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Succinyl-CoA ligase ADP-forming subunit beta promotes stress granule assembly to regulate redox and drive cancer metastasis

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By

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B.S., Public Health, Tulane University, 2015

M.P.H., Epidemiology, Tulane University, 2017

Advisor: Sumin Kang, Ph.D.

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Abstract

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By Austin Boese

Dysregulated cellular metabolism is a common characteristic of cancer, but recent studies highlight significant differences in metabolic phenotypes both within primary tumors, and in tumor cells that metastasize to distant organs. While highly proliferative primary tumor cells often exhibit the Warburg effect, marked by a preference for aerobic glycolysis to generate ATP, genes for mitochondrial biogenesis and oxidative phosphorylation are often upregulated in metastatic cancer cells. Therefore, detailed characterization of reprogrammed metabolic pathways in metastatic cancer may reveal useful targets to combat tumor progression and improve patient survival.

To successfully metastasize, cancer cells must first resist anoikis: an apoptotic cell death mechanism triggered by loss of proper contact with the extracellular matrix. Our lab has previously demonstrated that the mitochondrial enzyme glutamate dehydrogenase 1 (GDH1) contributes to anoikis resistance and metastasis by regulating the bioenergetic response through reactivation of AMPK in LKB1-deficient lung cancer. However, the role of other mitochondrial enzymes in anoikis resistance remains poorly understood. To identify other factors important for cancer cell anoikis resistance, we performed an unbiased RNAi screen targeting 120 mitochondrial enzymes in lung cancer cells and identified the ATP-specific Succinyl-CoA Synthetase beta subunit (SUCLA2) as an important factor for cancer cell survival after ECM detachment. Stable knockdown of SUCLA2 sensitized several cancer cell lines to anoikis when cultured under non-adherent conditions in vitro. Bioinformatic analysis of publicly available data revealed that higher tumor SUCLA2 mRNA expression is associated with poor cancer patient overall survival, and immunohistochemistry staining indicated that SUCLA2 protein levels are higher in metastatic lung and breast cancer tumors compared to their matched primary tumors. Detailed metabolic assays showed that the metabolic contribution of SUCLA2 during metastasis is independent of its role in the Krebs cycle and involves promoting protein expression of redox scavenging enzymes through stress granules in the cytosol. These findings provide a framework to identify and characterize novel metabolic features that selectively promote metastasis of solid tumors. Collectively, these data expand our understanding of the molecular mechanisms that drive cancer metastasis and identify SUCLA2 as a promising target that should be studied further for future anti-metastatic cancer therapy.

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Table of Contents

List of Figures

Abbreviations

ataxin-2-like (ATXN2L)

ATP-specific SCS (SCS-A)

electron transport chain (ETC)

extracellular matrix (ECM)

epithelial-mesenchymal transition (EMT)

extracellular acidification rate (ECAR)

glutamate dehydrogenase (GLUD1, GDH1)

inner mitochondrial membrane (IMM)

mitochondrial outer membrane permeabilization (MOMP)

N-acetylcysteine (NAC)

nuclear fragile X mental retardation interacting protein 2 (NUFIP2)

oxygen consumption rate (OCR)

oxidative phosphorylation (OXPHOS)

pentose phosphate pathway (PPP)

peroxiredoxin 1/2/4 (PRX1/2/4)

reactive oxygen species (ROS)

ras-GTPase-activating protein SH3 domain-binding protein 1 and 2 (G3BP1/2),

succinyl-CoA synthetase (SCS)

succinyl-CoA ligase ADP-forming subunit beta (SUCLA2)

ubiquitin specific protease 10 (USP10)

1. Background

Author's Contribution and Acknowledgement of Reproduction

This chapter is reproduced with minor edits from Boese AC, Kang S. Mitochondrial metabolism-mediated redox regulation in cancer progression. Redox Biol. 2021 Jun; 42:101870. A.C.B drafted the manuscript; A.C.B. and S.K. edited and revised the manuscript. This work was supported in part by NIH grants R01 CA207768 (S.K.), R01 CA175316 (S.K.), and F31 CA246889 (A.C.B.). A.C.B. is an NIH pre-doctoral fellow. S.K. is a Georgia Cancer Coalition Scholar and an American Cancer Society Basic Research Scholar.

1.1 Metabolic reprogramming is linked to cancer progression and metastasis.

Cancer cells exhibit abnormal metabolic activity when compared to their tissue of origin. One of the most well-known abnormal metabolic characteristics of cancer cells is the Warburg effect: a state of highly elevated glucose uptake and preference of glycolysis, rather than mitochondrial oxidative phosphorylation (OXPHOS) for ATP production, even in the presence of sufficient oxygen (1). Although the effects of glucose addiction and glycolytic dependency are still being actively researched, the Warburg effect benefits proliferative cancer cells through rapid ATP generation and simultaneous flux through the pentose phosphate pathway (PPP) to support redox homeostasis and biosynthesis (2). While the Warburg effect is commonly seen in proliferative cancer cells, evidence now suggests that the metabolic phenotypes between proliferative primary cancer cells and disseminated cancer cells are not the same (3).

In most epithelial and endothelial cells, detachment from the extracellular matrix (ECM) initiates a type of apoptotic cell death called anoikis. In recent years, several studies have demonstrated a strong connection between alterations in cellular metabolism and anoikis induction following matrix detachment (4-6). For instance, mitochondria are focal points of apoptotic signaling, since both the extrinsic and intrinsic pathways of apoptosis converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP) and liberate cytochrome c: a critical point in the execution of apoptosis (7). A direct example of the link between cell metabolism and apoptosis lies in the dual roles of cytochrome c. This small hemeprotein, which is associated with the inner mitochondrial membrane (IMM), plays an important role in bioenergetics and cellular ATP production because it mediates the transfer of an electron from respiratory complex III to complex IV in the mitochondrial electron transport chain (ETC). Cytochrome c also plays an essential role in the execution of apoptosis by activating caspases (8). Bcl-2 family

proteins are well-established regulators of cytochrome c in apoptosis by mediating its mitochondrial release into the cytosol, but the apoptotic activity of cytochrome c is also directly inhibited by reduced intracellular glutathione (GSH), an important antioxidant that can act on cytochrome c to keep it in its reduced state (9).

ECM-disengagement greatly disrupts cellular metabolic activity, including but not limited to diminished glucose uptake, reduced pentose phosphate pathway (PPP) flux, and elevated production of reactive oxygen species (ROS) to potentiate oxidative stress (7,17,18). These metabolic disturbances impair cellular bioenergetics and redox homeostasis, thereby contributing to anoikis susceptibility; mitigating oxidative stress is likely crucial for anoikis resistance following cancer cell ECM detachment. In fact, some evidence now suggests detached cancer cells upregulate metabolic pathways that support ROS detoxification and efficient bioenergetics and ATP production in conditions of low nutrients (10, 11). Several well-characterized oncogenic mutations are known to affect downstream signaling pathways that alter metabolic activity in cancer. Therefore, oncogene-mediated metabolic reprogramming likely promotes anoikis resistance and survival of disseminated cancer cells in the circulatory system.

1.2 Reactive oxygen species are a double-edged sword in cancer.

Reactive oxygen species (ROS) are naturally produced as metabolic byproducts from aerobic mitochondrial metabolism under normal physiological conditions (12). For instance, the ETC complexes generate superoxide anion when single electrons react with oxygen. In fact, it is estimated that up to 5% of oxygen used for mitochondrial respiration is converted to superoxide anions (13). It is important to note that ROS have dual functions within cells. For instance, a moderate increase in ROS can activate signaling pathways important for cell proliferation,

differentiation, and survival. However, excessive amounts of ROS can cause oxidative damage to cellular proteins, lipids and nucleic acids to a point that triggers cell death. In addition, ROS are involved in the execution of programmed cell death through the peroxidation of cardiolipin, which liberates cytochrome C from the inner mitochondrial membrane (14). Overall, ROS play vital roles in the induction of apoptosis and anoikis. Cancer cells have long been known to exhibit higher levels of ROS than their non-cancerous counterparts. ROS levels are increased in cells that have undergone matrix detachment, and antioxidant supplementation inhibits anoikis in certain cell types (15, 16). Therefore, cancer cells must adapt multiple metabolic strategies to balance efficient energy production and redox status to avoid oxidative stress and cell death.

Although it may seem counterintuitive, extensive evidence has indicated that chronic elevations in intracellular ROS levels below a certain threshold promote cancer cell survival, proliferation, anoikis resistance, and metastasis. For example, sublethal administration of hydrogen peroxide significantly upregulates Cav-1 which leads to anoikis resistance and anchorage-independent growth through increased Akt signaling in melanoma and lung carcinoma cells (17, 18). Of note, many cancers have chronically elevated intracellular ROS levels due to constitutive activation of ROS-producing enzymes such as 5-lipoxygenase and NADPH oxidase (NOX) (19). For instance, metastatic prostate cancer cells often exhibit increased intracellular ROS via constitutive activation of 5-lipoxygenase, which promotes Src-mediated ligand-independent firing of pro-survival EGFR signaling (20). Activation of Akt and EGFR signaling through ROS-induced Cav-1 and Src activity following matrix detachment may play a direct role in anoikis resistance through negative regulation of pro-apoptotic Bcl-2 family proteins, such as degradation of Bim (20). Indeed, treatment of aggressive prostate cancer cells with antioxidants erases anoikis resistance from Src-induced ligand-independent EGFR activation by restoring pro-apoptotic

signaling (20). In agreement, additional evidence suggests that elevated intracellular ROS can promote the activation of Src kinase and downstream ERK and PI3K/Akt signaling pathway to promote cell survival by inhibiting pro-apoptotic Bim and Bad (21-23).

Additional research has shown that cancer cells may utilize chronic ROS to inhibit anoikis through other mechanisms as well. For instance, expression of angiopoietin-like 4 protein has been shown to promote anoikis resistance in cancer by interacting with β1 and β5 integrins and stimulating NOX-mediated O_2^- production to mimic anchorage conditions (24). In addition, chronic moderate elevations of intracellular ROS may promote the expression of anti-apoptotic proteins such as Bcl-xL, c-FLIP and XIAP through activation of the transcription factor NF-κB (25). In summary, slightly elevated ROS levels can promote pro-growth and anti-apoptotic signaling, but cancer cells must employ antioxidant defenses and modulate metabolic pathways to keep ROS levels from rising too high and causing oxidative stress.

1.3 Glutaminolysis regulates redox in cancer.

Glutamine is the most abundant amino acid in blood plasma and serves as the major source of reduced nitrogen for cells. In addition to an increased demand for glucose, cancer cells also have high rates of glutamine consumption and glutaminolysis (26). Glutaminolysis is a mitochondrial metabolic pathway characterized by initial deamination of glutamine by glutaminase (GLS) to produce ammonia and glutamate. Glutamate is then further metabolized by glutamate dehydrogenase (GLUD1, GDH1) to its product, alpha ketoglutarate (α-KG): an important TCA intermediate used to fuel ATP production and anabolic biosynthesis of amino acids, lipids and nucleotides (27, 28). In addition, glutaminolysis is also involved in the production of antioxidant molecules to protect cells against oxidative stress (27, 29, 30). For instance, reduced

glutathione (GSH) is a tripeptide containing glutamate, glycine and cysteine, and is therefore heavily dependent on glutaminolysis for glutamate production and import of cysteine's precursor, cystine, via the X_C^- antiporter (xCT/SLC7A11) (31). In the context of cancer, xCT is known to be upregulated through the RAS-RAF-MEK-ERK signaling pathway and is essential for oncogenic Kras-mediated cellular transformation by mitigating oxidative stress through the GSH antioxidant defense system (32). Furthermore, xCT expression and environmental cystine are both necessary for increased glutamine TCA anaplerosis and glutaminase dependence often observed in cancer cells (33), and depletion of xCT is reported to subject pancreatic cancer cells to ferroptosis: a cell death outcome resulting from the accumulation of lipid ROS (34). Glutamine anaplerosis contributes to the regeneration of other antioxidant molecules as well. For instance, the conversion of glutamine to oxaloacetate (OAA) by malate dehydrogenase in the TCA cycle reduces NADP+ back to NADPH, which can then act either as a directly operating antioxidant on mitochondrial electron transport chain (ETC)-derived O_2^- and other ROS, or as an indirectly operating antioxidant through the re-reduction of glutathione disulfide (GSSG) to GSH via glutathione reductase (35, 36).

