of the above without the 2% Matrigel, was added every 4 subsequent days until fixing. At the indicated time, acinar structures were harvested with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Fixed structures then underwent three
glycine (100mM) washes in IF buffer (described above). Laminin-5 (Millipore, #MAB88198) primary antibody was used at 1:150 in IF buffer plus 10% goat serum. For secondary visualization, AlexaFluor 568 (Invitrogen, #A11031) was used at 1:200 in IF buffer plus 10% goat serum. Nuclei were counterstained with 5µg/mL DAPI and slides were mounted using Prolong Gold Antifade Reagent (Thermo). Luminal filling was examined using a Nikon A1R-HP microscope (Nikon, Melville, NY, USA) and scored as clear (~0-10% filled), most clear (~10-50% filled), mostly filled (~50-90% filled), or filled (~90-100% filled). Representative images are shown at 40X.

2.18 Analysis of RPPA Data

R studio version 3.1.3 was used to mine Colorectal Adenocarcinoma (TCGA, Provisional) for Reverse phase protein array (RPPA) clinical data from MD Anderson Cancer Center. KRas mutations and RPPA data for KRas, p27 phosphorylated at threonine 157 (phospho-p27/KIP1 T157) and p38/MAPK phosphorylated at threonine 180 (phospho-p38/MAPK T180) data was collected. The data were parsed into a data set that contained patients with highly expressed oncogenic KRas (samples with positive values of relative KRas levels). These patient data were then used to generate scatter plots in Excel separating KRas on the X-axis and either p27_pT157 or p38_pT180 on the Y-axis and a best fit line was drawn for visualization of the overall trend of relative protein levels. R studio was used to calculate the Pearson's coefficients (r-values) and p-values based on Fisher Z scores (* 95% confidence interval, p < 0.05 and ** 99% confidence interval, p < 0.001).
CHAPTER 3:
ONCOGENIC RAS DIFFERENTIALLY REGULATES METABOLISM AND ANOIKIS IN ECM-DETACHED CELLS


3.1 Abstract

In order for cancer cells to survive during metastasis, they must overcome anoikis, a caspase-dependent cell death process triggered by extracellular matrix (ECM) detachment, and rectify detachment-induced metabolic defects that compromise cell survival. However, the precise signals utilized by cancer cells to facilitate their survival during metastasis remain poorly understood. We have discovered that oncogenic Ras facilitates the survival of ECM-detached cancer cells by utilizing distinct effector pathways to regulate metabolism and block anoikis. Surprisingly, we find that while Ras-mediated PI(3)K signaling is critical for rectifying ECM-detachment-induced metabolic deficiencies,
the critical downstream effector is SGK-1 rather than Akt. Our data also indicate that oncogenic Ras blocks anoikis by diminishing expression of the phosphatase PHLPP1, which promotes anoikis through activation of p38 MAPK. Thus, our study represents a novel paradigm whereby oncogene-initiated signal transduction can promote the survival of ECM-detached cells through divergent downstream effectors.

3.2 Introduction

Cancer metastasis, the spread of cancer cells to distant parts of the body, accounts for approximately 90% of cancer-related deaths and represents an inherently difficult clinical challenge (Li et al., 2013; Nguyen and Massague, 2007). It has become clear that for successful metastasis to occur, cells must overcome a caspase-dependent cell death mechanism, anoikis, which is triggered by detachment from the extracellular matrix (ECM)(Frisch and Francis, 1994). In addition to anoikis evasion, cancer cells must also contend with anoikis-independent cellular alterations that can compromise cellular viability (Buchheit et al., 2014b). Chief amongst these cellular alterations are metabolic deficiencies that are induced by ECM-detachment (Buchheit et al., 2012; Davison and Schafer, 2010; Schafer et al., 2009). These metabolic alterations involve deficiencies in ATP generation, elevated levels of reactive oxygen species (ROS), and the induction of autophagy (Avivar-Valderas et al., 2011; Fung et al., 2008; Schafer et al., 2009). While recent studies have begun to unravel the strategies utilized by cancer cells to ameliorate metabolic deficiencies during ECM-detachment (Davison et al., 2013), the signal
transduction cascades responsible for regulating metabolism during ECM-detachment in cancer cells remain almost entirely unexplored.

The activation of oncogenic signaling pathways is critical to anchorage-independent growth and ultimately to the survival of a variety of distinct cancer cell types during ECM-detachment (Buchheit et al., 2015; Buchheit et al., 2014b). Presumably, this oncogenic signaling is also necessary for resolving the aforementioned ECM-detachment-induced metabolic deficiencies. To date, only ErbB2 has been demonstrated to promote metabolic changes during ECM-detachment. ErbB2 overexpression in mammary epithelial cells results in a stimulation of PI(3)K/Akt signaling to promote glucose uptake and ATP generation (Schafer et al., 2009). This increase in Akt-mediated ATP generation is sufficient to enhance luminal filling in 3-dimensional (3D) cell cultures of mammary acini and can promote anchorage-independent growth. These data raise the question as to how cancer cells that lack ErbB2 overexpression rectify metabolic deficiencies during ECM-detachment. Does activation of other oncogenic signaling pathways facilitate ATP generation during ECM-detachment, and are similar downstream effectors utilized? The design of novel chemotherapeutic approaches to eliminate ECM-detached cancer cells requires a better understanding of the signal transduction cascades that regulate metabolism during ECM-detachment.

The Ras oncogene is mutated in approximately 30% of all human cancers, typically resulting in constitutive activation via mutations in codon 12, 13, or 61 (Downward, 2003; Prior et al., 2012; Pylayeva-Gupta et al., 2011). Given the frequency with which Ras mutations arise in cancers, it seems reasonable to speculate that Ras signaling may be
involved in facilitating the survival of ECM-detached cells. Indeed, previous studies in intestinal epithelial cells suggest that Ras activation can promote anoikis evasion (McFall et al., 2001), although significant ambiguities exist with regards to the precise downstream signaling that is involved (Eckert et al., 2004). Once activated, Ras is well known to activate ERK and Akt, which have well documented roles in promoting cell survival in cancer cells (Downward, 2003). More specifically, the ability of Ras to modulate metabolism during ECM-detachment would be consistent with its known capabilities to activate PI(3)K and the proficiency with which PI(3)K signaling promotes glucose uptake and ATP generation (Bryant et al., 2002). Given this and the aforementioned studies demonstrating that ErbB2 promotes ATP generation during ECM-detachment by activating PI(3)K and Akt (Schafer et al., 2009), it seems reasonable to speculate that Ras promotes metabolic activity through a similar signal transduction cascade.

Here, we uncover a surprising and novel signal transduction pathway operating downstream of oncogenic Ras to promote the survival of ECM-detached cancer cells. Interestingly, rather than relying on PI(3)K/Akt to promote ATP generation, we have found that Ras overcomes ECM-detachment-induced ATP deficiencies through the activation of a distinct PI(3)K effector, SGK-1. Furthermore, anoikis resistance in cells harboring Ras mutations relies on the downregulation of the PHLPP1 phosphatase. Interestingly, despite the well-known proclivity of PHLPP1 to dephosphorylate and deactivate Akt, we have discovered that PHLPP1 mediates anoikis through the activation of the p38 MAPK pathway. These data identify novel downstream targets that could be utilized for the development of chemotherapeutic approaches aimed at antagonizing the
ability of Ras to eliminate deficits in ATP generation and block anoikis. Furthermore, our data substantially refine the current understanding of Ras signaling to suggest that Ras-mediated signal transduction can promote the survival of ECM-detached cells through divergent downstream effectors.

3.3 Results

3.3.1 Oncogenic Ras promotes ATP generation through a PI(3)K-dependent, Akt-independent signaling pathway in ECM-detached cells.

To examine the ability of oncogenic Ras to promote ATP generation and cell viability during ECM-detachment, we engineered MCF-10A cells to stably express constitutively active H-Ras (G12V, hereafter referred to as 10A HrasG12V) or K-Ras (G12V, hereafter referred to as 10A KrasG12V). Indeed, hyperactive Ras promotes ATP generation in ECM-detached (but not ECM-attached) cells (Figure 3.1A). This corresponds to an increase in the viability of ECM-detached cells (Figure 3.1B). Given that PI(3)K signaling is well known to operate downstream of Ras, and that PI(3)K signaling was previously shown to promote ATP generation during ECM-detachment (Schafer et al., 2009), we investigated if PI(3)K also operates downstream of oncogenic Ras to promote ATP generation. As expected, inhibition of PI(3)K signaling compromises ATP production in a dose-dependent fashion in both 10A HrasG12V and 10A KrasG12V cells (Figure 3.1C). However, when examining phosphorylation of the PI(3)K effector Akt, we surprisingly observe minimal changes in Akt (S473) phosphorylation (Figure 3.1C). In order to confirm the efficacy of the inhibitor, we blocked PI(3)K signaling in MCF-10A cells overexpressing
insulin growth factor receptor 1 (10A IGF-1R) and observed the expected loss of Akt phosphorylation (Figure 3.1D). These data therefore suggest that ATP generation in ECM-detached cells downstream of oncogenic Ras is occurring through a PI(3)K-dependent, but Akt-independent mechanism.
Figure 3.1 Oncogenic Ras promotes ATP generation through a PI(3)K-dependent, Akt-independent signaling axis.

A. ATP levels were measured using CellTiter Glo in the indicated cells 24 hours after plating in either attachment or detachment (poly-HEMA) B. Cell viability was measured after 24 hours in detached conditions in the indicated cells using AlamarBlue. C. ATP levels were measured using the ATP determination kit in the indicated cells 24 hours after plating in detachment. Detached cells were treated with the indicated dose of LY294002 (PI(3)K inhibitor) for 24 hours. Lysates were prepared and immunoblotted for phosphorylated (p)-Akt and GAPDH. D,E. Detached cells were treated with the indicated dose of LY294002 for 24 hours. Lysates were prepared and then underwent immunoblotting for IGF-1R, p-Akt, p-Sek1, GAPDH, or β-tubulin. F. The indicated (ECM-detached) cells were immunoblotted for Ras, p-Sek1, p-Akt, GAPDH, FLAG-tag (on K-Ras G12V), α-tubulin, or β-actin. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV).
3.3.2 SGK-1 activation is sufficient to promote ATP production and cell survival during ECM-detachment.

