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Migration and Invasion Enhancer 1 (MIEN1) is an oncogene which is involved in facilitating the migration and invasion of cancer cells through actin dynamics and gene expression. Increased MIEN1 expression in many types of tumors correlates with disease progression and metastatic propensity. The precise mechanism by which MIEN1 functions is yet to be understood. The goal of these studies is to progress toward determination of the mechanisms and genetic context in which MIEN1 functions contribute to cancer progression.

It was hypothesized that Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) mediated knockout of MIEN1 in metastatic breast cancer cells would result in reduced migration and invasion. CRISPR genome editing effectively produced specific genomic deletions in the MIEN1 gene which led to the elimination of its expression in these breast cancer cells. Migration in MDA-MB-231 (231) MIEN1 knockout (MIEN1-KO) cells exhibited no difference when compared to parental 231, which was in contrast with previous siRNA studies. Signaling in several MIEN1-KO pools was inconsistent. Knocking out MIEN1 in 231 derivative cell lines showed few significant alterations in the growth, migration, invasion, signaling, despite significant changes in metabolism. However, re-expression of the MIEN1 protein containing a mutant immunoreceptor tyrosine-based activation motif (ITAM) domain resulted in significantly

decreased invasion. This revealed that MIEN1-KO 231 derivative cells were susceptible to interference of compensatory mechanisms and demonstrates the importance of the migration and invasion pathways in which MIEN1 participates in breast cancer metastasis. These findings also suggest MIEN1 may still be a promising therapeutic target to inhibit metastasis if inhibitors can be developed which block ITAM function without affecting localization or expression.

EFFECT OF CRISPR MIEN1 KNOCKOUT IN

METASTATIC BREAST CANCER CELLS

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DISSERTATION

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By

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LIST OF ABREVIATIONS

ER ..... estrogen receptor

PR ..... progesterone receptor

Her2 ..... human epidermal growth factor receptor 2

TNBC ..... triple-negative breast cancer

ECM ..... extracellular matrix

MMP-9 ..... matrix metallopeptidase 9

EMT ..... epithelial-to-mesenchymal transition

SSP ..... serine synthesis pathway

FAK ..... focal adhesion kinase

SH2 ..... Src Homology 2

Akt ..... protein kinase B

MIEN1 ..... migration and invasion enhancer 1

ITAM ..... immunoreceptor tyrosine-based activation motif

Syk ..... spleen tyrosine kinase

VEGF ..... vascular endothelial growth factor

siRNA ..... small interfering RNA

CRISPR ..... Clustered Regularly Interspaced Short Palindromic Repeats

PAM ..... protospacer adjacent motif

Indel ..... insertion and/or deletion

sgRNA ..... single-guide RNA

NHEJ ..... non-homologous end joining

RNAi ..... RNA interference

shRNA ..... short-hairpin RNA

MIEN1-KO ..... MIEN1 knockout (used when simply referring to general knockout of MIEN1)

MKO ..... MIEN1 knockout (used in attachment with specific cell lines)

FACS ..... fluorescence activated cell sorting

PCR ..... polymerase chain reaction

GAPDH ..... Glycerol-3-phosphate dehydrogenase

231 ..... MDA-MB-231

EV ..... empty vector

WT ..... wild-type

CEV ..... MDA-MB-231 CRISPR empty vector clonal pooled cell line

MKO-B ..... MDA-MB-231 CRISPR MIEN1-KO clonal pooled cell line B

MKO-C ..... MDA-MB-231 CRISPR MIEN1-KO clonal pooled cell line C

MKO-D ..... MDA-MB-231 CRISPR MIEN1-KO clonal pooled cell line D

SAM ..... S-Adenosyl methionine

PHGDH ..... phosphoglycerate dehydrogenase

AMP ..... adenosine monophosphate

GMP ..... guanosine monophosphate

MELK ..... Maternal Embryonic Leucine Zipper Kinase

CHAPTER I

INTRODUCTION

Breast cancer

More than two hundred sixty thousand new cases of breast cancer were projected to be diagnosed in 2018 accounting for almost 30% of all cancers in women and making it the most diagnosed cancer in that demographic [1]. In addition, over forty thousand women were expected to die from this disease in the same year according to modeling performed by the American Cancer Society [1]. These statistics highlight the need for research to better understand this disease. Breast cancer as a whole is a complex, heterogeneous disease and is caused by a number of factors, which makes understanding breast cancer a difficult, but essential, task.

Breast cancer has been molecularly classified into four major subtypes, which are based largely on the immunohistochemical expression pattern of specific receptors found within the cancer tissue [2]. These are the hormone receptors, estrogen receptor (ER) and progesterone receptor (PR) [3], and the growth factor receptor, human epidermal growth factor receptor 2 (Her2) [4, 5]. Each subtype has its own biological features and histopathological characteristics. Additional large scale studies examining gene expression profiles of breast cancer subtypes have further divided the subtypes into subclasses [6-9]. Examining and categorizing breast cancers using protein or mRNA expression patterns as well as genomic gene amplification has been used for decades to distinguish cancer subtypes. Technological advances have increased the coverage,

sensitivity and resolution of these methods and identified additional criteria for distinction, including epigenetic alterations [10] and even correlating mutations in non-coding regions, such as promotors, with breast cancer subtypes [11]. These studies certainly demonstrate the complexity of breast cancer and indicate a need for a more complete understanding of this vast field of disease to improve diagnosis and treatment.

Triple-negative breast cancer

The most aggressive subtype of breast cancer is triple-negative breast cancer (TNBC) and is characterized by an absence of expression of the three major biomarker receptors which largely classify the other major subtypes. Although TNBC accounts for only 10-15% of breast cancer cases, this subtype has a disproportionate rate of distant relapse and mortality when compared to all other breast cancers [12, 13]. Since TNBC lacks established breast cancer biomarkers, it is difficult to effectively treat with targeted therapeutics, which necessitates non-specific, systemic radio or chemotherapy as the most common treatment modality [14]. The American Society of Clinical Oncology stated in their Clinical Practice Guidelines regarding breast cancer biomarkers that other than endocrine or Her2 status, no other biomarker has proven to be reliable as a prognostic biomarker for determination of adjuvant therapy, highlighting the importance of further studies to establish these markers, especially in TNBC [15]. Recent studies have attempted to identify additional markers, such as EGFR, cytokeratin 5/6 and androgen receptor status, within the TNBC subtype in an effort to more efficiently characterize the tumors and provide further insight during the primary and adjuvant treatment decision making process [16-20]. However, the heterogeneity and poor differentiation associated with TNBC makes the standardization of markers within this subtype a difficult task. More work still

needs to be done to identify the protein markers and underlying molecular mechanisms involved in the aggression and propagation of TNBC and its subclasses.

Metastasis

Although metastasis is well known to be the major cause of mortality in cancer patients, accounting for approximately 90% of cancer related deaths, it is perhaps one of the least understood aspects of cancer progression [21]. Because metastatic cancer is the reason for the majority of fatalities in breast cancer, it is actually preferential for the receptor status of the metastasis to be considered more than the primary tumor during evaluation of the patient [15, 22]. Metastasis is a complex process which involves a variety of proteins within the cancer cell acting in a concerted effort to leave the primary tumor and colonize other areas of the body. Cancer cells are often genetically unstable due to defects in DNA damage sensing or repair mechanisms which allow for the accumulation of mutations, deletions, and amplifications [23-26]. These alterations can affect tumor growth, cell survival, and ultimately, metastasis.

The process of metastasis begins with cancer cells acquiring enough alterations to allow them to break free from the primary tumor and invade the surrounding normal tissue. This may be accomplished by individual cells or in groups of cells [27-30]. This initial stage of metastasis classically includes downregulation or loss of cell-to-cell adhesion molecules, such as E-cadherin, as well as an upregulation of pro-migratory genes, such as extracellular matrix (ECM) degrading enzymes like matrix metallopeptidase 9 (MMP-9), to clear the path for the escaping cell(s) [31-34]. These changes are often associated with epithelial-to-mesenchymal transition (EMT) which was thought to be the gateway through which cancer cells must pass in order to gain metastatic potential [35, 36]. However, full EMT is not necessary for metastasis to take

place, which shows that cancer cells may initiate metastasis through a variety of mechanisms [28, 37, 38].

Locomotion by reorganization of the cytoskeleton and adherence to surrounding parenchyma or stroma through integrins and other adhesion molecules is also vital to metastatic invasion and dissemination, especially after the physical connection to the primary tumor has been severed. Once the cells have escaped the primary tumor, or if the primary tumor has developed its own vasculature, metastatic cells can continue to disseminate by squeezing between the endothelial cells encompassing the interstitium, lymphatic system or blood vessels and gaining access to the rest of the body through these aqueous circulatory systems [39]. Thousands of cells reach this point in the metastatic cascade; however, the extravasation of the cancer cells is a complex and selective process involving cellular cooperation and interaction with endothelial cells as the cancer cells flow through the vessel [40-44]. Once docked on the endothelium, the cells must once again undergo the same drastic cytoskeletal rearrangement required to enter circulation in order to extravasate. Upon entering the surrounding tissue, cancer cells must adapt to survive in the new tissue microenvironment. Metastatic cancer cells interact with surrounding endothelial cells, fibroblasts and immune cells to alter the environment and provide increased survival and growth signaling as well as immune suppression [45-50].

Each step of the metastatic cascade is associated with specific proteins that act to accomplish that step [51]. Initiator proteins are responsible for beginning the process and starting the preliminary invasion and dissemination. Progression proteins enable the cell to continue to move towards vasculature and communicate with the endothelium, both before and after intravasation. Finally, virulence genes are responsible for facilitating development of the secondary tumor through interaction with cells which make up the target organ.

The timeline of how early metastasis takes place in the course of the disease is becoming more clear. It has been shown that cancer cells can actually disseminate very early on in the development of the primary lesion [52, 53]. This leads to metastases which are genetically distinct compared to primary tumors, which impresses the need to characterize metastases in addition to primary tumors when developing treatment strategies. Early departure from the primary source and the selective pressure that the metastatic process places on tumor cells causes increased genomic alterations that lead to increased aggression and malignancy [52, 54]. The resulting secondary tumors may then actually start to initiate metastasis, which can bolster the primary tumor, parental metastatic tumor or seed additional satellite tumors elsewhere in the body [55, 56].

In order to be able to assess and treat metastatic cancer patients, a better understanding of the proteins which initiate, direct and drive this process is needed.

Organotropism and genetic context

Interestingly, many cancers have a propensity to metastasize to specific organs to the exclusion of others. This phenomenon was noticed as early as the late 1800's by Ernst Fuchs [57] and Dr. Stephen Paget who argued that "the distribution of the secondary growths is not a matter of chance" in post-mortem analysis of breast cancer patients [58]. Breast cancer specifically has a propensity to metastasize to the lung, bone, brain and liver [51, 59, 60]. Many breast cancer cell lines which are derived from patients via extraction from a malignant pleural effusion consist of a heterogeneous population of cells from multiple metastatic origins [61-64]. Because of this mixture of cells, it can be difficult to completely assess the genetic context of the cell line. Studies using serial *in vivo* selection of heterogeneous MDA-MB-231 breast cancer

cells have isolated sub-populations which possess organotropic capabilities. The metastatic derivative cell lines generated using this method have revealed gene signatures which mediate organotropism [65-67]. Cells exhibiting these signatures can be isolated from the parental population and when injected into mice follow the predicted metastatic tendency. Interestingly, these gene sets identified in these metastatic derivative cell lines did not completely overlap with other global metastatic gene sets, indicating that there are different factors required for metastasis to, and survival in, various target organs. Even exosomes isolated from these metastatic derivative cells contained unique proteomic signatures and showed a propensity for the same specific organ as the cells [68]. These exosomes were then able to interact with the endogenous cells of the target tissue to alter the environment within the organ in preparation for metastasis [68]. All of these findings indicate that genetic context is very important for the phenotypic outcome of organotropic metastasis. In the same way, the genetic context in which a gene is expressed is also very important. Some proteins may cause opposing phenotypes when expressed in different backgrounds [69]. For example, p21 expression was increased in several cancer types, including breast, and was associated with tumor progression [70-73], while reduced expression in other cancers, including cervical, correlated with tumor progression and negative prognosis [74, 75]. In addition, in bile duct carcinoma, both low and high p21 expression predicted shortened disease-free survival [76]. This shows that more factors than expression of single proteins influence the function of proteins within the context of cancer. Knowing genetic context is vital to understanding the complete picture of protein function. This information will allow for more accurate predictions of outcomes in cancer cases and more informed decisions during evaluation of treatment options.

Serine metabolism in cancer

Tumor metabolism is an area of study that has become more intriguing over the last several decades. Research has begun to show that the altered metabolism of cancer is not just the result of cellular damage nor are the changes solely for increased energy production. Instead, cancer metabolism is the product of intentional cellular reprogramming which promotes the production of biosynthetic precursors and signaling molecules required for rapid malignant cell growth [77]. Interestingly, undifferentiated embryonic cells also employ similar metabolic strategies during periods of active growth [78]. This 'metabolic re-wiring' is universal in tumors, but there is variation in the extent to which different biosynthetic pathways are either upregulated or downregulated based on the needs of the cancer for replication and propagation.

One pathway that has been shown to be elevated in breast cancer [79], human embryonic kidney and epithelial lung cancer cells [78], lymphosarcomas [80], and liver cancer [81, 82] is the serine synthesis pathway (SSP). This metabolic shunt is a branch of the glycolytic pathway in which 3-phosphoglycerate is first converted to serine through a series of oxidation and transamination reactions. Serine can then be converted to glycine through the donation of a methyl group to the folate pool. Flux through the SSP contributes to the production of many important biological molecules including amino acids, lipid precursors, and nucleotides. Moreover, this pathway is also the largest contributor of one-carbon units to the folic acid pool within the cell [83]. These methyl groups can then be used in important cellular processes such as DNA methylation, an essential part of DNA replication for actively proliferating cells [84]. The serine synthesis pathway also supports this methylation and other cellular processes through the *de novo* production of ATP [84]. Overall the SSP supports many anaplerotic reactions that

promote the highly anabolic activity of rapidly proliferating cells. In fact, recently investigators have even suggested this pathway may be oncogenic [85].

Focal adhesion kinase

One of the main effectors of focal adhesions, intracellular protein complexes which aid in connecting the cell's cytoskeleton to the ECM, is focal adhesion kinase (FAK). When FAK is recruited to focal adhesion complexes following integrin engagement with the ECM, it is activated through a combination of autophosphorylation and phosphorylation by other proteins including Src kinase (Fig 2) [86]. FAK then facilitates focal adhesion dynamics by regulating appropriate turnover of focal adhesion structures within the cell in response to stimuli [87, 88]. Autophosphorylation of tyrosine-397 (Y397) is the first step of FAK activation [89, 90]. Active FAK then interacts with Src which phosphorylates additional FAK tyrosine residues, including Y925 within the C-terminal Focal Adhesion Targeting (FAT) domain [86, 91]. Phosphorylated FAK Y925 then serves as a docking site for the Src Homology 2 (SH2)-domain containing protein Grb2, an upstream initiator of the Map Kinase pathway [86].

Protein kinase B

Protein Kinase B (PKB), more widely known as Akt, is a serine/threonine kinase which is generally activated by signaling mediated through extracellular mitogens binding receptor tyrosine kinases. Akt contains three major domains, a catalytic domain, a pleckstrin homology (PH) domain and a regulatory domain [92]. The PH domain mediates binding of Akt to phosphatidylinositol (3,4,5)triphosphate (PIP3) at the cell membrane, which is produced by phosphatidylinositol 3-kinase (PI3K) as a second messenger in response to the mitogenic

signaling [93]. Following Akt localization to the plasma membrane through binding to PIP3, Phosphoinositide dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), among others, are able to fully activate Akt through phosphorylation of specific serine and threonine residues[92, 94-97]. Akt is regulated by dephosphorylation which is mediated indirectly by dephosphorylation of PIP3 by PTEN and directly through other protein phosphatases such as protein phosphatase 2A [98-100].

Akt is involved in the regulation of a wide range of cellular processes. Apoptosis is inhibited by Akt phosphorylation of the pro-apoptotic protein Bad [101]. Akt regulates autophagy and cellular homeostasis by altering mitochondrial biosynthesis [102]. Global metabolism is significantly affected by Akt activity. Akt increases glycolytic flux through increased glucose intake as well as alterations to glycolytic enzymes [103]. Downstream gene expression is also modulated by Akt activity through direct and indirect phosphorylation of transcription factors [104, 105]. Many of these functions of Akt are favored in cancer, which is why at least one member of the Akt activation pathway is altered in many cancers [106].

MIEN1 structure and function

Migration and Invasion Enhancer I (*MIEN1*), previously known as C35, C17orf37, MGC14832, RDX12, ORB3 and XTP4, is an oncogene that is located on the long arm of the human chromosome 17, 0.5kb away from the *ERBB2* gene which encodes for Her2 [107]. The structure of the MIEN1 protein contains several functional domains: a prenylation motif, an immunoreceptor tyrosine-based activation (ITAM) motif, and a redox-active motif (Fig 1).

The C-terminal prenylation motif allows MIEN1 to insert into the inner-leaflet of the plasma membrane and interact with other membrane-associated proteins [108]. Mutation or

deletion of the prenylation motif, or blocking prenylation with chemical agents, results in loss of membrane association and ablation of MIEN1 function in directing migration and invasion of cancer cells, highlighting the fact that its function is dependent upon appropriate cellular localization. The redox-active motif of MIEN1 has been shown to be functional by two studies which identified glutathione peroxidase 1 [109] and glutathione peroxidase 4 [110], important regulators of oxidative stress response within the cell, as direct targets.

The redox-active motif is housed in a thioredoxin-like fold and is characterized by a "CxxC" amino acid sequence, where "x" refers to any amino acid (Fig 1) [109]. Though the redox active motif has been shown to be operational, no studies have been carried out to show its significance to MIEN1 function as a whole or the cellular context in which it functions.

The ITAM motif, a conserved amino acid sequence which was initially described in immune cell activation following recognition of antigen, mediates signaling and confers transformative properties to MIEN1 when overexpressed in normal mammary epithelial cells [111, 112]. This motif consists of two tyrosine residues which, when phosphorylated, can serve as docking sites for SH2 containing proteins, which can then mediate downstream signaling. The ITAM domain of MIEN1 has been shown to be phosphorylated and that mutation of either tyrosine residue results in decreased MIEN1 phosphorylation [113]. However, precise mechanism by which the MIEN1 ITAM mediates downstream signaling and the degree to which either tyrosine residue regulates this signaling is unknown. The ITAM motif of MIEN1 is able to activate spleen tyrosine kinase (Syk), which is responsible for MIEN1's ability to incite cellular transformation of immortalized normal breast epithelial cells [111].

MIEN1's mechanistic role in metastasis is becoming clearer, however much is still unknown about the exact mechanisms of action and the overall importance of MIEN1 in disease

Figure 1

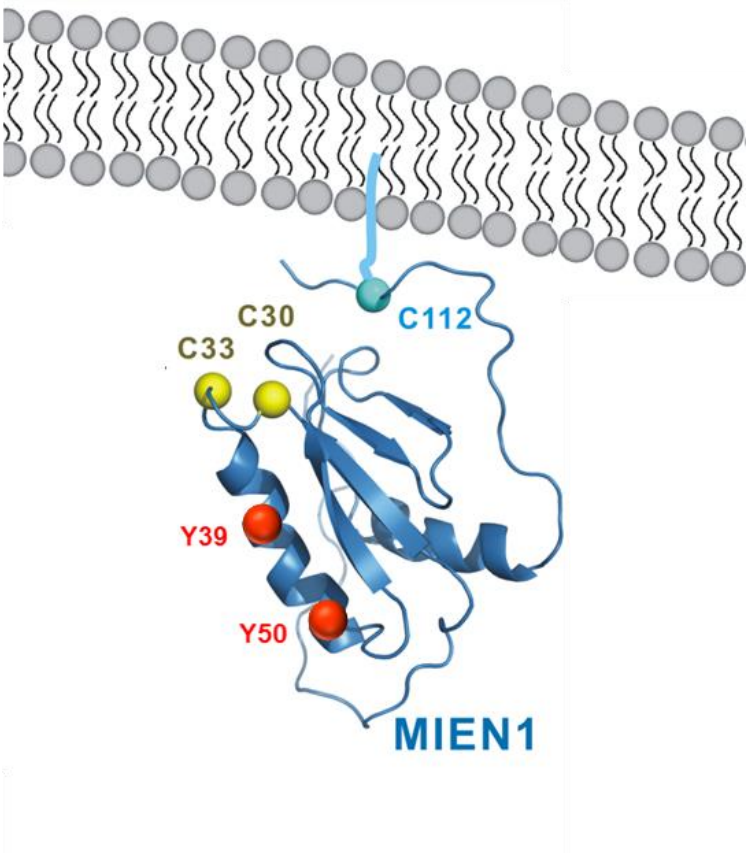


***Figure 1*. Structure of MIEN1.** Figure adapted from Hsu et al. [114]. Crystal protein structure of MIEN1 inserted into the inner leaflet of the plasma membrane. Location of key amino acids within MIEN1 functional domains are noted by spheres within the polypeptide chain. C30 and C33 indicate the location of the cysteine residues within the redox-active motif. Y39 and Y50 denote the tyrosine residues which are the main effectors of the ITAM motif. C112 is the first amino acid of the prenylation motif and becomes geranylgeranylated to allow MIEN1 to be anchored to the plasma membrane.

progression. MIEN1 increases expression of known metastatic cascade proteins such as MMP-9 and vascular endothelial growth factor (VEGF) through activation of Akt (Fig 2) [111, 115, 116]. Actin cytoskeletal dynamics, an important component of cellular locomotion, are also regulated by MIEN1 acting through FAK and cofilin, an actin binding protein involved in actin depolymerization, to stabilize actin filaments, particularly in the leading edge of the cell in the lamellum (Fig 2) [117, 118]. Knockdown of MIEN1 in breast cancer cells by small interfering RNA (siRNA) reduces FAK Y925 phosphorylation and filamentous actin within breast cancer cells leading to decreased migration [117]. This indicates that there is a relationship between MIEN1 signaling, FAK phosphorylation and downstream signaling pathways which favor actin filament formation.