Many cancers harbor oncogenic alterations that drive both increased glutamine uptake and metabolism. For instance, c-myc is a transcription factor commonly amplified in cancer that is known to drive glutamine import by upregulating expression of glutamine transporters such as system N transporter 2 (SN2) and alanine-serine-cysteine transporter 2 (ASCT2) (37). Of note, ASCT2 plays a critical role in leukemia initiation and maintenance driven by activation of the oncogene MLL-AF9 or deletion of Pten (38). In addition, c-myc activity also induces expression of several enzymes that participate in glutaminolysis, including glutaminase (39) and carbamoyl-phosphate synthetase 2 (40). The deletion or inactivation of tumor suppressor genes in cancer can

also promote glutaminolysis. For instance, deletion of the Rb gene leads to unchecked E2F transcription factor activity, which promotes both the uptake and usage of glutamine through increased mRNA expression of ASCT2 and GLS1 (41). In recent years, research has demonstrated that proliferative tumor cells utilize metabolites produced through glutaminolysis as cofactors to modulate the activity of ROS-scavenging enzymes and mitigate oxidative stress. For example, GDH1 is upregulated in many types of cancer and is important for redox homeostasis. Specifically, the metabolic product of GDH1, α-KG, is further metabolized into fumarate in the TCA cycle. Fumarate then binds to and enhances the activity of glutathione peroxidase 1 (GPx1), an enzyme that uses reduced glutathione to detoxify cellular ROS (42). Moreover, both chemical and genetic inhibition of GDH1 results in decreased proliferation of lung cancer, breast cancer and leukemia, but not of non-cancerous human proliferating cells (42). This suggests that glutaminolysis may be more important for redox regulation specifically in cancer cells.

Although most research on glutaminolysis in cancer focuses on cancer cell proliferation and tumor growth, emerging evidence suggests that glutaminolysis plays a pivotal role in promoting the metastatic progression of cancer. Farris et al. first identified that GLUD1 and its product α-KG are increased during the epithelial to mesenchymal transition (EMT), which is a key driver of metastasis, and are critical for suppressing hydrogen peroxide generation and protecting epithelial cells from ROS-induced anoikis (43). The transcription factor grainyhead-like 2 (GRHL2) is reported to downregulate GLUD1 expression and sensitize cells to anoikis (43), while the transcription factor pleomorphic adenoma gene 1 (PLAG1) is reported to drive GLUD1 expression after matrix detachment and promote anoikis resistance (44). Additional evidence demonstrates that GLUD1 can confer anoikis resistance by sustaining ATP production in cancer cells that are not able to properly monitor their bioenergetic state in nutrient-poor conditions. For

instance, approximately one-third of non-small cell lung cancers exhibit loss of the tumor suppressor liver kinase B1 (LKB1), a deficiency that is associated with metastasis and poor prognosis (45). LKB1 activates the master metabolic sensor, AMPK, through direct phosphorylation of its α subunit (AMPKα) at Thr 172. However, AMPK can also by activated by other kinases, including TGF-β-activated kinase 1 (TAK1) and calcium/calmodulin-dependent protein kinase kinase 2 (CamKK2) (46, 47). Importantly, AMPK contributes to cell survival under conditions of metabolic stress by phosphorylating and inhibiting the mTOR pathway, which promotes energy-consuming protein synthesis (46, 48). Therefore, AMPK activity may contribute to anoikis resistance in cancer cells under certain circumstances, such as in nutrient-deprived conditions post-ECM detachment. Indeed, evidence now demonstrates that, in LKB1-deficient lung cancer, glutaminolysis contributes to anoikis resistance and metastatic potential by reactivating AMPK signaling through GLUD1 and its metabolic product α-KG (44). Specifically, binding of α-KG to CamKK2 enhances the recruitment and subsequent activation of CAMKK2's substrate, AMPK, thereby sustaining mTOR pathway inhibition, balancing energy status, and eventually restoring anti-anoikis signaling (44). In agreement, other studies have also demonstrated that both LKB1- and CamKK2-mediated AMPK signaling contribute to anoikis resistance during detachment-induced metabolic stress (49). The differential contributions of GLUD1 and its metabolic product α-KG in both redox and energy homeostasis implies that the role of α-KG and other metabolites in cell metabolism and anoikis protection may depend on cell type and discrete cellular metabolic conditions.

In summary, a growing body of research highlights glutamine and glutaminolysis as indispensable for TCA anaplerosis, maintenance of bioenergetics, and regulation of redox status in diverse types of cancer. Unsurprisingly, small molecule inhibitors and other drugs that can target

glutamine metabolism have gained considerable interest as anti-cancer therapies. Glutamine blockade with the pro-drug form of the glutaminase inhibitor glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON) has recently been shown to disrupt glycolytic cancer metabolism and increase effector T cell oxidative metabolism to increase anti-tumor immunity (Figure 1) (50). Additionally, targeting GDH1 with the pupurin analog R162 attenuates proliferation and tumor growth in lung, breast, and leukemia cancer cells (42). Several preclinical studies have demonstrated that the glutaminase inhibitor Telaglenastat (CB-839) exhibits anti-tumor activity as a single agent in triple negative breast cancer cells (51), in combination with radiation therapy in NSCLC cell lines (52), and in combination with proteasome inhibitors in multiple myeloma (Figure 1) (53). The combination of Telaglenastat and standard-of-care chemoimmunotherapy is currently under investigation in clinical trials for non-squamous NSCLC (NCT04265534), multiple myeloma (NCT03798678), and IDH-mutated astrocytoma (NCT03528642). Nevertheless, more clinical research is needed to determine the efficacy of targeting glutamine metabolism in combination with other chemotherapeutic agents and immune checkpoint therapies for cancer.

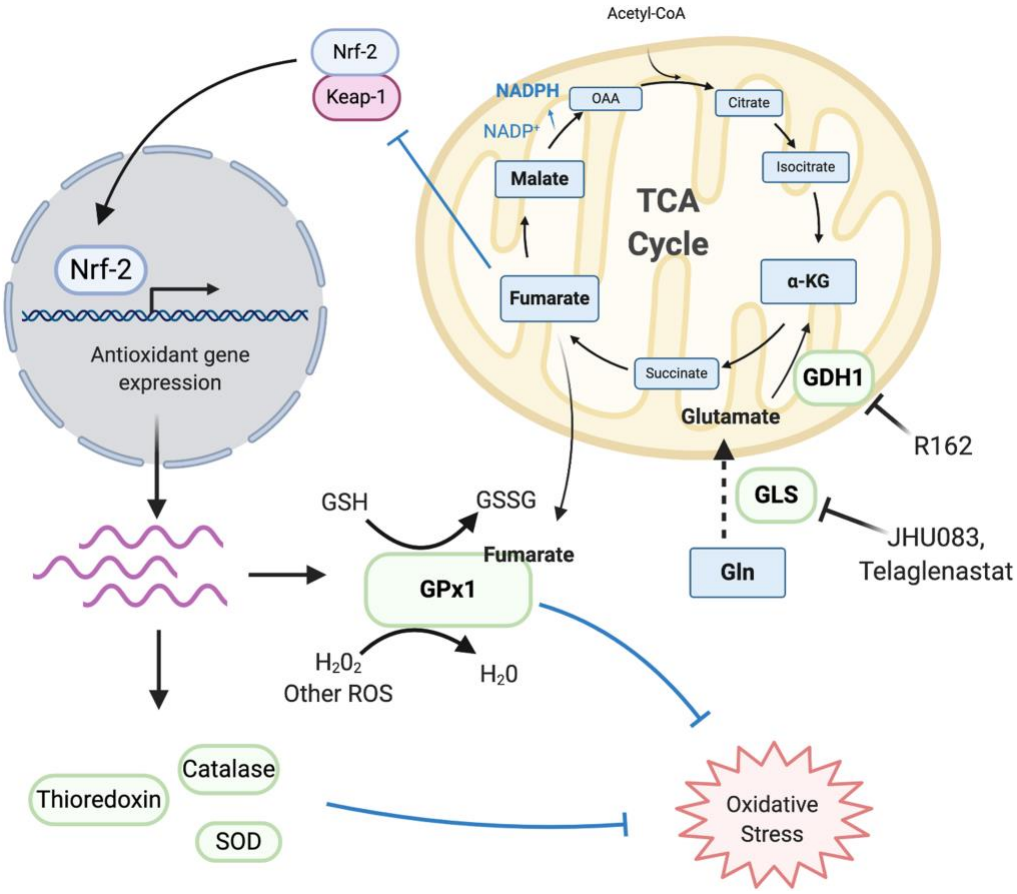


Figure 1. Glutaminolysis promotes ROS detoxification and redox homeostasis in cancer through TCA cycle intermediates. Upregulated GDH1 and other enzymes involved in glutaminolysis maintain increased levels of α-KG and downstream TCA metabolites such as fumarate and malate. Fumarate modulates cysteine residues on Keap-1 to promote antioxidant gene expression through the Nrf-2 transcription factor and enhances the antioxidant activity of GPx1. Conversion of malate to oxaloacetate via malate dehydrogenase regenerates NADP+ to NADPH, which can directly detoxify mitochondrial ROS or maintain intracellular pools of reduced glutathione.

1.4 Some cancers restrict glucose oxidation to prevent oxidative stress.

While decreased glucose-derived carbon influx can lead to bioenergetic stress after matrix detachment, matrix-detached cells may benefit from more limited glucose oxidation under certain circumstances. In normal epithelial and endothelial cells, detachment from the ECM leads to increased production of mitochondrial ROS (13, 15). To delay anoikis, detached cells must strike a balance between efficiently producing ATP via mitochondrial respiration in nutrient poor conditions and safeguarding against mitochondria-derived ROS overload. It is already well-established that matrix detachment is followed by reduced nutrient uptake, thereby limiting metabolic flux through glycolysis and the TCA cycle (16, 54, 55). However, untransformed epithelial cells are reported to restrict flux of glucose through the TCA cycle after matrix detachment by upregulating pyruvate dehydrogenase kinase 4 (PDHK4) expression to phosphorylate and inhibit pyruvate dehydrogenase (PDH), which is responsible for converting pyruvate to acetyl-CoA that flows into the TCA cycle (54, 55). Likewise, PDHK4 depletion and restoration of PDH activity increases oxidative metabolism of glucose and accelerates both ROS accumulation and downstream anoikis induction in detached cells (54). Taken together, although normal epithelial cells will generate ROS and eventually undergo anoikis when detached from matrix, this process can be delayed by limiting mitochondrial respiration through already decreased nutrient uptake and negative regulation of PDH activity to limit flux of glucose through the TCA cycle.

While normal epithelial cells are reported to restrict aerobic respiration of mitochondria after detachment, many cancer cells already preferentially limit glucose oxidation in mitochondria due to the Warburg effect. Therefore, the tendency of cancer cells to prefer aerobic glycolysis may give them an inherent survival advantage at suppressing mitochondrial ROS overload soon after

detachment. For instance, cancer cells are already known to express high levels of PDHKs under attached conditions to inhibit PDH and divert glucose metabolic flux away from the TCA cycle (54). Upregulation of PDHK1 gene expression by the transcription factors Myc and hypoxia inducible factor-1 (HIF-1), or activation of PDHK1 by post-translational modification such as tyrosine phosphorylation by diverse oncogenic tyrosine kinases are reported to further decrease pyruvate dehydrogenase complex (PDC) activity (56). Higher expression of PDHKs is also associated with poor cancer patient survival (57). Likewise, suppression of PDHK and activation of PDH has been shown to sensitize some cancer cells to anoikis and decreases their metastatic potential by stimulating glucose oxidation and ROS production (54). In addition, the M2 isoform of pyruvate kinase (PKM2), which is catalytically involved in the final key step of glycolysis and allosterically regulated by distinct tetrameric and dimeric states, is often overexpressed in cancer cells and allows glycolytic metabolites to divert away from the TCA cycle into other metabolic pathways such as the PPP, thereby limiting mitochondrial respiration (58). Taken together, this evidence demonstrates that although glucose uptake is important for bioenergetics and cell survival after matrix detachment, mitochondrial oxidation of glucose may trigger anoikis through the production and accumulation of ROS. In support of this possibility, treatment with metformin, which binds to complex I of the ETC and suppresses OXPHOS, has been shown to support the detachment and viability of breast cancer cells in vitro (59, 60). However, more research is needed to determine how anoikis resistant cells regulate carbon flux to balance bioenergetic needs and to maintain redox homeostasis.

1.5 Cancer cells upregulate their antioxidant defense systems to combat oxidative stress.

In normal epithelial cells, detachment from the ECM leads to downregulated expression of

EGFR and consequent decrease in cellular signaling through the pro-survival PI3K/Akt pathway (61). Since activation of PI3K and downstream Akt play essential roles in regulating both glucose uptake and glucose metabolism (62), detached cells often experience a significant reduction in levels of glucose-6-phosphate, an intermediate metabolite which fuels both glycolysis and the PPP, as a consequence of decreased glucose import. This, in turn, limits metabolic flux through the PPP and impaired regeneration of GSH, thereby potentiating an increase in intracellular ROS levels (63). This is important because increased ROS production after ECM detachment can initiate anoikis if levels become too high (15, 16, 64). While limited increases in ROS production can promote pro-survival signaling, ECM-detached cancer cells must be able to maintain adequate antioxidant capacity to prevent oxidative stress and initiation of anoikis. Indeed, circulating tumor cells often exhibit higher ROS levels in comparison to the primary tumors they were derived from (65). However, it is well-established that cancer cells actively engage multiple antioxidant defense systems to cope with high oxidative stress.

