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A Mechanistic Investigation of Insulin Receptor Substrate 2 Function in Breast Cancer Progression

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A MECHANISTIC INVESTIGATION OF INSULIN RECEPTOR SUBSTRATE 2 FUNCTION IN BREAST CANCER PROGRESSION

A Dissertation Presented

By

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Dedication

I would like to dedicate this work to my brother, Raul Mercado-Matos. His life has been the inspiration for this work. During his battle against cancer, my bother showed me the meaning of courage and optimism. His endless love and wisdom will always live with me.

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ABSTRACT

The advancement of cancer treatment depends on understanding the biological processes that contribute to disease progression. The spread of tumor cells from the primary site to distant organs is the biggest obstacle to efficacious treatment. The insulin receptor substrate (IRS) proteins IRS1 and IRS2 are cytoplasmic adaptor proteins that organize signaling events downstream of the Insulin receptor (IR) and the Insulin-like growth factor receptor 1 (IGF1R). Both of these receptors have been implicated in cancer progression. The IRS proteins share a significant level of homology and are both capable of recruiting and activating phosphatidylinositol-3 kinase (PI3K). Despite these similarities, signaling through IRS1 and IRS2 leads to distinct tumor cell outcomes *in vitro* and *in vivo*. *In vitro*, IRS1 regulates cell proliferation and growth and IRS2 regulates metabolism, survival and invasion. *In vivo*, Irs2 is a positive regulator of tumor metastasis, whereas Irs1 does not promote metastasis. The major objective of this thesis work was to further the understanding of the mechanism by which IRS2 signaling regulates tumor progression.

To investigate how IRS-1 and IRS-2 regulate distinct tumor cell outcomes, I examined the involvement of the microtubule cytoskeleton in IRS-dependent signaling. I determined that IRS2-mediated AKT activation is dependent upon an

intact microtubule cytoskeleton, whereas IRS1-mediated AKT signaling occurs independently of microtubules. As a result, drugs that disrupt microtubules promote apoptosis in cells that signal through IRS2, but cells that signal through IRS1 are resistant to the effects of microtubule disruption. However, AKT inhibition sensitizes IRS1-dependent cells to apoptotic cell death upon microtubule disruption. From a clinical perspective, my studies identify IRS2 as a potential biomarker for the response of breast cancer patients to anti-microtubule drug therapy. To investigate further the mechanism of IRS2 contributions to tumor progression, I employed a mutagenesis approach to identify structural requirements of IRS2 for its function. I established that the ability of IRS2 to activate PI3K is necessary for its regulation of both invasion and tumor initiating cell (TIC) self-renewal. I also identified two independent regions within the IRS2 C-terminus that are required for invasion and self-renewal, respectively. Characterization of the invasion-promoting region identified BMP2-induced protein kinase (BMP2K) as an interacting protein. Suppression of BMP2K expression in mammary tumor cells disrupts IRS2-mediated tumor cell invasion. Taken together, my work advances the understanding of how IRS2 contributes to breast cancer progression and provides a molecular understanding for the development of novel approaches for the treatment of breast cancer and other malignancies that rely upon IRS2.

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CHAPTER I

Introduction

Breast Cancer Epidemiology and Implications

Breast cancer is one of the most common malignancies affecting women in the USA, second only to skin cancer. Every year 250,000 new cases of invasive breast carcinoma are diagnosed, which accounts for 29% of the malignancies affecting women. In addition, 60,000 new cases of breast carcinoma in situ (lobular and ductal), pre-malignant lesions that increase the risk of developing breast cancer later in life, are diagnosed every year [1, 2]. Current screening methods such as mammography have significantly improved early detection of breast cancer and have helped advance successful treatment at early stages of the disease [3]. The use of surgical approaches, chemotherapy and radiation have been instrumental in the effective treatment of early stage breast cancer. Early detection of breast cancer is of extreme importance when it comes to effective treatment. Stages I and II, representing localized disease, have a 99% five-year survival rate, while stages III and IV, representing metastatic breast cancer, have a significant decrease in five-year survival at 73% and 23% respectively [4].

Breast cancer is a heterogenous disease that can be divided into five major molecular subtypes. These subtypes are classified as basal-like, ErbB2-overexpressing, luminal A and luminal B and normal breast tissue-like [5]. Survival analysis of each subtype shows different outcomes; Basal like breast cancers have a poor prognosis while estrogen receptor (ER) positive luminal

breast cancers have variable outcomes [6]. Although originally the ErbB2 subtype had poor outcomes, the discovery of targeted therapies against the ErbB2 receptor improved disease free survival for this subtype [7]. Many basal-like breast cancers lack the classic three markers ER, progesterone receptor (PR) and HER2, used in the histopathologic classification of breast cancer, and these breast cancers are classified as triple-negative breast cancers (TNBCs) [8]. TNBCs are highly aggressive and commonly diagnosed in a younger population [9]. This type of breast cancer has a very high tendency to metastasize and patients have an increased risk of relapse in comparison to patients with other molecular subtype tumors [9].

For cancer patients, metastasis is the leading cause of cancer related mortality. Annually, an estimated 40,000 women succumb to metastatic breast cancer [1, 10]. Common sites of breast cancer metastasis include regional lymph nodes, bone, brain, lung, liver and adrenal gland. The spread of breast cancer to distant organs represents a significant limitation to treatment. Metastatic disease selects for a more aggressive phenotype and is resistant to standard therapies, making treatment more difficult. Current treatment modalities for metastatic breast cancer include cytotoxic chemotherapies, however these drugs cause many side effects and provide little clinical benefit to patients [3, 4].

Despite a greater understanding of breast cancer in the past ten years, there has been no significant progress in the treatment of metastatic disease [11]. A randomized clinical trial over a period of 30 years showed no evidence of increased survival in patients with metastatic breast cancer [12]. Although we have made strides in the treatment of local disease, we need to pay attention to understanding how cancer progresses in order to better treat metastatic disease. Better understanding of processes that contribute to tumor progression will help the cancer community design more effective approaches for the treatment of advanced stage disease.

Cancer Metastasis: Overview of the Field

Despite all of the advances in clinical oncology and basic cancer research, metastasis continues to be a lethal trait of cancer. Metastasis is the process during which cancer cells leave the primary tumor and travel to distant organ sites to give rise to a secondary tumor. Metastasis is a multi-step process that consists of local invasion of tumor cells into adjacent stromal tissue, trans-endothelial migration into blood or lymphatic vessels, survival in the circulatory system, extravasation from the vessels and colonization at distal sites [13]. The process of colonization is very inefficient and is a rate limiting step for metastasis [14, 15]. Millions of cells can escape the primary tumor site but less than 0.1% of disseminated cells will give rise to a secondary tumor at a distant site [14, 15]. In

some cases, cells that reach distal organs remain in a state of growth arrest known as dormancy. However, the eventual growth of these cells will disrupt proper organ function. There are many complicating factors in the research of cancer progression and the onset of metastasis including the heterogeneous nature of tumors and multiple mechanisms implicated in the metastatic cascade. Understanding metastasis requires the dissection of molecular mechanisms that contribute to each step of the process.

Tumor cell invasion

Tumor cell invasion is essential for allowing cancer cells to move through non-permissive tissue barriers that normally restrain cells to specific locations. The ability of cancer cells to invade is required at many steps in the metastatic cascade: invasion through the basement membrane, through the extracellular matrix that surrounds the tumor and through the basement membrane supporting the blood vessels to both intravasate and extravasate [16]. Due to the complex nature of this process a better understanding of molecular mechanisms and the events that contribute to invasion can contribute to better treatment of cancers.

Cancer cells gain the ability to invade into the surrounding tissue by activating pathways used during development and wound healing. This is one of the reasons cancer is known as a “wound that never heals”. One process that

controls the regulation of a number of pathways is referred to as the epithelial to mesenchymal transition (EMT). During EMT, tumor cells downregulate epithelial markers like E-Cadherin resulting in the loss of cell adhesion junctions and cell-to-cell contact [17, 18]. In addition, cancer cells upregulate a host of mesenchymal markers, such as Vimentin or N-Cadherin, which result in the reorganization of the cytoskeleton and enhancement of cell motility [17]. Many studies have implicated the EMT as a driving force in breast cancer progression. In ER+ breast cancer cells, suppression of transforming growth factor-β (TGFβ) signaling inhibits the EMT, resulting in cells that are less invasive [19]. It has also been demonstrated that expression of TGFβ receptor I (TβIR) causes activation of EMT and induces migration and invasion of cancer cells [20]. In addition to the TGFβ signaling pathway, other developmental pathways that are dysregulated in cancer, like the Wnt and Notch signaling pathways, have been implicated in the induction of EMT and an increase in invasion [21]. In addition to its involvement in tumor cell invasion, EMT is also implicated in maintenance of tumor initiating cells, prevention of apoptosis and senescence, and resistance against chemotherapy [22, 23]. The involvement of EMT in so many biological processes implicated in cancer suggests that EMT plays a very important role in tumorigenesis and is a driver of metastasis.

While EMT has been shown to enhance a tumor's ability to metastasize, the principle that EMT is required for tumor metastasis is currently being challenged. It has not been definitively shown that all cells that have achieved the formation of

secondary tumors at a distant site have undergone EMT [24]. Several transgenic mouse models have been developed that suggest that EMT is not a requirement for invasion and metastasis *in vivo*. One group used the Polyoma middle T antigen under the mouse mammary tumor virus promoter (MMTV-PyMT) transgenic mouse model to irreversibly express green fluorescent protein (GFP) upon the induction of EMT by the activation of the *Fsp1* promoter [25]. Results from this study showed that cells that successfully formed lung metastases were GFP negative, suggesting that they did not undergo EMT. In a similar effort to investigate the role of EMT in metastasis, expression of the EMT transcription factors ZEB1 and ZEB2 was inhibited by expression of micro-RNAs in MMTV-PyMT derived cells. Inhibition of these transcriptions factors and the inability of cells to undergo the EMT failed to block metastasis upon orthotopic injection of cells into mice [24, 25]. These experiments provide evidence that there are alternative mechanisms to EMT that can aid carcinoma cells in invasion from the primary tumor.

To further understand tumor cell invasion, we need to understand the mechanism of invasion. There are two models for single cell invasion, the mesenchymal and the ameboid models [26]. Cells from connective tissue sarcomas or poorly differentiated carcinomas invade using the mesenchymal model. This model is characterized by the formation of focal adhesions, actin cytoskeleton rearrangement, integrin clustering and protease dependent ECM

degradation. In the mesenchymal model, the leading edge of invasive cells has a high concentration of focal adhesions [27]. These focal adhesions act as the recruitment sites for matrix metalloproteases and targets the degradation of ECM to places that will enhance invasion by the cancer cells [28]. In contrast, the amoeboid cell invasion model is characterized by a more diffuse cytoskeleton and less dependence on cell-matrix interactions [27]. This type of invasion is more common in low grade carcinomas. These cells squeeze through the matrix using small blebs present on the cell surface [29, 30]. The blebs are characterized by a low concentration of β1 integrins and reduced focal adhesions [31]. Due to the low dependence on focal adhesions, cells can move faster than cells that rely on mesenchymal invasion. Tumor xenograft studies using breast carcinoma cells have demonstrated that breast cancer cells predominantly invade using the amoeboid model, however some cancer cells can employ both models [32, 33].

Tumor cells can also invade as part of a multicellular cohort [28, 34-36]. This collective invasion mechanism relies upon tumor heterogeneity or tumor stromal cells in assisting the cancer cells to escape the tumor [37-40]. Tumors are composed of an array of cells that form a heterogenous tissue ranging from epithelial to mesenchymal characteristics. Mesenchymal cells with a higher invasive potential can help less invasive cancer cells overcome the limitations imposed by the primary tumor environment and invade the surrounding tissue [37]. In organoids derived from mouse mammary tumors, the more invasive cells can

invade the surrounding matrix and pull other epithelial cells with them [35, 41]. Additionally, stromal cells like fibroblasts can assist epithelial tumor cells to escape the primary tumor by degrading the extracellular matrix [38, 42]. These observations demonstrate that the tumor microenvironment and its cellular constituents can have an important role in the ability of tumor cells to invade the surrounding tissue and metastasize.

The tumor microenvironment is composed of both cellular and noncellular components and consists of ECM, fibroblasts, macrophages, neutrophils, lymphocytes, adipocytes and the blood and lymphatic vascular networks [43]. There is extensive evidence that components of the tumor microenvironment play a role in many aspects of tumorigenesis and tumor progression. The ECM plays a very important role in the ability of cancer cells to invade, mainly in its function as a barrier. One of the steps for tumor cells invasion, especially in the mesenchymal model, is the degradation of the ECM, so cells can move into the surrounding tissue [44, 45]. Matrix metalloproteases, enzymes that can break down and degrade ECM proteins, have been viewed as a good target for inhibiting invasion. However, upon inhibition of these proteases, cancer cells employing a mesenchymal model of invasion were able to switch to the amoeboid mode to overcome the protease inhibition [28, 46, 47]. Extensive phase III clinical trials of metalloprotease inhibitors not only failed, but in some cases worsened the disease [48, 49]. The use of metalloprotease inhibitors provided evidence that increased

tumor ECM and the stiffness of the ECM impacts invasion by allowing for the transition of cells from the mesenchymal to amoeboid model of invasion and enhanced tumor cell invasion.

In addition to the non-cellular components of the tumor microenvironment, normal stromal cells also take part in the regulation of tumor cell invasion. A stromal cell extensively implicated in the progression and metastasis of mouse mammary tumor cells is the macrophage [50]. Ablation of macrophage infiltration in mammary tumors does not impact primary tumor growth, but significantly inhibits tumor metastasis. The paracrine signaling of macrophage colony stimulating factor-1 (CSF-1) is required for human breast tumor metastasis *in vivo* [51]. Expression of the CSF-1 receptor (CSF-1R) in ovarian, endometrial and breast carcinomas has been associated with adverse clinicopathological outcomes [52-54]. *In vivo* imaging revealed that macrophages in breast tumors facilitate invasion by paving the way for breast tumor cells to reach endothelial cells where tumor cells can intravasate [39, 55]. These data suggest that macrophages play a significant role in the metastatic process of breast cancers.

Recent studies have implicated the importance of adipocytes and their role in tumorigenesis and cell invasion. Exposure of ER+ and TNBC cells to media conditioned by mouse and human adipocytes or direct co-culture with adipocytes enhanced the proliferation, migration and invasion of the cancer cells [56-58]. The

major adipokine released from adipocytes, adiponectin has been shown to stimulate migration and invasion in MDA-MB-231 cells upon incubation of cells with this hormone [59]. It is important to recognize the role adipocytes can play in tumorigenesis due to the current obesity epidemic in the country. Obesity is a risk factor for breast cancer and other malignancies and also predicts poor outcomes. This could play a role in tumor formation and progression, as well as affect the treatment of cancer.

Chemical changes to the tumor microenvironment can also impact tumor formation and progression. Alterations to the O_2 availability in the tumor has a profound effect on tumor cell invasion. As tumors grow at an accelerated pace they can surpass their capacity to obtain a proper blood supply, which in combination with excessive growth creates a hypoxic environment [60]. In human breast tumors *in situ* measurement of oxygen pressure (PO_2) revealed a pressure of 10 mm Hg while surrounding normal breast tissues have a PO_2 of 65mm Hg [61, 62]. Prolonged hypoxia can lead to regions of cell death as seen in advanced solid tumors [63]. The low oxygen level leads to gene regulatory changes mediated by the hypoxia-inducible factors (HIF), a protein complex composed of an O_2 regulated alpha subunit and a constitutively active HIF-1β subunit. The alpha subunits are subjected to constitutive ubiquitination and degradation that is only inhibited under hypoxic conditions [64]. Data from immunohistochemical studies performed in human tumor samples linked elevated levels of HIF-1α with increased

risk of metastasis [65]. This implicates a decrease in tumor oxygen tension with a more aggressive tumor behavior.

One of the main roles of HIF-1α is to enhance blood vessel formation in a tumor by transcription of the vascular endothelial growth factor (VEGF) gene [66]. It has been shown that knockout of HIF-1α in the MMTV-PyMT mouse model impairs tumor growth, vascularization and metastasis [67, 68]. In an orthotopic study using the breast cancer cell line MDA-MB-231, cells expressing shRNA against HIF-1α and HIF-2α were injected into mammary fat pads. Both knockdowns demonstrated a decrease in tumor lymphatic vessel density and metastasis to the regional lymph nodes [69]. Other studies using the same model demonstrated that injection of HIF-1α knockdown MDA-MB-231 cells can diminish the colonization of bone metastases [70]. Additionally, orthotopic injection of MDA-MB-231 HIF-1α knockdown cells into immunodeficient mice showed a decrease in spontaneous lung metastasis and decreased metastatic burden [71]. These data taken together suggest that the ability of cells to activate the HIF transcription factors under hypoxic conditions can result in increased metastasis.

Studies of tumor angiogenesis inhibition have contributed to the understanding of the impact of hypoxia on tumor cell invasion. In the pancreatic condtional β-VEGF knockout mice tumors develop regions of intense hypoxia. These regions of hypoxia correlate with an increase in invasion, and an increase

in metastasis [68, 72]. Also, in the pancreatic cancer model RIP1-Tag2 mice, treatment with anti-VEGFR2 antibodies or the VEGFR inhibitor sunitinib causes hypoxia in tumors, increases local invasion at the primary tumor site and increases metastasis [73]. Responses to VEGF inhibition in clinical trials have not been as predicted with regard to outcomes. Inhibition of this pathway has resulted in decreased time to progression, followed by resistance to therapy [73]. Many of the patients undergoing this therapy develop metastatic disease. The induction of tumor hypoxia contributes to a more invasive phenotype in these tumors and increases their metastatic potential.

Hypoxia can also impact tumor invasion by increasing the ECM stiffness by enhancing collagen hydroxylation. Studies investigating elevated fibrillar collagen in primary breast tumors revealed that increases in tumor fibrillar collagen are associated with recurrence and increased mortality [42]. The mechanism by which hypoxia increases tumor fibrillar collagen was studied in MDA-MB-231 cells. Under hypoxic conditions MDA-MB-231 cells have increased expression of prolyl-4-hydoxsylase enzymes, which are responsible for the generation of fibrillar collagen [74, 75]. Injection of MDA-MB-231 cells with prolyl-4-hydroxylase knockdown resulted in diminished tumor growth and a complete inhibition of lung metastasis [75]. These findings suggest that hypoxic environments not only increase the machinery for new formation of blood vessels to facilitate tumor cell extravasation, but also enhance tumor ECM stiffness by increasing collagen I

formation in tumors. A stiff environment has been implicated in enhanced invasion suggesting that hypoxia plays a role in creating the ideal environment for cancer cells to escape the primary tumor.

Tumor-initiating cells and their contribution to metastasis

In a cancer patient, millions of tumor cells escape into the circulatory system, but a very limited number of those cells can give rise to a tumor at a distant site [76]. Based on the “seed and soil” theory by Paget, the development of secondary growth depends on organs with the right growth conditions, only the proper soil will allow the growth of the newly resident cells [14, 77]. In the case of breast cancer, the major sites for metastasis are the bones, the lungs, the liver, the adrenal grands and the brain [76]. Even if tumor cells can travel to all of the organs in the human body, they may encounter conditions that restrict growth and/or survival and the formation of a secondary tumor. Upon arrival at a distant organ, many of the cells will become dormant until the proper conditions develop to allow growth again.

A conventional model of cancer propagation argues that all cells from a tumor have the potential to give rise to a metastatic tumor based on their ability to enter the cell cycle [78]. As we are familiar with the heterogeneous nature of tumors, not all cells in a tumor are created equal and only a limited number of cells

can initiate a tumor. The heterogeneity of tumors takes place due to the hierarchical nature of tumors with a population of cells known as the cancer stem cells (CSC), or as I will refer to them hereafter tumor-initiating cells (TICs), and transit amplifying cells that give rise to the bulk of the tumor [79-81]. Discoveries in the leukemia field first pointed to a small subset of cells as responsible for the propagation of the disease [82-84]. This subset of cells can be identified by cell surface markers, in the case of leukemia those markers are CD34+CD38- [84]. Serial transplantation of the CD34+CD38- cells demonstrates enhanced tumor formation activity in comparison to other tumor cells, and the ability of these TICs to self-renew. Self-renewal is the ability of these cells to maintain the TIC population while giving rise to differentiated cells that comprise the heterogeneous tumor [85].

In human breast cancer, there is a population of cells capable of giving rise to new tumors upon transplantation. Breast cancer cells isolated from human tumors using the markers $CD44^{+}CD24^{-/low}Lin^{-}$ have an increased tumor forming ability in NOD/SCID mice [86]. Serial transplantation of cells derived from tumors formed by $CD44^{+}CD24^{-/low}Lin^{-}$ cells can form heterogeneous tumors that mimic the original tumor [86]. Murine models of mammary cancer have also been tested for the identification of tumor-initiating cells. In the MMTV-Wnt1 model the THY+CD24+ cells were found to have enhanced tumor activity [87]. In the TP53-null mammary model $\beta1integrin^{hi}CD24+$ had the same tumor forming potential

[88]. Furthermore, work *in vitro* using the TNBC cells SUM149, MDA-MB-468 and MDA-MB-231 revealed a population of cells in each cell line with the markers CD44+CD49f+ that have tumor-initiating potential [89]. These cells not only possess enhanced self-renewal ability but also show increased resistance to the drug paclitaxel [89]. TIC populations represent a limitation to conventional treatment because they tend to be more resistant to therapeutic interventions and are responsible for the recurrence of cancers. These TICs cells have also been implicated in the ability of cancer cells to effectively colonize a distant organ during metastasis.

Direct evidence for the implication of TICs in metastasis came from patient derived xenograft (PDX) tumors. The most metastatic PDX tumors have a high percentage of TICs present in the primary tumors, while the least metastatic tumors have a lower percentage of these cells [90]. In the TNBC cell line SUM1315, inhibition of the Wnt signaling pathway caused a decreased in the percentage of $CD44^{+}/CD24^{-}$ cells and affected the ability of these cells to form a primary tumor and metastasize upon transplantation [91]. Analysis of breast cancer cells isolated from a bone marrow metastasis showed an increased number of TICs in comparison to the primary tumor [92]. In murine and human inflammatory breast cancer models, TICs have been correlated with more invasive, metastatic and aggressive disease and poor outcomes [93]. These findings support the idea that a small population of cells is responsible for tumor formation

and is also responsible for the successful colonization of distant organs during metastasis.

The environmental niche of TICs plays a vital role in their behavior. As discussed earlier, the hypoxic environment affects metastasis and studies have established that TICs are key players in metastasis. Inhibition of HIF-1α expression using shRNA in various TNBCs leads to inhibition of primary tumor growth and metastasis to the lungs supporting the importance of hypoxia in TICs [94]. SUM159 cells exposed to hypoxia *in vitro* for 48 hours caused an enrichment in TICs and the knockdown of HIF-1α in this cell line abrogated the hypoxia mediated TIC-induction [95]. Additional evidence from exposure of MCF-7 breast cancer cells to hypoxia shows an increase in the stem cell factors KLF4, NANOG, OCT4 and SOX2, which are important for maintenance of pluripotent stem cells and embryonic stem cells [96, 97]. The role hypoxia plays in mammary tumor progressions seems to be complex, not only can hypoxia influence the tumor microenvironment to enhance migration and invasion but it can also play a positive role in the maintenance of TICs, which can successfully colonize a distant site to form a metastatic lesion.

Pathophysiological conditions can trigger tumor cells to acquire tumor-initiating properties through the induction of EMT. Several studies have indicated that metastatic cells that have undergone EMT exhibit a TIC phenotype.

Disseminated breast cancer cells isolated from pleural effusions are enriched for CD44+CD24- cells [98]. Also, in pancreatic tumors, cells isolated from the invasive leading edge of a tumor are enriched for stem cell markers. Studies have shown that forced repression of E-cadherin by Snail expression, a step in the EMT process, triggered an enrichment in CD44+CD24- cells [99]. A study looking at the stem cell marker CD44 in human tumors and its association with EMT showed a positive correlation between CD44 expression and the EMT markers SNAI1, ZEB1, TWIST and VIMENTIN in human breast tumors [100]. The same study showed an association between the TIC marker CD44 expression and other stem cell markers like ALDH1, SOX2, NANOG, KLF4, MYC and OCT4, further implicating EMT with TICs. Of interest, this study reported a significant correlation between CD44 expression in breast cancer and poor overall survival rate [100]. Therefore, the developmentally conserved EMT program not only allows epithelial tumor cells to become more invasive but also contributes to the promotion of TIC properties in cancers.

TICs are not only a problem when it comes to cancer metastasis, these cells are also a big hurdle in the treatment and eradication of cancer. The presence of TICs in tumors can explain breast cancer treatment resistance and recurrence of disease ([89, 100, 101]). Upon treatment with paclitaxel and 5-fluorouracil, the breast carcinoma cell lines Sum159 and Sum149 undergo an enrichment in TICs and develop resistance to these therapies [102]. Although these treatments can

eliminate the bulk of the cancer cell population, as long as a subset of TICs remain they can repopulate the tumor after treatment has been withdrawn.

The identification of signaling pathways that are important in TICs can help identify strategies to target this cell population. In breast cancer, the Wnt/β-catenin, sonic hedgehog, notch and PTEN signaling pathways have been implicated in TIC self-renewal regulation [21]. One assay used to assess cancer stem cell activity *in vitro* is the mammosphere formation assay. This assay allows for cells with stem cell properties to grow in non-adherent growth conditions [103]. Expression of the Wnt activator Pygo2 in MDA-MB-231 cells enhances mammosphere formation suggesting an enrichment in TICs [104]. Deletion of the same activator in MMTV-Wnt1 tumor cells negatively affected mammosphere formation and tumor-forming capacity upon transplantation [105]. Identification of pathways that contribute to the ability of TICs to self-renew will contribute to better targeting of this population of cells in tumors and better treatment for localized and metastatic disease. As evidence of this, treatment of breast cancer cells with Wnt pathway inhibitors Salinomycin or CWP232228 induces the cell death of TICs [102, 106, 107]. Discoveries of novel regulators of TICs can provide alternative approaches for the eradication of metastatic cancers.

Insulin Receptor Substrate (IRS) Proteins

The IRS proteins are a family of cytoplasmic adaptor proteins that coordinate signaling downstream of cell surface receptors to regulate cellular outcomes. IRS1 and IRS2 are the main isoforms ubiquitously expressed in mammalian cells, whereas IRS4 is limited in its expression to kidney, brain, thymus and liver [108, 109]. Mice, but not humans, also express IRS-3 [108]. All the IRS proteins share significant homology in their sequence, specifically in their N-termini and to a lesser extent in their C-terminal tails. IRS1 and IRS2 are expressed in both normal mammary epithelial cells and breast cancer. However, the expression pattern of IRS1 and IRS2 in breast cancer is variable. IRS1 is highly expressed in localized tumors, while IRS2 is more highly expressed in invasive tumors [110]. Additional studies have also investigated the subcellular localization of these adaptor proteins in breast cancer. Immunohistochemical analysis of IRS1 and IRS2 expression in human breast tumor samples shows IRS1 is cytoplasmic and nuclear, whereas IRS2 is cytoplasmic and at the plasma membrane [111]. The nuclear localization of IRS1 correlates with enhanced survival in response to tamoxifen treatment and IRS2 membrane localization correlates with decreased overall survival [111, 112]. These studies support that not only the relative expression of the IRS proteins in breast tumors, but also their subcellular localization, can impact the clinical outcome for patients.

Murine mammary tumor models have contributed tremendously to the understanding of IRS biology in breast cancer. Transgenic mice overexpressing

IRS1 or IRS2 in the mammary glands develop mammary hyperplasia, tumors and metastases to the lungs [113]. Research performed by the Shaw Lab took advantage of the MMTV-PyMT model of murine mammary tumor metastasis and generated PyMT:Irs1-/- and PyMT:Irs2-/- mice to determine the role that the IRS proteins play in mammary tumor development and metastasis [114]. Knockout of Irs1 or Irs2 in this model did not impact primary tumor growth, but rather altered the ability of these tumors to metastasize (Figure 1.1) . PyMT:Irs2-/- mice exhibited a significant decrease in mammary tumor metastasis while PyMT:Irs1-/- mice, with elevated levels of IRS2 expression in the tumors, exhibited an increased metastatic rate [114]. Cell lines derived from these PyMT:Irs-/- tumors further demonstrated the disparities between these two proteins in breast cancer. Irs2-/- cells are more sensitive to cell death upon serum starvation, have decreased invasion and decreased aerobic glycolysis [115-117]. In contrast, Irs1-/- cells are more invasive, have greater survival upon serum starvation and maintain their ability to perform aerobic glycolysis. The ability of Irs2 to regulate glycolysis, invasion and tumor cell survival are likely important for its promotion of tumor metastasis *in vivo*.

IRS4 is emerging as a protein of interest in multiple cancers, however, there is limited information on IRS-4 in human breast cancer. Although IRS-4 is not expressed in the normal breast epithelium, it has tumorigenic potential, as expression of IRS4 in the non-transformed human mammary epithelial cell line

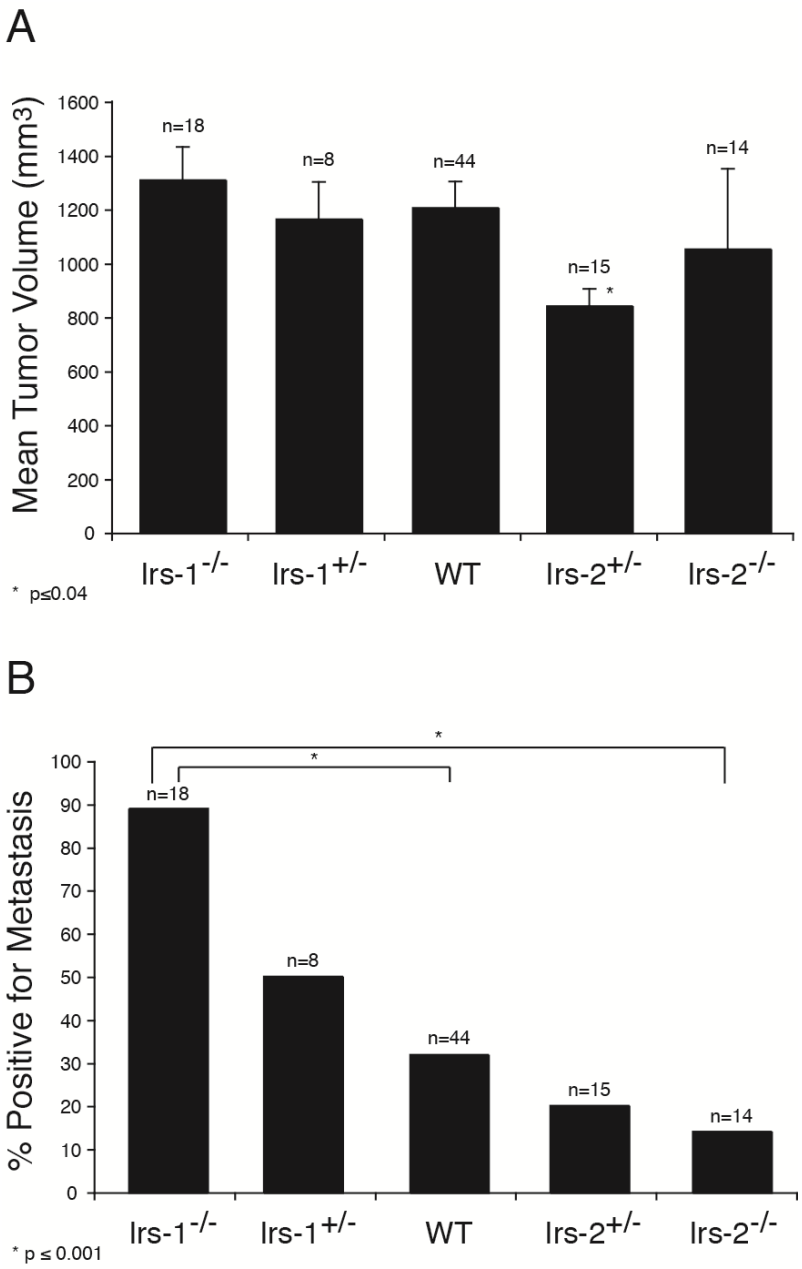

Figure 1.1 Analysis of IRS involvement in PyV-MT mammary tumor metastasis. (A) Female FVB/PyV-MT+/- mice were analyzed for their total tumor burden at 80 days of age. The mean tumor volume (±SEM) was determined for each Irs genotype using the formula: volume = 4/3p(length/2)(width/2)(length/2). The number of mice analyzed for each genotype is indicated. Statistical significance was determined using the Student's t-test. A p-value of ≤0.05 was considered statistically significant. (B) Lungs from 80-day-old female

FVB/PyV-MT+/- mice were sectioned and screened microscopically for the presence of metastatic lesions. Five representative H&E sections from each lung were analyzed. The percentage of mice that scored positively for metastatic lesions for each Irs genotype is shown and the number of mice analyzed is indicated. Statistical significance was determined using the chi square test. A p-value of ≤0.05 was considered statistically significant.

Adapted from:

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MCF-10A leads to anchorage-independent growth in a soft-agar assay similar to IRS1 and IRS2 [118]. In addition to proliferation effects, IRS4 has been linked to resistance to breast cancer therapies. Expression of IRS4 in the Her2+ breast cancer cells SKBR3 and BT474 leads to resistance to Trastuzumab or Lapatinib [118].

The IRS proteins also have very distinct roles in normal physiology as observed from studies of knockout mice. Irs1-/- mice are deficient in body growth, while Irs2-/- mice have defects in brain development and pancreatic beta-cell survival [119-122]. Loss of either Irs1 or Irs2 in mice leads to the development of insulin resistance, but only the loss of Irs2 causes diabetes [121, 123]. This difference is due to the loss of beta cells and insulin production in the pancreas in Irs2-/- mice, as Irs2 has been shown to regulate the survival of beta cells [123]. Irs1-/- mice develop pancreatic β-cell hyperplasia as they can only signal through Irs2 [120]. On the other hand, Irs4 knockout mice have only mild defects in growth, glucose homeostasis and reproduction [124]. Male Irs4-/- null mice are 10% smaller than their wild type counterparts while female Irs4-/- mice have no defects in growth. Also, Irs4-null mice exhibit lower levels of blood glucose in the fed and fasted states but have no alterations in insulin levels [124].

As demonstrated by the Irs1-, Irs2 and Irs4-null mice, the IRS proteins play an important role in normal metabolism. The IRS proteins also have additional

roles in murine physiology. In the mouse heart, myocardium specific loss of Irs1 and Irs2 leads to heart failure [125]. Irs2-/- deficient cells in the kidney tubules leads to a decrease in bicarbonate reabsorption, suggesting a role of the Insulin-Irs2 axis in renal regulation of bicarbonate [126]. In the mouse eye, Irs2 deletion triggers a decrease in signaling downstream of Irs2 and higher induction of cell death, a phenotype that cannot be compensated for by the expression of Irs1 [127]. These studies provide further proof of the differential roles the IRS proteins play in normal physiology and highlight the importance of understanding the regulation of IRS protein signaling.

The IRS proteins belong to a class of proteins known as the intrinsically disordered proteins (IDPs). These proteins are characterized by a low sequence complexity and a lack of a well-defined tertiary structure ([128]). A characteristic of such proteins is the high content of hydrophilic charged amino acids and low proportion of hydrophobic bulky amino acids ([128]). IDPs are completely functional but lack the ability to fold into stable globular three-dimensional structures and continually transition through multiple conformations that facilitate protein interactions and signaling [129]. Some IDPs are completely disordered while others contain combinations of structured globular domains and intrinsically disordered regions (IDRs). IDPs have the ability of functioning as central regulators in protein interaction networks and are involved in the regulation of complex signaling pathways [130]. The nature of these proteins allows them to

interact with other proteins with high specificity and modest affinity, allowing for the fast activation and termination of signaling events [131].

The IRS proteins contain two structured domains in their N-termini, the pleckstrin homology (PH) and phospho-tyrosine binding (PTB) domains, while their C-termini are comprised of the disordered sequence [132-135] (Figure1.2A). IRS2 also contains the KRLB region, which was identified from a peptide screen as a region that binds the IR and plays a role in the downregulation of insulin signaling [136]. This region has no confirmed tertiary structure. The PH domain contributes to the localization of the IRS proteins to activated receptors at the cell membrane by facilitating protein-protein interactions and the interaction between the protein and phospholipids at the cell membrane [132, 135]. The PTB domain mediates the interaction between the IRS proteins and activated receptors that harbor the NPXY motif [133, 134]. Recruitment of the IRS proteins to the activated receptors leads to the phosphorylation of multiple tyrosine residues in their C-termini. The IRS proteins do not exhibit intrinsic kinase activity but their phosphorylation leads to the recruitment and activation of downstream signaling effectors that include PI3K, Grb-2 and Shp-2 [137-140]. As we learn more about the role of these proteins in cancer, new evidence sheds light into their differential functions. We are interested in understanding the mechanism of IRS protein function as these adaptor proteins have been extensively implicated in many aspects of cancer biology.

Signaling through the IRS proteins: Upstream receptors

Irs1 was discovered in 1985 as a downstream substrate of the insulin receptor (IR) using a phosphotyrosine-specific antibody screen after insulin stimulation of hepatoma cells [119, 141]. Irs2 was discovered in 1990 as an alternative substrate of the IR in Irs1-/- mice in response to insulin stimulation [119, 142]. Subsequent studies revealed a role for the IRS proteins as signaling intermediates of the close family member Insulin-like growth factor-1 receptor (IGF-1R), as well as a subset of integrin and cytokine receptors [143]. In ER+ breast carcinoma cell lines, IRS1 mediates signaling downstream of the IL-4 cytokine receptor, but IRS2 does not interact with this receptor [144]. There is also limited evidence for the involvement of the IRS proteins downstream of the growth hormone (GH) receptor, VEGFR, EGFR, and the prolactin receptor [145]. In normal physiology, the IRS proteins have been most extensively characterized for their role in mediating IR regulation of glucose metabolism and IGF-1R mediated mitogenesis [146, 147].

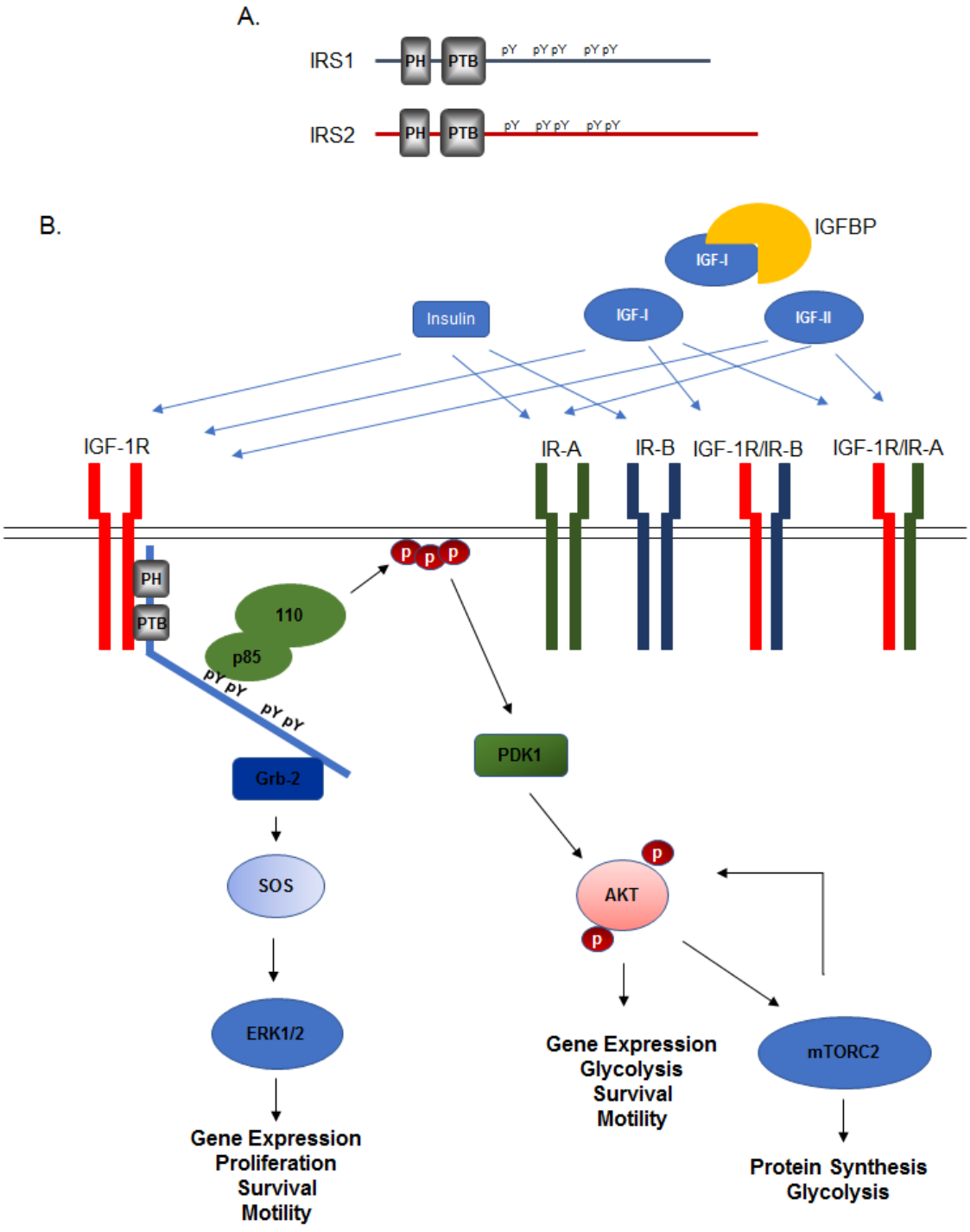

Figure 1.2 Summary of Insulin Receptor Substrate signaling. (A) Schematic of IRS1 and IRS2 depicting PH and PTB structural domains in grey and phosphotyrosine sites that bind PI3K. (B) Overview of the IR/IGF-1R/IRS signaling pathway.