The unique combination of functional domains combined with its subcellular context near the plasma membrane indicates that MIEN1 may be involved early in signal transduction which ultimately facilitates and drives metastasis of cancer cells.

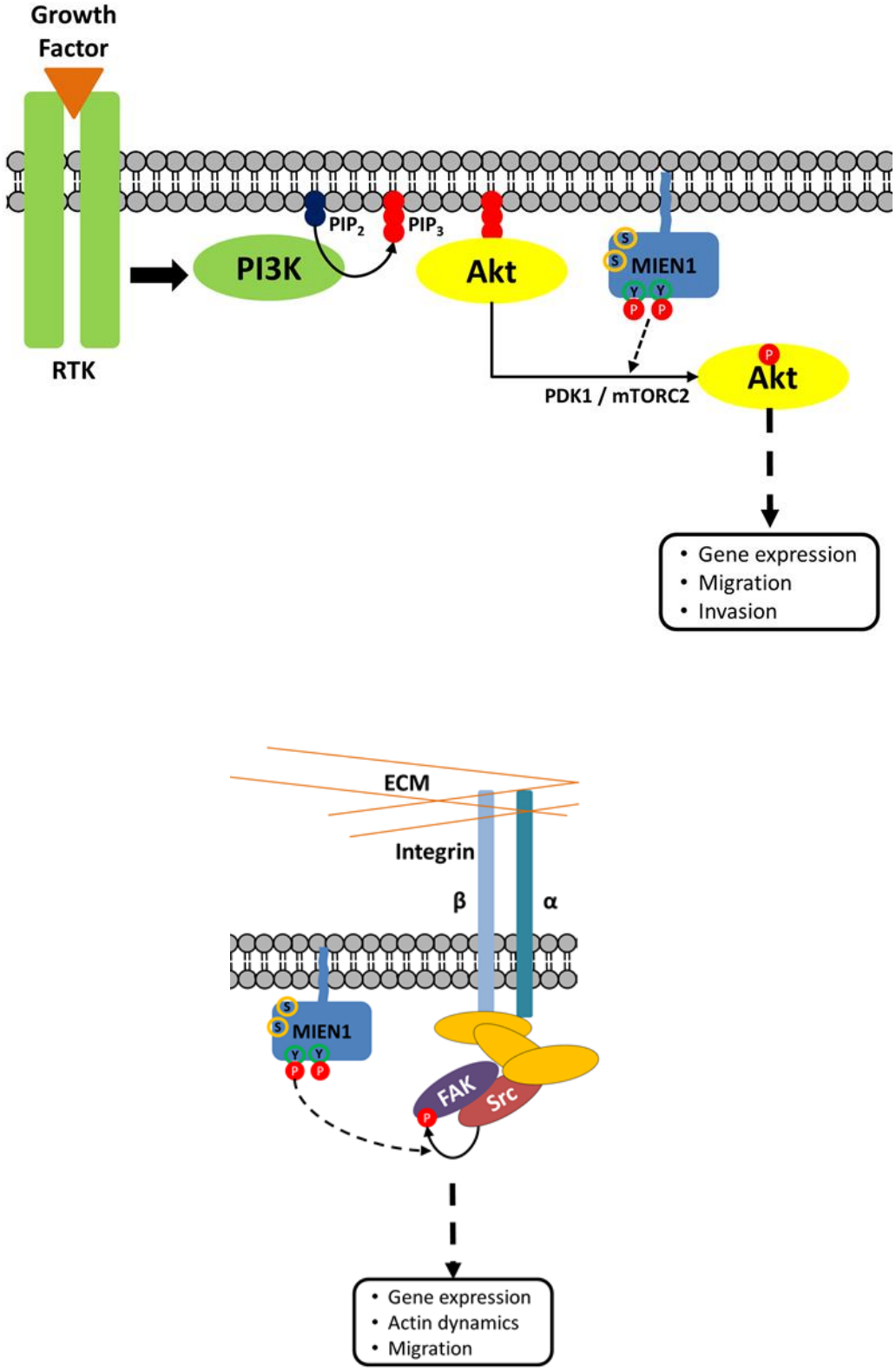
MIEN1 in cancer

MIEN1 is present in a wide range of tumors and plays a role in migration and invasion of a variety of cancer cell types. MIEN1 has been shown to be highly expressed in breast [107], ovarian [119], prostate [115], oral [116], gastric [120], and lung cancer [121] while showing only minor expression in leydig cells of the testis after examination of 37 normal tissues [107]. Due to its proximity to the *ERBB2* gene, *MIEN1* is commonly amplified in breast cancer along with *ERBB2* which leads to an overexpression of MIEN1 protein in many breast cancers as well as gastric cancer [107, 120]. MIEN1 mRNA is overexpressed in approximately 85% of Her2

Figure 2

MIEN1 function at the plasma membrane. (Top) Membrane associated MIEN1 facilitates the activation of Akt kinase. Growth factors and other mitogens bind transmembrane receptors, such as receptor tyrosine kinases, which dimerize and autophosphorylate to initiate downstream signaling. One protein activated by these receptors is phosphatidylinositol-3-kinase (PI3K). PI3K phosphorylates the membrane lipid phosphatidylinositol (3,4)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Akt can then bind PIP_3 with its PH domain to facilitate membrane associated and activation. MIEN1 modulates the activation of Akt in many different types of cancer cells. This activation results in signaling cascades which terminate in pro-migratory and pro-invasive gene expression. (Bottom) MIEN1 interacts with focal adhesion complexes to regulate the phosphorylation and activation of FAK. Integrin heterodimers interact with ECM proteins including fibronectin and collagen. This interaction leads to formation of focal adhesions within the cell as the ECM is linked to the cytoskeleton through these integrins. Focal adhesion complexes contain Src and FAK. FAK activation in focal adhesions triggers downstream gene expression and actin filament dynamics which favor migration of breast cancer cells.MIEN1 regulates FAK function by influencing FAK phosphorylation specifically at Y925.

Figure 2


Growth Factor
RTK
PIP2
PIP3
PI3K
Akt
MIEN1
PDK1 / mTORC2
Akt
• Gene expression
• Migration
• Invasion
ECM
Integrin
β
α
MIEN1
FAK
Src
• Gene expression
• Actin dynamics
• Migration

positive breast cancers [113]. However MIEN1 overexpression is not limited to Her2 positive breast cancer and can occur in all subtypes, which supports independent regulation of MIEN1 [113, 122]. In clinical samples of both prostate and breast cancer, MIEN1 expression correlates well with progression of the disease indicating MIEN1 may play a role in tumor progression [115, 123]. In congruence, MIEN1 overexpression was shown to increase the tumorigenicity of ovarian cancer cells both *in vitro* as well as *in vivo* [119]. Also, MIEN1 expression was shown to be elevated in oral cancer cells with higher dysplasia [116]. High mRNA levels were also observed in head and neck cancer to correlate with poor tumor grade and decreased overall survival [116]. MIEN1 mRNA expression has been shown to be a potential predictor Herceptin treatment efficacy in breast cancer patients and has also been identified as a potential driver gene within the *HER2* amplicon [124, 125].

MIEN1 was named due to its ability to increase the migration and invasion of prostate cancer cell lines when overexpressed [115]. MIEN1 has been shown to functionally increase the invasive and migratory phenotype of various types of cells including breast, prostate, oral, colon, fibroblasts and endothelial cells [108, 111, 115, 116, 126]. Knockdown of MIEN1 with siRNA also showed the expected result of decreasing the invasion of prostate cancer cells in a dose-dependent manner and breast cancer cells [115, 117]. MIEN1 function was also shown *in vivo* as prostate cancer cells overexpressing MIEN1 exhibited increased lung colonization in mice compared to vector controls [108].

It is important to continue to investigate the mechanisms by which MIEN1 mediates its function, but it is equally important to remember that the specific cancer type and genetic context is vital to understanding how these mechanisms are used by various cancer cells.

Clustered Regularly Interspaced Short Palindromic Repeats

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome editing is a technique that has been adapted from bacterial and archaea adaptive immune systems for use in research and treatment settings [127-131]. It is comprised of an RNA-guided CRISPR associated (Cas) endonuclease which mediates double-strand cleavage at a specified 20 base pair genomic locus (Fig 3) [127]. The exact location of cleavage is three base pairs upstream from the protospacer adjacent motif (PAM), a three nucleotide sequence composed of the "NGG" trinucleotide, which essential for Cas9 cleavage [127]. Double strand breaks in the DNA lead to either homologous recombination or non-homologous end-joining (NHEJ) DNA repair. The later repair is more error prone and can lead to insertions and deletions (indels) of DNA segments at the targeted locus. These indels generally result in a non-functional or non-existent gene product by causing disruption of transcription or altered transcripts due to frameshift. Frameshift alters the transcript to make it unreadable, cause premature termination of transcription through introduction of a stop codon, or non-functional gene products due to altered mRNA sequence resulting in a polypeptide which is unable to be properly folded and may lead to degradation. The ultimate result of most indels induced by CRISPR is the complete lack of protein expression.

The most important aspect of the CRISPR/Cas9 genome engineering system is design of the single-guide RNAs (sgRNAs). These RNA sequences are responsible for accurate targeting of the Cas9 endonuclease to the genomic region of interest. Much work has been done to understand how the composition of sgRNAs affects the targeting efficiency of the sgRNAs [132]. At each position in the sgRNA sequence, there are certain nucleotides which are more favored and increase targeting efficiency. For example, at the twentieth position, A and G

Figure 3

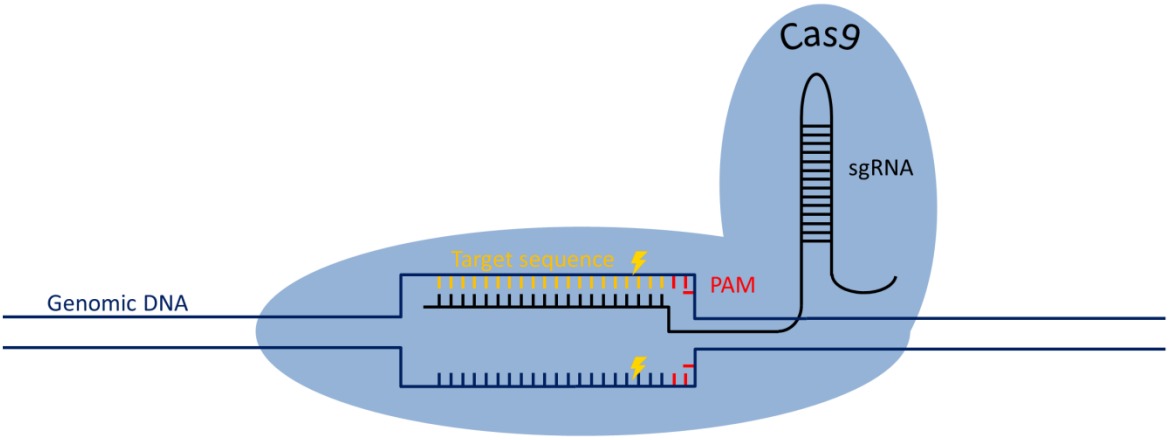


Figure 3. **CRISPR/Cas9 system.** Representation of Cas9 protein (blue structure) interacting with target sequence within the genome. SgRNA is loaded onto the Cas9 protein. This sgRNA directs Cas9/sgRNA complex to target loci. Helicase activity of Cas9 unwinds DNA and twenty nucleotides at the distal end of the sgRNA (black marks on end of sgRNA) anneals with the twenty nucleotides (yellow marks) upstream of the three nucleotide PAM sequence (red marks) within the chromosome. Endonuclease activity of Cas9 produces a double-strand break within the target sequence in the genomic DNA three nucleotides upstream (yellow lightning bolts) of the PAM sequence. Following cleavage, cellular DNA damage response facilitates NHEJ to repair break. This may result in indel mutations close to the cut site (yellow lightning bolts).

nucleotides are more highly favored, while C and T nucleotides are actually disfavored and adversely affect targeting [132].

Though it is important to pick sgRNA sequences with high-scoring on-target activities, even more crucial is the specificity of the sgRNA to your target. Editing the target genomic locus is only beneficial if it is the only alteration made to the cell, otherwise observed effects may be the result of off-target CRISPR cleavage and indel formation. There is much concern for the specificity of CRISPR/Cas systems and studies have shown that off-target mismatches at certain positions in the sgRNA sequence, generally more proximal to the PAM sequence, result in decreased off-target activity [133]. In addition to sgRNA design, work is being done to mutate the Cas9 endonuclease to decrease the tolerance for mismatches between the sgRNA sequence and off-target loci, or reduce the cutting of the DNA to one strand, necessitating two sgRNAs for complete cleavage [134, 135]. Also, it has been shown that shortened sgRNAs may provide increased specificity [136]. With these improvements, specificity of CRISPR genome editing is improving, which will allow for its use in therapeutic applications.

There are several methods which can be used to deliver the CRISPR/Cas9 system into cells. Transfection of plasmids with lipid based reagents, electroporation or transduction with lentiviral particles are standard methods, but may lead to stable expression of Cas proteins and increased off-target effects. Cells may also be transfected with Cas9 mRNA and pre-transcribed sgRNAs. Finally, Cas9 recombinant protein can be complexed with sgRNA prior to transfection. Messenger RNA and protein transfection will both be completely transient and should significantly reduce off-target effects.

The application of the CRISPR/Cas system is not limited to knocking out genes. Through homologous recombination, sequences such as point mutations, reporters or conditional

alleles can be inserted into the target locus [137, 138]. In addition, catalytically dead Cas proteins linked with transcription factors can be guided by sgRNAs to promoters to control gene expression or remodel chromatin [139]. The ability to specifically target sequences in the genome provides many possibilities for genetic manipulation and control in the research setting.

CRISPR vs. RNAi

Debate has arisen surrounding RNA interference (RNAi) and CRISPR in an effort to determine which technology is better for decreasing the expression of proteins within cells. RNAi uses short single-stranded RNA derived from either siRNA or short-hairpin RNA (shRNA) to target mRNA molecules [140]. These small exogenous RNA molecules are introduced through transfection or transduction into mammalian cells and are processed by Dicer and Drosha to produce mature siRNA which are loaded onto the RNA-induced silencing complex (RISC) [141, 142]. RISC is then directed to target mRNAs to either repress translation or degrade the transcript [143]. Studies have examined the on-target specificity of RNAi compared to CRISPR as well as the ability of each to identify essential genes through long term depletion [144-146]. CRISPR proved to be better at identifying genes necessary for cell survival, though some genes were also identified by the shRNA screen alone, indicating that there may be situations in which shRNA may be more suitable than CRISPR [144]. However, RNAi targeting is less specific than CRISPR and thus more susceptible to confounding off-target effects through knockdown of other transcripts [146]. Additionally, studies have shown that CRISPR knockout sometimes do not confirm the results shown by transient knockdown [147]. This indicates there are likely differences in the appropriate applications of these two technologies in the lab.

Hypothesis and specific aims

MIEN1 mediates the migration and invasion of cancer cells in a variety of cancers. MIEN1 is a unique protein involved in cancer progression which possesses both a prenylation and an ITAM domain. Though *MIEN1* is found within the genomic region containing *HER2*, and is commonly amplified with *HER2*, MIEN1 expression has been shown to be independently regulated and is highly expressed in many TNBC cell lines. Post-translational modification of MIEN1 by isoprenylation results in the translocation of the protein to the inner leaflet of the plasma membrane and facilitation of filopodia formation which drives breast cancer cell migration via actin cytoskeletal rearrangement. In addition, ITAM phosphorylation of MIEN1 is required for downstream signaling which leads to gene expression of proteases and growth factors such as MMP-9, urokinase plasminogen activator and VEGF. These proteins are the end-products of pro-invasion and pro-migratory signaling through MIEN1 which ultimately result in increased invasion and migration of cancer cells. These data indicate that MIEN1 is an important oncoprotein which stands at the crossroads of both metastatic signaling cascades and cytoskeletal dynamics. However, the genetic context, mechanism and extent to which MIEN1 contributes to, and is required for, the various steps of metastasis, including cellular migration and invasion, remains unclear. The above data lead to the hypothesis that knockout of MIEN1 using CRISPR genome editing in breast cancer cells will result in reduced migration and invasion.

The following specific aims will be pursued to investigate the validity of the hypothesis:

Aim #1: Develop an effective CRISPR system to target and delete *MIEN1*.

Aim #2: Evaluate the effect of MIEN1 knockout on breast cancer cell migration and invasion.

CHAPTER II

CRISPR DELETION OF *MIEN1* IN BREAST CANCER CELLS

Abstract

Migration and Invasion Enhancer 1 (MIEN1) is an oncogene which is involved in facilitating motility of cancer cells through actin dynamics and gene expression. Increased MIEN1 expression in many types of tumors correlates with disease progression and metastatic propensity. It is unclear precisely how MIEN1 is involved in these processes and more studies are required to tease out the mechanisms. Here it is shown that CRISPR genome editing effectively produced specific genomic deletions in the *MIEN1* gene, which led to the complete abrogation of its expression in breast cancer cells. The single guide RNA (sgRNA) mediated targeting of *MIEN1* was specific and none of the clones screened for off-target cleavage revealed any insertions or deletions (indels). Knocking out *MIEN1* in these breast cancer cells will allow future studies to determine the role MIEN1 plays in breast tumor metastasis, which might lead to production of novel therapeutics to treat this and other cancers in which MIEN1 has been shown to influence cancer development.

Introduction

MIEN1 is a gene that is located on the long arm of the human chromosome 17, 0.5kb away from the *ERBB2* gene which encodes for the epidermal growth factor receptor Her2 (Fig 1A) [107]. Due to its proximity to the *ERBB2* gene, *MIEN1* is commonly amplified in breast cancer along with *ERBB2* which leads to an overexpression of MIEN1 protein in many breast cancers [107]. MIEN1 has been shown to functionally increase the invasive and migratory phenotype of various types of cells including breast cancer, prostate cancer, oral cancer, fibroblasts and endothelial cells [108, 111, 115, 116]. MIEN1 increases expression of proteins known to be involved in metastatic processes such as MMP-9 and VEGF through activation of Akt and Syk [111, 115, 116]. Actin cytoskeletal dynamics, an important component of cellular locomotion, are also regulated by MIEN1 acting through cofilin and FAK, particularly in the leading edge of the cell in the lamella [117]. These data indicate that MIEN1 is an important molecule which sits at the crossroads of cytoskeletal dynamics and signaling cascades which culminate in metastatic function and gene expression. It is important to understand the context of the role of MIEN1 in increasing cell motility and aggression in order to estimate its use as a prognostic biomarker and therapeutic target in the future.

CRISPR based genome editing has been at the forefront of molecular biology in the last several years. This technique promises quick, efficient genome modification. The modified cells that result from CRISPR genome editing can then be used to study the effect long-term loss of a specific gene or temporally regulated expression has on signaling events and cellular function. Studying the role of MIEN1 in cancer progression can be accomplished by a variety of methods; however, genomic deletion using the CRISPR-Cas9 system offers the ability to

examine the functional significance of MIEN1 knockout (MIEN1-KO) in a background in which it is normally endogenously expressed.