Given that Akt does not appear to be a relevant mediator of ATP generation downstream of PI(3)K (in the presence of oncogenic Ras) during ECM-detachment, we assessed the contribution of serum and glucocorticoid regulated kinase-1 (SGK-1), another AGC family kinase known to operate downstream of PI(3)K (Schafer et al., 2009). SGK-1 shares significant sequence identity with Akt and has previously been linked to regulation of glucose transporter localization (Brickley et al., 2013; Bruhn et al., 2010; Park et al., 1999; Schafer et al., 2009). In our 10A HrasG12V and 10A KrasG12V cells, we see a decrease in SGK-1 activation upon PI(3)K inhibition as measured by phosphorylation status of Sek1 at serine 80 (Figure 3.1E), a canonical downstream SGK-1 target (Kim et al., 2007). In addition, we observe an increase in the phosphorylation of Sek1 in the presence of oncogenic Ras mutations (Figure 3.1F). Interestingly, this increase in Sek1 phosphorylation downstream of oncogenic Ras occurs in both ECM-detached and – attached cells with similar kinetics (Figures 3.2A, 3.2B).
Figure 3.2 SGK-1 activation does not change between attachment and detachment in cells harboring oncogenic Ras mutations.

A, B. The indicated cells were plated in either attachment or detachment for the indicated times. Lysates were prepared and the cells underwent immunoblotting for p-Sek1 and β-tubulin.
To examine if SGK-1 is sufficient to promote ATP generation and survival during ECM-detachment, we generated MCF-10A cells that express constitutively active SGK-1 (S422D, hereafter referred to as 10A S422D). Overexpression and constitutive activation of SGK-1 was confirmed via immunoblotting for SGK-1 and phospho-Sek1 (Figure 3.3A). When grown in ECM-detached conditions, we observe an increase in glucose uptake (Figure 3.3B), ATP production (Figure 3.3C), and cellular viability (Figure 3.3D) in 10A S422D cells. This increase in productive metabolism and ATP generation in ECM-detached 10A S422D cells does not seem to be a result of alterations in SGK-1 localization (Figure 3.4A) and occurs in both glucose-rich and glucose-limiting conditions (Figure 3.4B). Furthermore, using a 3D cell culture model of mammary acini where the hollow lumen is normally generated by ECM-detachment-induced cell death (Schafer et al., 2009), we see that constitutive activation of SGK-1 can promote luminal filling and cell survival (Figure 3.3E). Interestingly, constitutively active SGK-1 does not influence caspase activation in either ECM-attached or ECM-detached cells (Figure 3.3F), suggesting that the effects of SGK-1 activation on ATP generation and viability are independent of anoikis.

To ascertain the importance of SGK-1 kinase activity for the observed changes in ATP generation and cell viability during ECM-detachment, we expressed kinase-dead SGK-1 (K127M) in MCF-10A cells (hereafter referred to as 10A K127M). Immunoblotting for phospho-Sek1 confirmed the lack of SGK-1 kinase activity in these cells (Figure 3.3G). In contrast to constitutively active SGK-1 (S422D), the expression of kinase-dead SGK-1 (K127M) does not promote ATP production or cell viability in ECM-detached cells (Figure
3.3H, 3.3I). Thus, our data suggest that the SGK-1 mediated increase in ATP generation and cell viability during ECM-detachment is a direct consequence of SGK-1 kinase activity.
Figure 3.3 SGK-1 kinase signaling is sufficient to promote metabolic maintenance and survival in ECM-detached cells independently of modulating caspase-3/7 activity.

A. The indicated (ECM-detached) cells underwent immunoblotting for SGK-1, p-Sek1 or GAPDH. B. Glucose uptake levels were measured in detached MCF-10A and 10A S422D cells after 24 hours using the Amplex Red glucose assay kit. C. ATP levels were measured in detached MCF-10A cells and 10A S422D cells after 24 hours using the ATP determination kit. D. Cell viability was measured via AlamarBlue after 24 hours in detached MCF-10A cells and 10A S422D cells. E. MCF-10A and 10A S422D cells were plated in Matrigel and allowed to form acini for 7 days. Acini were stained with 4', 6-diamidino-2-phenylindole (DAPI, blue) and anti-Laminin 5 (red). Acini were scored as clear, mostly clear, mostly filled, or filled. Representative images of these categories are shown above at 40X. F. Caspase-3/7 activity was measured in MCF-10A and 10A S422D cells in attachment (left) and detachment (right) after 24 hours using the CaspaseGlo 3/7 assay. 10A S422D cells were treated with 1µM staurosporine (STS) at time of plating as a control for cell death. G. The indicated (ECM-detached) cells underwent immunoblotting for SGK-1, p-Sek1, and GAPDH. H. ATP levels were measured using the ATP determination kit following 24 hours of ECM-detachment in the indicated cells. I. Cell viability was measured via AlamarBlue after in the indicated cells following 24 hours of detachment. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV).
Figure 3.4 Detachment and glucose concentration does not affect SGK-1 localization or ATP generation.

A. 10A S422D cells were plated in either attachment or detachment for 24 hours. Following 24 hours, the cells underwent immunofluorescence for SGK-1. Representative images are shown at 60X. B. ATP levels were measured using the ATP determination kit in the indicated cells following 24 hours in ECM detachment in standard glucose concentration in DMEM/F12 media (left) or limiting glucose (0.4 mM) concentration in DMEM/F12 media (right). All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV).
3.3.3 SGK-1 is required for the promotion of ATP generation downstream of oncogenic Ras during ECM-detachment.

Given that SGK-1 kinase activity is sufficient to promote ATP generation during ECM-detachment, we next investigated the role of SGK-1 downstream of oncogenic Ras during ECM-detachment. Indeed, 10A HrasG12V and 10A KrasG12V cells have elevated SGK-1 activity (Figure 3.1F), ATP production (Figure 3.1A, C), glucose uptake (Figure 3.5A), and cellular viability (Figure 3.1B) during ECM-detachment. To more directly examine the role of SGK-1 in Ras-mediated regulation of ATP generation, we eliminated SGK-1 expression (and concomitant activity, Figure 3.5B) in 10A HrasG12V cells using shRNA (10A HrasG12V shSGK-1). Elimination of SGK-1 in these cells results in a loss of glucose uptake (Figure 3.5C), diminished ATP generation (Figure 3.5D), and a loss of cell viability during ECM-detachment (Figure 3.5E). Interestingly, the loss of SGK-1 signaling is only detrimental to ECM-detached cells, as SGK-1 knockdown does not alter ATP production in ECM-attached cells (Figure 3.5D). As we observed in our 10A S422D cells, the loss of ATP production in 10A HrasG12V shSGK-1 cells occurs in both glucose rich and glucose limiting conditions (Figure 3.6A).

To further validate the shRNA data, we used a pharmacological inhibitor of SGK-1 kinase activity, EMD638683, to assess the contribution of SGK-1 kinase activity to ATP generation and viability in 10A HrasG12V cells. Successful inhibition of SGK-1 in these cells was again confirmed via immunoblotting for phospho-Sek1 (Figure 3.5F). As expected, pharmacological inhibition of SGK-1 kinase activity resulted in a decrease in ATP production (Figure 3.5G) and viability (Figure 3.5H) in ECM-detached cells. Furthermore,
neither the shRNA mediated reduction in SGK-1 nor the inhibition of SGK-1 kinase activity altered caspase activation (Figure 3.6B, 3.6C). These data suggest that, analogous to the results observed in 10A S422D cells, loss of ATP generation and viability in ECM-detached cells with constitutively active Ras mutations occurs independently of anoikis induction.
Figure 3.5 SGK-1 is necessary for the promotion of ATP generation ECM-detached cells harboring oncogenic Ras.

A. Glucose uptake levels were measured at 24 hours in the indicated detached cells using the Amplex Red glucose assay kit. B. Immunoblotting against SGK-1, p-Sek1 and GAPDH was used to confirm the SGK-1 knockdown (shSGK-1) in detached 10A HrasG12V cells. C. Glucose uptake levels were measured using the Amplex Red glucose assay kit at 24 hours in detached 10A HrasG12V and shSGK-1 cells. D. ATP levels were measured in 10A HrasG12V and shSGK-1 cells after 24 hours in either attachment (left) or detachment (right) using CellTiter Glo. E. Cell viability was measured in detached 10A HrasG12V and shSGK-1 cells at 24 hours using AlamarBlue. F. Immunoblotting against p-Sek1 and β-tubulin was used following treatment of detached cells for 24 hours with 25µM of SGK-1 kinase inhibitor EMD638683 to confirm inhibitor efficacy. G. ATP levels were measured using the ATP determination kit in ECM-detached 10A HrasG12V cells following 24 hours of treatment with 25µM EMD638683. H. Cell viability was measured using AlamarBlue in ECM-detached 10A HrasG12V cells after being treated with 25µM EMD638683. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV, shEV) or DMSO (vehicle) treatment.
Figure 3.6 SGK-1-mediated ATP generation is not affected by glucose concentration and occurs independently of caspase-3/7 activity.

A. ATP levels were measured using the ATP determination kit in the indicated cells following 24 hours in ECM detachment in standard glucose concentration in DMEM/F12 media (left) or limiting glucose (.4mM) concentration in DMEM/F12 media (right). B. Caspase-3/7 activity was measured using the CaspaseGlo 3/7 assay kit following 24 hours in either attachment (left) or detachment (right) in the indicated cells. C. Caspase-3/7 levels were measured using the CaspaseGlo 3/7 assay in ECM-detached 10A HrasG12V cells following 24 hours of treatment with the SGK-1 kinase inhibitor EMD638683 (25μM). All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (shEV) or DMSO (vehicle) treatment.
To extend our studies into cancer cell lines with endogenous activating Ras mutations, we utilized a colorectal cancer cell line (HCT116), which contains a G13D mutation in K-Ras (Brink et al., 2003). HCT116 cells were engineered to have reduced SGK-1 (hereafter HCT116 shSGK-1 cells) using shRNA and knockdown was confirmed via immunoblotting for SGK-1 and phospho-Sek1 (Figure 3.7A). In line with our data acquired in MCF-10A cells, HCT116 shSGK-1 cells have diminished glucose uptake (Figure 3.7B), lower ATP generation (Figure 3.7C), and reduced viability during ECM-detachment (Figure 3.7D). Furthermore, the loss of ATP generation in HCT116 shSGK-1 cells occurs in both glucose-rich and glucose-limiting conditions. (Figure 3.8A). Interestingly, the loss of SGK-1 signaling also leads to a significant reduction in anchorage-independent growth in HCT116 cells (Figure 3.7E). Building on these findings, we treated HCT116 cells with the SGK-1 kinase inhibitor (EMD638683) (Figure 3.7F) and discovered a decrease in ATP generation (Figure 3.7G) and viability (Figure 3.7H) in ECM-detached cells. In line with our data in MCF-10A cells, the loss of ATP generation and viability observed during ECM-detachment in HCT116 shSGK-1 cells and HCT116 cells treated with the SGK-1 inhibitor occurs independently of alterations in caspase activation (Figure 3.8B, 3.8C). These data suggest that the ability of SGK-1 kinase activity to promote the survival during ECM-detachment is also present in cancer cells with endogenous activating mutations in Ras.
Figure 3.7 SGK-1 is required for ATP maintenance and survival of ECM-detached colon cancer cells.