IR and IGF-1R signaling play a role in the growth and development of many tissues. During fetal development, IGF-1 and IGF-1R mutations can lead to developmental delays and postnatally children with these mutations display a growth deficiency [148]. In the mammary gland, disruption of IGF-1R signaling causes reduced gland growth [149]. The IR/IGF-1R signaling network is comprised of the ligands insulin, IGF-1 and IGF-2, the cell surface receptors IR-A, IR-B, IGF-1R and IGF-2R, and the IGF-binding proteins [150]. IR-A and IR-B are splice variants that differ in the inclusion (IR-B) or exclusion (IR-A) of exon 11 resulting in a 12 amino acid difference in the β-subunit. The IR-A isoform is expressed prenatally and is also expressed aberrantly in many cancers accounting for 60-100% of IR expression in tumor cells [151]. The IR-B isoform is the predominant receptor expressed in liver, muscle and adipocytes and is responsible for the metabolic effects of insulin [151]. The affinity of each receptor for ligands is different, with IR-A binding insulin, IGF-1 and IGF-2 and IR-B only binding insulin [152, 153]. The IR and IGF1R receptors can form homodimers as well as heterodimers in many tissues, including in cancer cells.

Elevated levels of IGF ligands in the circulation are considered risk factors for the development of breast cancer [154, 155]. Low levels of IGF1-binding proteins (IGFBP) are also considered risk factors for cancer. The IGFBPs bind free IGF-1 ligand in the circulation and decrease the level of bioavailable IGF-1. Upon a decrease in IGFBP expression, the level of free IGF ligands in the serum

rises, enhancing IGF-1 signaling in peripheral tissues and affecting cancers that depend on this signaling pathway. The IGF ligands at high concentration are not only risk factors for breast cancer but also risk factors for prostate and colon cancer [156]. Additionally, elevated insulin levels in breast cancer patients correlate with more aggressive disease and poor outcomes [157].

The IGF-1R has been implicated in both tumor initiation and progression in breast cancer. Expression of constitutively active IGF-1R in breast epithelial MCF-10A cells increases growth in soft agar and promotes tumor formation in nude mice [158]. Transgenic overexpression of the IGF-1R also results in the induction of mammary tumors *in vivo* [159]. IGF-1R receptor signaling is crucial for tumor growth by mediating mitogenesis, maintaining the transformed phenotype and protecting tumor cells from apoptosis [160]. The IGF-1R pathway has also been implicated in progression of tumors from localized to metastatic disease. In a murine metastatic model [161], inhibition of the IGF-1R did not inhibit primary tumor growth, but caused significant inhibition of metastasis *in vivo*. Expression of dominant negative IGF-1R in human breast carcinoma cells decreased invasion and anchorage independent growth, and upon injection into mice, inhibited metastasis [161, 162]. Also, in a bone metastatic clone of MDA-MB-231 cells, IGF-1 blockade leads to a decrease in tumor cell motility and inhibits tumor metastasis *in vivo* [163]. In breast cancer patients, IGF-1R signaling, as determined by phosphorylation of IGF-1R at Tyr 1135/1136, is associated with metastasis of

breast and lung cancers to the brain [164, 165]. Moreover, upregulation of IGF-1R in breast cancer patients after neoadjuvant therapy predicts poor outcome [166], further supporting a role for the IGF-1R in breast cancer progression.

In MCF-10A cells, overexpression of the IGF-1R leads to tumor growth in nude mice by the induction of EMT via the upregulation of Snail and inhibition of E-cadherin expression [158]. Several studies have shown that the expression of the IGF-1R receptor is elevated in TICs in comparison to the bulk population of tumor cells [167, 168]. Also, IGF-1 signaling has been shown in MDA-MB-231 cells to be a positive regulator of the EMT transcription factor ZEB1, causing the induction of invasion and metastasis in these cells [167]. The involvement of IGF-1R signaling in resistance to tumor therapy, EMT and TICs suggests that the IGF-1R plays an important role in tumor progression by regulating both tumor initiation and invasion. This evidence makes the IGF-1R signaling pathway an important target in breast cancer.

Inhibition of IGF-1R signaling in cancer cells *in vitro* and in mouse mammary tumor models has demonstrated the benefits of inhibiting this pathway in cancer. Several inhibitors are under clinical development to target this signaling pathway in patients. However, targeting of the IGF-1R in clinical trials has demonstrated to be a complex task. Inhibitors against this pathway did not show the predicted outcomes based on their preliminary preclinical studies. Failure was in part due to

upregulation of the Insulin receptor (IR-A), downregulation of the IGF-1R and upregulation of other tyrosine kinase receptors in the tumor cells [169]. Also, the constitutive activation of signaling molecules downstream of the receptor can limit the efficacy of this approach [170]. The dismal performance of these inhibitors does not take away from the importance of IGF-1R signaling in cancer. Poor clinical trial design could have contributed to the lack of response in phase III trials as none of the patients participating in the trials were screened for IGF-1R expression in their tumors. In the case of Ewing sarcoma, a cancer with high levels of IGF-1R expression, complete responses were observed upon inhibition of IGF-1R signaling [169]. These results suggest that further understanding of this pathway, specifically understanding the IRS proteins as mediators of IR/IGF-1R signaling, can aide in the design of improved clinical trials that target key aspects of IR/IGF-1R signaling in a more efficient way.

Signaling through the IRS proteins: Downstream effectors

The IR and IGF-1R are composed of two extracellular alpha subunits and two transmembrane and intracellular beta subunits, which are joined by disulfide bonds [171]. Binding of the ligands IGF-1, IGF-2 or insulin to the extracellular subunits of the receptors causes activation of the kinase domains and autophosphorylation of the intracellular subunits. These phosphorylation events create docking sites for the IRS proteins [172]. Phosphorylation of the IRS proteins

by the receptor kinases leads to the recruitment of SH2 domain containing proteins like GRB2/SOS and phosphatidyl inositol 3'-kinase (PI3K) resulting in activation of the mitogen-activated protein kinase (MAPK) and PI3K pathways, respectively, that are important mediators of the IGF-1/insulin bioactivities [173-175]. Both of these signaling pathways have been implicated in cancer extensively, but their activation downstream of the IR or the IGF-1R can have very different outcomes. One possible mechanism for their differential outcomes is the recruitment of unique effectors by either IRS1 or IRS2 to activate different downstream signaling pathways. Additionally, as mentioned previously the differential cellular localization of the adaptors may contribute to their divergent outcomes [111].

A very important signaling molecule that is activated downstream of the IRS proteins is PI3K. PI3K is one of the major pathways activated by receptor tyrosine kinases and is extensively implicated in cancer. Upon activation, PI3K generates phosphatidylinositol 3,4,5-triphosphate (PIP_3) at the cell membrane leading to the recruitment of effectors and amplification of intracellular signaling [176, 177]. There are three different classes of PI3K (Class I-III) with distinct lipid substrates and effectors. Class I PI3K generates PIP_3 and includes two subclasses: Class IA is activated in response to receptor tyrosine kinases like the IGF-1R and Class IB is activated by G-protein coupled receptors (GPCRs) [177]. As this work focuses on the IR/IGF-1R/IRS mediated regulation of cancer progression, further discussion of Class IA PI3K is required.

Class IA PI3K is formed by a heterodimer consisting of a regulatory subunit and a catalytic subunit [178]. There are 5 isoforms of the regulatory subunit (p85α, p55α, p50α, p85β and p55γ) and three isoforms of the catalytic subunit (p110α, p110β, and p110δ) [177]. In basal conditions the association of p85 with p110 leads to inhibition of the lipid kinase activity of the catalytic subunit [179]. Upon receptor tyrosine kinase phosphorylation, the p85 subunit binds to phosphorylated tyrosine residues on the receptor or adaptor proteins, releasing the inhibition of the p110 catalytic subunit and rendering the kinase active [179]. Ligand stimulation of the IR or IGF-1R by insulin/IGF1 leads to receptor mediated phosphorylation of the IRS proteins and activation of PI3K. Loss of IRS expression abrogates the activation of PI3K following stimulation of these receptors, indicating that these adaptors are required to activate the PI3K signaling pathway [180, 181]. The generation of PIP_3 by PI3K activation leads to the recruitment of AKT to the cell membrane through its PH domain [182]. This event regulates processes like cell survival, growth, proliferation, transcription, glucose metabolism, cell motility and protein translation [183]. In addition, PIP_3 can activate the RAC signaling pathway and regulate cell-to-cell contact, actin cytoskeleton rearrangements, cell adhesion, transcription, translation, cell survival and cell cycle [184]. Activation of AKT and RAC signaling by PI3K leads to the regulation of a variety of cellular processes that are essential for normal cell biology. If these processes are dysregulated they

can also contribute to many aspects of tumorigenesis. Therefore, the dysregulation of PI3K signaling is a hallmark of many cancers.

In many cancers including colon, lung, gastric, breast and brain, PI3K is mutated [185, 186]. Activating mutations of the p110α catalytic subunit of PI3K (PI3KCA) take place in 25% of all breast cancers and the mutations occur mostly in exon 9 and exon 20, which encode the helical and catalytic domains of the protein [187]. These gain of function mutations cause increased PI3K activity and constitutive activation of downstream targets such as AKT, ribosomal protein S6 kinase (S6K) and inactivation of glycogen synthase kinase-3 (GSK-3) [188]. The PI3K pathway can also be regulated by dephosphorylation of PIP_3 by the phosphatase PTEN [189]. Loss of the tumor suppressor PTEN leads to deregulation of the PI3K pathway in cancer. Germline mutations in the PTEN gene result in Cowden's Syndrome and predispose patients to a number of different cancers, including breast cancer [189, 190]. Beyond mutations in PTEN, PI3K can be dysregulated through constitutively active signaling from upstream tyrosine kinase receptors. In breast cancers and Ewing sarcomas, the IGF-1R pathway is commonly amplified causing the activation of PI3K signaling [169]. The requirement of the IRS proteins for activating PI3K in response to IR/IGF-1R signaling is an additional justification to support understanding the function of these adaptor proteins.

Regulation of IRS expression

The regulation of IRS protein expression likely contributes to the differential impact of IRS-1 and IRS-2 in breast cancer. The IRS genes are hormone responsive, with IRS1 regulated by the estrogen receptor (ER) and IRS2 regulated by the progesterone receptor (PR) [191, 192]. This differential regulation plays a role in response to therapy. In the context of the ER antagonist tamoxifen that is used for treatment of ER+ breast tumors, inhibition of IRS1 contributes to a favorable response to this inhibitor [171]. IRS1 knockdown in MCF-7 cells enhances tamoxifen mediated cell death supporting the role of IRS1 expression in ER+ tumors [193]. Treatment of PR+ breast cancer cells with progestin increases the expression and phosphorylation of IRS2 [192, 194, 195]. Moreover, other growth factor receptors have been shown to regulate IRS protein expression including the EGFR and FGFR [196]. These receptors have also been implicated in breast cancer, suggesting a collaboration between the EGFR, FGFR and IGF1R/IR pathways in cancer.

The tumor microenvironment can contribute to the regulation of IRS protein expression. As previously discussed, hypoxia plays an important role both in promoting tumor cell invasion and increasing tumor-initiating cells. Hypoxia is also a regulator of IRS2 expression in breast carcinoma cells. Exposure of breast carcinoma cells to hypoxia increases IRS2 expression at the mRNA and protein

level, while IRS1 protein expression decreases in hypoxic conditions. The expression of siRNA against HIF1α and HIF2α represses IRS2 expression in hypoxic conditions [197]. The induction of IRS2 during hypoxia promotes enhanced tumor cell survival and invasion. These findings provide evidence for a mechanism by which IRS2 and hypoxia can contribute to cancer metastasis.

Another mechanism for regulating IRS expression is the post-transcriptional targeting of IRS mRNA by microRNAs (miRNAs). miRNAs, small (18-24 nucleotides) single-stranded non-coding RNAs, bind the 3'UTR region of mRNAs and target them for degradation or inhibit their translation [17]. miRNAs have been implicated in many biological processes such as cell proliferation, cell cycle, apoptosis, migration, invasion and differentiation. In lung cancer two miRNAs, miR146a and miR338-3p, have been demonstrated to inhibit non-small cell lung cancer metastasis by targeting IRS2 expression [198, 199]. In the lung cancer cell lines H460 and H1299, miR146a repression of IRS2 inhibited migration and invasion of these cells, which was further validated with siRNA against IRS2 [199]. In A549 lung carcinoma cells, miRNA-338-3p decreases the expression of IRS2 and inhibits tumor cell invasion. Overexpression of IRS2 using a 3'UTR deficient mRNA restored invasion in these cells [198]. miR-141 also plays a role in the inhibition of tumor cell invasion *in vitro* in thyroid carcinoma cell lines and inhibits primary tumor growth *in vivo* by downregulating IRS2 expression [200]. All of these studies support the importance of IRS protein regulation for tumor progression.

In addition to transcriptional and post-transcriptional regulation, IRS protein levels can also be regulated through post-translational modifications. Several studies have demonstrated that the IRS proteins are ubiquitinated by several ubiquitin ligases and targeted for degradation by the 26S proteasome [201]. The IRS proteins can also associate with the deubiquitinating enzyme ubiquitin specific protease 7 (USP7) [202]. This interaction plays a role in the stabilization of the IRS proteins and in turn the maintenance of IGF1R/IR signaling. Ligand stimulation, IGF-1 for IRS2 and insulin for IRS1, leads to the dissociation of USP7 from the IRS proteins and their subsequent degradation. Stimulation of these pathways in a ligand dependent manner can have differential regulation of expression at the IRS protein level and produce different responses in cells depending on which IRS protein remains expressed.

The prolonged stimulation of signaling downstream of the IR/IGF-1R can activate a negative feedback loop that is dependent upon the mechanistic target of rapamycin (mTOR) ([203, 204]. Downstream of mTOR, S6 kinase phosphorylates the IRS proteins on serine/threonine residues and targets them for ubiquitination and degradation by the ubiquitin proteasome system (UPS) [109, 201]. In addition, the phosphorylation of serine residues near the PI3K binding motifs can lead to a decrease in the association of p85 with the adaptor proteins and inhibition of signaling downstream of PI3K [205]. Most of the negative

feedback regulatory events for the IRS proteins have been studied in IRS1. In the breast carcinoma cell line MDA-MB-468, the use of inhibitors against the PI3K pathway caused an increase in the expression of IRS2, but also resulted in increased association with the regulatory subunit of PI3K and allowed the further activation of PI3K signaling [206]. These results suggest that upon the activation of IR/IGF-1R signaling, one mechanism that cells can employ to turn off this pathway is degradation of the IRS proteins.

In addition to stimulating protein degradation, phosphorylation of serine residues in the IRS proteins can contribute to inhibition of insulin mediated IRS protein signaling. Increased serine phosphorylation of IRS1 is a common finding during insulin resistance and diabetes [204, 207]. Insulin resistance and diabetes are known for the induction of inflammation. In inflammatory states, the proinflammatory cytokine TNFα is secreted by macrophages and other immune cells. TNFα causes activation of the c-Jun NH_2-terminal kinase (JNK) and the phosphorylation of IRS1 at Ser307 [207]. This residue is located near the PTB domain and inhibits the interaction between the IR catalytic domain and the PTB domain causing inhibition of IR/IRS1 signaling [207]. The tumor microenvironment is rich in inflammatory cells that could contribute to the negative regulation of IRS1 activity in tumors. In support of this possibility, IRS1 serine phosphorylation is elevated in metastatic mammary tumors and this correlates with reduced activity as meaured by tyrosine phosphorylation [116].

The microtubule cytoskeleton

The microtubule cytoskeleton is composed of heterodimers of α- and β-tubulin that polymerize in a head-to-tail array to form protofilaments [208]. These protofilaments have polarity with two distinct ends, a plus end and a minus end. The polarity is important in the trafficking of motor proteins along the microtubules [209]. 13 protofilaments come together to give rise to the hollow cylindrical tubes that we know as microtubules. Beyond their role in mitosis, microtubules are important players in intracellular trafficking, cell size and shape, protein signaling, cell division and cell migration and motility [210-212]. Microtubules are very dynamic structures with constant polymerization and depolymerization taking place simultaneously. The regulation of polymerization and depolymerization rates in the cells determine the net growth of microtubules and plays a role in mitosis and cell motility and migration [213]. Independent of their role in mitosis the ability of microtubules to regulate cellular trafficking, motility and invasion can contribute to their role in tumor progression.

One of the hallmarks of cancer is uncontrolled cell proliferation and the microtubules are very important in this process due to their role in cellular division. Due to this central role in mitosis, disruption of microtubules has been an exciting target in cancer therapeutics. Two major approaches to targeting the microtubules

exist, the vinca alkaloid therapy that leads to the disruption of microtubules and the taxanes that cause the stability of the microtubules [214, 215]. The vinca alkaloids, which include the agents vinblastine, vincristine and vinorelbine, cause the disruption of the microtubule cytoskeleton [214]. On the contrary, taxane drugs like paclitaxel and docetaxel stabilize the microtubules and prevent the progression of the cell cycle [215]. The disruption or the stabilization of microtubules in cancer cells causes cell death and is one of the reasons these drugs are widely used in cancer therapy.

In order to understand more about the role of microtubules in the cell it is necessary to understand their interacting proteins. An important group of proteins that interact with microtubules are the motor proteins, which are essential in the trafficking of vesicles and organelles along the microtubules. One of the motor proteins, Dyenin, allows retrograde movement towards the minus ends of microtubules, while kinesin motor proteins can facilitate anterograde movement towards the positive ends of the microtubules [216]. Kinesins are a family of microtubule motor proteins that mediate a diverse set of functions within the cell that include the transport of vesicles, organelles, chromosomes, protein complexes and even move microtubules [216, 217]. These enzymes are characterized by having a motor domain that contains the ATP binding site, a stalk and a tail. The stalk and the tail are important for dimerization of the kinesins and confers specificity of the cargo binding. There are 45 kinesins identified in humans

and 12 have been shown to be implicated in many aspects of mitosis including the segregation of the chromosomes, formation of the mitotic spindle, kinetochore-microtubule dynamics, and cytokinesis [217, 218].

The role of microtubule motor proteins in mitosis has made them an attractive target in cancer therapy. The use of taxanes and vinca alkaloids for cancer treatment has severe neurotoxicity as a side effect due to the importance of microtubules in the trafficking of neurotransmitter vesicles along tubulin in axons and dendrites [210]. If mitotic kinesins are targeted instead, the broad side effects from microtubule disruption can be avoided. A kinesin that has been extensively studied in cancer is KIF11 (also known as Eg5 or kinesin-5) and inhibitors against this motor protein have already progressed to clinical trials [219]. In pancreatic cancer cells, overexpression of KIF11 is associated with tumor cell invasion [219]. Other kinesins involved in tumor cell invasion are KIF20 in pancreatic cancer and KIF2A in human glioma cells [220, 221]. Elevated levels of KIF20 has also been identified in cancers of the breast, lung and bladder [222].

Treatment of cancer cells with microtubule disrupting agents has been a well exploited therapeutic intervention in cancers, but not a perfect treatment. In breast cancers, taxanes are one of the first line therapies used in the clinic and among the mechanisms of taxane resistance, the kinesin motors seem to play an important role. In breast cancer, the overexpression of KIFC3, KIF1C and KIF5A

caused resistance of breast cancer cells to docetaxel by opposing the stabilizing effects of the drug [223]. The effects of kinesin overexpression during vinca alkaloid treatment has yet to be determined. Moreover, limitations to the inhibition of kinesins in the treatment of cancers have already appeared. In clinical trials KIF11 failed to work against slow or non-proliferating tumor cells [224]. As the understanding of microtubules improves, better approaches to cancer therapy continue to be developed.

Cell Death: Apoptosis

The main goal of cancer therapies is the elimination of cancer cells through the induction of cell death. In mammals, the predominant mechanism of cell death throughout development and tissue homeostasis is apoptosis. In the case of cancer, cells have an acquired ability to evade apoptotic cell death [225]. The ability of cancer cells to escape apoptosis provides cells with an advantage to continue uncontrolled cell proliferation and also provides cells with the capability of resisting therapeutic interventions [226]. Apoptotic cell death is regulated by two pathways: one determined by intrinsic death stimuli and the other by extrinsic death stimuli. Intrinsic stimuli include excessive oncogene activation, DNA damage or the unfolded protein response (UPR). Extrinsic stimuli involve the activation of the Fas or TNFα receptors on the cell surface [227]. These pathways

converge at the mitochondrial outer membrane (MOM) with the Bcl-2 proteins regulating the fate of apoptosis.

The Bcl-2 family of proteins consists of pro-apoptotic and anti-apoptotic proteins and it is divided into three classes based on the presence of the conserved Bcl-2 homology (BH) regions 1-4. The anti-apoptotic proteins Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and A1 have the four BH regions. Pro-apoptotic proteins are divided into the multi region BH proteins, Bax, Bak Bok and the BH-3 only proteins, Bad, Bim, Bid, Noxa, Puma, Bik/Blk, Bmf, Hrk/DP5, Beclin-1 and Mule [226]. BH-3 only proteins are essential initiators of the intrinsic apoptotic pathway and are important for the integration of many cellular processes into the regulation of apoptosis. One of the BH-3 only proteins Bim associates with the microtubule motor protein dynein and localizes to the microtubules in healthy cells [228]. Upon apoptotic stimuli, release of Bim from microtubules leads to the binding of Bim to Bax and Bak, followed by the permeabilization of the mitochondrial membrane, release of cytochrome C and cell death.

The multiplicity of apoptotic regulatory proteins that are present in mammalian systems allows for a fine-tuned control of the process and the integration of multiple signals into this pathway. Some of the BH-3 only proteins are regulated by growth factor receptor signaling. Bim and Bad are transcriptionally upregulated by growth factor receptor signaling deprivation [229,

230]. Alterations in the regulation of the Bcl-2 family proteins have been demonstrated in multiple cancers. Upregulation of anti-apoptotic proteins like BCL2 causes inhibition of BH-3 only pro-apoptotic proteins as seen in Chronic lymphocytic leukemia with BCL2-mediated sequestration of BIM and inhibition of apoptosis [226].

Other important proteins in the apoptotic process are the cysteine-aspartic proteases (Caspases). These are the proteins responsible for dismantling cells and causing changes in cell morphology and composition such as chromatin condensation and DNA fragmentation, loss of cell adhesion, cell shrinkage, membrane blebbing and the formation of apoptotic bodies [231]. Caspases are expressed in the cell as inactive zymogens, the procaspases, and their cleavage is necessary for their full activation. There are two groups of caspases, the initiator caspases and the effector caspases. The initiator caspases include Caspase-9, Caspase-8, Caspase-10 and Caspase 2 and the effectors consist of Caspase-3, Caspase-6 and Caspase 7. Alterations in the expression of caspases have been reported in many cancers including breast cancer. A study looking at surgically resected breast tumors demonstrated a 75% decrease in Caspase-3 transcript and protein levels [232]. The reduction in Caspase-3 suggests that deregulation of apoptosis contributes to breast cancer. In colon cancer, somatic mutations that cause a decrease in expression of Capase-3 have also been identified [233]. As deregulation of apoptosis plays a key role in cancer development and

maintenance, there are continuing efforts to understand how to target this pathway to enhance the induction of cell death.

Rationale for Thesis Work

Understanding cellular processes that contribute to the progression of tumors from localized disease to metastasis is required for the development of better therapeutic approaches to target or prevent metastatic disease. In the last decade, very little progress has been made when it comes to targeting disease that progresses beyond the primary tumor site. The Insulin/IGF-1 pathway plays a role in development, metabolism and tumorigenesis. The IRS proteins are key signaling intermediates downstream of these growth factor receptors. Previous studies of the IRS proteins have demonstrated that IRS2, but not IRS1, plays a role in breast cancer metastasis. Despite evidence for the divergent roles of IRS1 and IRS2 in breast cancer, a mechanistic explanation of how they function differentially is still lacking. Additional studies in multiple human malignancies have demonstrated a correlation between IRS2 expression and function and metastatic disease. *In vitro* work has revealed that IRS2 plays a role in regulating cellular functions such as invasion, cell survival and glycolysis, all processes that contribute to tumor progression and metastasis. For such an important player in tumor progression, the intricate biological details of its mechanism of action continue to be unknown.

The goal of my thesis research has been to develop a more detailed understanding of how IRS2 mediates its important functions to promote breast cancer metastasis. I set out to understand structural and functional differences between IRS1 and IRS2 that would explain how these two similar adaptor proteins have markedly different roles in cancer progression. My long-term goal of elucidating the unique mechanisms of IRS2 function is to aide in the rational design of novel therapeutic approaches for targeting IRS2 in cancer.

CHAPTER II

Differential involvement of the microtubule cytoskeleton in Insulin Receptor Substrate-1 (IRS-1) and IRS-2 signaling to AKT determines response to microtubule disruption in breast carcinoma cells

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Jennifer L. Clark designed and performed experiments for Figure 2.1 and contribute with manuscript writing.

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Jenny Janusis designed and performed experiment for Figures 2.2, and 2.3 and contributed to manuscript writing.

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ABSTRACT

The insulin receptor substrate (IRS) proteins serve as essential signaling intermediates for the activation of PI3K by both the insulin-like growth factor-1 receptor (IGF-1R) and its close family member the insulin receptor (IR). Although IRS-1 and IRS-2 share significant homology, they regulate distinct cellular responses downstream of these receptors and play divergent roles in breast cancer. To investigate the mechanism by which signaling through IRS-1 and IRS-2 results in differential outcomes, we assessed the involvement of the microtubule cytoskeleton in IRS-dependent signaling. Treatment with drugs that either stabilize or disrupt microtubules reveal that an intact microtubule cytoskeleton contributes to IRS-2, but not IRS-1, mediated activation of AKT by IGF-1. Proximal IGF-1R signaling events including IRS tyrosine phosphorylation and recruitment of PI3K are not inhibited by microtubule disruption, indicating that IRS-2 requires the microtubule cytoskeleton at the level of downstream effector activation. IRS-2 colocalization with tubulin is enhanced upon Taxol-mediated microtubule stabilization, which together with the signaling data suggests that the microtubule cytoskeleton may facilitate access of IRS-2 to downstream effectors such as AKT. Of clinical relevance, our data reveal that expression of IRS-2 sensitizes breast carcinoma cells to apoptosis in response to treatment with microtubule disrupting drugs, identifying IRS-2 as a potential biomarker for the response of breast cancer patients to vinca alkaloid drug treatment.

INTRODUCTION

Insulin receptor substrate-1 (IRS-1) and IRS-2 are cytoplasmic adaptors for the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R), and they play a major role in determining the cellular response to stimulation by these ligands [108]. Notably, the IRS proteins are required for the activation of PI3K downstream of the IR and IGF-1R, which activate AKT and mTOR to promote proliferation, survival, motility, protein synthesis, and glucose metabolism [180, 234-236]. IRS-1 and -2 are expressed ubiquitously in humans, including the normal and malignant mammary epithelium [108]. Despite their considerable sequence homology however, IRS-1 and IRS-2 play divergent roles in breast cancer. *In vitro*, studies to assess IGF-1-dependent signaling through the IRS proteins in breast carcinoma cells have revealed that IRS-1 primarily regulates proliferation and survival, whereas IRS-2 regulates motility, invasion, and glycolysis [115, 117, 237-239]. *In vivo*, overexpression of either IRS-1 or IRS-2 in the mouse mammary gland promotes mammary tumorigenesis [113]. However, metastasis is diminished in the absence of Irs-2 expression and increased in the absence of Irs-1 expression [115, 116].

Differential localization patterns of IRS-1 and -2 in human breast tumors suggest one explanation for their divergent functions in breast cancer [111]. In normal breast tissue, ductal carcinoma *in situ* (DCIS), and invasive breast tumors,

IRS-1 is primarily localized in the nucleus, and also diffusely in the cytoplasm, frequently correlating with nuclear expression of estrogen receptor (ER) [193, 240, 241]. Nuclear expression of IRS-1 also correlates with response to tamoxifen in breast cancer patients [112]. IRS-1 has been implicated in the regulation of estrogen response genes through its interaction with the ER at estrogen response elements (ERE) in gene promoters [193]. The interaction of IRS-1 with β-catenin and its regulation of genes such as c-Myc and Cyclin D1 likely contribute to its role in stimulating proliferation [242, 243]. In contrast to IRS-1, IRS-2 is absent from the nucleus in normal breast and breast tumors, and is expressed either in the cytoplasm or at the cell membrane [111]. Diffuse cytoplasmic IRS-2 staining is associated with better overall survival of breast cancer patients, whereas membrane localization of IRS-2 in breast tumors is associated with decreased overall survival, particularly in patients with progesterone receptor negative tumors [111].

The localization pattern of IRS-1 and IRS-2 in human tumors suggests that their trafficking to distinct subcellular compartments contributes to their ability to elicit unique signaling responses. However, the mechanism by which the intracellular localization of IRS-1 and IRS-2 is determined is not known. The microtubule cytoskeleton, which plays an important role in intracellular trafficking, has been implicated in insulin-dependent regulation of glucose uptake, and this role for microtubules occurs distal to IRS phosphorylation [210, 212, 244]. These

observations support a role for microtubules in IRS-mediated downstream signaling, potentially through targeting of these adaptor proteins to unique subcellular compartments. We examined the role of the microtubule cytoskeleton in determining differential signaling and functional outcomes for IRS-1 and IRS-2. The data we obtained in the current study reveal that IRS-2 co-localizes with microtubules and that the microtubule cytoskeleton contributes to IRS-2, but not IRS-1, dependent signaling to AKT. The differential involvement of the microtubule cytoskeleton in IRS-1 and IRS-2-mediated AKT signaling influences the apoptotic sensitivity of breast carcinoma cells to microtubule disrupting drugs.

EXPERIMENTAL PROCEDURES

Cell lines, shRNA and transfection. The MDA-MB-231 cell line was obtained from the ATCC Cell Biology Collection. SUM-159 cells were a kind gift from Art Mercurio (UMass Medical School, Worcester, MA). Wildtype (WT), Irs-1$^{-/-}$, and Irs-2$^{-/-}$ mammary tumor cell lines were established from MMTV-PyV-MT-derived tumors as previously described [115]. *Irs-1$^{fl/fl}$* and *Irs-2$^{fl/fl}$* mammary tumor cells were isolated from female *FVB MMTV-PyMT::Irs-1$^{fl/fl}$* and *MMTV-PyMT::Irs-2$^{fl/fl}$* mice, respectively, and *Irs$^{-/-}$* cells were generated by infection with adenoviral Cre-recombinase as described previously [180]. Lentiviral vectors containing small hairpin RNAs (shRNAs) targeting GFP and human IRS-2 were obtained from Open Biosystems (Hunstville, AL). MDA-MB-231 cells were infected with virus, and stably expressing cells were selected by the addition of 2μg/ml puromycin. IRS expression was restored in the *Irs$^{-/-}$* mammary tumor cells by transfection with HA-tagged human IRS-1 or IRS-2 (kindly provided by Adrian Lee; University of Pittsburgh, Pittsburgh, PA) and selection in G418 (0.5mg/ml) [113].

Immunoblotting and immunoprecipitation. Cells were either serum starved overnight (MDA-MB-231) or for 4 hours (PyMT cells) in serum-free medium. Drugs were added to the medium prior to stimulation with IGF-1. MDA-MB-231 cells were treated with nocodazole or vinblastine for 30 minutes and Taxol for 2 hours. The mouse tumor cell lines were treated with nocodazole, vinblastine or vinorelbine for 1 hour. The concentrations (see Figure Legends) and time periods of incubation

were determined to stabilize or disrupt the microtubule cytoskeleton as assessed by immunofluorescence staining for tubulin. For microtubule altering drugs, Paclitaxel (#T7402) was obtained from Sigma-Aldrich (St. Louis, MO), and Nocodazole (#S2775), Vinblastine (#S1248) and Vinorelbine (#S4269) were obtained from Selleckchem (Houston, TX). MK2206 (#S1078) was obtained from Selleckchem. Cells were stimulated for 5-30 minutes with human recombinant IGF-1 (R&D Systems, Minneapolis, MN) prior to extraction.

For total cell extract immunoblots, cells were solubilized at 4^{o}C in RIPA lysis buffer (25 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium orthovanadate) containing protease inhibitors (Roche, Basel, Switzerland). Cell extracts containing equivalent amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20, and 5% (wt/vol) dry milk or 5% bovine serum albumin (BSA), incubated overnight at 4^{o}C in the same buffer containing primary antibodies and then incubated for 1 h in 5% blocking buffer with milk containing peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Biorad, Hercules, CA) using a ChemiDoc XRS+ (Bio-Rad) with Image lab software. Only signals within a linear range were used for quantitation and signals were normalized to total protein and/or housekeeping genes. The following antibodies were used for immunoblotting: IRS-1 (human:

#C20, Santa Cruz Biotechnology, Santa Cruz, CA; mouse: Bethyl Custom Immunochemistry Services), IRS-2 (#4502, Cell Signaling, Danvers, MA), p85 (#05-212, Millipore, Billerica, MA), IGF-1Rβ (#3025, Cell Signaling), phospho-AKT S473 (#9271 and #4060, Cell Signaling), phospho-AKT T308 (#2965, Cell Signaling), AKT (#sc-8312, Santa Cruz; #9272, Cell Signaling), α-tubulin (#T5168, Sigma-Aldrich), phospho-ERK (#9106, Cell Signaling), ERK (#9102, Cell Signaling), GAPDH (#A300-642A, Bethyl), Cleaved Caspase-3 (#9664, Cell Signaling), BIM (#2933, Cell Signaling), peroxidase-conjugated goat anti-rabbit IgG (#111-035-144, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and peroxidase-conjugated goat anti-mouse IgG (#711-035-151, Jackson ImmunoResearch Laboratories, Inc).

For immunoprecipitations, cells were extracted using a 20 mM Tris, pH 7.4 buffer containing 1% Nonidet P-40, 0.137 M NaCl, 10% glycerol, 10 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitors (Roche). Aliquots of cell extracts containing equivalent amounts of protein were pre-cleared for 30 min with protein A sepharose beads and then incubated overnight at 4°C with antibodies and protein A sepharose beads (Amersham Biosciences, Piscataway, NJ) with constant agitation. The beads were washed three times in extraction buffer. Laemmli sample buffer was added to the samples and immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described above. The following antibodies were used for immunoprecipitation: IRS-1 (#C20, Santa Cruz), IRS-2 (Bethyl Custom

Immunochemistry Services), IGF-1R (#3025, Cell Signaling), rabbit IgG (#sc-2027, Santa Cruz) and mouse IgG_{2b} (#ab18421, Abcam).

Immunofluorescence microscopy. Subconfluent, adherent cells plated on glass coverslips were treated with nocodazole (1μM) for 1 hour or Taxol (20 μM) for 2 hours. Cells were washed three times with Dulbecco's PBS and fixed in 3.8% paraformaldehye in Dulbecco's PBS with 0.5% Tween (PBST) for 1 hr. Permeabilized cells were blocked for 1 hr using 3% BSA in PBST. Primary antibodies diluted in blocking buffer were added to cells and incubated at room temperature for 1 hr. Secondary antibodes were diluted in the same buffer and cells were incubated at room temperature for an additional 30 minutes. Cells were washed three times with PBST after each antibody incubation. Coverslips were then mounted on glass slides using Prolong Gold containing DAPI (Cell signaling) and the slides were viewed by confocal microscopy (Ziess LSM700; 63X oil immersion objective). All images were adjusted equally for brightness/contrast using ImageJ software. Antibodies used for immunofluorescence include IRS-1 (#H165, Santa Cruz, Santa Cruz, CA), IRS-2 (#NB110-57138, Novus, Littleton, CO), α-tubulin (#T5168, Sigma-Aldrich, St. Louis, MO), Alexafluor-488 Donkey anti-rabbit IgG (#A-21206; ThermoFisher Scientific, Waltham, MA) and Alexafluor-568 Donkey anti-mouse IgG (#A10037; ThermoFisher Scientific). The specificity of the IRS-1 and IRS-2 antibody staining was validated by staining PyMT:Irs1-/-

,Irs2-/- double null cells that were transfected with either empty vector, IRS-1 or IRS-2 (Supplemental Fig. 1).

Cell cycle analysis. MDA-MB-231 cells were treated with nocodazole (1μM), vinblastine (20nM), vinorelbine (20nM), Taxol (10μM) or MK2206 (250nM) for 48 hours. The mouse PyMT mammary tumor cell lines were treated with nocodazole (1μM) or Taxol (1 or 10μM) for 48 hours. Adherent cells were collected by trypsinization after treatment and combined with non-adherent cells from the culture medium for cell cycle analysis. After centrifugation, the cell pellet was washed once in cold PBS, and the cells were then fixed in 70% methanol and stored overnight at -20^{o}C. The fixed cells were washed once in PBS and then resuspended in PBS containing 0.1% Triton-X-100, 0.1 mM EDTA, 0.05 mg/ml RNAse A (50 U/mg) and 50 μg/ml propidium iodide. The cells were analyzed by flow cytometry using a Becton Dickinson (Franklin Lakes, NJ) FACSCalibur after a 1 hour incubation at room temperature.

RESULTS

IGF-1R signaling is dependent upon the microtubule cytoskeleton.

To investigate the role of the microtubule cytoskeleton in IRS-dependent IGF-1R signaling, MDA-MB-231 cells were stimulated with IGF-1 after short-term (30 min - 1hr) treatment with Paclitaxel (Taxol) or nocodazole. Taxol, a taxane drug commonly used in cancer treatment, stabilizes microtubules, whereas nocodazole causes depolymerization of the tubulin cytoskeleton [208, 211, 215]. IRS signaling was measured by assessing the phosphorylation status of AKT, a downstream signaling effector of PI3K, because the IRS proteins are required for the recruitment and activation of PI3K by the IGF-1R [180, 236, 245]. Although microtubule stabilization by Taxol treatment did not alter the level of AKT activation (Fig. 2.1A), disruption of the microtubule cytoskeleton in response to nocodazole treatment significantly reduced phosphorylation of AKT at both threonine 308 (T308) and serine 473 (S473) (Fig. 2.1B). By contrast, the activation of ERK1/2 by IGF-1, which can occur independently of the IRS proteins [180], was not decreased by nocodazole treatment (Fig. 2.1B). IGF-1-dependent AKT activation was also reduced when cells were treated with the vinca alkaloid drug vinblastine, which also disrupts microtubules and is used clinically in chemotherapy regimens (Fig. 2.1C) [215]. Taken together, these results indicate that an intact microtubule cytoskeleton contributes to IRS-dependent activation of AKT by the IGF-1R.