Figure 1

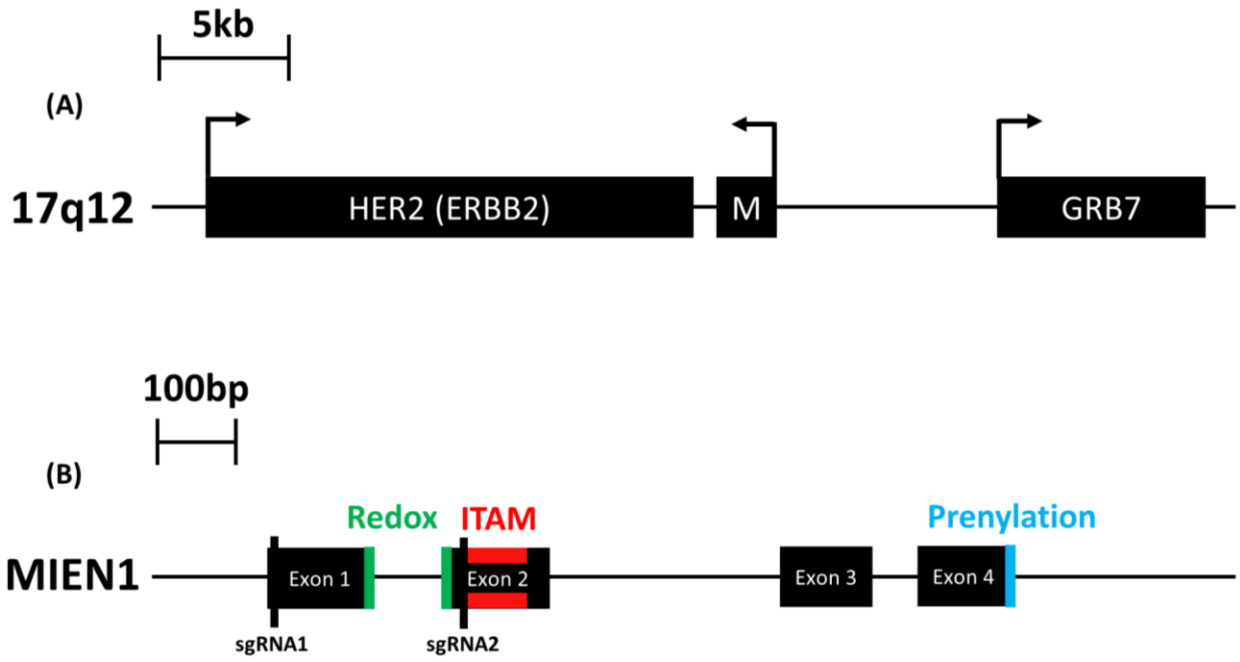


***Figure 1. MIEN1* gene location and structure.** (A) Chromosome 17q12 genomic locus/*HER2* amplicon. Genomic location and orientation of *MIEN1* gene (M) in relation to *HER2* and *GRB7* within the *HER2* amplicon. Scale bar indicates length of five kilo bases or five thousand base pairs. (B) Structure of *MIEN1* gene and sgRNA loci. Exons of *MIEN1* denoted by black boxes with locations of the redox, immunoreceptor tyrosine activation motif (ITAM) and prenylation domains. Location of sgRNA target sequences within the *MIEN1* gene are denoted by vertical black lines. Scale bar indicates length of 100 base pairs. The full-length *MIEN1* transcript is approximately 730bp long.

Materials and methods

Cell lines and culture conditions. The human epithelial breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA, USA). The MDA-MB-231 derived organotropic metastatic variants 831 (brain) [67], 1833 (bone) [66], and 4175 (lung) [65] were provided as gift from Dr. Joan Massagué, Memorial Sloan Kettering Cancer Center (New York City, NY, USA). Before shipment, the cell lines were authenticated by STR analysis with the PowerPlex® Fusion V1.0 (Promega). All three cell lines tested negative for mycoplasma infection when tested with MycoAlert PLUS (Lonza). The cell lines were confirmed to be mycoplasma free prior to use. All cell lines were cultured in DMEM high-glucose (HyClone) supplemented with 10% FBS, 4.05mM glutamine, 100IU penicillin, 100IU streptomycin and 0.25ug/ml Amphotericin B. Cultures were maintained in a humidified incubator at 37˚C with 5% CO_2.

sgRNAs, plasmids and cloning. Sequences for sgRNA oligos are shown in Table 1. SgRNA oligos were cloned into vectors according to the provided protocols from Addgene (Cambridge, Massachusetts, USA). sgRNA-1 and sgRNA-3 were cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid, which contains both Cas9 endonuclease/EGFP and sgRNA expression cassettes, and was a gift from Feng Zhang (Addgene plasmid # 48138) [148]. SgRNA-2 and sgRNA-4 were cloned into the MLM3636 plasmid, which contains an sgRNA expression cassette, was a gift from Keith Joung (Addgene plasmid # 43860).

Transfection and CRISPR genome editing. Cells were co-transfected with PX458 and MLM3636 plasmids using jetPRIME (VWR) as recommended by the manufacturer. 24-hours post-transfection, GFP positive cells were isolated using fluorescence activated cell sorting (FACS) on an SH800 Cell Sorter (Sony Biotechnology Inc.). Single cell clones were grown

Table 1

sgRNA oligos	**Oligo1** (5'…….3')	**Oligo2** (5'…….3')
sgRNA1	CACCGGGAGCCGGCCGCGATGAGCG	AAACCGCTCATCGCGGCCGGCTCCC
sgRNA2	ACACCTAGGTCGCCTCGAAGCCGCAG	AAAACTGCGGCTTCGAGGCGACCTAG
sgRNA3	CACCGATCGAGATCGAGTCGCGCCT	CTAGCTCTAGCTCAGCGCGGACAAA
sgRNA4	ACACCGCATCAGACAGGTATTACCGG	GCGTAGTCTGTCCATAATGGCCAAAA

Table 1. **SgRNA oligo sequences.** List of sgRNA oligos which were cloned into appropriate vectors as outlined in Materials and Methods. Oligo1 and Oligo2 are annealed

and screened via polymerase chain reaction (PCR) using AmpliTaq Gold 360 PCR Master Mix (Invitrogen) and immunoblotting for MIEN1-KO. Primers used for PCR are shown in Table 2.

Sequencing. Regions surrounding sgRNA target/off-target sites within the *MIEN1*, *LRP8* and *SQSTM1* gene segments were amplified by PCR. PCR reactions were cleaned using a GeneJET PCR Purification Kit (Thermo Scientific). Amplicons were then analyzed by Sanger sequencing (ACGT, Inc.). Primer sequences are shown in Table 2.

Western blot and antibodies. Proteins were harvested by lysing cells with RIPA lysis buffer (150mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 50mM Tris) supplemented with Protease Inhibitor Cocktail Set I (Millipore). Cell lysates were sonicated and centrifuged for 15 minutes at 4800 rpm at 4˚C. Protein concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). Cell extracts were separated using a 4–12% Bis-Tris NuPAGE gel (Life Technologies Corporation) before being transferred to a nitrocellulose membrane (iBlot, Invitogen, USA). Membranes were blocked with 5% Bovine Serum Albumin (Sigma) in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature for 1 hour. Membranes were then incubated with agitation either 2.5 hours at room temperature or overnight at 4˚C with primary antibody in 2.5% BSA in TBST. Following four 5 minute washes, membranes were then incubated with the appropriate HRP conjugated secondary antibody (SouthernBiotech) in 5% milk in TBST at room temperature for 1 hour before being washed four times for 5 minutes each. Finally membranes were developed using Immobilon Western Chemiluminescent HRP substrate (Millipore). Images were captured using and Alpha-imager Fluoretech HD2 (ProteinSimple). Images were transformed using Fiji biological image analysis software [149]. Antibodies: 1) mouse anti-MIEN1 MO2 monoclonal raised against full-length recombinant MIEN1 (Abnova. 1:1000 dilution), 2) mouse Guide-it™

Cas9 polyclonal raised against full-length Cas9 from *Streptococcus pyogenes* (Clonetech, 1:1000 dilution), 3) mouse anti-β-actin C4 monoclonal raised against chicken actin (Santa Cruz Biotechnology. 1:2000 dilution).

Table 2

PCR/Sequencing(*) Primers	**Forward Primer** (5'…….3')	**Reverse Primer** (5'…….3')
MIEN1 Deletion (sgR1/2)	GTCAAAACGTAGCAGTGGCG (*)	GGAACTCATGTTGGCCGGA
MIEN1 Deletion (sgR3/4)	GTCAAAACGTAGCAGTGGCG	TAGCGCCACTTTACTGCCAA
LRP8	TTCGCTATGCCTAACTGCAC	GACACAGATACCAGTGGAGG (*)
SQSTM1	GTTCGCTACAAAAGCCGCG (*)	GGGCATTATCTGAACCCACCA

Table 2. **PCR and sequencing primer oligo sequences.** Sequences of primers used for PCR amplification across CRISPR induced genomic deletions. Primers used for sequencing are denoted with an asterisk.

Results

Selection of sgRNA sequences

In order to efficiently target the *MIEN1* gene for knockout, CRISPR guide sequences were selected using CRISPR Design Tool [150] and Benchling biology software [151] based on the following criteria: the single-guide RNA (sgRNA) sequence should A) have an on-target score ≥ 50 [133], B) have a specificity score ≥ 50 (low off-target effects) [150] C) be found within an exon of *MIEN1*, D) be found within an exon specific to *MIEN1*, E) be found within an exon integral to the function of MIEN1, F) be located close to the N-terminus. Four high-scoring candidate sgRNAs which met a variety of these criteria were selected for *MIEN1* deletion analysis (Table 3).

Table 3

sgRNA	On-target [133, 151]	Off-target [150, 151]	Quality Score [150]
sgRNA1	61.4	83.8	85
sgRNA2	68.4	95.3	95
sgRNA3	54	97.1	98
sgRNA4	78.2	47.8	92

Table 3. SgRNA scores. On-target, off-target and quality scores for all four sgRNAs used to delete portions of the *MIEN1* gene. All scores range from 1-100. Higher scores in all measures indicate better predicted performance of sgRNAs.

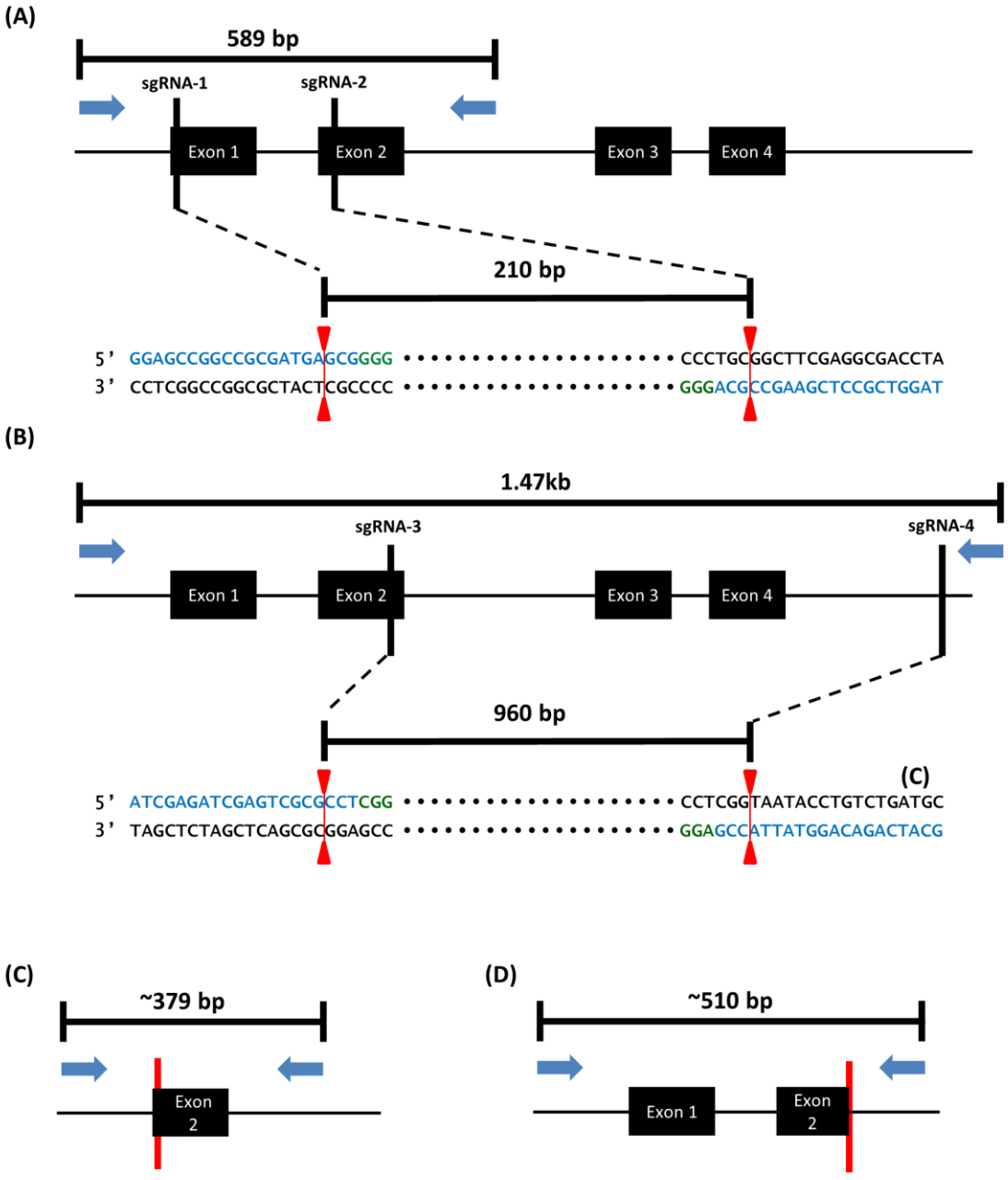
Evaluation of sgRNA deletion efficiency by PCR screening

To screen FACS sorted single cell clones, a deletion strategy using two sgRNA sequences was used to enable rapid, high-throughput analysis of genomic alterations [152]. Two pairs of sgRNAs were tested for knockout efficiency in breast cancer cells. Pair-1 consisted of sgRNA-1 and sgRNA-2 and pair-2 consisted of sgRNA-3 and sgRNA-4 (Fig 1B). Primers were designed flanking each pair of sgRNA which yielded a 589bp amplicon for pair-1 and a 1.47kb amplicon for pair-2 (Fig 2A, B). Following deletion and NHEJ, the deletion PCR amplicon is reduced to approximately 379bp for pair-1 and 510bp for pair-2 (Fig 2C, D). In order to determine which pair of sgRNAs would be most efficient, MDA-MB-231 (231) and derivative cells were co-transfected with PX458 and MLM3636 plasmids and single cells were isolated by FACS and clones were expanded before being subjected to deletion PCR screening. SgRNA pair-1 showed deletion bands in 47% (71/151) of clones screened, while sgRNA pair-2 only showed a deletion band in 24% (54/222).

Figure 2

***MIEN1* Deletion PCR**. (A) *MIEN1* deletion schematic for sgRNA pair-1. SgRNA sequences and predicted cleavage locations resulting in a 210bp deletion. (B) *MIEN1* deletion schematic for sgRNA pair-2. SgRNA sequences and predicted cleavage locations resulting in a 960bp deletion. (C) SgRNA pair-1 genomic deletion of *MIEN1* exon 1. PCR product size following deletion of sequence between sgRNA-1 and sgRNA-2. (D) SgRNA pair-2 genomic deletion of *MIEN1* exons 3 and 4. PCR product result following deletion of sequence between sgRNA-3 and sgRNA-4. Blue arrows represent location of primers. Blue sequences are 20-nucleotide sgRNA sequences. Green sequences are PAM sequences. Red line indicates approximate location of NHEJ to form deletion.

Figure 2


(A)
589 bp
sgRNA-1
sgRNA-2
Exon 1
Exon 2
Exon 3
Exon 4
210 bp
5' GGAGCCGGCCGCGATGAGCGGGG CCCTGCGGCTTCGAGGCGACCTA
3' CCTCGGCCGGCGCTACTCGCCCC GGGACGCCGAAGCTCCGCTGGAT
(B)
1.47kb
sgRNA-3
sgRNA-4
Exon 1
Exon 2
Exon 3
Exon 4
960 bp
(C)
5' ATCGAGATCGAGTCGCGCCTCGG CCTCGGTAATACCTGTCTGATGC
3' TAGCTCTAGCTCAGCGCGGAGCC GGAGCCATTATGGACAGACTACG
(C)
~379 bp
Exon 2
(D)
~510 bp
Exon 1
Exon 2

Indel PCR screening

In addition to simultaneous cleavage, deletion, and NHEJ, there are several other possible Cas9 cleavage mechanisms (Fig 3). First, simultaneous cleavage occurs, but the intervening segment in not deleted and is instead re-inserted during NHEJ, which introduces indels at both target sites. Second, cleavage occurs at one of the target sites and NHEJ is performed before cleavage occurs at the other target site. Third, simultaneous cleavage occurs, however the intervening segment is inverted and then re-inserted during NHEJ. Fourth, cleavage and NHEJ occurs at only one of the two sites. All of these scenarios should also result in gene product knockout, but none of them are able to be detected using the deletion PCR alone as they will all most likely yield an amplicon close to the wild-type length. In order to be able to identify clones which were edited by these alternate mechanisms without deletion, an indel PCR was designed using one of the sgRNA oligos which was cloned into the PX458 vector (sgRNA1 Oligo1 Table 1). Since this oligo contains the sgRNA sequence, it straddles the Cas9 cleavage site and its annealing should be sensitive to indels (Fig 3). The absence of a band in the indel reaction indicates CRISPR cleavage and NHEJ occurred which produced indels in both alleles. Single cell clones generated using sgRNA pair-1were screened by indel PCR reactions to identify additional potential MIEN1-KO clones (Fig 4). Only one additional clone was found using this method which only produced the wild-type band in the deletion reaction while producing no band in the indel reaction. All sgRNA pair-1 clones which exhibited genomic lesions either by presence of the smaller band in the deletion reaction or absence of a band in the indel reaction were selected as candidates for western blot screening (Fig 4).

Figure 3

Indel PCR. (A) Schematic of indel PCR reaction. (B) Diagrams of additional possible CRISPR cleavage scenarios. Green arrow indicates sgRNA1 Oligo1 which acts as the forward primer. Blue arrow is the same reverse primer from the deletion reaction. Red vertical lines indicate possible indels resulting from cleavage and NHEJ. Red 'X' indicates an inability for the forward primer to anneal.

Figure 3

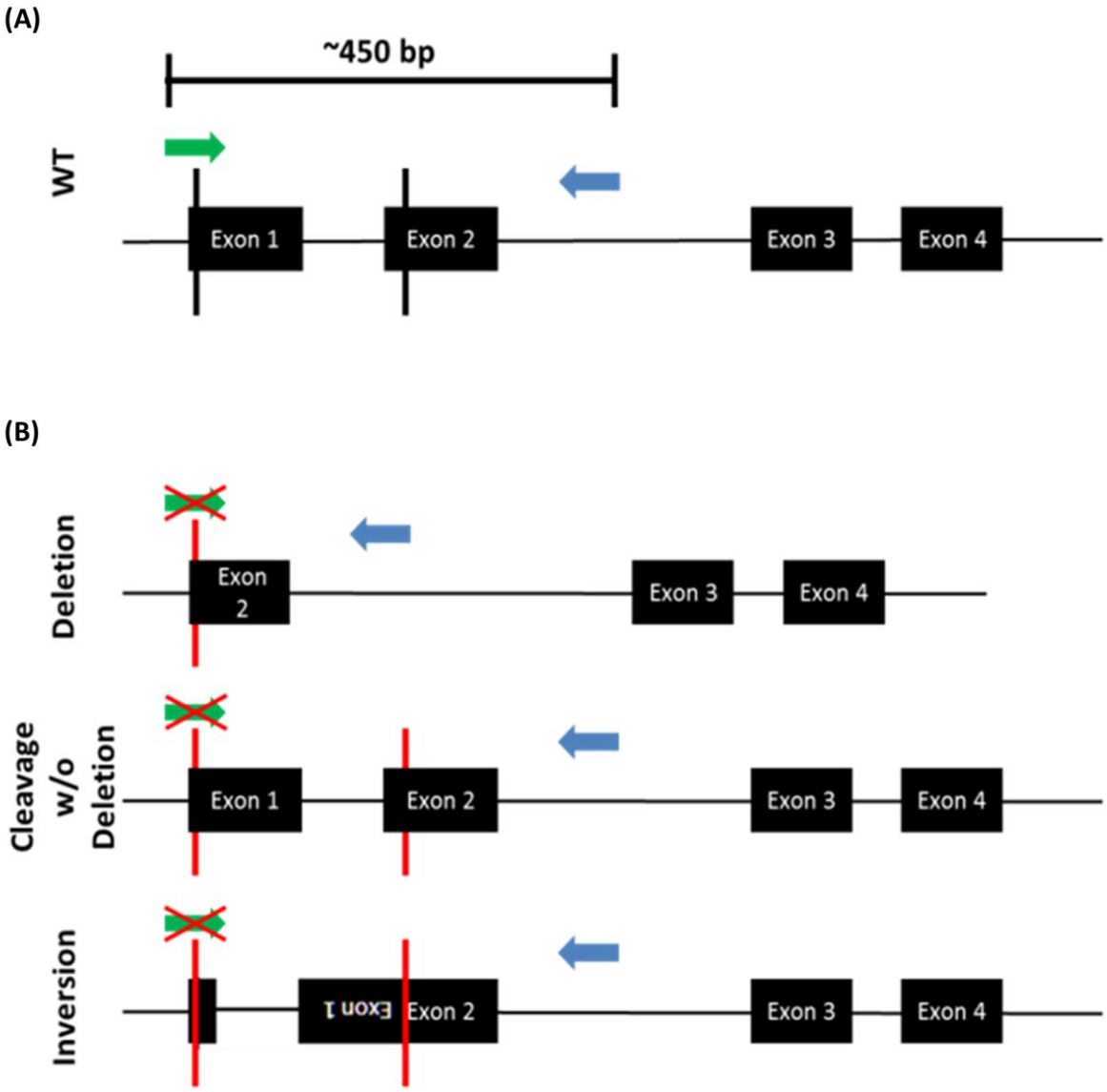

(A)
~450 bp
WT
Exon 1
Exon 2
Exon 3
Exon 4
(B)
Deletion
Exon 2
Exon 3
Exon 4
Cleavage w/o Deletion
Exon 1
Exon 2
Exon 3
Exon 4
Inversion
Exon 1
Exon 2
Exon 3
Exon 4

Figure 4

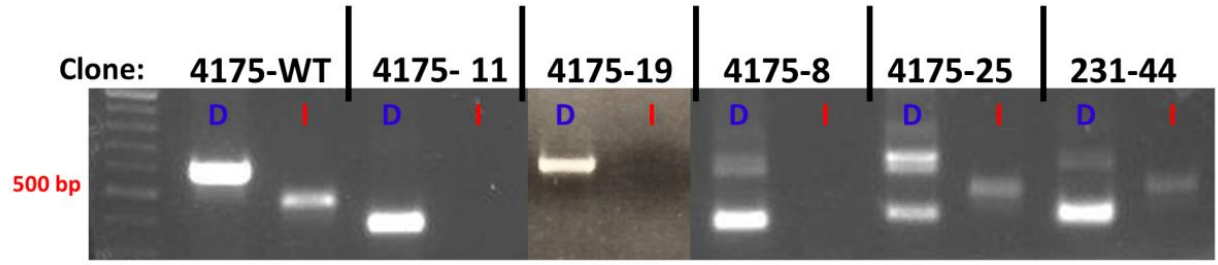


Figure 4. **PCR screening of MIEN1-KO clones**. (A) Agarose gel of deletion and indel PCR screening. 4175-WT is parental wild-type cells used as a control. Other lanes are representative clones exhibiting all possible band combinations. These cells exhibit expected deletion band of ~589bp and indel band of ~450bp, which indicates no deletions or indels are present. Clone 4175-11 shows only the shorter ~379bp band in the deletion reaction and no indel band, which means this clone is a homozygous deletion clone. Clone 4175-19 shows the longer deletion band, but no indel band, which suggests that this clone may be a knockout. The 210 bp segment between sgRNA-1 and sgRNA-2 was not deleted, but cleavage did occur and resulted in indels at the sgRNA-1 target site which did not allow PCR in the indel reaction. Clone 4175-8 shows both a long band and a more prominent short band and no indel band. The lack of a band in the indel PCR suggests knockout, even though both alleles were not deleted. Clone 4175-25 and 231-44 both have long and short bands in the deletion reaction and both show weak bands in the indel reaction. Presence of indel bands may indicate one intact allele or single nucleotide indel which still results in primer binding during PCR WT: wild-type. D: Deletion. I: Indel.