In addition to limiting the amount of ROS produced from mitochondrial respiration, aspects of the Warburg effect may help to reduce matrix-detachment induced oxidative stress by shunting glucose into the PPP. For instance, aerobic glycolysis in cancer cells diverts more glucose-derived carbon into the oxidative phase of the PPP, which produces antioxidant NADPH and GSH (66). In addition, acute increases in intracellular concentrations of ROS inhibits the glycolytic enzyme PKM2 through oxidation of Cys^{358}, which in turn diverts glucose flux into the PPP to generate reducing power for ROS detoxification (67). Inhibition of PDH though overexpression of PDKs in cancer may have similar effects as well (54, 56). While flux through the PPP can contribute to indirect inhibition of anoikis through the detoxification of ROS, it is also worth mentioning that production of reduced glutathione through the PPP can directly inhibit anoikis and apoptosis by

keeping cytochrome c in its reduced state, thereby inhibiting its apoptogenic role in caspase activation (9). However, it is important to mention that many of these aspects Warburg effect have historically been observed in proliferative primary solid tumors. Therefore, diversion of glucose into the PPP by PKM2 and PDHK upregulation needs to be further tested in detached cancer cells to determine whether these mechanisms are main drivers of anoikis resistance.

Evidence to suggest that cancer cells are also able to employ reductive carboxylation of glutamine to keep ROS levels in check after ECM detachment. For instance, in anchorage-independent lung cancer tumor spheroids, oxidation of glutamine and glucose is suppressed, while reductive formation of citrate from glutamine-derived α-KG via cytosolic isocitrate dehydrogenase-1 (IDH1) is increased (10). This reductively produced citrate in the cytosol is then able to enter the mitochondria and participate in the TCA cycle, thereby generating NADPH to combat mitochondria-derived ROS such as O_2^- (10). In addition to glutamine, fatty acid oxidation (FAO) also appears to confer anoikis resistance to certain types of cancer through modulation of redox status. For example, colorectal cancer cells have been reported to upregulate the rate-limiting enzyme of fatty acid oxidation, CPT1A, when cultured in suspension and CPT1A expression is also upregulated in colorectal cancer metastases (68). Specifically, CPT1A-mediated FAO has been demonstrated to suppress anoikis in colorectal cancer by providing acetyl-CoA for the TCA cycle to maintain adequate NADPH/NADP+ and GSH/ GSSG ratios (68). Taken together, this evidence also highlights that cancer cells can utilize nutrients other than glucose to maintain antioxidant defense after matrix detachment.

While NADPH and GSH constitute important antioxidant molecules for the detoxification of intracellular ROS, several antioxidant enzymes may be used by matrix-detached cells in attempts to maintain redox homeostasis as well (69). Manganese superoxide dismutase (MnSOD),

which is localized to the mitochondria, plays an important role in detoxifying superoxide into the less reactive hydrogen peroxide (H_2O_2), which is then further broken down into water and dioxygen. Cell disengagement from the ECM is reported to induce expression of MnSOD to combat detachment-induced elevations in mitochondrial ROS (13). Accordingly, cells with reduced levels of MnSOD are reported to be hypersensitive to anoikis following matrix detachment due to ROS overload leading to oxidative stress (13). While chronically elevated ROS levels are often observed in cancer, many cancer cells also upregulate antioxidant enzymes to make sure ROS levels do not become high enough to cause cell death. MnSOD upregulation is reported to suppress anoikis in human mammary epithelial cells and metastatic breast cancer cells through dismutation of mitochondria-produced superoxide radicals (13). Other studies have also demonstrated that superoxide dismutase and the antioxidant enzyme catalase grant anoikis resistance to breast cancer cells after ECM disengagement by reducing ROS and therefore indirectly supporting ATP production (64). While MnSOD gene expression is reported to be under transcriptional control of NF-κB (13), its expression, along with other antioxidant genes, has also been reported to be induced by the Nrf-2 transcription factor, which is known to be overactivated via upstream signaling by oncogenic Ras, Raf and Myc (70, 71). Under normal conditions, Nrf-2 is bound to its inhibitor, Keap-1, and remains in the cytoplasm, but increases in ROS from oncogenic growth factor receptor signaling or ECM detachment lead to the dissociation of Nrf-2 from Keap-1, and Nrf-2 is able to enter the nucleus where it binds to antioxidant response elements (ARE) in DNA. This promotes gene expression of several ROS-scavenging enzymes including catalase, thioredoxin, glutathione peroxidase and superoxide dismutase (72). In addition, metabolites produced by glutaminolysis can directly modulate the antioxidant Nrf-2 response as well. Fumarate, which is derived from α-KG, is known to modify cysteine residues on Keap-1

which impairs its ability to repress Nrf-2 by keeping it in the cytosol (Figure 1) (73, 74). Therefore, glutaminolysis may play a central role in upregulating expression of antioxidant genes to keep ROS levels in check in both adherent and disseminated cancer cells.

Eukaryotic cellular metabolism is composed of an incredibly complex network of catabolic and anabolic pathways that are tightly regulated to balance energy production with redox homeostasis and biosynthesis. It is now well-established that epithelial and endothelial cellular detachment from the extracellular matrix causes widespread disturbances in metabolism. Furthermore, cell fate is highly influenced by the availability of nutrients and activity of diverse metabolic pathways that produce ATP, antioxidants, biosynthetic precursors, and metabolites used in cell signaling. Therefore, it is unsurprising that mitochondrial metabolic and apoptotic pathways are intertwined. Indeed, preclinical research is now beginning to delineate how cancer cells modulate mitochondrial metabolic pathways in a context-dependent manner to maintain a redox status that promotes tumor growth, apoptosis resistance and survival in circulation. In general, evidence thus far indicates that oncogenic alterations promote diversion of glucose away from the mitochondria and a heavy reliance on glutaminolysis to facilitate rapid ATP production and biosynthesis while simultaneously keeping ROS production in check. Continued research on the links between mitochondrial metabolic reprogramming and cancer metastasis is critical to understand the biology of metastatic dissemination and identify molecular targeted therapies for metastatic cancers.

1.6 Detection and treatment of metastatic cancer remains a significant clinical challenge.

The spread of cancer cells from a primary tumor to a distant organ and subsequent recolonization encompasses cancer metastasis. Common sites of cancer metastasis include the

brain, bones, lungs, and liver (75). Targeting cancer metastasis in the clinic is difficult for several reasons. For instance, metastatic tumors are often not detected until they grow large enough to be visualized using computed tomography (CT) or magnetic resonance imaging (MRI); at this point, metastatic tumors are often incurable. In addition, treatment of primary tumors can promote metastatic spread. For instance, radiation followed by angiogenic inhibitor therapy can unintentionally promote metastasis by increasing hypoxia which reprograms tumor metabolism and increases expression of metastatic gene products (76). These data further highlight altered cellular metabolism as a driver of cancer metastasis.

Another barrier to treating cancer metastasis is a lack of molecular targeted therapies specifically for metastatic tumors. Treatment options are usually the same for primary and metastatic cancer, even though gene expression profiles between metastatic and matched primary tumors are different (77). Future therapies need to consider the unique biology of metastatic cells to control metastatic burden in cancer patients. For example, cancer cells that metastasize to the bone interact with osteoblasts and osteoclasts and change their behavior (78). Thus, compounds that inhibit osteoclast differentiation or activity such as bisphosphonates and receptor activator of nuclear factor kappa-B ligand (RANK-L) inhibitors may be useful to manage bone metastases (79). It is important to note that clinical trials for antimetastatic cancer therapies are also logistically challenging. Cancer clinical trials are currently conducted in a manner that isn't conducive to assess the efficacy of potential anti-metastatic therapies, and many patients enrolled in clinical trials already present with metastatic disease. Furthermore, cancer clinical trials are expensive and too short to include metastasis as an endpoint. To determine the anti-metastatic efficacy of new therapies, future trials need to include metastasis as a primary endpoint. Nevertheless, development of targeted therapies for metastatic tumors is still in its infancy due to

our incomplete understanding of the molecular drivers of metastasis (80). A better understanding of the molecular mechanisms that underly each step of the metastatic cascade is required to find new biomarkers and better therapies to target metastasis (81).

1.7 In vitro screening identifies mitochondrial metabolic targets that promote anoikis resistance and cancer metastasis.

Research highlights differences in mitochondrial metabolism between metastatic and primary tumors, but the specific mitochondrial enzymes and pathways that promote metastasis of cancer to distant organs remain incompletely characterized. Since the metastatic cascade is a complex process involving several steps, the Kang lab established an in vitro screening system to assess mitochondrial factors that might be associated with a specific step of metastasis, such as anoikis resistance. Chapter 2 presents data in which we validate and characterize SUCLA2, a mitochondrial TCA cycle enzyme subunit, is identified from RNAi screening as a factor that is critical for anoikis resistance and tumor metastasis. We demonstrate that SUCLA2 regulates redox homeostasis through stress granule-mediated antioxidant enzyme expression in disseminated cancer cells to provide a metabolic advantage that promotes cancer cell anoikis resistance and tumor metastasis.

Chapter 2: Succinyl-CoA ligase ADP-forming subunit beta promotes stress granule assembly to regulate redox and drive cancer metastasis.

Abstract

Although recent studies demonstrate active mitochondrial metabolism in cancers, the mechanism through which mitochondrial factors contribute to cancer metastasis is largely elusive. Through a customized mitochondrion RNAi screen, we identified succinyl-CoA synthetase [ADP-forming] subunit beta (SUCLA2) as a critical anoikis resistance and metastasis driver in human cancers. Upon detachment, SUCLA2, but not the subunit alpha in the complex, relocates from mitochondria to cytosol where it binds to and promotes the formation of stress granules. SUCLA2-mediated stress granules facilitate the protein translation of antioxidant enzymes including catalase, which mitigates oxidative stress and renders cancer cells resistant to anoikis. We provide clinical evidence that SUCLA2 expression correlates with catalase levels as well as metastatic potential in lung and breast cancer patients. These findings not only implicate SUCLA2 as an antimetastatic cancer target, but also provide insight into a unique non-canonical function of SUCLA2 that cancer cells exploit to metastasize.

2.1 Introduction

Altered metabolism is a hallmark of cancer, but growing evidence highlights dynamic changes in the metabolism of metastasizing cancer cells that are essential to progress through the metastatic cascade (3, 11, 42, 82, 83). Although the requirement for mitochondrial ATP production is reduced in glycolytic tumor cells, the demand for TCA cycle-derived biosynthetic precursors and NADPH is unchanged or even increased (84). Studies also suggest that metastatic cancer cells are more reliant on mitochondrial metabolic pathways (10, 44, 85). Nevertheless, only a few mitochondrial enzymes among a large panel of metabolic targets have been implicated in metastasis prevention and treatment so far, and other mitochondrial metabolic pathways essential for metastasis have not been extensively explored (86).

Reports estimate that primary tumors can shed millions of cancer cells into the circulation every day, most of which are eliminated by anoikis, a programmed cell death induced by extracellular membrane detachment, and thus never metastasize (87, 88). Therefore, cancer cells must become resistant to anoikis to metastasize, but how mitochondrial metabolic reprogramming allows tumor cells to survive in the circulatory system during metastasis remains largely unclear. It is widely accepted that ROS play distinct roles in metastasis depending on their concentrations, distributions, and subcellular location. For instance, ROS can induce epithelial-mesenchymal transition (EMT) and MAPK activation in cancer cells, thereby playing a crucial role in metastasis (89, 90). However, many studies demonstrate that oxidative stress sensitizes cancer cells to anoikis and upregulation of a ROS scavenger superoxide dismutase promotes anoikis resistance and cancer metastasis, suggesting a link between redox metabolism and anoikis resistance (13, 91).

Succinyl-CoA synthetase (SCS) is a mitochondrial enzyme that catalyzes the reversible conversion of succinyl-CoA and ADP or GDP to succinate and ATP or GTP (92). SCS functions

as a heterodimer composed of an invariable α subunit (SUCLG1) and a variable β subunit (SUCLA2 or SUCLG2). SUCLA2 encodes for the succinyl-CoA synthetase ATP-specific β subunit, which together with the α subunit forms ATP-specific SCS (SCS-A), the enzyme in the TCA cycle that can generate ATP *via* direct substrate-level phosphorylation. SCS-A can generate two ATPs per molecule of glucose that enters the TCA as two molecules of pyruvate-derived acetyl-CoA. Mutations in SUCLA2 that impair SCS activity cause succinyl-CoA accumulation and global succinylation of proteins in mitochondria (93). The SUCLA2 gene is located 302K base pairs away from the RB1 gene and a strong positive correlation is observed in copy number between RB1 and SUCLA2 genes in prostate cancers (94). Nonetheless, little is known about the biological function of SUCLA2 in human cancers and beyond its classical role in the TCA cycle.