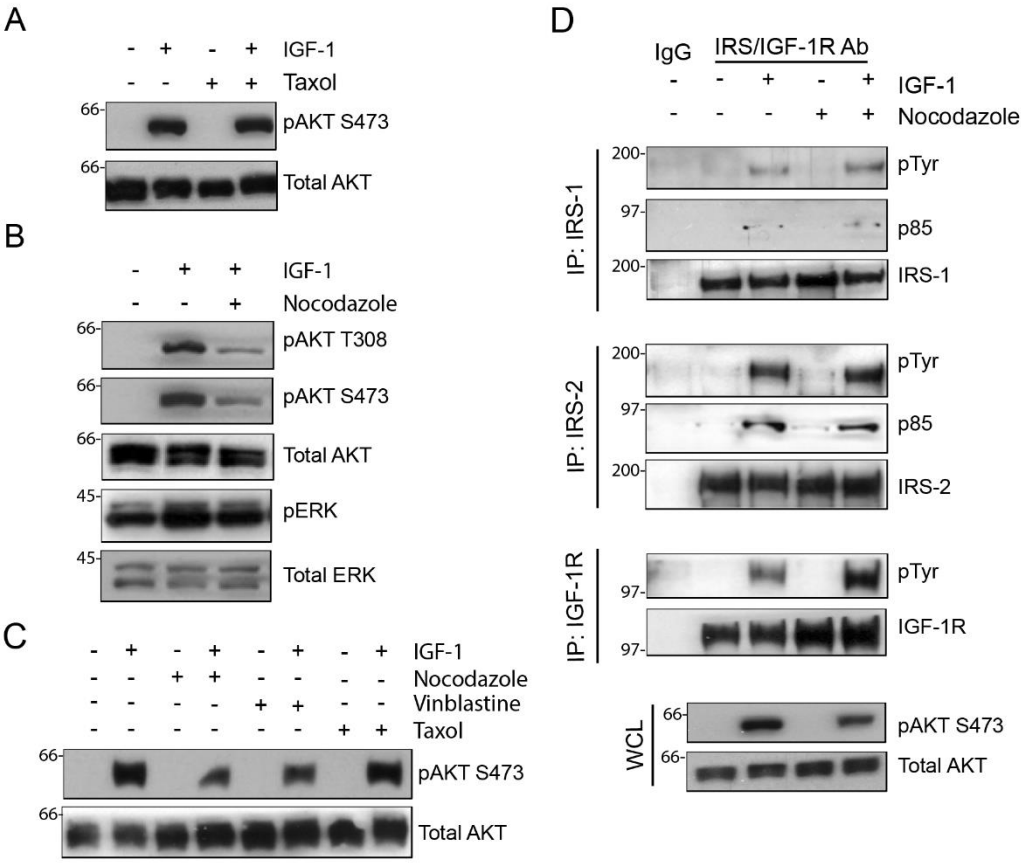

Figure 2.1. Involvement of the microtubule cytoskeleton in IGF-1 stimulated AKT activation. MDA-MB-231 cells were treated with DMSO, Taxol (20 uM), nocodazole (1 uM) or vinblastine (20 nM) for 30 minutes and then stimulated with IGF-1 (20ng/ml) for 5 min. (A-C) Aliquots of cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for phospho-S473AKT (pAKT S473), phospho-T308AKT (pAKT T308) or phospho-Thr202/Tyr204ERK (pERK). The immunoblots were subsequently stripped and reprobed with total AKT and ERK-specific antibodies. (D) Aliquots of cell extracts containing equivalent amounts of total protein were immunoprecipitated with antibodies specific for IRS1, IRS2 or IGF-1Rβ subunit and immunoblotted with antibodies specific for phosphotyrosine (pTyr) and the p85 subunit of PI3K (p85). The pTyr immunoblots were subsequently stripped and reprobed with IRS1, IRS2 or IGF-1Rβ-specific antibodies. Total cell extracts were also immunoblotted with antibodies specific for phospho-S473AKT and total AKT (WCL, bottom panels). The immunoblots shown for all panels are representative of three independent experiments.

To investigate the mechanism by which nocodazole inhibits AKT activation, phosphorylation of the IGF-1R and IRS proteins, and IRS/PI3K association were examined. Treatment with nocodazole did not decrease IRS-1 or -2 tyrosine phosphorylation or association with the p85 regulatory subunit of PI3K in response to IGF-1 stimulation, indicating that activation of PI3K upstream of AKT was not inhibited by microtubule disruption (Fig. 2.1D). Of note, expression of the IGF-1R β-subunit and its IGF-1-dependent tyrosine phosphorylation were increased following nocodazole treatment (Fig. 2.1D). We attribute this increase to the accumulation of the activated receptor at the cell surface or in early endosomes, resulting in sustained expression and activation [246].

IRS-2, but not IRS-1, requires the microtubule cytoskeleton to activate AKT

To determine if there is a selective role for the microtubule cytoskeleton in IRS-1 or IRS-2 mediated signaling, mammary tumor cells derived from PyMT:WT, PyMT:Irs-1$^{-/-}$ or PyMT:Irs-2$^{-/-}$ tumors were treated with nocodazole and stimulated with IGF-1 (Fig. 2A). WT cells that express both Irs-1 and Irs-2 demonstrated a 40% reduction in Akt activation after treatment with nocodazole. PyMT:Irs-1$^{-/-}$ cells, which signal exclusively through Irs-2, exhibited a further reduction in IGF-1-dependent Akt activation (~50%) following treatment with nocodazole. In contrast, Akt activation was equivalent in PyMT:Irs-2$^{-/-}$ cells, which signal exclusively through Irs-1, in response to IGF-1 stimulation following DMSO or nocodazole treatment.

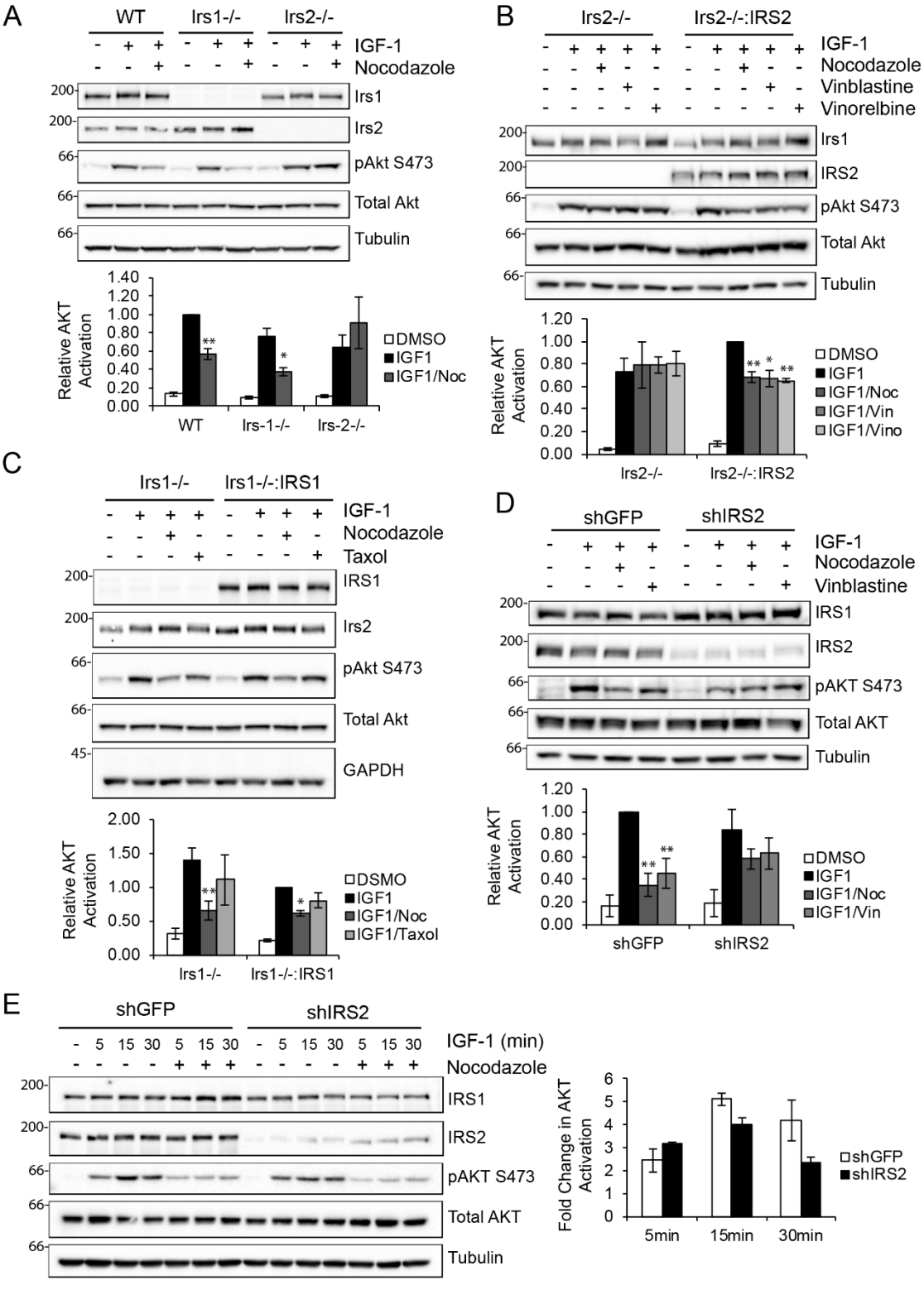
A
WT
Irs1-/-
Irs2-/-
IGF-1
Nocodazole
Irs1
Irs2
pAkt S473
Total Akt
Tubulin
Relative AKT Activation
DMSO
IGF1
IGF1/Noc
WT
Irs-1-/-
Irs-2-/-
B
Irs2-/-
Irs2-/-:IRS2
IGF-1
Nocodazole
Vinblastine
Vinorelbine
Irs1
IRS2
pAkt S473
Total Akt
Tubulin
Relative AKT Activation
DMSO
IGF1
IGF1/Noc
IGF1/Vin
IGF1/Vino
Irs2-/-
Irs2-/-:IRS2
C
Irs1-/-
Irs1-/-:IRS1
IGF-1
Nocodazole
Taxol
IRS1
Irs2
pAkt S473
Total Akt
GAPDH
Relative AKT Activation
DSMO
IGF1
IGF1/Noc
IGF1/Taxol
Irs1-/-
Irs1-/-:IRS1
D
shGFP
shIRS2
IGF-1
Nocodazole
Vinblastine
IRS1
IRS2
pAKT S473
Total AKT
Tubulin
Relative AKT Activation
DMSO
IGF1
IGF1/Noc
IGF1/Vin
shGFP
shIRS2
E
shGFP
shIRS2
IGF-1 (min)
Nocodazole
IRS1
IRS2
pAKT S473
Total AKT
Tubulin
Fold Change in AKT Activation
shGFP
shIRS2
5min
15min
30min

Figure 2.2. Selective requirement of the microtubule cytoskeleton for IRS-2 mediated signaling. (A) *PyMT:WT*, *PyMT:Irs-1*$^{-/-}$, and *PyMT:Irs-2*$^{-/-}$ cells were treated with DMSO or 20 uM nocodazole for 1 hour and then stimulated with IGF-1 (10ng/ml) for 5 min. (B) *PyMT:Irs-2*$^{-/-}$ cells transfected with empty vector (Irs2-/-) or IRS2 (Irs2-/-:IRS2) were treated with DMSO, 1 uM nocodazole, 20nM vinblastine or 20nM vinorelbine for 1 hour and then stimulated with IGF-1 (10ng/ml) for 15 min. (C) *PyMT:Irs-1*$^{-/-}$ cells transfected with empty vector (Irs1-/-) or IRS1 (Irs1-/-:IRS1) were treated with DMSO, 1 uM nocodazole or 10 uM Taxol for 1 hour and then stimulated with IGF-1 (10ng/ml) for 5 min. (D) MDA-MB-231 cells expressing either an shRNA targeting *GFP* (shGFP) or *IRS2* (shIRS2) were treated with DMSO, 1 uM nocodazole or 20nM vinblastine for 30 min and then stimulated with IGF-1 (10ng/ml) for 30 min. (E) MDA-MB-231 cells expressing either an shRNA targeting *GFP* (shGFP) or *IRS2* (shIRS2) were treated with DMSO or 1 uM nocodazole for 30 min and then stimulated with IGF-1 (10ng/ml) for the time periods indicated. The data in the graph represent the fold change in phospho-AKT between DMSO and Nocodazole treated cells for each cell type. Aliquots of cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for IRS1, IRS2, phospho-S473AKT, total AKT, tubulin or GAPDH. The data shown in the graphs for each immunoblot represent the mean of three (±SEM) independent experiments. *p≤0.05 relative to shGFP; **p≤0.01 relative to shGFP.

The role of IRS-2 in the sensitivity of cells to microtubule disruption was explored further using *PyMT:Irs-1*$^{-/-}$ and *PyMT:Irs-2*$^{-/-}$ cells that were derived from *PyMT:Irs-1*$^{fl/fl}$ and *PyMT:Irs-2*$^{fl/fl}$ cell lines, respectively, after acute adenoviral-Cre infection. *PyMT:Irs-2*$^{fl/fl}$ cells with or without restored IRS-2 expression were stimulated with IGF-1 after treatment with nocodazole and vinblastine. An additional vinca alkaloid drug vinorelbine, which is used to treat breast cancer patients [215, 247], was also assayed [248]. As observed previously (Fig. 2.2A), disruption of microtubules did not decrease Akt activation in cells lacking Irs-2 expression (Irs2-/-), whereas the increase in Akt activation that was observed upon restoration of IRS-2 expression was eliminated by microtubule disruption (Irs2-/-:IRS2) (Fig. 2.2B). In contrast, the fold-change reduction in Akt activation observed in Irs1-/- cells after nocodazole treatment (50%) was diminished (30%) upon restoration of IRS-1 expression (Irs-/-:IRS1) (Fig. 2.2C). Taxol treatment did not inhibit Akt activation in the mouse mammary tumor cells (Fig. 2.2C) as was observed for the MDA-MB-231 cells (Fig. 2.1A).

To assess the role of microtubules in IRS-2-mediated signaling in human breast carcinoma cells, *IRS2* expression was suppressed by shRNA-targeting in MDA-MB-231 cells (Fig. 2.2D). Total AKT activation in response to IGF-1 stimulation was reduced in shIRS-2 cells compared to shGFP cells (lanes 2 and 6). Treatment of shGFP cells with either nocodazole or vinblastine significantly reduced AKT activation (lanes 3 and 4) (Fig. 2.2D), as was observed for the

parental cells (Fig. 2.1C). In contrast, no significant reduction in AKT phosphorylation occurred in the shIRS2 cells, which signal predominantly through IRS-1, upon treatment with either nocodazole or vinblastine (lanes 7-8) (Fig. 2.2D). The fold difference in AKT activation observed in the shGFP and shIRS2 cells upon microtubule disruption increased with time of stimulation indicating that microtubules are required to sustain IRS2-dependent signaling (Fig. 2.2E). Taken together with the mouse cell line data, these results support that an intact microtubule cytoskeleton contributes to IGF-1 signaling through IRS-2, but not IRS-1,

The dependency of IRS2 signaling to Akt on an intact microtubule cytoskeleton indicates a potential interaction of IRS-2 with microtubules. To investigate this possibility, the localization of IRS-1, IRS-2 and tubulin was examined by immunofluorescent staining and confocal imaging. For these experiments, SUM-159 breast carcinoma cells were used because they express both IRS-1 and IRS-2, spread well on coverslips and retain their spread morphology upon treatment with Taxol and nocodazole, which facilitates detection of co-localization. Both IRS-1 and IRS-2 were expressed in a punctate manner throughout the cytoplasm, with a modest enhancement in the perinuclear region (Fig. 2.3; left panels). Although no specific pattern of staining was observed for IRS-1, the punctate staining for IRS-2 was more organized, with some apparent

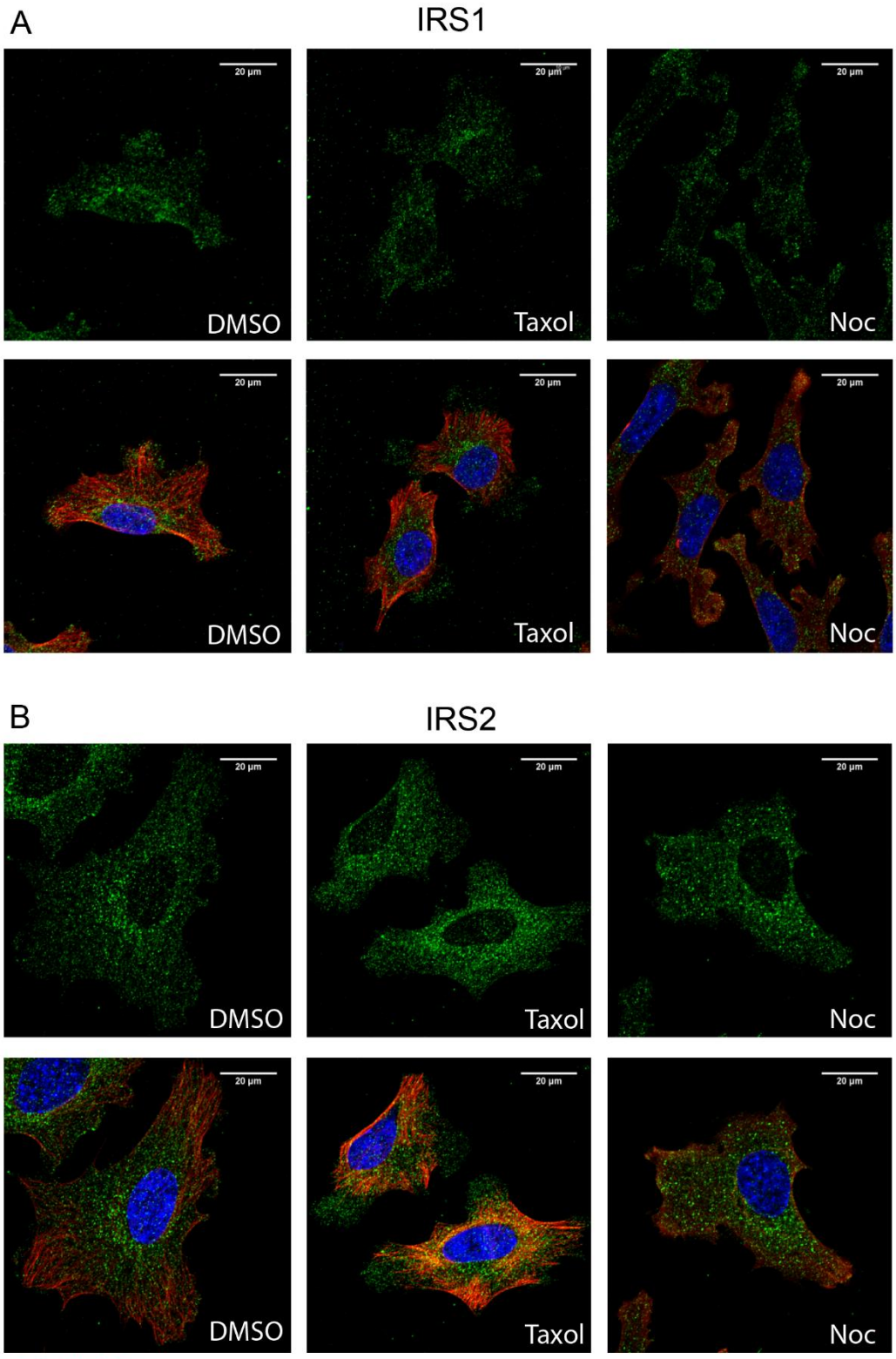
A
IRS1
20 µm
DMSO
Taxol
Noc
B
IRS2
IRS1/IRS2
Tubulin

Figure 2.3. Intracellular localization of IRS proteins and co-localization with microtubules. SUM159 cells were treated with either DMSO, 20 μM Taxol or 1 uM nocodazole for 1-2 hours before fixation. Fixed cells were co-stained for IRS-1 or IRS-2 (green) and tubulin (red). Individual images for IRS-1/IRS-2 and merged IRS/tubulin images are shown (magnification 63x). Bar = 20μM

alignment along microtubules. To assess further the association of IRS-2 with microtubules, cells were stained after a short-term incubation with either Taxol or nocodazole to evaluate their impact on IRS localization. The organized, linear pattern of IRS-2 staining was more apparent upon Taxol stabilization of the microtubules, and a subset of IRS-2 puncta co-localized with tubulin under these conditions (Fig. 2.3B; middle panels). IRS-2 was dispersed throughout the cytoplasm with no tubulin co-localization upon disruption of microtubules by nocodazole (Fig. 2.3B; right panels). In contrast, IRS-1 staining was not modified in response to Taxol or nocodazole treatment (Fig. 2.3A).

IRS-2 determines cellular responses to microtubule disruption

Drugs that target the microtubule cytoskeleton are used clinically for the treatment of cancer [215]. To investigate how IRS-2 may impact the response of tumors to microtubule stabilizing or disrupting drugs, MDA-MB-231 cells were treated for 48hours with either nocodazole or Taxol and then analyzed for viability by propidium iodide staining and flow cytometry analysis. A significant increase in the sub-G1 population occurred in response to both nocodazole and Taxol treatment in the shGFP control cells (Fig. 2.4A), consistent with an induction of cell death. When compared with the shGFP cells, cell death was significantly diminished in the shIRS-2 cells in response to nocodazole treatment (Fig. 2.4A). In contrast, when cells were treated with Taxol, which does not inhibit IRS2-mediated AKT signaling (Figs. 2.1A and 2.2C), cell death levels were similar for

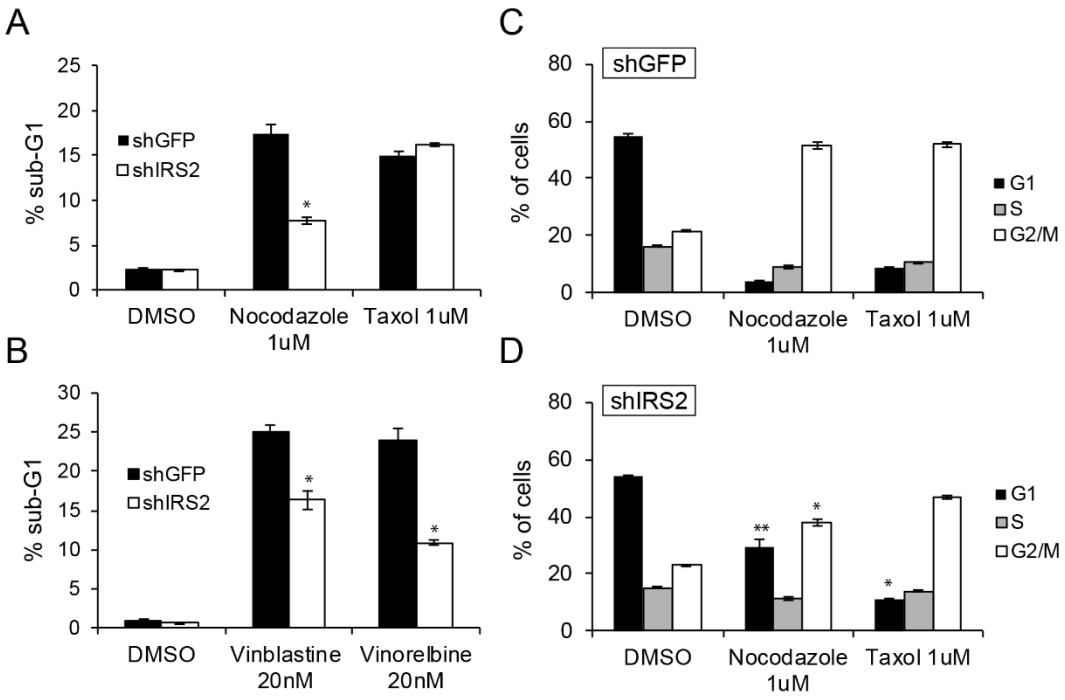

Figure 2.4. Role of IRS-2 in the cellular response to microtubule disruption. MDA-MB-231 cells were treated with DMSO or the indicated drugs for 48 hrs. The cells were stained with propidium iodide and analyzed by flow cytometry. Shown are the % of cells in the sub-G1 peak (A, B) or cell cycle stages (C,D). The data shown represent the mean of representative experiments performed three (±SEM) (nocodazole, vinblastine and vinorelbine) or two (±SD) (Taxol) independent times. *p≤0.05 relative to shGFP; **p≤0.01 relative to shGFP.

the shGFP and shIRS2 cells. To investigate further the clinical relevance of the IRS2-dependent sensitivity of breast carcinoma cells to microtubule disruption, MDA-MB-231 cells were treated with vinblastine or vinorelbine, both of which are used in the treatment of solid tumors, including breast cancer [215]. As was observed for nocodazole treatment, cell death was significantly diminished in the shIRS2 cells treated with either vinblastine or vinorelbine when compared with the shGFP treated cells (Fig. 2.4B).

A similar resistance to cell death upon treatment with nocodazole was observed for PyMT:Irs2-/- cells when compared with parental cells (PyMT:Irs2fl/fl), or $Irs2^{-/-}$ cells in which WT Irs-2 expression was restored (Irs2-/-:IRS2) (Fig. 5A). In contrast, restoration of IRS1 expression to PyMT:Irs1-/- cells reduced cell death in response to nocodazole treatment (Fig. 2.5B). Similar to the MDA-MB-231 cells, cell death in response to Taxol treatment was not dependent upon Irs expression in the PyMT mammary tumor cells (Fig. 2.5A and 2.5B).

As has been reported previously, cells undergo a G2/M arrest in response to microtubule disruption or stabilization [249]. Cell cycle profiles of the cells treated with nocodazole or Taxol were analyzed to determine if IRS2 expression influences the cell cycle response to microtubule-targeting drugs. MDA-MB-231:shGFP cells exhibited an increase in G2/M arrest when treated with nocodazole (Fig. 2.4C). In contrast, a significantly higher percentage of MDA-MB-

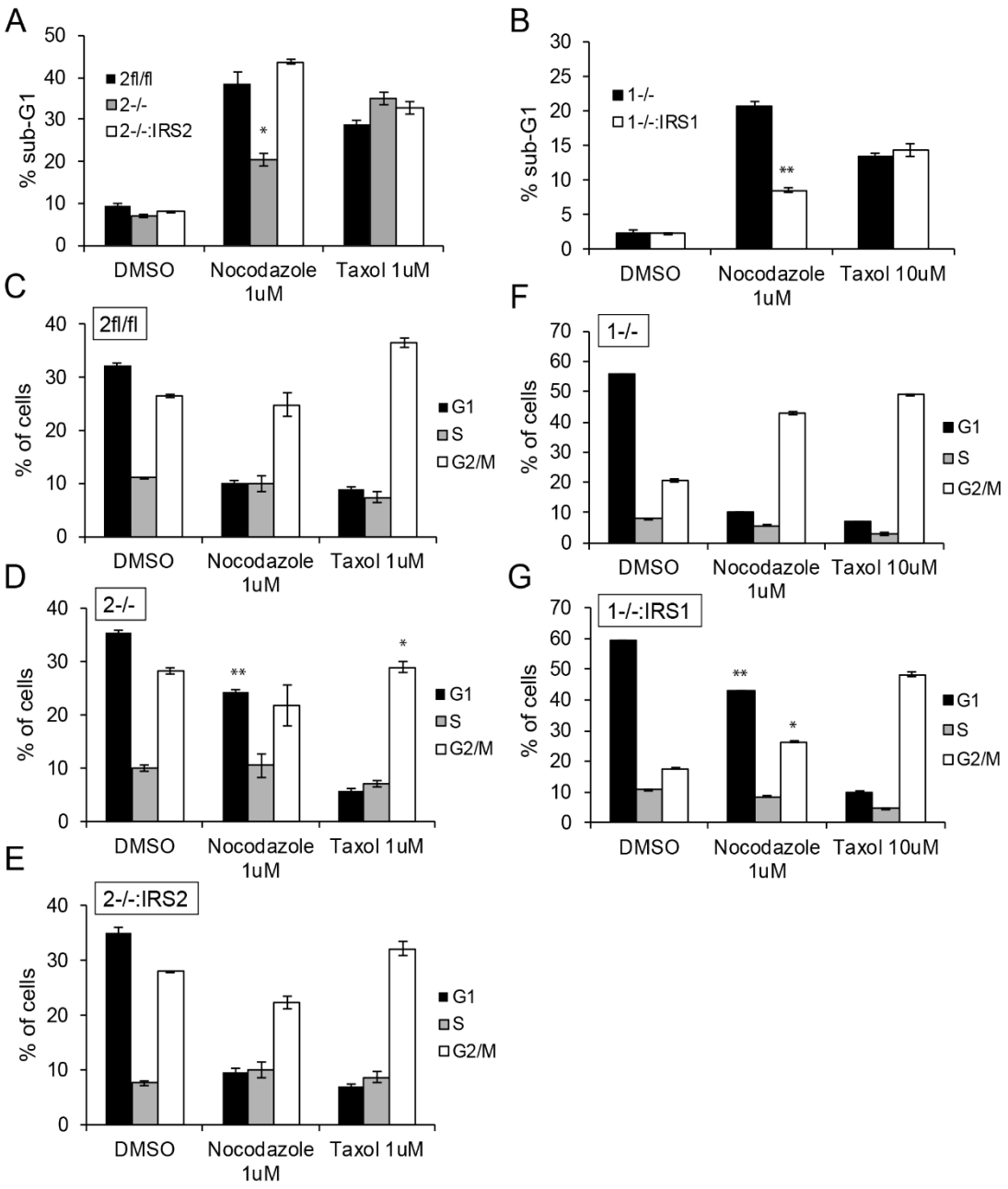

Figure 2.5. Differential impact of Irs1 and Irs2 on the cellular response to microtubule disruption. PyMT cells were treated with DMSO or the indicated drugs for 48 hrs. The cells were stained with propidium iodide and analyzed by flow cytometry. Shown are the % of cells in the sub-G1 peak (A, B) or cell cycle stages (C-G). The data shown represent the mean (±SEM) of representative experiments performed three or two (Taxol;Irs2 cells) independent times. 2fl/fl,

PyMT:Irs2fl/fl cells; 2-/-, *PyMT;Irs2-/-* cells; 2-/-:IRS2, *PyMT:Irs2-/-:IRS2* cells. 1-/-, *PyMT;Irs1-/-* cells; 1-/-:IRS1, *PyMT:Irs1-/-:IRS1* cells. *p≤0.05 relative to Irsfl/fl; **p≤0.001 relative to Irsfl/fl.

231:shIRS2 cells remained in G1/G0 upon disruption of microtubules (Fig. 2.4D). This increase in G1/G0 cells was also observed in PyMT:Irs2-/- cells (Fig. 2.5D), and

rescue of IRS-2 expression restored the G2/M arrest profile to that of the parental PyMT:Irs2fl/fl cells (Fig. 2.5C,E). In contrast, PyMT:Irs1-/- exhibited a G2/M arrest, and restoration of IRS-1 expression promoted in an increase in G1/G0 cells (Fig. 2.5F,G), similar to the profile of the IRS2 deficient cells that signal only through IRS1. All cells treated with Taxol exhibited a similar G2/M arrest profile (Fig. 2.4 and 2.5). These data indicate that breast carcinoma cells that express and signal through IRS-2 are more sensitive to drugs that depolymerize microtubules and respond by undergoing cell cycle arrest and increased cell death, whereas Taxol response is not determined by IRS-2 expression.

Given that AKT signaling is selectively resistant to microtubule disruption in cells deficient for IRS2 expression, we hypothesized that the resistance of these cells to nocodazole-mediated cell death was the result of sustained AKT signaling in these cells. To test this hypothesis, cells were treated with nocodazole for 48hrs in the presence or absence of an AKT-specific inhibitor, MK2206. Treatment of MDA-MB-231 shGFP and shIRS2 cells with MK2206 alone did not alter the % of sub-G1 cells (Fig. 2.6A) or cell cycle profile (Fig. 2.6B,C), as has been reported previously for parental MDA-MB-231 cells at the concentration of inhibitor used in these assays [247]. This concentration of MK2206 was, however, sufficient to

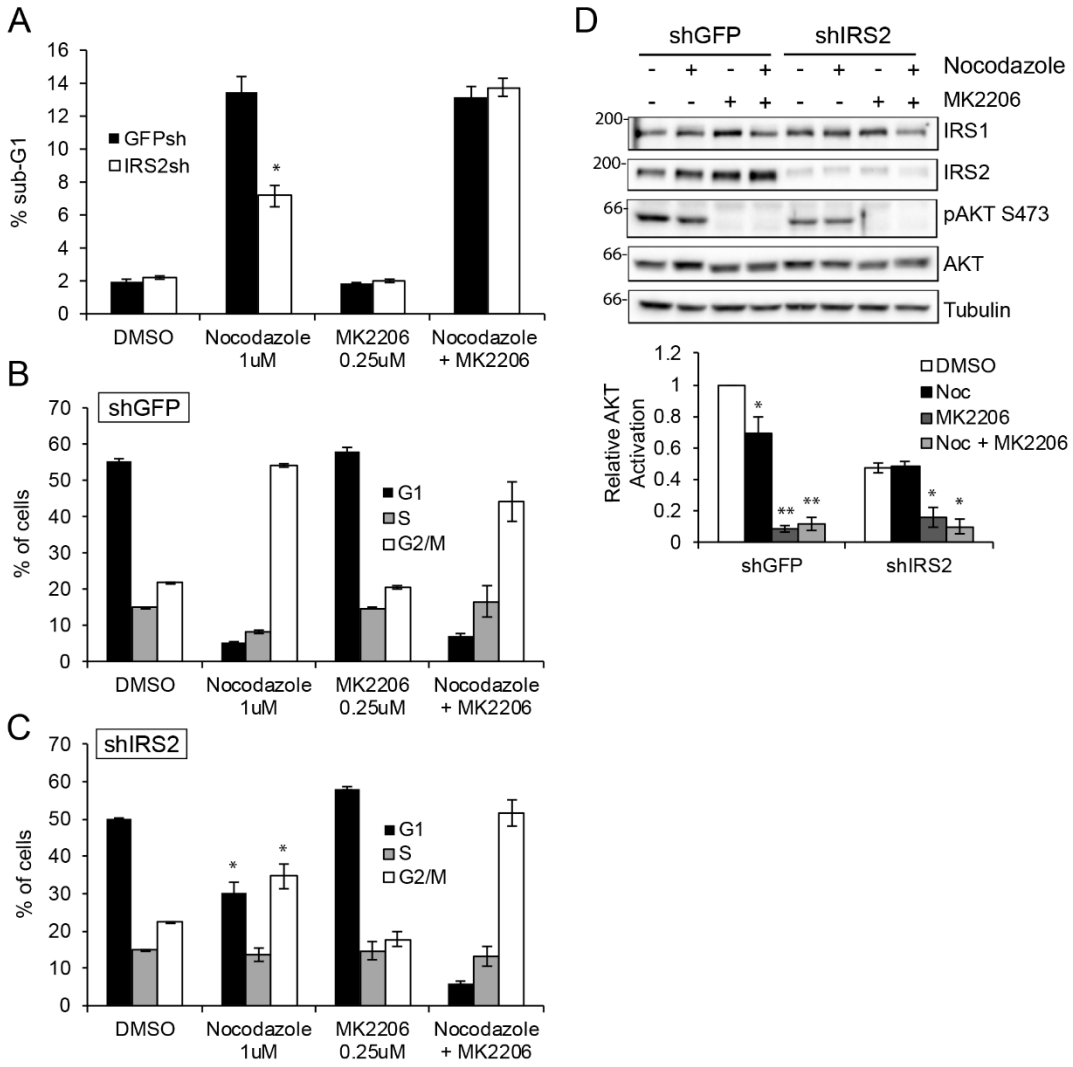

Figure 2.6. Role of AKT in the IRS-2-dependent response to microtubule disruption. MDA-MB-231 cells were treated with DMSO or the indicated drugs for 48 hrs. (A-C) Cells were stained with propidium iodide and analyzed by flow cytometry. Shown are the % of cells in the sub-G1 peak (A) or cell cycle stages (B,C). The data shown represent the mean (±SEM) of representative experiments performed three independent times. *p≤0.05 relative to shGFP (D) Aliquots of cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for IRS1, IRS2, phospho-S473AKT, total AKT or Tubulin. The data shown in the graph below represent the mean (±SEM) of three independent experiments. *p≤0.05 relative to DMSO; **p≤0.001 relative to DMSO.

inhibit AKT activation (Fig. 2.6D). Combined treatment of the MDA-MB-231:shGFP cells with MK2206 and nocodazole did not increase cell death (Fig. 6A) or alter cell cycle progression (Fig. 2.6B). In contrast, addition of MK2206 to the nocodazole treated MDA-MB-231:shIRS2 cells increased the % of sub-G1 cells to that observed for the shGFP cells treated with nocodazole alone (Fig. 2.66A). Combined treatment with MK2206 and nocodazole also promoted a G2/M arrest in these cells, restoring the cell cycle profile to that of the nocodazole-treated IRS2-expressing cells (Fig. 2.6C).

To investigate the mechanism of cell death in response to microtubule disruption, cell extracts from MDA-MB-231 cells treated with nocodazole for 48hrs in the presence or absence of MK2206 were immunoblotted for cleaved-caspase 3. Caspase 3 cleavage increased significantly upon treatment of shGFP cells with nocodazole, confirming that these cells undergo an apoptotic cell death (Fig. 2.7A) [225]. Cleaved caspase 3 levels were significantly lower in the shIRS2 nocodazole-treated cells. However, combined treatment of shIRS2 cells with both nocodazole and MK2206 increased caspase 3 cleavage, supporting a role for AKT signaling in the enhanced viability of these cells. Analysis of upstream apoptotic effectors identified the BCL2 homology 3 (BH3)-only protein BIM as a potential regulator of caspase 3 activation in response to microtubule disruption [225]. Specifically, the BIM-EL and BIM-L isoforms of BIM were expressed at elevated

levels in shGFP cells when compared with shIRS2 cells, and expression increased upon co-treatment with MK2206 in shIRS2 cells (Fig. 2.7B).

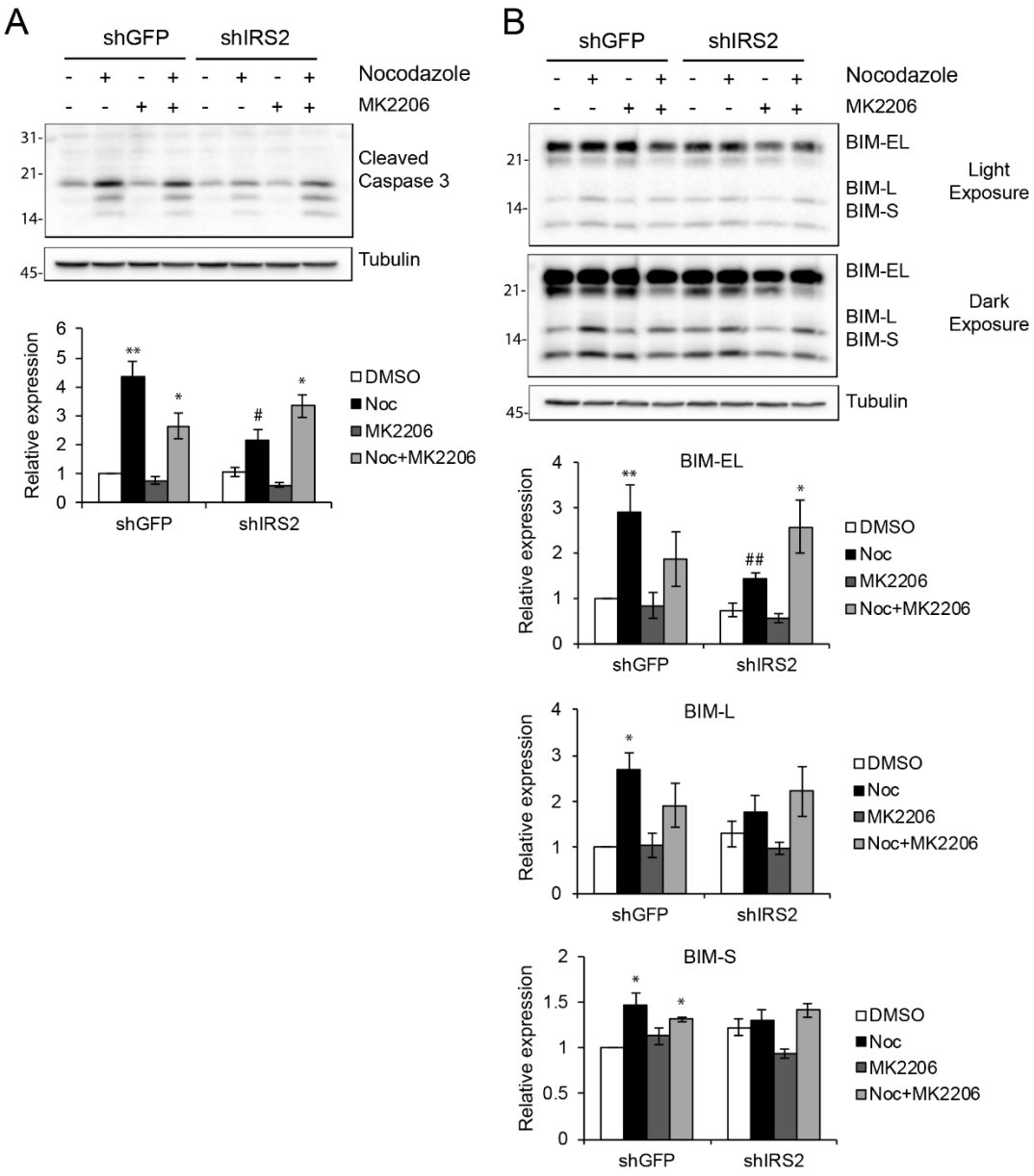

Figure 2.7. Involvement of the apoptotic effector BIM in the response to microtubule disruption. MDA-MB-231 cells were treated with DMSO or the indicated drugs for 48 hrs. Aliquots of cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for Cleaved Caspase-3 (A) or BIM (B) and Tubulin. The data shown in the graphs below each panel of immunoblots represent the mean (±SEM) of three independent experiments. *p≤0.05 relative to DMSO; **p≤0.01 relative to DMSO; #p≤0.05 relative to shGFP-Nocodazole; ## p≤0.01 relative to shGFP-Nocodazole.