On-target and off-target sequencing

Several clones which were chosen for western blotting were sequenced to examine the genomic lesion at the *MIEN1* locus following deletion as well as potential off-target indels. *MIEN1* deletion bands were PCR amplified and sequenced (Fig 5A). Several of the deletion bands showed perfect deletion and repair without any indels. There were also several single nucleotide deletions of the adenine immediately upstream of the sgRNA1 cut site. This validated the efficient NHEJ that allowed for the determination of the *MIEN1* gene deletion by PCR. The sgRNA sequences which were used to target the *MIEN1* gene both had high overall off-target scores, indicating low probability of off-target effects. To examine potential off-target CRISPR-mediated cleavage and indel formation, the highest scoring off-target locus within a gene for each sgRNA was sequenced (Fig 5B-D). All 19 *MIEN1* deletion clones which were sequenced at these potential off-target loci showed no indels at either the *LRP8* or *SQSTM1* locus, indicating no off-target effects (Fig 5D).

Figure 5

On-target and off-target sequencing. (A) *MIEN1* deletion sequences. Representative sequences of several clones which showed the smaller band in the deletion PCR reaction resulting from deletion and NHEJ. Underlined sequences correspond to remains of sgRNA target sequences. Red sequences correspond to PAM sequences. (B and C) SgRNA off-target. SgRNA sequences with on-target activity score (0-100, higher is better). Second line is sequence of highest scoring off-target location within a gene. Red letters denote mismatches from the *MIEN1* targeting sgRNA sequence. (D) Off-target sequencing. Representative sequences of several clones which show no indels at the off-target loci.

Figure 5

(A)

MIEN1 Exon1/2

4175-WT	agcaggggcccgagcGGAGCCGGCCGCGATGAGCGGGGagccgggg…//….ccagtgaaCCCTGCGGCTTCGAGGCGACCTAcctggagctggccag
231-82 Allele 1	agcaggggcccgagcGGAGCCGGCCGCGATG……GGCTTCGAGGCGACCTAcctggagctggccag
231-82 Allele 2	agcaggggcccgagcGGAGCCGGCCGCGATGA……GGCTTCGAGGCGACCTAcctggagctggccag
4175-1	agcaggggcccgagcGGAGCCGGCCGCGATG……GGCTTCGAGGCGACCTAcctggagctggccag
4175-4	agcaggggcccgagcGGAGCCGGCCGCGATG……GGCTTCGAGGCGACCTAcctggagctggccag
4175-11	agcaggggcccgagcGGAGCCGGCCGCGATG……GGCTTCGAGGCGACCTAcctggagctggccag
4175-29 Allele 1	agcaggggcccgagcGGAGCCGGCCGCGATGA……GGCTTCGAGGCGACCTAcctggagctggccag
4175-29 Allele 2	agcaggggcccgagcGGAGCCGGCCGCGATG……GGCTTCGAGGCGACCTAcctggagctggccag
4175-33 Allele 1	agcaggggcccgagcGGAGCCGGCCGCGATGA……GGCTTCGAGGCGACCTAcctggagctggccag
4175-33 Allele 2	agcaggggcccgagcGGAGCCGGCCGCGATG……GG…TTCGAGGCGACCTAcctggagctggccag

(B)

Genomic location	Sequence	% OTA
MIEN1 Exon1 (sgRNA1)	**GGAGCCGGCCGCGATGAGCGGGG**	61.4
LRP8 Exon5	**GGTGACGGCAGCGATGAGCGCGG**	2.5

(C)

Genomic location	Sequence	% OTA
MIEN1 Exon2 (sgRNA2)	**TAGGTCGCCTCGAAGCCGCAGGG**	68.4
SQSTM1 Exon1	**TAGTGCGCCTGGAAGCCGCCAGG**	0.5

(D)

LRP8 Exon5

4175-WT	ctggccgccgtgttcgtgtgcgacggcgacgacgactgtGGTGACGGCAGCGATGAGCGCGGctgtgcagacccggcctgcgggccccgcgagttccgctgc
4175-1	ctggccgccgtgttcgtgtgcgacggcgacgacgactgtGGTGACGGCAGCGATGAGCGCGGctgtgcagacccggcctgcgggccccgcgagttccgctgc
4175-16	ctggccgccgtgttcgtgtgcgacggcgacgacgactgtGGTGACGGCAGCGATGAGCGCGGctgtgcagacccggcctgcgggccccgcgagttccgctgc
4175-29	ctggccgccgtgttcgtgtgcgacggcgacgacgactgtGGTGACGGCAGCGATGAGCGCGGctgtgcagacccggcctgcgggccccgcgagttccgctgc

SQSTM1 Exon1

4175-WT	gtcaccgcccccgccgctccccggcccgctcaccgcggTAGTGCGCCTGGAAGCCGCCAGGccgcagcgcggggaacagggcggccacccggctcagcagc
4175-1	gtcaccgcccccgccgctccccggcccgctcaccgcggTAGTGCGCCTGGAAGCCGCCAGGccgcagcgcggggaacagggcggccacccggctcagcagc
4175-16	gtcaccgcccccgccgctccccggcccgctcaccgcggTAGTGCGCCTGGAAGCCGCCAGGccgcagcgcggggaacagggcggccacccggctcagcagc
4175-29	gtcaccgcccccgccgctccccggcccgctcaccgcggTAGTGCGCCTGGAAGCCGCCAGGccgcagcgcggggaacagggcggccacccggctcagcagc

MIEN1 knockout screening

Clones selected based on deletion and indel PCR reactions were screened by western blot for MIEN1 protein knockout (Fig 6A). 85% (50/59) of the clones screened were negative for MIEN1 protein. Some clones which amplified both small and large bands during PCR, clone 231-44 for instance (Fig 4A), still showed MIEN1 expression following western blot, indicating that some clones retained one unmodified allele following CRISPR editing. Additionally, it is important to ensure that knockout clones are Cas9-negative to reduce the potential for off-target cleavage, which would be the case if the nuclease was stably expressed through genomic insertion. Only 16% (8/50) of those clones which were MIEN1 negative expressed Cas9 (Fig 6B). These clones were not used in subsequent experiments. The remaining 42 clones which were both MIEN1-negative and Cas9-negative will be used in future experiments.

Figure 6

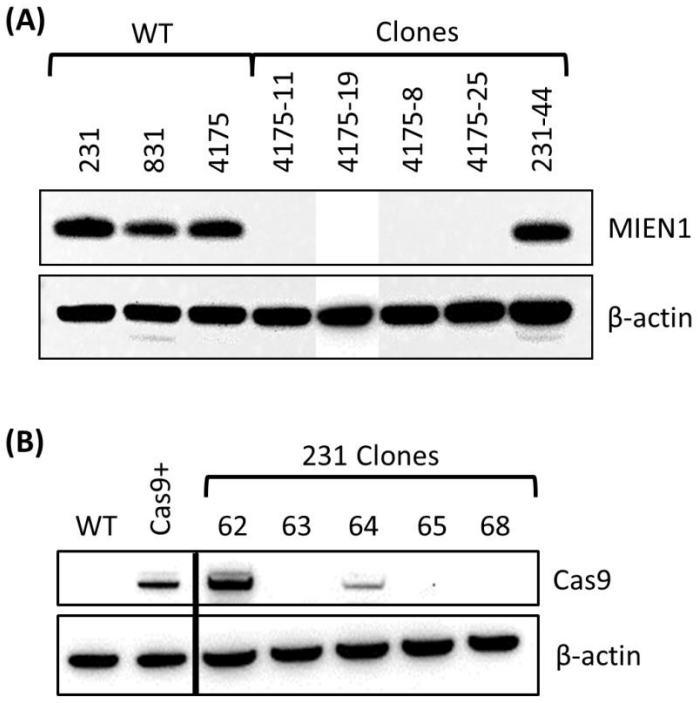


Figure 6. **MIEN1 knockout western blot**. (A) MIEN1 western blot. Parental wild-type (WT) cell lines with clones which match the PCR gel from Fig 3A. (B) Cas9 western blot. Representative western blot from Cas9 screening of MIEN1-KO clones. Cas9+ sample was pool of cells transfected and selected with Cas9 expressing plasmid.

Discussion

CRISPR genome engineering has become a staple of molecular biology research over the last several years. There is still debate about the benefits and applications of CRISPR vs other gene expression regulation systems, such as RNAi. However, CRISPR has been shown to yield more reproducible data and minimize global off-target effects compared to RNAi [146, 147]. While there are concerns about the specificity and potential off-target effects of CRISPR-Cas systems [153, 154], much work has been done to improve the specificity of sgRNAs and reduce the off-target cleavage of Cas proteins through genetic mutation [133, 134, 155]. Here, the CRISPR-Cas9 system was used to eliminate MIEN1 protein from breast cancer cells which normally have high MIEN1 expression. Two sgRNAs were used to delete a segment from the *MIEN1* gene. Co-transfection of the two sgRNAs allows for simultaneous Cas9 endonuclease-mediated cleavage at both target sites. During the NHEJ repair process following simultaneous cleavage, the intervening DNA sequence is lost. Therefore, the deletion of DNA can be easily resolved on an agarose gel following PCR amplification across the deleted segment. In the event that deletion does not occur and an alternate sequence of cleavage and NHEJ occurs, an indel PCR reaction may be used to identify those clones which may contain indels at sgRNA target sites rather than deletion of the desired segment. Also, It has been shown that NHEJ after deletion using two sgRNAs often results in precise repair without large indels [156]. Precise NHEJ was also observed in this study upon sequencing deletion clones (Fig 5A).

This study has shown that the co-expression of sgRNA-1 and sgRNA-2 with the Cas9 endonuclease resulted in a high on-target editing efficiency (47%) of the *MIEN1* gene in four breast cancer cell lines with no detected off-target effects. The editing efficiency was nearly twice as high using this pair of sgRNAs as compared to the combination of sgRNA-3 and

sgRNA-4. The majority of these edited cells were identified by the presence of the smaller sized DNA band in the deletion PCR, indicating a high likelihood of MIEN1 protein knockout. Almost all of the clones selected through PCR screening revealed no MIEN1 protein expression when screened by western blot indicating that the strategy of co-expression of two high-scoring sgRNAs is an efficient system for producing MIEN1-KO cell lines for future experimentation.

Due to the high on-target efficiency of sgRNA-1 and sgRNA-2, only one additional clone was identified using the indel PCR reaction which would not have been identified by the deletion reaction alone. This approach may be more helpful when used for other targets in the genome where multiple high-scoring sgRNA target sites may not be present. The indel reaction may also be used to identify cells edited using a single sgRNA and may be more helpful than the traditional Surveyor or T7 endonuclease screening method [157]. These endonucleases cleave double stranded DNA when mismatches are present following PCR amplification and re-annealing. However, if a single sgRNA is used and the same indel is produced at both genomic target loci, the T7 endonuclease-based strategy will not be able to identify these types of clones, unlike the indel PCR. Using the indel PCR in combination with the deletion reaction is a high-throughput strategy for identification of CRISPR edited clones.

It has been previously shown that MIEN1 is an oncogene involved in the migration, invasion and progression of breast, prostate and oral cancers and is associated with decreased overall survival [108, 115-117, 123]. MIEN1 mediates many pro-metastatic signals including activation of Akt kinase which facilitates downstream gene expression through NF-kB [115] and modulation of actin cytoskeleton to facilitate cell motility [117]. Because of the importance of MIEN1 in cancer progression and metastatic events, use of CRISPR mediated MIEN1-KO cells will provide a better understanding of the role of MIEN1 protein in these various processes.

Knocking out MIEN1 in these cancer cells affords the opportunity to study the effect of its absence from a background in which it is normally expressed rather than using an overexpression approach in cell lines which do not have endogenous expression as this may lead to undesirable pleiotropic effects unrelated to normal MIEN1 function within cancer cells. This platform will also allow for further study of the contributions of MIEN1 protein to the metastatic processes and validation of its potential as a therapeutic target and prognostic biomarker.

Acknowledgements

I would like to thank Priyanka Desai for her help with cell culture and sample preparation during screening of clones.

CHAPTER III

EFFECT OF MIEN1 KNOCKOUT IN METASTATIC BREAST CANCER CELLS

Abstract

Genetic context is a vital factor which dictates how cancer cells behave. Certain cancers have a genetic and proteomic predisposition to metastasize to specific organs in the body. Breast cancer predominantly metastasizes to the brain, bone, lungs and liver and this organotropism is marked by a specific gene expression signature which facilitates this metastatic targeting. MIEN1 has been implicated in several steps of the metastatic process, including invasion and extravasation, however, it is unknown how this protein behaves in different organotropic contexts. In an effort to better understand the dependence of specific genetic contexts of organotropic metastases on MIEN1 protein function, CRISPR genome editing was utilized to delete the *MIEN1* gene in the aggressive, triple-negative breast cancer cell line MDA-MB-231 (231) as well as several of its metastatic derivative cell lines. Migration in 231 MIEN1-KO cells exhibited disparate results when compared to previous siRNA studies and signaling in MIEN1-KO pools was inconsistent. Knocking out MIEN1 in metastatic derivative cell lines showed few significant alterations with regard to growth, migration, invasion and signaling of these breast cancer metastasis cell lines. Substantial metabolic changes were observed in lung metastatic breast cancer cells. Expression of an MIEN1 protein containing a mutant ITAM domain in lung metastatic cells, though it failed to significantly influence signaling, revealed that they were susceptible to its hindrance of compensatory mechanisms within unknown MIEN1 signaling

pathways. This demonstrates the importance of the migration and invasion pathways in which MIEN1 participates in breast cancer metastasis as well as the functional significance of these pathways within the broader context of breast cancer.

Introduction

MIEN1 is an oncogene that has been shown to have transformative properties in mammary epithelial cells and increase migration, invasion and metastasis in various cancer cells [108, 111, 116, 117]. *MIEN1* is found in a tail-to-tail arrangement with the *ERBB2* gene, which codes for Her2 [107]. Her2 amplification and/or overexpression has been observed in many different cancers including lung [158], gastric [159], ovarian [160], and breast cancers [5]. In breast cancer, Her2 overexpression or amplification was seen in as high as 34% of tumors [161, 162]. The amplification of Her2 leads in many cases to both amplification and increased expression of MIEN1 protein; however, MIEN1 retains independent transcriptional and translational mechanisms which enable its expression irrespective of Her2 expression patterns. The MIEN1 promoter, which is hypermethylated in normal cells, can become hypomethylated in cancer cells, leading to binding of upstream stimulatory factor (USF) transcription factors and increased transcription [122]. MIEN1 mRNA is also negatively regulated post-transcriptionally by microRNAs, which are decreased in prostate and gastric cancer cells [126, 163]. MIEN1 expression is high in many TNBC cell lines, which do not express the Her2 receptor [113]. This indicates that MIEN1 function is not dependent upon the expression of its genomic neighbors. TNBC is the most aggressive subtype of breast cancer and the contribution of MIEN1 to the progression of this disease is not yet clear. Studying the function of MIEN1 in Her2-negative cell lines will allow investigation of the distinct mechanisms by which MIEN1 mediates migration and invasion.

CRISPR genome editing is a technique that has been adapted from bacterial and archaea adaptive immune systems [127]. It is comprised of an RNA-guided Cas9 endonuclease which mediates double strand cleavage at the specified 20 base pair genomic locus which leads to either

NHEJ or homologous recombination [127]. This system can be employed to selectively knockout genes in cells to potentially allow for investigation of cellular functions in the absence of a protein of interest without worrying about transfection or knockdown efficiency using siRNA. The effects of permanent gene loss can be studied using CRISPR to determine the long-term dependency on a specific protein.

Certain cancers have a proclivity to metastasize to specific organs in the body. The proclivity of breast cancer cells to metastasize to lung [65], bone [66], and brain [67] has been shown to be due to distinct sets of genes, indicating that organ-specific metastasis is guided by differential gene expression. Overexpression of these genes in parental cell lines resulted in increased cancer cell homing to the predicted organs in mice. The individual effect of genes on increasing metastasis was varied, indicating an interdependency of some genes to elicit a phenotype. It is unknown if MIEN1 requires the expression of other genes in order to increase the migration and invasion of cancer cells or if MIEN1's actions are largely autonomous. MIEN1 is highly expressed in 231 cells as well as the organotropic metastatic variants: 831 (brain), 1833 (bone), and 4175 (lung). Knocking-out MIEN1 in these cells will enable the investigation of the dependency of these organotropic metastatic cell lines on the expression of MIEN1 for migration and invasion.

In this study, the functional significance of MIEN1 knockout in TNBC cells was examined. The long-term effect of MIEN1 deletion in these cell lines produced few alterations in the phenotypic pattern with regard to migration, invasion and signaling. However, MIEN1-KO did produce metabolic changes in lung metastatic cells, indicating long term loss of MIEN1 did result in significant alterations within these cells. The functional importance of MIEN1 within these cells was demonstrated by expression of ITAM mutant MIEN1 within the lung

metastatic MIEN1-KO cells, which caused significantly reduced invasion through ECM. This points to MIEN1 being involved in alternative invasive signaling pathways, which have not yet been identified.

Materials and methods

Cell lines and culture conditions. The human epithelial breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA, USA). The MDA-MB-231 derived organotropic metastatic variants 831 (brain) [67], 1833 (bone) [66], and 4175 (lung) [65] were provided as a gift from Dr. Joan Massagué, Memorial Sloan Kettering Cancer Center (New York City, NY, USA). Before shipment, the cell lines were authenticated by STR analysis with the PowerPlex® Fusion V1.0 system (Promega). All three cell lines were negative for mycoplasma infection when tested with MycoAlert PLUS (Lonza). All cell lines were cultured in DMEM high-glucose (HyClone) supplemented with 10% FBS, 4.05mM glutamine, 100IU penicillin, 100IU streptomycin and 0.25ug/ml Amphotericin B. Cultures were maintained in a humidified incubator at 37˚C with 5% CO_2.

MIEN1-KO cell lines were generated by pooling MIEN1-KO clones generated as outlined in Chapter II. A list of cell lines complete with the number of clones in each pooled cell line is described in Table 1.

An MDA-MB-231 derivative cell line was produced by pooling clones generated from cells transfected with empty vector PX458 plasmid without any sgRNA (Table 1) and sorted as outlined in Chapter II.