A. Immunoblotting against SGK-1, p-Sek1 and β-tubulin was used to confirm the SGK-1 knockdown (shSGK-1) in detached HCT116 cells. B. Glucose uptake levels were measured using the Amplex Red glucose assay kit in HCT116 and shSGK-1 cells after 24 hours in detachment. C. ATP levels were measured using CellTiter Glo after 24 hours in either attachment (left) or detachment (right) in HCT116 and shSGK-1 cells. D. Cell viability for HCT116 and shSGK-1 cells was measured via AlamarBlue following 24 hours in detachment. E. HCT116 and shSGK-1 cells were plated in soft agar. After 5 days, images were taken following iodonitrotetrazolium chloride (INT-violet) staining. Colony count was determined using ImageJ. F. Immunoblotting against p-Sek1 and β–tubulin was used following treatment of detached cells for 24 hours with 25µM EMD638683 to confirm the efficacy of the inhibitor. G. ATP levels were measured using the ATP determination kit in ECM-detached HCT116 cells after 24 hours of treatment with 25µM EMD638683. H. Cell viability was measured in ECM-detached HCT116 cells using AlamarBlue following 24 hours of treatment with 25µM EMD638683. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (shEV) or DMSO (vehicle) treatment.
Figure 3.8 SGK-1-mediated ATP generation in ECM-detached cancer cells occurs independently of anoikis and is not altered in limiting glucose conditions.

A. ATP levels were measured using the ATP determination kit following 24 hours of ECM detachment in the indicated cells in standard glucose concentration in DMEM media (left) or limiting glucose (.4mM) concentration in DMEM media (right). B. Caspase-3/7 activity was measured following 24 hours of attachment (left) or detachment (right) in the indicated cells using the CaspaseGlo 3/7 assay. C. Caspase-3/7 levels were measured following treatment with 25μM EMD638683 for 24 hours in ECM-detached HCT116 cells. EMD638683 was added at the time of plating. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (shEV) or DMSO (vehicle) treatment.
3.3.4 Oncogenic Ras downregulates PHLPP1 to evade anoikis.

The survival of cancer cells during ECM-detachment requires rectifying ECM-detachment-induced loss of ATP generation (accomplished in this case by Ras-mediated SGK-1 activation) and blocking caspase activation (anoikis) (Buchheit et al., 2014b). Given that we did not discern a role for Ras-mediated SGK-1 signaling in anoikis regulation (Figure 3.3E, 3.6B, 3.6C, 3.8B, 3.8C), we hypothesized that oncogenic Ras may utilize a distinct signaling pathway to block anoikis. Indeed, oncogenic Ras (either H-Ras or K-Ras) can effectively inhibit caspase-3/7 activation (Figure 3.9A) and promote cell viability (Figure 3.9B) during ECM-detachment. These changes in caspase activation are specific to ECM-detached cells, as the introduction of oncogenic Ras does not inherently alter caspase activation in ECM-attached cells (Figure 3.9A). While contemplating Ras-mediated signaling pathways that may be responsible for this anoikis inhibition, we revisited our surprising results from Figure 3.1C: Akt phosphorylation in cells with oncogenic Ras is only marginally affected when PI(3)K is inhibited. We reasoned that this might be due to diminished phosphatase activity and hence the resulting phosphorylated (and activated) Akt may play an important role in anoikis inhibition. The phosphatase PHLPP1 (PH Domain and Leucine Rich Repeat Protein Phosphatase 1) is known to negatively regulate Akt by dephosphorylating the hydrophobic motif (S473)(Gao et al., 2005; Newton and Trotman, 2014). To examine if PHLPP1 is negatively regulated by Ras, we assessed PHLPP1 levels in the presence and absence of oncogenic Ras. Indeed, the expression of oncogenic Ras (either H-Ras or K-Ras) is sufficient to lower the quantities of PHLPP1 in MCF-10A cells (Figure 3.9C). To examine if changes in PHLPP1 have the capacity
to regulate anoikis, we engineered MCF-10A cells to be deficient in PHLPP1 (10A shPHLPP1) and confirmed (by immunoblotting) that PHLPP1 reduction did lead to an increase in Akt phosphorylation (Figure 3.9D). Interestingly, reduction of PHLPP1 in MCF-10A cells is sufficient to inhibit caspase-3/7 activation during ECM-detachment (Figure 3.9E) and to promote the viability of ECM-detached cells (Figure 3.9F). Furthermore, acinar structures derived from MCF-10A cells deficient in PHLPP1 have substantially increased luminal filling which is indicative of enhanced survival during ECM-detachment (Figure 3.9G).
Figure 3.9 Loss of PHLPP1 leads to anoikis evasion and enhanced viability in ECM-detached cells.

A. Caspase-3/7 activity was measured using the CaspaseGlo 3/7 assay in the indicated cells following 48 hours in either attachment (left) or detachment (right). B. Cell viability was measured at 48 hours in detachment in the indicated cells using AlamarBlue. C. The indicated (ECM-detached) cells underwent immunoblotting for PHLPP1 and GAPDH. D. Detached MCF-10A and 10A shPHLPP1 (shPHLPP1) cells underwent immunoblotting for PHLPP1 and β-actin. p-Akt levels were measured as a functional control. E. Caspase-3/7 activity was measured in MCF-10A and shPHLPP1 cells after 48 hours in either attachment (left) or detachment (right) using the CaspaseGlo 3/7 assay. F. Cell viability was measured using AlamarBlue in MCF-10A and shPHLPP1 cells following 48 hours in detachment. G. MCF-10A and shPHLPP1 cells were plated in Matrigel and allowed to form acini for 10 days. Acini were stained DAPI (blue) and anti-Laminin 5 (red), and scored as clear, mostly clear, mostly filled, or filled. Representative images are shown above at 40X. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV, shEV).
To examine if the Ras-mediated elimination of PHLPP1 is necessary for anoikis inhibition, we rescued PHLPP1 expression in MCF-10A derivatives with activating Ras mutations and HCT116 cells, and confirmed PHLPP1 expression in each cell line via immunoblotting (Figure 3.10A). Indeed, when PHLPP1 expression is rescued in these cell lines, there is a substantive increase in caspase-3/7 activation (Figure 3.10B). Furthermore, in HCT116 cells with PHLPP1 overexpression, there is a marked decrease in anchorage-independent growth in soft agar (Figure 3.10C). Treatment with the pan-caspase inhibitor z-VAD-fmk rescued anchorage independent growth in HCT116 cells engineered to express PHLPP1 (Figure 3.10C), but had a negligible effect on HCT116 EV cells. These data suggest that PHLPP1-mediated caspase activation is responsible for the loss of colony formation in soft agar. Interestingly, the increase in anoikis induction as a result of PHLPP1 expression occurs independently of the regulation of ATP generation (Figure 3.11A, B, C).
Figure 3.10 PHLPP1 downregulation is necessary for Ras-induced anoikis evasion

A. The indicated cells were immunoblotted for PHLPP1, α-tubulin, or β-actin. B. Caspase-3/7 levels were measured using the CaspaseGlo 3/7 assay in the indicated cells after 48 hours in detachment. C. HCT116 EV and HCT116 PHLPP1 cells treated with 20μM of caspase inhibitor z-VAD-fmk (or DMSO vehicle) were plated in soft agar and images were taken after 5 days following staining with INT-violet. 20μM z-VAD-fmk and DMSO was added at plating and every day as the cells were fed with new media. Colony count was determined using ImageJ. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV).
Figure 3.11 PHLPP1 overexpression does not affect ATP levels in ECM-detached cells.

A, B, C. ATP levels were measured using the ATP determination kit in the indicated cells following 24 hours of detachment. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV).
3.3.5 PHLPP1-mediated rescue of anoikis in cells with Ras mutations is dependent on p38 MAPK.

Much to our surprise, when we examined the phosphorylation of Akt following PHLPP1 expression in either 10A HRasG12V or HCT116 cells, we did not observe an appreciable loss of phospho-Akt (Figure 3.12A). This unexpected result suggested that the ability of PHLPP1 to promote anoikis might be independent of its ability to dephosphorylate Akt. In support of this possibility, we found that the levels of FKBP5, which has been shown to function as a scaffolding protein to target PHLPP1 to Akt (Park et al., 1999), are substantially reduced in the presence of oncogenic Ras (Figure 3.12B). This loss of FKBP5 would render PHLPP1 obsolete in its ability to target and dephosphorylate Akt, suggesting that PHLPP1-mediated anoikis in cells with oncogenic Ras is unlikely to be due to Akt regulation. Another common target of PHLPP1 is the ERK/MAPK pathway (Qiao et al., 2010; Suljagic et al., 2010) but we observe no change in phosphorylated ERK when PHLPP1 is overexpressed (Figure 3.12A).

Given that neither Akt nor ERK seem to be the relevant target of PHLPP1 responsible for anoikis induction, we next investigated the role of the p38 MAPK pathway in PHLPP1-mediated anoikis. Recently, Mst-1 has been identified as a substrate of PHLPP1 and desphosphorylation of Mst-1 leads to its activation and ultimately to stimulation of p38 MAPK activity (Qiao et al., 2010; Webster et al., 1993). Indeed, in 10A HrasG12V and HCT116 cells, the expression of PHLPP1 results in an increase in p38 MAPK activation (that can be blocked by treatment with the p38 MAPK inhibitor SB203580) as measured by phosphorylation of the downstream target MAPKAPK2 (Xu et al., 2006) (Figure 3.12C).
Furthermore, inhibition of p38 MAPK with SB203580 leads to a significant reduction in PHLPP1-mediated caspase-3/7 activation (Figure 3.12C). Together, these data demonstrate that the rescue of anoikis by PHLPP1 in cells harboring mutations in oncogenic Ras is dependent on activation of the p38 MAPK pathway.

3.3.6 SGK-1 and PHLPP1 cooperatively function to promote the survival of ECM-detached cancer cells.

Given that our data support a model by which Ras facilitates cell survival during ECM detachment through distinct downstream signaling pathways, we sought to investigate if there is any synergy between SGK-1 and PHLPP1/p38 MAPK in promoting anchorage-independent growth. To examine this possibility, we engineered HCT116 shSGK-1 cells to transiently express PHLPP1 and confirmed this expression via immunoblot (Figure 3.7D). Indeed, in HCT116 shSGK1 cells we see a decrease in anchorage independent growth that is further diminished by the addition of PHLPP1 (Figure 3.12D). These data suggest that the simultaneous modulation of both SGK-1 and PHLPP1/p38 MAPK signaling during ECM-detachment can function cooperatively to influence anchorage-independent growth.