DISCUSSION

We demonstrate in the current study a differential involvement of the microtubule cytoskeleton in IRS-dependent activation of AKT. AKT activation in response to IGF-1 stimulation is maintained when the microtubule cytoskeleton is disrupted in cells that signal only through IRS-1. In contrast, microtubule disruption significantly diminishes AKT activation when the IGF-1R signals through IRS-2. Proximal IGF-1R signaling events including receptor tyrosine phosphorylation, IRS tyrosine phosphorylation and recruitment of PI3K are not inhibited by microtubule disruption, indicating that IRS-2 requires the microtubule cytoskeleton at the level of downstream effector activation. The co-localization of IRS-2, but not IRS-1, with tubulin is enhanced upon Taxol-mediated microtubule stabilization, which in concert with the signaling data suggests an interaction of IRS-2 with the microtubule cytoskeleton that may facilitate its access to effectors such as AKT. Functionally, IRS-2 sensitizes breast carcinoma cells to apoptotic cell death in response to treatment with microtubule disrupting drugs through a mechanism involving the inhibition of AKT signaling and regulation of the BH3-only apoptotic activator BIM. Our data identify IRS-2 as a potential biomarker for the response of breast cancer patients to vinca alkaloid drug treatment.

The IRS proteins function as signaling intermediates for both the IGF-1R and IR. Previous studies have investigated the importance of the microtubule cytoskeleton in signaling through the IR in insulin-responsive cell types such as

adipocytes and muscle [212, 244]. Similar to our findings with IGF-1R signaling, proximal IR signaling events are not impacted by microtubule disruption, while distal events such as GLUT4 translocation to the plasma membrane are inhibited [244]. The impact of microtubule disruption on AKT activation in response to insulin stimulation is cell type dependent. Insulin-induced AKT activation was modestly reduced in 3T3-L1 adipocytes, maintained in CHO cells that express IR and IRS-1 (CHO.IR.IRS-1), and inhibited in skeletal muscle cells upon treatment with microtubule disrupting drugs [212, 250]. Importantly, the involvement of either IRS-1 or IRS-2 was not investigated in these different cell models. The differential expression and activation of IRS-1 and IRS-2 in each cell type may explain the differential responses of these cells to microtubule disruption.

Our demonstration that IRS-1 and IRS-2 differ in their dependence upon an intact microtubule cytoskeleton for their downstream signaling adds to the mechanistic understanding of how signaling by these homologous proteins regulates distinct functional outcomes. IRS-1 and IRS-2 are both capable of recruiting PI3K and activating AKT, yet the functional response to this activation is quite different for each adaptor protein [108]. Stimulation of cells with insulin or IGF-1 promotes proliferation when IRS-1 is the dominant signaling adaptor [239, 251]. Although a direct role for AKT in this response has not been demonstrated, it is dependent upon activation of PI3K [251]. In contrast, stimulation of breast carcinoma cells that express IRS-2 as the dominant signaling adaptor respond by

increasing migration/invasion and glycolytic metabolism [115, 117, 238, 239]. IRS-2-dependent activation of PI3K is also required for these cellular responses [180]. With regard to metabolism, the ability of IRS-2 to selectively regulate distinct downstream AKT effectors contributes to this differential outcome. IRS-2-dependent activation of AKT results in the phosphorylation and inactivation of GSK3 and this inactivation is required for IRS-2-mediated regulation of glucose uptake [180]. In the current study, expression of the apoptotic activator BIM increased in response to disruption of IRS-2-dependent AKT activation, a mechanism that may involve the selective regulation of another AKT effector pathway. Specifically, BIM expression is positively regulated by FOXO transcription factors, which are inactivated by AKT phosphorylation [252-254]. IRS-2 has been previously shown to mediate insulin regulation of Foxo1 activity and Bim expression in neonatal mouse hepatocytes [255].

Our current data support the hypothesis that the ability of IRS-2 to interact with and potentially traffic along microtubules may determine its access to distinct subsets of effectors to elicit unique functional responses. Of note, a selective role for IRS-1 and IRS-2 dependent signaling in skeletal muscle has been reported that involves differential AKT isoform activation. Insulin stimulated myoblast differentiation and glucose metabolism are regulated by IRS-1/AKT2 signaling, whereas signaling through IRS-2/AKT1 controls lipid metabolism [256]. The possibility that AKT isoforms are selectively sensitive to microtubule disruption

could contribute to the differential sensitivity of IRS-1 and IRS-2 to microtubule loss.

The results of our study are consistent with the work of other groups that suggest a central role for AKT in the tumor cell response to microtubule-disrupting drugs [257-259]. Specifically, AKT promotes the phosphorylation of microtubule binding proteins that stabilize microtubules, and in doing so increases the resistance of tumor cells to drugs that function by disrupting the microtubule cytoskeleton. Our data now reveal that the mechanism by which tumor cells regulate AKT activity will also influence response to these drugs. Our results have implications for the use of microtubule-disrupting drugs, such as vinblastine and vinorelbine, for the treatment of breast cancer. The differential responses of IRS-1 and IRS-2-dependent signaling to these chemotherapeutic drugs raise the possibility that IRS-2 may influence how breast tumors respond to vinca alkaloid drug treatment. We reported previously that expression of IRS-2 at the cell membrane is associated with a statistically significant decrease in overall survival in breast cancer patients [111]. We hypothesize that IRS-2 at the cell membrane is indicative of active signaling, and patients that have tumors with this staining pattern may be more sensitive to microtubule-disrupting drugs than patients without active IRS-2 signaling. Moreover, breast tumors with low IRS2 expression/function could be responsive to combination therapies that pair an AKT

inhibitor with a vinca alkaloid drug. Therefore, IRS-2 could be used as a biomarker to identify patients for targeted treatment with these drugs.

CHAPTER III

Mechanistic dissection of IRS-2: Structural functional analysis identifies regions that regulate tumor cell invasion and tumor-initiating cell self-renewal

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Author contributions:

Jenny Janusis performed experiments for Figures 3.2, 3.4 and 3.6.

Samuel Chen performed experiments for Figure 3.5.

Sha Zhu assisted with cloning, design of the mutagenesis approach and cell line models.

Abstract

The insulin receptor substrate (IRS) proteins IRS1 and IRS2 play divergent roles in breast cancer. Although IRS1 and IRS2 share considerable homology and activate PI3K signaling downstream of the insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF-1R), they mediate very different outcomes. IRS2, but not IRS1, is a positive regulator of mammary tumor metastasis and IRS2 loss decreases mammary carcinoma cell invasion, survival and glycolysis. To investigate the mechanism by which IRS2 regulates tumor metastasis, we took a mutagenesis approach to identify structural features of IRS2 that are required for regulating invasion and self-renewal, two essential functions for cancer progression. Our studies reveal that the ability of IRS2 to promote invasion is dependent upon upstream IGF-1R/IR activation and the recruitment and activation of PI3K. Additional sequences within the C-terminus of IRS2 are also required for IRS2 -mediated tumor cell invasion, and these sequences are sufficient to confer invasion-promoting ability when swapped into IRS1. We demonstrate for the first time that IRS2, but not IRS1, regulates tumor-initiating cell (TIC) self-renewal of breast carcinoma cells, and the ability of IRS2 to activate PI3K, as well as the IRS2 C-terminus, are also required for this function. Additional deletion analysis of the IRS2 C-terminus identified two distinct regions that regulate tumor cell invasion and self-renewal, respectively. Further analysis of the invasion region revealed an interaction with bone morphogenic protein-2 inducible kinase (BMP2K), a serine

threonine kinase of the numb associated kinase (NAK) family. Suppression of BMP2K expression decreases IRS2-dependent invasion. Taken together, our data contribute to the mechanistic understanding of how IRS2 regulates breast carcinoma cell invasion and self-renewal, two important processes for metastasis.

Introduction

The insulin receptor substrate (IRS) proteins IRS1 and IRS2 are cytoplasmic adaptor proteins that contribute to the growth and progression of breast cancer [108]. Although they share significant homology and the ability to activate similar signaling pathways, these adaptor proteins play distinct functional roles in breast cancer. IRS1 is an estrogen receptor (ER) regulated gene and it is expressed at high levels in the ER+, luminal subtype of breast cancer [260]. IRS1 interacts with the ER to positively regulate its transcriptional activity at estrogen response genes [112]. In this regard, tamoxifen response in breast cancer patients positively correlates with nuclear IRS1 expression [112]. *In vitro* studies implicate a role for IRS1 in the regulation of proliferation and survival in luminal breast carcinoma cells [110]. IRS1 expression decreases as ER expression or function is lost in more poorly differentiated, invasive breast tumors [110]. In contrast, IRS2 is expressed at higher levels in ER- breast carcinoma cells of the basal-like/triple negative breast cancer (TNBC) subtypes and it regulates tumor cell invasion and glycolytic metabolism [117, 197]. The differential functions of IRS1 and IRS2 in breast cancer are further evidenced by the fact that mouse mammary tumors that lack IRS2 are significantly diminished in their ability to metastasize to the lungs, whereas tumors lacking IRS1, but expressing elevated IRS2, have enhanced metastatic potential [114]. IRS2 expression at the cell membrane in human breast tumors correlates with decreased overall survival, a

finding that further supports a role for IRS2 in more aggressive tumor behavior [111].

The IRS proteins are recruited to cell surface receptors through N-terminal PH and PTB domains where they are phosphorylated on multiple tyrosine residues within their C-termini, either directly by receptor tyrosine kinases or by associated non-receptor kinases [261-263]. These phosphorylation events generate binding sites for the recruitment and activation of signaling effectors to amplify signaling downstream of the receptors and modify cell behavior. The IRS proteins were first characterized as regulators of signaling downstream of the insulin receptor (IR) and the insulin-like growth factor receptor (IGF-1R), but they can also serve as signaling intermediates of additional growth factor, cytokine and integrin receptors [145, 262, 264]. Many of these receptors have been implicated in tumor development, growth and metastasis, highlighting the importance of understanding the mechanism by which the IRS proteins mediate their distinct downstream signaling outcomes [145, 265, 266].

The IRS proteins are “intrinsically disordered” proteins (IDP) that contain structured N-terminal PH and PTB domains followed by a predominantly disordered C-terminal extension, which likely contributes to the divergent function of these proteins. The lack of structure within the C-terminal region of these adaptor proteins is thought to allow for flexibility in coordinating multiple

interactions in response to upstream signals through the formation of “loops” that interact with distinct binding partners to stimulate different downstream outcomes [130]. Both IRS1 and IRS2 recruit and activate the PI3K/AKT pathway *in vitro* and *in vivo*. Additional common binding proteins include growth factor receptor-bound protein 2 (GRB-2) and SHP2 [137, 138]. To date, unique binding partners have not been identified for IRS1 and IRS2 to explain their functional differences. Although both IRS1 and IRS2 are capable of activating the PI3K pathway, they have different mechanisms of AKT activation downstream of PI3K, with IRS2 requiring an intact microtubule cytoskeleton for full AKT activation and IRS1 activating AKT independently of microtubules [267]. Selective regulation of downstream AKT effectors by IRS1 and IRS2, such as GSK-3 Beta, has also been demonstrated [180]. The differential sensitivity to microtubule disruption for signaling suggests trafficking and intracellular localization of the IRS proteins contributes to their distinct functional outcomes.

Metastasis is the leading cause of mortality in breast cancer patients. Although there have been significant advances made in the treatment of primary breast tumors, there remains a relative lack of effective therapeutic approaches for metastatic disease [11, 12]. Metastasis is a complex, multi-step process. The ability to invade from the primary tumor through the underlying basement membrane into the surrounding stromal microenvironment and to access the vasculature is an early pre-requisite of metastatic cells [268, 269]. Dissemination

through the blood or lymphatic vessels, extravasation and colonization of secondary organ sites is also required. The cancer stem cell properties of self-renewal and pluripotency, as well as their ability to differentiate and repopulate the heterogeneity of a tumor, facilitate the metastatic colonization of distant organs [89, 270]. The prevention of tumor cell invasion and secondary tumor formation are major goals of therapy aimed at treating metastatic disease. In the current study, we sought to investigate the mechanism by which IRS2 selectively regulates mammary tumor metastasis. Our structural dissection of IRS2 reveals discrete regions within the C-terminus that are required for the ability of IRS2 to regulate both invasion and self-renewal.

EXPERIMENTAL PROCEDURES

Cells, shRNA, transfection and infection. MDA-MB-231 cells were obtained from the ATCC Cell Biology Collection. SUM-159 cells were a kind gift from Art Mercurio (UMass Medical School, Worcester, MA). Mammary tumor cells were isolated from female FVB MMTV-PyMT:Irs1$^{f/f}$/Irs2$^{f/f}$ mice and Irs1$^{-/-}$,Irs2$^{-/-}$:PyMT cells were generated by infection with adenoviral Cre-recombinase and subcloning to isolate cells with complete knockout of both Irs1 and Irs2 as described previously [180]. IRS1/IRS2 double null SUM-159 cells were generated by CRISPR/Cas9-mediated gene editing. gRNAs were designed using MIT CRISPR DESIGN (http://crispr.mit.edu/) to target an early 5' exon region for either IRS1 (sequence of gRNA: GCATGCTCTTGGGTTTGCGCAGG) or IRS2 (sequence of gRNA: AACCACAGCGTGCGCAAGTGCGG). The gRNAs were subcloned into the pSpCas9(BB)-2A-GFP plasmid (Addgene #48138). Cells were transfected with the CRISPR plasmid containing the IRS1-gRNA using lipofectamine 2000 (Invitrogen) and sorted by flow cytometry for the GFP-high population to obtain IRS1-/- cells. IRS1-/- cells were transfected with the CRISPR plasmid containing the IRS2-specific gRNA and sorted for GFP high cells to generate SUM-159:IRS1-/- ,IRS2-/- cells. Lentiviral vectors containing shRNAs targeting GFP, IRS2 and mouse BMP2K were obtained from the UMass RNAi core (Worcester, MA). Cells were infected viruses and stable cell lines were generated by selection in 100 ug/ml puromycin. PyMT cells were transfected with Lipofectamine 2000 (Invitrogen)

according to manufacturer's protocol. For stable selection, cells were grown in G418 (0.5mg/ml) or puromycin (100 ug/ml).

Mutagenesis and Cloning. Primers for human IRS2 were purchased from Integrated DNA technologies (IRS1Δ942, fwd 5' TACCCCTACGACGTCCC 3' and rev 5' CTGAGCAGCTGTGTCCAC 3'; IRS2Δ917, fwd 5' TACCCCTACGACGTCCC 3' and rev 5' AGGCACTACAGGGTGAGG 3'; IRS2Δ1014, 5' TACCCCTACGACGTCCC 3' and rev 5' ATACGGGGAGGAGGCCT 3'; IRS2Δ1188, fwd 5' TACCCCTACGACGTCCC 3' and rev 5' GCCCTCGCTGCTTTTCCT 3'; IRS2ΔSR, 5' CCGCCGTTGCCCCCG 3' and rev 5' GGGCTCGCCAAAGTCGATG 3'; IRS2ΔINV, fwd 5' GGCGTGGGTGTCGGC 3' and rev 5' ATACGGGGAGGAGGCCT 3'). IRS mutants were generated from pCDNA-IRS2-HA and pCDNA-IRS1-HA by PCR amplification with Q5 Hot Start High-Fidelity DNA Polymerase (#M0493S, New England Biolabs) and PCR products were ligated using the Quick Ligation Kit (#M2200S, New England Biolabs). IRS protein chimeras were generated using PCR (IRS1/S2 chimera, IRS2 C-terminus insert fwd 5' GACCAGTACGTGCTCAT GAGCT 3' and rev 5' CACAGTCGAGGCTGATCAGCTCAGCTGGCGTAGTCGGG 3'; IRS1 vector fwd 5' GCTGATCAGCCTCGACTGTG 3' and rev 5' AGCTCATGAGCACGTA CTGGTCGTATCCCCCACCCAGGCT 3'; IRS2/S1 chimera, IRS1 C-terminus insert fwd 5' TGCGGGGCTAGGCTGGAG 3' and rev 5'

GCACAGTCGAGGCTGATCAGCCTAGCTGGCGTAGTCGGGGAC 3', IRS2 vector fwd 5' GCTGATCAGCCTCGACTGTGC 3' and rev 5' CTCCAGCC TAGCCCCGCAGCTGTCCCCGCCACAGGT 3'). C-terminal inserts and IRS1 and IRS2 N-termini with vector were generated by PCR using High-Fidelity Phusion DNA Polymerase (#M0530S, New England Biolabs). IRS1 and IRS2 C-termini were transferred to IRS2 and IRS1 vectors respectively using Phusion PCR.

Individual IRS2 regions were generated by PCR amplification from pCDNA-IRS2-HA (CONT region, fwd 5' CTCAAGCT TGGCGTGGGTGTCGGC 3' and rev 5' ACCGTCGACCCACCGCCTCCGGACTCTTTCACGATGGTGGC CT 3'; SR region, fwd 5' CTCAAGCTTGGGGCCCGCCTGTCG 3' and rev 5' ACCGTCGACCCACCGCCTCCGGAATACGGGGAGGAGGCCTC 3'; INV region, fwd 5' CTCAAGCTTCCGCCGTTGCCCCCG 3' and rev 5' ACCGTCGACCCACCGCCTCCGGAGCCCTCGCTGCTTTTCCT 3'; INV region for pulldown, fwd 5' CTCTCTAGAATGCCGCCGTTGCCCCCG 3' and rev 5' CTAGGAATTCGCCCTCGCTGCTTTTCCT 3'). After amplification, regions were subcloned into the pCDH-mVenus vector. The INV region was also subcloned into a tandem affinity purification vector pCDNA-3xFLAG-6His (INV fwd 5' CTCTCTAGAATGCCGCCGTTGCCCCCG 3' and rev 5' CTAGGAATTCG CCCTCGCTGCTTTTCCT 3')

Immunoblotting and immunoprecipitation. Cells were serum starved for 4 hours (PyMT cells) in serum-free medium before stimulation with IGF-1 for 10 minutes. Cells were pretreated with the IR/IGF1R inhibitor BMS754807 (#S1124, Selleckchem) for 4 hours before serum starvation (4 hours) and stimulation with IGF-1. For total cell extract immunoblots, cells were solubilized at 4ºC in RIPA lysis buffer (25 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium orthovanadate) containing protease inhibitors (Roche, Basel, Switzerland). Cell extracts containing equivalent amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20, and 5% (wt/vol) dry milk or 5% bovine serum albumin (BSA), incubated overnight at 4ºC in the same buffer containing primary antibodies and then incubated for 1 h in 5% blocking buffer with milk containing peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Biorad, Hercules, CA) using BIORAD ChemiDoc XRS+ with Image Lab Software. The following antibodies were used for immunoblotting: HA (#11867423001, Roche), IRS-2 (#4502, Cell Signaling, Danvers, MA), p85 (#05-212, Millipore, Billerica, MA), IGF-1Rβ (#3025, Cell Signaling), pIGF-1Rβ Y1135/1136 (#3024, Cell Signaling), phospho-AKT S473 (#9271 and #4060, Cell Signaling), phospho-AKT T308 (#2965, Cell Signaling), AKT (#9272, Cell Signaling), α-tubulin (#T5168, Sigma-Aldrich), peroxidase-conjugated goat anti-rabbit IgG (#111-035-

144, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and peroxidase-conjugated goat anti-mouse IgG (#711-035-151, Jackson ImmunoResearch Laboratories, Inc).

For immunoprecipitations, cells were extracted using a 20 mM Tris, pH 7.4 buffer containing 1% Nonidet P-40, 0.137 M NaCl, 10% glycerol, 10 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitors (Roche). Aliquots of cell extracts containing equivalent amounts of protein were pre-cleared for 1hr with protein A sepharose beads and then incubated overnight at 4°C with antibodies and protein A sepharose beads (Amersham Biosciences, Piscataway, NJ) with constant agitation. The beads were washed three times in extraction buffer. Laemmli sample buffer was added to the samples and immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described above. An HA-specific Ab 9#, Roche) was used for immunoprecipitation.

Lactate and Glucose Uptake assays. Cells were grown in 12-well plates to near confluence, washed with PBS, and then incubated in 0.1% BSA/DMEM (1g/liter glucose) for 24 hours. Lactate levels in the conditioned media were measured using a lactate assay kit (Trinity Biotech), and glucose levels were measured using a glucose assay kit (Sigma), according to the instructions of the manufacturer. Cell density per well was determined using a crystal violet staining, and lactate

production and glucose uptake were expressed as a rate measurement (millimolar/milligram/hour) normalized to cell density.

Transwell Invasion assay. Matrigel (5ug; Corning) was diluted in sterile water and dried overnight onto the upper side of Transwell chambers (6.5-mm diameter, 8-um pore size; Corning). One hour before the assay, Matrigel was rehydrated with Dulbecco's modified Eagle's medium (DMEM; 1g/liter glucose) containing 0.1% Bovine serum albumin (BSA). Cells (10^4) were placed into the top chamber and conditioned media from NIH-3T3 cells was placed in the bottom chamber. After 4 hours, non-invading cells in the top chamber were removed using a cotton swab, and cells on the bottom surface of the filter were fixed with methanol and allowed to dry overnight at room temperature. Nuclei were stained with DAPI quantified using ImageJ.

3D Matrigel-Collagen I assay. Growth factor reduced Matrigel (#354230, Corning) and rat tail collagen I (#354236, Corning) were mixed to a final concentration of 2mg/ml and 1mg/ml, respectively, and 150 ul was added to each chamber of an 8 chamber slide to form an underlayer (#354118, Corning). For the cell suspension, Matrigel was mixed with Collagen I to a final concentration of 2.17mg/ml Matrigel and 1.09mg/ml collagen I and 230ul of the Matrigel/Collagen I was mixed with 20 ul of a cell suspension containing 3000 cells. 250ul of the Matrigel/Collagen I/cell suspension, with a final concentration of Matrigel (2mg/ml)

and Collagen I (1mg/mg), was added to the chambers. After a 3 hour incubation at 37ºC, 300ul of 10%FBS-DMEM media was added to the chambers. The media was changed every 2-3 days for a total of 8 days. The number of invasive and non-invasive colonies was scored.

Mammosphere serial passage assay. Single cells in suspension were plated in ultralow attachment plates (#Corning) at a density of 25,000 viable cells/ml. Cells were grown in serum-free HAM-F12 media (#Corning) supplemented with B27 (Invitrogen), 20 ng/ml EGF, 20 ng/ml bFGF (BD Biosciences) and 50ng/ml insulin (Sigma). After 4 days, the number of mammospheres 50 um or larger were scored. For serial passage, mammospheres were collected by centrifugation (1,500rpm) and dissociated enzymatically for 15 min in 0.05% trypsin, 0.53 mM EDTA (Invitrogen). Trypsin was inhibited by adding a 1:1 volume of 1X Soybean Trypsin inhibitor (Sigma), after which cells were centrifuged and washed once in 1X PBS. Cells were resuspended in mammosphere media and 25,000 cells were plated into new ultralow attachment plates. The collection and dissociation of mammospheres was performed for 3 passages.

Microscopy. SUM-159 cells were infected with lentiviruses containing pCDH-vector, pCDH-SR-mVenus, pCDH-INV-mVenus or pCDH-CONT-mVenus. Cells were plated in 60mm cell culture dishes at a subconfluent density. Fluorescent

and phase contrast images were taken using a Olympus IX71 microscope. All images were adjusted equally for brightness and contrast using ImageJ.

Tandem affinity purification and mass spectrometry. Six 15cm plates of SUM-159 cells were lysed after transient transfection of pCDNA-INV-3XFlag-6His, washed twice in PBS and lysed by scrapping cells in lysis buffer (50mM Tris, [pH 7.4], 250mM NaCl, 0.1% Tx-100, plus 10 mM sodium fluoride, 1 mM sodium orthovanadate) containing protease inhibitors (Roche, Basel, Switzerland). The lysate was incubated on ice for 20 minutes, clarified twice by centrifugation and incubated for 3 hours in 200ul of a FLAG M2 Agarose bead slurry (Sigma) that had been previously equilibrated in lysis buffer. Beads were washed five times and then the INV region was eluted five times with 0.5mg/ml 3x FLAG peptide (Sigma) in lysis buffer, with 15 minutes of incubation prior to recovery of each elution. Pooled FLAG elutions were incubated with 100ul of pre-equilibrated TALON (Millipore) agarose beads for 2 hours, washed five times in lysis buffer, and eluted five times with lysis buffer plus 250mM imidazole (15 minutes incubation per elution).

Pooled elutions from the TALON beads were Trichloroacetic acid precipitated by adding 0.11 volumes of ice cold 100% TCA and incubation on ice for 10 minutes before adding an additional 500 ul of cold 10% TCA and incubation on ice for another 20 minutes. Samples were centrifuged for 30 minutes at 14,000 rpm, after which the supernatant was removed, the pellet was washed with 500ul

cold acetone, centrifuged at 14,000 rpm for 10 minutes and then the pellet was dried in a vacuum evaporator for 5 minutes. The protein pellet was reconstituted in 1x Laemmli sample buffer and resolved on an SDS-PAGE 2 cm short gel. Gels were stained with Comassie blue and then destained. The samples were cut out of the gel and sent for mass spectrometry analysis at the UMASS Proteomics Core (Shrewsbury, Massachusetts).

Quantitative RT-PCR. Total RNA was isolated using the Qiagen RNeasy Plus Mini kit. Reverse transcription was performed with the qScript cDNA Supermix kit (Quantabio) using 1 ug RNA. RT-qPCR was performed in a 20 uL reaction with primers designed from the Harvard PrimerBank. Mouse Actin primers (control) and BMP2K primers (fwd 5' GCTTCCGTCCCTTTCATTTCT 3'Rev 5'AGCCTCCATTTTTGGTAAGGTTT 3') were used. RT-qPCR was performed with the Applied Biosystems QuantStudio 6 Flex apparatus. The RT-qPCR program consisted of an initial step of 20 seconds at 95°C followed by 40 cycles of 95°C for 1 second with 60°C at 20 seconds. The delta –delta Ct method was used to determine relative mRNA expression. Actin was used as a control for normalization.

Statistics. For the densitometry analysis of immunoblots, the results were compared using the Student t-test and the values were represented as means ± standard errors (SEM) for each group. Student t-test was also used for analysis

of the invasion and mammosphere serial passage assay data. In all analyses, a P value of 0.05 was considered statistically significant. Corresponding significance levels are indicated in the Figure Legends.

RESULTS

The IGF1R/PI3K axis contributes to IRS2-mediated tumor cell invasion.

We have previously demonstrated that mammary tumor cells lacking Irs2 expression are deficient in their ability to invade [115]. To further our understanding of how IRS2 regulates tumor cell invasion, we used double Irs1/Irs2 null (PyMT:Irs1-/-/Irs2-/-) mouse mammary tumor cells to assess Irs2 function in the absence of Irs1 expression. As shown in Figure 3.1A, restoration of Irs2 expression in the double null cells significantly increases invasion. The IRS proteins are regulated by tyrosine phosphorylation in response to upstream receptor activation. To determine if the IGF-1R or IR regulate IRS2-dependent invasion, assays were performed in the presence of the dual IGF1R/IR small molecule inhibitor BMS-754807 [271-273]. Cells were pretreated for 4 hours and then incubated with inhibitor throughout the Transwell Matrigel invasion assay. Inhibition of IR/IGF1R did not alter the invasion of Irs1-/-Irs2-/- cells expressing empty vector. In contrast, the enhanced IRS2-dependent tumor cell invasion was inhibited in a dose dependent manner (Fig. 3.2A). Cells treated in parallel with BMS-754807 and then acutely stimulated with IGF-1 were assayed for IGF-1R/IR phosphorylation and downstream activation of AKT, as a measure of PI3K activity, to confirm inhibition of the pathway. BMS-754807 efficiently blocked both IGF-1R/IR and AKT phosphorylation (Fig. 3.1B).

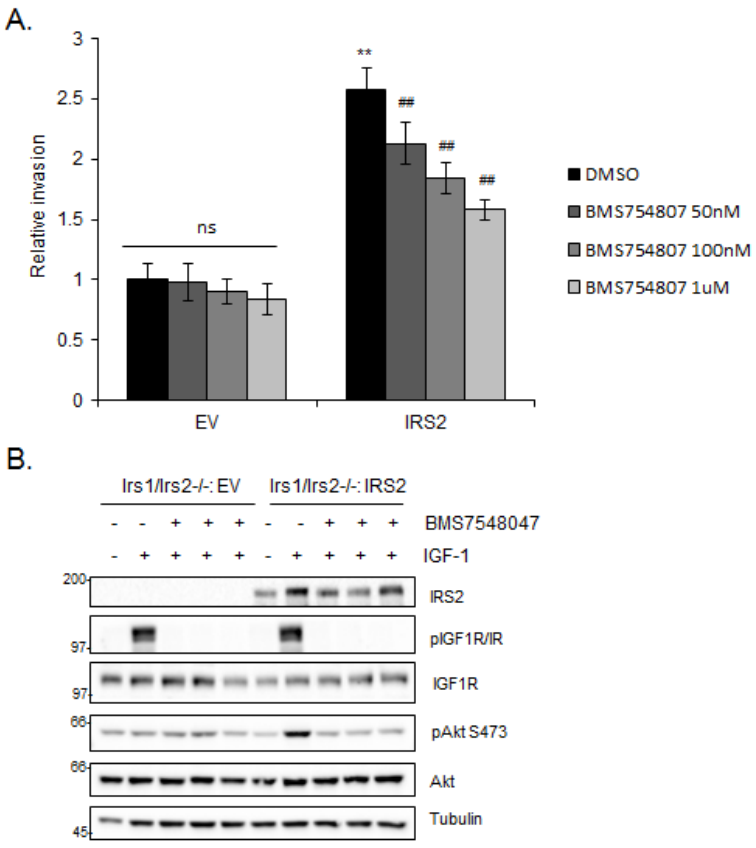

Figure 3.1. The IR/IGF-1R contributes to IRS2-mediated tumor cell invasion. PyMT:Irs1-/-/Irs2-/- cells expressing EV or IRS2 were treated with DMSO or BMS754807 at the concentrations indicated for 4 hours. (A) Matrigel Transwell invasion assays performed for 5 hours. The data shown represent the mean ± S.E.M of three experiments. ns, no significant difference; **, $p<0.01$ relative to EV-DMSO; ##, $p<0.01$ relative to IRS2-DMSO. (B) Cells were stimulated with IGF-1 (50 ng/ml) for 10 minutes in the presence or absence of BMS7548047. Aliquots of cell extracts containing equivalent amount of proteins were immunoblotted with antibodies specific for IRS2, p-IGF1R (Y1135/1136), IGF1R, pAKT (S473), AKT, or tubulin.

Both IRS1 and IRS2 can recruit and activate PI3K, but only IRS2 promotes invasion. We next wanted to examine if the ability of IRS2 to activate PI3K signaling is required for IRS2-mediated tumor cell invasion. Previous work from our group identified four essential tyrosines in IRS2 (Y649, Y671, Y734, Y814) that are required for the recruitment of PI3K and activation of the downstream PI3K/AKT pathway in response to IGF-1 and insulin stimulation [180]. PyMT:Irs1-/-/Irs2-/- cells expressing equivalent levels of Irs2 or Irs2-Y5F, an IRS2 mutant in which the essential tyrosines have been mutated to phenylalanine (Irs2-Y5F) to prevent PI3K recruitment (Fig. 3.2B), were examined for their invasive potential in a Transwell Matrigel invasion assay. As observed previously, expression of Irs2 resulted in a 2-fold increase in tumor cell invasion. Cells expressing Irs2-Y5F showed a modest increase in invasion, but they were significantly less invasive than cells expressing Irs2 (Fig. 3.2C). To confirm the results of the 2D Matrigel Transwell assay, cells were grown within a Matrigel/Collagen I matrix to assess invasive potential in a 3D environment that mimics the tumor matrix microenvironment *in vivo* [36]. Poorly/less invasive cells grow as spherical colonies in this 3D-matrix, whereas invasive cells invade into the matrix and form branched colonies (Fig. 3.2E). Although Irs2 and Irs2-Y5F expressing cells formed similar numbers of colonies, cells expressing Irs2 were significantly more invasive than cells expressing Irs2-Y5F (Fig. 3.2D). The partial reduction of tumor cell invasion in the Irs2-Y5F mutant raises the possibility that in addition to the ability of Irs2 to

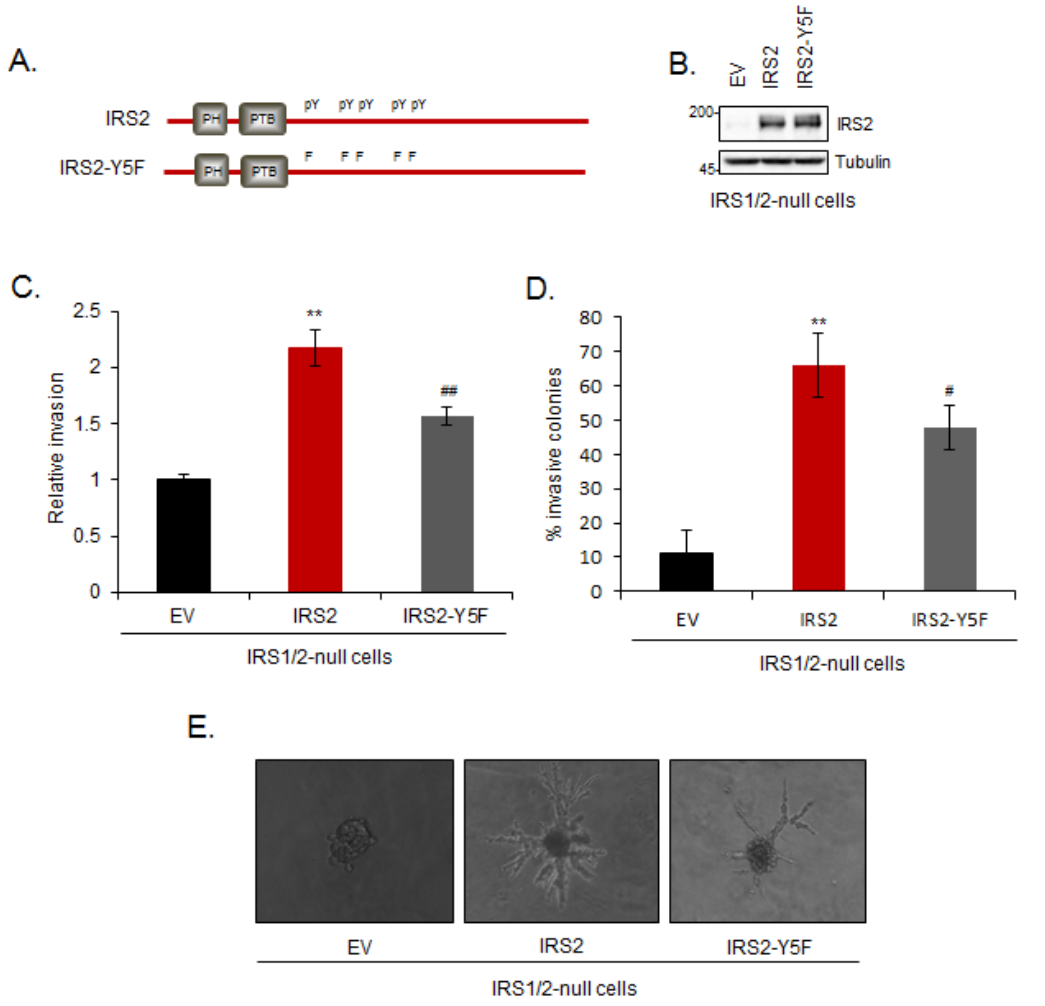

Figure 3.2. IRS2 activation of PI3K contributes to IRS2-mediated tumor cell invasion. (A) Schematic of Irs2 and Irs2-Y5F proteins. (B) Aliquots of cell extracts from PyMT:Irs1-/-/Irs2-/- cells expressing EV, Irs2 or Irs2-Y5F were immunoblotted with antibodies specific for IRS2 and Tubulin. (C) Matrigel Transwell invasion assays. The data shown represents the mean ± S.E.M of three experiments. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2. (D,E) Matrigel-Collagen I 3D invasion assay. Representative images of colonies formed in EV, Irs2 and Irs2-Y5F expressing cells are shown below (magnification X10). The data shown represent the mean ± S.E.M of a representative experiment performed three times independently. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2.

activate the PI3K pathway, additional mechanisms contribute to IRS2 regulation of tumor cell invasion.