For generation of cells stably overexpressing EGFP (pEGFP-C1 vector), MDA-MB-231-4175 cells were transfected using jetPRIME (VWR) as recommended by the manufacturer with pEGFP-C1 plasmids: EGFP empty vector (EV), EGFP-MIEN1^{WT} or EGFP-MIEN1$^{Y/39/50F}$ for 24 hours. Cultures were subjected to selection with complete medium supplemented with 700 μg/ml G418 (Invitrogen) for 2 weeks. Cells stably expressing GFP were FACS sorted on an SH800 Cell Sorter (Sony Biotechnology Inc.). The resulting pool of

Table 1

Cell line	Label	Parent	Description	# of clones
MDA-MB-231	231		Parental cell line	-
MDA-MB-231-CEV	CEV	231	Pooled clones transfected with EV-PX458	22
MDA-MB-231-MKO-B	MKO-B	231	1st set. Pooled MIEN1-KO clones	12
MDA-MB-231-MKO-C	MKO-C	231	Set 1 + 2. Pooled MIEN1-KO clones	18
MDA-MB-231-MKO-D	MKO-D	231	Set 2. Pooled MIEN1-KO clones	6
MDA-MB-231-831	831		Brain metastatic derivative of 231	-
MDA-MB-231-831-MKO	831-MKO	831	Pooled MIEN1-KO clones	9
MDA-MB-231-1833	1833		Bone metastatic derivative of 231	-
MDA-MB-231-1833-MKO	1833-MKO	831	Pooled MIEN1-KO clones	4
MDA-MB-231-4175	4175		Lung metastatic derivative of 231	-
MDA-MB-231-4175-MKO	4175-MKO	4175	Pooled MIEN1-KO clones	19
- - - EGFP Overexpression cell lines - - -				
MDA-MB-231-4175-EGFP-EV	4175-EV	4175	4175 cells stably expressing EGFP	
MDA-MB-231-4175-MKO-EGFP-EV	4175-MKO-EV	4175-MKO	4175-MKO cells stably expressing EGFP	
MDA-MB-231-4175-MKO-EGFP-MIEN1-WT	4175-MKO-WT	4175-MKO	4175-MKO cells stably expressing wild-type MIEN1 N-terminally tagged with EGFP	
MDA-MB-231-4175-MKO-EGFP-MIEN1-Y39/50F	4175-MKO-Y39/50F	4175-MKO	4175-MKO cells stably expressing ITAM mutant MIEN1 N-terminally tagged with EGFP	

***Table 1*. List of cell lines.** All cell lines used in this study are outlined below. Full name of the cell line is displayed along with the label that will be used for the cell line throughout this study. The number of clones used to generate MIEN1-KO pooled cell lines is noted.

GFP positive cells were cultured in complete medium supplemented with 400 μg/ml G418 (Invitrogen) for selective pressure to maintain gene expression.

Western blot and antibodies. Proteins were harvested by lysing cells with RIPA lysis buffer (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50mM Tris) supplemented with Protease Inhibitor Cocktail Set I (Millipore), Phosphatase Inhibitor Cocktail Set II (Millipore), and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Cell lysates were sonicated and centrifuged for 10 minutes at 10,000 rpm at 4˚C. Protein concentrations were estimated using a Pierce BCA Protein Assay Kit (Thermo Scientific). Seven micrograms of protein was separated using a 4–12% Bis-Tris NuPAGE gel (Life Technologies Corporation) before being transferred to a nitrocellulose membrane using an iBlot (Invitogen). Membranes were blocked with 5% Bovine Serum Albumin (Sigma) in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature for 1 hour. Membranes were then incubated on a rocker overnight at 4˚C with primary antibody in 2.5% BSA in TBST. Five minute washes were completed four to six times, followed by incubation with the appropriate HRP-conjugated secondary antibody (SouthernBiotech) in 5% milk in TBST at room temperature for 1 hour before being washed 4-6 times for 5 minutes each. Finally membranes were developed using Immobilon Western Chemiluminescent HRP substrate (Millipore). Images were captured using and Alpha-imager Fluoretech HD2 (ProteinSimple). Images were transformed and densiometric analysis was performed on images using Fiji biological image analysis software [149]. Lanes were normalized to corresponding Glycerol-3-phosphate dehydrogenase (GAPDH) band density prior to subsequent statistical analysis. Antibodies: 1) mouse anti-MIEN1 MO2 mAb (monoclonal antibody) raised against full-length recombinant MIEN1 (Abnova. 1:1000 dilution), 2) mouse anti-GAPDH mAb raised against full-

length human GAPDH (Santa Cruz Biotechnology. A-3. 1:2000 dilution), 3) rabbit anti-phospho-FAK Tyr925 polyclonal raised against a synthetic phosphopeiptide corresponding to residues surrounding pTyr925 of human FAK (Thermo Fisher Scientific. PA5-17733. 1:1000 dilution), 4) rabbit anti-FAK mAb raised against a synthetic peptide corresponding to residues surrounding Val793 of human FAK (Cell Signaling. (D5O7U) XP® Rabbit mAb #71433. 1:1000 dilution). 5) rabbit anti-panAkt mAb antibody raised against a synthetic peptide at the carboxy-terminal sequence of mouse Akt (Cell Signaling. C67E7. 1:1000 dilution), 6) rabbit anti-phospho-Akt Ser473 mAb antibody raised against a synthetic phosphopeptide corresponding to residues around Ser473 of human Akt (Cell Signaling. D9E. 1:1000 dilution), 7) rabbit anti-Green Fluorescent Protein mAb antibody raised against a synthetic peptide corresponding to the amino terminus of GFP (Cell Signaling. D5.1. 1:1000 dilution).

Morphology. Cells were grown on treated cell culture dishes and pictures were captured on an inverted light microscope using an attached Canon digital camera.

Scratch wound healing migration assay. 231, 831, 1833, and 4175 cells were grown to confluency in 6-well dishes. Cell monolayers were then wounded with a sterile micropipette tip. The cells were then washed twice with serum-free medium to remove floating cells and residual FBS and then incubated in serum-free medium. Wound was imaged at indicated time points. Images were collected using a Cannon digital camera mounted on an inverted light microscope. Total area of wounds was measured using the "MRI Wound Healing Tool" macro in Fiji [164]. Multiple locations within each well were recorded for each experiment. Results represent at least three independent experiments. Averages were generated from each well/independent experiment, which were then used for subsequent statistical analyses.

Cell proliferation. Five thousand cells were plated into each well of a 96-well plate with 200ul of complete medium. Plates were spun down at 500 rpm in a swinging tray to ensure even plating of the cells. Every 24 hours, 10ul of 10.5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the wells. Cells were incubated with MTT at 37˚C for 2 hours to allow formation of formazan. Media was carefully removed and 150ul of DMSO was added to each well. Wells were mixed well to ensure formazan crystals were solubilized. Absorbance was read at 570nm. DMSO blank was subtracted from the absorbance of each sample. To measure the initial time point (T0), MTT was added to cells 2 hours after seeding experiment to allow for cell attachment. Media in other wells was changed every 48 hours. Results represent at least four independent experiments performed in at least quadruplicate. Averages were generated from each independent experiment, which were then used for subsequent statistical analyses.

Cell survival. Twenty thousand cells were plated into each well of a 96-well plate with 200ul of complete medium. The next day, media was changed to serum free medium. MTT assay was then performed as outlined above. Results represent five independent experiments performed in quadruplicate. Averages were generated from each independent experiment, which were then used for subsequent statistical analyses.

Spheroid growth. One thousand cells in 50 µl of media were plated into each well of a low-attachment U-bottom 96-well plate. Plates were briefly centrifuged at 500 rpm to collect cells at the bottom of the well. With plates on ice, 50 µl of 5% Matrigel (Corning) was added to each well. Plates were again spun at 500 rpm at 4 ˚C. Plates were then placed at 37˚C for 30 minutes before 100 µl of complete media was added to the top of the solidified Matrigel. Spheroids formed overnight and images were collected on a Cannon digital camera mounted on an

inverted light microscope every 24 hours. Total area of spheroids was measured using the "Analyze Spheroid Cell Invasion In 3D Matrix" macro in Fiji [164]. Results represent at least eight total spheroids for each cell line.

Spheroid invasion. Spheroids were formed as outlined above. On the following day, spheroids were collected in complete medium. Spheroids were then washed twice with Cell Recovery Solution (Corning) to remove residual Matrigel from the spheroids. Spheroids were washed three times with complete medium to remove residual Cell Recovery Solution. Spheroids were then individually plated in 10 µl of complete medium in wells of a low-attachment U-bottom 96-well plate. Forty microliters of 100% Matrigel matrix was added into each well on ice and plates were briefly centrifuged at 500 rpm at 4 ˚C. Plates were then placed at 37˚C for 30 minutes before 100 µl of complete media was added to the top of the solidified Matrigel. Images of invasion were captured on a Cannon digital camera mounted on an inverted light microscope every 24 hours. Total area of spheroids was measured using the "Analyze Spheroid Cell Invasion In 3D Matrix" macro in Fiji [164]. Results represent at least eight combined different spheroids taken from three separate experiments.

Metabolomic analysis. Cells were harvested with trypsin EDTA and washed twice with PBS prior to counting. Four samples for each cell line, consisting of five million cells each, were spun down in a microcentrifuge tube before the PBS was removed and cell pellets were flash frozen in liquid nitrogen. Metabolite extraction, sample preparation and chromatography were carried out at the Metabolomics Core at the Baylor College of Medicine in Houston, Texas as previously outlined [165-168]. Chromatograms were manually reviewed and peak area was integrated based on the retention time. Each metabolite peak area was normalized with relation to the peak area of a spiked internal standard and $\log_2$ transformed prior to statistical analysis.

Statistical analysis. The Student's t-test was used for statistical analysis. Data are presented as mean ± standard error of the mean. For metabolomics analysis, differential metabolites were identified by adjusting the p-values for multiple testing at a False Discovery Rate (FDR) threshold of <0.25 according to the Benjamini-Hochberg procedure [169]. This set of metabolites was used for generating the heat map. * denotes a p-value of less than 0.05 and was considered to be significant. ** denotes a p-value of less than 0.01. *** denotes a p-value of less than 0.005.

Results

Effect of MIEN1-KO on morphology

MIEN1 CRISPR knockout cell lines were produced by pooling all clones which lacked MIEN1 expression following western blotting. In order to see the effect of MIEN1-KO on general cellular characteristics, the morphology of the cells was first examined (Fig 1). There was no observable difference in the morphology of the MIEN1-KO cell lines when compared to their parental cell lines.

Figure 1

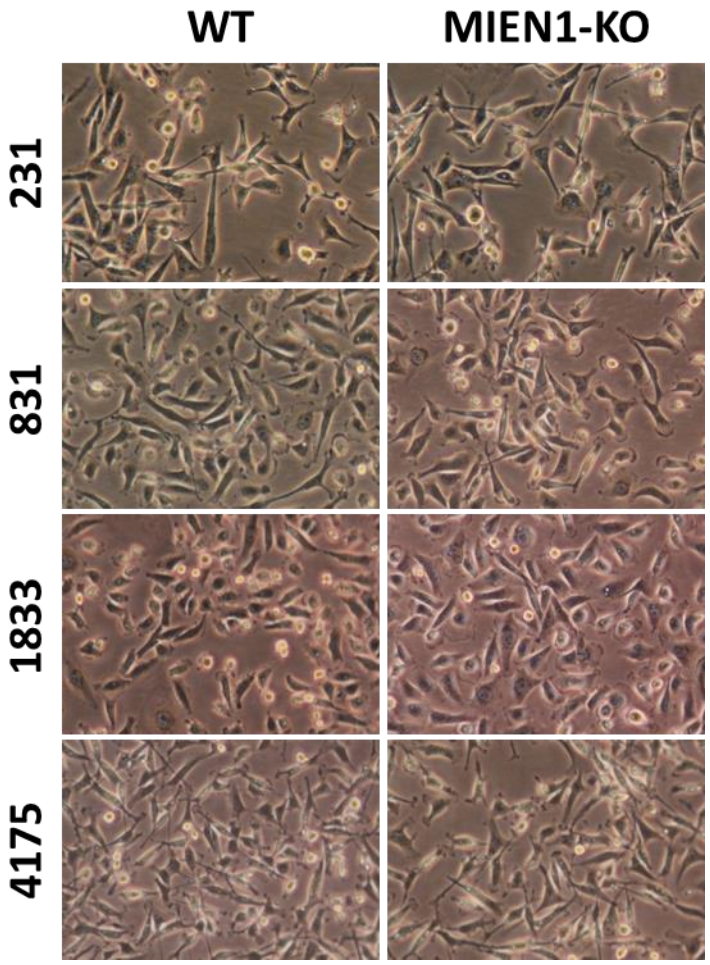


Figure 1. **MIEN1-KO cell morphology**. Representative 10x magnification phase contrast images of MIEN1-KO cells and corresponding parental cells.

Effect of MIEN1-KO in MDA-MB-231 cells

In order to examine how the process of knockout clone selection affects cellular function and signaling in a heterogeneous cell line, multiple pooled cell lines were generated from clones derived from parental 231 cells (Table 1). Two distinct sets of MIEN1-KO pools were produced (MKO-B and MKO-D) and these pools were combined to create a third pool (MKO-C). In addition to the MIEN1-KO pooled cell lines, clones were isolated from 231 cells transfected with PX458 plasmid, which did not contain any sgRNA to control for the clonal isolation process, and pooled to generate a control cell line (CEV). These cell lines were compared to parental 231 cells in a wound healing assay (Fig 2A). After 12 hours of migration, no significant difference was observed in any pooled cell line compared to 231 cells (Fig 2B). The two separate pools of 231 MIEN1-KO cells, MKO-B and MKO-D, showed disparate migration. The combination of these two pools in MKO-C showed an intermediate migration between MKO-B and MKO-D. MKO-C migration was also the most similar to 231 cells.

Next, known signaling mediators of MIEN1 in 231 were examined (Fig 2C). Phosphorylation status of Akt at Ser473 compared to parental 231 cells significantly increased in MKO-B cells, significantly decreased in MKO-D and remained relatively unchanged in MKO-C and CEV cells (Fig 2D). FAK activation as determined by phosphorylation of Y925 was significantly decreased in MKO-B and also slightly decreased in MKO-C compared to parental 231 cells. FAK phosphorylation was increased in CEV cells and unchanged in MKO-D cells (Fig 2E).

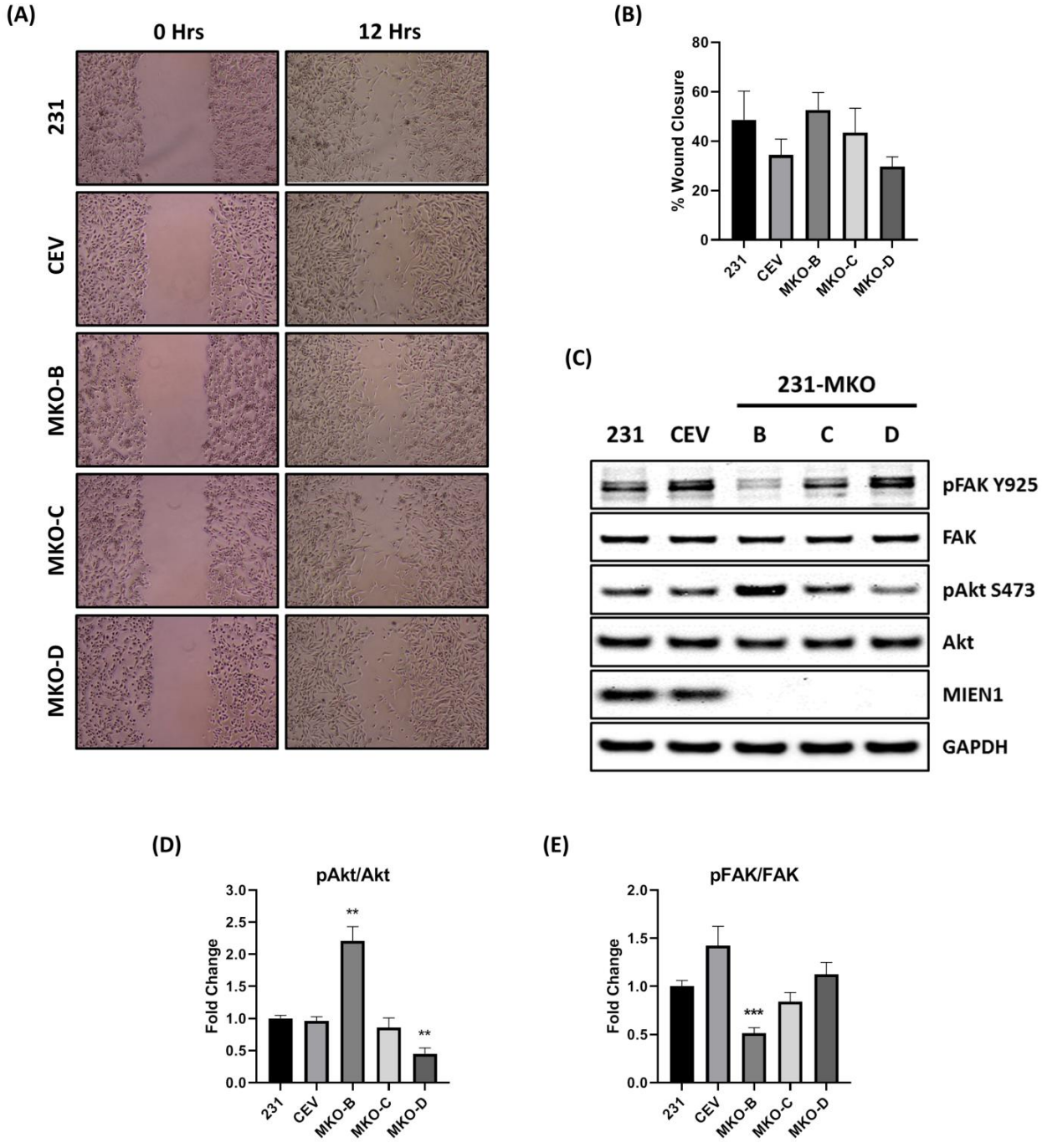
Figure 2

Effect of MIEN1-KO in MDA-MB-231 cells. (A) Wound healing assay. MDA-MB-231 cells and all pooled cell lines were scratched and allowed to migrate to close the wound during 12 hours of incubation. Representative images taken at 0 and 12 hours. (B) Quantification of wound healing. Results are expressed as % of original wound area covered by migrating cells. (C) Western blot of the phosphorylation status of FAK and Akt in cell extracts. (D) Quantification of FAK and Akt phosphorylation normalized to GAPDH.

CEV: CRISPR empty vector, MKO-B: 231-MIEN1-KO-B, MKO-C: 231-MIEN1-KO-C, MKO-D: 231-MIEN1-KO-D.

Error bars indicate standard error. The p-values were computed using Student's t-test. *p-value <0.05. **p-value <0.01. ***p-value <0.005

Figure 2


(A)
0 Hrs
12 Hrs
231
CEV
MKO-B
MKO-C
MKO-D
(B)
% Wound Closure
80
60
40
20
0
231
CEV
MKO-B
MKO-C
MKO-D
(C)
231-MKO
231
CEV
B
C
D
pFAK Y925
FAK
pAkt S473
Akt
MIEN1
GAPDH
(D)
pAkt/Akt
Fold Change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
231
CEV
MKO-B
MKO-C
MKO-D
(E)
pFAK/FAK
Fold Change
2.0
1.5
1.0
0.5
0.0
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231
CEV
MKO-B
MKO-C
MKO-D

Effect of MIEN1-KO on proliferation and 3D growth in metastatic breast cancer cells

In order to decrease the variable of heterogeneity, 831, 1833 and 4175 metastatic derivative cells were examined to see how MIEN1-KO affected cell proliferation. MTT assays were performed to compare rates of proliferation between wild-type and MIEN1-KO cell lines (Fig 3A-C). MIEN1-KO had no discernable effect on the proliferation of these metastatic cell lines. Next, to test if MIEN1-KO had an effect on three-dimensional spheroid growth, metastatic derivative cell lines and their MIEN1-KO counterparts were grown in Matrigel to facilitate spheroid formation (Fig 3D). In both the 1833 and 4175 sets, no difference in spheroid growth was observed (Fig 3F-G). A slight increase in growth was observed in 831-MKO cells when compared to wild-type 831 (Fig 3E).