Given the aforementioned data, it is reasonable to surmise that simultaneous activation of SGK-1 and inhibition of p38 MAPK may be able to enhance the viability of cells treated with PI(3)K inhibitors. Indeed, overexpression of constitutively active SGK-1 (S422D) and inhibition of p38 MAPK (with SB203580), rescues cell death induced by PI(3)K inhibition (Figure 3.12E). We next sought to further assess the individual contributions of
the SGK-1 and p38 MAPK pathway. Interestingly, while p38 MAPK inhibition alone was not sufficient to promote viability of ECM-detached cells upon PI(3)K-inhibition, p38 MAPK inhibition did cooperate with constitutive activation of SGK-1 to elevate viability (Figure 3.12F). Together, these data suggest that simultaneous activation of SGK-1 and inhibition of p38 MAPK can promote the viability of cells treated with a PI(3)K inhibitor.

We next sought to ascertain if patients afflicted with colorectal adenocarcinomas containing Ras mutations harbored evidence of concomitant regulation of SGK-1 and PHLPP1 in their tumors. Indeed, in these patients, there is a statistically significant negative correlation between levels of oncogenic K-Ras and phosphorylation of p38 MAPK at threonine 180 (Figure 3.12G). Furthermore, in these same patients, there exists a statistically significant positive correlation between levels of oncogenic K-Ras and phosphorylation of p27/KIP1 at threonine 157, a site well known to be phosphorylated by SGK-1 (Hong et al., 2008) (Figure 3.12G). In aggregate, these data suggest that differential and concurrent regulation of ATP production and caspase activation (anoikis) downstream of oncogenic K-Ras through SGK-1 and PHLPP1/p38 MAPK is occurring in human colorectal adenocarcinoma.
Figure 3.12 PHLPP1 mediates anoikis through a p38-dependent signaling axis.

A. The indicated (ECM-detached) cells underwent immunoblotting for p-Akt, p-ERK, GAPDH, α-tubulin, or β-actin. B. Immunoblotting for FKBP5 and β-actin was performed on the indicated (ECM-detached) cells. C. The indicated (ECM-detached) cells were treated with SB203580 (p38 inhibitor) at 10µM (HCT116 PHLPP1) or 20µM (10A HrasG12V PHLPP1) concentrations. Caspase-3/7 activity levels were measured in the indicated cells using the CaspaseGlo 3/7 assay after 24 hours (10A HrasG12V) or 48 hours (HCT116) in detachment. Immunoblotting for p-MAPKAPK2 and GAPDH was performed to confirm the efficacy of the inhibitor. D. Immunoblotting for PHLPP1, β-tubulin, and p-Sek1 was performed on the indicated (ECM-detached) cells. The indicated cells were plated in soft agar and images were taken after 5 days following staining with INT-violet. Colony count was calculated using ImageJ. E. Cell viability was measured using AlamarBlue in the indicated attached cells following treatment for 24 hours with DMSO, 10µM LY294002 (PI(3)K inhibitor), or 10µM SB203580 (p38 inhibitor). F. Cell viability was measured by AlamarBlue in ECM-detached 10A EV and 10A S422D cells treated with the indicated inhibitor (DMSO, 10µM LY294002, or 10µM SB203580) for 24 hours. G. RPPA data for colorectal adenocarcinoma patients was analyzed. Top, phosphorylated p27/Kip1 (T157) vs oncogenic K-Ras (Pearson’s coefficient = 0.47; p < 0.01). Bottom, phosphorylated p38 MAPK (T180) vs oncogenic K-Ras (Pearson’s coefficient = -0.37; p < 0.05). All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. a.u., arbitrary units. Fold change is calculated as a ratio compared to empty vector (EV, shEV).
3.4 Discussion

Given that it is now appreciated that cancer cells must rectify metabolic defects (e.g. ATP generation) in addition to blocking caspase activation (anoikis) in order to survive during ECM-detachment (Buchheit et al., 2014b), it is critically important to better understand the precise molecular mechanisms involved in mediating both anoikis and metabolic changes. The data described here outline a unique, multi-faceted signaling pathway operating downstream of oncogenic Ras to permit anoikis inhibition and stimulate ATP production (Figure 3.13). Our data place Ras signaling in contrast to ErbB2 with regards to metabolic regulation during ECM-detachment, as previous studies have shown that ErbB2 promotes ATP generation through PI(3)K-mediated activation of Akt (rather than SGK-1) (Schafer et al., 2009). These data suggest that the strategies utilized by cancer cells to alleviate deleterious metabolic changes (and block anoikis) may vary considerably across tumor types and contexts (Buchheit et al., 2014a; Rayavarapu et al., 2015; Weigel et al., 2014). Therefore, additional studies examining the relationship between oncogenic signaling and metabolism in ECM-detached cancer cells are warranted to better ascertain the best strategies to facilitate their elimination.

The identification of SGK-1 as a primary mediator of ATP generation downstream of oncogenic Ras (and PI(3)K) adds to the growing body of literature suggesting that molecules other than Akt can function downstream of PI(3)K to modulate distinct cellular functions in cancer cells (Bruhn et al., 2010; Gasser et al., 2014). Our studies suggest that ECM-detached cells with oncogenic Ras mutations could be compromised by SGK-1 inhibition. Indeed, the development of SGK-1 antagonists has already commenced in
other cancers (Sherk et al., 2008; Skor et al., 2013), and our results suggest similar inhibitors may be efficacious in eliminating ECM-detached cells with hyperactivating Ras mutations. Interestingly, our findings are also consistent with data suggesting that SGK-1 activity can mediate the sensitivity of cancer cells to targeted therapies (Sommer et al., 2013). In addition, it is noteworthy that other investigators have discovered that SGK-1 can interact with proteins at the mitochondria and can inhibit the induction of necrotic cell death (Brickley et al., 2013; O’Keeffe et al., 2013). Our data implicating SGK-1 in ATP generation may provide additional insight into these connections between SGK-1 and mitochondrial integrity.

Much like SGK-1, interest in understanding PHLPP1 regulation during tumorigenesis has intensified over the past few years. Studies from a number of labs have now implicated loss of PHLPP1 activity as an important facet of cancer cell survival (Cai et al., 2013; Newton and Trotman, 2014; Wang et al., 2014; Wen et al., 2013). That being said, despite the fact that PHLPP1 has a predicted Ras-associated domain (Warfel and Newton, 2012), our study is the first (to our knowledge) to directly link downregulation of PHLPP1 to oncogenic Ras signaling. In addition, the vast majority of studies aimed at understanding PHLPP1 in cancer cells have focused on the ability of PHLPP1 to dephosphorylate Akt. However, our data suggest that PHLPP1-mediated anoikis is not reliant on Akt dephosphorylation but is instead dependent on activation of the p38 MAPK pathway. Our data provide an important and novel physiological setting by which PHLPP1-mediated activation of p38 MAPK could be important in the context of tumor progression.
Novel therapeutic strategies aimed at reactivating or stimulating p38 MAPK may be an effective strategy to eliminate ECM-detached cancer cells harboring Ras mutations.

In fact, our data suggest that the most effective way to eliminate ECM-detached cells with Ras mutations would be through stimulating p38 MAPK (and inducing anoikis) while simultaneously antagonizing SGK-1 (and blocking ATP generation). Indeed, our analysis of publically available RPPA data from colorectal cancer patients (Figure 7G) demonstrates a correlation between mutant K-Ras levels and simultaneous activation of SGK-1 and inactivation of p38 MAPK. However, while these data are consistent with the notion that simultaneous activation of p38 MAPK and inhibition of SGK-1 might be an effective therapeutic strategy, future experiments aimed at assessing the efficacy of such a regimen will be critical to the development of novel chemotherapies designed to eradicate ECM-detached cancer cells.
Figure 3.13 Model for oncogenic Ras mediated cell survival during ECM-detachment.
CHAPTER 4:

SGK-1-MEDIATED ATP GENERATION: A NOVEL METABOLIC PATHWAY THAT SUPPORTS ECM-DETACHED CELL SURVIVAL

These data are unpublished and part of a collaboration with the following students I have been fortunate to work with: Jordan Cockfield (graduate student), Daniel Pape (undergraduate student), and Hannah Meissner (undergraduate student).

4.1 Abstract

Successful metastasis requires cancer cells to overcome both anoikis - caspase-dependent cell death triggered by extracellular matrix (ECM) detachment - and ECM-detachment-induced metabolic defects that compromise cell survival. While studies have begun to elucidate signal transduction cascades responsible for anoikis evasion, less is known about the precise signals cancer cells use to overcome ECM-detachment-induced metabolic deficiencies. Previously, we discovered that oncogenic Ras utilizes a PI(3)K/SGK-1 signaling cascade in order to promote glucose-mediated ATP generation and survival of ECM-detached cells. We have expanded on these studies and found that SGK-1 signaling is required in a variety of cell types and oncogenic backgrounds (specifically during ECM-detachment) for glucose-derived ATP production - via regulating GLUT1 levels and localization - and anchorage independent growth. When examining the mechanism by which SGK-1 promotes ATP generation, we surprisingly found that
treatment with the mitochondrial uncoupler CCCP did not impact the ability of SGK-1 to promote ATP generation suggesting that the TCA cycle is not required for SGK-1 mediated ATP generation. Intriguingly, ATP generation instead requires flux through the pentose phosphate pathway (PPP) and consequent production of glyceraldehyde-3-phosphate (G3P). PPP-derived G3P is then shuttled back to glycolysis where ATP production can robustly occur. This metabolic pathway appears to be critical for the anchorage independent growth of cancer cells as genetic or pharmacological disruption of glucose flux through the PPP significantly abrogates colony formation in a variety of distinct cell lines. Overall, this study represents a novel metabolic pathway downstream of SGK-1 that is highly conserved across multiple epithelial cancer cell lines during ECM detachment. Our data suggest that SGK-1 may act as a master regulator of glucose metabolism and energy production during ECM-detachment that may be amenable to novel targeted therapies aimed at eliminating ECM-detached cancer cells through disruption of metabolism.

4.2 Introduction

Clinically, 90% of cancer-related deaths are attributed to metastasis, or the spread of cancer from the location of origin to distant sites (Li et al., 2013; Nguyen and Massague, 2007). During each step of the metastatic cascade, cancer cells face periods of extracellular matrix (ECM) detachment, which canonically triggers anoikis, or caspase-dependent cell death (Frisch and Francis, 1994). Thus, it is crucial for cancer cells to overcome anoikis at each step during the metastatic cascade to ultimately survive.
However, overcoming anoikis alone is insufficient to promote long-term survival of cells in ECM detachment (Debnath et al., 2002).

Independently of anoikis induction, ECM detachment triggers additional programs that compromise the viability of cells, such as changes in cellular metabolism (Buchheit et al., 2014b). In particular, ECM-detached cells oftentimes lose the capacity to generate ATP, which is essential to cell survival during detachment (Buchheit et al., 2012; Buchheit et al., 2014b; Davison and Schafer, 2010; Mason et al., 2016; Schafer et al., 2009). While recent studies have begun to elucidate how cancer cells can overcome ECM-detachment-induced metabolic deficiencies (Davison et al., 2013; Jiang et al., 2016; Mason et al., 2016; Schafer et al., 2009), the precise molecular mechanisms governing these processes has remained elusive.