The IRS2 C-terminus regulates tumor cell invasion

The IRS proteins are intrinsically disordered proteins that lack defined domain structure with the exception of N-terminal PH and PTB domains that mediate recruitment to upstream receptors. To dissect additional structural requirements of IRS2 involved in promoting tumor invasion, we sought to identify regions of the protein that are required for this functional outcome while preserving the ability of IRS2 to be recruited to upstream receptors and activate the PI3K/AKT pathway. To do so, we generated an IRS2 truncation mutant lacking the C-terminal portion of the protein 3' to the PI3K binding sites (IRS2Δ917) (Fig 3.3A). A corresponding truncation mutant was also generated for IRS1 (IRS1Δ942) (Fig 3.3A). Wild type and mutant proteins were expressed in the double-null cells and their phosphorylation and ability to interact with the PI3K regulatory subunit p85 in response to IGF-1 stimulation was examined. Both truncation mutants maintained their ability to be phosphorylated and recruit PI3K in response to IGF-1 stimulation (Fig 3.3B). To examine further the role of the C-terminus in the function of the IRS proteins, IRS1/S2 and IRS2/S1 chimeras were generated by swapping the C-termini of each adapter protein (Fig 3.3A). The chimeric IRS proteins were

phosphorylated and interacted with PI3K to a similar level as the WT proteins. The IRS proteins are required for IGF-1R-dependent stimulation of PI3K activation as

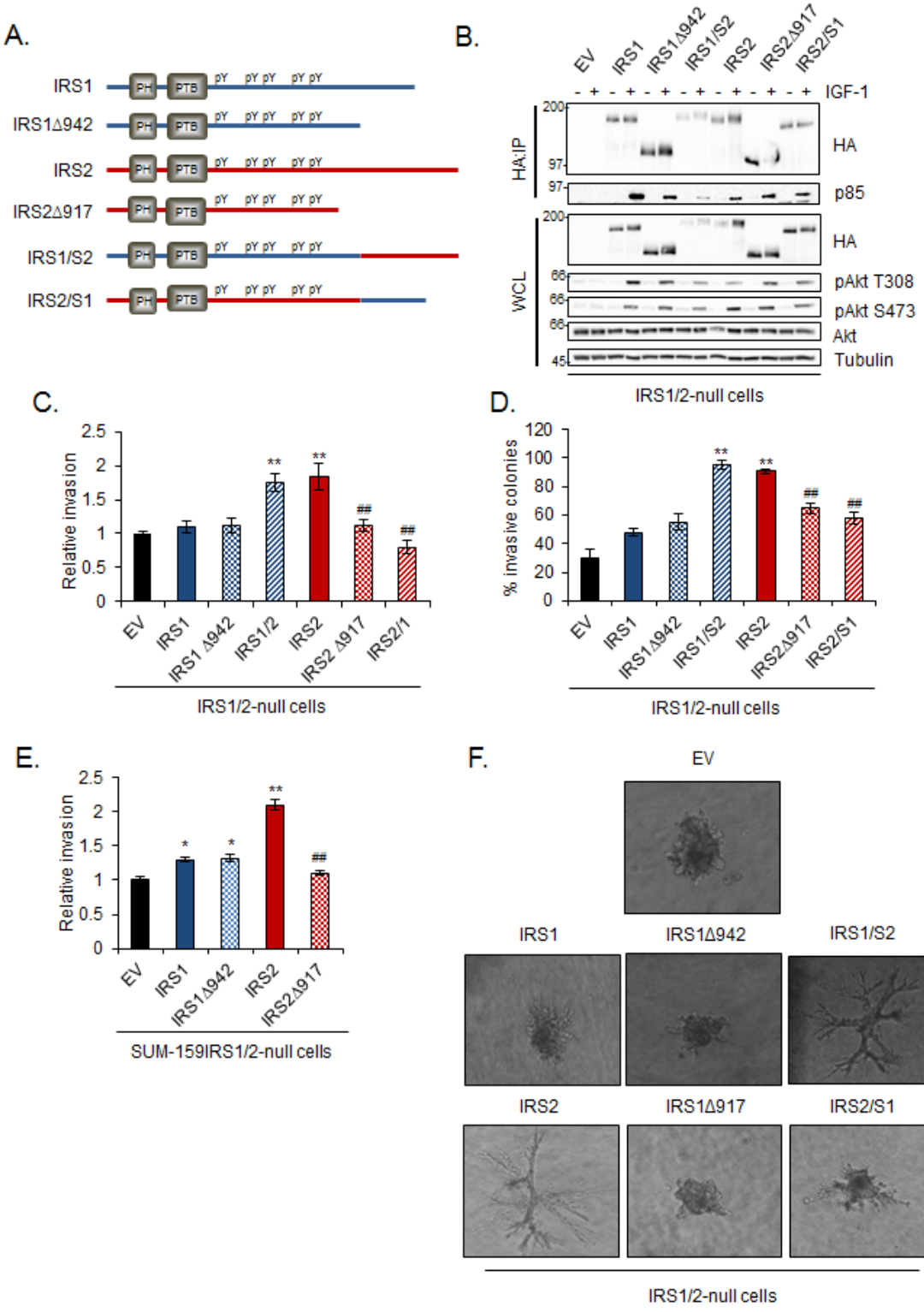
A.
IRS1
IRS1Δ942
IRS2
IRS2Δ917
IRS1/S2
IRS2/S1
PH
PTB
pY
B.
EV
IRS1
IRS1Δ942
IRS1/S2
IRS2
IRS2Δ917
IRS2/S1
IGF-1
HA:IP
HA
p85
WCL
HA
pAkt T308
pAkt S473
Akt
Tubulin
IRS1/2-null cells
C.
Relative invasion
EV
IRS1
IRS1 Δ942
IRS1/2
IRS2
IRS2 Δ917
IRS2/1
IRS1/2-null cells
D.
% invasive colonies
EV
IRS1
IRS1Δ942
IRS1/S2
IRS2
IRS2Δ917
IRS2/S1
IRS1/2-null cells
E.
Relative invasion
EV
IRS1
IRS1Δ942
IRS2
IRS2Δ917
SUM-159IRS1/2-null cells
F.
EV
IRS1
IRS1Δ942
IRS1/S2
IRS2
IRS1Δ917
IRS2/S1
IRS1/2-null cells

Figure 3.3. The IRS2 C-terminus regulates tumor cell invasion. (A) Schematic depicting WT IRS1, WT IRS2 and the IRS mutant proteins. (B) PyMT:Irs1-/-/Irs2-/- cells were serum starved for 4 hours and stimulated with IGF-1 (50 ng/ml) for 10 minutes. Aliquots of cell extracts containing equivalent amounts of total protein were immunoprecipitated (IP) with an HA-specific antibody and immunoblotted with antibodies specific for HA or the p85 subunit of PI3K (p85). Total cells extracts were also immunoblotted with antibodies specific for HA, pAKT (S473), pAKT (T308), AKT and Tubulin. (C) Matrigel Transwell invasion assays. The data shown represent the mean ± S.E.M of three experiments. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2. (D) Matrigel-Collagen I 3D invasion assays. (F) Representative images of colonies formed are shown below (magnification10X). The data shown represent the mean ± S.E. of a representative experiment performed three times independently. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2. (E) Matrigel Transwell invasion assay with SUM-159 cells expressing EV, WT IRS1, IRS1Δ942, WT IRS2 and IRS2Δ917. The data shown represent the mean ± S.E.M of three experiments. *, $p<0.05$ relative to EV; **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2.

evidenced by the lack of AKT activation in the double IRS1/IRS2 null cells in response to IGF-1 stimulation (EV; Fig 3.3B). Expression of either IRS1 or IRS2 restores the ability of IGF-1 to stimulate AKT phosphorylation at both Thr308 and Ser473 sites. Truncation or swapping of the IRS C-termini resulted in a similar level of AKT activation (Fig 3.3B). These results demonstrate that the C-terminal regions of IRS1 and IRS2 are not required for the IGF-1-dependent activation of the PI3K/AKT pathway.

Previous studies have implicated IRS2, but not IRS1, in the promotion of tumor cell invasion. Consistent with these previous findings, the invasion of cells expressing IRS1 or the IRS1 truncation mutant (IRS1Δ942) was equivalent to the invasion observed for cells expressing empty vector (pCDNA) (Fig 3.3C). Truncation of the IRS2 C-terminus prevented the IRS2-dependent increase in invasion (Fig 3.3C), indicating that sequences contained within this region are important for IRS2-dependent promotion of invasion. To examine the sufficiency of the IRS2 C-terminus to promote invasion, cells expressing the IRS1/S2 chimera, which contains the N-terminal portion of IRS1 and the C-terminal portion of IRS2, were assayed for their invasive potential. Cells expressing the IRS1/S2 chimera exhibited a similar increase in invasion that was observed for cells expressing WT IRS2. In contrast, cells expressing the IRS2/S1 chimera, which contains the N-terminal portion of IRS2 and the C-terminal portion of IRS1, failed to increase invasion above vector control cells (Fig 3.3C). Cells expressing either WT IRS2 or

the IRS1/S2 chimera also grew in a highly invasive manner within the Matrigel/Collagen I matrix (Fig3.C-F). In contrast, cells expressing the IRS2 truncation mutant or the IRS2/S1 chimera exhibited little to no invasion and the colonies formed were similar to cells expressing empty vector or IRS1. Similar invasion results were obtained when the WT-IRS proteins and their truncation mutants were expressed in an IRS1/IRS2 double null SUM-159 human breast carcinoma cell line that was generated by CRISPR/Cas9 knockout (Fig 3.3E). Taken together, these data demonstrate that the C-terminus of IRS2 is both necessary and sufficient for IRS-mediated tumor cell invasion.

The IRS2 C-terminus plays a role in the regulation of tumor-initiating cell self-renewal.

Once a tumor cell invades from the primary tumor and spreads to distant organs it must establish a secondary tumor in these foreign microenvironments. Tumor cells that have the ability to form successful metastatic lesions are thought to possess the properties of cancer stem cells (CSC), also referred to as tumor initiating cells (TIC) [270, 274]. One important property of TICs is their ability to self-renew, which sustains the stem cell population ([85]). To determine if IRS2 regulates TIC self-renewal, serial passage mammosphere assays were performed [103]. PyMT:Irs1-/-/Irs2-/- cells expressing either empty vector, IRS1 or IRS2 were plated in non-adherent conditions and grown for 4 days, at which time the mammospheres were counted and then dissociated to re-plate for additional

passages. A similar number of mammospheres were observed for cells expressing either empty vector, IRS1 or IRS2 in the first plating. Upon serial passage, cells that express IRS2 maintained higher mammosphere numbers than cells expressing either empty vector or IRS1 (Fig 3.4A).

To determine if the ability of IRS2 to activate PI3K signaling is required for TIC self-renewal, cells expressing the Irs2-Y5F PI3K-binding mutant were evaluated for mammosphere formation. The number of mammospheres formed by cells expressing Irs2-Y5F at each passage was similar to empty vector expressing cells. These data support that activation of PI3K signaling by IRS2 plays a role in the regulation of self-renewal. IRS1/IRS2 null PyMT cells expressing IRS truncations and chimeras were also evaluated by mammosphere serial passage to determine if the IRS2 C-terminus contributes to the regulation of TIC self-renewal. The number of mammospheres formed by cells expressing IRS2Δ917 and IRS2/S1 at each passage was similar to cells expressing empty vector and IRS1 (Fig 3.3C). In contrast, cells expressing both WT-IRS2 and IRS1/S2 sustained an enhanced ability to form mammospheres over several passages, supporting a positive role for the IRS2 C-terminus in the regulation of self-renewal (Fig 3.3C).

Identification of distinct regions within the IRS2 C-terminus that regulate tumor cell invasion or TIC self-renewal.

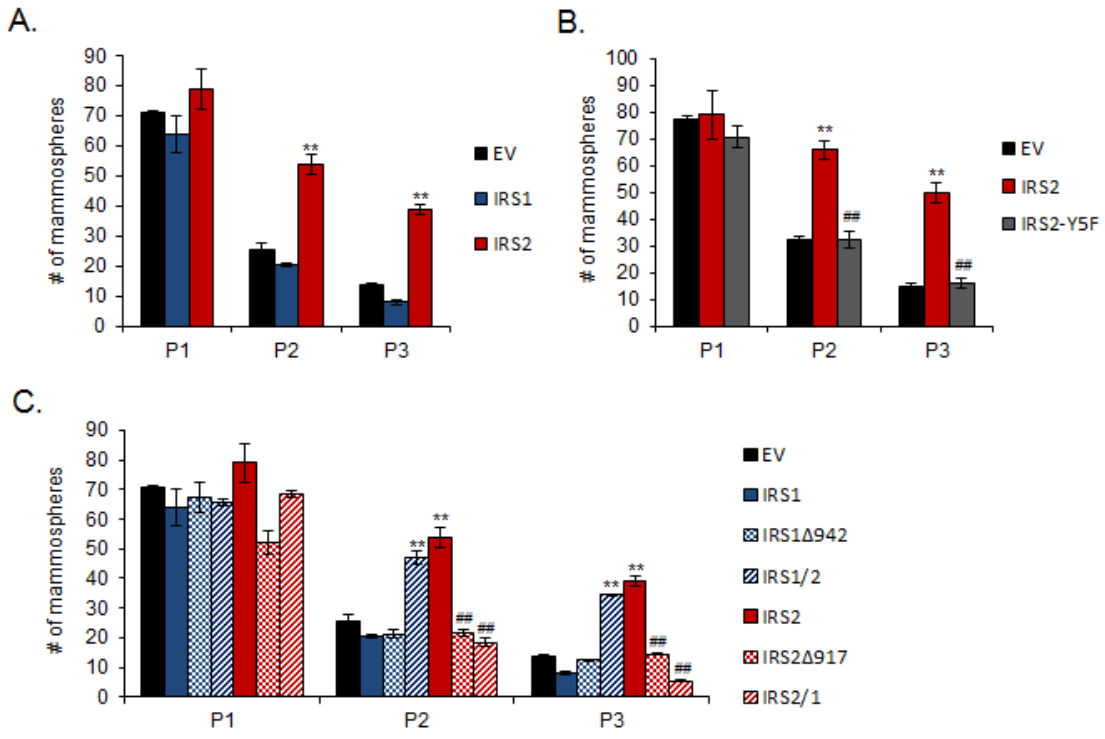

Figure 3.4. The IRS2 C-terminus regulates tumor-initiating cell self-renewal. (A-C) PyMT:Irs1-/-/Irs2-/- expressing the indicated IRS proteins were assayed for their ability to form mammospheres over three serial passages. The data shown represent the mean ± S.E.M of representative experiments performed three times independently. **, $p<0.01$ relative to EV; ##, $p<0.01$ relative to IRS2.

The IRS2Δ917 truncation mutant lacks the final 421 amino acids of the IRS2 protein. To dissect further how this region contributes to the regulation of tumor cell invasion and self-renewal, smaller truncation mutants were generated that lack either 324 amino acids (IRS2Δ1014) or 150 amino acids (IRS2Δ1188) (Fig 3.5A). The mutants were expressed in the IRS1/IRS2 null PyMT cells and assayed for their response to IGF-1 stimulation. As was observed for the larger truncation mutant (IRS2Δ917), deletion of smaller regions of the IRS2 C-terminus did not impair tyrosine phosphorylation, PI3K recruitment or downstream AKT signaling (Fig. 3.5B).

Cells expressing the IRS2Δ1014 and IRSΔ1188 IRS2 truncation mutants were assayed for their invasive and self-renewal potential. Deletion of the C-terminal 150 amino acids (IRS2Δ1188) did not inhibit the ability of IRS2 to promote either invasion (Fig. 3.5C) or self-renewal (Fig. 3.5D) when compared with full length IRS2 (Fig. 3.5C-D). The IRS2Δ1014 mutant was deficient in promoting tumor cell invasion (Fig. 3.5C), but retained the ability to regulate self-renewal (Fig 3.5D). Taken together, these data identify discrete regions within the IRS2 C-terminus that are important for its functional outcomes. A 174 amino acid region (1014-1188) is required for the ability of IRS2 to enhance tumor cell invasion, whereas a 97 amino acid region (917-1014) is essential for the ability of IRS2 to regulate of self-renewal (Fig. 3.5A).

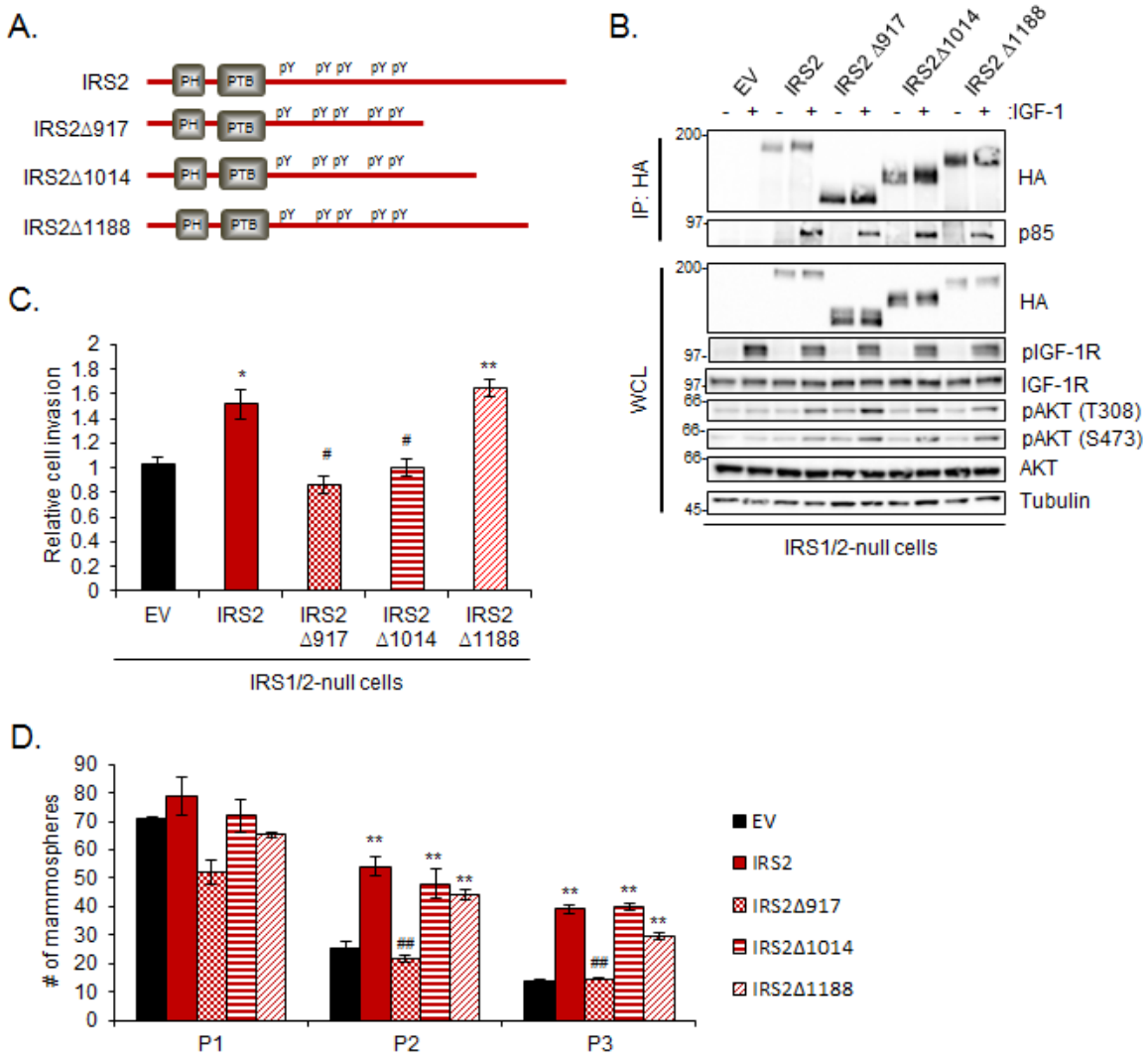

Figure 3.5. Identification of distinct regions that regulate tumor cell invasion and tumor-initiating cell self-renewal. (A) Schematic depicting WT IRS1, WT IRS2 and the IRS mutant proteins. (B) PyMT:Irs1-/-/Irs2-/- cells expressing the indicated IRS2 proteins were serum starved for 4 hours and stimulated with IGF-1 (50 ng/ml) for 10 minutes. Aliquots of cell extracts containing equivalent amounts of total protein were immunoprecipitated (IP) with an HA-specific antibody and immunoblotted with antibodies specific for HA or the p85 subunit of PI3K (p85). Total cells extracts were also immunoblotted with antibodies specific for HA, pAKT (S473), pAKT (T308), AKT and Tubulin. (C) Matrigel Transwell invasion assays. The data shown represent the mean ± S.E.M of three experiments. **, $p<0.01$ relative to EV; ##, $p<0.01$ relative to IRS2. (D) Mammosphere serial passage assay. The data shown represent the mean ± S.E.M of a representative experiment performed three times independently. **, $p<0.01$ relative to EV; ##, $p<0.01$ relative to IRS2.

To confirm the contribution of these independent regions to the regulation of tumor cell invasion and self-renewal by IRS2, internal deletion mutants were generated in which either the 174 amino acids between residues 1014 and 1188 (IRS2ΔINV) or the 97 amino acids between residues 917 and 1014 (IRS2ΔSR) were deleted (Fig. 3.6A). Both internal deletion mutants maintained their ability to associate with PI3K and activate the AKT pathway in response to IGF-1 stimulation (Fig. 3.6B). Consistent with the results obtained with the IRS2 truncation mutants, deletion of the 174 amino acid region alone rendered IRS2 incapable of promoting tumor cell invasion as measured by both Matrigel Transwell (Fig. 3.6C) and 3D Matrigel-Collagen I (Fig. 3.6D) assays. Deletion of the 97 amino acid region alone inhibited serial passage mammosphere formation (Fig. 3.6E). Importantly, IRS2ΔINV retained the ability to regulate self-renewal and IRS2ΔSR retained the ability to promote tumor cell invasion (Fig. 3.6C-E).

In previous work, we identified a role for IRS2 in the regulation of aerobic glycolysis that is dependent upon the recruitment and activation of PI3K [180]. To determine if the IRS2 C-terminus also contributes to the regulation of aerobic glycolysis, PyMT:Irs1-/-/Irs2-/- cells expressing WT-IRS2, IRS2Δ917, IRS2ΔSR and IRSΔINV were assayed for their level of glycolytic metabolism. As demonstrated previously, expression of WT-IRS2 significantly increased glucose uptake and lactate production in comparison to empty vector control cells. All of the IRS2 deletion mutants supported equivalent glucose uptake and lactate production as WT-IRS2, demonstrating that the C-terminus of IRS2 is not required

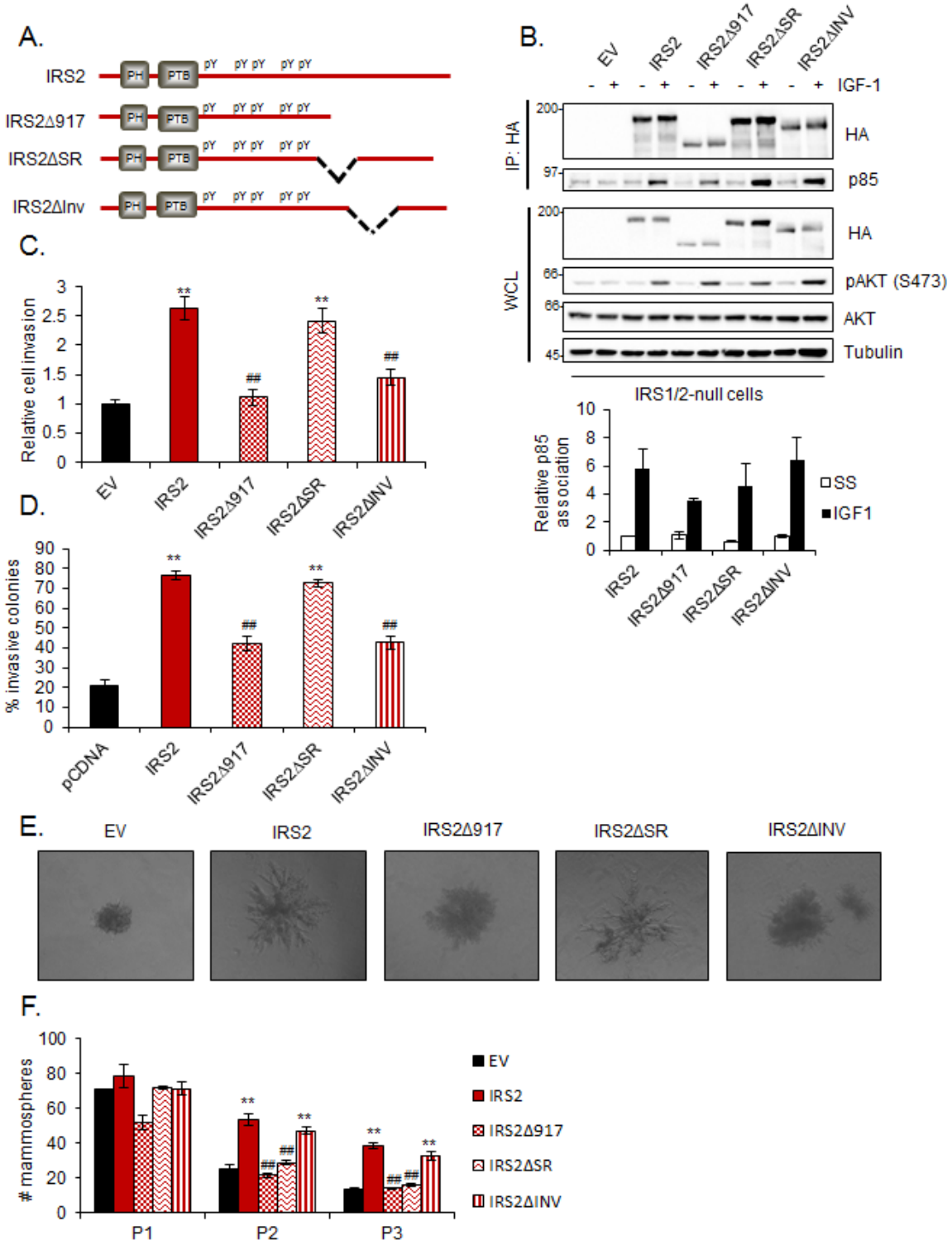
A.
IRS2
IRS2Δ917
IRS2ΔSR
IRS2ΔInv
PH
PTB
pY
B.
EV
IRS2
IRS2Δ917
IRS2ΔSR
IRS2ΔINV
IGF-1
IP: HA
HA
p85
WCL
pAKT (S473)
AKT
Tubulin
IRS1/2-null cells
Relative p85 association
SS
IGF1
C.
Relative cell invasion
D.
% invasive colonies
pCDNA
E.
F.
mammospheres
P1
P2
P3

Figure 3.6. Internal deletion of the SR and INV regions impairs their respective functions. (A) Schematic depicting IRS2ΔSR and IRS2ΔINV mutants. (B) PyMT:Irs1-/-/Irs2-/- cells expressing the indicated IRS2 proteins were serum starved for 4 hours and stimulated with IGF-1 (50 ng/ml) for 10 minutes. Aliquots of cell extracts containing equivalent amounts of total protein were immunoprecipitated (IP) with an HA-specific antibody and immunoblotted with antibodies specific for HA or the p85 subunit of PI3K (p85). Total cells extracts were also immunoblotted with antibodies specific for pAKT (S473), AKT and Tubulin. The data shown in the graph below shows quantification of p85 association with the IRS proteins and represents the mean ± S.E.M. of three independent experiments. No statistical significance was observed among the groups. (C) Matrigel Transwell invasion assays. The data shown represent the mean ± S.E.M. of three experiments. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2. (D) Matrigel-Collagen I 3D invasion assay. (E) Representative images of colonies formed are shown below (magnification10X). The data shown represent the mean ± S.E.M. of a representative experiment performed three times independently. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2. (F) Cells were assayed for their ability to form mammospheres over three serial passages.The data shown represent the mean ± S.E.M of a representative experiment performed three times independently. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2.

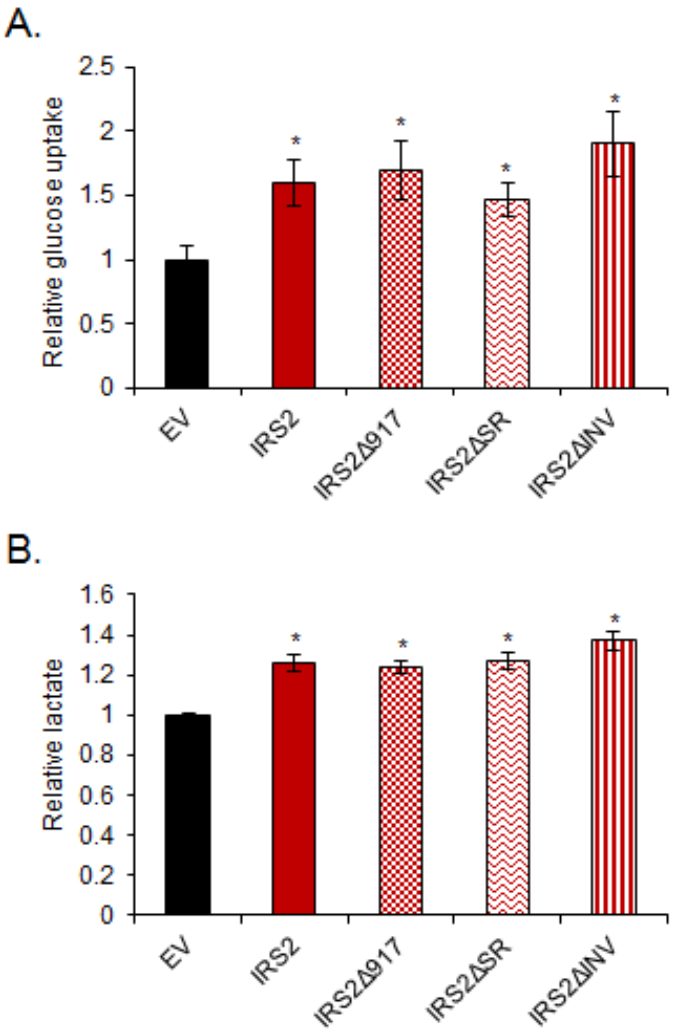

Figure 3.7. The SR and INV regions are not required for the IRS2-dependent regulation of aerobic glycolysis. PyMT:Irs1-/-/Irs2-/- cells expressing EV, WT IRS2, IRS2Δ917, IRS2ΔSR and IRS2ΔINV were grown in 0.1%BSA/DMEM for 24 hours. (A) Glucose uptake and (B) lactate production were measured and normalized to cell density. The data shown represent the mean ± S.E.M. of three independent experiments. *, $p<0.05$ relative to EV.

for the regulation of glycolysis. Therefore, the inhibition of tumor cell invasion and self-renewal observed for the IRS2ΔINV and IRSΔSR mutants does not result from defects in aerobic glycolysis in these cells.

The invasion region (INV) of IRS2 inhibits IRS2-mediated cell invasion.

IRS2 is an adaptor protein with no intrinsic kinase activity that is responsible for coordinating signaling downstream of the IR/IGF-1R. We hypothesized that the INV and SR regions in the IRS2 C-terminus contribute to IRS2 function through specific inter- or intramolecular interactions that alter IRS2 downstream signaling. To investigate these potential interactions, we generated green fluorescent protein (GFP)-tagged constructs containing the 97 aa self-renewal region (SR), the 174 aa invasion region (INV) or the final 150 aa of the IRS2 C-terminus (CONT) that we demonstrated was not required for either invasion or self-renewal (Fig. 3.8A). These domains were expressed at equivalent levels after lentiviral infection and stable selection of SUM-159 breast carcinoma cells (Fig. 3.8B). Expression of the SR, INV and CONT domains did not inhibit the expression of endogenous IRS2 or interfere with IGF-1R-dependent PI3K/AKT activation (Fig. 3.8C). Cells expressing the C-terminal domain constructs were assayed for their ability to invade in a Matrigel Transwell assay. Expression of the INV region decreased invasion significantly, while expression of the SR or CONT regions had no impact upon invasion (Fig. 3.8D). Similar results were obtained when the IRS2-INV, SR

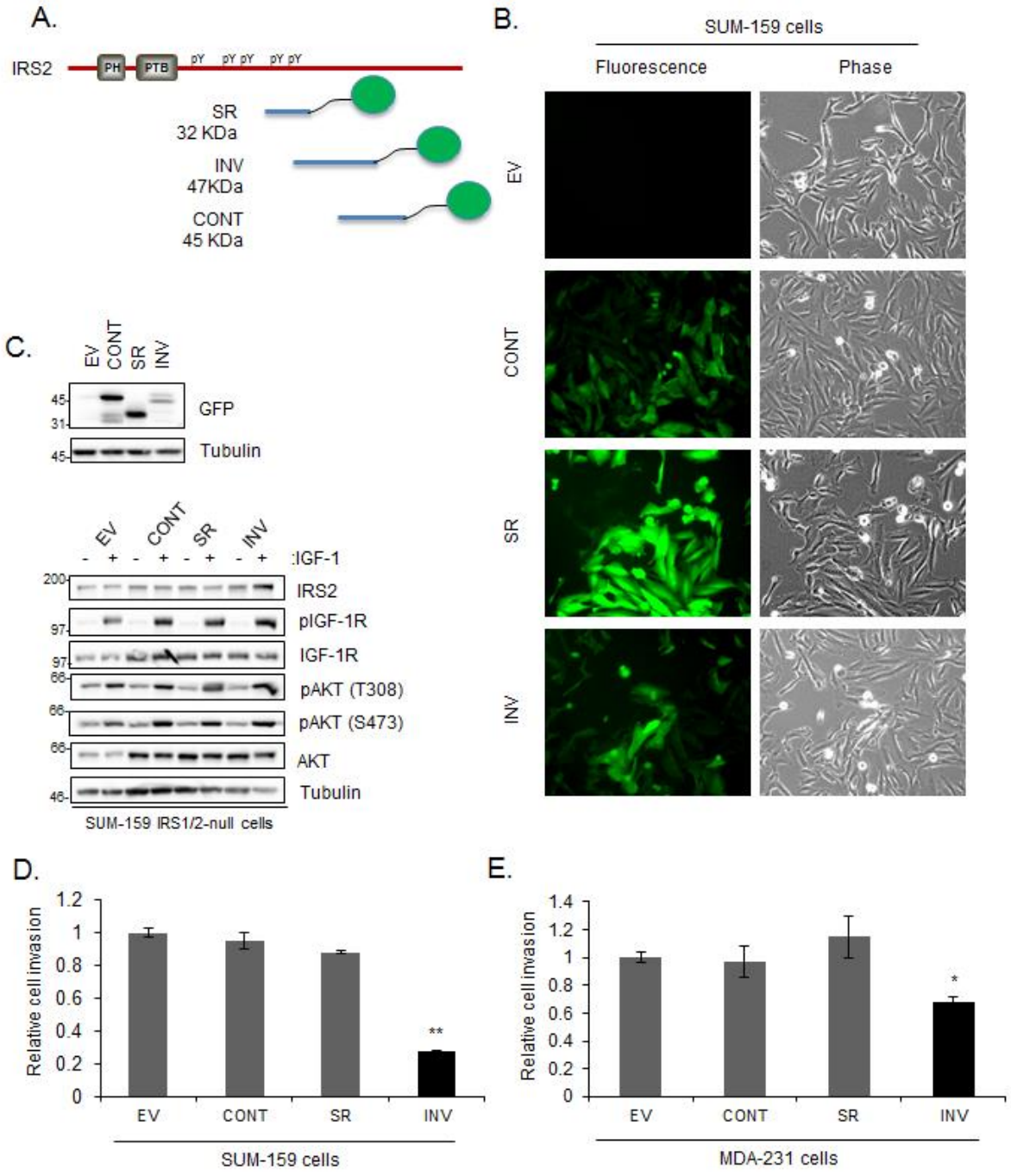

Figure 3.8. The IRS2 INV region behaves in a dominant negative manner. (A) Schematic depicting the SR, INV and CONT regions tagged with mVenus. SUM-159 cells expressing EV, CONT, SR or INV regions of IRS2 were imaged. (B) Fluorescence and phase contrast images of SUM-159 cells expressing the

individual domains (Magnification X10). (C) Total cell extracts were immunoblotted with antibodies specific for GFP and Tubulin. SUM-159 expressing IRS2 CONT, SR and INV were serum starved for 4 hours and stimulated with IGF-1 (50 ng/ml) for 10 minutes. Aliquots of cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies specific for IRS2, pIGF-1R (Y1135/1136), IGF-1R, pAKT (T308), pAKT (S473), AKT and tubulin. (D) SUM-159 cells and (E) MDA-MB-231 cells expressing CONT, SR and INV regions were evaluated for invasion in Matrigel Transwell invasion assays. The data shown represent the mean ± S.E.M. of three experiments. **, $p<0.01$ relative to EV. *, $p<0.01$ relative to EV.

and CONT constructs were expressed in human triple negative MDA-MB-231 breast carcinoma cels (Fig. 3.8E). The ability of the INV region to act in a dominant negative manner and inhibit invasion suggests that there are interactions in this region of IRS2 that are important for the regulation of cell invasion.

To identify proteins that interact with the INV region, tandem affinity purification followed by mass spectrometry was performed. To do so, the INV region was tagged with a flexible triple-Flag-His tag (INV-3XFLAG-His) and transiently transfected into SUM-159 cells. Mass spectrometry analysis identified bone morphogenetic protein-2-inducible kinase (BMP2K) as an interacting protein for the INV region (Fig. 3.9A). BMP2K is a member of the numb associated kinase (NAK) family of serine-threonine kinases and it has been implicated in osteoblast activation during bone formation [275]. To investigate the role of BMP2K in IRS2-dependent cell invasion, BMP2K expression was suppressed by shRNA targeting in IRS1/2-null PyMT cells expressing WT-IRS2. Knockdown of BMP2K resulted in a significant decrease in BMP2K mRNA (Fig. 3.9B). As a positive control, an shRNA targeting IRS2 was also expressed in these cells (Fig. 3.9C). Suppression of either IRS2 or BMP2K expression decreased tumor cell invasion (Fig. 3.9C). Taken together these data suggest that the interaction of BMP2K with the invasion region plays a role in the regulation of tumor cell invasion by IRS2.

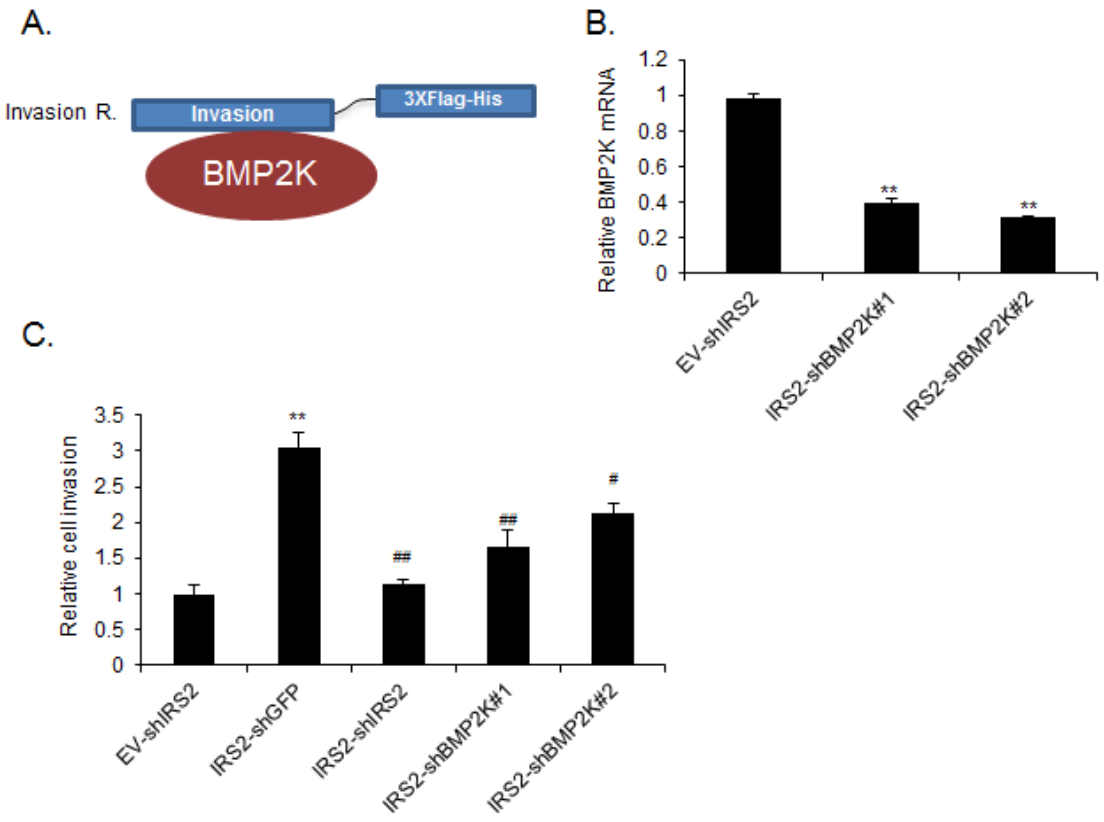

Figure 3.9. BMP2K contributes to IRS2-mediated tumor cell invasion. (A) Schematic of BMP2K binding. (B) Two shRNAs targeting BMP2K (shBMP2K#1 and shBMP2K#2) were expressed in PyMT:Irs1-/-/Irs2-/- cells expressing IRS2. and BMP2K expression was assessed by quantitative PCR. The data shown are expressed as BMP2K mRNA expression relative to control EV control cells expressing shIRS2 and represent the mean ± S.E.M of one experiment. $^{\#\#}$, $p<0.05$ relative to EV. (C) PyMT:Irs1-/-/Irs2-/- cells expressing EV or IRS2 were infected with lentiviruses expressing shRNA targeting shGFP, shIRS2, shBMP2K#1 or shBMP2K#2 and Matrigel Transwell invasion assays were performed. The data shown represent the mean ± S.E.M of three experiments. **, $p<0.01$ relative to EV; $^{\#\#}$, $p<0.01$ relative to IRS2.