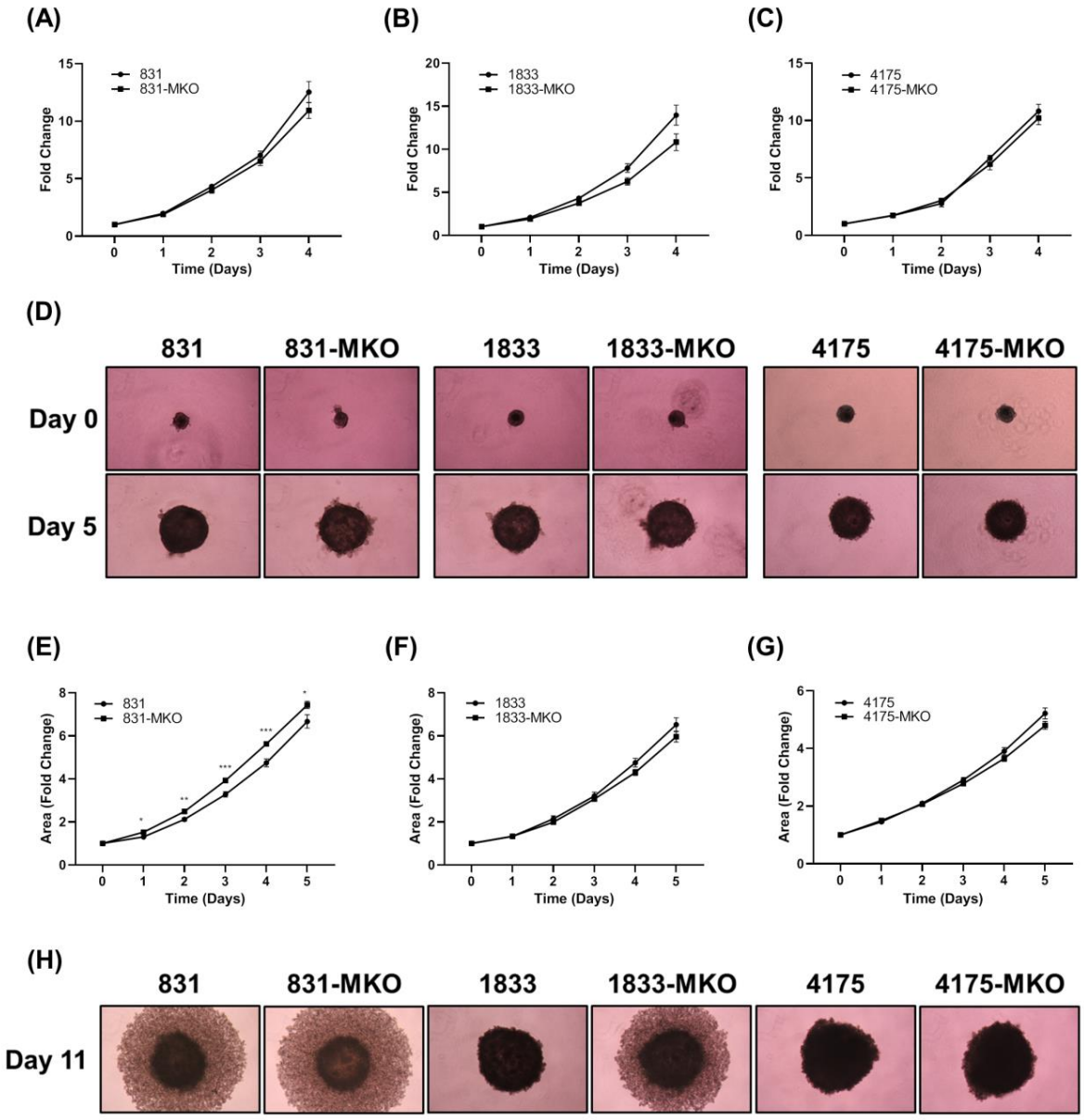
Interestingly, after 11 days in culture, 831 and 831-MKO spheroids began to disintegrate and shed cells. This was not due to cell death as cells harvested from the low-attachment culture plate attached and grew when re-plated in a treated culture dish. 4175 and 4175-MKO did not exhibit spheroid disruption during extended culture. However, while 1833 cells retained the majority of cells within the spheroids, 1833-MKO cells showed disintegration of spheroid cultures similar to what was seen with both 831 and 831-MKO. Together these data indicate that knocking out *MIEN1* does not affect the proliferation of these breast cancer cell lines in a monolayer but may play a context dependent role in three-dimensional spheroid growth and maintenance.

Figure 3

Cell proliferation and spheroid growth of metastatic breast cancer cells. (A-C) MTT assay comparing fold change of proliferation of wild-type (A) 831, (B) 1833, (C) 4175 cells and their corresponding MIEN1-KO cell lines. (D) Spheroid growth. Representative 4x magnification phase contrast images of spheroids grown in Matrigel for 5 days. (E-G) Spheroid growth quantification. Fold change of area of spheroid growth. (H) Representative 4x magnification phase contrast images of spheroids grown in Matrigel for 11 days.

Error bars indicate standard error. The p-values were computed using Student's t-test. *p-value <0.05. **p-value <0.01. ***p-value <0.005

Figure 3


(A)
831
831-MKO
Fold Change
Time (Days)
(B)
1833
1833-MKO
Fold Change
Time (Days)
(C)
4175
4175-MKO
Fold Change
Time (Days)
(D)
831
831-MKO
1833
1833-MKO
4175
4175-MKO
Day 0
Day 5
(E)
831
831-MKO
Area (Fold Change)
Time (Days)
(F)
1833
1833-MKO
Area (Fold Change)
Time (Days)
(G)
4175
4175-MKO
Area (Fold Change)
Time (Days)
(H)
831
831-MKO
1833
1833-MKO
4175
4175-MKO
Day 11

Effect of MIEN1-KO on signaling, migration, and invasion in metastatic breast cancer cells

Alterations to established MIEN1 signaling cascades within MIEN1-KO cells were investigated by western blot. No significant changes were observed in the phosphorylation status of Akt S473 or FAK Y925 following normalization to GAPDH in any of the MIEN1-KO cell lines when compared to their wild-type counterparts (Fig 4).

In order to test the migratory and invasive abilities of these metastatic derivative cell lines, wild-type cell lines and MIEN1-KO counterparts were subjected to a wound healing assay (Fig 5A-D). Notably, the migration ability of the wild-type cell lines differed as 831 cells had the highest wound closure capabilities across all cell types. Both 1833 and 4175 cells had similar migratory abilities compared to their corresponding MIEN1-KO cell lines (Fig 5C-D). However, knockout of MIEN1 in 831 cells significantly reduced migration at both 12 and 24 hours (Fig 5B). This indicates that genetic context may play a role in how cells are able to respond to deletion of *MIEN1*.

Three-dimensional spheroid invasion through an ECM was then tested. Spheroids were suspended in Matrigel and allowed to invade into the surrounding matrix for four days. Once again, all metastatic derivative cell lines showed different capacity for invasion. Wild-type 831, 1833 and 4175 cells showed 8.3-fold, 9.3-fold, and 22.5-fold invasive abilities respectively (Fig 5E-H). Knocking out MIEN1 in 831 and 4175 cells revealed no difference in the invasion when compared to wild type (Fig 5F, H). However, MIEN1-KO in 1833 resulted in a modest increase in invasion, reiterating the importance of genetic context for MIEN1-KO functional significance (Fig 5G).

Figure 4

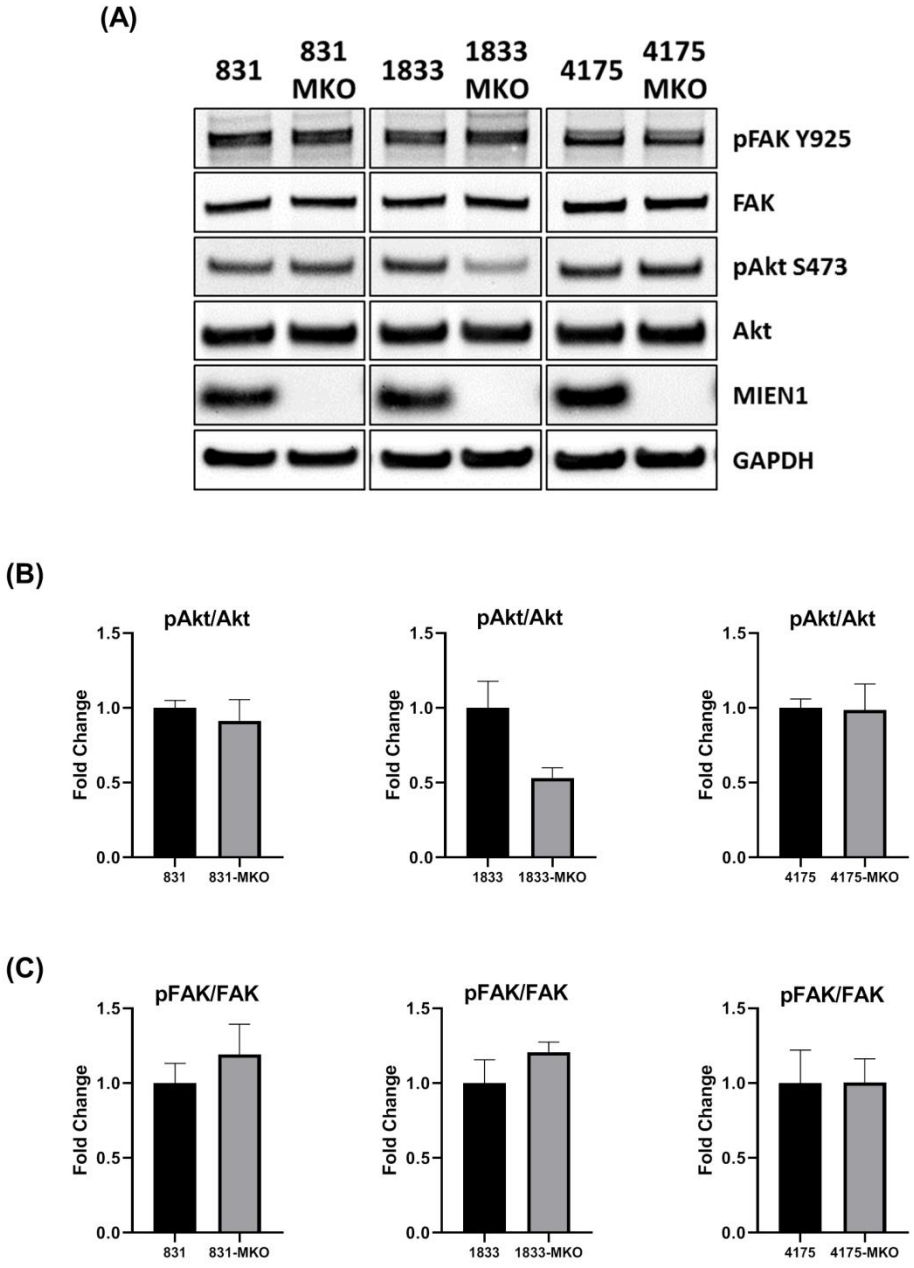


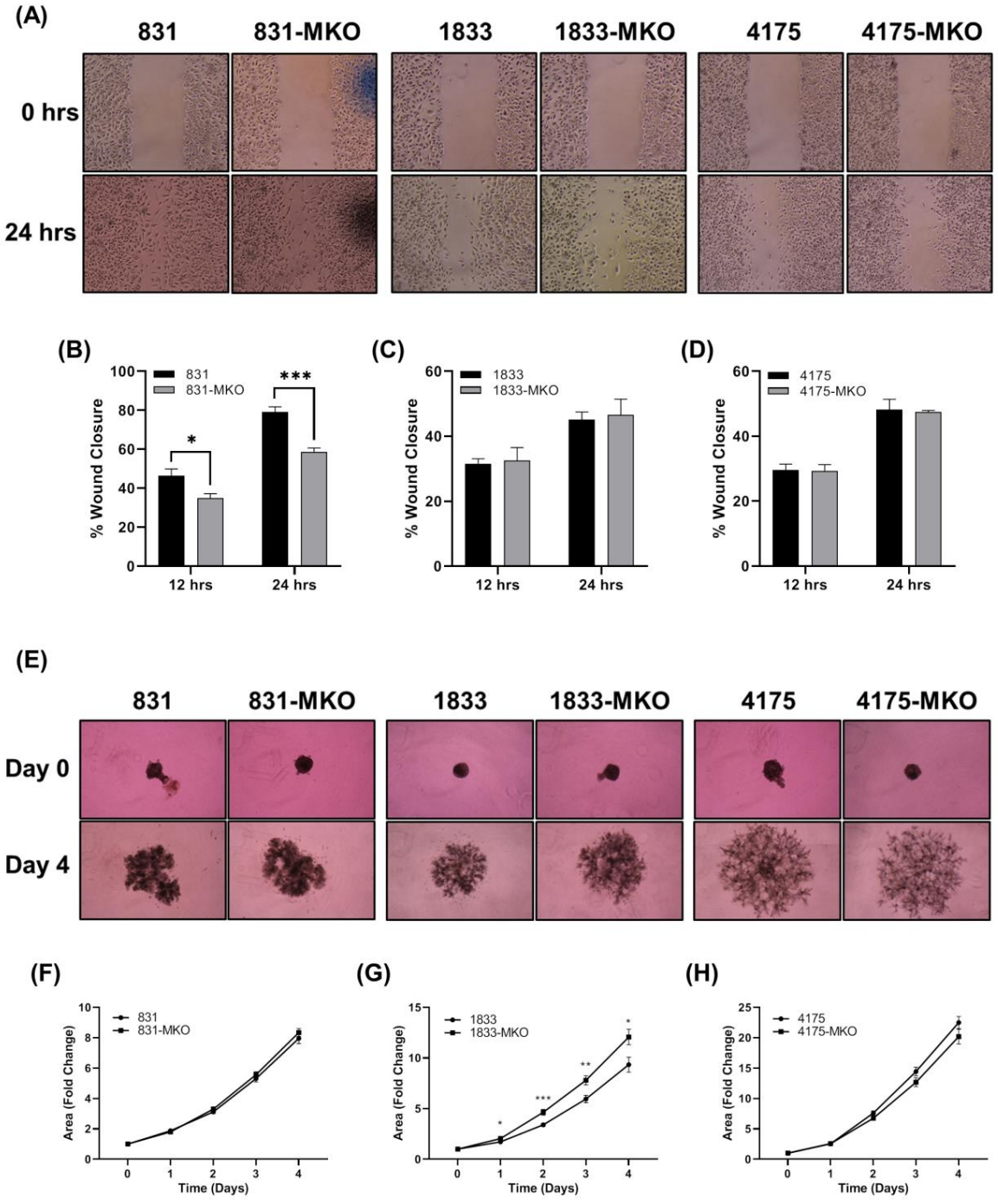
Figure 4. **Signaling in metastatic breast cancer cells.** (A) Western blots of extracts from wild-type and MIEN1-KO cell lines. Each lane was normalized to GAPDH prior to analysis. (B) Comparison of the phosphorylation status of Akt. Expressed as fold change. (C) Comparison of the phosphorylation status of FAK. Expressed as fold change. Error bars indicate standard error.

Figure 5

Migration and invasion of metastatic breast cancer cells. (A) Wound healing assay. MDA-MB-231 cells and all pooled cell lines were scratched and allowed to migrate to close the wound for 24 hours. Representative images taken at 0 and 24 hours. (B-D) Quantification of wound healing at 12 and 24 hours post-wound. Results are expressed as % of original wound area covered by migrating cells. (E-H) Spheroids embedded in a 100% Matrigel matrix were allowed to invade the surrounding matrix for 4 days. (E) Pictured are representative images of spheroids from Days 0, 1, 2, and 3. (F-H) Spheroid invasion quantification. Fold change of area of spheroid invasion.

Error bars indicate standard error. The p-values were computed using Student's t-test. *p-value <0.05. **p-value <0.01. ***p-value <0.005

Figure 5


(A)
831
831-MKO
1833
1833-MKO
4175
4175-MKO
0 hrs
24 hrs
(B)
831
831-MKO
% Wound Closure
12 hrs
24 hrs
(C)
1833
1833-MKO
(D)
4175
4175-MKO
(E)
Day 0
Day 4
(F)
Area (Fold Change)
Time (Days)
(G)
(H)

Effect of MIEN1-KO on cell survival and metabolism in lung metastatic breast cancer cells

The 4175 cells were examined to see if MIEN1-KO played any role in cell survival. No difference was observed between 4175 and 4175-MKO cells upon serum deprivation (Fig 6A). Next, alterations in the metabolic profile of 4175 following MIEN1-KO were investigated using liquid chromatography/mass spectrometry (LC/MS). Samples were analyzed for changes in amino acids and many other metabolic intermediates. Notably, several changes were observed in the serine synthesis pathway (SSP), a shunt off of glycolysis. Serine exhibited the second highest differential abundance and Log_2 transformed levels were over three-fold higher in 4175-MKO cells compared to 4175 cells (Fig 6B). Glycine, another SSP intermediate, was also increased in MIEN1-KO cells (Fig 6C). Both adenosine monophosphate (AMP) and guanosine monophosphate (GMP) were among the top six most increased metabolites in 4175-MKO cells (Fig 6D-E), while the non-phosphorylated, nucleoside forms of these nucleotides were significantly decreased in 4175-MKO cells compared to wild type (Fig 6F-G). Another member of the SSP responsible for one-carbon metabolism within the cell, S-Adenosyl methionine (SAM), though significantly altered, did not show the same magnitude of change observed in other pathway intermediates (Fig 6H).

Figure 6

Cell survival and metabolism in lung metastatic breast cancer cells. (A) MTT assay comparing fold change of cell survival during serum deprivation of 4175 and 4175-MKO cells. (B-I) Analysis of metabolites from 4175 and 4175-MKO cells lines. Comparison of fold change of $\log_2$ transformed difference of metabolites from spiked internal standard. (B) serine, (C) glycine, (D) Adenosine monophosphate (AMP), (E) Guanosine monophosphate (GMP), (F) Adenosine, (G) Guanosine, and (H) S-adenosyl methionine (SAM). (I) Heat map of hierarchical clustering of 117 metabolites in 4175 and 4175-MKO cells. Red arrows denote metabolites in the serine/glycine shunt compared in B-H. Columns represent individual cell extracts, with technical replicates, and rows refer to distinct metabolites. Map shows a colored representation of the correlation based on the number of standard deviations each sample is from the average of all log transformed values. Blue indicates a decrease compared to average of all samples. Yellow indicates an increase compared to average of all samples. 4175 samples are labeled as WT-A/B/C/D and are grouped together on the left of the heat map underneath the light blue bar at the top of the heat map. 4175-MKO samples are labeled as MKO-A/B/C/D and are grouped together under the red bar at the top of the heat map. Metabolites listed have been filtered based on a false discovery rate of 0.25 according to Benjamini–Hochberg procedure.
Error bars indicate standard error. The p-values were computed using Student's t-test. *p-value <0.05. **p-value <0.01. ***p-value <0.005

