Spring 5-7-2016

# Role of AKT2 in Cell Survival, Establishment and/or Maintenance of Colorectal Cancer Metastasis

Ekta Agarwal
*University of Nebraska Medical Center*

# ROLE OF AKT2 IN CELL SURVIVAL , ESTABLISHMENT AND/OR MAINTENANCE OF COLORECTAL CANCER METASTASIS

By

**Ekta Agarwal**

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska Medical Center
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Biochemistry and Molecular Biology

**Under the supervision of Professor Michael G. Brattain**

University of Nebraska Medical Center
Omaha, Nebraska
April 2016

Supervisory Committee:

| | |
|---|---|
| **Kaustubh Datta, Ph.D.(Chair)** | **Sanjib Chowdhury, Ph.D.(Co-Chair)** |
| Jenny Wang, Ph.D. | Jennifer Black, Ph.D. |
| Joyce Solheim, Ph.D. | Punita Dhawan, Ph.D. |

**TABLE OF CONTENTS**

**LIST OF TABLES AND FIGURES**

**ABSTRACT**

**ACKNOWLEDGEMENT**

**TABLE OF CONTENTS**

# LIST OF FIGURES

**Chapter IV:**



**Chapter V:**

**ROLE OF AKT2 IN CELL SURVIVAL, ESTABLISHMENT AND/OR MAINTENANCE OF COLORECTAL CANCER METASTASIS**

**Ekta Agarwal, Ph.D.**

**University of Nebraska Medical Center, 2016**

**Advisor: Michael G. Brattain, Ph.D.**

There is extensive evidence for the role of aberrant cell survival signaling mechanisms in cancer progression and metastasis. Akt acts as a key signaling node that bridges oncogenic receptors to many essential pro-survival cellular functions, and is perhaps the most commonly activated signaling pathway in human cancer. Akt has three isoforms, Akt1, 2 and 3. Variable phenotypic differences are observed following the genetic inactivation and/or removal of the Akt isoforms, which suggests that the isoforms have distinct non-redundant functional characteristics despite sharing a high level of structural homology and similar mechanisms of activation.

The major goal of the work presented in this dissertation was to identify the role of Akt isoforms on cell survival, establishment and/or maintenance of metastasis in colorectal cancer (CRC). Although there is an increase in the 5-year survival rate of CRC during early stages, the progress in the survival rate during the metastatic stage is still dismal, suggesting the need to develop anti-metastatic therapy. The work presented in this dissertation has led to the identification of Akt2, among Akt isoforms, as a major player for establishment and/or maintenance of metastasis. shRNA-mediated knockdown of Akt2, not Akt1, causes reduction in metastatic burden in CRC. We show that loss of Akt2 upregulates Metastasis Suppressor 1 (MTSS1) and inhibits the expression of anti-apoptotic genes, XIAP and survivin thus inhibiting cell survival which in turn could lead to reduction in metastatic potential of the cells. It has been shown that activated Akt stabilizes XIAP by S87 phosphorylation, leading to survivin/XIAP complex formation, caspase inhibition and cytoprotection of cancer cells. Extensive drug

development efforts and clinical evaluations are underway targeting the aberrant cell survival properties associated with PI3K/Akt signaling in regulating cancer progression and metastasis. Inhibition of Akt activation by small molecule kinase inhibitors is an attractive candidate for targeting aberrant cell survival associated with malignant progression and metastasis and could be effective in the treatment of CRC. In this dissertation work, we have used a kinase inhibitor of Akt, MK-2206, to inhibit phosphorylation of Akt. We provide novel mechanistic insights on MK-2206-mediated cell death. MK-2206 showed an anti-tumorigenic role and led to a dual mechanism of cell death by inhibiting XIAP and survivin and by inducing Apoptosis Inducing Factor.

Additionally, strategies to inhibit Akt2 as opposed to the other two isoforms may provide a therapeutic approach for treatment of metastases. A negative correlation between Akt2 and MTSS1 in human primary colorectal cancer samples might be useful in identification of metastatic patients.

The work presented in this dissertation may assist in understanding whether loss of Akt2 could be a mechanism of increasing cell death, thus leading to reduction in metastasis. Additionally, this work provides a new paradigm for MK-2206-mediated control of aberrant cell survival associated with IGF1R-dependent CRC that may offer new targets for enhancing cell death in cancer cells.

## ACKNOWLEDGEMENTS

I would like to thank my PhD. advisor Dr. Michael G. Brattain for his mentorship, guidance and teaching during the period of my graduate studies. I greatly appreciate his encouragement to think independently. He gave me the freedom to explore scientific ideas and ensured I never lost focus of my main research goals. I thank him whole-heartedly for his advice, ideas and criticisms throughout the journey of my graduate program of 4 years.

I extend my special thanks to my Co-Advisor, Dr. Sanjib Chowdhury for all his guidance and encouragement. I thank him sincerely for his advice and discussions which helped me in completing my thesis work. He has been a great teacher, friend and colleague, who has extended his hand for help every time I needed.

My sincere thanks to my supervisory committee Chair Dr. Kaustubh Datta for his valuable advice in the past 4 years and for critical evaluation of my thesis.

I thank my supervisory committee members, Dr. Jennifer Black, Dr. Jenny Wang, Dr. Joyce Solheim and Dr. Punita Dhawan for their positive feedback and guidance. I would also like to thank my supervisory committee members for my comprehensive examination, Dr. Stanley Cox, Dr. P.Wang Cheng and Dr. David Klinkebiel.

I would like to acknowledge my lab members. My special thanks to Katie Bailey, with whom I have shared a very special relationship. I have shared my personal and professional things of life with her. My thanks to Dr. Catherine Murari, Premila Leiphrakpam, Caroline Robb, Brittany Poeleart and Shane Laschanzky for being excellent lab mates. I would like to thanks our lab manager Meghan Mendick for making all the resources available to me when ever needed.

I would like to thank everyone at the University of Nebraska Medical Center, especially the Department of Biochemistry and Molecular Biology for their support. I would like to thank everyone in the BMB office, Amy Dodson, Karen Hankins and Jeanette. I would like to also thank Colby, office associate at the Eppley Institute. I would like to thank Graduate Studies at UNMC for providing funding support throughout my graduate program.

I would extend my thanks to Dr. Anathbandu Chaudhuri and Dr. Xiaolin for patiently teaching me all the animal surgeries.

Last but not the least I thank my family, friends and relatives for their moral support during the tough period of graduate studies.