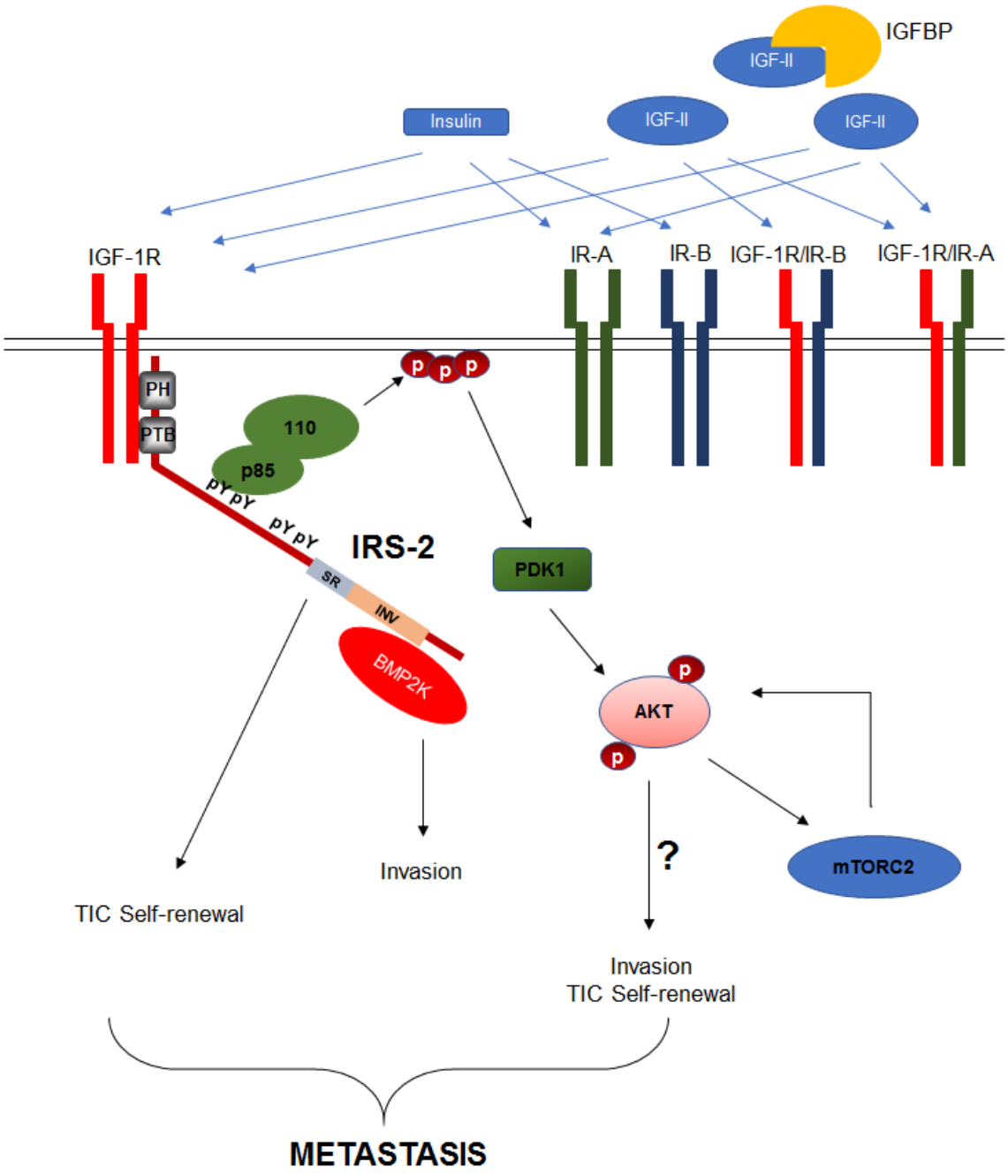

Figure 3.10. Model of Insulin Receptor Substrate-2 signaling. Overview of IRS2 signaling and its contributions to metastasis.

Discussion

In this study, we investigated mechanisms by which IRS2 regulates breast carcinoma cell functions that are important for metastasis. Our mutagenesis analysis identified structural features of IRS2 that are required for regulating invasion and self-renewal, two essential functions of metastatic tumor cells. Although it was previously established that IRS2 regulates invasion, we demonstrate for the first time that IRS2, but not IRS1, regulates TIC self-renewal. Our studies reveal that the ability of IRS2 to promote invasion is dependent upon upstream IGF-1R/IR activation and the recruitment and activation of PI3K. Additional sequences within the C-terminus of IRS2 are also required for IRS2-mediated tumor cell invasion, and these sequences are sufficient to confer invasion-promoting ability when swapped into IRS1. The ability of IRS2 to activate PI3K and the IRS2 C-terminus are also required for the regulation of self-renewal. Importantly, we identified two independent regions within the IRS2 C-terminus that regulate either tumor cell invasion or self-renewal, respectively, supporting that IRS2 regulates these functions through distinct signaling mechanisms. We demonstrate that the C-terminal region required for invasion inhibits invasion when expressed exogenously, indicating that essential binding interactions occur within this region. Tandem affinity purification and mass spectrometric analysis of this invasion region revealed an interaction with BMP2K, and suppression of BMP2K decreases IRS2 dependent invasion. Our study demonstrates that the C-terminus

of IRS2 plays an important role in determining the functional outcomes of IRS2 signaling.

Our structure/function analysis of IRS2 reveals important mechanistic information regarding the differential functions of IRS1 and IRS2. An open question about IRS1 and IRS2 has been how these adaptor proteins can each activate PI3K and downstream AKT signaling in response to common upstream receptor stimuli but regulate diverse cellular outcomes. The intrinsically disordered nature of these proteins has made it difficult to compare their sequences to identify unique domains that would explain these differences. IDPs tend to be highly dynamic and their function is dependent on their ability to acquire specific protein conformations during intramolecular and intermolecular interactions [276], suggesting that specific binding interactions are likely to occur with the IRS proteins. Our structure/function analysis demonstrated that sequences within the IRS2 C-terminus are both necessary and sufficient for the ability of the IRS proteins to regulate tumor invasion and self-renewal. It will be informative in future studies to determine if sequences in the IRS1 C-terminus also contribute to the ability of this adaptor protein to uniquely regulate breast carcinoma cell proliferation. The ability to activate PI3K is important for the function of both IRS1 and IRS2 and their C-termini are not required for the recruitment or activation of this signaling pathway. These findings suggest that intramolecular interactions or intermolecular interactions with unique binding partners that occur within the C-terminus are likely

to modify the outcomes of or cooperate with PI3K to regulate downstream signaling. The differential requirement for an intact microtubule cytoskeleton in the IRS-dependent activation of AKT suggests an additional mechanism by which selective interactions within the C-termini could alter signaling outcomes through the regulation of the intracellular localization of these adapter proteins.

Our study is the first to demonstrate a role for IRS2 in the regulation of tumor initiating cell function. The TIC properties of self-renewal and pluripotency, the ability to differentiate and repopulate the heterogeneity of a tumor, are thought to facilitate the metastatic colonization of distant organs. In support of this role for TICs in metastasis, a single cell analysis of metastatic breast cells revealed that early stage metastatic cells have a stem-like gene expression signature, whereas late stage metastatic cells (high tumor burden) are more heterogeneous and similar to the primary tumor in their gene expression, likely reflecting the division of the early arriving stem cells to generate more differentiated, proliferative progeny. IRS2 promotes the formation of mammospheres upon serial passage, supporting that it regulates self-renewal to sustain the tumor initiating cell population. This ability of IRS2 to regulate self-renewal would support metastatic colonization of distant organs. IRS2 promotion of both tumor cell invasion and self-renewal is reminiscent of the epithelial to mesenchymal transition (EMT). EMT is a developmental program whereupon epithelial cells lose their cell-adhesion and polarity and become mesenchymal in morphology. Cells that have undergone an

EMT lose expression of epithelial genes such as E-cadherin and gain mesenchymal genes such as vimentin and fibronectin. In cancer, cells that have undergone an EMT acquire both invasive and self-renewal capabilities. To date, there are limited reports on IRS function in EMT. In H1299 lung cancer cells, IRS2 expression decreases the epithelial marker E-cadherin and enhances the mesenchymal marker vimentin [199]. The regulation of EMT by IRS2 in this model leads to increased cell invasion. Loss of IRS2 expression in a model of renal tubular fibrosis increases E-cadherin expression. In the same model, IRS1 inhibits TGFβ induction of EMT [277]. The inverse impact of IRS1 and IRS2 on the EMT is consistent with the differential regulation of invasion, self-renewal and metastasis by the IRS proteins in breast cancer [114-116].

The dominant negative behavior of the INV region suggests that important binding interactions occur within this region that contribute to IRS2-mediated invasion. Deletion of this region could impact IRS2 function by preventing IRS2 from acquiring specific protein conformations required for the regulation of tumor cell invasion or by affecting the interaction of IRS2 with key proteins that facilitate invasion. Our identification of BMP2K as a novel IRS2-interacting protein supports the latter mechanism, although it doesn't negate the possibility that additional intramolecular interactions are also important. BMP2K is a relatively unstudied serine threonine kinase that has been implicated in osteoblast activation and differentiation during osteogenesis [275]. In this regard, BMP2K plays a role in

TGF-β/BMP signaling downstream of BMP-2 [278]. Additionally, BMP2K is part of the NUMB endocytic adaptor protein complex [279]. The NUMB complex plays a role in the endocytic trafficking of the transmembrane receptors Notch, E-cadherin and β1-integrin [280-282]. Of interest, NUMB has been implicated in TGF-β signaling in renal fibrosis [283], the same model system in which IRS2 regulates E-cadherin expression. The common involvement of NUMB and IRS2 in fibrosis implicates IRS2-BMP2K in the regulation of EMT that promotes tumor cell invasion. Future studies are necessary to elucidate the mechanistic role of BMP2K in the IRS2 regulation of tumor invasion.

A key outcome from our study is the elucidation of the role of the IRS2 C-terminus in regulating each of the metastasis promoting functions of IRS2. The ability to invade, self-renew, and support glycolytic metabolism are important properties of metastatic tumor cells. All three functions share a common requirement for IRS2-dependent PI3K activation, as demonstrated by the inability of cells expressing the IRS2-Y5F mutant that is deficient in PI3K recruitment to invade, form mammospheres after serial passage or enhance glucose uptake and lactate production. However, the contribution of the IRS2 C-terminus to each of these important functions is distinct. The C-terminus is not required for regulating glycolysis and mutually exclusive sequences contribute to the promotion of invasion and self-renewal. These findings reveal that IRS2 regulates each of these functions through distinct mechanisms. Importantly, these mechanistic differences

can be exploited to dissect the importance of individual IRS2 functions for breast cancer metastasis.

The IGF-1R pathway has been implicated in many aspects of tumorigenesis and continues to be a studied target for cancer therapy. Our data demonstrate that IRS2 is the mediator of IGF-1R signaling in tumor cell invasion and may also regulate self-renewal. Targeting the IGF-1R receptor has proved unsuccessful clinically as multiple specific inhibitors have failed to inhibit tumor growth [169]. One reason for the failure of IGF-1R inhibitors is the upregulation of the insulin receptor to compensate for the loss of IGF-1R signaling [170]. A concern with targeting both receptors is the disruption of normal metabolic homeostasis. Our results provide a rationale for considering IRS2 and IRS2-interacting proteins as a viable alternative approach for the inhibition of the IGF-1R/IR pathway in cancer. We have identified regions that isolate the regulation of invasion and self-renewal from the regulation of glycolysis. The identification of the INV and SR regions opens the door to possible therapeutic approaches that can allow the exclusive targeting of IRS2-mediated tumor cell processes that contribute to cancer metastasis without affecting IRS2-mediated metabolic processes in normal tissues.

CHAPTER IV

Discussion

Summary of Findings

IRS1 and IRS2 are adaptor proteins downstream of the insulin and the IGF-1 receptors. The expression of these proteins in cancer has been investigated and their role in cancer is well known. These proteins play very distinct roles in cancer and there is a need to understand how they differentially regulate essential tumor processes such as aerobic glycolysis, cell survival and cellular invasion. Since the IRS proteins share considerable sequence homology and regulate the activation of similar pathways, I sought to identify possible differential signaling mechanisms of IRS1 and IRS2. I determined through manipulation of the microtubule cytoskeleton that IRS-2 requires the microtubule cytoskeleton to fully activate the PI3K/AKT pathway. In contrast, cells that signal through IRS1 are able to maintain PI3K/AKT signaling upon disruption of microtubules. More important, I determined that IRS-2-dependent cells are more sensitive to microtubule disruption induced cell death while cells that express IRS-1 are more resistant to the disruption of the microtubules. We analyzed the mechanism of IRS2-dependent microtubule disruption induced apoptosis and determined that the disruption of microtubules decreases AKT activation causing the upregulation of the BH3-only proapoptotic protein BIM. The differential regulation of AKT activation by the IRS proteins and the ability of IRS2 to regulate BIM levels in cancer cells is one mechanism that contributes to IRS2 regulation of cell survival.

Our structural/functional studies of IRS2 significantly further our understanding of IRS2 in cancer. To investigate the mechanism by which IRS-2 regulates tumor metastasis, I took a mutagenesis approach to identify structural features of IRS-2 that are required for functions that contribute to cancer progression. My studies reveal that the ability of IRS-2 to promote invasion is dependent upon upstream IGF-1R/IR activation and the recruitment and activation of PI3K. I demonstrate for the first time that IRS2, but not IRS1, regulates tumor-initiating cell (TIC) self-renewal of breast carcinoma cells, and the ability of IRS-2 to activate PI3K, as well as the IRS-2 C-terminus, are required for both invasion and self-renewal. One of my most significant discoveries was the identification of two distinct regions within the IRS2 C-terminus that regulate tumor cell invasion or self-renewal. Further analysis of the invasion region revealed an interaction with bone morphogenic protein-2 inducible kinase (BMP2K), a serine threonine kinase of the numb associated kinase (NAK) family. I also determined that suppression of BMP2K expression decreases IRS-2 dependent invasion.

Implications of the differential signaling downstream of the IRS proteins

One of the roles of IRS2 in cancer cells is the regulation of cell survival. In Chapter 2, we determine that signaling downstream of IRS2 requires the microtubule cytoskeleton and that disruption of the ability of IRS2 to signal upon disruption of the microtubule cytoskeleton causes apoptosis by increased

expression of the pro-apoptotic protein BIM. Further inhibition of AKT phosphorylation by treatment with a pan-AKT inhibitor (MK2206) does not enhance cell death. These data suggest to us that there is a pool of AKT inhibited by disruption of the microtubule cytoskeleton that is responsible for the death phenotype that I observe. AKT has three isoforms, AKT1, AKT2 and AKT3, and these isoforms are known to play distinct roles in cancer. Selective roles for IRS1 and IRS2 signaling downstream of the IR in skeletal muscle cells have been shown to require the specific regulation of AKT isoforms by the IRS proteins. Insulin mediated myoblast differentiation and glucose metabolism are mediated by IRS1/AKT2 signaling, whereas signaling through IRS2/AKT1 regulates lipid metabolism [256]. In the mammary epithelial cell line MCF-10A, differential roles for AKT1 and AKT2 isoforms have been identified. AKT1 expression represses cell migration and invasion, while AKT2 increases cell survival and increases EMT through the downregulation of E-cadherin [284]. In the breast cancer cell line MDA-MB-435, AKT2 plays a role in tumor cell invasion and metastasis upon injection of these cells into mice [285]. These data suggest the possibility that in mammary carcinoma cells the IRS protein can selectively regulate different AKT isoforms for the regulation of cell survival, invasion and metabolism. I hypothesize, that the selective effect of microtubule disruption in IRS2-expressing cells could be due to the differential requirement of AKT isoforms on an intact microtubule cytoskeleton. Given the known function of AKT2, I propose that for IRS2 regulation of AKT2 is important for cell survival.

To further identify a mechanism responsible for the differential effects of microtubule disruption in IRS1- and IRS2-expressing cells, determining if IRS2 requires the microtubule cytoskeleton to access a specific AKT isoform would be important. Previous work suggesting that IRS1 and IRS2 differentially regulate the AKT isoforms for the regulation of aerobic glycolysis, makes me believe that the effects of microtubule disruption induced cell death in IRS2-dependent cells can be a product of IRS2 requiring microtubules for activation of a specific AKT isoform. In order to determine the impact of microtubule disruption in IRS2-dependent AKT isoform activation, PyMT:WT, PyMT:Irs1-/- and PyMT:IRS2-/- cells could be treated with either DMSO or Nocodazole. These samples would be used for immunoprecipitation (IP) of AKT1, AKT2 and AKT3 to assess the phosphorylation of individual AKT isoforms after microtubule disruption.

The ability of IRS to regulate specific AKT isoforms can be implicated in the ability of IRS2 to selectively regulate distinct AKT effectors. IRS2 dependent activation of AKT results in the phosphorylation and inactivation of GSK3 and this inactivation is required for IRS2-mediated regulation of glucose uptake [180]. In my current study, IRS2 activation of AKT resulted in the regulation of the pro-apoptotic protein BIM. The expression of BIM is regulated by the FOXO transcription factors, which are inactivated by AKT phosphorylation [253]. IRS2 has been previously shown to regulate Foxo1 activity and Bim expression in

mouse hepatocytes [255]. I hypothesize in breast carcinoma cells that IRS2 is dependent on the microtubule cytoskeleton to activate AKT2, and inhibit the FOXO transcription factors resulting in inhibition of BIM expression. Upon disruption of the microtubule cytoskeleton IRS2 is unable to activate AKT2 releasing inhibition in FOXO transcription factors leading to increase expression of BIM and apoptosis [252, 255].

Based on our work in IRS protein signaling and the IRS2 dependency on microtubules to activate the PI3K/AKT pathway, the next step would be to determine the role of IRS1 and IRS2 in response to microtubule disruption *in vivo.* To do so, PyMT:IRS1-/- cells that signal through Irs2 and PyMT:Irs2-/- cells that signal through Irs1 would be injected into the mammary fat pads of female NOD/SCID mice. Upon tumor formation mice will be treated with vehicle or Vinorelbine. This study would help address the impact of microtubule disruption in primary tumor growth and metastasis of tumors that signal through Irs1 or Irs2.

The requirement of an intact microtubule cytoskeleton for IRS2 activation of AKT in breast carcinoma cell suggests that there is an interaction between IRS2 and microtubules. The IRS proteins do not contain any known microtubule binding sequences. The only reported interaction between the IRS proteins and the microtubule cytoskeleton is the interaction between IRS proteins and the C-terminus NH2-terminal kinase (JNK) interacting protein 1 (JIP1) [286]. JIP1

interacts with the kinesin light chain (KLC) of kinesin-1 for trafficking along the microtubules [287]. This is evidence of IRS interaction with microtubules. However, both IRS proteins can interact with JIP1/kinesin-1 which would suggest a sensitivity of both adaptor proteins to disruption of microtubules. Other potential mediators of IRS protein interactions with microtubules are the 14-3-3 proteins. 14-3-3 protein interact with kinesin-1 to regulate trafficking in neuronal cell axons [288]. There is evidence of 14-3-3 proteins regulating IRS1/PI3K signaling [289]. The interaction between IRS1 and 14-3-3 proteins requires IRS1 phosphorylation at Ser-270, Ser374 and Ser641 [290]. This interaction inhibits IRS1 signaling upon insulin stimulation in skeletal muscle [290]. The interaction between IRS2 and 14-3-3 proteins was determined in the same study but the role of this interaction was not elucidated. There is a possibility that the interaction of IRS2 with the microtubule cytoskeleton mediates the trafficking along the microtubule cytoskeleton for the localization of IRS2/PI3K/AKT signaling complex. A differential role of IRS2 and IRS1 interaction with 14-3-3 protein could explain the selective inhibition of IRS2 signaling upon the disruption of the microtubule cytoskeleton.

I have developed a set of tools in my IRS2 structural/functional analysis that I can employ to further identify a mechanism of IRS2-microtubule dependent signaling. First, PyMT:IRS2-/- cells expressing EV, WT IRS1, IRS1Δ942, WT IRS2 and IRS2Δ917 could be used to determine the response of these mutants to

microtubule disruption upon IGF-1 stimulation and if the IRS2 C-terminus is required for IRS2 dependency on the microtubule cytoskeleton for signaling. Also, IRS1/S2 and IRS2/S1 chimeras can be used to determine if transferring the IRS2 C-terminus to IRS1 can sensitize IRS1 expressing cells to microtubule disruption. We can use other mutants like the IRS2ΔSR and IRS2ΔINV to determine if the effects we are seeing in tumor cell invasion and TIC self-renewal can be due to a loss in IRS2-microtubule interaction.

To further understand the differential requirement of the IRS proteins for the microtubule cytoskeleton to activate the PI3K/AKT pathway I need to determine the mediators of the interaction between IRS proteins and microtubules, I want to determine if the interaction is direct, mediated by microtubule associated proteins (MAPS) or other unidentified proteins. Using either purified recombinant IRS2 and IRS1 (My BioSource) or cytosolic extracts from MDA-231 cells (high IRS2 expression), I will perform an in vitro binding assay incubating the cytosolic extract and the recombinant IRS proteins with MAPS containing tubulin (Cytoskeleton) or MAPS free tubulin as described in [291]. These mixtures will allow me to determine if the interaction between IRS and microtubules is direct, in the absence of MAPS and other cytosolic proteins, or mediated by a third protein that might be part of the MAPS or present in the cytosolic extract. IRS2-null cells extracts, purified JNK-interacting protein 1 (JIP1) (EzBiolab) a well-known protein that does not interact directly with microtubules), will be used as negative control [286, 287]. Purified

recombinant RBl2p/cofactor A (EzBiolab) a well-known direct microtubule binding protein will serve as a positive control, JIP1 can serve as a positive control of indirect microtubule interaction because it binds microtubules in a MAPS-dependent manner. Binding to tubulin will be examined by microtubule co-sedimentation assays follow by western blot analysis for the detection of IRS proteins or other proteins binding tubulin. The identification of the proteins that mediate the interaction between IRS proteins and microtubules can advance our understanding of IRS1 and IRS2 signaling. The mediators of the IRS-microtubule interaction can be important regulators of IRS protein signaling and help understand the differential roles IRS1 and IRS2 have in cancer.

In addition to the differential requirement of the microtubule cytoskeleton for IRS1 and IRS2 to signal to the AKT pathway, there are other possible mediators of IRS2 and IRS1 that required closed attention. There are three PI3K p110 catalytic subunits and these are frequently mutated in cancer [177, 185, 186]. More work needs to be performed to elucidate the interactions between IRS1 and IRS2 with the different PI3K catalytic subunits and if differential interaction between the adaptors with different mutant p110 mutants accounts for the differential effects in IRS proteins functions. Moreover, the activation of PI3K by the IRS proteins not only activates the PI3K/AKT pathway but can also contribute to the activation of the RAC signaling pathway. This pathway regulates cell-to-cell contact and actin cytoskeleton rearrangements [184]. The RAC pathway is implicated in tumor cell

invasion and there is a need to address if the differential activation of RAC downstream of IRS proteins contributes to the ability of IRS2 to regulate tumor cell invasion. Additionally, I have shown that mutations in the IRS2 C-terminus does not impair the ability of this adaptor to activate the PI3K pathway. I need to investigate if our mutants IRS2Δ917 and IRS2ΔINV do not have an effect in the activation of RAC signaling. The inability of these mutants to activate RAC signaling can contribute to the effects of these mutants in tumor cell invasion.

New discoveries: IRS2-dependent regulation of tumor cell invasion and TICs self-renewal

IRS2 significantly contributes to the metastatic process by the regulation of tumor cell invasion and we recently identified additional contributions of IRS2 to the regulation of breast carcinoma TIC self-renewal. Data from previous studies suggest that IGF-1R activation of AKT is required for the regulation of tumor cell invasion. In glioma cells, miRNA-383 downregulates IGF-1R and decreases AKT activation and causes a decrease in tumor cell invasion [292]. Prostate cancer cells DUI45 increase IGF-1R activity upon stimulation increases AKT activity and causes an increase in tumor cell invasion [293]. Inhibition of IGF-1R signaling by monoclonal antibody EM146 against the IGF-1R receptor in MDA-MB-435 cells inhibits tumor cell invasion. My data provides evidence that the ability of IGF-1R to recruit IRS2 and activate the PI3K pathway contributes to the regulation of tumor

cell invasion. In human mammary epithelial cells MCF-10A ablation of AKT1 enhances tumor cell invasion and metastasis in vivo. Loss of AKT1 decreases miRNA-200 expression and these miRNAs are responsible for the regulation of the E-cadherin transcription repressors ZEB1 and ZEB2 [294, 295]. The knockdown of AKT1 and TGFβ stimulation of these cells enhances mammosphere formation [296]. These data suggest that AKT2 signaling contribute to the regulation of EMT in MCF-10A cells and increases invasion in this cell line. In a glioblastoma cell line CD133+ cells require AKT activation for mammosphere formation and inhibition of AKT ablates the ability of these cells to form mammospheres. We have seen that inhibition of AKT activation by the Irs2-Y5F mutant shows a defect in TIC self-renewal, suggesting that AKT activation downstream of IRS2/PI3K may be required for both invasion and self-renewal. The ability of IRS2 to activate specific AKT isoforms may also contribute to its ability to regulate tumor cell invasion and TIC self-renewal.

In addition to the requirement of IRS2/PI3K/AKT signaling for tumor cell invasion and self-renewal, I identified two distinct regions in the IRS2 C-terminus that play a role in the regulation of self-renewal (SR) and invasion (INV). Deletions of each of these regions renders IRS2 unable to regulate their respective functions while maintaining the ability of the protein to activate PI3K/AKT signaling. These results suggest that additional requirements in IRS2 protein are needed to regulate these processes. The IRS2 is an adaptor protein with no intrinsic kinase activity

and understanding protein interactions with these regions can help elucidate mechanistic regulation of tumor cell invasion and TICs self-renewal by IRS2. I used tandem affinity purification and mass spectrometry to identify interactions of the INV-region with protein that could be responsible for the role of this region in tumor cell invasion. Our protein purification approach contained nuclear and cytosolic fractions, the presence of the nuclear fraction results in the presence of many nuclear proteins in our mass spectrometry. I believe that these are artifacts as we know that IRS2 is not a nuclear protein. Future experiments would require an isolation of the cytosolic fraction to diminish the number of non-specific binding detected in the whole cell extract.

Among the interactors identified in this approach were, intersectin, desmoplakin and BMP2K. Intersectin and desmoplakin have previously been implicated in tumor cells invasion and metastasis. These proteins remain important targets to investigate as we continue to determine the regulation of tumor cell invasion by IRS2 and the role of the INV region. The association of IRS2 with BMP2K is a novel finding that reveals potential mechanistic information regarding IRS2 regulation of invasion. Although there is relatively little information about BMP2K function in cancer, according to the human protein atlas, BMP2K is overexpressed in lymphoma, breast, pancreatic, prostate and skin cancers. Potential contributions of BMP2K to IRS2 function in cancer can be postulated from its known function in normal cell biology.

BMP2K is part of the Numb endocytic adaptor protein complex [279]. The Numb complex plays a role in the endocytic trafficking of the transmembrane receptors Notch, E-cadherin and β1-integrin [280-282]. All these receptors play a role in cancer suggesting that BMP2K as a Numb associated kinase (NAK) could also play a role in tumorigenesis. Structural analysis of NAKs revealed that BMP2K, along with other members of this kinase family, can bind with high affinity to inhibitors that were previously thought to be selective for their targets [297]. The JNK inhibitor SP600125, JAK inhibitors momelotinib and baricitinib, Aurora kinase A inhibitor AT9283 and a dual MAPK and TAK1 inhibitor have all been identified as strong inhibitors of BMP2K. There is a need to be determine if the inhibition of BMP2K by these different drugs contributes to their effects in cancer. Additionally, these data suggest that BMP2K can be a druggable target in cancer.

The biology of BMP2K remains to be elucidated but the biology of its binding partner Numb is well studied. There is evidence that implicates Numb independent of its role in endocytosis in the regulation of breast tumor cell invasion. Expression of the Numb isoform Numb6 in D-17 breast carcinoma cell lines leads to the regulation of EMT factors Vimentin and Slug while suppressing the expression of E-cadherin [298]. Inhibition of the PI3K/AKT pathway in this model inhibits the regulation of EMT and tumor cell invasion by Numb6. Based on the requirement of PI3K/AKT for Numb6-dependent regulation of tumor cell invasion we can

hypothesize that IRS2 activation of the PI3K pathway and its interaction with Numb can contribute to the regulation of tumor cell invasion. Studies to determine the interaction between Numb, BMP2K and IRS2 need to be elucidated and this complex can play a role in the regulation of tumor cell invasion.

The role Numb plays in endocytosis have been shown to have a role in the regulation of Notch signaling in *Drosophila* and mammalian models [280, 299]. In drosophila neural progenitor cells and colon cancer stem cells, Numb has been implicated in the differential regulation of Notch signaling and endosomal trafficking to regulate assymetric cell divison and maintenance of the stem cell population [300]. These suggest that in addition to a role in tumor cell invasion, the ability of Numb to regulate endocytosis can play an impact in breast cancer TICs. The ability of IRS2 to interact with BMP2K a Numb-associated kinase can implicate that the interaction of IRS2 with BMP2K can play a role in the ability Numb stem cell biology.

Of interest, Numb is also implicated in TGF-β signaling in renal fibrosis [283]. This implicates BMP2K in TGF-β signaling regulation during renal fibrosis as part of the Numb endocytic complex. In the same model of renal fibrosis IRS2 negatively regulates E-cadherin expression. There is a possibility that IRS2-BMP2K interaction could be playing a role in the regulation of EMT in renal fibrosis, as well as in breast cancer tumor cell invasion. I hypothesize that the disruption

of the IRS2 interaction with BMP2K in our IRS2ΔINV mutant renders IRS2 unable to regulate EMT, causing a defect in cellular invasion.

The conditions we used in our pulldown experiment allowed us to identify BMP2K as an interactor with the IRS2 INV-region. Given that the IRS proteins are intrinsically disordered, the interactions of the IRS proteins with binding partners are predicted to be weak, and transient interactions might not have been preserved during our pulldown. Scansite analysis of the 174 amino acids in the INV region revealed possible interactions between this region and the protein Intersectin and Cortactin. Both proteins have been extensively implicated in cancer cell migration, invasion and tumor metastasis due to their role in cytoskeletal remodeling [301, 302]. Interestingly, Intersectin, Cortactin and the Numb complex are implicated in endocytosis of clathrin coated vesicles [282, 303, 304]. The role of these proteins in endocytosis and their interaction with the INV region of IRS2 point to a role of IRS2 as a regulator of endocytosis, and this role in endocytosis as part of its ability to regulate tumor cell invasion. Additional studies to validate the interaction between IRS2 and BMP2K are warranted. In order to do so, immunoprecipitation of WT IRS2, IRS2Δ917 and IRS2ΔINV should be performed to validate the interaction with the INV region. Immunoblotting for Intersectin and Cortactin in these experiments would also help uncover other novel IRS2-interacting proteins.

Beyond the validation of the IRS2 and BMP2K interaction we want to determine how the interaction of IRS2 and BMP2K is contributing to tumor-cell invasion. BMP2K is a serine/threonine kinase and the targets of this proteins remain to be elucidated. This proteins kinase domain is well identified and inhibitors against it are available. Among our future studies we need to identify the targets of BMP2K as part of the mechanism of IRS2-BMP2K regulation of tumor cell invasion. Additionally, we need to determine if IRS2 is phosphorylated by BMP2K and if this is of importance for the regulation of IRS2 biology. Generation of BMP2K kinase mutant will help determine if the kinase activity of BMP2K is required for IRS2-mediated tumor cell invasion. Also, this mutant and a phospho proteomics approach can help identify BMP2K targets. We identified BMP2K as an interactor with the INV region but mammosphere formation assays in BMP2K knockdown cells are needed to determine if this protein has an impact in IRS2-mediated TIC-self-renewal.

Additional studies of the SR region are required to fully understand how IRS2 regulates TIC self-renewal. Pulldown of the SR region and mass spectrometry, as done with the INV region, will help elucidate novel protein interactions in this region that could help determine the mechanism of IRS2 regulation of TICs self-renewal. Scansite analysis of the SR region predicts the AMP-activated protein kinase (AMPK) phosphorylation of IRS2 in this region. Activation of AMPK by the diabetic drug metformin in the fibrosarcoma cell line

FSaII has shown a decrease in the TICs fraction and a decrease in the ability of these cells to form mammospheres [305, 306]. Metformin also inhibits serial-passage mamosphere formation in thyroid carcinoma cells lines suggesting that AMPK activation plays a role in regulation of self-renewal [307]. Further studies to determine if AMPK phosphorylation of the SR region in IRS2 and the effects of this phosphorylation on IRS2-mediated TIC self-renewal are warranted.

To determine the role of the SR and the INV regions in tumor formation and metastasis *in vivo*, PYMT:IRS2-/- cells expressing EV, WT IRS2, IRS2ΔSR and IRS2ΔINV can be injected orthotopically into mice to determine their role in tumor formation. These studies could also serve to identify which IRS2-regulated process, cellular invasion, TIC self-renewal or both are required for primary tumor growth or IRS2-mediated metastasis. Also, limiting dilution assay and serial transplantation assays *in vivo* should be performed to determine the effects of WT IRS2, IRS2ΔSR and IRS2ΔINV on the frequency of tumor initiating cells and the regulation of self-renewal *in vivo.*

As stated in the introduction, the immune system plays a very important role in the regulation of tumor metastasis. To elucidate the importance of IRS2 SR and INV regions in breast carcinoma metastasis I would like to have a mouse model with an intact immune system. The generation of mammary gland conditional knock-in IRS2ΔSR and IRS2ΔINV mutants will facilitate determining the impact of

just the loss of IRS2's ability to regulate self-renewal or tumor cell invasion without altering the rest of the tumor microenvironment. We can also cross IRS2ΔSR and IRS2ΔINV mutant mice with MMTV-PyMT mice to determine the impact of these mutants on tumor formation and metastasis in a spontaneous tumor model. The use of orthotpic models or immunocompromised models represented a limitation due to the artificial environment that is created during tumor cells injections and the lack of an intact immune system. Generation of conditional knock-in under the endogenous promoter will mimic the condition that take place during the tumor formation. If in the event of aberrant mammary gland development due to the expression of IRS2 mutant proteins we can generate and inducible system and induce our mutant proteins after the glad has developed.

The conditional knock-in models can be utilized not only to study the impact of IRS2ΔSR and IRS2ΔINV in tumor formation and metastasis but also in cancer stem cells self-renewal. Using the mouse stem cell markers $CD29^+$ and $CD24^+$ we can isolate tumor initiating cells for serial transplantation to determine the effects of these mutants on TIC-self-renewal *in vivo*. Based on our mammosphere formation assays in vitro, upon initial injection of isolated TICs we might not see a difference in tumor formation as this is equivalent to passage one in our *in vitro* assay and we do not see a difference in tumor formation. Upon serial transplantation *in vivo,* we can be able to detect a difference in tumor formation consistent with depletion of stem cells.

Additionally, IRS2 plays an important role in glucose metabolism, especially in the regulation of pancreatic β-cell functions, and glycolysis in peripheral tissues. As we try to dissect IRS2-dependent regulation of cancer specific functions it will be important to determine if deletion of the SR and INV regions affects the role of IRS2 during development and metabolism. To elucidate the effect of IRS2ΔSR and IRS2ΔINV in development and metabolism *in vivo,* we require the generation of CRISPR/Cas9 mice with these regions deleted. These animals will help investigate the effects of SR and INV region loss in development and in adult tissue physiology. Is of vital importance that we determine the role of these regions in adult tissue physiology and we look to target them in cancer treatment. Ideally, we can disrupt the SR and INV region functions in cancers without derailing the normal metabolic functions of IRS2.

One of the biggest limitations to our understanding of IRS protein biology is a lack of structural information. The structure of proteins is very important when it comes to understanding function and the development of drugs to target them [130]. The intrinsically disordered nature of IRS1 and IRS2 gives the proteins a tremendous advantage as efficient signaling molecules, but limits our ability to study their structure. Better understanding of IRS protein structure can help understand their role in multiple cellular processes that contribute to their biology in cancer. My work and the works of others demonstrate the important role IRS2

plays in breast cancer metastasis. To further elucidate the biology of this protein a better understanding of its structure can be of significance. Based on structural models, protein-protein interactions can be discovered and this is a significant accomplishment in understanding how this protein works. Additionally, future approaches to target IRS2 in cancer can benefit from the known structure of this protein for the development of agents that can inhibit its cancer related functions and not metabolic functions.

The experimental approaches of X-ray and electron crystallography that are amenable to stably folded proteins do not work on IDPs. Nuclear magnetic resonance (NMR) has also been used to determine IDPs structural analysis with limited success due to the fast changes in conformation IDPs undergo and the limitation in detection of distance interactions among atoms [308]. This makes extensive computer modeling needed for the elucidation of average structural conformations. Cryo-electron microscopy (Cryo-EM) can be used to look at the structure of proteins as single molecules or complexes in their natural environment with no need of crystal structures [309]. Also, this technique allows capturing multiple conformation of proteins and the flexibility in conformational changes that IDPs undergo do not represent a problem for structural determination [309]. Currently, Cryo-EM is the best approach available to determine structure in IDPs. A combination of Cryo-EM and mass spectrometry can help determine the structures of protein complexes and determine the proteins involved in these

complexes. We can use WT IRS2, IRS2ΔSR and IRS2ΔINV mutants and determine how loss of the SR or INV region affects the conformation and the interaction with other proteins using Cryo-EM.

How to develop novel approaches for cancer treatment

The IR/IGF-1R signaling pathway has been extensively implicated in many aspects of cancer. Due to their contributions to tumorigenesis and progression, targeting these signaling molecules was hypothesized to represent a benefit for cancer. Due to the importance of insulin signaling in metabolism and glucose homeostasis, targeting the IGF-1R was the first approach taken. Monoclonal antibodies that block receptor/ligand binding and small molecule inhibitors targeting the receptor's active site showed promising results in vitro and in preclinical animal studies [310]. On the contrary, the performance of these agents was disappointing in clinical trials. Many of the studies performed did not achieve a clinical response with the exception of some complete responses in Ewing sarcomas [169, 311]. The biggest question that the scientific community needs to address is, why did these trials fail?

There is a lot to learn from this experience and with our current findings we hope to improve future trial design. Poor clinical trial design contributed significantly to the poor performance of these trials. For phase III clinical trials, patients were not screened for the expression of IGF-1R in their tumors. If the

target of the drug is not present in the cancer, using the inhibitors in these patients is like treating them with a placebo. In the case of HER2 positive tumors and the Herceptin trials, proper patient selection and screening for the target of interest ensured successful trials [312]. The best example of IGF-1R inhibition efficacy was in Ewing Sarcoma tumors that express high levels of the IGF-1R receptor and are very dependent on this pathway for tumor cell survival [311]. In addition to poor trial design, inhibition of the IGF-1R receptor resulted in downregulation of IGF-1R and upregulation of the IR-A isoform of the insulin receptor in tumors [170]. The IGF-1R and IR-A can form heterodimers at the cell surface. Many of the IGF-1R inhibitors fail to block IGF-1R/IR-A heterodimers, also contributing to the failure of IGF-1R inhibition. These data demonstrate that targeting IGF-1R alone is not sufficient for cancer treatment.

In a mouse model of Her2-mediated breast cancer on a background of hyperinsulinemia (MTB/TAN/MKR$^{+/+}$ mice), larger tumors developed and there was an increase in metastasis to the lungs in comparison to non-hyperinsulinemic mice. Tumors from this animal model have elevated phosphorylation of the IR/IGF1R, suggesting activation of these receptors during hyperinsulinemia. Also, tumors from these animals have elevated levels of vimentin (EMT marker) and are more aggressive with extensive pulmonary metastasis [313]. Additional *in vivo* studies looking at the impact of IR/IGF-1R signaling in breast cancer metastasis looked at TIC markers in Mvt-1 mouse mammary tumor cells [314]. In this model,

IR knockdown decreases the expression of CD24, a maker of tumor initiating cells in murine mammary malignancies. Taken together, the studies from Ferguson and Rostoker implicate IR/IGF-1R signaling with tumor progression by the induction of EMT and regulation of tumor-initiating cells in hyperinsulinemia conditions. Downstream of IGF-1R or IR homodimers and IGF-1R/IR heterodimers the IRS proteins function as important signaling molecules. Moreover, previous studies have not determined which IRS protein is mediating signaling downstream of the receptors. Based on my studies, I hypothesize that IRS2 is contributing to the effects of IR/IGF-1R in cancer metastasis and is doing so by contributing to regulation of tumor cell invasion and TIC self-renewal.

IGF-1R and IR play a critical role in metabolism and the IRS1 proteins are necessary mediators. Irs1-/- mice develop insulin resistance in peripheral tissues but fail to develop diabetes, while Irs2-/- mice develop diabetes due to failure of β-cells in the pancreas [109, 123]. When targeting IRS2 to disrupt IR/IGF-1R signaling we must consider the fundamental role this pathway plays in metabolism and try to separate the role of IR/IGF-1R/IRS2 in metabolism and cancer. My identification of two distinct regions in IRS2 that regulate tumor cell invasion and TIC self-renewal could facilitate targeting IRS2-specific cancer functions that contribute to metastasis. I demonstrated that IRS2ΔSR and IRS2ΔINV maintain their ability to activate PI3K/Akt signaling and do not impair aerobic glycolysis in cancer cells. Additional studies are required to investigate the role these IRS2

mutants will have in other tissues. Disrupting cancer-specific functions of IRS2 may lead to better disruption of the IR/IGF-1R pathway in cancer without causing tremendous side-effects.