Figure 6

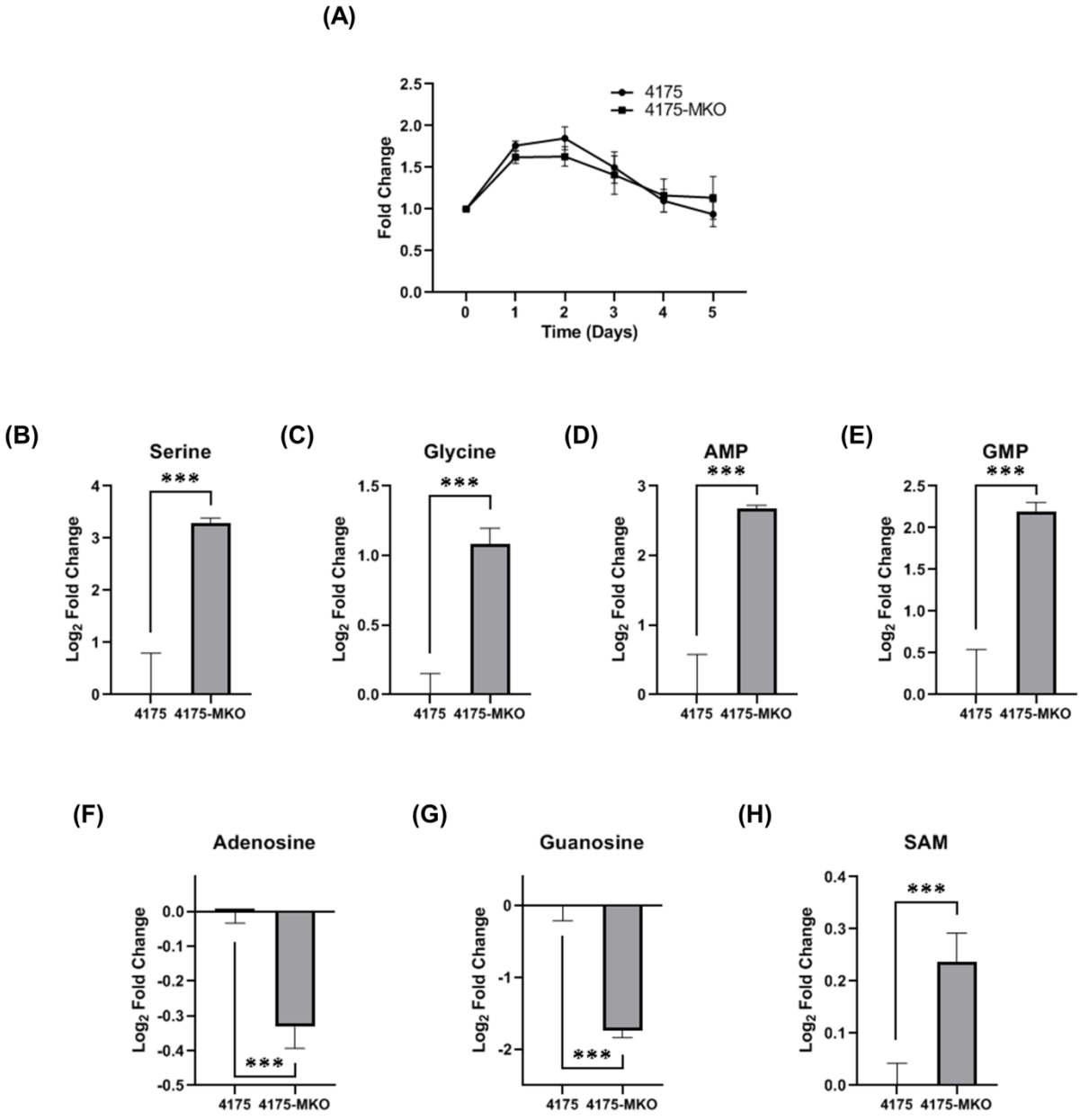
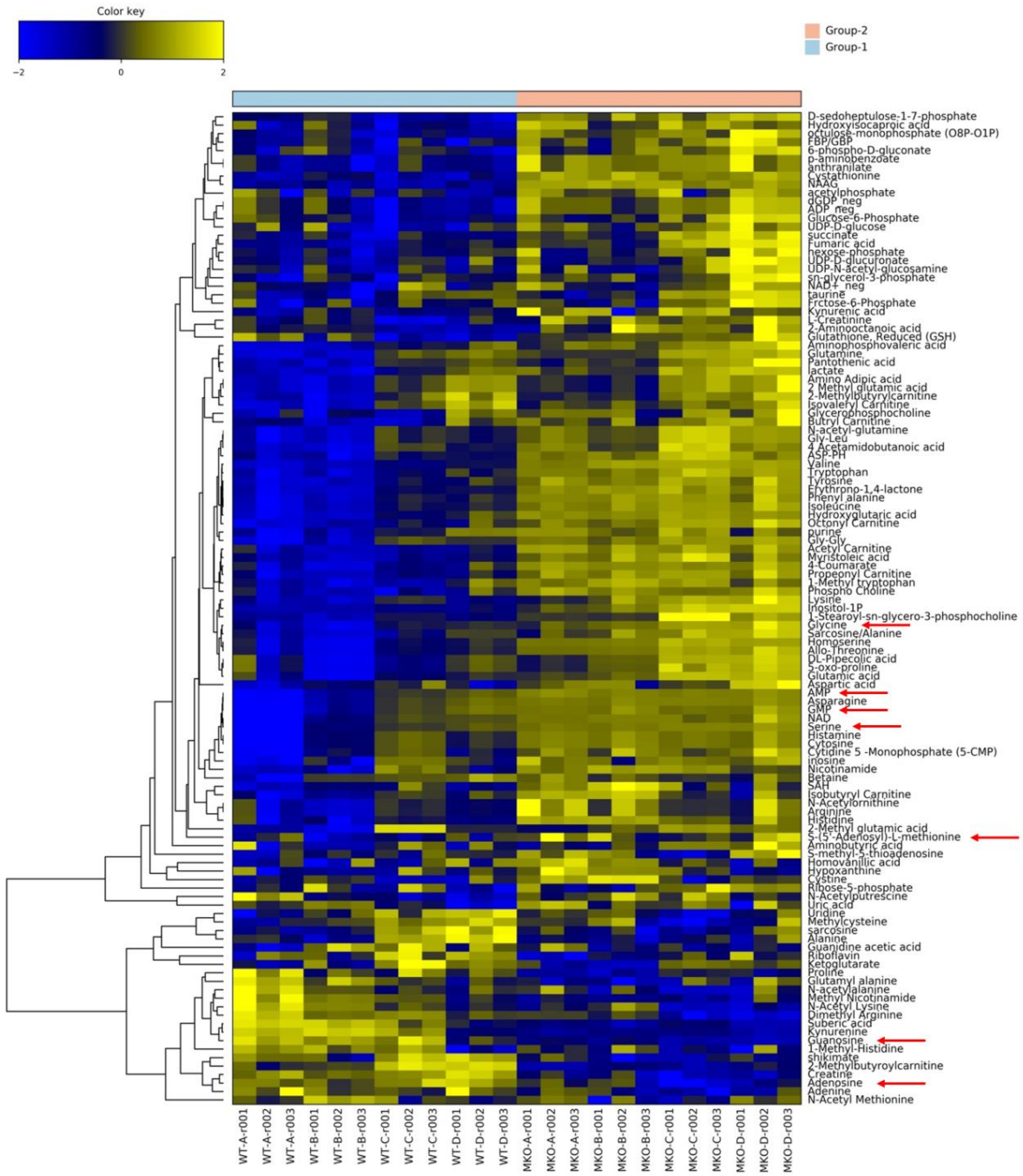

(A)
4175
4175-MKO
Fold Change
Time (Days)
(B)
Serine
(C)
Glycine
(D)
AMP
(E)
GMP
(F)
Adenosine
(G)
Guanosine
(H)
SAM
Log2 Fold Change
***
4175
4175-MKO

Figure 6 cont.

(I)



MIEN1 ITAM mutant reduces invasion

Wild-type MIEN1 (MIEN1^{WT}) and ITAM mutant MIEN1 (MIEN1$^{Y39/50F}$) proteins were expressed in 4175-MKO cells to observe the effect of re-introduction of MIEN1 in these cells. Fold change in spheroid growth was not altered in 4175-MKO-MIEN1^{WT} or 4175-MKO-MIEN1$^{Y39/50F}$ cells when compared to either the 4175-EV (empty vector) or 4175-MKO-EV, indicating that MIEN1 seems to be dispensable for spheroid growth and that interfering with normal MIEN1 signaling through ITAM tyrosine residue mutation does not alter growth signaling (Fig 7A-B).

The effect of MIEN1 ITAM mutation on Matrigel invasion in an MIEN1 knockout background was also tested (Fig 7C-D). Re-expression of MIEN1^{WT} resulted in a slight decrease in the invasive capacity of 4175-MKO-MIEN1^{WT} cells compared to 4175-MKO-EV control (Fig 7D). However, when MIEN1$^{Y39/50F}$ was introduced into the 4175-MKO cells, invasion was significantly inhibited (Fig 7D).

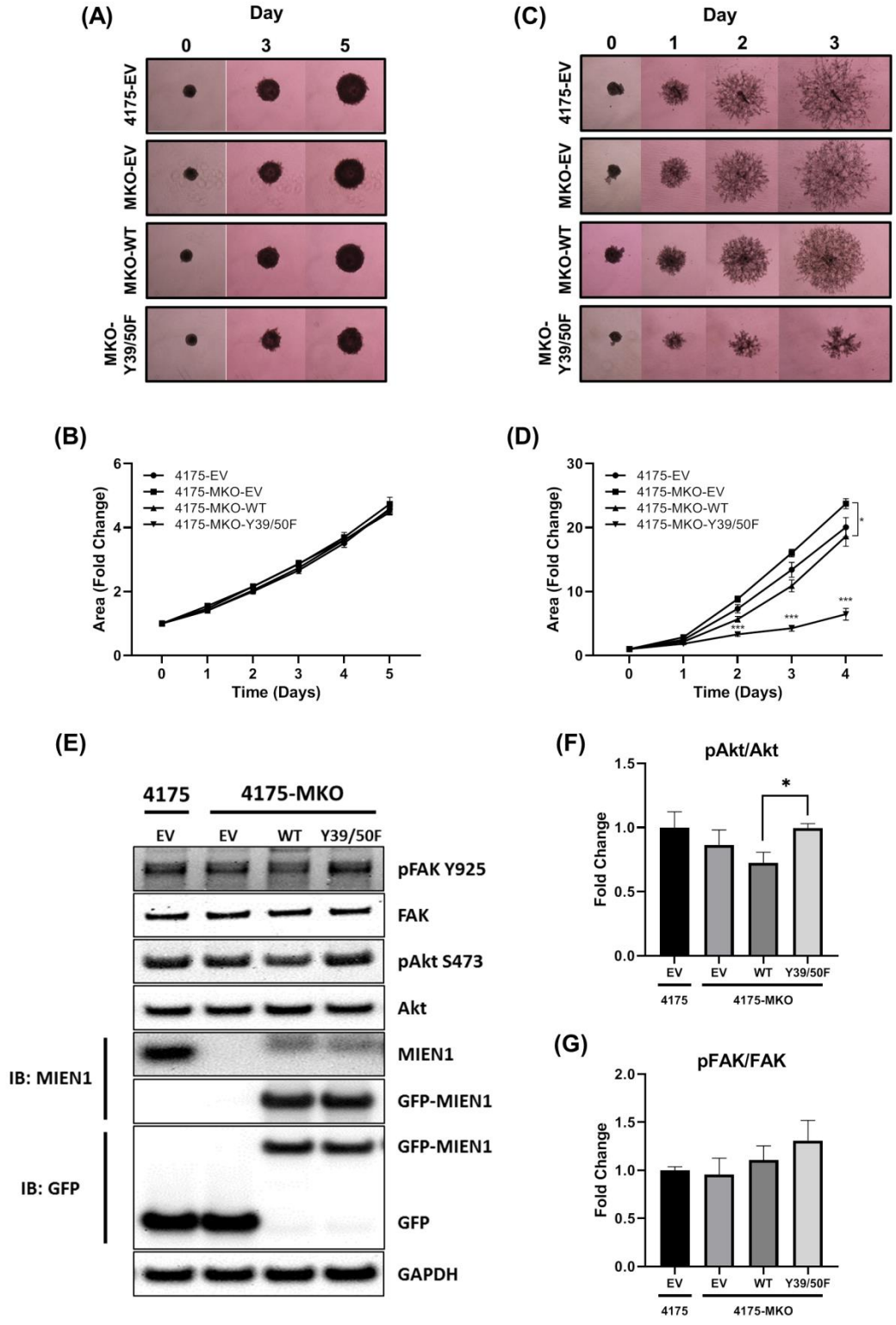
This suppression of invasion was not due to altered signaling through Akt or FAK as both pathways were maintained in all 4175-MKO overexpression cell lines relative to 4175-EV (Fig 7E-G).

Figure 7

MIEN1 ITAM domain mutant in growth, invasion, and signaling of lung metastatic breast cancer cells. (A) Spheroids of 4175 cells expressing EGFP (4175-EV) and 4175-MKO cells stably expressing EGFP (MKO-EV), EGFP- MIEN1^{WT} (MKO-WT), and EGFP- MIEN1$^{Y39/50F}$ (MKO-Y39/50F) grown in 2.5% Matrigel. Pictured are representative images of spheroids taken on days 0, 3, and 5. (B) Spheroid growth quantification. Fold change of area of spheroid growth over the course of 5 days. (C) Spheroids embedded in a 100% Matrigel matrix were allowed to invade the surrounding matrix for 4 days. Pictured are representative images of spheroids from days 0, 1, 2, and 3. (D) Spheroid invasion quantification. Fold change of area of spheroid invasion. Significant differences were observed between MKO-EV and MKO-WT at all time points (1: *, 2: ***, 3: ***). The comparisons between MKO-Y39/50F and all other cell lines on days 2, 3, and 4 are equally significant as indicated. (E) Western blot analysis of signaling following MIEN1 expression in 4175-MKO cell line. Each lane was normalized to GAPDH prior to analysis. (F) Comparison of the phosphorylation status of Akt. Expressed as fold change compared to 4175-EV. (G) Comparison of the phosphorylation status of FAK. Expressed as fold change compared to 4175-EV.

Error bars indicate standard error. The p-values were computed using Student's t-test. *p-value <0.05. **p-value <0.01. ***p-value <0.005

Figure 7


(A)
Day
0
3
5
4175-EV
MKO-EV
MKO-WT
MKO-Y39/50F
(C)
Day
0
1
2
3
4175-EV
MKO-EV
MKO-WT
MKO-Y39/50F
(B)
Area (Fold Change)
Time (Days)
4175-EV
4175-MKO-EV
4175-MKO-WT
4175-MKO-Y39/50F
(D)
Area (Fold Change)
Time (Days)
4175-EV
4175-MKO-EV
4175-MKO-WT
4175-MKO-Y39/50F
*
***
***
***
(E)
4175
4175-MKO
EV
EV
WT
Y39/50F
pFAK Y925
FAK
pAkt S473
Akt
MIEN1
GFP-MIEN1
GFP-MIEN1
GFP
GAPDH
IB: MIEN1
IB: GFP
(F)
pAkt/Akt
Fold Change
*
EV
EV
WT
Y39/50F
4175
4175-MKO
(G)
pFAK/FAK
Fold Change
EV
EV
WT
Y39/50F
4175
4175-MKO

Discussion

Metastasis is a complex process involving cancer cells overcoming many barriers in order to colonize other organs in the body and establish secondary tumors. Two of the most important aspects of metastasis are the migration and invasion of the cancer cells. Following the creation of the first cancer cells through mutations and epigenetic changes, some cells also gain the ability to metastasize through similar processes. Select progeny of these metastatic cancer cells then break away from the tumor and begin to infiltrate the surrounding tissue. This process requires an avenue of escape from the primary tumor and means to be able to utilize the available conduit. Avenues of escape are created by cancer cells through production and secretion of tissue altering proteins, such as MMPs to degrade ECM or VEGF to recruit endothelial cells for the development of new vasculature. In addition, metastasizing cells can interact with tumor associated normal cells, such as macrophages and fibroblasts, to aid in tumor development, facilitate ECM remodeling, activate chemokine production, and influence blood vessel formation to expedite migration and invasion [45, 48, 170-174]. After making a way of escape, cancer cells must then migrate and move away from the primary tumor. This dissemination is accomplished either as individual cells or groups of cells [27, 29]. Both of these methods require coordinated detachment from the primary tumor followed by reorganization of the cytoskeleton and interaction with the extracellular environment to mediate metastasis.

In this study, the migratory and invasive capacity of MIEN1-KO cells was investigated. *MIEN1* is an oncogene that has been implicated in the progression of many different types of cancer and has been shown to functionally increase the migration, invasion and extravasation of metastatic cancer cells [108, 115]. Specifically, MIEN1 has been shown to regulate migration of the TNBC cell line MDA-MB-231 through actin cytoskeletal rearrangement. MIEN1 induces

phosphorylation and activation of FAK, a major regulator of focal adhesion complexes, and reduces activity of cofilin [117]. Knockdown of MIEN1 with siRNA leads to reduced migration due to reduced FAK, Akt, ERK and NF-kB phosphorylation [117]. CRISPR mediated knockout of MIEN1 in 231 cells revealed altered functional characteristics and signaling compared to previous siRNA knockdown studies. While previous studies have shown a two-fold decrease in migration following siRNA knockdown of MIEN1 in 231 cells [117], MIEN1-KO in 231 cells showed no significant change in migratory capacity in a wound healing assay. This was tested in multiple pooled MIEN1-KO cell lines. The absence of a decrease in migration and the MIEN1-KO cell lines and the differing wound closure abilities of the different MIEN1-KO pools indicate two things. First, the process of generating and expanding single-cell knockout clones and in culture is a selective process. Second, the parental 231 cell population is heterogeneous [65].

When generating clones, transfection of plasmid is not 100% efficient [175] and even results in some cell death, which excludes some cells at the earliest stage of the process. Transfected cells are then sorted by FACS and plated as a single cell (or at extremely low densities) in a culture dish, which is a strenuous process involving prolonged detachment of the cells and high turbulence during sorting. Following plating, single cells must proliferate into colonies. Cells in culture normally interact with the cells that are around them and are affected by the density of the culture [52, 176-178]. Single-cell expansion is a large selective pressure few cells are able to overcome. Isolation of clones selects for more robust cells with distinct characteristics, to the exclusion of the majority of the original population.

Another indication of the heterogeneity of the cell line is evidenced by the signaling in the 231 MIEN1-KO pools. MKO-B cells showed increased phosphorylated Akt at S473, which is normally decreased following MIEN1 siRNA knockdown. MKO-B cells also had decreased

FAK phosphorylation of Y925 relative to wild-type 231, which is consistent with siRNA knockdown [117]. However, a second pool of MIEN1-KO cells, MKO-D, revealed the exact opposite signaling pattern with significantly decreased Akt phosphorylation combined with slightly increased FAK phosphorylation. Combining these two pools to produce MKO-C resulted in minor decreased signaling in both Akt and FAK, which was more consistent with siRNA studies, though to a far lesser magnitude. This indicates that perhaps increasing the number of clones in pooled knockout cell lines may be able to overcome some of the selection incurred by clonal isolation and enable more accurate comparisons to parental cell lines.

Fundamentally, some have questioned whether the breast cancer cell lines most widely used are representative of the true heterogeneity seen in breast cancer patients [62, 179-181]. Derivation of cell lines from patients is a selective pressure and eliminates those cells which are unable to grow on culture treated plastic dishes or survive *in vitro* conditions, so a comprehensive model of breast cancer pathology is most likely impossible to recreate in culture.

In order to eliminate some of the uncertainty of heterogeneity, the effect of MIEN1 was examined in metastatic derivative cell lines of 231. These cell lines were generated by consecutive rounds of *in vivo* selection through injection of 231 cells into athymic mice [67]. Metastases were then isolated and cell lines were subcultured. 831 was generated from a brain metastasis [67]. 1833 was generated from a bone metastasis [66]. 4175 was generated from a lung metastasis [65]. Using these derivative cell lines provided a more homogenous pool of cells from which to generate MIEN1-KO cells. The extent of selection incurred by generation of single-cell clones is greatly reduced due to the preliminary selection that was carried out in the mice. Thus, it is reasonable that the comparison between parental metastatic derivative cell lines and MIEN1-KO pooled cell lines is more appropriate than comparisons between parental 231

cells and the matching MIEN1-KO cell lines, unless the number of clones generated from 231 can be greatly increased.

Though these derivative cell lines may not accurately represent clinical tumors, they can still be appropriate model systems for understanding the genetic context of metastasis to specific organs. Differential gene expression drives these cell lines to show propensity toward specific organs to the exclusion of others [65-67]. Comparing pooled MIEN1-KO cell lines to their corresponding metastatic derivative cell line provides the opportunity to examine how genetic context may influence MIEN1 function in a TNBC background. This comparison also allows for evaluation of the extent of dependency each genetically distinct cell line has on MIEN1 protein in the context of metastatic function. Deletion of *MIEN1* in all three metastatic derivative cell lines, 831, 1833, and 4175, did not affect the proliferation of these cells in either two-dimensional monolayer or three-dimensional spheroid growth, with the lone exception of spheroid growth in 831. However, this difference appears to be due to the compactness of 831-MKO spheroids as the border between the spheroid and the surrounding area is not as smooth as 831. Though the difference in the area of spheroid growth was statistically significant between 831 and 831-MKO at the majority of the time points, the final time point showed the least significance, indicating that the increased area of total spheroid growth lessened the impact of the ruffled surface caused by decreased compaction. These results are in agreement with available literature as MIEN1 has yet to be implicated in cellular division.

MDA-MB-231 cells require integrin β1 interaction with ECM proteins, like those contained in Matrigel, in order to form compact spheroids [182]. These metastatic derivative cell lines have been shown to have differing patterns of integrin expression in exosomes, with 4175 cells exhibiting high levels of integrin β1, while 831 cells express low levels of integrin β1 [68].

This explains the dissolving of 831 and 831-MKO spheroids as well as the stability of 4175 and 4175-MKO spheroids. MIEN1-KO in 1833 cells may affect cellular contact with ECM proteins by altering cell surface expression of integrins.

Interestingly, MIEN1-KO only reduced the migratory capacity of 831 cells, while having no discernable effect on the migration of 1833 or 4175 cells. This finding indicates that there is indeed a genetic component regulating dependency on MIEN1 for migratory function. Therefore, 1833 and 4175 must have unique compensatory mechanisms which are able to account for the lack of MIEN1. The pattern of MIEN1 dependency did not persist in a three-dimensional Matrigel invasion assay. MIEN1-KO in both 831 and 4175 cells showed no change in invasion capabilities compared to wild type; however, 1833-MKO cells surprisingly displayed increased invasion compared to wild-type 1833. One explanation for this behavior may be the low number of clones used for the 1833-MKO pooled cell line as only four MIEN1-negative/Cas-9-negative clones were generated for this cell line. The four knockout clones used may not be enough to accurately represent the heterogeneity present within parental wild-type 1833 cells.

The highest number of MIEN1-KO clones was generated from 4175 cells, which means that the 4175-MKO cell line was the most representative of its parental cell line, so these cells were selected for metabolic profiling. Though MIEN1 has not previously been linked to cellular metabolism, numerous changes were observed in the abundance of metabolites upon MIEN1-KO. Most notably, several SSP intermediates and end-products were significantly elevated in 4175-MKO cells. The SSP is an anaplerotic shunt of glycolysis that leads to the production of several macromolecules essential for cellular function, including nucleotides, amino acids and lipid precursors. SSP flux was shown to be essential in some breast cancers [79, 183]. Further,

high SSP flux has shown association with metastasis in melanoma cells [184]. This increased SSP activity is often due to increased expression or activity of the gatekeeper enzyme of the pathway, phosphoglycerate dehydrogenase (PHGDH). PHGDH is amplified in TNBC and expression of PHGDH is increased in ER-negative breast cancer cells compared to ER-positive [78, 183]. Elevated levels of phosphoserine aminotransferase, the second enzyme of the SSP, was found in ER-negative metastases [185]. These studies indicate that TNBCs may have a tendency to develop high flux through the SSP. It appears that the selective pressure of MIEN1-KO on 4175 lung metastatic breast cancer cells stimulated SSP flux, which was maintained in the cells even following adaptation. High levels of serine in 4175-MKO cells may not come from glycolysis as asparagine is also elevated. In fact, one study showed that asparagine functioned as an amino acid exchange factor and intracellular asparagine facilitated the import of serine in cells derived from patient liposarcoma [186]. Irrespective of the source of serine, end-products of serine metabolic pathways seem to play a role in various cancer processes.