Upon detachment from the ECM, oncogenic signaling is oftentimes critical for cell survival (Buchheit et al., 2015; Buchheit et al., 2014b; Mason et al., 2016). In line with this, oncogenic signaling – specifically ErbB2 and Ras – has the capacity to overcome metabolic deficiencies by enhancing glucose uptake and ATP generation, which leads to cell survival during detachment (Mason et al., 2016; Schafer et al., 2009). Intriguingly, ErbB2 and Ras work through differing downstream PI(3)K effectors, ErbB2 through Akt and Ras through SGK-1, to promote ATP generation and survival during detachment. These studies highlight how different oncogenic insults may utilize different downstream effectors to overcome ECM-detachment-induced metabolic deficiencies. Given that SGK-1 was required downstream of oncogenic Ras for ATP generation and survival during
detachment, it leaves the question if SGK-1 has the capacity to regulate ATP generation in cancer cells that do not harbor oncogenic Ras mutations.

SGK-1 is a serine/threonine kinase that serves as a downstream PI(3)K effector. SGK-1 is similar to the canonical downstream PI(3)K effector Akt – their catalytic domains are approximately 50% similar – however SGK1 does not have a pleckstrin homology domain (Bruhn et al., 2010). SGK-1 activity is regulated via an mTORC2-dependent phosphorylation in the hydrophobic motif (S422), which provides a binding site for PDK1 to phosphorylate the activation loop (T256) (Bruhn et al., 2010; Collins et al., 2003; Pearce et al., 2010). SGK-1 has been linked to facilitating glucose transporters (GLUT1/4) to the membrane to promote glucose uptake (Jeyaraj et al., 2007; Palmada et al., 2006), the pathology of metabolic syndromes that can regulate blood pressure and body weight (Lang et al., 2006), and regulating the major effector of the small GTPase RAN (Amato et al., 2013). Additionally, many recent studies indicate SGK-1 expression and activation are upregulated in malignant tumors and it is critical for tumorigenesis in numerous different cancer types (Castel et al., 2016; Ciriello et al., 2015; Conza et al., 2017; Fagerli et al., 2011; Hall et al., 2012; Kach et al., 2015; Luo et al., 2011; Szmulewitz et al., 2012; Talarico et al., 2015; Talarico et al., 2016a; Talarico et al., 2016b). However, the precise signal transduction cascades regulated by SGK-1, particularly in cancer, remain poorly understood.

Here, we report a novel and unique signaling cascade that is regulated by SGK-1 to promote glucose-mediated ATP generation and anchorage independent growth. Intriguingly, SGK-1-mediated ATP generation is specific for ECM detachment and is
required in a variety of cancer types with differing oncogenic backgrounds. Interestingly, we have found that SGK-1 regulates glucose metabolism through regulating GLUT1 (but not GLUT4) levels and localization at the plasma membrane. Glucose is then shunted into the pentose phosphate pathway (PPP), where it produces glyceraldehyde 3-phosphate (G3P). G3P then re-enters glycolysis and promotes ATP generation and anchorage independent growth. Surprisingly, SGK-1-mediated ATP generation occurs independently of mitochondrial oxidative phosphorylation. These data identify a novel SGK-1-mediated signaling axis that indicates SGK-1 may be amenable to chemotherapeutic intervention. Further, our data substantially refine our current understanding of PI(3)K-mediated glucose metabolism (for ATP production) and anchorage independent growth, and suggest that alternative downstream effectors other than Akt are critical for survival of cancer cells during detachment.

4.3 Results

4.3.1 SGK-1, but not Akt, promotes ATP generation during ECM detachment and anchorage independent growth

Our previous data suggests that different oncogenes can utilize different downstream PI(3)K effectors, namely SGK-1 and Akt (Mason et al., 2016; Schafer et al., 2009), for ATP generation and anchorage independent growth. Thus, we were interested in elucidating the capacity of both SGK-1 and Akt to promote ATP generation during ECM detachment and anchorage independent growth in multiple different cancer cell lines with distinct oncogenic signatures. Using retroviral transduction, we overexpressed
constitutively active SGK-1 (S422D) and Akt (Myr-Akt) and confirmed overexpression and activation (p-NDRG1 for SGK-1 (Heikamp et al., 2014), p-Akt for Akt) via immunoblot in multiple cancer cell lines (Figure 4.1A). Intriguingly, only overexpression of constitutively actively SGK-1, but not Akt, was sufficient to promote ATP generation during detachment and anchorage independent growth in each of these cell lines (Figure 4.1A, 4.1B). This result is not due to an increased activity of SGK-1 compared to Akt (measured via the shared downstream target GSK-3β (Ser9 (Sakoda et al., 2003))) (Figure 4.2A). These data suggest that SGK-1, and not Akt, is sufficient to promote ATP generation during ECM detachment and anchorage independent growth in different cancer types with varying oncogenic backgrounds. Additionally, SGK-1-mediated ATP generation during detachment occurs independently of anoikis regulation, as SGK-1 does not alter anoikis levels (Figure 4.3A). Intriguingly, SGK-1-mediated ATP generation is specific to ECM-detached cancer cells, as SGK-1 is unable to promote ATP generation during ECM attachment (Figure 4.4A). Overall, these data suggest that SGK-1 is a potent inducer of ATP generation, specifically during ECM detachment, and anchorage independent growth – independently of modulating anoikis – in multiple distinct cancer cell types.
Figure 4.1 SGK-1, but not Akt, promotes ATP generation during ECM detachment and anchorage independent growth.

**A.** ATP levels were measured in the indicated cells following 24 hours in ECM detachment. Immunoblotting against p-NDRG1 and SGK-1 confirmed SGK-1 overexpression and activation, and p-Akt was used to confirm activation of Akt in Myr-Akt cells. **B.** The indicated cells were plated in soft agar. After 4 (4T07), 6 (HCT116), or 8 (MDA-MB-468) days, images were taken following INT-violet staining. Colony count was determined using ImageJ. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (EV).
Figure 4.2 Constitutive active SGK-1 does not have higher activity level than constitutive active Akt.

A. The indicated cells underwent immunoblotting for p-GSK3β following 24 hours in detachment.
Figure 4.3 SGK-1-mediated ATP generation occurs independently of anoikis regulation

A. The indicated cells were plated in ECM detachment for 24 hours and caspase-3/7 levels were measured. B. The indicated cells were treated with 25μM EMD638683 for 24 hours in detachment. Following 24 hours, caspase activity was measured. Immunoblotting against p-NDRG1 confirmed inhibitor efficacy. C. Caspase-3/7 activity was measured in the indicated cells following 24 hours in detachment. D. Following 24 hours in detachment, caspase-3/7 levels were measured. Prior to plating cells were treated for 9 days with 10ng/mL doxycycline to induce knockdown of mitochondrial DNA (time matched with Figure 3.5G). All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (EV, shEV) or vehicle treatment (DMSO, No Dox).
Figure 4.4 SGK-1-mediated ATP generation is specific to ECM-detached cells

A. The indicated attached cells were plated in ECM attachment for 24 hours and ATP levels were measured. B. The indicated attached cells were treated for 24 hours with 25μM EMD638683 and ATP levels were measured. Immunoblotting against p-NDRG1 confirmed inhibitor efficacy. C. The indicated cells were grown in ECM attachment for 24 hours and ATP levels were measured. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (EV, shEV) or vehicle treatment (DMSO).
4.3.2 SGK-1 kinase signaling is required to promote ATP generation during detachment and anchorage independent growth

Given our data suggesting constitutively active SGK-1 is sufficient to promote ATP generation during detachment and anchorage independent growth, whereas Akt is not, we elucidated the requirement of SGK-1 for ATP generation during detachment and anchorage independent growth. Loss of SGK-1 signaling using either a small molecule kinase inhibitor of SGK-1, EMD638683, or shRNA-mediated knockdown of SGK-1 (denoted shSGK-1) resulted in a significant decrease in ATP generation during ECM detachment (Figure 4.5A, 4.5B). Immunoblotting was used to confirm the efficacy of EMD638683 and shRNA hairpins (Figure 4.5A, 4.5B). Intriguingly, the requirement for SGK-1 is specific to ECM detachment, as ECM-attached cancer cells do not require SGK-1 signaling for ATP generation (Figure 4.4B, 4.4C). Additionally, loss of SGK-1 signaling, through either pharmacological inhibition or shRNA, resulted in a significant decrease in anchorage independent growth (Figure 4.5C, 4.5D). Additionally, and in line with our data overexpressing SGK-1, inhibition of SGK-1 (either by small molecule or shRNA) does not result in anoikis modulation (Figure 4.3B, 4.3C). Taken together, these data suggest that SGK-1 is required, specifically during ECM detachment, in multiple distinct cancer cells with different sites of origin and oncogenic backgrounds to promote ATP generation and anchorage independent growth. Additionally, these data indicate that SGK-1-mediated changes in metabolism occur independently of anoikis regulation.
Figure 4.5 SGK-1 kinase signaling is required to promote ATP generation during detachment and anchorage independent growth.

A. ATP levels were measured in the indicated cells following 24 hours in ECM detachment in the presence of the SGK-1 kinase inhibitor EMD638683 (25μM). Immunoblotting against p-NDRG1 was used to confirm the efficacy of the EMD638683. B. ATP levels were measured in the indicated cells following 24 hours in detachment. Immunoblotting for SGK-1 confirmed efficacy of shRNA-mediated inhibition. C, D. The indicated cells were plated in soft agar and treated with 25μM EMD638683 (C) or shRNA towards SGK-1 (D). After 4 (4T07 shSGK-1), 5, (4T07 EMD638683, HCT116 EMD638683, 6 (HCT116 shSGK-1), 9 (MDA-MB-468 EMD638683), or 14 (KPL4 shSGK-1) days, images were taken following INT-violet staining. Colony count was determined using ImageJ. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (shEV) or vehicle treatment (DMSO).
4.3.3 SGK-1 signaling regulates glucose uptake in ECM-detached cells

Given that SGK-1 regulates ATP production during detachment, and a large percentage of ATP in cells is generated by glucose catabolism, we investigated if SGK-1 signaling had the capacity to regulate glucose uptake. In agreement with SGK-1-mediated ATP generation occurring as a result of increased glucose metabolism, constitutive active SGK-1, and to a much lesser extent Akt, is sufficient to promote glucose uptake during detachment (Figure 4.6A). While these data suggest that Akt has the capacity to - albeit to a lower magnitude compared to SGK-1 - regulate glucose uptake, this regulation is insufficient to lead to changes in ATP generation during detachment or anchorage independent growth (Figure 4.1A-D). Moreover, loss of SGK-1 signaling through small molecule inhibition (confirmed via immunoblot) or shRNA leads to a significant decrease in glucose uptake during detachment (Figure 4.6B, 4.6C). Overall, these data suggest that SGK-1-mediated ATP generation occurs as a result of SGK-1 being sufficient, and required, to regulate glucose uptake during ECM detachment.
Figure 4.6 SGK-1 signaling regulates glucose uptake in ECM-detached cells

A. Glucose uptake levels were measured at 24 hours in the indicated detached cells. B. Glucose uptake levels were measured in the indicated detached cells after 24 hours. The indicated cells were treated with 25μM EMD638683 at the time of plating. Immunoblotting against p-NDRG1 was used to confirm the efficacy of EMD638683. C. Glucose uptake levels were measured in the indicated cells following 24 hours in ECM detachment. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (EV, shEV) and/or vehicle treatment (DMSO).
4.3.4 SGK-1-mediated glucose metabolism occurs via regulation of GLUT1

Given that SGK-1 regulates glucose metabolism, we began seeking the mechanism in which this occurs. As glucose uptake is regulated by glucose transporters (GLUTs), we reasoned that SGK-1 may regulate GLUTs to promote the observed glucose uptake. In particular, we examined SGK-1 regulation of GLUT1 and GLUT4, which have been shown to be regulated by SGK-1 (Jeyaraj et al., 2007; Palmada et al., 2006). In line with GLUT1 regulation by SGK-1, overexpression of SGK-1 leads to an increase in GLUT1 abundance, whereas loss of SGK-1 via shRNA leads to a decrease in GLUT1 abundance via immunoblot (Figure 4.7A, 4.7B).