Today, this country is facing an epidemic of obesity. Obesity leads to insulin resistance in peripheral tissues like fat and muscle. One of the responses to insulin resistance is the increased production of insulin by β-cells in the pancreas. Eventually the β-cells in the pancreas are depleted leading to the development of diabetes. IRS2 plays a role in the activation of PI3K and AKT activation and promotes the regeneration of β-cells in the pancreas. New approaches are being developed to induce the expression of IRS2 in pancreatic cells using agents like Trimeprazine [315]. Special consideration needs to be paid to this approach as it can lead to induction of IRS2 in tumor tissues and have a negative impact in tumorigenesis.

Our data provides the rationale for IRS2 as a biomarker in breast cancer tumors for the design of new therapeutic approaches. Based on our microtubule disruption data, disrupting the microtubule cytoskeleton can specifically target IRS2-expressing cells. IRS2-expressing cells, based on our discoveries in this study and extensive work by our group have a more aggressive phenotype [114, 198, 200]. Disruption of microtubules will ensure disruption of IRS2 signaling independent of the upstream activator of the protein. Retrospective studies looking

at IRS2 expression in tumors from patients treated with vinca alkaloids and correlating clinical response with IRS2 expression would provide information towards the clinical benefits of this approach in cancer. Vinca alkaloids, more specifically vinorelbine, are used in breast cancer for treatment of advanced disease with limited clinical benefits [248]. Our data supports the introduction of vinorelbine earlier in treatment for patients with tumors that express high levels of IRS2, as eliminating tumor cells that express IRS2 can provide more clinical benefits earlier in the course of disease.

When designing clinical interventions for cancer therapeutics we need to always keep in mind the heterogeneous nature of tumors [316]. Targeting one specific pathway in cancer might not always be the best approach when it comes to this complex disease. The design of combination therapies based on the dependence of tumors on multiple signaling pathways can improve the clinical benefit of these interventions. The IR/IGF-1R signaling has been proven to cause resistance to Her2 inhibition in breast tumors and EGFR inhibition in lung tumors [317-319]. These results suggest that for those specific cases mentioned above, combination of Her2 or EGFR inhibitors with IR/IGF-1R/IRS2 targeted inhibitors could result in better clinical responses.

Overall significance

IRS2 has gained notoriety as a regulator of metastasis in cancers of the breast, pancreas, lungs, liver, brain and thyroid. This protein regulates metastasis by contributing to the regulation of TIC self-renewal, invasion, glycolysis and cell survival in tumor cells, all processes that are important in the metastatic cascade. The results of my study looking into the requirement of microtubules for IRS2 activation of AKT provided us information to understand differential regulation of PI3K/AKT signaling by the IRS proteins in breast carcinoma cells. In addition, these data provided us with the rationale for novel approaches to target IRS2 signaling and IRS2-expressing cells in cancers. We provide the rationale for the use of IRS2 as a biomarker for response to vinorelbine therapy in cancer. Vinorelbine is currently used for the treatment of advance disease, but could be repositioned as a first-line therapy in IRS2-dependent malignancies. In this study, we also provide the rationale for IRS1 as a biomarker for response to combination therapy consisting of microtubule disruption and AKT inhibition.

Finally, while the role of IRS2 in cancer continues to gain attention the need to further understand mechanistic inputs of IRS2 into processes that contribute to metastasis has become a priority. My study dissecting IRS2 and determining the contribution from different protein regions to the regulation of tumor cell invasion and TIC self-renewal permitted the identification of two distinct regions that regulate each process without affecting the ability of IRS2 to regulate PI3K/AKT signaling or aerobic glycolysis. I demonstrated that these regions are required for

IRS2 dependent regulation of TIC self-renewal and invasion *in vitro.* We have also determined that the INV region requires the interaction with the Ser/threonine kinase BMP2K for the regulation of IRS2-dependent tumor cell invasion. BMP2K has a potential to become a target for the inhibition of IRS2-dependent tumor cell invasion in cancer.

APPENDIX

The Insulin receptor substrate-2 (IRS-2) C-terminus regulates its subcellular localization

Previous studies from the Shaw lab and other labs have demonstrated that the intracellular localization of the IRS proteins in human breast tumors plays a role in patient response to therapy (IRS1) and overall patient survival (IRS2). IRS1 is expressed predominantly in the nucleus or diffusely in the cytoplasm, and its nuclear expression correlates with positive response to tamoxifen treatment. IRS2 is expressed either in the cytoplasm, diffusely or in a punctate pattern, or at the cell membrane. The membrane staining of IRS2 correlates with a significant decrease in overall patient survival [111]. These correlations of response and survival with subcellular localization suggest that the localization of IRS1 and IRS2 impacts their downstream signaling outcomes. In support of this role for localization, in Chapter 2 of this thesis, I determined that the ability of IRS2, but not IRS1, to signal efficiently to AKT was dependent upon an intact microtubule cytoskeleton.

In Chapter 3, I developed a series of IRS1 and IRS2 deletion mutants and chimeras to investigate the contribution of the C-terminus of each protein to their function. My studies demonstrated that the IRS2 C-terminus is necessary and sufficient for the regulation of tumor cell invasion and TIC self-renewal. Moreover, I identified discrete regions within the IRS2 C-terminus that are required for either invasion or self-renewal. As a next step in understanding how these C-terminal sequences contribute to IRS2 functions, I investigated their impact on the subcellular localization of IRS2.

To perform the localization experiments, I expressed the WT and mutant/chimeric proteins in IRS1/IRS2-null Sum159 cells (Fig. A1A). These cells spread well in cell culture allowing for good visualization of the nuclear and cytoplasmic compartments. Cells were plated on glass coverslips and fixed for 1 hour in 4% formalin followed by permeabilization with 0.1% Triton-X. Following permeabilization, cells were incubated in rabbit anti-HA antibody (CS#3724), washed 3 times in 1XDPBS with 0.1%Tween 80, and incubated with mouse anti-Rabbit 488 conjugated secondary antibody. After washing, glass coverslips were mounted on glass slides in Prolong Gold mounting medium with DAPI for nuclear staining. Images were taken using a (Zeiss LSM700). The percentage of nuclear staining was determined using the imaging software ImageJ.

I first examined the localization of WT-IRS1 and WT-IRS2. Consistent with previous reports in human breast tumors, IRS1 is expressed in the cytoplasm and nucleus and IRS2 is only expressed in the cytoplasm in SUM-159 cells. Truncation of the IRS1 C-terminus (IRS1Δ942) does not alter the localization of IRS1 when compared to the wild type protein (Fig. A.1B). In contrast, truncation of the IRS2 C-terminus (IRS2Δ917) resulted in the redistribution of IRS2 to both the nucleus and cytoplasm (Fig. A.1B) These data suggest that the C-termini of the IRS proteins are important for the regulation of protein localization, a finding that was confirmed by the fact that a chimeric IRS1 protein with the N-terminus of IRS1 and C-terminus of IRS2 (IRS1/S2) is restricted to the cytoplasm while an IRS2/S1 chimera localizes to both compartments (Fig. A1B). The percentage of IRS protein

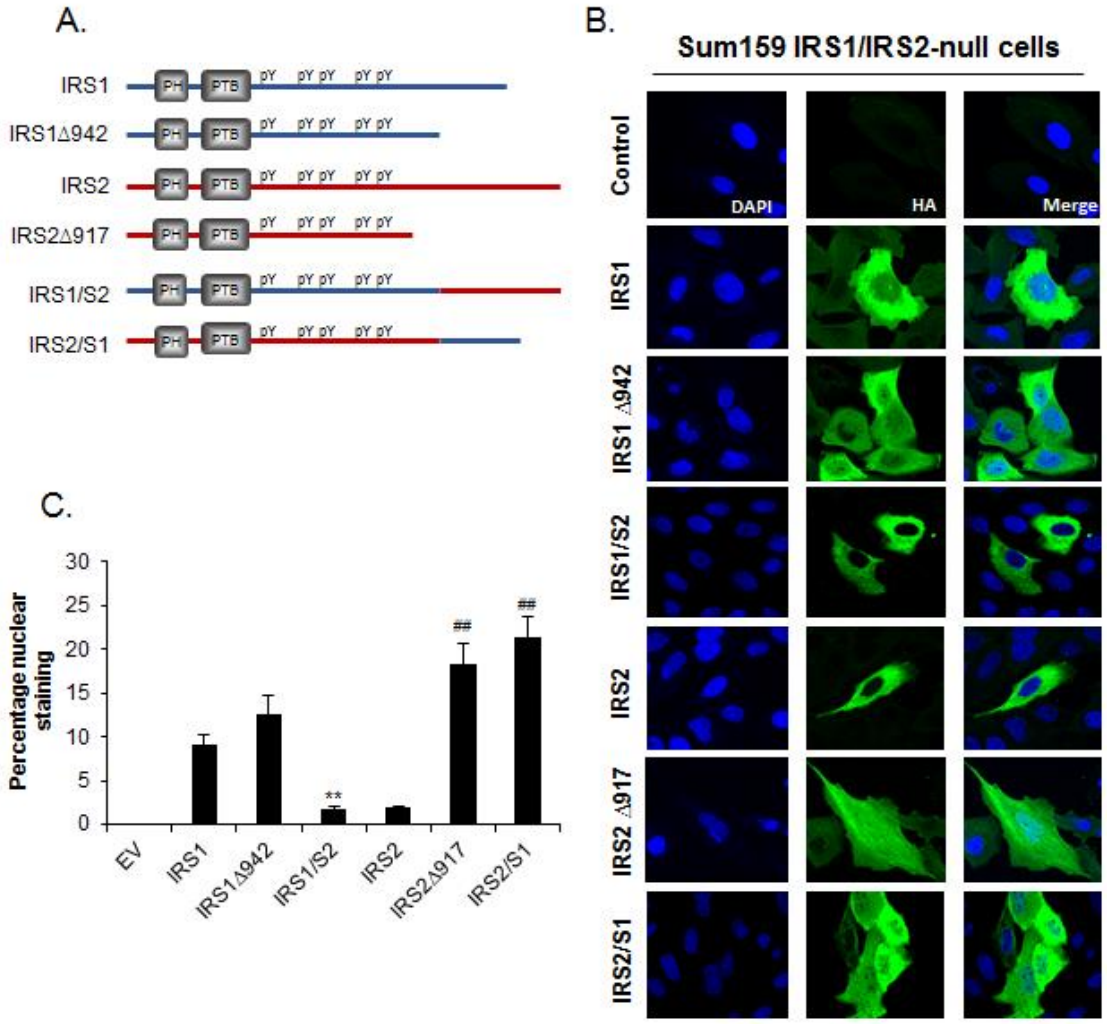

Figure A.1. The IRS2 C-terminus is sufficient for the regulation of IRS2 localization. (A) Schematic depicting WT IRS1, IRS1Δ942, IRS1/S2, WT IRS2, IRS2Δ917 and IRS2/S1 proteins. SUM-159 IRS1/IRS2-null cells expressing the proteins depicted in (A) were plated on glass coverslips. (B) Cells were stained for HA and mounted in Prolong Gold medium with DAPI. Representative DAPI (blue), HA (green) and merged HA/DAPI images are shown (magnification X63). (C) The percentage of nuclear staining was quantified and the data shown represent the mean ± S.E.M of 10 cells. **, $p<0.01$ relative to IRS1; $^{\#\#}$, $p<0.01$ relative to IRS2.

that is localized to the nucleus was quantified (Fig. A1C). IRS1 contains two nuclear localization sequences that are not located within the C-terminal region that was deleted. These data suggest that IRS2 can traffic to the nucleus, but the C-terminus is responsible for retaining IRS2 in the cytoplasm.

To further dissect the contribution of the IRS2 C-terminus to subcellular localization, I examined two additional IRS2 C-terminal deletion mutants in which 324 amino acids (IRS2Δ1014) or 150 amino acids (IRS2Δ1188) were deleted (Fig A.2A). The IRS2Δ1014 mutant localizes to the nucleus and cytoplasm, whereas the IRS2Δ1188 mutant remains cytoplasmic (Fig. A.2B-A.2C). The final 150 amino acids of IRS2 are not required for retaining IRS2 in the cytoplasm However, deletion of an additional 174 amino acids (IRS2Δ1014) or 271 amino acids (IRS2Δ917) causes a redistribution of IRS2 to the nucleus, suggesting that sequences within this 271 amino acid region control the cytoplasmic retention of IRS2.

In Chapter 3, I identified discrete regions within the IRS2 C-terminus that are required for either self-renewal (SR) or invasion (INV) (Fig. A.3A). To determine if these regions regulate subcellular localization of IRS2 to contribute to their respective functions, the SR and INV deletion mutants, IRS2ΔSR and IRS2ΔINV, were expressed in Irs1/Irs2-null PyMT cells. In contrast to the full IRS2 C-terminal deletion mutant (IRS2Δ917) which localizes to both the nucleus and cytoplasm, the internal SR and INV deletion mutants were expressed exclusively in the cytoplasm (Fig. A.3B-A.3C). From these data, I conclude that the defects in

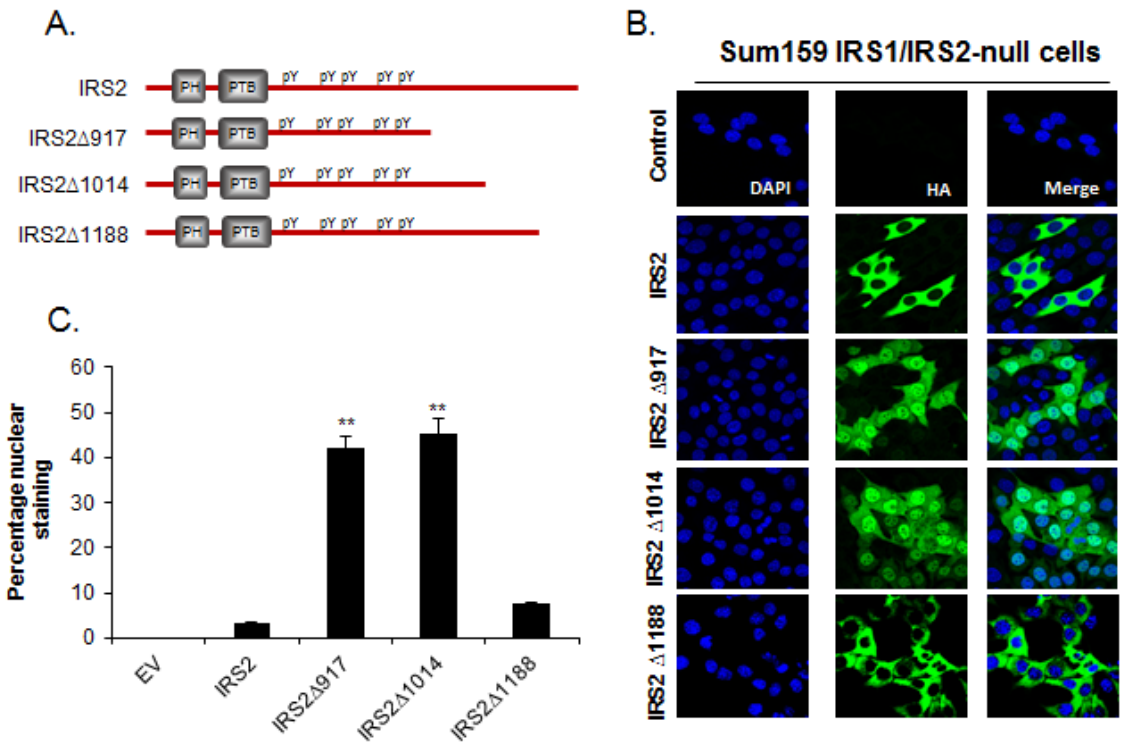

Figure A.2. Identification of a region within the IRS2 C-terminus that regulates IRS2 subcellular localization. (A) Schematic depicting WT IRS2, IRS2Δ917, IRS2Δ1014 and IRS2Δ1188. SUM-159 IRS1/IRS2-null cells expressing the proteins depicted in (A) were plated on glass coverslips. (B) Cells were stained for HA and mounted in Prolong Gold medium with DAPI. Representative DAPI (blue), HA (green) and merged HA/DAPI images are shown (magnification X63). (C) The percentage of nuclear staining was quantified and the data shown represent the mean ± S.E.M. of 10 cells. **, $p<0.01$ relative to IRS2.

IRS2ΔSR self-renewal and IRS2ΔINV invasion are not related to aberrant protein subcellular localization as both deletion mutants localize to the cytoplasm similar to wild type IRS2.

IRS2 does not contain any canonical nuclear localization sequences, and yet it is able to traffic into the nucleus upon truncation of the C-terminus. Of interest, the IRS1/S2 chimera is cytoplasmic even with the nuclear localization sequences in the IRS1 N-terminus. These data suggest that a role of the IRS2 C-terminus is to maintain IRS2 in the cytoplasmic compartment. To investigate further how the IRS2 C-terminus regulates the trafficking of IRS2, and restricts the protein from the nuclear compartment, I treated cells expressing WT-IRS2, IRS2Δ917, IRS2Δ1014 and IRS2Δ1188 with the nuclear export inhibitor Leptomycin B. WT-IRS2 and IRS2Δ1188, which are normally restricted to the cytoplasm, exhibited increased expression in the nucleus upon inhibition of nuclear export (Fig. A.4A). In contrast, treatment of cells expressing IRS2Δ917 or IRS2Δ1014, which exhibit elevated nuclear expression in the absence of treatment, did not increase the percentage of nuclear IRS expression in response to leptomycin B (Fig. A.4B). Taken together, these data suggest that IRS2 is capable of trafficking into the nuclear compartment, but it is rapidly exported out of the nucleus.

Taking into consideration my data from the internal deletion mutants and IRS2Δ1188, I conclude that the final 150 amino acids of IRS2 (1189-1338) are not required for cytoplasmic retention of the protein. Neither the loss of this region in

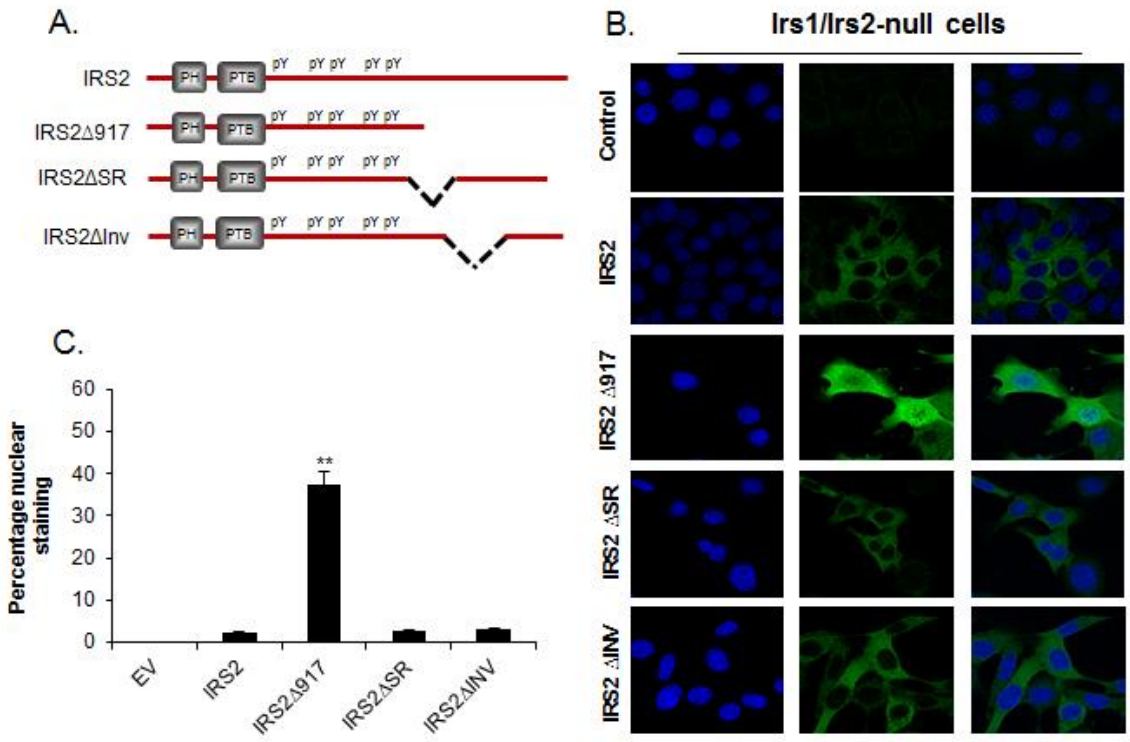

Figure A.3. Internal deletion of the IRS2 SR and INV regions does not alter IRS2 subcellular localization. (A) Schematic depicting WT IRS2, IRS2Δ917, IRS2ΔSR and IRS2ΔINV. PyMT:Irs1-/-/Irs2-/- cells expressing the proteins depicted in (A) were plated on glass coverslips. (B) Cells were stained for HA and mounted in Prolong Gold medium with DAPI. Representative DAPI (blue), HA (green) and merged HA/DAPI images are shown (magnification X63). (C) The percentage of nuclear staining was quantified and the data shown represent the mean ± S.E.M. of 10 cells. **, $p<0.01$ relative to IRS2.

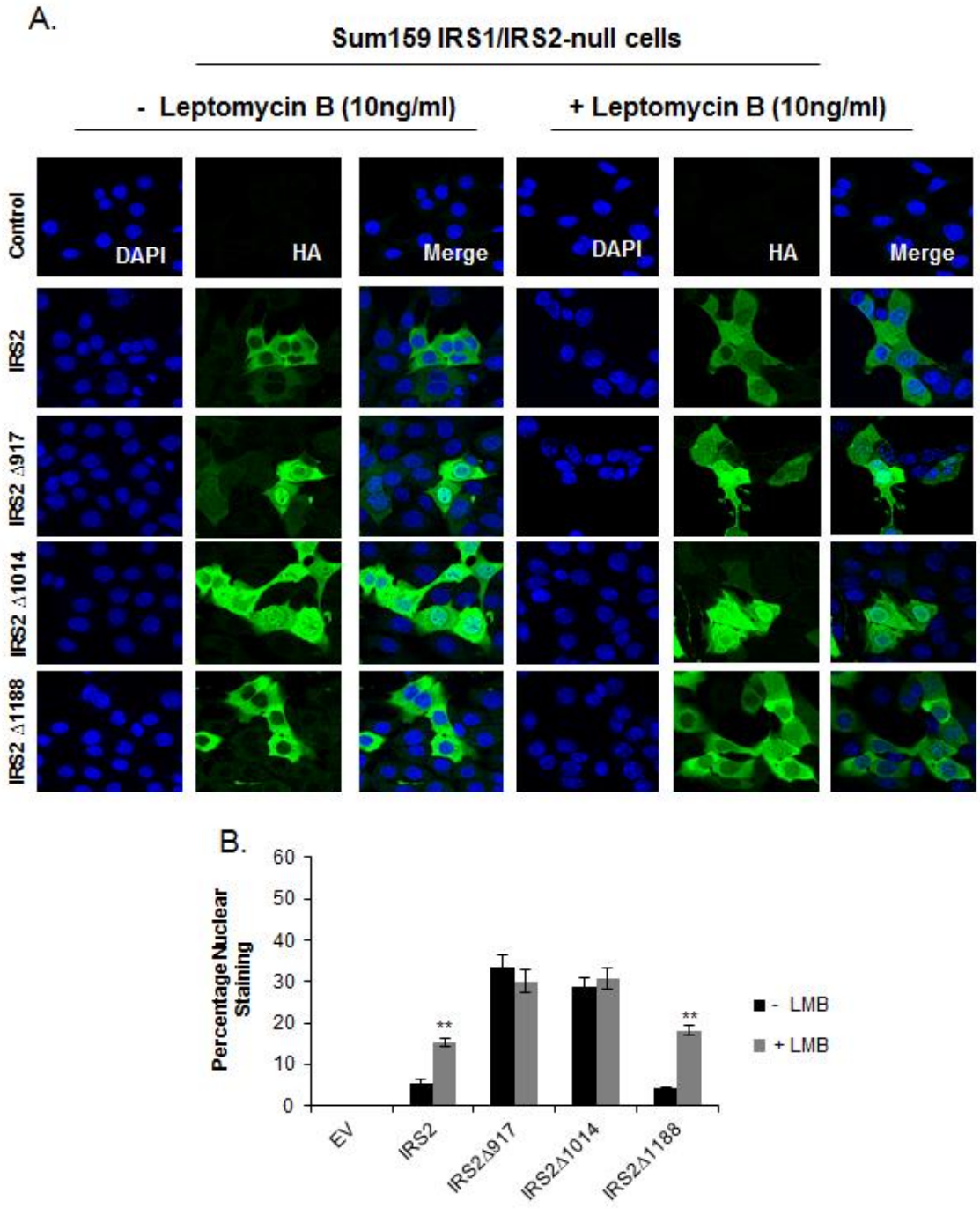

Figure A.4. The IRS2 C-terminus is required for the nuclear export of IRS2. SUM-159 IRS1/IRS2-null cells expressing WT IRS2, IRS2Δ917, IRS2Δ1014 and IRS2Δ1188 were plated on glass coverslips and treated with DMSO or Leptomycin B (10 ng/ml) for 24 hours. (A) Fxed cells were stained for HA and mounted in

Prolong Gold medium with DAPI. Representative DAPI (blue), HA (green) and merged HA/DAPI images are shown (magnification X63). (B) The percentage of nuclear staining was quantified and the data shown represent the mean ± S.E.M of 10 cells. **, $p<0.01$ relative to IRS2.

IRS2Δ1188 nor its presence in IRS2ΔSR and IRS2ΔINV alters the subcellular localization of IRS2. Internal deletion of the SR or INV regions alone also does not impair IRS2 localization. However, in apparent conflict with the IRS2ΔINV mutant, IRS2Δ1014, which also lacks the INV domain, localizes to the nucleus. I hypothesize that the combined loss of the INV region with the final 150 amino acids may alter intramolecular interactions and impair proper protein folding that could affect important protein interactions that regulate IRS2 localization within the cytoplasm. This misfolding could interfere with interactions that restrict IRS2 to the cytoplasm or interactions that are required for interaction with exportin proteins that regulate nuclear export of IRS2.

REFERENCES

1. American Cancer Society., *Cancer facts & figures*. The Society: Atlanta, GA. p. volumes.
2. American Cancer Society., *Breast cancer facts & figures*. The Society: Atlanta, Ga. p. v.
3. *Breast cancer survivorship : consequences of early breast cancer and its treatment*. 2016, New York, NY: Springer Berlin Heidelberg. pages cm.
4. *Breast cancer prevention and treatment*. 2016, New York, NY: Springer Science+Business Media. pages cm.
5. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
6. Rouzier, R., et al., *Breast cancer molecular subtypes respond differently to preoperative chemotherapy.* Clin Cancer Res, 2005. **11**(16): p. 5678-85.
7. Yu, D. and M.C. Hung, *Overexpression of ErbB2 in cancer and ErbB2-targeting strategies.* Oncogene, 2000. **19**(53): p. 6115-21.
8. Schneider, B.P., et al., *Triple-negative breast cancer: risk factors to potential targets.* Clin Cancer Res, 2008. **14**(24): p. 8010-8.
9. Jitariu, A.A., et al., *Triple negative breast cancer: the kiss of death.* Oncotarget, 2017.
10. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA Cancer J Clin, 2016. **66**(1): p. 7-30.
11. Steeg, P.S., *Targeting metastasis.* Nat Rev Cancer, 2016. **16**(4): p. 201-18.
12. Tevaarwerk, A.J., et al., *Survival in patients with metastatic recurrent breast cancer after adjuvant chemotherapy: little evidence of improvement over the past 30 years.* Cancer, 2013. **119**(6): p. 1140-8.
13. Geiger, T.R. and D.S. Peeper, *Metastasis mechanisms.* Biochim Biophys Acta, 2009. **1796**(2): p. 293-308.
14. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited.* Nat Rev Cancer, 2003. **3**(6): p. 453-8.
15. Gomis, R.R. and S. Gawrzak, *Tumor cell dormancy.* Mol Oncol, 2016.
16. Brooks, S.A., et al., *Molecular interactions in cancer cell metastasis.* Acta Histochem, 2010. **112**(1): p. 3-25.
17. Valastyan, S., *Roles of microRNAs and other non-coding RNAs in breast cancer metastasis.* J Mammary Gland Biol Neoplasia, 2012. **17**(1): p. 23-32.
18. Valastyan, S. and R.A. Weinberg, *Tumor metastasis: molecular insights and evolving paradigms.* Cell, 2011. **147**(2): p. 275-92.
19. Yin, K., et al., *MiR-206 suppresses epithelial mesenchymal transition by targeting TGF-beta signaling in estrogen receptor positive breast cancer cells.* Oncotarget, 2016. **7**(17): p. 24537-48.

20. Zhang, H., et al., *KLF8 involves in TGF-beta-induced EMT and promotes invasion and migration in gastric cancer cells.* J Cancer Res Clin Oncol, 2013. **139**(6): p. 1033-42.
21. Takebe, N., et al., *Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways.* Nat Rev Clin Oncol, 2011. **8**(2): p. 97-106.
22. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-54.
23. Thiery, J.P., et al., *Epithelial-mesenchymal transitions in development and disease.* Cell, 2009. **139**(5): p. 871-90.
24. Christiansen, J.J. and A.K. Rajasekaran, *Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis.* Cancer Res, 2006. **66**(17): p. 8319-26.
25. Fischer, K.R., et al., *Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance.* Nature, 2015. **527**(7579): p. 472-6.
26. Pandya, P., J.L. Orgaz, and V. Sanz-Moreno, *Modes of invasion during tumour dissemination.* Mol Oncol, 2017. **11**(1): p. 5-27.
27. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: diversity and escape mechanisms.* Nat Rev Cancer, 2003. **3**(5): p. 362-74.
28. Wolf, K., et al., *Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion.* Nat Cell Biol, 2007. **9**(8): p. 893-904.
29. Sahai, E. and C.J. Marshall, *Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis.* Nat Cell Biol, 2003. **5**(8): p. 711-9.
30. Sanz-Moreno, V. and C.J. Marshall, *The plasticity of cytoskeletal dynamics underlying neoplastic cell migration.* Curr Opin Cell Biol, 2010. **22**(5): p. 690-6.
31. Bergert, M., et al., *Force transmission during adhesion-independent migration.* Nat Cell Biol, 2015. **17**(4): p. 524-9.
32. Giampieri, S., et al., *Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility.* Nat Cell Biol, 2009. **11**(11): p. 1287-96.
33. Pinner, S. and E. Sahai, *Imaging amoeboid cancer cell motility in vivo.* J Microsc, 2008. **231**(3): p. 441-5.
34. Friedl, P. and D. Gilmour, *Collective cell migration in morphogenesis, regeneration and cancer.* Nat Rev Mol Cell Biol, 2009. **10**(7): p. 445-57.
35. Cheung, K.J., et al., *Collective invasion in breast cancer requires a conserved basal epithelial program.* Cell, 2013. **155**(7): p. 1639-51.
36. Nguyen-Ngoc, K.V., et al., *3D culture assays of murine mammary branching morphogenesis and epithelial invasion.* Methods Mol Biol, 2015. **1189**: p. 135-62.
37. Almendro, V., A. Marusyk, and K. Polyak, *Cellular heterogeneity and molecular evolution in cancer.* Annu Rev Pathol, 2013. **8**: p. 277-302.

38. Gaggioli, C., et al., *Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells.* Nat Cell Biol, 2007. **9**(12): p. 1392-400.
39. Dovas, A., et al., *Imaging interactions between macrophages and tumour cells that are involved in metastasis in vivo and in vitro.* J Microsc, 2013. **251**(3): p. 261-9.
40. Condeelis, J. and J.W. Pollard, *Macrophages: obligate partners for tumor cell migration, invasion, and metastasis.* Cell, 2006. **124**(2): p. 263-6.
41. Ilina, O. and P. Friedl, *Mechanisms of collective cell migration at a glance.* J Cell Sci, 2009. **122**(Pt 18): p. 3203-8.
42. Hasebe, T., et al., *Highly proliferative fibroblasts forming fibrotic focus govern metastasis of invasive ductal carcinoma of the breast.* Mod Pathol, 2001. **14**(4): p. 325-37.
43. Chen, F., et al., *New horizons in tumor microenvironment biology: challenges and opportunities.* BMC Med, 2015. **13**: p. 45.
44. Allavena, P., et al., *The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages.* Crit Rev Oncol Hematol, 2008. **66**(1): p. 1-9.
45. Lu, P., et al., *Extracellular matrix degradation and remodeling in development and disease.* Cold Spring Harb Perspect Biol, 2011. **3**(12).
46. Wolf, K., et al., *Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis.* J Cell Biol, 2003. **160**(2): p. 267-77.
47. Wyckoff, J.B., et al., *ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo.* Curr Biol, 2006. **16**(15): p. 1515-23.
48. Coussens, L.M., B. Fingleton, and L.M. Matrisian, *Matrix metalloproteinase inhibitors and cancer: trials and tribulations.* Science, 2002. **295**(5564): p. 2387-92.
49. Fingleton, B., *Matrix metalloproteinase inhibitors for cancer therapy:the current situation and future prospects.* Expert Opin Ther Targets, 2003. **7**(3): p. 385-97.
50. Lin, E.Y. and J.W. Pollard, *Tumor-associated macrophages press the angiogenic switch in breast cancer.* Cancer Res, 2007. **67**(11): p. 5064-6.
51. Patsialou, A., et al., *Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor.* Cancer Res, 2009. **69**(24): p. 9498-506.
52. Kacinski, B.M., *CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract.* Mol Reprod Dev, 1997. **46**(1): p. 71-4.
53. Toy, E.P., et al., *The activated macrophage colony-stimulating factor (CSF-1) receptor as a predictor of poor outcome in advanced epithelial ovarian carcinoma.* Gynecol Oncol, 2001. **80**(2): p. 194-200.

54. Filderman, A.E., et al., *Macrophage colony-stimulating factor (CSF-1) enhances invasiveness in CSF-1 receptor-positive carcinoma cell lines.* Cancer Res, 1992. **52**(13): p. 3661-6.
55. Condeelis, J. and R. Weissleder, *In vivo imaging in cancer.* Cold Spring Harb Perspect Biol, 2010. **2**(12): p. a003848.
56. Carter, J.C. and F.C. Church, *Mature breast adipocytes promote breast cancer cell motility.* Exp Mol Pathol, 2012. **92**(3): p. 312-7.
57. Santander, A.M., et al., *Paracrine Interactions between Adipocytes and Tumor Cells Recruit and Modify Macrophages to the Mammary Tumor Microenvironment: The Role of Obesity and Inflammation in Breast Adipose Tissue.* Cancers (Basel), 2015. **7**(1): p. 143-78.
58. Grisouard, J., et al., *Targeting AMP-activated protein kinase in adipocytes to modulate obesity-related adipokine production associated with insulin resistance and breast cancer cell proliferation.* Diabetol Metab Syndr, 2011. **3**: p. 16.
59. Falk Libby, E., et al., *Globular adiponectin enhances invasion in human breast cancer cells.* Oncol Lett, 2016. **11**(1): p. 633-641.
60. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression.* Nature, 2006. **441**(7092): p. 437-43.
61. Vaupel, P. and L. Harrison, *Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response.* Oncologist, 2004. **9 Suppl 5**: p. 4-9.
62. Vaupel, P., A. Mayer, and M. Hockel, *Tumor hypoxia and malignant progression.* Methods Enzymol, 2004. **381**: p. 335-54.
63. Bacon, A.L. and A.L. Harris, *Hypoxia-inducible factors and hypoxic cell death in tumour physiology.* Ann Med, 2004. **36**(7): p. 530-9.
64. Ohh, M., et al., *Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein.* Nat Cell Biol, 2000. **2**(7): p. 423-7.
65. Dales, J.P., et al., *Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients.* Int J Cancer, 2005. **116**(5): p. 734-9.
66. Forsythe, J.A., et al., *Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1.* Mol Cell Biol, 1996. **16**(9): p. 4604-13.
67. Liao, D., et al., *Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression.* Cancer Res, 2007. **67**(2): p. 563-72.
68. Liao, D. and R.S. Johnson, *Hypoxia: a key regulator of angiogenesis in cancer.* Cancer Metastasis Rev, 2007. **26**(2): p. 281-90.
69. Wang, F., et al., *Down-regulation of hypoxia-inducible factor-1 suppresses malignant biological behavior of triple-negative breast cancer cells.* Int J Clin Exp Med, 2014. **7**(11): p. 3933-40.

70. Hiraga, T., et al., *Hypoxia and hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer.* Cancer Res, 2007. **67**(9): p. 4157-63.
71. Zhang, X.H., et al., *Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma.* Cell, 2013. **154**(5): p. 1060-73.
72. Paez-Ribes, M., et al., *Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis.* Cancer Cell, 2009. **15**(3): p. 220-31.
73. Paez-Ribes, M., et al., *Potential Proinvasive or Metastatic Effects of Preclinical Antiangiogenic Therapy Are Prevented by Concurrent Chemotherapy.* Clin Cancer Res, 2015. **21**(24): p. 5488-98.
74. Gilkes, D.M., et al., *Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts.* J Biol Chem, 2013. **288**(15): p. 10819-29.
75. Gilkes, D.M., et al., *Collagen prolyl hydroxylases are essential for breast cancer metastasis.* Cancer Res, 2013. **73**(11): p. 3285-96.
76. Chambers, A.F., A.C. Groom, and I.C. MacDonald, *Dissemination and growth of cancer cells in metastatic sites.* Nat Rev Cancer, 2002. **2**(8): p. 563-72.
77. Langley, R.R. and I.J. Fidler, *The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs.* Int J Cancer, 2011. **128**(11): p. 2527-35.
78. Kreso, A. and J.E. Dick, *Evolution of the cancer stem cell model.* Cell Stem Cell, 2014. **14**(3): p. 275-91.
79. Clarke, M.F., et al., *Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells.* Cancer Res, 2006. **66**(19): p. 9339-44.
80. Clarke, M.F. and M. Fuller, *Stem cells and cancer: two faces of eve.* Cell, 2006. **124**(6): p. 1111-5.
81. Vermeulen, L., et al., *The developing cancer stem-cell model: clinical challenges and opportunities.* Lancet Oncol, 2012. **13**(2): p. e83-9.
82. Bonnet, D. and J.E. Dick, *Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell.* Nat Med, 1997. **3**(7): p. 730-7.
83. Bonnet, D., et al., *CD8(+) minor histocompatibility antigen-specific cytotoxic T lymphocyte clones eliminate human acute myeloid leukemia stem cells.* Proc Natl Acad Sci U S A, 1999. **96**(15): p. 8639-44.
84. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-8.
85. O'Brien, C.A., A. Kreso, and C.H. Jamieson, *Cancer stem cells and self-renewal.* Clin Cancer Res, 2010. **16**(12): p. 3113-20.
86. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.

87. Cho, R.W., et al., *Isolation and molecular characterization of cancer stem cells in MMTV-Wnt-1 murine breast tumors.* Stem Cells, 2008. **26**(2): p. 364-71.
88. Zhang, M., et al., *Identification of tumor-initiating cells in a p53-null mouse model of breast cancer.* Cancer Res, 2008. **68**(12): p. 4674-82.
89. To, K., et al., *Y-box binding protein-1 induces the expression of CD44 and CD49f leading to enhanced self-renewal, mammosphere growth, and drug resistance.* Cancer Res, 2010. **70**(7): p. 2840-51.
90. Lawson, D.A., et al., *Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells.* Nature, 2015. **526**(7571): p. 131-5.
91. DiMeo, T.A., et al., *A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer.* Cancer Res, 2009. **69**(13): p. 5364-73.
92. Balic, M., et al., *Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype.* Clin Cancer Res, 2006. **12**(19): p. 5615-21.
93. Charafe-Jauffret, E., et al., *Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer.* Clin Cancer Res, 2010. **16**(1): p. 45-55.
94. Zhang, H., et al., *HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs.* Oncogene, 2012. **31**(14): p. 1757-70.
95. Conley, S.J., et al., *Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia.* Proc Natl Acad Sci U S A, 2012. **109**(8): p. 2784-9.
96. Semenza, G.L., *Regulation of the breast cancer stem cell phenotype by hypoxia-inducible factors.* Clin Sci (Lond), 2015. **129**(12): p. 1037-45.
97. Semenza, G.L., *Dynamic regulation of stem cell specification and maintenance by hypoxia-inducible factors.* Mol Aspects Med, 2016. **47-48**: p. 15-23.
98. Morel, A.P., et al., *Generation of breast cancer stem cells through epithelial-mesenchymal transition.* PLoS One, 2008. **3**(8): p. e2888.
99. Mani, S.A., et al., *The epithelial-mesenchymal transition generates cells with properties of stem cells.* Cell, 2008. **133**(4): p. 704-15.
100. Xu, H., et al., *Enrichment of CD44 in basal-type breast cancer correlates with EMT, cancer stem cell gene profile, and prognosis.* Onco Targets Ther, 2016. **9**: p. 431-44.
101. Borah, A., et al., *Targeting self-renewal pathways in cancer stem cells: clinical implications for cancer therapy.* Oncogenesis, 2015. **4**: p. e177.
102. Gupta, P.B., et al., *Identification of selective inhibitors of cancer stem cells by high-throughput screening.* Cell, 2009. **138**(4): p. 645-59.
103. Rota, L.M., et al., *Determining mammosphere-forming potential: application of the limiting dilution analysis.* J Mammary Gland Biol Neoplasia, 2012. **17**(2): p. 119-23.