# CHAPTER I

## Introduction

### 1.1 Colorectal cancer and current therapies

Colorectal cancer (CRC) is the third most common type of cancer and the second leading cause of cancer related deaths in United States. As per the reports of National Cancer Institute (NCI) it has been estimated that about 134,490 (70,820 men and 63,670 women) new cases of colorectal cancer will be diagnosed in 2016. This accounts for about 8% of all new cancer cases that will be reported in the year 2016. The number of new cases of CRC is 42.4 per 100,000 men and women per year. It is expected that there will be about 49,190 CRC related deaths this year, which accounts for 8.4% of all the cancer related deaths and 15.5 per 100,000 men and women per year. As per the reports of Surveillance Epidemiology and End Results (SEERS) the median age at the time of diagnosis is estimated to be 68 years. The relative survival of cancer patients compared to general patients shows a 5-year survival of about 64.9%.

Reports from SEERS show an inverse relationship between stage distribution of CRC and its corresponding 5-year survival rate. A localized case of CRC where the tumor is confined to the primary site has a stage distribution of 39% and the corresponding 5-year survival rate is 90.1%. Regional CRC stage, when the tumor spreads to regional lymph nodes has a stage distribution of 36% and 5- year survival rate of 70.8%. Metastatic cases of CRC, where the tumor has spread to distant sites as liver and lung has stage distribution of 20% and the 5-year survival rate drastically reduces to 13%. It is known that the lifetime risk for CRC is 51.2%. Thus, it becomes evident that 1 out of 20 individuals would be diagnosed with CRC in their lifetime. Race and ethnicity are two additional factors that affect CRC incidence and mortality. NCI has reported that the mortality rates amongst whites and African Americans are much higher than all other people of different races and ethnicity living in United States.

The etiological and pathogenetic mechanisms responsible for CRC development are complex and heterogeneous. Dietary and life style risk factors in addition to inherited and somatic

mutations also contribute to development of CRC. A diet rich in unsaturated fats and red meat, excess alcohol consumption and reduced physical activity are some of the lifestyle risk factors for CRC (Potter 1999, Slattery 2000, Hawk and Levin 2005). Commonly colon cancer begins with formation of polyp in the inner lining of colon or rectum. Figure 1.1 shows the stages of progression of colon cancer. Over time this polyp grows into a tumor but it still remains confined to the inner wall in Stage 0 of CRC. This stage is completely curable and surgical removal of the polyp is the standard therapy. In Stage 1 the tumor grows to outer layer of the colon wall and invades the submucosa; however surgery is the standard therapy in this stage also. It is known that there is a high 5 year survival rate both stages 0 and 1 of CRC. In Stage 2 tumor grows out of the wall but does not spread to the lymph nodes. Surgical resection of the colon is the common therapy for this stage; however some recent studies are focusing on adjuvant chemotherapy in this stage of colon cancer, although it is still controversial as it depends on the condition of the patient. Some reports have shown that 5 Fluorouracil (5FU) based chemotherapy causes a better overall survival of the patient as compared to surgery alone. Stage 3 is evident by presence of tumor cells in the lymph nodes. Surgery and Adjuvant chemotherapy is the standard treatment in this stage. During this stage the 5 year survival rate is 70%. Stage 4 is characterized by presence of cancer cells at distant sites such as liver and lung. This stage denotes distinct metastasis. The standard treatment options for this stage are surgery (surgical ablation of liver and lung tumors), chemotherapy and /or targeted therapy.

There are several screening tools to detect CRC as fecal occult blood test (FOBT), sigmoidoscopy, colonoscopy and digital rectal examination. Despite all these advancements about 20% CRC cases are diagnosed at stage 4 i.e. when the tumor has already undergone metastasis. There are only 10 active and approved drugs for metastatic colon cancer patients as per the reports of NCI. These drugs can be used alone or in combination with other drugs. Most commonly used drugs are 5-FU, Oxaliplatin, Bevacizumab, FOLFOXIRI (a combination of

Fluorouracil, Leuvocorin, Oxaliplatin and Irinotecan) and Cetuximab. It has been reported that combination of these drugs has improved the survival rate of patients with stage 3 CRC. Small molecule inhibitors and monoclonal antibodies targeted against specific molecular pathways are also being used in combination with traditional treatments. Bevacizumab, which targets the angiogenic signaling and has been approved for the treatment of CRC. It has been shown to increase the 5-year survival rate. Although there has been increase in the number of drugs to treat CRC, we still need to identify more molecular pathways, so that small molecule inhibitors can be designed against them, that can then be used to treat CRC.

Figure 1.2 shows the model for colon cancer progression, which depicts a loss in the tumor suppressor and a gain in oncogenes leading to progression from stage 1 to stage 4 of CRC, however not much is known about the signaling pathways leading to distant metastasis. In this dissertation, we have identified one such molecular pathway, which can be targeted to inhibit maintenance of metastasis in CRC.

**1.2 Metastasis**

Metastasis is considered as a complex process, that occurs through a series of steps, which includes invasion, intravasation, transport through the circulatory system, arrest at secondary site, extravasation and growth in secondary organs (Mehlen and Puisieux 2006). The temporal course of metastasis varies in different cancer conditions, thus making it more difficult to diagnose cancer at early stages. It is believed that several genetic and epigenetic alterations help the cells to undergo metastasis. Cancer cells become addicted to certain processes as unlimited proliferative potential, tolerating cell division defects, maintaining progenitor like phenotypes thus leading to formation of oncogenically transformed cells (Hanahan and Weinberg 2000). Cancer cell metastasis progress through multiple steps as shown in Figure 1.4.

Metastasis is the end product of an evolutionary process, in which interactions between cancer cells and their microenvironment lead to alterations in the behavior of the cells (Chiang and Massague 2008). We know that surgical resection in addition to therapy can cure primary tumors, however it is difficult to target metastasis because of its systemic nature and increased resistance to therapeutic agents. Metastases is responsible for 90% of cancer deaths, thus it is important to understand the molecular pathways involved in metastasis to develop effective, targeted approaches to prevent and treat cancer metastasis. It is known that a very small population of cells from the primary tumor succeeds in infiltrating, surviving and ultimately overtaking a distant organ (Fidler 2003) thus making a metastasis a highly inefficient process as shown in Figure 1.3.

There are different classes of genes that play an important role in metastasis. They have been discussed as follows:

1. **Metastasis initiation genes:** These genes could be present already in the primary tumor. The metastasis initiating genes help these transformed cells to invade the surrounding tissue and attract a supportive environment thus facilitating the dispersion of the cancer cells. These genes promote cell motility, epithelial to mesenchymal transition (EMT), extracellular matrix degradation, angiogenesis and evasion from immune system (Yang and Weinberg 2008). EMT markers Kiss-1 are some of the metastasis initiation genes (Nguyen, Bos et al. 2009)
2. **Metastasis progression genes:** These are also a part of both primary tumor and metastasis, however unlike metastasis initiation genes, they play different role in primary tumor as they support tumor growth through one effect, whereas they enhance metastasis through other effect. MMP-1, COX-2, EGFR are some of the metastasis progression genes (Nguyen, Bos et al. 2009).