As GLUTs must be localized to the plasma membrane to promote glucose uptake, we examined the localization of GLUT1 upon addition of constitutively active SGK-1 or upon shRNA-mediated knockdown of SGK-1. Overexpression of SGK-1 leads to an increase in not only the abundance of GLUT1, but also the percentage at the plasma membrane, whereas loss of SGK-1 leads to significant decreases in abundance and percentage at the plasma membrane (Figure 4.7C-E). Surprisingly, while SGK-1 can regulate GLUT1, neither overexpression of SGK-1 or loss of SGK-1 leads to significant changes in the abundance of GLUT4 (Figure 4.7F-H). Overall, these data suggest that SGK-1 regulates the abundance, and localization to the plasma membrane, of the glucose transporter GLUT1, but not GLUT4, during ECM detachment.
Figure 4.7 SGK-1-mediated glucose metabolism occurs via regulation of GLUT1.

**A, B.** The indicated cells underwent immunoblotting for GLUT1 levels following 24 hours in detachment. **C, D, E.** Following 24 hours in detachment, the indicated cells underwent immunofluorescence for GLUT1. Representative images are shown at 100x magnification (left) and GLUT1 levels and percent at membrane are quantified on right. **F, G, H.** The indicated cells underwent immunofluorescence for GLUT4 following 24 hours in detachment. Representative images at 100x magnification (left) are shown and GLUT4 abundance is quantified below.
4.3.5 Mitochondrial oxidative phosphorylation is disposable for SGK-1-mediated ATP generation

Since SGK-1 regulates glucose metabolism and ATP production during detachment, we explored whether the majority of ATP was being produced via glycolysis or oxidative phosphorylation. As expected, loss of glycolytic flux (and glucose-derived carbons entering the mitochondria) by 2-deoxyglucose (2-DG), leads to substantial decreases in ATP generation during detachment and anchorage independent growth (Figure 4.8A, 4.8B). To examine if this loss of ATP generation during detachment upon 2-DG treatment was due to oxidative phosphorylation, we added cell permeable methyl pyruvate that bypasses glycolysis and enters the mitochondria for oxidative phosphorylation (Nutt et al., 2005). Surprisingly, addition of methyl pyruvate was unable to overcome the loss of ATP production by addition of 2-DG (Figure 4.8C), even though methyl pyruvate alone has the capacity to promote ATP generation during detachment in non-transformed mammary epithelial cells (Figure 4.9A). These data suggest that glycolytic-derived ATP generation is imperative for promoting ATP generation during detachment and anchorage independent growth.

We next sought to identify if SGK-1-mediated ATP generation was also occurring due to glycolytic flux and not oxidative phosphorylation. In line with this being the case, addition of methyl pyruvate was unable to overcome the loss of ATP production upon inhibition of SGK-1 (inhibition confirmed via immunoblot) (Figure 4.8D). Further indicating that SGK-1-mediated ATP generation during detachment is occurring independently of oxidative phosphorylation, treatment of 4T07 S422D cells with the mitochondrial
uncoupler CCCP does not mitigate SGK-1-mediated ATP generation, whereas 2-DG abrogates SGK-1-mediated ATP generation during detachment (Figure 4.8E). This result is not because CCCP not being functional, as control cells see marked decrease in ATP generation during detachment upon both CCCP and 2-DG administration (Figure 4.8E). Additionally, to further confirm glycolysis-dependent, oxidative phosphorylation-independent, SGK-1-mediated ATP generation during detachment, we overexpressed constitutively active SGK-1 (S422D) and Akt (Myr-Akt) in ρ(o) cells (ATPIF1 KO). These cells have an inducible dominant negative polymerase gamma, which depletes mitochondrial DNA (the electron transport chain), as well as an ATPIF1 knockout to maintain mitochondrial membrane potential (Martinez-Reyes et al., 2016) (Figure 4.8F). Intriguingly, only overexpression of SGK-1, and not Akt, was sufficient to increase glucose uptake and ATP generation during detachment in ρ(o) cells, and this was maintained upon depletion of mitochondrial DNA (Figure 4.8G). Additionally, SGK-1-mediated ATP generation does not occur because of anoikis regulation (Figure 4.3D). Overall, these data suggest that SGK-1 regulates glucose metabolism for ATP generation during detachment via glycolytic flux and independently of oxidative phosphorylation.
Figure 4.8 Mitochondrial oxidative phosphorylation is disposable for SGK-1-mediated ATP generation.

A. ATP levels were measured following 24 hours in detachment and treatment with 10mM 2-DG. B. 4T07 cells were plated in soft agar and treated with 10mM 2-DG. After 5 days, images were taken following iodonitrotetrazolium chloride (INT-violet) staining. Colony count was determined using ImageJ. C. ATP levels were measured after 24 hours in detached 4T07 cells following treatment with 10mM 2-DG and 10mM methyl pyruvate (MP). D. ATP levels were measured after 24 hours in ECM detachment. Cells were treated with 25μM EMD638683 and the indicated dose of methyl pyruvate (MP) at plating. Immunoblotting of p-NDRG1 was used to confirm inhibitor efficacy. E. The indicated cells were plated in detachment for 3 hours with 10mM 2-DG or 25μM CCCP treatment. F. Immunoblotting against SGK-1 and p-NDRG1 was used to confirm overexpression and activation of SGK-1 in S422D cells. Immunoblotting against p-Akt was used to confirm activation and overexpression of Akt in Myr-Akt cells. G. Glucose uptake levels (left) and ATP levels (right) were measured in the indicated detached cells after 24 hrs. 10ng/mL doxycycline was used to induce ATPIF1 knockout in ATPIF1 KO cells for 9 days prior to plating. Immunoblotting for mtCOXII was used to confirm loss of mitochondrial DNA. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (EV) or vehicle treatment (DMSO, EtOH, H20).
Figure 4.9 Methyl pyruvate promotes ATP generation in ECM-detached MCF-10A cells

A. MCF-10A cells were grown for 24 hours in detachment and treated with the indicated dose of methyl pyruvate (MP). Following 24 hours, ATP levels were measured. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with vehicle treatment (EtOH).
4.3.6 PPP flux is required for SGK-1-mediated ATP generation and anchorage independent growth

As SGK-1 regulates glucose-mediated ATP generation independently of oxidative phosphorylation, we explored if any glycolytic shunts were required for ATP generation during detachment. Of particular interest was the PPP, which has been shown previously to be required for ATP generation during detachment (Schafer et al., 2009). In line with SGK-1-mediated ATP generation occurring due to PPP flux, small molecule inhibition of the PPP using 6-AN significantly decreased ATP generation downstream of SGK-1 signaling during detachment (Figure 4.10A). To confirm that our data with the inhibitor was not due to off-target effects, we used siRNA-mediated knockdown (and confirmed via immunoblot) towards the rate limiting PPP enzyme G6PD (Figure 4.10B). Upon even minimal decreases of G6PD via siRNA, there was a significant decrease in ATP generation during detachment downstream of SGK-1 signaling (Figure 4.10B). These data suggest that SGK-1-mediated ATP generation during detachment requires PPP flux.

The PPP serves as a large producer of NADPH in cells, which serves to maintain redox homeostasis and support anaplerosis/biosynthesis (Patra and Hay, 2014). Additionally, PPP-mediated ATP generation during detachment was previously linked to NADPH production for mitigating oxidative stress (Schafer et al., 2009). To examine if the loss of ATP generation during detachment upon PPP inhibition was due to a loss in NADPH production, we exogenously added methyl malate, which is converted to NADPH via malic enzyme independently of PPP flux (Schafer et al., 2009). Intriguingly, addition of methyl malate was unable to restore ATP generation upon inhibition of the PPP by either small
molecule or siRNA (confirmed via immunoblot) (Figure 4.10C, 4.10D). In agreement with loss of NADPH not being the cause of the decreased ATP generation observed upon PPP inhibition, PPP inhibition does not lead to increases in oxidative stress during detachment, nor does methyl malate mitigate oxidative stress in these conditions (Figure 4.10C, 4.10D). These results were not due to PPP regulation of anoikis (Figure 4.11A) or methyl malate being generically unable to regulate redox homeostasis for ATP generation, as methyl malate was sufficient to increase ATP generation during detachment and mitigate oxidative stress in MCF-10A cells (Figure 4.11B, 4.11C)(Schafer et al., 2009). Overall, these data suggest that SGK-1-mediated ATP generation during detachment is occurring through PPP flux, yet is independent of anoikis regulation, PPP-mediated NADPH production, or oxidative stress mitigation.
Figure 4.10 PPP flux is required for SGK-1-mediated ATP generation and anchorage independent growth.