104. Chen, J., et al., *Pygo2 associates with MLL2 histone methyltransferase and GCN5 histone acetyltransferase complexes to augment Wnt target gene expression and breast cancer stem-like cell expansion.* Mol Cell Biol, 2010. **30**(24): p. 5621-35.
105. Watanabe, K., M. Fallahi, and X. Dai, *Chromatin effector Pygo2 regulates mammary tumor initiation and heterogeneity in MMTV-Wnt1 mice.* Oncogene, 2014. **33**(5): p. 632-42.
106. Jang, G.B., et al., *Wnt/beta-Catenin Small-Molecule Inhibitor CWP232228 Preferentially Inhibits the Growth of Breast Cancer Stem-like Cells.* Cancer Res, 2015. **75**(8): p. 1691-702.
107. Jang, G.B., et al., *Blockade of Wnt/beta-catenin signaling suppresses breast cancer metastasis by inhibiting CSC-like phenotype.* Sci Rep, 2015. **5**: p. 12465.
108. Mardilovich, K., S.L. Pankratz, and L.M. Shaw, *Expression and function of the insulin receptor substrate proteins in cancer.* Cell Commun Signal, 2009. **7**: p. 14.
109. White, M.F., *IRS proteins and the common path to diabetes.* Am J Physiol Endocrinol Metab, 2002. **283**(3): p. E413-22.
110. Porter, H.A., et al., *IRS1 is highly expressed in localized breast tumors and regulates the sensitivity of breast cancer cells to chemotherapy, while IRS2 is highly expressed in invasive breast tumors.* Cancer Lett, 2013. **338**(2): p. 239-48.
111. Clark, J.L., et al., *Membrane localization of insulin receptor substrate-2 (IRS-2) is associated with decreased overall survival in breast cancer.* Breast Cancer Res Treat, 2011. **130**(3): p. 759-72.
112. Migliaccio, I., et al., *Nuclear IRS-1 predicts tamoxifen response in patients with early breast cancer.* Breast Cancer Res Treat, 2010. **123**(3): p. 651-60.
113. Dearth, R.K., et al., *Mammary tumorigenesis and metastasis caused by overexpression of insulin receptor substrate 1 (IRS-1) or IRS-2.* Mol Cell Biol, 2006. **26**(24): p. 9302-14.
114. Gibson, S.L., Z. Ma, and L.M. Shaw, *Divergent roles for IRS-1 and IRS-2 in breast cancer metastasis.* Cell Cycle, 2007. **6**(6): p. 631-7.
115. Nagle, J.A., et al., *Involvement of insulin receptor substrate 2 in mammary tumor metastasis.* Mol Cell Biol, 2004. **24**(22): p. 9726-35.
116. Ma, Z., et al., *Suppression of insulin receptor substrate 1 (IRS-1) promotes mammary tumor metastasis.* Mol Cell Biol, 2006. **26**(24): p. 9338-51.
117. Pankratz, S.L., et al., *Insulin receptor substrate-2 regulates aerobic glycolysis in mouse mammary tumor cells via glucose transporter 1.* J Biol Chem, 2009. **284**(4): p. 2031-7.
118. Ikink, G.J., et al., *IRS4 induces mammary tumorigenesis and confers resistance to HER2-targeted therapy through constitutive PI3K/AKT-pathway hyperactivation.* Nat Commun, 2016. **7**: p. 13567.
119. Araki, E., et al., *Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene.* Nature, 1994. **372**(6502): p. 186-90.

120. Tamemoto, H., et al., *Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1.* Nature, 1994. **372**(6502): p. 182-6.
121. Withers, D.J., et al., *Disruption of IRS-2 causes type 2 diabetes in mice.* Nature, 1998. **391**(6670): p. 900-4.
122. Schubert, M., et al., *Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation.* J Neurosci, 2003. **23**(18): p. 7084-92.
123. Withers, D.J., et al., *Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signalling.* Nat Genet, 1999. **23**(1): p. 32-40.
124. Fantin, V.R., et al., *Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis.* Am J Physiol Endocrinol Metab, 2000. **278**(1): p. E127-33.
125. Qi, Y., et al., *Myocardial loss of IRS1 and IRS2 causes heart failure and is controlled by p38alpha MAPK during insulin resistance.* Diabetes, 2013. **62**(11): p. 3887-900.
126. Zheng, Y., et al., *Roles of insulin receptor substrates in insulin-induced stimulation of renal proximal bicarbonate absorption.* J Am Soc Nephrol, 2005. **16**(8): p. 2288-95.
127. Yi, X., et al., *Insulin receptor substrate 2 is essential for maturation and survival of photoreceptor cells.* J Neurosci, 2005. **25**(5): p. 1240-8.
128. Babu, M.M., et al., *Intrinsically disordered proteins: regulation and disease.* Curr Opin Struct Biol, 2011. **21**(3): p. 432-40.
129. Uversky, V.N., C.J. Oldfield, and A.K. Dunker, *Intrinsically disordered proteins in human diseases: introducing the D2 concept.* Annu Rev Biophys, 2008. **37**: p. 215-46.
130. Fong, J.H., et al., *Intrinsic disorder in protein interactions: insights from a comprehensive structural analysis.* PLoS Comput Biol, 2009. **5**(3): p. e1000316.
131. Dunker, A.K., et al., *The unfoldomics decade: an update on intrinsically disordered proteins.* BMC Genomics, 2008. **9 Suppl 2**: p. S1.
132. Voliovitch, H., et al., *Tyrosine phosphorylation of insulin receptor substrate-1 in vivo depends upon the presence of its pleckstrin homology region.* J Biol Chem, 1995. **270**(30): p. 18083-7.
133. Sawka-Verhelle, D., et al., *Insulin receptor substrate-2 binds to the insulin receptor through its phosphotyrosine-binding domain and through a newly identified domain comprising amino acids 591-786.* J Biol Chem, 1996. **271**(11): p. 5980-3.
134. Backer, J.M., C. Wjasow, and Y. Zhang, *In vitro binding and phosphorylation of insulin receptor substrate 1 by the insulin receptor. Role of interactions mediated by the phosphotyrosine-binding domain and the pleckstrin-homology domain.* Eur J Biochem, 1997. **245**(1): p. 91-6.

135. Yenush, L., et al., *The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1.* J Biol Chem, 1996. **271**(39): p. 24300-6.
136. Wu, J., et al., *Structural and biochemical characterization of the KRLB region in insulin receptor substrate-2.* Nat Struct Mol Biol, 2008. **15**(3): p. 251-8.
137. Myers, M.G., Jr., et al., *The COOH-terminal tyrosine phosphorylation sites on IRS-1 bind SHP-2 and negatively regulate insulin signaling.* J Biol Chem, 1998. **273**(41): p. 26908-14.
138. Myers, M.G., Jr., et al., *Role of IRS-1-GRB-2 complexes in insulin signaling.* Mol Cell Biol, 1994. **14**(6): p. 3577-87.
139. Lee, C.H., et al., *Nck associates with the SH2 domain-docking protein IRS-1 in insulin-stimulated cells.* Proc Natl Acad Sci U S A, 1993. **90**(24): p. 11713-7.
140. Myers, M.G., Jr., et al., *Insulin receptor substrate-1 mediates phosphatidylinositol 3'-kinase and p70S6k signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation.* J Biol Chem, 1994. **269**(46): p. 28783-9.
141. White, M.F., R. Maron, and C.R. Kahn, *Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells.* Nature, 1985. **318**(6042): p. 183-6.
142. Tobe, K., et al., *Identification of a 190-kDa protein as a novel substrate for the insulin receptor kinase functionally similar to insulin receptor substrate-1.* J Biol Chem, 1995. **270**(11): p. 5698-701.
143. White, M.F., *The IRS-signalling system in insulin and cytokine action.* Philos Trans R Soc Lond B Biol Sci, 1996. **351**(1336): p. 181-9.
144. Jackson, J.G., M.F. White, and D. Yee, *Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells.* J Biol Chem, 1998. **273**(16): p. 9994-10003.
145. Yamauchi, T., et al., *Growth hormone and prolactin stimulate tyrosine phosphorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase.* J Biol Chem, 1998. **273**(25): p. 15719-26.
146. Previs, S.F., et al., *Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo.* J Biol Chem, 2000. **275**(50): p. 38990-4.
147. Myers, M.G., Jr. and M.F. White, *Insulin signal transduction and the IRS proteins.* Annu Rev Pharmacol Toxicol, 1996. **36**: p. 615-58.
148. Abuzzahab, M.J., et al., *IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation.* N Engl J Med, 2003. **349**(23): p. 2211-22.

149. Kleinberg, D.L., M. Feldman, and W. Ruan, *IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis.* J Mammary Gland Biol Neoplasia, 2000. **5**(1): p. 7-17.
150. LeRoith, D. and C.T. Roberts, Jr., *The insulin-like growth factor system and cancer.* Cancer Lett, 2003. **195**(2): p. 127-37.
151. Belfiore, A., et al., *Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease.* Endocr Rev, 2009. **30**(6): p. 586-623.
152. Sacco, A., et al., *Differential signaling activation by insulin and insulin-like growth factors I and II upon binding to insulin receptor isoform A.* Endocrinology, 2009. **150**(8): p. 3594-602.
153. Denley, A., et al., *The insulin receptor isoform exon 11- (IR-A) in cancer and other diseases: a review.* Horm Metab Res, 2003. **35**(11-12): p. 778-85.
154. Renehan, A.G., M. Harvie, and A. Howell, *Insulin-like growth factor (IGF)-I, IGF binding protein-3, and breast cancer risk: eight years on.* Endocr Relat Cancer, 2006. **13**(2): p. 273-8.
155. Renehan, A.G., J. Frystyk, and A. Flyvbjerg, *Obesity and cancer risk: the role of the insulin-IGF axis.* Trends Endocrinol Metab, 2006. **17**(8): p. 328-36.
156. Pollak, M.N., *Insulin-like growth factors and neoplasia.* Novartis Found Symp, 2004. **262**: p. 84-98; discussion 98-107, 265-8.
157. Singh, P., J.M. Alex, and F. Bast, *Insulin receptor (IR) and insulin-like growth factor receptor 1 (IGF-1R) signaling systems: novel treatment strategies for cancer.* Med Oncol, 2014. **31**(1): p. 805.
158. Kim, H.J., et al., *Constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary epithelial cells and is accompanied by an epithelial-to-mesenchymal transition mediated by NF-kappaB and snail.* Mol Cell Biol, 2007. **27**(8): p. 3165-75.
159. Franks, S.E., et al., *Transgenic IGF-IR overexpression induces mammary tumors with basal-like characteristics, whereas IGF-IR-independent mammary tumors express a claudin-low gene signature.* Oncogene, 2012. **31**(27): p. 3298-309.
160. Xie, Y., et al., *Expression of insulin-like growth factor-1 receptor in synovial sarcoma: association with an aggressive phenotype.* Cancer Res, 1999. **59**(15): p. 3588-91.
161. Sachdev, D., et al., *The type I insulin-like growth factor receptor regulates cancer metastasis independently of primary tumor growth by promoting invasion and survival.* Oncogene, 2010. **29**(2): p. 251-62.
162. Sachdev, D., et al., *A dominant negative type I insulin-like growth factor receptor inhibits metastasis of human cancer cells.* J Biol Chem, 2004. **279**(6): p. 5017-24.
163. Yoneda, T., et al., *A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and*

a brain-seeking clone in vivo and in vitro. J Bone Miner Res, 2001. **16**(8): p. 1486-95.
164. Law, J.H., et al., *Phosphorylated insulin-like growth factor-i/insulin receptor is present in all breast cancer subtypes and is related to poor survival.* Cancer Res, 2008. **68**(24): p. 10238-46.
165. Improta, G., et al., *Protein pathway activation mapping of brain metastasis from lung and breast cancers reveals organ type specific drug target activation.* J Proteome Res, 2011. **10**(7): p. 3089-97.
166. Heskamp, S., et al., *Upregulation of IGF-1R expression during neoadjuvant therapy predicts poor outcome in breast cancer patients.* PLoS One, 2015. **10**(2): p. e0117745.
167. Nantajit, D., D. Lin, and J.J. Li, *The network of epithelial-mesenchymal transition: potential new targets for tumor resistance.* J Cancer Res Clin Oncol, 2015. **141**(10): p. 1697-713.
168. Duru, N., et al., *HER2-associated radioresistance of breast cancer stem cells isolated from HER2-negative breast cancer cells.* Clin Cancer Res, 2012. **18**(24): p. 6634-47.
169. Garofalo, C., et al., *Efficacy of and resistance to anti-IGF-1R therapies in Ewing's sarcoma is dependent on insulin receptor signaling.* Oncogene, 2011. **30**(24): p. 2730-40.
170. Chen, H.X. and E. Sharon, *IGF-1R as an anti-cancer target--trials and tribulations.* Chin J Cancer, 2013. **32**(5): p. 242-52.
171. Surmacz, E., *Function of the IGF-I receptor in breast cancer.* J Mammary Gland Biol Neoplasia, 2000. **5**(1): p. 95-105.
172. Valverde, A.M., et al., *Insulin receptor substrate (IRS) proteins IRS-1 and IRS-2 differential signaling in the insulin/insulin-like growth factor-I pathways in fetal brown adipocytes.* Mol Endocrinol, 1998. **12**(5): p. 688-97.
173. Jones, J.I. and D.R. Clemmons, *Insulin-like growth factors and their binding proteins: biological actions.* Endocr Rev, 1995. **16**(1): p. 3-34.
174. Taniguchi, C.M., et al., *Divergent regulation of hepatic glucose and lipid metabolism by phosphoinositide 3-kinase via Akt and PKClambda/zeta.* Cell Metab, 2006. **3**(5): p. 343-53.
175. Taniguchi, C.M., B. Emanuelli, and C.R. Kahn, *Critical nodes in signalling pathways: insights into insulin action.* Nat Rev Mol Cell Biol, 2006. **7**(2): p. 85-96.
176. Ruderman, N.B., et al., *Activation of phosphatidylinositol 3-kinase by insulin.* Proc Natl Acad Sci U S A, 1990. **87**(4): p. 1411-5.
177. Engelman, J.A., J. Luo, and L.C. Cantley, *The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism.* Nat Rev Genet, 2006. **7**(8): p. 606-19.
178. Carpenter, C.L., et al., *Purification and characterization of phosphoinositide 3-kinase from rat liver.* J Biol Chem, 1990. **265**(32): p. 19704-11.

179. Yu, J., et al., *Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit.* Mol Cell Biol, 1998. **18**(3): p. 1379-87.
180. Landis, J. and L.M. Shaw, *Insulin receptor substrate 2-mediated phosphatidylinositol 3-kinase signaling selectively inhibits glycogen synthase kinase 3beta to regulate aerobic glycolysis.* J Biol Chem, 2014. **289**(26): p. 18603-13.
181. Dong, X., et al., *Irs1 and Irs2 signaling is essential for hepatic glucose homeostasis and systemic growth.* J Clin Invest, 2006. **116**(1): p. 101-14.
182. Burgering, B.M. and P.J. Coffer, *Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction.* Nature, 1995. **376**(6541): p. 599-602.
183. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream.* Cell, 2007. **129**(7): p. 1261-74.
184. Etienne-Manneville, S. and A. Hall, *Rho GTPases in cell biology.* Nature, 2002. **420**(6916): p. 629-35.
185. Samuels, Y., et al., *High frequency of mutations of the PIK3CA gene in human cancers.* Science, 2004. **304**(5670): p. 554.
186. Samuels, Y., et al., *Mutant PIK3CA promotes cell growth and invasion of human cancer cells.* Cancer Cell, 2005. **7**(6): p. 561-73.
187. Bachman, K.E., et al., *The PIK3CA gene is mutated with high frequency in human breast cancers.* Cancer Biol Ther, 2004. **3**(8): p. 772-5.
188. Isakoff, S.J., et al., *Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells.* Cancer Res, 2005. **65**(23): p. 10992-1000.
189. Sansal, I. and W.R. Sellers, *The biology and clinical relevance of the PTEN tumor suppressor pathway.* J Clin Oncol, 2004. **22**(14): p. 2954-63.
190. Liaw, D., et al., *Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome.* Nat Genet, 1997. **16**(1): p. 64-7.
191. Lee, A.V., et al., *Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo.* Mol Endocrinol, 1999. **13**(5): p. 787-96.
192. Cui, X., et al., *Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer.* Mol Endocrinol, 2003. **17**(4): p. 575-88.
193. Morelli, C., et al., *Nuclear insulin receptor substrate 1 interacts with estrogen receptor alpha at ERE promoters.* Oncogene, 2004. **23**(45): p. 7517-26.
194. Cui, X., et al., *Progesterone crosstalks with insulin-like growth factor signaling in breast cancer cells via induction of insulin receptor substrate-2.* Oncogene, 2003. **22**(44): p. 6937-41.

195. Vassen, L., W. Wegrzyn, and L. Klein-Hitpass, *Human insulin receptor substrate-2 (IRS-2) is a primary progesterone response gene.* Mol Endocrinol, 1999. **13**(3): p. 485-94.
196. Lassarre, C. and J.M. Ricort, *Growth factor-specific regulation of insulin receptor substrate-1 expression in MCF-7 breast carcinoma cells: effects on the insulin-like growth factor signaling pathway.* Endocrinology, 2003. **144**(11): p. 4811-9.
197. Mardilovich, K. and L.M. Shaw, *Hypoxia regulates insulin receptor substrate-2 expression to promote breast carcinoma cell survival and invasion.* Cancer Res, 2009. **69**(23): p. 8894-901.
198. Zhang, P., et al., *MiR-338-3p inhibits the growth and invasion of non-small cell lung cancer cells by targeting IRS2.* Am J Cancer Res, 2017. **7**(1): p. 53-63.
199. Park, D.H., et al., *MicroRNA-146a inhibits epithelial mesenchymal transition in non-small cell lung cancer by targeting insulin receptor substrate 2.* Int J Oncol, 2015. **47**(4): p. 1545-53.
200. Dong, S., et al., *microRNA-141 inhibits thyroid cancer cell growth and metastasis by targeting insulin receptor substrate 2.* Am J Transl Res, 2016. **8**(3): p. 1471-81.
201. Rui, L., et al., *Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2.* J Biol Chem, 2001. **276**(43): p. 40362-7.
202. Yoshihara, H., et al., *Insulin/insulin-like growth factor (IGF) stimulation abrogates an association between a deubiquitinating enzyme USP7 and insulin receptor substrates (IRSs) followed by proteasomal degradation of IRSs.* Biochem Biophys Res Commun, 2012. **423**(1): p. 122-7.
203. Gual, P., Y. Le Marchand-Brustel, and J.F. Tanti, *Positive and negative regulation of insulin signaling through IRS-1 phosphorylation.* Biochimie, 2005. **87**(1): p. 99-109.
204. Copps, K.D. and M.F. White, *Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2.* Diabetologia, 2012. **55**(10): p. 2565-2582.
205. Lee, J.Y., et al., *Inhibition of PI3K binding to activators by serine phosphorylation of PI3K regulatory subunit p85alpha Src homology-2 domains.* Proc Natl Acad Sci U S A, 2011. **108**(34): p. 14157-62.
206. Simpson, L., et al., *PTEN expression causes feedback upregulation of insulin receptor substrate 2.* Mol Cell Biol, 2001. **21**(12): p. 3947-58.
207. Aguirre, V., et al., *Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action.* J Biol Chem, 2002. **277**(2): p. 1531-7.
208. Schiff, P.B., J. Fant, and S.B. Horwitz, *Promotion of microtubule assembly in vitro by taxol.* Nature, 1979. **277**(5698): p. 665-7.
209. Lu, W. and V.I. Gelfand, *Moonlighting Motors: Kinesin, Dynein, and Cell Polarity.* Trends Cell Biol, 2017. **27**(7): p. 505-514.

210. Granger, E., et al., *The role of the cytoskeleton and molecular motors in endosomal dynamics.* Semin Cell Dev Biol, 2014. **31**: p. 20-9.
211. Samson, F., et al., *Nocodazole action on tubulin assembly, axonal ultrastructure and fast axoplasmic transport.* J Pharmacol Exp Ther, 1979. **208**(3): p. 411-7.
212. Liu, L.Z., et al., *Microtubule network is required for insulin-induced signal transduction and actin remodeling.* Mol Cell Endocrinol, 2013. **365**(1): p. 64-74.
213. Joo, E.E. and K.M. Yamada, *Post-polymerization crosstalk between the actin cytoskeleton and microtubule network.* Bioarchitecture, 2016. **6**(3): p. 53-9.
214. Fanale, D., et al., *Stabilizing versus destabilizing the microtubules: a double-edge sword for an effective cancer treatment option?* Anal Cell Pathol (Amst), 2015. **2015**: p. 690916.
215. Dumontet, C. and M.A. Jordan, *Microtubule-binding agents: a dynamic field of cancer therapeutics.* Nat Rev Drug Discov, 2010. **9**(10): p. 790-803.
216. Hirokawa, N., et al., *Kinesin superfamily motor proteins and intracellular transport.* Nat Rev Mol Cell Biol, 2009. **10**(10): p. 682-96.
217. Yu, Y. and Y.M. Feng, *The role of kinesin family proteins in tumorigenesis and progression: potential biomarkers and molecular targets for cancer therapy.* Cancer, 2010. **116**(22): p. 5150-60.
218. Liu, X., H. Gong, and K. Huang, *Oncogenic role of kinesin proteins and targeting kinesin therapy.* Cancer Sci, 2013. **104**(6): p. 651-6.
219. Venere, M., et al., *The mitotic kinesin KIF11 is a driver of invasion, proliferation, and self-renewal in glioblastoma.* Sci Transl Med, 2015. **7**(304): p. 304ra143.
220. Stangel, D., et al., *Kif20a inhibition reduces migration and invasion of pancreatic cancer cells.* J Surg Res, 2015. **197**(1): p. 91-100.
221. Hirokawa, N. and R. Takemura, *Kinesin superfamily proteins and their various functions and dynamics.* Exp Cell Res, 2004. **301**(1): p. 50-9.
222. Zhang, W., et al., *High Expression of KIF20A Is Associated with Poor Overall Survival and Tumor Progression in Early-Stage Cervical Squamous Cell Carcinoma.* PLoS One, 2016. **11**(12): p. e0167449.
223. De, S., et al., *Overexpression of kinesins mediates docetaxel resistance in breast cancer cells.* Cancer Res, 2009. **69**(20): p. 8035-42.
224. Chandrasekaran, G., P. Tatrai, and F. Gergely, *Hitting the brakes: targeting microtubule motors in cancer.* Br J Cancer, 2015. **113**(5): p. 693-8.
225. Taylor, R.C., S.P. Cullen, and S.J. Martin, *Apoptosis: controlled demolition at the cellular level.* Nat Rev Mol Cell Biol, 2008. **9**(3): p. 231-41.
226. Chipuk, J.E., et al., *The BCL-2 family reunion.* Mol Cell, 2010. **37**(3): p. 299-310.
227. Eimon, P.M., et al., *Delineation of the cell-extrinsic apoptosis pathway in the zebrafish.* Cell Death Differ, 2006. **13**(10): p. 1619-30.

228. Puthalakath, H., et al., *The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex.* Mol Cell, 1999. **3**(3): p. 287-96.
229. Tsang, T.Y., et al., *Downregulation of hepatoma-derived growth factor activates the Bad-mediated apoptotic pathway in human cancer cells.* Apoptosis, 2008. **13**(9): p. 1135-47.
230. Reginato, M.J., et al., *Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis.* Nat Cell Biol, 2003. **5**(8): p. 733-40.
231. Jager, R. and R.M. Zwacka, *The enigmatic roles of caspases in tumor development.* Cancers (Basel), 2010. **2**(4): p. 1952-79.
232. Devarajan, E., et al., *Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance.* Oncogene, 2002. **21**(57): p. 8843-51.
233. Ghavami, S., et al., *Apoptosis and cancer: mutations within caspase genes.* J Med Genet, 2009. **46**(8): p. 497-510.
234. Myers, M.G., Jr., et al., *IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10350-4.
235. Hadari, Y.R., et al., *Insulin and insulinomimetic agents induce activation of phosphatidylinositol 3'-kinase upon its association with pp185 (IRS-1) in intact rat livers.* J Biol Chem, 1992. **267**(25): p. 17483-6.
236. Myers, M.G., Jr., et al., *IRS-1 is a common element in insulin and insulin-like growth factor-I signaling to the phosphatidylinositol 3'-kinase.* Endocrinology, 1993. **132**(4): p. 1421-30.
237. Jackson, J.G. and D. Yee, *IRS-1 expression and activation are not sufficient to activate downstream pathways and enable IGF-I growth response in estrogen receptor negative breast cancer cells.* Growth Horm IGF Res, 1999. **9**(5): p. 280-9.
238. Jackson, J.G., et al., *Regulation of breast cancer cell motility by insulin receptor substrate-2 (IRS-2) in metastatic variants of human breast cancer cell lines.* Oncogene, 2001. **20**(50): p. 7318-25.
239. Byron, S.A., et al., *Insulin receptor substrates mediate distinct biological responses to insulin-like growth factor receptor activation in breast cancer cells.* Br J Cancer, 2006. **95**(9): p. 1220-8.
240. Koda, M., et al., *Expression of insulin receptor substrate 1 in primary breast cancer and lymph node metastases.* J Clin Pathol, 2005. **58**(6): p. 645-9.
241. Sisci, D., et al., *Expression of nuclear insulin receptor substrate 1 (IRS-1) in breast cancer.* J Clin Pathol, 2006. **60**: p. 633-41.
242. Chen, J., et al., *Functional significance of type 1 insulin-like growth factor-mediated nuclear translocation of the insulin receptor substrate-1 and beta-catenin.* J Biol Chem, 2005. **280**(33): p. 29912-20.
243. Wu, A., J. Chen, and R. Baserga, *Nuclear insulin receptor substrate-1 activates promoters of cell cycle progression genes.* Oncogene, 2007.

244. Olson, A.L., A.R. Trumbly, and G.V. Gibson, *Insulin-mediated GLUT4 translocation is dependent on the microtubule network.* J Biol Chem, 2001. **276**(14): p. 10706-14.
245. Franke, T.F., et al., *Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate.* Science, 1997. **275**(5300): p. 665-8.
246. Rohatgi, R.A., et al., *Beclin 1 regulates growth factor receptor signaling in breast cancer.* Oncogene, 2015. **34**(42): p. 5352-62.
247. Sangai, T., et al., *Biomarkers of response to Akt inhibitor MK-2206 in breast cancer.* Clin Cancer Res, 2012. **18**(20): p. 5816-28.
248. Aapro, M. and J. Finek, *Oral vinorelbine in metastatic breast cancer: a review of current clinical trial results.* Cancer Treat Rev, 2012. **38**(2): p. 120-6.
249. Tishler, R.B., et al., *Microtubule-active drugs taxol, vinblastine, and nocodazole increase the levels of transcriptionally active p53.* Cancer Res, 1995. **55**(24): p. 6021-5.
250. Thomas, E.C., et al., *The subcellular fractionation properties and function of insulin receptor substrate-1 (IRS-1) are independent of cytoskeletal integrity.* Int J Biochem Cell Biol, 2006. **38**(10): p. 1686-99.
251. Myers, M.G., Jr., et al., *YMXM motifs and signaling by an insulin receptor substrate 1 molecule without tyrosine phosphorylation sites.* Mol Cell Biol, 1996. **16**(8): p. 4147-55.
252. Linseman, D.A., et al., *Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons.* J Neurosci, 2002. **22**(21): p. 9287-97.
253. Urbich, C., et al., *FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells.* FASEB J, 2005. **19**(8): p. 974-6.
254. De Bruyne, E., et al., *IGF-1 suppresses Bim expression in multiple myeloma via epigenetic and posttranslational mechanisms.* Blood, 2010. **115**(12): p. 2430-40.
255. Valverde, A.M., et al., *IRS-2 mediates the antiapoptotic effect of insulin in neonatal hepatocytes.* Hepatology, 2004. **40**(6): p. 1285-94.
256. Huang, C., et al., *Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in l6 myotubes.* J Biol Chem, 2005. **280**(19): p. 19426-35.
257. Gagnon, V., et al., *Akt and XIAP regulate the sensitivity of human uterine cancer cells to cisplatin, doxorubicin and taxol.* Apoptosis, 2008. **13**(2): p. 259-71.
258. Yang, S.X., et al., *Akt phosphorylation at Ser473 predicts benefit of paclitaxel chemotherapy in node-positive breast cancer.* J Clin Oncol, 2010. **28**(18): p. 2974-81.
259. Page, C., et al., *Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis.* Anticancer Res, 2000. **20**(1A): p. 407-16.

260. Reiss, K., et al., *Nuclear IRS-1 and cancer.* J Cell Physiol, 2012. **227**(8): p. 2992-3000.
261. Kellerer, M., et al., *Leptin activates PI-3 kinase in C2C12 myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) dependent pathways.* Diabetologia, 1997. **40**(11): p. 1358-62.
262. Johnston, J.A., et al., *Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases.* J Biol Chem, 1995. **270**(48): p. 28527-30.
263. de Melo Campos, P., et al., *IRS2 silencing increases apoptosis and potentiates the effects of ruxolitinib in JAK2V617F-positive myeloproliferative neoplasms.* Oncotarget, 2016. **7**(6): p. 6948-59.
264. Shaw, L.M., *Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the alpha6beta4 integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion.* Mol Cell Biol, 2001. **21**(15): p. 5082-93.
265. Chen, M., et al., *Integrin alpha6beta4 controls the expression of genes associated with cell motility, invasion, and metastasis, including S100A4/metastasin.* J Biol Chem, 2009. **284**(3): p. 1484-94.
266. Sun, X.J., et al., *Role of IRS-2 in insulin and cytokine signalling.* Nature, 1995. **377**(6545): p. 173-7.
267. Mercado-Matos, J., et al., *Differential involvement of the microtubule cytoskeleton in insulin receptor substrate 1 (IRS-1) and IRS-2 signaling to AKT determines the response to microtubule disruption in breast carcinoma cells.* J Biol Chem, 2017. **292**(19): p. 7806-7816.
268. Wittekind, C. and M. Neid, *Cancer invasion and metastasis.* Oncology, 2005. **69 Suppl 1**: p. 14-6.
269. van Zijl, F., G. Krupitza, and W. Mikulits, *Initial steps of metastasis: cell invasion and endothelial transmigration.* Mutat Res, 2011. **728**(1-2): p. 23-34.
270. Shiozawa, Y., et al., *Cancer stem cells and their role in metastasis.* Pharmacol Ther, 2013. **138**(2): p. 285-93.
271. Hou, X., et al., *Dual IGF-1R/InsR inhibitor BMS-754807 synergizes with hormonal agents in treatment of estrogen-dependent breast cancer.* Cancer Res, 2011. **71**(24): p. 7597-607.
272. Carboni, J.M., et al., *BMS-754807, a small molecule inhibitor of insulin-like growth factor-1R/IR.* Mol Cancer Ther, 2009. **8**(12): p. 3341-9.
273. Awasthi, N., et al., *BMS-754807, a small-molecule inhibitor of insulin-like growth factor-1 receptor/insulin receptor, enhances gemcitabine response in pancreatic cancer.* Mol Cancer Ther, 2012. **11**(12): p. 2644-53.
274. Mimeault, M. and S.K. Batra, *Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells.* J Cell Mol Med, 2013. **17**(1): p. 30-54.

275. Lisse, T.S., et al., *Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts.* J Bone Miner Res, 2013. **28**(6): p. 1478-88.
276. Forman-Kay, J.D. and T. Mittag, *From sequence and forces to structure, function, and evolution of intrinsically disordered proteins.* Structure, 2013. **21**(9): p. 1492-9.
277. Tanaka, S. and J.R. Wands, *Insulin receptor substrate 1 overexpression in human hepatocellular carcinoma cells prevents transforming growth factor beta1-induced apoptosis.* Cancer Res, 1996. **56**(15): p. 3391-4.
278. Chen, G., C. Deng, and Y.P. Li, *TGF-beta and BMP signaling in osteoblast differentiation and bone formation.* Int J Biol Sci, 2012. **8**(2): p. 272-88.
279. Krieger, J.R., et al., *Identification and selected reaction monitoring (SRM) quantification of endocytosis factors associated with Numb.* Mol Cell Proteomics, 2013. **12**(2): p. 499-514.
280. McGill, M.A., et al., *Numb regulates post-endocytic trafficking and degradation of Notch1.* J Biol Chem, 2009. **284**(39): p. 26427-38.
281. Nishimura, T. and K. Kaibuchi, *Numb controls integrin endocytosis for directional cell migration with aPKC and PAR-3.* Dev Cell, 2007. **13**(1): p. 15-28.
282. Sato, K., et al., *Numb controls E-cadherin endocytosis through p120 catenin with aPKC.* Mol Biol Cell, 2011. **22**(17): p. 3103-19.
283. Zhu, F., et al., *Numb contributes to renal fibrosis by promoting tubular epithelial cell cycle arrest at G2/M.* Oncotarget, 2016. **7**(18): p. 25604-19.
284. Irie, H.Y., et al., *Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition.* J Cell Biol, 2005. **171**(6): p. 1023-34.
285. Chau, N.M. and M. Ashcroft, *Akt2: a role in breast cancer metastasis.* Breast Cancer Res, 2004. **6**(1): p. 55-7.
286. Standen, C.L., et al., *Signal transduction cross talk mediated by Jun N-terminal kinase-interacting protein and insulin receptor substrate scaffold protein complexes.* Mol Cell Biol, 2009. **29**(17): p. 4831-40.
287. Verhey, K.J., et al., *Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules.* J Cell Biol, 2001. **152**(5): p. 959-70.
288. Geiger, J.C., et al., *The GRIP1/14-3-3 pathway coordinates cargo trafficking and dendrite development.* Dev Cell, 2014. **28**(4): p. 381-93.
289. Kosaki, A., et al., *14-3-3beta protein associates with insulin receptor substrate 1 and decreases insulin-stimulated phosphatidylinositol 3'-kinase activity in 3T3L1 adipocytes.* J Biol Chem, 1998. **273**(2): p. 940-4.
290. Ogihara, T., et al., *14-3-3 protein binds to insulin receptor substrate-1, one of the binding sites of which is in the phosphotyrosine binding domain.* J Biol Chem, 1997. **272**(40): p. 25267-74.
291. Cao, B. and C. Mao, *Identification of microtubule-binding domains on microtubule-associated proteins by major coat phage display technique.* Biomacromolecules, 2009. **10**(3): p. 555-64.

292. He, Z., et al., *Downregulation of miR-383 promotes glioma cell invasion by targeting insulin-like growth factor 1 receptor.* Med Oncol, 2013. **30**(2): p. 557.
293. Saikali, Z., et al., *Role of IGF-1/IGF-1R in regulation of invasion in DU145 prostate cancer cells.* Cancer Cell Int, 2008. **8**: p. 10.
294. Gregory, P.A., et al., *An autocrine TGF-beta/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition.* Mol Biol Cell, 2011. **22**(10): p. 1686-98.
295. Gregory, P.A., et al., *The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.* Nat Cell Biol, 2008. **10**(5): p. 593-601.
296. Iliopoulos, D., et al., *MicroRNAs differentially regulated by Akt isoforms control EMT and stem cell renewal in cancer cells.* Sci Signal, 2009. **2**(92): p. ra62.
297. Sorrell, F.J., et al., *Family-wide Structural Analysis of Human Numb-Associated Protein Kinases.* Structure, 2016. **24**(3): p. 401-11.
298. Karaczyn, A.A., et al., *Human NUMB6 Induces Epithelial-Mesenchymal Transition and Enhances Breast Cancer Cells Migration and Invasion.* J Cell Biochem, 2017. **118**(2): p. 237-251.
299. Johnson, S.A., D. Zitserman, and F. Roegiers, *Numb regulates the balance between Notch recycling and late-endosome targeting in Drosophila neural progenitor cells.* Mol Biol Cell, 2016. **27**(18): p. 2857-66.
300. Hwang, W.L. and M.H. Yang, *Numb is involved in the non-random segregation of subcellular vesicles in colorectal cancer stem cells.* Cell Cycle, 2016. **15**(20): p. 2697-703.
301. Jeganathan, N., et al., *Rac1-mediated cytoskeleton rearrangements induced by intersectin-1s deficiency promotes lung cancer cell proliferation, migration and metastasis.* Mol Cancer, 2016. **15**(1): p. 59.
302. MacGrath, S.M. and A.J. Koleske, *Cortactin in cell migration and cancer at a glance.* J Cell Sci, 2012. **125**(Pt 7): p. 1621-6.
303. Tian, C.Y., et al., *[Function of intersectin in endocytosis and exocytosis].* Sheng Li Xue Bao, 2012. **64**(4): p. 489-94.
304. Belleudi, F., et al., *Polarized endocytosis of the keratinocyte growth factor receptor in migrating cells: role of SRC-signaling and cortactin.* PLoS One, 2011. **6**(12): p. e29159.
305. Lee, C.E., et al., *Long-Term Consumption of Platycodi Radix Ameliorates Obesity and Insulin Resistance via the Activation of AMPK Pathways.* Evid Based Complement Alternat Med, 2012. **2012**: p. 759143.
306. Sung, J.Y. and H.C. Choi, *Metformin-induced AMP-activated protein kinase activation regulates phenylephrine-mediated contraction of rat aorta.* Biochem Biophys Res Commun, 2012. **421**(3): p. 599-604.
307. Chen, G., et al., *Metformin inhibits growth of thyroid carcinoma cells, suppresses self-renewal of derived cancer stem cells, and potentiates the*

effect of chemotherapeutic agents. J Clin Endocrinol Metab, 2012. **97**(4): p. E510-20.
308. Marion, D., *An introduction to biological NMR spectroscopy.* Mol Cell Proteomics, 2013. **12**(11): p. 3006-25.
309. Fernandez-Leiro, R. and S.H. Scheres, *Unravelling biological macromolecules with cryo-electron microscopy.* Nature, 2016. **537**(7620): p. 339-46.
310. Burtrum, D., et al., *A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo.* Cancer Res, 2003. **63**(24): p. 8912-21.
311. Scotlandi, K., et al., *Expression of insulin-like growth factor system components in Ewing's sarcoma and their association with survival.* Eur J Cancer, 2011. **47**(8): p. 1258-66.
312. Emens, L.A. and N.E. Davidson, *Trastuzumab in breast cancer.* Oncology (Williston Park), 2004. **18**(9): p. 1117-28; discussion 1131-2, 1137-8.
313. Ferguson, R.D., et al., *Hyperinsulinemia promotes metastasis to the lung in a mouse model of Her2-mediated breast cancer.* Endocr Relat Cancer, 2013. **20**(3): p. 391-401.
314. Rostoker, R., et al., *CD24 cell surface expression in Mvt1 mammary cancer cells serves as a biomarker for sensitivity to anti-IGF1R therapy.* Breast Cancer Res, 2016. **18**(1): p. 51.
315. Kuznetsova, A., et al., *Trimeprazine increases IRS2 in human islets and promotes pancreatic beta cell growth and function in mice.* JCI Insight, 2016. **1**(3).
316. Gerlinger, M., et al., *Intratumor heterogeneity and branched evolution revealed by multiregion sequencing.* N Engl J Med, 2012. **366**(10): p. 883-92.
317. Yeo, C.D., et al., *Expression of insulin-like growth factor 1 receptor (IGF-1R) predicts poor responses to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in non-small cell lung cancer patients harboring activating EGFR mutations.* Lung Cancer, 2015. **87**(3): p. 311-7.
318. Park, E., et al., *Membranous Insulin-like Growth Factor-1 Receptor (IGF1R) Expression Is Predictive of Poor Prognosis in Patients with Epidermal Growth Factor Receptor (EGFR)-Mutant Lung Adenocarcinoma.* J Pathol Transl Med, 2015. **49**(5): p. 382-8.
319. Lu, D., et al., *Simultaneous blockade of both the epidermal growth factor receptor and the insulin-like growth factor receptor signaling pathways in cancer cells with a fully human recombinant bispecific antibody.* J Biol Chem, 2004. **279**(4): p. 2856-65.