3. **Metastasis virulence genes:** These genes are required for metastatic colonization in a specific organ. These genes increase the metastasis ability of the disseminated cancer cells that have achieved the stages of initiation and progression. It has been reported that these genes can lead to increase proliferation and survival solely during colonization at metastatic sites and would not be represented in the primary tumor. IL-11, TNK alpha, IL-16 are metastasis virulence genes (Nguyen, Bos et al. 2009). The different steps of metastasis are as follows:

1. **Local invasion:** Cancer cells invade the surrounding tissue and attract a supportive stroma thus facilitating the dispersion of cancer cells. Recent studies show that well maintained architecture of normal epithelium tends to be a barrier for invasion. The metastatic cells have to overcome this barrier so that they can develop evident malignancies (Hu, Yao et al. 2008). There are different ways of undergoing local invasion by cancer cells: collective invasion is the most common mechanism where cancer cells invade as multicellular units. Alternatively they can invade as individual cells via an integrin dependent or an integrin-independent (Rho/ROCK dependent) mechanism. The metastasis initiating genes play an important role in this step of metastasis as they promote cell motility, EMT and extracellular matrix degradation, so that the cancer cells can infiltrate the distant tissues (Yang and Weinberg 2008). EMT leads to breakdown of tight junctions leading to loss in polarity of cell and dissociation of epithelial cell sheets leading to increased invasion of cells (Thiery, Acloque et al. 2009). Once the cancer cells have crossed the basement membrane they encounter a variety of tumor associated stromal cells which further augments the aggressiveness of the cells (Joyce and Pollard 2009), thus the stroma helps the direct entry of the tumor cells into the circulation so that they propagate to distant sites.

2. **Intravasation:** In this step of metastasis, the cancer cells invade and move through the walls of the nearby blood vessels. This step is facilitated by molecular changes that promote the ability of cancer cells to cross the endothelial cells of the blood vessels (Gupta and Massague 2006). Cancer cells show increased cell survival during this step thus suggesting that entry of cells into the vasculature can be an important rate-limiting step of metastasis (Wyckoff, Jones et al. 2000). During this stage, vascular endothelial growth factors lead to formation of new blood vessels. This neovasculature is leaky and is very dynamic. The weak interaction between the endothelial cells and the microvasculature enables the cells to undergo intravasation (Carmeliet and Jain 2011).
3. **Circulation:** In this stage the cancer cells move through the lymphatic system and blood stream, get arrested in the capillary bed and proliferate to reach the distant sites. The cells during this stage are referred as Circulation Tumor Cells (CTCs). The CTCs represent the cells that are between primary tumor and sites of dissemination and are also known as metastatic intermediates (Nagrath, Sequist et al. 2007). The lifespan of CTCs varies with type of cancer, but overall they spend a very small time in the circulation and get embedded in the capillaries (Meng, Tripathy et al. 2004). During this stage the cancer cells have to pass through various types of stressful and selective steps as matrix detachment, shear forces and resistance from immune system (Joyce and Pollard 2009). Additionally, some recent reports show that CTCs are predetermined to enter certain tissues. In case of colorectal cancer, the entry of cells in the liver initiates a proinflammatory response that triggers the Kupffer cells to release certain molecules that activate the vascular adhesion receptors, thus leading to increased adhesion of CTC in the liver (Auguste, Fallavollita et al. 2007).
4. **Extravasation:** When the CTCs reach distant organs they rupture the walls of the

surrounding vessels thus placing the tumor in direct contact with the tissue. Normally this step is considered to be a reverse of intravasation.

5. **Proliferation and Angiogenesis:** After extravasation, the cancer cells multiply at distant sites of the body and form micrometastases, which then lead to growth of new blood vessels. The formation of new blood vessels helps to supply oxygen and nutrients which are necessary for continuous tumor growth. During this step, the cancer cells adjust to the unfavorable foreign microenvironment by establishing a "pre-metastatic niche" (Psaila and Lyden 2009). This mechanism helps the cancer cells to evade the stress significantly but not completely. The proliferation potential of the cells is still very low and there is a fine balance between proliferation and apoptosis, thus leading to attrition of metastatic colonization (Chambers, Groom et al. 2002). The metastatic colonization is highly dependent on the ability of the cells to undergo neoangiogenesis and self-renewal. Thus overall these steps are affected by various signaling pathways (Valastyan and Weinberg 2011).

Thus various genetic and epigenetic alterations help the carcinoma cells to complete these complex, biological steps that lead to formation of metastases. The mechanisms that help the cancer cells to survive after they reach distant organs and after being exposed to foreign microenvironments are not well known. Cell survival at this stage is considered as a rate-limiting step for the establishment of metastasis (Wong, Lee et al. 2001). A better understanding of the survival mechanisms is required to develop therapeutic strategies to eliminate metastasis after primary tumor has been removed.

### 1.3 Cell survival regulators

Apoptosis or 'programmed cell death' has been extensively studied over last few years. It is known that apoptosis is a barrier to metastasis. During the process of metastasis the cells have to pass through various stresses as loss of adhesion: leading to detachment from extracellular matrix,

nutrient deprivation and hypoxia, all of which can lead to apoptosis. Cell death by the process of apoptosis involves the activation of cysteine proteases called caspases (Salvesen and Dixit 1997). Caspases have been divided into: initiator caspases and effector caspases. Effector caspases are cleaved and activated by initiator caspases thus leading to activation of apoptosis. There are two main pathways for apoptosis: Extrinsic pathway (Death receptor pathway) and Intrinsic pathway (Mitochondrial-mediated pathway) (Zimmermann and Green 2001).

In the extrinsic pathway, activation of CD95 receptor or tumor- necrosis factor receptor (TNF) recruits the death-inducing signaling complex (DISC), which then activates initiator caspase: caspase-8. Cleavage of downstream effector caspases by caspase 8 leads to cell death.

The intrinsic pathway involves the release of proteins from the mitochondria such as cytochrome c to induce cell death. Cytochrome c stimulates the formation of another caspase activating complex, apoptosome, which then activates the initiator caspase-9 leading to activation of downstream signaling pathway resulting in apoptosis. The BCL2 family members include both pro-apoptotic (BAX and BH3 only proteins) and anti-apoptotic (BCL2 and BCL-$X_L$) proteins which play an important role in the release of cytochrome c. Activation of p53 (tumor suppressor) also lead to increase in the transcription of BCL2 family member proteins, leading to release of cytochrome c. The two pathways can function independently but there can be a cross talk between the two pathways in certain conditions. Activation of caspase-8 via the extrinsic pathway sometimes fails to activate effector caspases but can efficiently cleave BID (BH3 interacting domain death agonist), which then activates the intrinsic pathway of apoptosis. In addition to BCL-2 and Caspases, Inhibitor of Apoptosis proteins (IAPs) also contributes to apoptosis (Srinivasula and Ashwell 2008).