Stress granules and P-bodies are dense aggregations in the cytosol composed of RNAs and proteins that appear to help cells cope with specific stress stimuli (95, 96). While stress granules assemble key components of the translation machinery, P-bodies assemble the essential enzymes of cytoplasmic RNA degradation (97). Cellular stress leads to mRNA translation reprogramming and emerging evidence indicates that transcripts localized to stress granules are selectively translated to deal with stress conditions (98). Ras-GTPase-activating protein SH3 domain-binding protein 1 and 2 (G3BP1/2), ubiquitin specific protease 10 (USP10), and ataxin-2-like (ATXN2L) are components of stress granules that play a role in SG formation (99-101). In addition, nuclear fragile X mental retardation-interacting protein 2 (NUFIP2) is known to re-localize to stress granules upon exposure to stress (102). Stress granules have emerged as systems that can integrate oncogenic signals and tumor-related stress stimuli to promote cancer cell fitness (95), and upregulation of stress granules has been observed in breast and prostate cancer (103, 104). However, the molecular interactions and mechanisms that modulate stress granule assembly and

how these may become altered in human diseases remain largely unknown.

To explore the relationship between mitochondrial metabolism and cancer metastasis, we generated a customized RNAi library targeting mitochondrial enzymes. Using this approach, we have obtained evidence that SUCLA2 promotes anoikis resistance. SUCLA2 contributes to stress granule assembly, enhancing the translation of cytosolic ROS scavengers managing redox balance to promote survival of disseminated cancer cells during metastasis. These studies uncover the non-classical function of the Krebs cycle enzyme SUCLA2 in cancer metastasis that is associated with stress granules and redox metabolism.

2.2 Materials and Methods

Reagents: Short hairpin RNAs (shRNAs) for SUCLA2, SUCLG1, SUCLG2, and mitochondria metabolic enzymes in pLKO.1-based lentiviral vector were obtained from Horizon Discovery. NUFIP2 shRNA clones were from Genecopoeia. The sense strand sequences of the shRNAs were CCAGCCAACTTCCTTGATGTT (SUCLA2 #1), CCCAGGAGAGAATACTACTTT (SUCLA2 #2), TGGAATGGATCACGTAGACAT (SUCLG1 #1), CGGCAACATCTCTATGT TGAT (SUCLG1 #2). CCTGCTTCATTTACAAGAATT (SUCLG2 #1), AGGTGTCTTCAAT AGTGGTTT (SUCLG2 #2), and CCACCATTATTATTTCTACAA (NUFIP2). SUCLA2 and catalase were flag tagged at the C and N terminus, respectively by PCR and cloned into pLHCX-Gateway vector (42). pLX304-SUCLA2-V5 (HsCD00438652) was purchased from DNASU. Lentiviral vector for G3BP1-GFP expression (119950) was obtained from Addgene. The general oxidative stress indicator CM-H_2DCFDA (C6827) was from Invitrogen. Mito-TEMPO (SML0737), N-acetyl-L-cysteine (NAC; A7250), and dimethyl-succinate (W239607) were

obtained from Sigma-Aldrich. Annexin V staining kit (556547) was from BD Biosciences. Mitochondria isolation kit for cultured cells (89874) was obtained from Pierce. TransIT-LT1 (MIR2304) was from MirusBio. ROS-Glo H_2O_2 Assay (G8820) and Caspase-Glo 3/7 Assay Systems (G8091) were obtained from Promega. EZClick Global Protein Synthesis Assay (K459) and Catalase Activity Colorimetric/Fluorometric Assay (K773) were purchased from Biovision. Succinate Assay Kit (ab204718) was obtained from Abcam. All other chemicals not specified were obtained from Sigma-Aldrich.

Antibodies: Antibodies against SUCLA2 (A-9/sc-374107), SUCLG2 (C-1/sc-393756), V5-Probe (H-9/sc-271926), α-tubulin (B-5-1-2/sc-23948), Tom40 (H-300/sc-11414), PRX (B-11/sc-137222), and FASN (A-5/sc-55580) were purchased from Santa Cruz Biotechnology. Anti-SUCLG1 antibody (NBP1-32728) was obtained from Novus Biologicals. Antibodies against Cox IV (3E11/4850), G3BP2 (31799), phospho-eIF2α Ser51 (119A11/3597), eIF2α (D7D3/5324), and catalase (D4P7B/12980) were obtained from Cell Signaling Technology. Anti-β-actin antibody (AC-15/A1978) and anti-flag antibody (M2/F3165) were purchased from Sigma-Aldrich. Antibody against Hsp90α (ab2928) was from Abcam and anti-NUFIP2 antibody (17752-1-AP) from Proteintech.

Cell culture: A549, H460, MDA-MB231, and MDA-MB468 cell lines were purchased from American Type Culture Collection (ATCC) and Lenti-X 293T cell line was obtained from Takara Bio USA. A549 and H460 cells were cultured in RPMI1640 media supplemented with 10% fetal bovine serum (FBS). MDA-MB231, MDA-MB468, and Lenti-X 293T cells were cultured in Dulbecco Modified Eagle Media (DMEM) supplemented with 10% FBS. Lentivirus shRNA

production, viral infection, and stable knockdown cell selections were performed using psPAX2 and pMD2.G packaging system as described (105).

RNAi screen: The customized shRNA library targeting 119 human mitochondria metabolic genes was generated using the pLKO.1 lentiviral vectors designed by The RNAi Consortium (TRC). Lentivirus from each shRNA clone was produced and pooled to infect 1 x 10^5 A549 cells for 48 hours in 6 well-plates to target each mitochondrial gene. Cells were then split and re-seeded on 1% agarose coated 6 well-plates or into 4 replicates in 96 well-plates and half of the replicates were treated with 2 mg/ml puromycin. Post 48-hour incubation, detachment-induced cell death was assessed by annexin V staining and cell proliferation rate was determined using CellTiter Glo Luminescent Cell Viability Assay. Candidate genes that resulted in <15% cell death by knockdown alone or had >25% shRNA virus infection efficacy by puromycin selection were selected for analysis.

Anoikis and cell proliferation assays: Anoikis was induced by culturing cells on 1% agarose coated plates for 48 hours unless specified. Apoptotic cell death was determined by FITC-conjugated annexin V/propidium iodide staining or Caspase Glo 3/7 activity assay based on the manufacturer's instructions. Cell viability was determined at indicated time points using CellTiter-Glo Luminescent Cell Viability Assay. Numbers of viable cells were estimated using cell number standard curves.

Metabolic analyses: Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were acquired in cells with or without SUCLA2 knockdown by sequential treatment with

500 nM oligomycin, 500 nM FCCP, and 1 µM antimycin A/rotenone and analyzed using Agilent Seahorse XFe24 flux analyzer. Cells were cultured under hypoxic conditions using sealed hypoxia incubation chambers (StemCell Technologies). The ATP present in cells was determined using ATP luminescent somatic cell assay kit (FLASC, Sigma-Aldrich). General ROS and hydrogen peroxide levels in cells were quantified using chloromethyl derivative of H_2DCFDA (Invitrogen) and ROS-Glo H_2O_2 Assay (Promega), respectively. Levels of intracellular succinate, catalase activity, and global protein synthesis were monitored in detached cancer cells with or without SUCLA2 loss using commercial assay kits indicated in the Reagents section according to the manufacturers' protocols. In brief, fixed and permeabilized cells were treated with O-propargyl-puromycin to stop translation and synthesized peptides were quantified by reaction with the fluorescent azide at Excitation 494/Emission 521 nm for protein synthesis assay. To detect activity of catalase in cells, unconverted substrate H_2O_2 that reacts with OxiRed probe was measured at 570 nm. Quantitative analysis of 116 hydrophilic and ionic metabolites involved in central energy metabolism in A549 cells with SUCLA2 knockdown was performed by Human Metabolome Technologies C-SCOPE using capillary electrophoresis and mass spectrometry (CE-MS) platform.

Immunofluorescence staining: 5×10^4 cells cultured in suspension were washed, diluted in 100 µl of PBS, and placed on sample chambers made with Cytoslide and Cytofunnel. The cells were then centrifuged using Shandon Cytospin 4 (Thermo Scientific) at 800 rpm for 5 min and applied to immunofluorescence staining. In brief, cells on glass slides were fixed in PHEMO buffer (25 mM HEPES, 68 mM PIPES, 0.5% Triton X-100, 15 mM EGTA, 3 mM $MgCl_2$, 3.7% formaldehyde, 0.05% glutaraldehyde), blocked in 10% goat serum, and stained with MitoTracker and anti-SUCLA2 antibody followed by anti-mouse IgG antibody conjugated with Alexa 488.

Cells were applied with antifade mountant with DAPI and imaged on a SP8 confocal microscope.

Mass spectrometry-based proteomics: SUCLA2 interacting proteins were identified by SUCLA2 co-immunoprecipitation followed by micro-capillary LC-MS/MS analysis. In brief, 3 mg of cell lysates obtained from detached A549 cells with or without SUCLA2 knockdown were pre-cleared with Protein G Sepharose 4 Fast Flow (Millipore Sigma). Pre-cleared lysates were incubated with 10 μg of monoclonal anti-SUCLA2 antibody followed by protein G Sepharose incubation. The bead bound proteins were extracted and denatured in 0.12 M Tris (pH 6.8), 3.33% SDS, 10% glycerol, and 3.1% dithiothreitol, followed by precipitation using ProteoExtract Protein Precipitation Kit (Calbiochem). The precipitates were applied for trypsin digestion and mass spectrometry analysis at the Taplin Mass Spectrometry Facility at Harvard Medical School.

Animal study: In vivo xenograft mouse model study was performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. Athymic nude mice (Hsd:Athymic Nude Foxn1^{nu}, female, 5-week-old, Envigo) were intravenously injected with 1 x 10^6 A549 cells with or without SUCLA2 shRNA through the tail vein. The mice harboring SUCLA2 knockdown cells were randomly divided into two groups, and antioxidant was administered to one group by supplementing the drinking water with 10 mg/ml NAC from 1 day after xenograft for 9 weeks.

Immunohistochemistry: Usage of de-identified human tissue specimens was approved by the Institutional Review Board (IRB) of Emory University. Clinical samples were obtained with informed consent under the protocols that are approved by the Health Insurance Portability and

Accountability Act. Formalin fixed-paraffin-embedded breast and lung cancer tissue samples composed of primary and matched lymph node metastasized tumors were obtained from US Biomax (BR1005b, LC817, and LC814). Immunohistochemistry staining was performed as previously described using anti-SUCLA2 antibody (1:1000) and anti-catalase antibody (1:500) (106). The staining intensities were scored in the range of 0 to +3.

Publicly available database analysis: Expression of SUCLA2 mRNA (RNAseq RSEM) and clinical data for each cancer type were downloaded from cBioPortal (http://cbioportal.org/) or firebrowse (http://firebrowse.org/) and Kaplan-Meier plots were generated in R environment using survival packages (https://cran.r-project.org). Patients were dichotomized into two groups which are upper and lower quartiles by SUCLA2 expression.

Statistics: Statistical analyses and graphic presentations were conducted using GraphPad Prism version 9. Data presented are from a representative experiment of multiple biological replicates. Error bars indicate mean ± SEM for Figure 3G and mean ± SD for all other graphs. Statistical analyses of significance were based on two-tailed Student's *t* test for Figures 2C, 2E, 2F, 4B, 5D, 6A, 6C, 6D, chi-square test for Figure 3H, Wilcoxon signed-rank test for Figures 7A and 7B, Spearman's Rho for Figure 7C, log-rank test for Figure 7D, and one-way or two-way ANOVA for all other data figures. Results of one representative experiment from at least two independent experiments are shown. Precise numbers of independent repeats are stated in each figure legend.

2.3 Results

The Krebs cycle enzyme SUCLA2 is important for cancer cells to resist anoikis. To better picture how mitochondrial metabolism contributes to tumor metastasis in human cancer, we performed an unbiased RNAi screen using a customized shRNA library targeting 119 mitochondrial metabolic enzyme genes represented by 605 lentiviral vector-based shRNA constructs. Lung cancer A549 cells were transduced with a pooled virus harboring 2~5 shRNA clones targeting each individual gene and cultured under detachment conditions. We identified succinyl-CoA ligase subunit beta (SUCLA2), an enzyme in the Krebs cycle as a lead that may be an important factor for anchorage independent survival of cancer cells (Figure 2A and Table 1). To further substantiate the role of SUCLA2 in cancer cell anoikis resistance, we generated diverse human cancer cell lines with stable SUCLA2 knockdown. These cell lines include lung cancer A549 and H460 cells, and breast cancer MDA-MB231 and MDA-MB468 cells. Loss of SUCLA2 resulted in enhanced detachment-induced apoptotic cell death in all the cell lines tested assessed by annexin V staining and caspase 3/7 activity (Figures 2B and 2C). However, SUCLA2 knockdown did not significantly affect the cell proliferation rate of these cells, suggesting that SUCLA2 is involved in the regulation of cell death mediated by detachment but not proliferation (Figure 2D). We next assessed whether the overexpression of SUCLA2 could confer anoikis resistance potential. We observed that transiently enforced SUCLA2 expression significantly reduced anoikis in all the cancer cells tested (Figures 2E and 2F). These data suggest that SUCLA2 is a potential anoikis resistance driver in lung and breast cancer cell lines.