A consequence of the SSP is the production of single-carbon methyl groups, which are able to be used in a variety of biological reactions. One important application of these methyl groups, especially within the context of cancer, is the generation of SAM. SAM is responsible for donating these single-carbon units during DNA and histone methylation [187]. Hypermethylation in cancer is a major regulator of gene expression. Tumor suppressor genes and their promotors can become methylated, which facilitates tumorigenesis and development of cancer [188]. Though SAM levels were not especially elevated in MIEN1-KO cells, this may not be an indication of the SAM levels shortly after deletion of the *MIEN1* gene. Knockout of MIEN1 may have prompted SSP flux to alter gene expression and contribute to adaptation. Another interesting finding revealed that SSP flux may help resist anoikis in breast cancer cells

[78]. Anoikis is a mode of programmed cell death that is brought about by detachment of cells from ECM proteins [189]. Integrins form a link between the ECM and the cytoskeleton and provide pro-survival and other signals to the cell [189-191]. Overcoming this dependence on cellular contact with surrounding tissue is essential for tumor progression and especially metastasis as cells must not only invade through tissue with limited attachment, but also survive completely devoid of attachment in circulation [192]. Cancer cells utilize the EMT program to alter gene expression and signaling within the cell to combat this reliance. MIEN1 has already been connected with anoikis resistance in prostate cancer cells [163]. Transfection with microRNA-940, which targets MIEN1 transcripts for both degradation and translational repression, reduced anchorage-independent growth of these prostate cancer cells. Also, E-cadherin was increased following microRNA-940 treatment, while vimentin was decreased, indicating that perhaps MIEN1 functions in maintaining a mesenchymal phenotype and aids in anoikis resistance. Due to loss of MIEN1 through genomic deletion, 4175 cells may have responded by increasing SSP flux to counteract reversion back to a more epithelial-like state. More work is necessary to understand the role of MIEN1 in EMT and cell survival in breast as well as prostate cancer.

In agreement with maintenance of migration and invasion in 831, 1833, and 4175 cells upon MIEN1 deletion, known downstream MIEN1 signaling targets revealed no change in activation. Neither phosphorylation of Akt at S473 nor phosphorylation of FAK at Y925 were significantly altered following MIEN1-KO, indicating that adaptation led to alternative means of maintaining activation of these signaling mediators. However, though not statistically significant, Akt phosphorylation at S473 was slightly reduced in 1833-MKO cells, which may also be due to the lower number or clones used to generate this cell line. Together, this suggests

that compensatory mechanisms exist in all three genetic contexts which are able to overcome loss of MIEN1 and maintain these signaling pathways.

Spheroid growth was unaffected following MIEN1^{WT} or MIEN1$^{Y39/50F}$ proteins being introduced into 4175-MKO cells. This confirms that MIEN1 does not seem to play a role in cell proliferation and spheroid growth, at least in the context of lung metastatic TNBC. However, when spheroids of 4175-MKO cells expressing MIEN1$^{Y39/50F}$ were embedded in Matrigel, invasion was significantly reduced compared to all other cell lines. This finding, combined with the observation of Akt and FAK activation maintenance in 4175-MKO cells expressing MIEN1$^{Y39/50F}$ indicates MIEN1 involvement in pathways not previously reported. In spite of compensatory mechanisms activating alternative signaling within these cells to overcome the loss of MIEN1 and maintain signaling, migration, and invasion, dysfunctional MIEN1 is still able to interfere with signaling pathways and significantly decrease cellular invasion.

Overall, this study demonstrates that in breast cancer cells, MIEN1 function is dependent upon genetic context. This study also shows that complete loss of MIEN1, which can alter intracellular signal transduction and global metabolism, is not deleterious to breast cancer cells as they are able to compensate for the injury and maintain migratory and invasive potential. Furthermore, though these breast cancer cells were able to overcome knockout of MIEN1, they remained susceptible to mutant MIEN1 which interfered with signaling cascades responsible for invasion. These data necessitate further study to understand the significance of the relationship between MIEN1 and genetic context and elucidate the alternative signaling pathways which are activated upon loss of MIEN1. Future studies will enable a more accurate understanding of the consequence of MIEN1 expression in patient tumors and provide valuable insight into potential therapies which target metastatic propensity of breast cancer cells.

Acknowledgements

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CHAPTER IV

FINAL DISCUSSION, SUMMARY, AND CONCLUSIONS

Metastasis is a complex process which requires many effectors to initiate, propagate, and culminate this event in the development of a new tumor in a distant site of the body. There are many roadblocks to this process, which prevent the majority of disseminated cells from forming satellite tumors [193]. It is estimated that only 0.01% of cells which leave the primary tumor are able to form metastases [194, 195]. Cancer cells gain new abilities and characteristics through mutations, deletions, and amplifications and gene regulation [23-26]. All facets of tumor growth are affected by these changes; however, as these changes accumulate, they initiate and sustain dissemination and metastasis. Many genes have been identified which lead to and/or facilitate metastatic events. MIEN1 has been identified as an important mediator of signaling cascades responsible for directing gene expression and cytoskeletal dynamics and reorganization. A much clearer understanding of MIEN1's functional significance and mechanisms of action within the migration, invasion and metastasis of cancer cells is still needed. Studies addressing this gap in knowledge could help to establish MIEN1 as a useful protein for predicting metastatic potential of early diagnosed patients or forecasting prognosis for late-stage cancers. Greater insights into MIEN1 mechanistic function can lead to novel targeted therapeutics against MIEN1 in combination with standard treatments that could reduce metastatic burden in patients.

CRISPR knockout of MIEN1 in cancer cells is an effective tool for understanding the role of MIEN1 within various contexts. However, it is important to ensure that the correct research question is asked when using CRISPR for genomic alterations so the observed results are interpreted accurately. Since the discovery of CRISPR and its adaptation and implementation into research in mammalian cells earlier this decade, debate has surfaced regarding CRISPR's benefits compared to RNAi. It is important to note that none of the alterations made by RNAi are permanent. Specifically, RNAi only impacts translation, whereas transcription is not interrupted. This means that upon depletion of siRNA or removal of the stimulus for inducible shRNA, cells may recover from the short interruption in protein production.

The transient nature of RNAi lends itself to the analysis of short-term adaptive responses in cells. This has enabled researchers to tease out various mechanisms and pathways through brief knockdown of protein expression. However, this method does not examine the long-term effects of removing a protein from the system and the response generated by the cells as they must adapt and compensate for complete loss of the protein. Even in cells with constitutive expression of shRNA molecules, low levels of protein may still persist and function, leading to inconclusive or false results. CRISPR instead may be the technology of choice for investigating the long term dependency on the expression of individual proteins or combinations of proteins. CRISPR genome-editing results in irreversible elimination of protein expression and forces the cells to respond and adapt for a longer period of time. This forced adaptation can lead to permanent gene expression changes and altered signaling within the cells.

Genome editing by CRISPR can provide insight into the progression of various cancers as the adaptive response to genetic lesions can be studied. These studies could enable a better

understanding of tumor development and metastasis as the relationship between loss of specific genes and propensity toward these cancer processes can be examined. Due to the different responses of cells to RNAi and CRISPR, it is no surprise that some studies utilizing siRNA show different results when compared directly to CRISPR. Maternal Embryonic Leucine Zipper Kinase (MELK) was previously identified by RNAi to be required for proliferation of a variety of cancers, including TNBC [196-198]. MELK inhibitors were even advanced to clinical trials based on these RNAi studies [147]. However, subsequent CRISPR based studies of MELK have invalidated previous studies which indicated cancer dependency on MELK for proliferation and other cellular processes [199, 200]. This epitomizes the major difference between these two technologies and demonstrates the importance of ensuring a robust research strategy and selection of the appropriate tool. Though CRISPR and RNAi phenotypic outcomes do not always corroborate each other, there are instances where they are show similar effects [144].

Because of the selective pressure and changes induced by CRISPR knockout, it is conceivable that this technology might be utilized in the investigation of combination or sequential therapy in cancer. It is well established that drugs administered in combination are more effective than monotherapies in the treatment of cancer [201-203]. Co-administration of CRISPR and therapeutics with different targets may provide insight into the relationship between the gene targeted by CRISPR and the treatment. These studies could identify genes which are required for resistance to certain treatments in various cellular contexts [204]. Also, CRISPR may be used to understand how prolonged treatment targeting a specific protein, as in antibody therapy, may alter the tumor biology. CRISPR can artificially simulate long term treatment and allow for a platform to identify how this may infer chemoresistance or susceptibility to other treatments.

In Chapter III, the result of CRISPR knockout of MIEN1 in the context of several metastatic breast cancer cells lines was evaluated. MIEN1-KO did not produce the hypothesized result of reduced migration and invasion; however, this lesion may have produced an altered susceptibility to therapeutics due to other changes induced by deletion of *MIEN1*. This is an important consideration to keep in mind in the event that treatments are designed against MIEN1 in the future. Due to the absence or low expression of MIEN1 in normal tissues, targeting MIEN1 could reduce side-effects often associated with chemotherapeutic drugs [107].

Established MIEN1 function involves MIEN1 signaling through Akt and FAK in order to affect pro-metastasis gene expression and cytoskeletal dynamics. These pathways culminate in migration and invasion of breast, prostate, and oral cancers (Fig 1A) [115-117]. MIEN1-KO alters cellular signaling and results in adaptation to maintain FAK and Akt signaling (Fig 1B). MIEN1$^{Y39/50F}$ expression in MIEN1-KO cells indicates that in addition to these pathways, alternative pathways, which may be downstream from Akt and/or FAK, are activated and regulate cellular invasion since FAK and Akt signaling are maintained despite inhibition of invasion (Fig 1C). It is interesting that these signaling targets of MIEN1 are unable to overcome the decreased invasion incited by MIEN1$^{Y39/50F}$ expression. This may also point to Akt and FAK perhaps being further downstream from MIEN1 than previously thought or the effector pathway of migration and invasion being downstream from Akt and FAK. Another possibility for altered cellular invasion despite maintenance of Akt signaling could be the alternative activation of Akt isoforms. Akt is expressed as three isoforms, which can each by activated by distinct mechanisms and are able to phosphorylate isofom specific targets, despite having similar serine residues required for activation [92]. Akt1 and Akt2 have both been investigated in migration and invasion of cancer cells, but it has been shown that cellular context is the key to determining

the effect of activation of specific isoforms [205-211]. MIEN1's ability to regulate activation of individual isoforms and its relation to cellular context is unknown.

These findings indicate a potential updated mechanism of MIEN1 function (Fig 1D). In addition to FAK and Akt, it is proposed that MIEN1 may also mediate migration and invasion through alternative pathways which have yet to be identified. Also, the lack of altered migration and invasion following MIEN1-KO shows MIEN1 may be somewhat dispensable for these processes; however, MIEN1 may be instrumental in other aspects of metastasis not tested herein. MIEN1 has been shown to regulate extravasation and colony formation in prostate cancer cells [108]. Also, MIEN1 directly interacts with the pro-migratory protein Annexin A2 and enhances its cell surface expression [113]. MIEN1 may regulate other cell surface proteins which enable interaction with surrounding cancer cells, normal tissue and endothelial cells. This was further indicated by the disintegration of 1883-MKO cells following extended spheroid culture. MIEN1 may specifically regulate cell-cell adhesion proteins in bone metastatic breast cancer. This indicates that MIEN1-KO may prove to be more deleterious in the wider context of metastasis as a whole than is fully demonstrated in this study.

Finally, MIEN1 has not yet been connected to cancer cell metabolism; however, many metabolites were altered in 4175-MKO cells compared to 4175 cells, most notably, several members of the SSP. This finding suggests that knocking out MIEN1 has far reaching effects on 4175 cells and also demonstrates the functional significance of MIEN1 may be more extensive than previously understood.

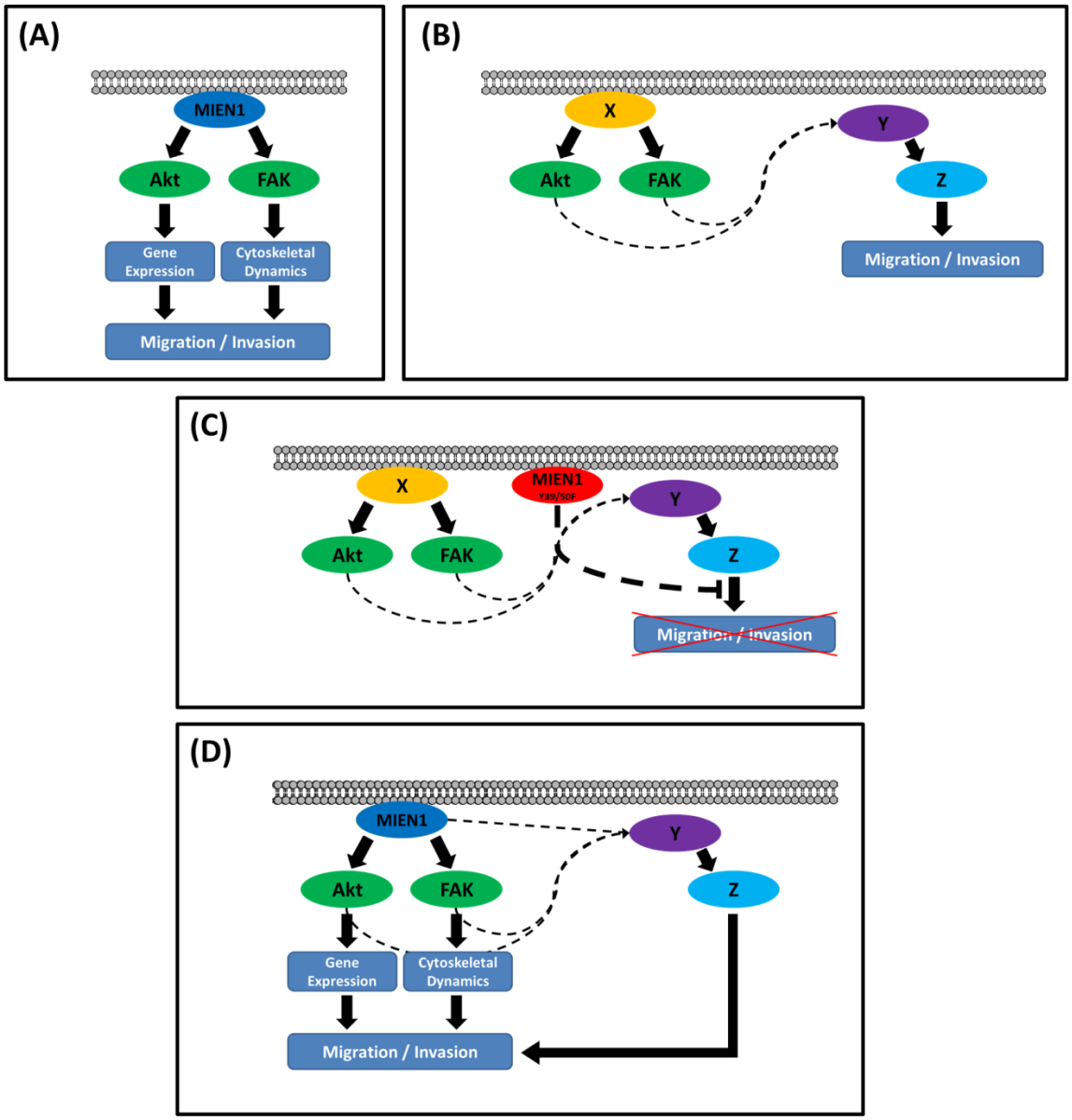
This dissertation further contributes to the discussion about CRISPR genome editing, its comparison to siRNA, and its applications, especially in the study of MIEN1. Generation of MIEN1-KO cells produced no observable phenotype changes when migration and invasion were

investigated, indicating that MIEN1 is not required for these cellular functions. However, the lack of inhibition of migration and invasion in MIEN1-KO cells did not equate with a lack of effect of MIEN1-KO on these cells. Alterations accumulated within MIEN1-KO cells due to the selective pressure of both the clonal generation process and loss of MIEN1 protein. These changes were evidenced by the differences observed in metabolism resulting in increased SSP flux. In addition, expression of MIEN1$^{Y39/50F}$ ITAM mutant protein in 4175-MKO cells did not affect known MIEN1 signaling pathways, but did significantly inhibit invasion. This suggests MIEN1 is involved in alternative signaling pathways which have not yet been identified, or that MIEN1-KO instigated compensatory invasion pathways, which mutant MIEN1 is able to obstruct.

Figure 1

MIEN1 mechanism. (A) Current understanding of MIEN1 signaling and function within cancer cell migration and invasion. MIEN1 facilitates the activation of Akt and FAK, which leads to gene expression and altered cytoskeletal dynamics in order to propagate cellular migration and invasion. (B) Proposed adaptive response following MIEN1 deletion from breast cancer cells. Breast cancer cells are able to activate compensatory mechanisms ("X") to maintain Akt and FAK signaling after MIEN1 knockout. These cells may also have adopted alternative signaling pathways to regulate cellular migration and invasion, which may be downstream of Akt and FAK ("Y" and "Z"). (C) Re-expression of MIEN1 ITAM mutant. Following overexpression of MIEN1$^{Y39/50F}$ ITAM mutant protein, Akt and FAK signaling are maintained. However, invasion of cancer cells is significantly inhibited. (D) Proposed model of MIEN1 function. MIEN1$^{Y39/50F}$ expression indicated that MIEN1 may be involved in additional signaling pathways which regulate cellular motility. In addition to Akt and FAK, MIEN1 activates a yet unknown, separate pathway which also leads to increased migration and invasion.

Figure 1


(A)
MIEN1
Akt
FAK
Gene Expression
Cytoskeletal Dynamics
Migration / Invasion
(B)
X
Y
Akt
FAK
Z
Migration / Invasion
(C)
X
MIEN1
Y39/50F
Y
Akt
FAK
Z
Migration / Invasion
(D)
MIEN1
Y
Akt
FAK
Z
Gene Expression
Cytoskeletal Dynamics
Migration / Invasion

CHAPTER V

FUTURE DIRECTIONS

In this dissertation, the functional significance of loss of MIEN1 in breast cancer cells was examined. The studies suggest dependence upon MIEN1 for cancer cell functions is determined by the genetic and proteomic context. Future studies should focus on understanding how genetic context influences MIEN1 function to determine if there are certain cancers or cancer subtypes which are not affected by MIEN1 expression.

Cancer cells are notorious for adaptation and reprograming. Although there was no reduction of invasion of metastatic derivative cell lines following MIEN1-KO, this study revealed that long term depletion of MIEN1 in these cell lines led to context dependent reduction in migration and global metabolism changes. Also, introduction of MIEN1$^{Y39/50F}$ ITAM mutant protein into 4175-MKO cells revealed a hindrance of alternative signaling pathways which were activated to maintain migratory and invasive phenotypes in MIEN1-KO cell lines. It will be important to discern these compensatory mechanisms to enable identification of potentially druggable pathways to target in tandem with already established MIEN1-involved pathways.

Much of the function of MIEN1 has been attributed to the ITAM domain. Phosphorylation of the two tyrosine residues within the MIEN1 ITAM has even been shown to have transformative properties within normal breast epithelial cells [111]. Though the importance of this domain is apparent from the current literature, the genetic context of MIEN1 ITAM function still requires further study. Also, the kinase(s) responsible for MIEN1

phosphorylation and activation is not known. Further studies should explore MIEN1 activators in multiple cancer types to determine if there is a common activator, or if this too is context dependent. Further, the contribution of the redox-active motif to the activation and function of the ITAM domain is not known.

MIEN1 requires binding partners in order to mediate its function within the cell. Some proteins have been reported to interact with the redox-active motif [109, 110], however, the functional significance of these interactions and its relation to cancer cell biology is not known. ITAM signaling requires binding of SH2 containing proteins which can then become activated and mediate downstream signaling. Binding to activated ITAM motifs is determined by the sequences around the tyrosine residues [212]. This also appears to be dependent upon cellular context as Syk has been implicated in mediating MIEN1 function in mammary epithelial cells and ER^+/PR^+ breast cancer cells [111]. However, the effector of MIEN1 signaling in TNBC is likely different as Syk overexpression decreased migration in 231 cells by inhibiting PI3K and NFkB activity [213, 214]. Identifying novel interactors and effectors of MIEN1 in TNBC and other cancer types will be important areas of study to show precisely how MIEN1 influences migration and invasion of these cells.

Re-expression of $MIEN1^{Y39/50F}$ in 4175-MKO cells revealed that this mutant protein was able to inhibit the invasive capacity of these cells even after adaptation, which points to MIEN1 as a potential therapeutic target. Due to the low expression of MIEN1 in normal tissues, targeting MIEN1 could reduce side-effects often associated with chemotherapeutic drugs [107]. It would seem that inhibitors which alter MIEN1 ITAM mediated signaling might be efficacious and reduce the migration and invasion of cancer cells. It would appear to be important to ensure that these drugs targeted the ITAM domain, without affecting expression or cellular localization

as MIEN1-KO showed no phenotypic response. This certainly requires further study as no inhibitors of MIEN1 have been produced to date. Additionally, it would be beneficial to examine the effect of MIEN1-KO on chemoresistance in these breast cancer cells. Treatment with standard chemotherapeutics may reveal that the forced adaptation incurred by MIEN1-KO may increase susceptibility to these drugs. Other drugs which target actin filaments and reduce cellular motility may also have increased efficacy following MIEN1-KO. These studies could lead to a basis for combination treatment including MIEN1 ITAM inhibitors.

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