### 1.3.1 Inhibitor of Apoptosis Proteins (IAPs)

Originally IAPs were identified in viruses as cellular proteins with a Baculoviral IAP Repeat (BIR) motif, a sequence of 70 amino acids that coordinates a zinc ion with histidine and cysteine residues (Srinivasula and Ashwell 2008). They were found to be involved in suppressing the host cell death response to viral infection (Deveraux and Reed 1999). Additionally, they also participate in mitotic chromosome segregation, cellular morphogenesis, copper homeostasis and intracellular signaling. IAP proteins are implicated in multiple ways in cell death regulation, ranging from inhibition of apoptosis and necrosis to the regulation of cell cycle and inflammation.

**Structural features of IAPs**

The BIR domain is the basic structural feature of IAPs. The IAP molecules may have one or multiple BIR domains at the N terminus. On the basis of presence of BIR domains, the IAP family has 8 members as shown in Figure 1.5. The C terminus of IAPs has the RING domain and the CARD domain (Caspase-associated recruitment domain). The Ring domain regulates cell death and functions as an E3 ubiquitin ligase. The anti-apoptotic role of the CARD domain has not been well studied, although it is known that IAPs with BIR domains are capable of blocking apoptosis. This implies that CARD domain is not essentially required for IAP function (Roy, Deveraux et al. 1997).

### 1.3.2 X linked Inhibitor of Protein (XIAP)

The XIAP member of IAP family is very well characterized. The gene encoding XIAP has a molecular weight of 57kDa. It is present on chromosome Xq24-25. Biochemical and structural analyses have revealed that XIAP has 3 BIR domains (Eckelman, Salvesen et al. 2006). These domains are 40% homologous to each other structurally but have striking differences in their functions. It is known that the BIR1 domain inhibits caspase 3 and 7, whereas the BIR 3 domain inhibits caspase 9 (Deveraux, Leo et al. 1999). BIR 1 and 2 domains have a conserved sequence known as the IAP binding motif (IBM) interacting domain, whereas this domain is absent in the

BIR 1 domain (Scott, Denault et al. 2005). XIAP is known to be the only IAP, that can inhibit caspase activity by direct physical interaction. On induction of apoptosis, Smac (also known as DIABLO) is released from the mitochondria and interacts with the BIR domains of XIAP to inhibit caspase inhibition (Chai, Shiozaki et al. 2001). The RING domain of XIAP has E3 ubiquitin ligase activity, which is responsible for recognizing the substrates that are ubiquitinated by XIAP (Obexer and Ausserlechner 2014). The RING domain also controls the stability of XIAP and induces proteasomal degradation of bound proteins (Vaux and Silke 2005). The overall mechanism of inhibition of cell death by XIAP is shown in Figure 1.6.

In addition to the above-mentioned function, XIAP also plays an important role in NF-kappa B and MAP kinase activation during TGF beta and BMP receptor signaling (Birkey Reffey, Wurthner et al. 2001). XIAP acts as a cofactor and bridges the TGF beta and BMP type 1 receptors to TAK1. TAK1, a MAP3K, activates the MAPK and NF-kappa B signaling (Brown, Vial et al. 2005). Additionally, XIAP also regulates the Jun N- terminal kinase (JNK), Myc and PI3K/Akt pathways. The activation of these pathways by XIAP is mediated via TNF (Karin and Gallagher 2009). This signaling pathway is essential for diverse developmental processes, regulation of apoptosis, and vascular development (Jadrich, O'Connor et al. 2003).

XIAP is differentially upregulated in various cancer conditions such as breast cancer, melanoma, clear-cell renal carcinoma, and colon cancer and it confers resistance to chemotherapy-induced cell death (Wilkinson, Cepero et al. 2004, Berezovskaya, Schimmer et al. 2005), however it has been also shown that there is a downregulation of XIAP on treatment with chemotherapy, as it undergoes autoubiquitination in response to DNA damage caused by chemotherapeutic agents (Deveraux and Reed 1999, Cheng, Jiang et al. 2002). Treatment with XIAP siRNA in combination with chemotherapy enhances chemosensitivity, thus suggesting that XIAP can be considered as a target for anticancer therapy (Andersen, Becker et al. 2005). Recent studies show that phosphorylation of XIAP by Akt regulates its ubiquitination and degradation.

Akt phosphorylates XIAP at Serine 87, thus stabilizing it by inhibiting its degradation, leading to inhibition of cell death (Dan, Sun et al. 2004).

Increased expression of XIAP has been associated with cancer progression (Fong, Liston et al. 2000, Gordon, Mani et al. 2007).The biological significance of increased expression of XIAP in cancer invasion and metastasis is still unknown. In hepatocellular carcinoma, loss of XIAP inhibits metastasis (Shi, Ding et al. 2008). Similarly, in colon cancer it has been reported that knockdown of XIAP inhibits invasion of cancer cells. XIAP shows its metastatic role as it affects cell motility via regulation of beta-actin polymerization and cytoskeleton formation. XIAP inhibits the biological effect of RhoGDI in regulation of Arp2/3 recruitment and beta actin polymerization (Liu, Zhang et al. 2011). Thus XIAP increases metastasis by inhibiting cell survival and increasing motility of cancer cells. Thus targeting XIAP can be a lead target to inhibit both survival and metastasis of cancer cells.

### 1.3.3 Survivin

Survivin is the smallest of the known IAP family proteins, as it contains a single BIR domain. It has a molecular weight of 16 kDa and is present on chromosome 17q25. Survivin prevents apoptosis by binding to caspases and inhibiting them. This IAP protein family member has been implicated both in cell survival and regulation of mitosis in cancer (Ambrosini, Adida et al. 1997, Giodini, Kallio et al. 2002).

Survivin is highly expressed in multiple cancer types skin, pancreatic, colon cancer and lymphoma; however it has a very restricted expression in normal tissues (Ambrosini, Adida et al. 1997). Loss of survivin leads to an increase in apoptosis and increased sensitization to anti-cancer drugs in cancer cells, thus suggesting that survivin may be required for cell survival and chemoresistance in various cancer conditions (Nakahara, Kita et al. 2007) and making survivin a target for tumor diagnosis, prognosis and therapy. The mechanism by which survivin inhbits

apoptosis is still not clearly known; however, some recent studies have delineated the mechanism of cell death inhibition by survivin. According to one study, it has been demonstrated that, in response to cell death, survivin physically associates with XIAP; this complex of XIAP and survivin increases the stability of XIAP thus leading to inhibition of caspase activity. It has been shown that survivin binds to the RING domain of XIAP and inhibits its polyubiquitination and proteosomal degradation. Increased stability of IAPs increase cell viability, as the stabilized survivin-XIAP complex suppresses caspase 9 activity, thus blocking apoptosis. Furthermore, survivin can sequester SMAC/DIABLO (mitochondrial activator of caspases), thus preventing inhibition of XIAP (Sun, Nettesheim et al. 2005) as shown in Figure 1.7.