**A.** ATP levels were measured in the indicated SGK-1 overexpressing cells following 24 hours in detachment. Cells were treated with 150μM 6-AN at time of plating. **B.** ATP levels were measured in the indicated cells transfected with siRNA for G6PD following 24 hours in ECM detachment. Immunoblotting for G6PD confirmed siRNA efficacy. **C, D.** ATP levels were measured (left) and ROS levels were measured (right) following 24 hours in detachment. Cells were treated with either 150 μM 6-AN (C), or siRNA towards G6PD (D) and the indicated concentration of methyl malate (MM). Immunoblotting against G6PD confirmed siRNA knockdown. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with non-targeting siRNA (siNT) or vehicle treatment (DMSO, EtOH).
Figure 4.11 SGK-1/PPP-mediated ATP generation occurs independently of anoikis and ROS regulation

A. Caspase-3/7 levels were measured following 24 hours in detachment and treatment with 150μM 6-AN. B. MCF-10A cells were plated in ECM detachment for 24 hours. Cells were treated with 5mM methyl malate (MM) at the time of plating, and ATP levels were measured. C. Oxidative stress (ROS) levels were measured following 24 hours in detachment in MCF-10A cells treated with 5mM methyl malate (MM). All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with vehicle treatment (DMSO, EtOH).
### 4.3.7 SGK-1/PPP-mediated ATP generation and anchorage independent growth is due to G3P production

Given that PPP-mediated ATP generation is not due to producing NADPH for redox homeostasis, we interrogated what other PPP product was responsible for SGK-1-mediated ATP generation during detachment. Apart from NADPH, the PPP produces G3P, which is able to re-enter glycolysis (Patra and Hay, 2014), presumably leading to ATP generation. To test the possibility of SGK-1-mediated ATP generation through PPP flux being dependent upon G3P production (and subsequent metabolism through glycolysis), we treated our 4T07 S422D cells with 6-AN or siRNA towards G6PD (confirmed via immunoblot) and saw the expected decrease in ATP generation during detachment (Figure 4.12A, 4.12B). Surprisingly, loss of ATP generation via PPP inhibition could be overcome by the addition of nicotinamide mononucleotide (NMN) (Figure 4.12A, 4.12B), an NAD+ precursor that stimulates GAPDH activity to metabolize G3P (Yun et al., 2015). These data suggest that G3P metabolism is being limited upon PPP inhibition and is required for SGK-1-mediated ATP generation during detachment.

To test the requirement of G3P production downstream of SGK-1 by PPP flux for ATP generation during detachment more directly, we introduced exogenous G3P – a non-cell permeable metabolite – via use of the detergent saponin (Chang et al., 2013). In agreement with our NMN data, addition of G3P was sufficient to rescue the loss of ATP generation during detachment upon PPP inhibition by either small molecule or siRNA (confirmed via immunoblot) (Figure 4.12C, 4.12D). Overall, these data suggest that SGK-
1/PPP-mediated ATP generation during ECM detachment growth requires the production, and subsequent metabolism, of G3P via the PPP.

We next sought to further confirm the requirement of G3P production downstream of SGK-1 via addition of NMN or exogenous G3P. In line with our previous data suggesting SGK-1 requires G3P production and metabolism via glycolysis for ATP generation during detachment, addition of NMN was sufficient to overcome loss of ATP generation during detachment and anchorage independent growth upon SGK-1 inhibition via small molecule (confirmed via immunoblot) or shRNA in multiple distinct cancer cell lines (Figure 4.12E-H). Additionally, exogenous G3P was sufficient to rescue the loss of ATP generation during detachment upon inhibition of SGK-1 via shRNA or small molecule (confirmed via immunoblot) (Figure 4.12I, 4.12J). Overall, these data suggest that SGK-1-mediated ATP generation during detachment and anchorage independent growth is reliant upon G3P production (from the PPP), which re-enters glycolysis for ATP generation, in multiple cancer cell lines with different oncogenic backgrounds.
Figure 4.12 SGK-1/PPP-mediated ATP generation and anchorage independent growth is due to G3P production.

A. ATP levels following 24 hours in detachment. Cells were treated with 150μM 6-AN and 1mM NMN. B. ATP levels were measured in the indicated cells following 24 hours in detachment and treatment with siRNA towards G6PD and 1mM NMN. Immunoblotting for G6PD confirmed siRNA efficacy. C, D. ATP levels were measured after 24 hours in detachment. Cells were treated with 150μM 6-AN (C) or siG6PD (D). Following 24 hours, cells were treated with .01% saponin on ice for 30 minutes, followed by incubation with 100μM G3P at 37 C. Immunoblotting against G6PD confirmed siRNA knockdown. E. The indicated cells were plated in ECM detachment for 24 hours. ATP levels were measured following treatment with 25μM EMD638683 and 1mM NMN. Immunoblotting for p-NDRG1 confirmed inhibitor efficacy. F. ATP levels were measured in the indicated detached cells following 24 hours. Cells were treated with 1mM NMN. G. The indicated cells were plated in soft agar and treated with 25μM EMD638683 and 1mM NMN. Images were taken following 5 (HCT116) or 9 (MDA-MB-468) days and stained using INT-violet. Colony count was determined using ImageJ. H. The indicated cells were plated in soft agar and treated with 1mM NMN for 4 (4T07) or 6 (HCT116) days. Colonies were then stained with INT-violet and colony count was determined via ImageJ. I, J. ATP levels were measured following 24 hours in ECM detachment and treatment with 25μM EMD638683 (I) or shSGK-1 (J). Following 24 hours, cells were treated with .01% saponin on ice for 30 minutes, followed by incubation with 100μM G3P at 37 C. Immunoblotting of p-NDRG1 confirmed inhibitor efficacy. All data are presented as mean ± S.D. and statistical significance was calculated using a two-tailed t-test. Fold change is calculated as a ratio compared with empty vector (shEV, siNT) or vehicle treatment (H2O, DMSO).
4.4 Discussion

Upon detachment from the ECM, cancer cells must be able to overcome ECM-detachment-induced metabolic deficiencies – such as ATP generation – to promote survival and anchorage independent growth (Buchheit et al., 2014b; Mason et al., 2016; Schafer et al., 2009). However, the precise molecular mechanisms governing this process remain poorly understood. The data described here indicate a unique and novel SGK-1 signaling axis that is crucial to the survival of a variety of different ECM-detached cancer cell lines (Figure 4.13). These data suggest that, while studies have indicated different oncogenes signal through differing downstream effectors to promote metabolic maintenance during detachment (Buchheit et al., 2012; Buchheit et al., 2014b; Davison et al., 2013; Mason et al., 2016; Schafer et al., 2009), SGK-1 may be a common downstream regulator of many different oncogenes that is required for ATP generation, specifically during ECM detachment, and anchorage independent growth.

These data add to the literature suggesting that downstream PI(3)K effectors other than Akt, such as SGK-1, are crucial to the survival of cancer cells and can promote chemotherapeutic resistance (Bruhn et al., 2010; Castel et al., 2016; Gasser et al., 2014; Talarico et al., 2016b). In line with this, SGK-1 has been implicated to be critical for promoting resistance to PI(3)K and androgen inhibitors in the clinic (Arora et al., 2013; Castel et al., 2016; Isikbay et al., 2014; Sommer et al., 2013). These data have led to the rapid development of SGK-1 inhibitors for the therapeutic purposes (Sherk et al., 2008; Skor et al., 2013; Talarico et al., 2015; Talarico et al., 2016a; Talarico et al., 2016b), and our data suggests the potential for broad applicability of these inhibitors to many cancers.
from different origins and oncogenic backgrounds. Additionally, our data provide insight into the molecular mechanisms underlying SGK-1-mediated survival of cancer cells, and highlight how inhibitors of SGK-1 may be advantageous to specifically eliminate ECM-detached cancer cells.

Our data are the first, to our knowledge, to identify the SGK-1 axis through GLUT1/PPP/G3P as critical for the survival of ECM-detached cancer cells. Thus, apart from SGK-1, our data also highlight the possibility of targeting GLUT1 localized at the plasma membrane to eliminate metastatic cancer cells, which has been under investigation (Liu et al., 2012; Yun et al., 2015). Further, our data uncover a novel metabolic pathway that could be antagonized at multiple locations using targeted therapy, such as simultaneously targeting SGK-1 and GLUT1. However, more studies are needed to test the efficacy, and specificity, of this type of combinatorial therapy.

Intriguingly, SGK-1-mediated ATP generation during detachment and anchorage independent growth occurs independently of mitochondrial oxidative phosphorylation. While aerobic glycolysis has been shown to be preferentially supported by cancer cells since the 1920’s (Warburg et al., 1924), most cancer cells do not have defects in oxidative phosphorylation (Dupuy et al., 2015; Moreno-Sanchez et al., 2007; Wallace, 2012). One possibility is that aerobic glycolysis accounts for the high production of ATP (DeBerardinis et al., 2008), as even though the yield of ATP per glucose consumed is low, the high glycolytic flux exceeds that of oxidative phosphorylation (Guppy et al., 1993; Warburg, 1956b). Further, bypassing oxidative phosphorylation may lead to the reduction of oxidative stress-inducing free radicals. However, additional experiments are needed to
identify why SGK-1-mediated ATP generation occurs independently of oxidative phosphorylation, as well as the utilization of TCA intermediates during this signaling cascade.

Our data also suggest that SGK-1-mediated ATP generation occurs via flux through the PPP, however the precise molecular mechanisms underlying this process remain elusive. Given that the PPP is a key provider of NADPH for redox homeostasis/biosynthesis, D-ribose-5-phosphate for nucleotide synthesis, and G3P for ATP generation and serine/glycine metabolism (Cantor and Sabatini, 2012; Patra and Hay, 2014; Schulze and Harris, 2012; Ward and Thompson, 2012), it is feasible to believe that SGK-1-mediated increases in glucose uptake promotes PPP flux to meet the many metabolic demands of proliferating cells. Additionally, SGK-1 may have the capacity to upregulate PPP enzymes to enhance PPP flux downstream of SGK-1. Further studies are necessary to understand the link between SGK-1 signaling and PPP flux, however.

Overall, our data highlight the dependence upon SGK-1 signaling in promoting the survival of many different cancer cell types with different oncogenic backgrounds, indicating that SGK-1 may be a master regulator of ATP generation specifically during ECM detachment. Thus, these data add substantially to the underpinnings of SGK-1-mediated survival in cancer cells. Our data also further indicate, and highlight, SGK-1 as a potentially viable target for the development of targeted therapies for the clinic to specifically eliminate metastatic disease.
Figure 4.13 Model for SGK-1-mediated ATP production in ECM-detached cancer cells.
CHAPTER 5:
CONCLUSIONS AND PERSPECTIVES

5.1 Targeting Ras-driven cancers through downstream effectors

Since the 1980’s, it has been well appreciated that mutations in Ras occur in a large percentage of human cancers (Bos, 1989). However, specifically targeting Ras has proven troublesome, and Ras has been described as elusive and undruggable (Bates, 2015; Downward, 2003; Gysin et al., 2011). At least part of the difficulties faced when targeting Ras are that GTP-competitive inhibitors must be used at too high of concentrations (Becher et al., 2013; John et al., 1993), the fluidity of the surface of the Ras protein hinders identifying proper binding sites for orthosteric inhibitors with high enough affinity and potency (Spiegel et al., 2014), variable responses to covalent locking of Ras-GDP binding (Lim et al., 2014), and differential post-translational processing of Ras isoforms (Ahearn et al., 2011).