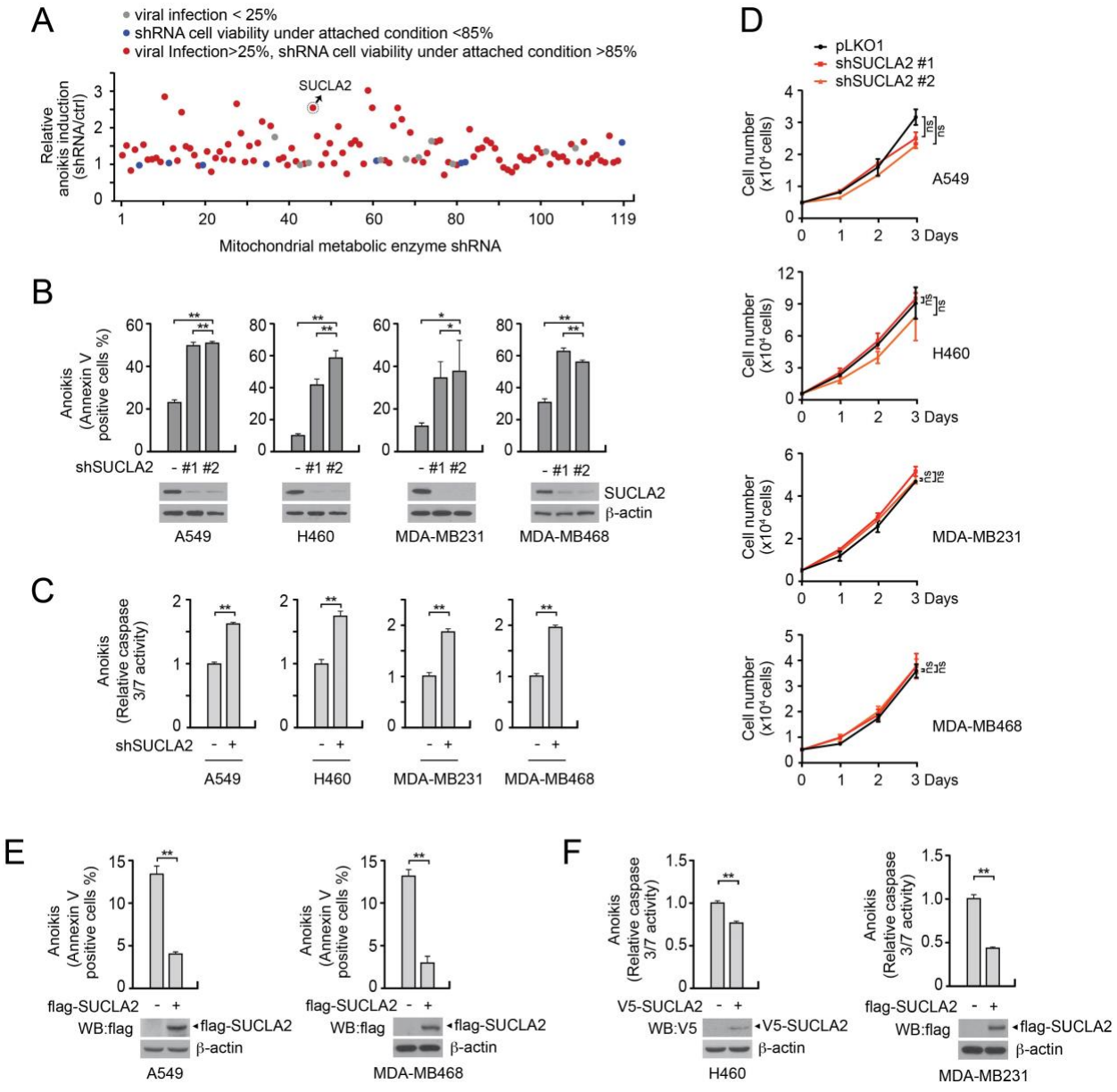


Figure 2. SUCLA2 is important for anoikis resistance in cancer cells. (A) RNAi screen evaluating the effect of targeting 119 mitochondrial genes on anoikis induction was carried out using A549 cells on 1% agarose coated dishes for 48 h. Cell death induced by detachment was assessed by annexin V staining. Candidates with viral infection rate less than 25% (gray) or shRNAs that induce cell death greater than 15% under attached condition (blue) were excluded.

(B and C) Effect of SUCLA2 target downregulation on anoikis induction in lung and breast cancer cells. Cells were transduced with SUCLA2 shRNA clones followed by anoikis induction by culturing on 1% agarose. Anoikis was assessed after 48 h by annexin V staining (B) and caspase 3/7 activity assay (C). (D) Effect of SUCLA2 knockdown on cell proliferation rates were determined by bioluminescent cell viability assay. (E and F) Effect of flag or V5 c-terminal tagged SUCLA2 overexpression on anoikis resistance in diverse cancer cell lines. Anoikis rates were determined by annexin V staining (E) and caspase 3/7 activity assay (F). Data shown are mean ± SD from 2 replicates for the screen shown in (A) and 3 replicates for the rest. One-way ANOVA (B, D) and two-tailed Student's t test (C, E, and F) were used for statistics (ns: not significant; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$). See also Table 1.

Table 1. Relative anoikis induced by target downregulation of 119 mitochondrial metabolic genes.

No.	Gene name	Relative anoikis (shRNA/pLKO1)
1	ACAD8	1.2244
2	ACSM3	1.4930
3	AK3	0.8006
4	ATP5I	1.3760
5	COX6B1	0.9449
6	GLUD1	1.5147
7	MDH2	1.1009
8	NDUFB3	1.1125
9	NDUFS7	1.1461
10	PPM1K	1.0392
11	TK2	2.8470
12	COX7C	1.0058
13	PDK3	1.4096
14	ACAA2	1.0726
15	ACSM1	2.4161
16	AGMAT	1.4698
17	ATP5E	1.4151
18	BCKDHB	1.2282
19	CMPK2	0.9320
20	COX5A	0.9490
21	DMGDH	1.3273
22	HCCS	0.7636
23	MCEE	1.1470
24	NDUFA1	1.3221
25	NDUFB10	1.1094
26	NDUFS4	1.5281
27	OXCT1	1.0824
28	TXNDC12	2.6497
29	ATP5C1	1.8355
30	AASS	1.0330
31	ACSS1	1.4791
32	ARG2	1.0731
33	AUH	1.5614
34	CA5B	2.1578
35	COX5B	0.9768
36	CRLS1	2.0355
37	CYP27A1	1.7275
38	GPT2	1.1084
39	MARS2	1.1556
40	MUT	0.9485
41	NDUFA7	1.4183
42	NDUFB9	1.0544
43	OGDHL	0.9521
44	SDHA	0.9880
45	PDK1	1.0106
46	SUCLA2	2.5388
47	ACAT1	1.7547
48	AGXT2	0.9620
49	ATP5D	1.2715
50	ATP5L	1.5535
51	COX11	0.9976
52	CYP11B2	2.0203
53	GPAM	1.2890
54	HEMK1	0.7061
55	NDUFA4	1.5414
56	NDUFC1	1.6320
57	NUDT2	1.5186
58	PDP2	0.9819
59	UQCRQ	3.0200
60	UQCRH	2.5410
61	COX6C	1.0667
62	NDUFS8	1.0931
63	ABAT	1.0792
64	ACADSB	1.0099
65	ACO2	2.0404
66	ACOT2	2.5373
67	ACP6	2.2205
68	ACSM5	1.1163
69	ACSS3	1.8535
70	ALAS2	1.0759
71	ALDH5A1	1.1614
72	AMT	1.0316
73	ATP5G3	1.2820
74	ATP5J	1.6162
75	BCAT2	1.5953
76	BCKDK	1.6364
77	CHDH	0.6772
78	COQ3	1.0316
79	COX15	0.9830
80	COX17	0.9539
81	COX6A2	1.0058
82	COX7A2	1.0319
83	COX8A	1.9424
84	CYP11A1	1.1855
85	CYP27B1	1.3913
86	DBT	1.4312
87	DLAT	1.6366
88	ECHS1	1.5621
89	GATM	1.4131
90	GPD2	1.0903
91	HADHA	0.8847
92	HMGCS2	0.8184
93	HSD17B8	0.7522
94	L2HGDH	0.8991
95	MAOA	1.1873
96	MMAB	1.0917
97	MTHFD1L	1.0805
98	MTHFD2	1.1633
99	NAGS	1.4116
100	NDUFA10	1.2931
101	NDUFA6	1.3221
102	NDUFA8	0.9719
103	NDUFB7	1.2592
104	NDUFS1	1.1675
105	NDUFS6	1.1858
106	NDUFV2	1.0479
107	NME4	1.5299
108	NT5M	1.4202
109	OAT	1.0399
110	OGDH	1.5904
111	PPA2	0.9970
112	PRODH2	1.1416
113	PYCR2	1.2259
114	SARDH	1.0723
115	SDHD	1.0211
116	UQCRB	1.7627
117	SLC25A21	1.0211
118	PDK4	1.0693
119	FECH	1.5756

*Relative anoikis induction (shRNA/pLKO.1) of candidate genes with viral infection rate less than 25% and shRNAs that induce cell death greater than 15% under attached condition are marked in gray and blue, respectively.

SUCLA2 contributes to anoikis resistance through controlling redox status. We next explored whether SUCLA2 contributes to anoikis resistance by offering metabolic advantages in cancer cells. As SUCLA2 is associated with mitochondrial energy production, we first assessed whether SUCLA2 alters bioenergetics flux and the total ATPs under anchorage-independent conditions. Although SUCLA2 can generate two ATP per molecule of glucose that enters the TCA cycle, removal of SUCLA2 did not generally alter the oxygen consumption rate (OCR), extracellular acidification rate (ECAR), or intracellular ATP level in detached cells (Figures 3A and 3B). Moreover, knockdown of SUCLA2 similarly induced anoikis under hypoxia compared to cells under normoxia conditions (Figure 3C). These data suggest that there exists a non-bioenergetic role of SUCLA2 during tumor dissemination and metastasis.

We hypothesized that other metabolic advantages such as redox balance may drive the SUCLA2-mediated anoikis resistance. Indeed, we noticed that the loss of SUCLA2 resulted in a significant increase in ROS levels when cells were under detachment conditions but not when cells were adherent, suggesting that SUCLA2 contributes to ROS scavenging in cancer cells upon detachment (Figure 3D). In line with this observation, a metabolic profiling monitoring levels of 91 metabolites involved in central metabolism including glycolysis, pentose phosphate pathway, TCA cycle,

amino acid metabolism, and nucleic acid metabolism in detached lung cancer cells with SUCLA2 loss demonstrated that the knockdown of SUCAL2 resulted in decreases in antioxidants, whereas oxidative forms increased (Figure S1). Treatment with N-acetylcysteine (NAC), a pharmacological antioxidant, significantly attenuated the elevated ROS and increased anoikis that were mediated by SUCLA2 loss in a panel of cancer cell lines including A549, H460 and MDA-MB231 (Figure 3E). We next functionally validated the effect of SUCLA2 on tumor progression *in vivo* by xenograft mouse study. Mice were intravenously injected with A549 cells that harbor control empty vector or SUCLA2 shRNA (Figure 3F). Loss of SUCLA2 resulted in dramatic reduction of metastatic potential, whereas treatment with NAC significantly restored the number of colonized tumor nodules reduced in the mice that harbor cells lacking SUCLA2 (Figures 3G-3I). These results suggest that SUCLA2 contributes to tumor metastasis predominantly by controlling redox state of disseminated cancer cells.