Survivin has also been known to play a role in mitosis. It is known to affect the assembly/stability of metaphase and anaphase microtubules and spindle check point function (Giodini, Kallio et al. 2002). The chromosome passenger complex (CPC) is a key regulator of mitosis and survivin is one of the members of this complex, which includes Borealin, INCENP and Aurora B kinase (Jeyaprakash, Basquin et al. 2011). The expression of survivin varies in different stages of cell cycle, with highest expression during G2/M phase and declining rapidly in G1 phase (Li, Ambrosini et al. 1998). It has been shown that survivin-depleted cells can exit mitosis prior to segregation of sister chromatids.

Recently, it has been determined that survivin undergoes phosphorylation at serine and threonine sites. Threonine phosphorylation of survivin inhibits its mitotic functions and increases its cytoprotective role (Barrett, Osborne et al. 2009), whereas phosphorylation at serine is required for alignment of chromosomes during mitosis (Colnaghi and Wheatley 2010). A phosphomimetic mutant of survivin (T34A) is not able to bind to and inhibit caspases, thus leading to an increase in cell death (O'Connor, Grossman et al. 2000). In addition to caspase-dependent cell death, loss of survivin is also responsible for caspase-independent cell death. Knockdown of survivin induces the translocation of AIF (Apoptosis Inducing factor) from the

cytoplasm to the nucleus, leading to caspase-independent cell death (Pavlyukov, Antipova et al. 2011), whereas overexpression of survivin prevents the release of AIF thus leading to increased cell survival (Okuya, Kurosawa et al. 2009) as depicted in Figure 1.8. Identification of survivin signaling and its novel targets can lead to a better understanding of its role in cancer and its potential as an important cancer drug target.

### 1.3.4 Apoptosis Inducing Factor (AIF)

The AIF gene is mapped to chromosome Xq25-26 region in humans. It is translated to a 67 kDa precursor molecule which then undergoes folding to become mature AIF (Susin, Lorenzo et al. 1999). It is a flavin adenine dinucleotide-containing, NADH-dependent oxidoreductase, that resides in the mitochondrial intermembrane space. The enzymatic activity of AIF is still unknown. AIF has three domains: an N-terminal mitochondrial localization sequence (MLS), a spacer of 27 amino acids, and a Carboxy-terminus with an oxidoreductase domain including the nuclear localization sequence (NLS) as shown in Figure 1.9 (Modjtahedi, Giordanetto et al. 2006).

On induction of apoptosis, AIF undergoes proteolysis and translocates to the nucleus where it triggers chromatin condensation and large scale DNA degradation in a caspase independent manner (Susin, Lorenzo et al. 1999) as shown in Figure 1.10. Presence of AIF in the cytosol triggers the release of more AIF from the mitochondria, forming a positive feedback loop thus leading to increased apoptosis (Cai, Yang et al. 1998). The release of AIF from the mitochondria is also guarded by anti-apoptotic Bcl-2 family protein (Daugas, Susin et al. 2000). These anti-apoptotic proteins reduce the permeabilization of the outer mitochondrial membrane thus preventing the release of cytochrome c and AIF and inhibiting both caspsase-dependent and caspase-independent cell death (Cande, Cecconi et al. 2002). Additionally, PAR polymer, the major product of PARP -1 activation, is a pro-death signaling molecule that causes release of AIF from the mitochondria, leading to nuclear condensation and cell death (Yu, Andrabi et al. 2006).

In breast (Ostrakhovitch and Cherian 2005), lung (Kuhar, Sen et al. 2006), colon (Huerta, Harris et al. 2003), and prostate cancer (Fu, Zhang et al. 2006), it has been observed that treatment with chemotherapeutic agents induces apoptosis by increasing the depolarization of the mitochondrial membrane, leading to increased release of AIF (Lee, Jeong et al. 2006). Previous studies have shown that AIF plays a role in chemosensitization of cells resistant to cytotoxic induced apoptosis. Overexpression of AIF increases the sensitivity to chemotherapy, thus leading to chemotherapeutic drug-induced cell death (Fu, Qiu et al. 2006). All these studies indicate that AIF can be a prognostic marker and a target for chemotherapeutic intervention and an important molecule for increasing the chemosensitization in various cancer types (Millan and Huerta 2009).

### 1.3.5 Ezrin

Ezrin is a member of the ERM (Ezrin, Radixin, Moesin) protein family (Bretscher, Edwards et al. 2002). These proteins share 70% sequence homology and have a common structure (Turunen, Wahlstrom et al. 1994). This structure consists of an NH2- terminal domain, an alpha-helical domain and an actin-binding COOH-terminal domain (Gould, Bretscher et al. 1989). In native state, the carboxyl domain and the amino-terminal domains self-associate, thus masking the F actin-binding domain (Pestonjamasp, Amieva et al. 1995). Ezrin is expressed in normal tissues and has been shown to play an important role in embryogenesis (Dard, Louvet-Vallee et al. 2004, Polesello and Payre 2004). In normal cells, Ezrin has diverse roles in villar organization in the gut, maintenance of photoreceptors, control of cortical stiffening during mitosis, and regulation of RhoA activity in epithelial cells (Saotome, Curto et al. 2004, Chorna-Ornan, Tzarfaty et al. 2005, Kunda, Pelling et al. 2008). ERM knockout mice can survive only for 21 days after birth (Kivela, Jaaskelainen et al. 2000).

Ezrin links F-actin to the cell membrane on phosphorylation, thus acting as a linker between plasma membrane and cytoskeleton (Sato, Funayama et al. 1992). In addition to F actin, it can also bind to CD44, phosphotidyl inositol 4,5 bisphosphate and intercellular adhesion

molecules (ICAMs), and the scaffolding protein ERM binding phosphoprotein 50 (EBP50) (Pakkanen 1988, Tsukita, Oishi et al. 1994, Heiska, Alfthan et al. 1998). Interaction of ezrin with ICAMs, suggest that it also plays a role in immune responses as ICAMs are responsible for initiating immune responses when stimulated with inflammatory cytokines (Fawcett, Holness et al. 1992, Vazeux, Hoffman et al. 1992). CD44 plays a role in migration of invasive tumors by inducing intracellular signaling, and interaction of ezrin with CD44 suggests that ezrin might be implicated in metastasis (Ponta, Sherman et al. 2003). Phosphorylation of the C terminus of ERM is required for their activation. Phosphorylation at threonine sites leads to an "open/active" conformation so that they can directly affect the cells (Geller and Gorlick 2011). In osteosarcoma, it has been reported that high expression of phosphor-ezrin is observed only at the invasive front of large metastatic lesions (Ren, Hong et al. 2011).