These studies highlight that therapeutic options that inhibit specific downstream Ras effectors that are crucial for tumorigenesis may be a more viable option for targeting Ras-driven cancers. In line with this, there have been many attempts to inhibit downstream Ras signaling, but inhibiting major downstream effects (such as the PI(3)K and MAPK pathway) have shown minimal efficacy in the clinic, in part because of negative
feedback loops (Manning et al., 2005; O'Reilly et al., 2006), toxic side effects (Foukas et al., 2006; Shi et al., 2005), inhibition of normal cellular function (Alloatti et al., 2004), and resistance to treatment (Kobayashi et al., 2005; Lito et al., 2013). Thus, more specific inhibitors that function downstream of major Ras effectors may be more efficacious therapeutic targets.

Of particular interest downstream of PI(3)K is SGK-1, a serine/threonine kinase that shares 50% similarity with Akt in the catalytic domain (Bruhn et al., 2010). SGK-1 activity is regulated via an mTORC2-dependent phosphorylation in the hydrophobic motif (S422), which provides a binding site for PDK1 to phosphorylate the activation loop (T256) (Bruhn et al., 2010; Collins et al., 2003; Pearce et al., 2010). SGK-1 has been linked to facilitating glucose transporters (GLUT1/4) to the membrane to promote glucose metabolism (Jeyaraj et al., 2007; Palmada et al., 2006), the pathology of metabolic syndromes that can regulate blood pressure and body weight (Lang et al., 2006), and regulating the major effector of the small GTPase RAN (Amato et al., 2013). Additionally, many recent studies indicate SGK-1 expression and activation are upregulated in malignant tumors and it is critical for tumorigenesis in numerous different cancer types (Castel et al., 2016; Ciriello et al., 2015; Conza et al., 2017; Fagerli et al., 2011; Hall et al., 2012; Kach et al., 2015; Luo et al., 2011; Szmulewitz et al., 2012; Talarico et al., 2015; Talarico et al., 2016a; Talarico et al., 2016b). Our data suggest that SGK-1 is required downstream of oncogenic Ras to promote ATP generation, anchorage independent growth, and survival specifically during detachment (Mason et al., 2016; Mason and Schafer, 2016, 2017). While more studies are necessary to test the efficacy of targeting
SGK-1 in the clinic, our data suggest that in cancers harboring oncogenic Ras mutations (30% of all cancers), targeting SGK-1 may be able to specifically eliminate ECM-detached cancer cells.

While SGK-1-mediated ATP generation is crucial for the survival of ECM-detached cells with oncogenic Ras mutations, it is well-known that cancer cells must overcome both ECM-detachment-induced metabolic deficiencies (in this case via SGK-1 signaling) and anoikis induction (Buchheit et al., 2014b). However, we found that SGK-1, while it regulates metabolism, does not regulate anoikis in ECM-detached cells harboring oncogenic Ras mutations (Mason et al., 2016). Thus, an alternative downstream effector must be responsible to promote anoikis evasion downstream of oncogenic Ras.

Intriguingly, our data suggested that upon inhibition of the PI(3)K pathway, while ATP generation is lost during detachment (due to PI(3)K/SGK-1 signaling), Akt activity is maintained. These data indicated that oncogenic Ras may have the capacity to downregulate the phosphatase for Akt (S473). The phosphatase PHLPP1 is known to negatively regulate Akt by dephosphorylating the hydrophobic motif (S473) (Gao et al., 2005; Newton and Trotman, 2014). Further, PHLPP1 regulation during tumorigenesis has intensified over the past few years, as many labs have now implicated loss of PHLPP1 activity as an important facet of cancer cell survival (Cai et al., 2013; Newton and Trotman, 2014; Wang et al., 2014; Wen et al., 2013). We find that in ECM-detached cells, oncogenic Ras downregulates PHLPP1 to overcome p38 MAPK-mediated anoikis induction (Mason et al., 2016). However, the precise mechanism by how oncogenic Ras downregulates PHLPP1, or how PHLPP1 activates p38 MAPK is still uncertain. Presumably, PHLPP1-
mediated activation of p38 MAPK occurs through Mst-1, which has been identified as a substrate of PHLPP1. Desphosphorylation of Mst-1 leads to its activation and ultimately to stimulation of p38 MAPK activity (Qiao et al., 2010; Webster et al., 1993). Future experiments are needed to confirm if Mst-1 is, indeed, acting downstream of PHLPP1 to activate p38 MAPK.

Overall, our data suggest that ECM-detached cells that harbor oncogenic Ras mutations utilize divergent downstream effectors to overcome ECM-detachment-induced metabolic deficiencies (ATP generation) and anoikis. In particular, our data show that simultaneous inhibition of SGK-1 signaling and activation of PHLPP1 signaling is potentially the most effective way to eliminate ECM-detached cancer. In agreement with this, RPPA data from colon cancer patients harboring oncogenic K-Ras mutations see simultaneous activation of SGK-1 and inhibition of p38 MAPK signaling. More studies specifically looking at this potential therapeutic combination are needed. Our data suggesting that oncogenic Ras utilizes divergent downstream effectors during ECM detachment significantly adds to the field and provides novel therapeutic approaches to potentially specifically target, and eliminate, metastatic cancer cells harboring oncogenic Ras mutations.

5.2 SGK-1: A potential master regulator of ATP generation during ECM detachment

Given that SGK-1 is critical downstream of oncogenic Ras to promote ATP generation and survival during detachment, we began exploring if SGK-1 was also required for ATP generation and survival during detachment for cancer cells that do not
harbor oncogenic Ras mutations. Intriguingly, we find that SGK-1 is crucial to produce ATP during detachment and anchorage independent growth in a panel of cancer cells of varying tissues of origin and oncogenic insults. These data suggest that SGK-1 is not only required downstream of oncogenic Ras, but it is also essential downstream of many other activating oncogenic insults.

When looking at the downstream SGK-1 signaling axis, we were intrigued to see that SGK-1 regulates the glucose transporter GLUT1, but not GLUT4, as it has been previously shown to have the capacity to directly promote both transporters translocation to the plasma membrane (Jeyaraj et al., 2007; Palmada et al., 2006). These data suggest the possibility that targeting GLUT1, either in combination with SGK-1 or independently, may be an efficacious therapeutic target. Preliminary data in K-Ras and B-Raf mutant cancers suggest that GLUT1 presence at the plasma membrane can be utilized for therapeutic intervention (Yun et al., 2015). Additionally, small molecule inhibitors of GLUT1 have shown promise in preliminary studies in vitro and in vivo (Liu et al., 2012). Further, interrogating the requirement of GLUT1 downstream of SGK-1 by either shRNA, siRNA, or small molecule is necessary to additionally link SGK-1 and GLUT1. It is also essential to understand how SGK-1 regulates GLUT1 levels and localization. Examples of pathways involved could be SGK-1 promoting phosphorylation of GLUT1, increasing transcription of GLUT1, or inhibiting GLUT1 turnover via the proteasome or lysosome.

Downstream of glucose uptake is the catabolism of glucose into pools of metabolites that fit the bioenergetic, redox, and biosynthetic needs of a proliferating cancer cells (Cantor and Sabatini, 2012; Schulze and Harris, 2012; Ward and Thompson,
Upon SGK-1 constitutive activation, we find a need for flux through the PPP to promote ATP generation during detachment and anchorage independent growth. The PPP is a major source of the reducing/biosynthetic equivalent NADPH, as well as D-ribulose-5-phosphate for nucleotide synthesis, and G3P that can re-enter glycolysis (Bensaad et al., 2006; Herrero-Mendez et al., 2009; Pandolfi et al., 1995; Patra and Hay, 2014; Schafer et al., 2009) and produce either ATP or be shunted through the serine/glycine metabolism to meet other needs of the cell (such as NADPH production or nucleotide synthesis). We find that, downstream of SGK-1 activation, the PPP is essential for producing G3P that can re-enter glycolysis and promote ATP generation. Preliminary results (not included) from metabolite flux analysis suggest this is the case, but PPP flux in these cells also produces an increase in the abundance of D-ribose-5-phosphate, as well as reduced glutathione (indicative of NADPH production). Thus, SGK-1-mediated PPP flux may be essential as it allows the cell to make the biosynthetic/redox homeostasis molecule NADPH, D-ribose-5-phosphate for nucleotide synthesis, and promote ATP generation; all from a single molecule of glucose. However, more studies are essential to see if this is indeed the case. Further studies elucidating whether SGK-1-mediated PPP is due to the increased abundance of glucose uptake, or if it is targeted (such as through inhibition of phosphofructokinase, glucose 6-phosphate isomerase, etc.), are needed.

One interesting component of SGK-1 promoting high levels of glucose uptake and PPP flux that is essential for ATP generation during detachment and anchorage independent growth is that it occurs independently of mitochondrial oxidative phosphorylation. While aerobic glycolysis has been shown to be preferentially supported
by cancer cells since the 1920’s (Warburg et al., 1924), most cancer cells do not have defects in oxidative phosphorylation (Dupuy et al., 2015; Moreno-Sanchez et al., 2007; Wallace, 2012). This increase in aerobic glycolysis accounts for the high production of ATP (DeBerardinis et al., 2008), as even though the yield of ATP per glucose consumed is low, the high glycolytic flux is high enough that the percent of ATP produced from glycolysis exceeds that of the much slower oxidative phosphorylation (Guppy et al., 1993; Warburg, 1956b). Further, bypassing oxidative phosphorylation may lead to the reduction of oxidative stress-inducing free radicals. With this in mind, while we see an independence of oxidative phosphorylation for ATP generation during detachment, preliminary data (not shown) suggest that TCA intermediates are also increased in the presence of constitutively active SGK-1 (constitutively active SGK-1 also leads to increases in the abundance of alanine and lactate produced downstream of pyruvate). One possible explanation is that the majority of TCA cycle intermediates are being used for anaplerosis rather than bioenergetics, which has been shown to occur frequently in cancer (Cetinbas et al., 2016; DeBerardinis and Chandel, 2016). Additional experiments are needed to identify the utilization of TCA intermediates in cells downstream of SGK-1 signaling. Further, experiments aimed at elucidating why SGK-1-mediated ATP generation occurs independently of oxidative phosphorylation may aid in elucidating the role of TCA intermediates downstream of SGK-1. Possibilities of SGK-1-mediated inhibition of glucose-derived carbons into the mitochondria could be the phosphorylation, and inactivation, of pyruvate dehydrogenase or negatively regulating the mitochondrial pyruvate carrier.
Overall, our data suggest that SGK-1 is required in a plethora of different cancer cell lines with varying oncogenic backgrounds to promote ATP generation and survival during ECM detachment. Additionally, our studies highlight a novel and unique mechanism utilized by SGK-1 that could provide promise to the development of combinatorial therapeutic approaches to specifically target, and eliminate, metastatic cancer cells. Thus, while more studies are needed, we believe that SGK-1 may serve as a master regulator for ATP generation and survival during ECM detachment, and may provide an ideal candidate for therapeutic intervention. In vivo studies directly targeting SGK-1 (via novel small molecules or genetic inhibition) could begin to elucidate therapeutic efficacy.
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