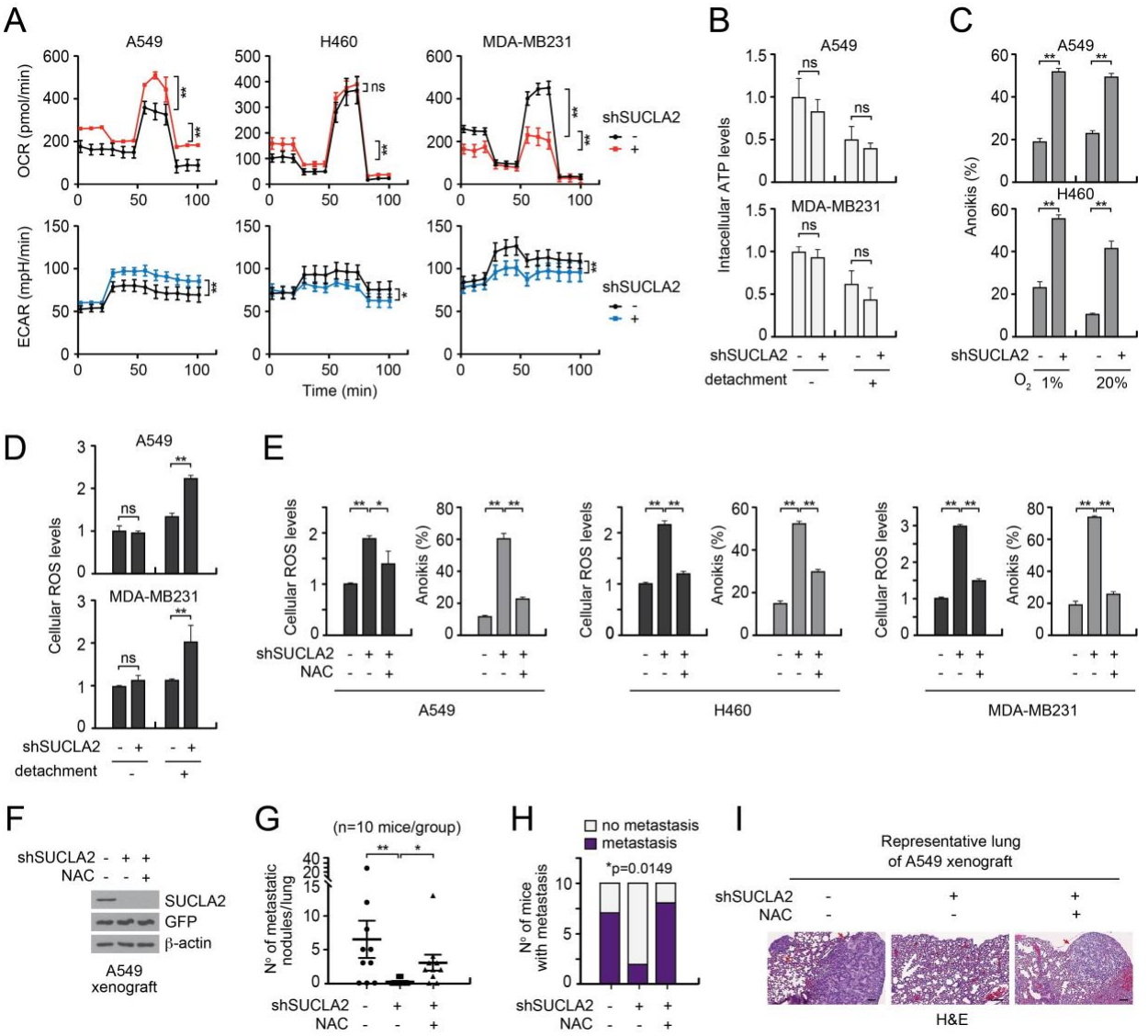


Figure 3. SUCLA2 contributes to cancer metastasis by controlling redox status. (A) Bioenergetics profiles including oxygen consumption rate (OCR; *top panels*) and extracellular acidification rate (ECAR; *bottom panels*) were obtained over time using extracellular flux analyzer in detached A549, H460, and MDA-MB231 cells with SUCLA2 knockdown or an empty vector. (B) SUCLA2 knockdown effect on ATP levels in attached or detached cells. (C) Outcome of SUCLA2 loss on anoikis resistance under hypoxic (1% oxygen) or normoxic (20% oxygen)

conditions. (D) SUCLA2 knockdown effect on cellular ROS levels under attached or detached culture conditions. (E) Detached cancer cells with SUCLA2 knockdown were treated with 0.5 mM N-acetyl cysteine (NAC) for 48 h and cellular ROS and anoikis rates were determined. (F-I) NAC was administered through daily drinking water at 10 mg/ml in A549 xenograft mice with SUCLA2 knockdown and changes in metastasis potential were monitored. SUCLA2 expression in injected cells was confirmed by immunoblotting in (F). Number of metastatic nodules in each mouse (G), number of mice with metastasis in each group (H), and representative images of hematoxylin and eosin (H&E) stained lung section from each group (I) at week 9 are shown. Scale bars shown in (I) represent 100 μm. Data shown are mean ± SD from 3 replicates of each group for (A-E). Data shown in (G) are mean ± SEM from 10 mice. P values were obtained by one- or two-way ANOVA (A-E and G) and Chi-square test (H) (ns: not significant; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$).

SUCLA2-mediated anoikis resistance is independent of the TCA cycle. To explore the mechanism by which SUCLA2 arranges cells to resist anoikis, we first examined whether SUCLA2 remains in the mitochondria with other subunits of succinyl CoA synthetase (SCS) for the Krebs cycle reaction upon cell detachment. Subcellular fractionation and immunofluorescence staining revealed that detached cell culture resulted in translocation of SUCLA2 from mitochondria to cytosol, whereas another A-SCS subunit SUCLG1 remained in the mitochondria (Figures 4A and 4B). Knockdown of SUCLG1 or SUCLG2, other components that form the SCS complex, did not induce anoikis as in SUCLA2 targeted cells (Figures 4C and 4D). SUCLA2 knockdown did not change the intracellular level of succinate, the metabolic product or substrate of SUCLA2 depending on cells undergoing oxidation or reductive carboxylation. Furthermore, treatment with cell-permeable succinate did not rescue the anoikis resistance lost by SUCLA2

knockdown in A549 and MDA-MB231 cells (Figures 4E and 4F). These data suggest that SUCLA2 confers anoikis resistance through a mechanism that is independent of its known role in the Krebs cycle.

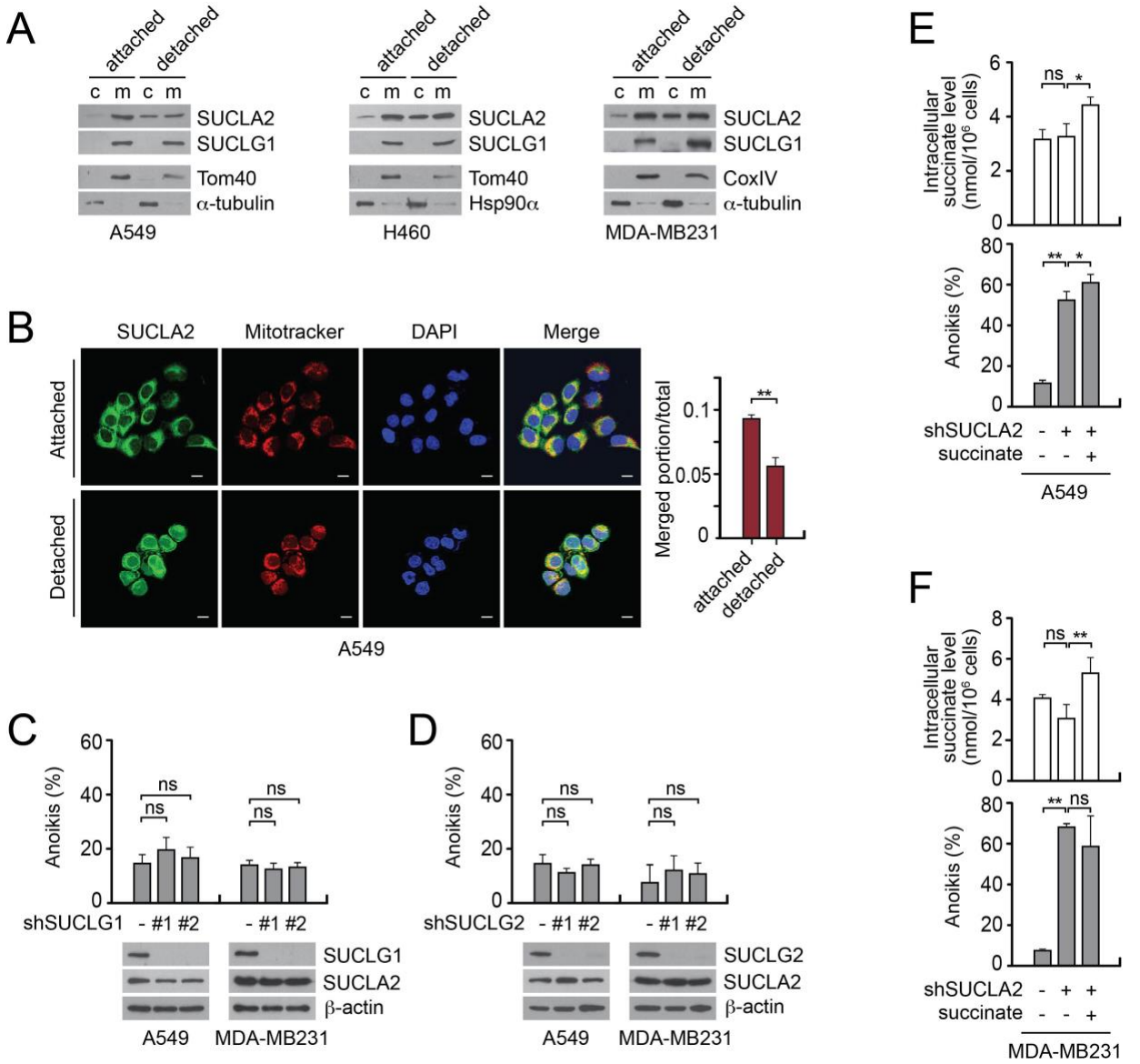


Figure 4. SUCLA2 translocates from mitochondria to the cytosol upon detachment. (A) Western blot analyses demonstrate the cytosolic and mitochondrial localization of SUCLA2 and SUCLG1 in attached and detached cancer cells. α-tubulin and hsp90α were used as control markers for cytosol. Tom 40 and Cox IV were used as markers for mitochondria. c: cytosol, m: mitochondria. (B) Immunofluorescence staining shows the localization of SUCLA2 with mitochondrion-selective probe MitoTracker in A549 cells. Quantitative colocalization analysis is shown on the right. Scale bars represent 10 μm. (C and D) Effect of SUCLG1 (C) and SUCLG2 (D) knockdown on anoikis resistance in A549 and MDA-MB231 cells. (E and F) Effect of succinate supplementation on anoikis resistance in cells with SUCLA2 knockdown. A549 (E) and MDA-MB231 (F) cells were cultured on 1% agarose plates in the presence of 3 mM dimethyl-succinate for 48 h. Intracellular succinate levels (upper panels) and anoikis rates (lower panels) were determined. Representative images are shown from at least 3 replicates for (A and B left). Error bars represent ± SD from 3 replicates for (B-F). P values were obtained by two-tailed Student's t test for (B) and one-way ANOVA for the rest (ns: not significant; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$).

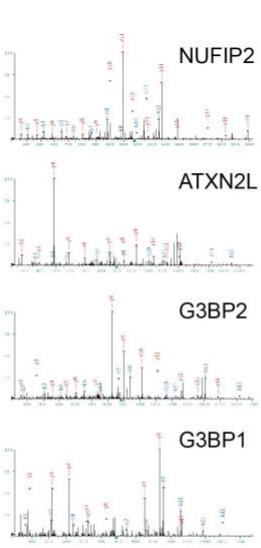
SUCLA2 interacts with and facilitates stress granule assembly in disseminated cancer cells. To better understand the molecular mechanism of the enzyme-independent role of SUCLA2 in redox management, we uncovered the proteins that interact with SUCLA2 in detached cancer cells by mass spectrometry analysis. Endogenous SUCLA2 was enriched from detached A549 cells, and the identity and quantity of the proteins pulled down with SUCLA2 were compared to that in cells with SUCLA2 knockdown. Proteomics profiling revealed that SUCLA2 binds to a series of

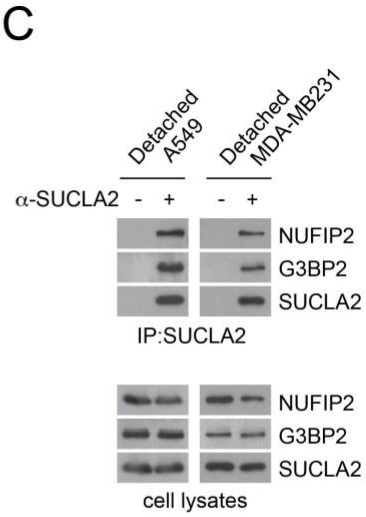
stress granule proteins including NUFIP2, ATXN2L, G3BP1 and G3BP2 (Figures 5A and 5B). Co-immunoprecipitation analysis further confirmed that SUCLA2 interacts with stress granule components in detached A549 and MDA-MB231 cells (Figure 5C).

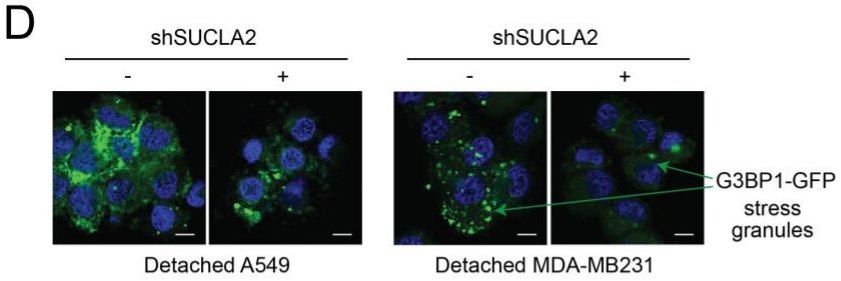
We next examined whether SUCLA2 contributes to assembly of stress granules. Knockdown of endogenous SUCLA2 in detached A549 and MDA-MB231 cells significantly attenuated stress granules, monitored by analyzing cells expressing the stress granule marker GDBP1 tagged with GFP (Figure 5D). To further study whether SUCLA2 controls anoikis through stress granules in detached cells, we first target downregulated NUFIP2, one of the main stress granule components that interacts with SUCLA2 and tested its effect on anoikis. As shown in Figure 5E, knockdown of NUFIP2 mimicked the effect of targeting SUCLA2, leading to enhanced induction of anoikis (Figure 5E). Phosphorylation of eIF2a on serine 51, a prerequisite for canonical stress granules, was unaltered by SUCLA2 knockdown, suggesting that the effect of SUCLA2 on stress granules is not related to eIF2a signaling but its functional assembly that is followed by eIF2a phosphorylation (Figure 5F). These data suggest that SUCLA2-mediated anoikis resistance is mechanistically linked with stress granule formation.