Ezrin is expressed on the cell surface to maintain the polarity of endothelial cells. It is involved in cell-cell and cell-matrix interactions and thus plays an important role in tumorigenesis, development, invasion, and metastasis (Chen, Wang et al. 2011). Recent reports show that ezrin also participates in cell survival, proliferation, and migration processes. It is necessary for some signaling pathways that play an important role in metastasis, such as MAPK, Akt, Rho kinase, and CD44 (Chen, Wang et al. 2011). Active Rho A can recruit ROCK family members that phosphorylate cytoskeletal proteins such as members of ERM family, thus leading to increased membrane-cytoskeletal interaction (Bretscher, Edwards et al. 2002). In addition to coordinating-metastasis associated signaling events, ezrin also plays a role in cell adhesion and cell-cell communication (Pujuguet, Del Maestro et al. 2003). Ezrin mediated formation of cell-cell adhesion leads to increase in cell survival and helps to pass growth signals between the surrounding tissue and invading metastatic cells (Tsukita, Oishi et al. 1994). Ezrin leads to increased metastasis of cancer cells by various mechanisms, as it inhibits anoikis during

migration of cells (Bretscher, Edwards et al. 2002), or it sends sustenance signals to the cells when they enter in the harsh environment of the metastatic sites (Tsai, Mendoza et al. 2007).

Previous work from our lab has shown that there is an increase in the expression of phosphor-ezrin (T567) in liver metastasis in vivo as well as in human colorectal cancer patients. It has been reported that expression of phosphor-ezrin may be regulated by the IGF-1R signaling pathway and thus activation of this pathway may lead to increase in cell survival by modulating XIAP and survivin (Leiphrakpam, Rajput et al. 2014). Additionally, increased expression of phospho ezrin in the metastatic lesions of various cancers such as pediatric sarcoma (Khanna, Wan et al. 2004), breast (Li, Wu et al. 2008), colon (Leiphrakpam, Rajput et al. 2014) and ovarian cancer, brain tumors, and soft tissue sarcoma (Song, Fadiel et al. 2005) suggests that it can be a potential biomarker as well as a lead target for anti-metastatic therapy.

**1.4 Metastasis Suppressor Gene 1 (MTSS1)**

MTSS1, also known as MIM (missing in metastasis), has been identified as a tumor suppressor in various cancer conditions (Lee, Macoska et al. 2002, Ma, Guan et al. 2007). Figure 1.11 depicts the domain structure of MTSS1. The N terminal portion of MIM contains an IMD (IRSp53 MIM domain) that can bind to actin filaments and interact with the GTPase Rac. MIM overexpression induces Rac activation. MIM interacts with PTP (Protein Tyrosine Phosphatase) delta near its proline-rich region, thus regulating tyrosine phosphorylation-dependent signaling. MTSS1 regulates the activity of Rac1 during cell-cell junction formation, thus localizing Rac1 GTP to these sites and strengthening cell –cell contacts, leading to reduced cell dissociation. It acts as a scaffold protein, which binds with actin binding protein thus regulating lamellipodia formation (Lin, Liu et al. 2005). It has been shown that MTSS1 affects cytoskeleton and actin filament organization (Mattila, Salminen et al. 2003). It induces actin-rich protrusions resembling micro spikes and lamelliopodia at the plasma membrane and promotes assembly of actin fibers. The actin cytoskeleton plays a key role in regulating essential cellular processes such as cell

migration. Additionally, MTSS1 enhances Arp2/3-mediated actin polymerization by interacting with Cortactin. Overexpression of MTSS1 leads to disassembly of actin based cytoskeleton organization characterized by an increase in lamellipodia and filopodia plasma membrane protrusion and actin depolymerization whereas MTSS1 depletion disrupts the actin cytoskeleton thus increasing the ability of cells to disaggregate. Thus, overall, MTSS1 increases the strength of cell-cell junction by blocking EMT (22479308).

MTSS1 is associated with higher motility and invasiveness of cancer cells, thus resulting in faster infiltrative tumor growth and metastatic behavior (Loberg, Neeley et al. 2005). It is considered as a co-transcription factor which binds to GLI proteins and increases their transcription, thus leading to an increase in tumor cell growth and proliferation (Gonzalez-Quevedo, Shoffer et al. 2005). It appears that MTSS1 is first overexpressed to promote carcinogenesis and then with ongoing tumor progression its downregulation results in more aggressive biological behavior (Kayser, Csanadi et al. 2015). MTSS1 overexpression results in reduction in tumor cell migration, invasion, and growth, and an increase in cell adhesion. It is believed that it also maintains the polarity of the cell by affecting cell adhesion (Mattila, Salminen et al. 2003) thus contributing to the maintenance of the integrity of epithelial tissues. MTSS1-deficient mice have defect in intercellular junctions of epithelial cells (Saarikangas, Mattila et al. 2011).

It has been shown that patients expressing high levels of MTSS1 have a better prognosis (Parr and Jiang 2009), thus, indicating that MTSS1 is a survival indicator. Patients with oesophageal squamous cell carcinoma show high expression of MTSS1 and have a favorable prognosis in comparison to patients with low expression of MTSS1. In breast cancer, it has been observed that MTSS1 overexpression suppresses invasion, migration, and adherence, and governs metastasis (Parr and Jiang 2009).

There are 3 p63 binding sites on the promoter of MTSS1, as determined by CHIP-Seq assay (Giacobbe, Compagnone et al. 2015). It was observed that MTSS1 and delta NP63 are positively correlated and are negative prognostic factor in human tumors. Breast cancer patients with positive correlation show a decrease of overall survival of the patients as compared to those with no correlation between p63 and MTSS1, indicating that p63 and MTSS1 axis is a negative prognostic factor on patient survival and is important for tumor regression. Delta NP63 is associated with inhibition of migration and invasion of tumor cells but some reports indicate that it can be an oncogene and pro-invasive factor in breast cancer (Giacobbe, Compagnone et al. 2015).

The expression of MTSS1 is reduced in bladder, breast, and prostate cancer. This metastasis suppressor gene can be targeted by methylation and ubiquitination by DNMT3A (Yan, Yao et al. 2015) and beta-TRCP (Zhong, Shaik et al. 2013), respectively, which then leads to reduction in the expression of MTSS1. Additionally, it has been also shown that it can also be targeted by microRNAs (Guo, Ren et al. 2015). All these studies suggest that MTSS1 can be an important marker for metastasis, although further studies are required to confirm it as a metastasis suppressor.

**1.5 Cortactin (CTTN)**

Cortactin is a cytoskeletal protein that interacts with F-actin, Arp2/3 complex and with other cytoskeletal proteins. It is composed of 3 major domains as shown in figure 1.12 (A): N terminal acidic (NTA) region that is responsible for binding and activation of Arp2/3 complex, a central tandem repeat of 37 amino acid sequence, and a C terminal domain which interacts with N-WASP and MTSS1 (Shvetsov, Berkane et al. 2009). The CTTN gene is present on chromosome 11, which normally shows altered expression in most cancers. It is ubiquitiously expressed in most hematopoietic cells (van Rossum, Schuuring-Scholtes et al. 2005). Its undergoes post translational modifications such as alternative splicing, phosphorylation and acetylation (Ohoka

and Takai 1998) leading to alterations in its functions. Loss of domains by alternate splicing leads to reduced affinity for F actin (Ohoka and Takai 1998), reduced localization with actin and reduced motility. Similarly acetylation by HDAC6 inhibits F actin binding and cell motility (van Rossum, de Graaf et al. 2003). CTTN is a substrate of Src tyrosine kinase, which phosphorylates it at Y421 and Y466. Phosphorylation of the tyrosine residues stimulates the cleavage of CTTN by calpain and this cleavage appears to mediate CTTN-regulated protrusion of the cell edge (Perrin, Amann et al. 2006). Tyrosine phosphorylation of CTTN correlates with aggressiveness of cancer cells and is required for metastasis whereas inhibition of phosphorylation decreases cancer cell metastasis. Disruption of tyrosine phosphorylation sites prevents cell edge protrusion, cancer cell invasion and actin polymerization (Kelley, Hayes et al. 2010). Tyrosine phosphorylation increases focal adhesion dynamics and leads to stress fiber disassembly (Kruchten, Krueger et al. 2008), leading to increased motility of cancer cells.

CTTN is also phosphorylated by several serine/threonine kinases. The serine/threonine-protein kinase PAK3 phosphorylates it on S113 in response to growth factor receptor stimulation (Webb et al., 2006). PAK1 phosphorylates CTTN on S405 and S418 downstream of Rac1 and CDC42, which are members of the Rho family of small GTPases that are required for its localization to the cell edge and its increased association with N-WASP (Grassart et al., 2010; Vidal et al., 2002; Weed et al., 1998). Mitogen-activated protein kinase kinases (MEKs, also known as MAP2Ks) and extracellular signal-regulated kinases (ERKs, also known as MAPKs) have also been shown to phosphorylate CTTN on residues S405 and S418 (Campbell et al. 1999). ERK-mediated phosphorylation enhances its association with N-WASP, leading to increased actin polymerization mediated by the Arp2/3 complex (Martinez-Quiles et al., 2004) as shown in Figure 1.12 (B). Protein kinase D (PKD) phosphorylates CTTN on S298, which appears to increase cell migration owing to activation of the Arp2/3 complex (Eiseler et al., 2010). In

addition to S298, PKD phosphorylates CTTN at S348 in breast cancer cells, but the role of this second phosphorylation site is unclear (De Kimpe et al., 2009).

Another mechanism to affect cell motility is by affecting cell adhesion; CTTN null cells have more prominent focal adhesion (Lai, Szczodrak et al. 2009) thus affecting cell spreading, which is an adhesion-dependent process. CTTN regulation of adhesion is a likely mechanism to regulate motility (Owen, Pixley et al. 2007). Hyper phosphorylation of CTTN causes increased cell migration. Interestingly, it appears that tyrosine and serine phosphorylation can occur simultaneously, suggesting that it can integrate signals from diverse upstream signaling cascades (Kelley et al., 2010b; Kelley et al., 2011) to mediate its action.

Expression of CTTN mRNA in bladder cancer is associated with metastasis and poor prognosis, implicating its role in invasiveness of cancer cells. Overexpression of this protein has been observed in 15% of primary metastatic breast carcinomas and in nearly 30% of head and neck squamous cell carcinomas (Akervall, Jin et al. 1995, Buday and Downward 2007). Increased expression of CTTN has been linked to invasive cancers such as melanoma, colorectal, and glioblastoma making it an important biomarker for invasive cancers (Hirakawa, Shibata et al. 2009, Kirkbride, Sung et al. 2011).

**1.6 Akt signaling**

The serine threonine kinase Akt is the central cell survival signaling node in cancer. Aberrant loss or gain of function of Akt leads to various pathological conditions (Bellacosa, Testa et al. 1991). Activation of receptor tyrosine kinases triggers PI3K signaling. This is generally mediated by the adaptor protein IRS1, which binds and activates PI3K. Activated PI3K converts PIP2 to PIP3, which then causes translocation of Akt from the cytoplasm to the plasma membrane. Akt at the plasma membrane comes in close proximity to PDK1 and mTORC2 which then phosphorylate it at Thr 308 and Ser 473 respectively (Manning and Cantley 2007). Akt signaling is inhibited by

phosphatases as PTEN and PHLPP1/2 (Gao, Furnari et al. 2005). The PI3K/Akt signaling has been illustrated in detail in Figure 1.13.

Akt has three isoforms: Akt1, Akt2 and Akt3. These isoforms are highly homologous structurally. They each have four basic domains: PH domain, linker region, catalytic domain and regulatory domain. The PH domain, catalytic domain, and regulatory domain share a high level of homology amongst the isoforms, however the linker region varies in the three isoforms (Clark and Toker 2014). Phenotypes of individual Akt isoform knockouts suggest that these isoforms have non-redundant functions (Cho, Thorvaldsen et al. 2001, Garofalo, Orena et al. 2003, Dummler and Hemmings 2007). Although the role of Akt isoforms in cell survival and metastasis of colorectal cancer cells has been described in detail in the next chapter, we will discuss some of the major downstream substrates of Akt. Some of the Akt substrates are known to control more than one function.

Akt plays a critical role in promoting cell survival by inhibiting pro-apoptotic proteins. It negatively regulates Bcl-2 family proteins. It phosphorylates BAD at Ser 136, thus leading to an increase in its binding with 14-3-3 and increased cell survival (Datta, Katsov et al. 2000). Akt is also known to inhibit BH3-only proteins by affecting the expression of FOXO and p53. Akt phosphorylates FOXO proteins thus inhibiting their function of promoting apoptosis, cell cycle arrest and metabolic processes (Tran, Brunet et al. 2003). Additionally, Akt phosphorylates GSK3 thereby activating it which in turn are known to inhibits the pro-survival Bcl-2 fmaily member MCL-1 (Maurer, Charvet et al. 2006). Pro-caspase-9 is directly phosphorylated by Akt, leading to a reduction in its protease activity (Cardone, Roy et al. 1998). Akt also exerts cell survival effects through cross talk with other pathways such as NF-kappa B pathway and the JNK/p38 pathway (Ozes, Mayo et al. 1999, Kim, Khursigara et al. 2001).

The mTOR1 complex is activated by Akt. mTORC1 regulates translation initiation and ribosome biogenesis, thus affecting the growth of the cells (Wullschleger, Loewith et al. 2006).