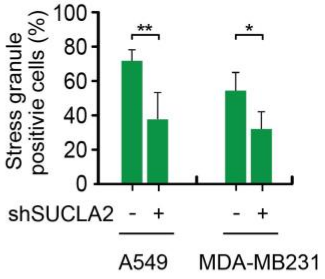
A

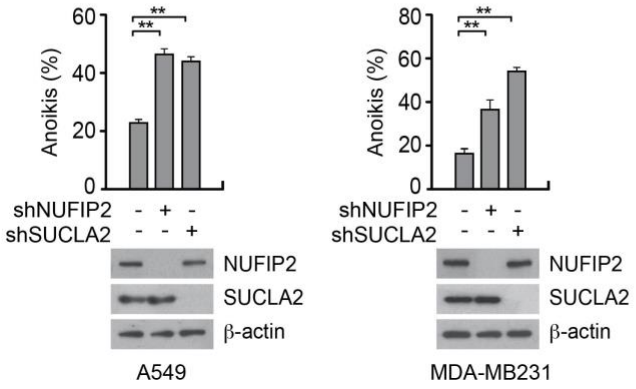
Gene symbol	Relative scan intensity: Detached A549 shSUCLA2 -	+
NUFIP2	81.0	1.0
MRPS5	55.6	1.0
SLIRP	27.3	1.0
SUCLA2	24.8	1.0
EPRS	18.2	1.0
ATXN2L	16.7	1.0
MPHOSPH10	16.2	1.0
G3BP2	7.0	1.0
PABP4	4.1	1.0
USP10	3.9	1.0
G3BP1	2.8	1.0











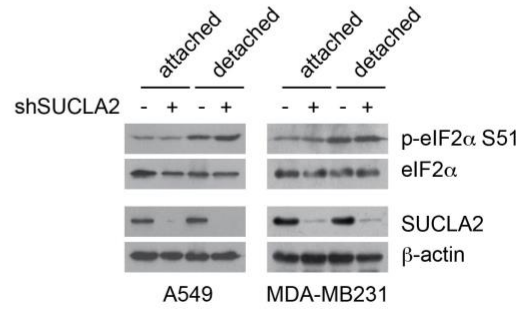


Figure 5. SUCLA2 binds to and promotes formation of stress granule complexes. (A) Identification of SUCLA2 interacting proteins by proteomics analysis. Detached A549 cells with or without SUCLA2 knockdown were applied to SUCLA2 immunoprecipitation followed by LC-MS/MS analysis. Summed intensities of peptides in each sample were compared. (B) MS spectra of stress granule protein fragments identified by LC-MS/MS are shown. (C) Endogenous interaction between stress granule proteins and SUCLA2 was determined by SUCLA2 coimmunoprecipitation in detached cancer cells. (D) Stress granules assembled in detached cells with or without SUCLA2 knockdown are shown by G3BP1-GFP. Stress granule positive cells are quantified using image J software and shown on the right. Scale bars represent 10 μm. (E) Effect of NUFIP2 knockdown on anoikis induction was compared to the effect of SUCLA2 knockdown. (F) EIF2a phosphorylation at S51 that elicits stress granule assembly was unaltered by SUCLA2 knockdown. Levels of phospho-eIF2a were assessed by immunoblotting in attached and detached cells with or without SUCLA2 knockdown. Error bars represent ± SD from 3 replicates for (D and E). P values were obtained by two-tailed Student's t test for (D) and one-way ANOVA for (E) (*: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$).

SUCLA2-mediated stress granules promote catalase expression Stress granules are known to promote protein synthesis when translation initiation is inhibited by stress responses. We found that loss of SUCLA2 when cells are under detachment stress results in significant decrease in global protein biosynthesis (Figure 6A). Based on our finding that SUCLA2 promotes metastasis by handling intracellular ROS, we next determined whether SUCLA2 knockdown attenuates metabolic enzymes associated with redox scavenging systems including catalase, peroxiredoxin

1/2/4 (PRX1/2/4), and superoxide dismutase 2 (SOD2). Intriguingly, protein levels of the cytosolic enzymes involved in scavenging hydrogen peroxide, specifically catalase, markedly decreased, whereas the levels of mitochondrial SOD2 and fatty acid synthase (FASN) were unaltered in detached cells lacking SUCLA2 (Figure 6B). In agreement with this result, the enzyme activity of catalase was significantly attenuated, and the substrate H_2O_2 accumulated when SUCLA2 was target downregulated in detached cancer cells (Figures 6C and 6D). Overexpression of a cytoplasmic H_2O_2 scavenger (catalase), but not mito-TEMPO (a mitochondria-targeted antioxidant), reversed the elevated ROS and anoikis induced by SUCLA2 knockdown in detached cancer cells (Figures 6E and 6F). These data suggest that SUCLA2 contributes to anoikis-resistant cell survival through catalase by removing cytosolic H_2O_2 rather than managing mitochondrial ROS.

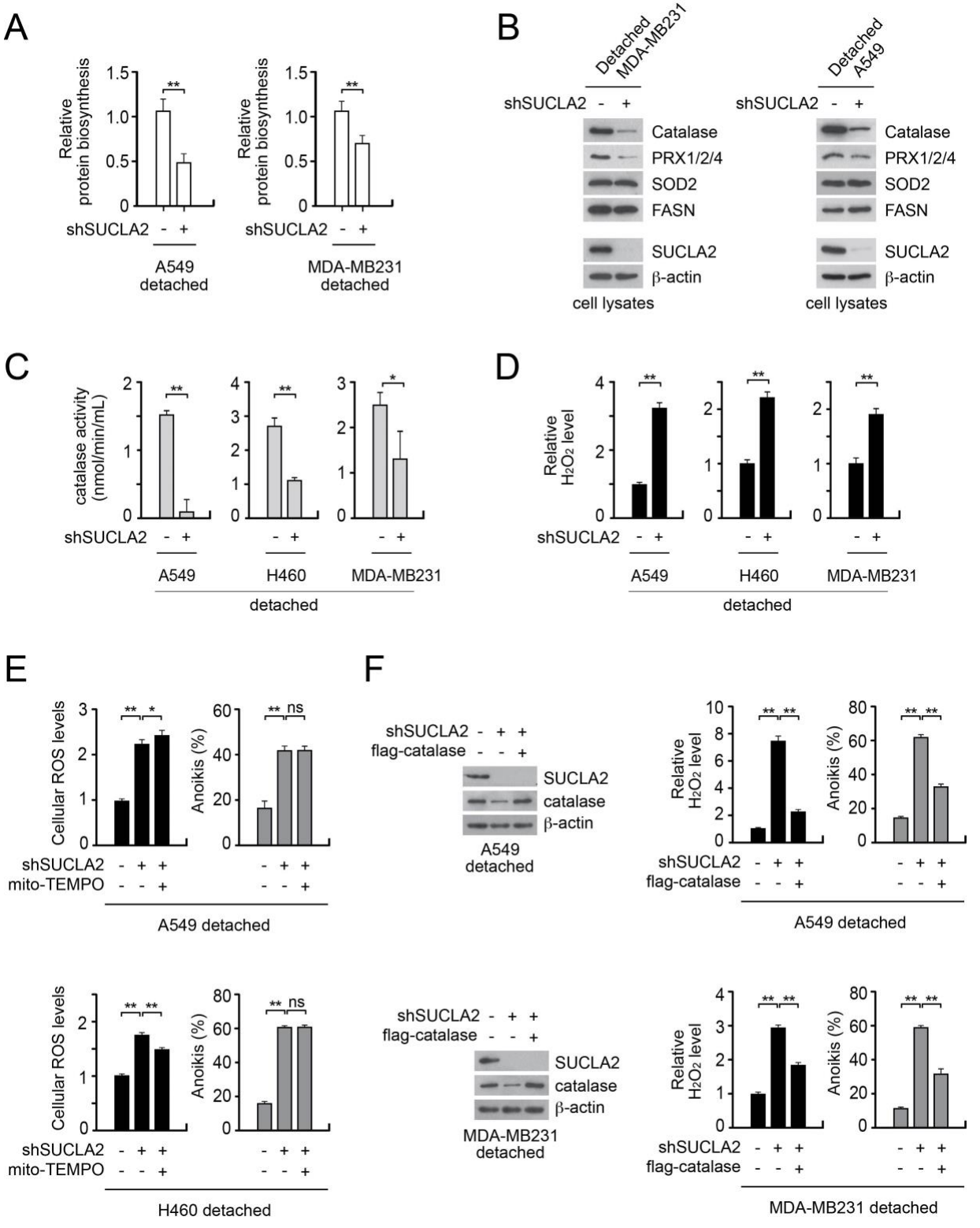

A
Relative protein biosynthesis
shSUCLA2
A549 detached
MDA-MB231 detached
B
Detached MDA-MB231
Detached A549
shSUCLA2
Catalase
PRX1/2/4
SOD2
FASN
SUCLA2
β-actin
cell lysates
C
catalase activity (nmol/min/mL)
shSUCLA2
A549
H460
MDA-MB231
detached
D
Relative H2O2 level
shSUCLA2
A549
H460
MDA-MB231
detached
E
Cellular ROS levels
Anoikis (%)
shSUCLA2
mito-TEMPO
A549 detached
H460 detached
F
shSUCLA2
flag-catalase
SUCLA2
catalase
β-actin
A549 detached
MDA-MB231 detached
Relative H2O2 level
Anoikis (%)

Figure 6. SUCLA2 contributes to redox balance and anoikis resistance in part by mediating catalase expression. (A) Effect of SUCLA2 loss on global protein biosynthesis. Cells with or without SUCLA2 cultured under detachment conditions were applied to fluorescent-based protein synthesis assay. (B) Levels of metabolic enzymes catalase, PRX1/2/4, SOD2, and FASN in detached SUCLA2 knockdown cells. (C and D) Effect of SUCLA2 knockdown on the enzyme activity of catalase (C) and intracellular hydrogen peroxide levels (D) in A549, H460, and MDA- MB231 cells. (E and F) Effect of mito-TEMPO (E) or flag-catalase overexpression (F) on the cellular ROS or H_2O_2 levels (*left panels*) and anoikis induction (*right panels*) in A549, H460, and MDA-MB231 cells with SUCLA2 knockdown. 10 μM mito-TEMPO was supplemented in the culture media, or flag-catalase was transiently overexpressed followed by 48 h detachment. SUCLA2 knockdown and total catalase level change by flag-catalase expression are shown by immunoblotting analysis. Error bars represent ± SD from 3 replicates. P values were obtained by two-tailed Student's *t* test for (A, C, D) and one-way ANOVA for (E and F) (ns: not significant; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$).

Expression of SUCLA2 positively correlates with catalase protein level and metastatic potential in cancer patients. To demonstrate the clinical significance of our findings, we first examined whether SUCLA2 positively correlates with metastatic progression and with protein expression of catalase in primary human lung cancer and breast cancer tumor samples. A total of 124 cases of paired primary tumors and metastatic lymph nodes were applied to immunohistochemistry (IHC) staining to assess SUCLA2 and catalase expression. The IHC study revealed that the protein levels of both SUCLA2 and catalase were significantly higher in metastatic lymph nodes compared to the matched primary tumor samples (Figures 7A and 7B).

Furthermore, staining scores of SUCLA2 and catalase positively correlated in these lung and breast cancer patient tumor specimens (Figure 7C). In line with our results from the histological study of patient tumors, analyses of a publicly available transcriptomic database of a larger pool of human cancers including lung, breast, bladder, cervical, sarcoma, and ovarian cancers further demonstrated that high levels of SUCLA2 correlated with poor outcome in patients with various types of cancers (Figure 7D). These data support the role of SUCLA2 signaling as a common metastasis driver in cancers, which may be linked with SUCLA2-mediated, stress granule-driven catalase expression.

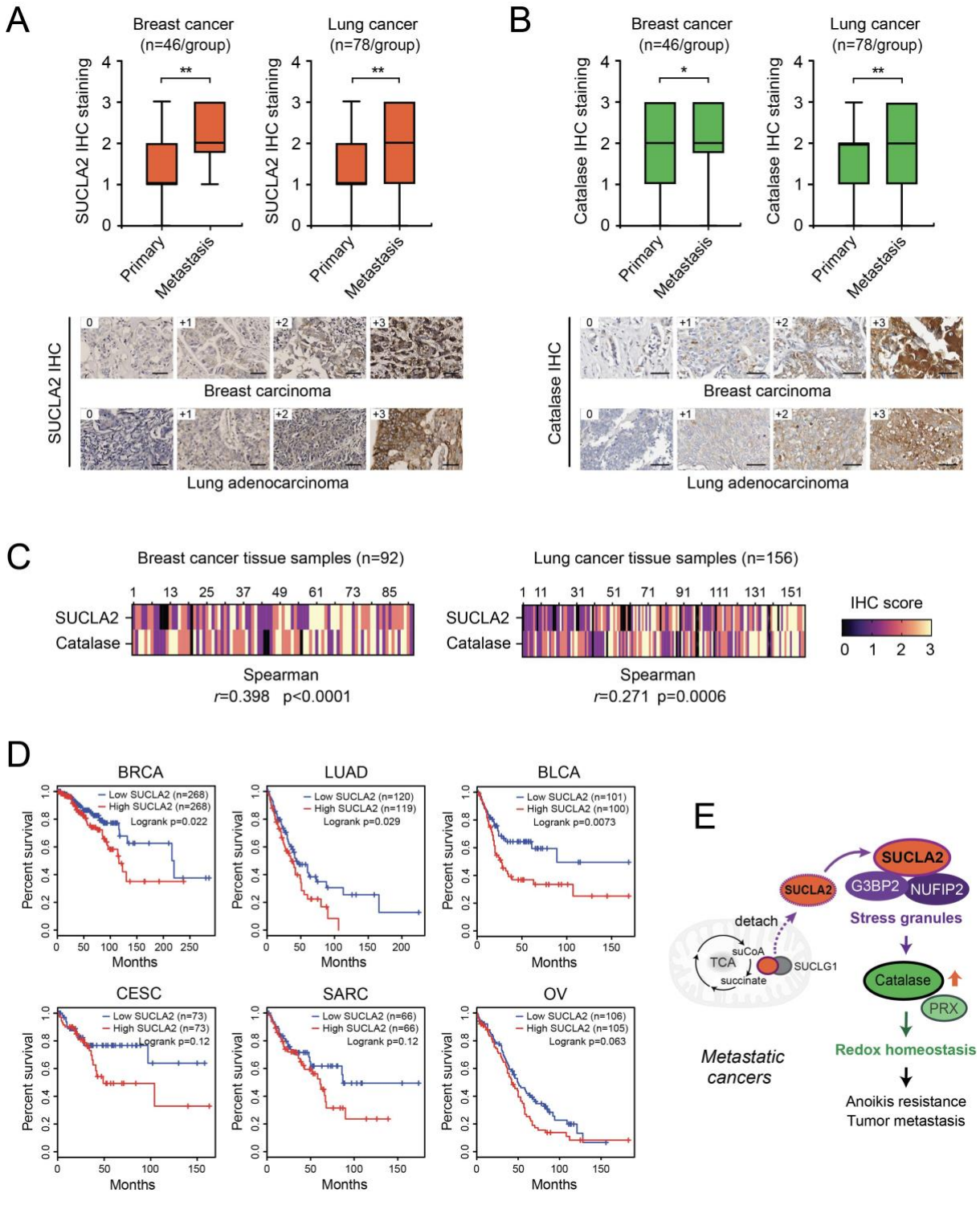

A
Breast cancer (n=46/group)
Lung cancer (n=78/group)
SUCLA2 IHC staining
Primary
Metastasis
SUCLA2 IHC
Breast carcinoma
Lung adenocarcinoma
B
Catalase IHC staining
Catalase IHC
C
Breast cancer tissue samples (n=92)
Lung cancer tissue samples (n=156)
SUCLA2
Catalase
IHC score
Spearman
r=0.398 p<0.0001
r=0.271 p=0.0006
D
BRCA
LUAD
BLCA
CESC
SARC
OV
Percent survival
Months
Low SUCLA2 (n=268)
High SUCLA2 (n=268)
Logrank p=0.022
Low SUCLA2 (n=120)
High SUCLA2 (n=119)
Logrank p=0.029
Low SUCLA2 (n=101)
High SUCLA2 (n=100)
Logrank p=0.0073
Low SUCLA2 (n=73)
High SUCLA2 (n=73)
Logrank p=0.12
Low SUCLA2 (n=66)
High SUCLA2 (n=66)
Logrank p=0.12
Low SUCLA2 (n=106)
High SUCLA2 (n=105)
Logrank p=0.063
E
SUCLA2
G3BP2
NUFIP2
Stress granules
detach
TCA
suCoA
succinate
SUCLG1
Catalase
PRX
Redox homeostasis
Anoikis resistance
Tumor metastasis
Metastatic cancers

Figure 7. SUCLA2-catalase signaling is associated with metastatic progression in human cancers. (A and B) The levels of SUCLA2 (A) and catalase (B) in paired primary and metastatic tumors from patients with lung cancer (left panels) and breast cancer (right panels). Representative IHC images for 0, +1, +2, and +3 scores are shown on the bottom for each cancer type. Scale bars represent 50 μm. (C) The correlation between protein expression levels of SUCLA2 and catalase in tumor samples analyzed in (A) and (B). (D) Kaplan-Meier survival analysis demonstrating high expression of tumor SUCLA2 mRNA is associated with poor overall survival in patients with diverse types of cancer. RNA-seq data were downloaded from the TCGA and patients were stratified as SUCLA2 high (≥ upper quartile) and SUCLA2 low (≤ lower quartile). P values were determined by Wilcoxon signed-rank test for (A and B), Spearman's Rho for (C) and log-rank test for (D) (*: $0.01 < p < 0.05$; **: $p < 0.01$). (E) Proposed working model of enzyme-independent SUCLA2-mediated anoikis resistance and cancer metastasis. SUCLA2 dissociates from the succinyl-CoA synthetase complex and moves from mitochondria to the cytosol upon cancer cell detachment from the extracellular matrix. SUCLA2 binds to and facilitates stress granule assembly that mediates protein translation of antioxidant enzymes including catalase and peroxiredoxin, and this provides metabolic advantages for cancer cells to survive during detachment and metastasize.

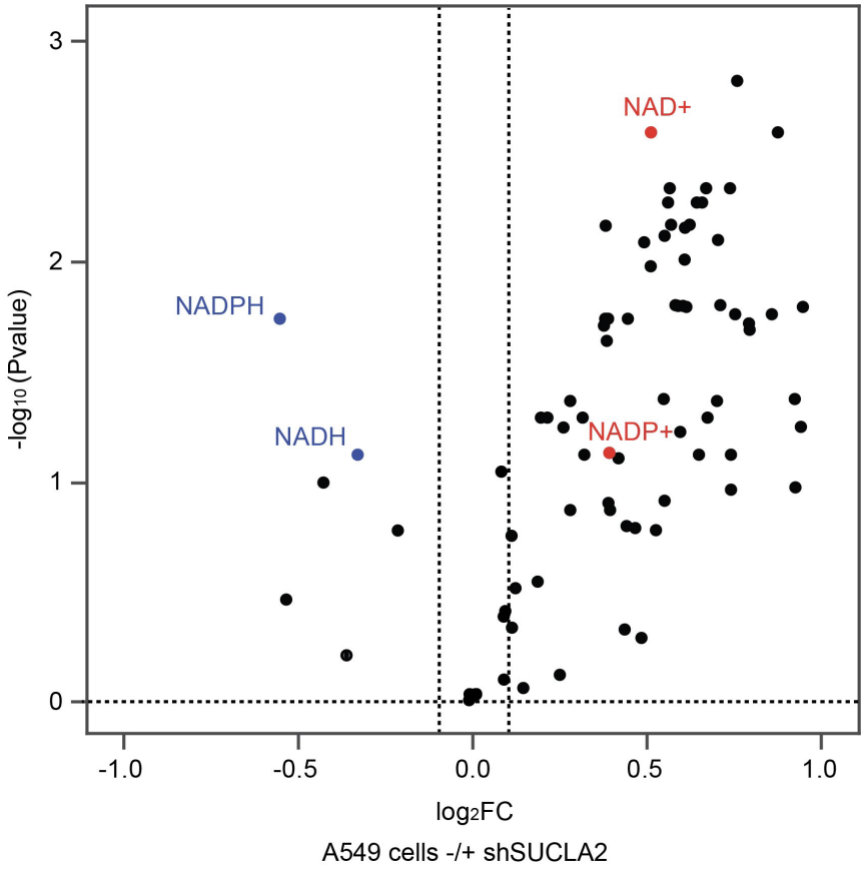


Figure S1. Metabolomics profiling reveals redox metabolite level changes in detached lung cancer cells with SUCLA2 knockdown. A volcano plot showing comparison of metabolites engaged in central metabolism in A549 cells with or without SUCLA2. 116 metabolites were measured by capillary electrophoresis mass spectrometry (CE-TOFMS and CE-QqQMS; Human Metabolome Technologies). Twenty-five metabolites from the C-SCOPE panel were not detected. Representative reduced forms that increase the antioxidant status are highlighted in blue and oxidant forms in red.

3. General Discussion and Future Directions

The assumption that mitochondrial metabolism isn't essential for cancer progression has been disputed by numerous recent human and animal studies. Although mitochondria have been shown to be associated with metastasis, the precise molecular mechanisms by which mitochondria foster cancer cell survival during the different stages of the metastatic cascade remain unclear. This study identified a Krebs cycle enzyme SUCLA2 as a critical factor that gives disseminated cancer cells a metabolic advantage to resist anoikis and metastasize. We show that SUCLA2 uncouples from another complex subunit, SUCLG1, in the Krebs cycle and moves to the cytosol upon cell detachment, where it binds to and promotes the formation of stress granules that are composed of proteins such as G3BP2 and NUFIP2. Stress granules associated with SUCLA2 promote redox homeostasis by augmenting translation of ROS eliminating enzymes including catalase, which dampens oxidative stress and promotes anoikis resistance (Figure 7E). This study deciphers a unique, non-canonical role of SUCLA2 in stress granules in the cytoplasm that modulates redox metabolism for cell survival, which is completely independent of the metabolic function of the SCS complex in the mitochondria.

Dynamic protein-protein interactions and protein-RNA interactions drive stress granule assembly. For instance, the interaction of G3BP with 40S ribosomal subunits is required for G3BP2-mediated stress granule formation (100). Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 in the stress granules and the level of ataxin-2 is important for the assembly of stress granules and P-bodies (107). The stress granule protein NUFIP2 is known to interact with DDX6 (102). It is plausible that the interaction of SUCLA2 with proteins in the stress granules such as G3BP and NUFIP2 facilitates the assembly of stress granules to perform their function as

protein translation machinery. Detailed future structural dynamics analyses on interactions among these molecules are warranted.

This study indicates that SUCLA2 contributes to redox metabolism and eventually anoikis resistance by enhancing the protein levels of catalase and peroxiredoxins 1, 2, and 4, which are cytosolic enzymes that scavenge hydrogen peroxide. Manganese superoxide dismutase (MnSOD), an antioxidant enzyme that regulates hydrogen peroxide fluxes, is known to mediate anoikis resistance and cancer metastasis in nasopharyngeal cancer (91). Furthermore, GDH1 is reported to suppress hydrogen peroxide *via* its metabolic product a-ketoglutarate, which protects human mammary epithelial cells from anoikis (43). Interestingly, hydrogen peroxide is also reported to inhibit human large cell lung carcinoma from anoikis by inhibiting caveolin-1 degradation, which was performed in a cell line with intact SUCLA2 that controls hydrogen peroxide by making ROS scavenging enzymes (18). These studies together suggest that the homeostasis of hydrogen peroxide is a major factor that determines cell fate in disseminated tumors. On the other hand, antioxidant NAC treatment or overexpression of catalase partially but not fully restored the anti-anoikis potential in cells lacking SUCLA2. Thus, although cytosolic ROS scavengers may be the predominant downstream factors in SUCLA2 signaling, it is likely that additional proteins beyond redox enzymes that are generated by SUCLA2-induced stress granules also provide anti-anoikis signaling. Global proteomics analysis using cells with SUCLA2 modulation is further warranted.

Few studies to date have identified molecules as SUCLA2 inhibitors. Vanadate and vanadyl ions attenuate the activity of ATP-dependent SCS, which is composed of SUCLA2 and SUCLG1, isolated from rat brain mitochondria (108). Tartryl-CoA and propionate have been identified to inhibit GTP-specific SCS (109, 110). However, the present study delineates that the role of SUCLA2 in anoikis resistance is enzyme-independent and not associated with the functional SCS

complex in the Krebs cycle, but with stress granules in the cytosol. Therefore, molecules that interfere with the interactions between SUCLA2 and the stress granule complex would offer a strategy to target anoikis resistance and metastasis that is mediated by SUCLA2 specifically in human cancers without affecting its canonical metabolic role in the mitochondria of other cells and tissues. Further research is warranted to validate SUCLA2 as a promising target for anticancer therapy.

4. Acknowledgements

The Taplin Biological Mass Spectrometry Facility at Harvard Medical School conducted proteomics analysis and Human Metabolome Technologies performed metabolite profiling. This work was supported in part by NIH grant F99 CA264407, R01 CA175316, R01 CA266613, R01 CA207768, R37 CA249305, R01 CA269782, DoD W81XWH-21-1-0213, and the Integrated Cellular Imaging Core of Winship Cancer Institute of Emory University under NIH P30 CA138292.

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