Activation of Akt is known to affect the downstream targets of the mTORC1 complex, such as S6K1 and eukaryotic initiation factor 4E (elF4E) binding protein (Sekulic, Hudson et al. 2000). Akt is also known to phosphorylate and inhibit the tumor suppressor tuberous sclerosis complex 2 (TSC2), one of the negative regulators of mTORC1 signaling (Manning, Tee et al. 2002). Akt can also stimulate cell proliferation by regulating the cell cycle. It phosphorylates p27, a cyclin-dependent inhibitor thus leading to increased binding to 14-3-3, which then sequesters p27 in the nucleus, thereby reducing its cell cycle inhibitory effects (Medema, Kops et al. 2000). Similarly Akt is also known to inhibit p21, thus leading to increased progression of cell cycle (Heron-Milhavet, Franckhauser et al. 2006). Akt also regulates cyclin D, and cyclin E, thus affecting the G1-S phase of cell cycle transition (Yeh, Cunningham et al. 2004).

PI3K/Akt signaling also leads to increased vasodilation, vascular modeling and angiogenesis by increasing the expression of endothelial nitric oxide synthase (eNOS) (Dimmeler, Fleming et al. 1999). Additionally, Akt signaling increases the production of HIF1alpha through mTORC1 translation. Activation of HIF1alpha increases the expression of VEGF and other angiogenic factors, leading to increased vascular growth (Gordan and Simon 2007).

Recently, a role for Akt in cell migration and invasion has also been determined. Akt increases epithelial to mesenchymal transition (EMT) thus leading to increased metastasis (Irie, Pearline et al. 2005). Additionally, Akt also affects the stability of nuclear factor of activated T cells (NFAT), thus regulating metastasis (Yoeli-Lerner, Yiu et al. 2005). It has been identified recently that the 3 isoforms of Akt affect cell migration in an opposing way, in which Akt1 inhibits migration but Akt2 is known to enhance the metastatic phenotype (Arboleda, Lyons et al. 2003).

Understanding the downstream signaling pathways of Akt help us to understand the mechanism of how Akt affects various cell biological processes. We have gained a better

understanding of Akt functions by knockout, knockdown or by pharmacological inhibition of Akt or specific Akt isoforms, however still more needs to be done to confirm which isoform of Akt plays the most important role. The information provided in the following chapters of this dissertation helps us to better answer this question.

**1.7 Figures**

**Figure 1.1:** Different stages of colorectal cancer from stage 1 to stage 5. Stage 0 is marked with the presence of a polyp, which then grows progressively to form a tumor, which in turn undergoes metastasis and migrates to distant sites. This figure has been adapted from Bailey (2014) "The role of EPA in chemoprevention of colorectal cancer" with permission.

**Figure 1.1:**

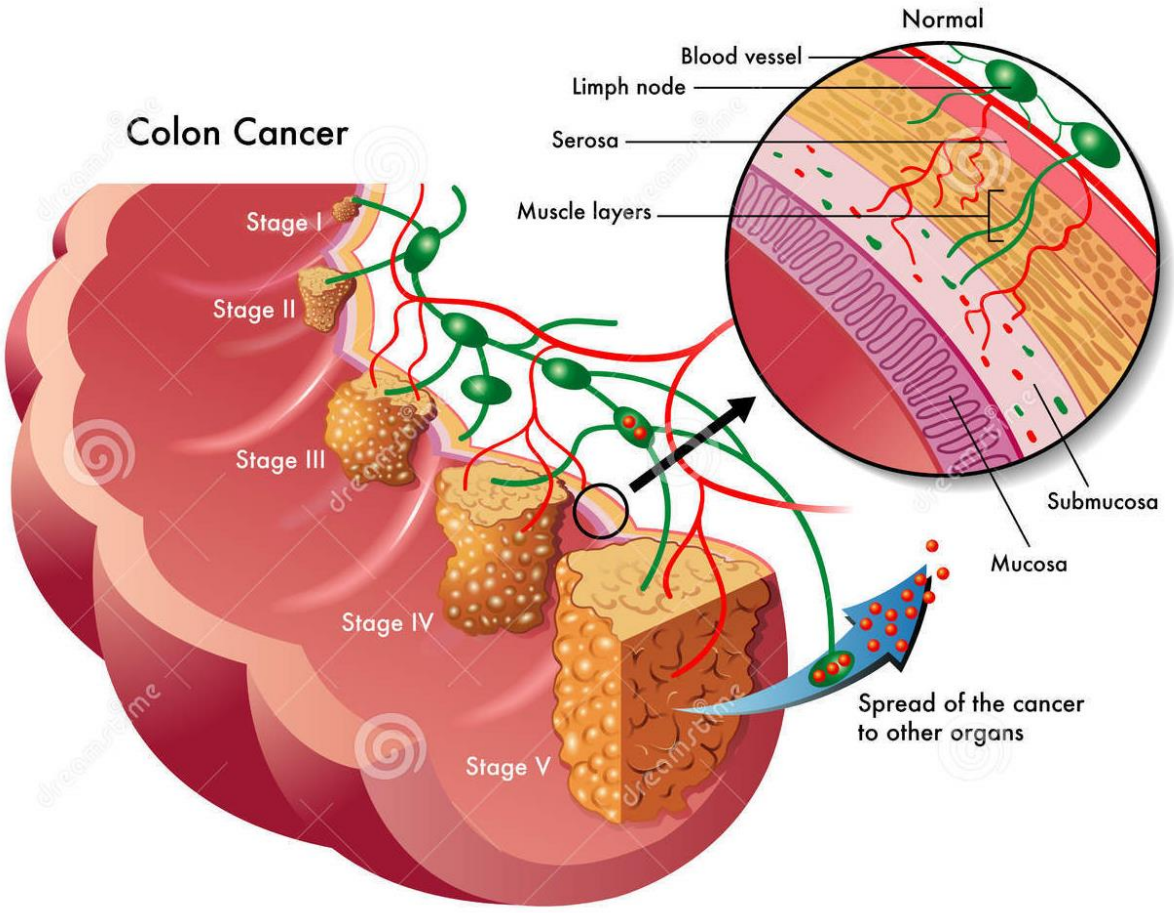

Normal
Blood vessel
Limph node
Serosa
Muscle layers
Submucosa
Mucosa
Colon Cancer
Stage I
Stage II
Stage III
Stage IV
Stage V
Spread of the cancer
to other organs

**Figure 1.2:** Progression model representing the genes and growth factors that are altered in colon cancer. The genes responsible for transition from final stage of colon cancer to metastasis (depicted by question mark) are still unknown. This figure has been adapted from Markowitz and Bertangnolli (2009) N Eng J Med 2009: 361:2449-60 with permission.

**Figure 1.2:**

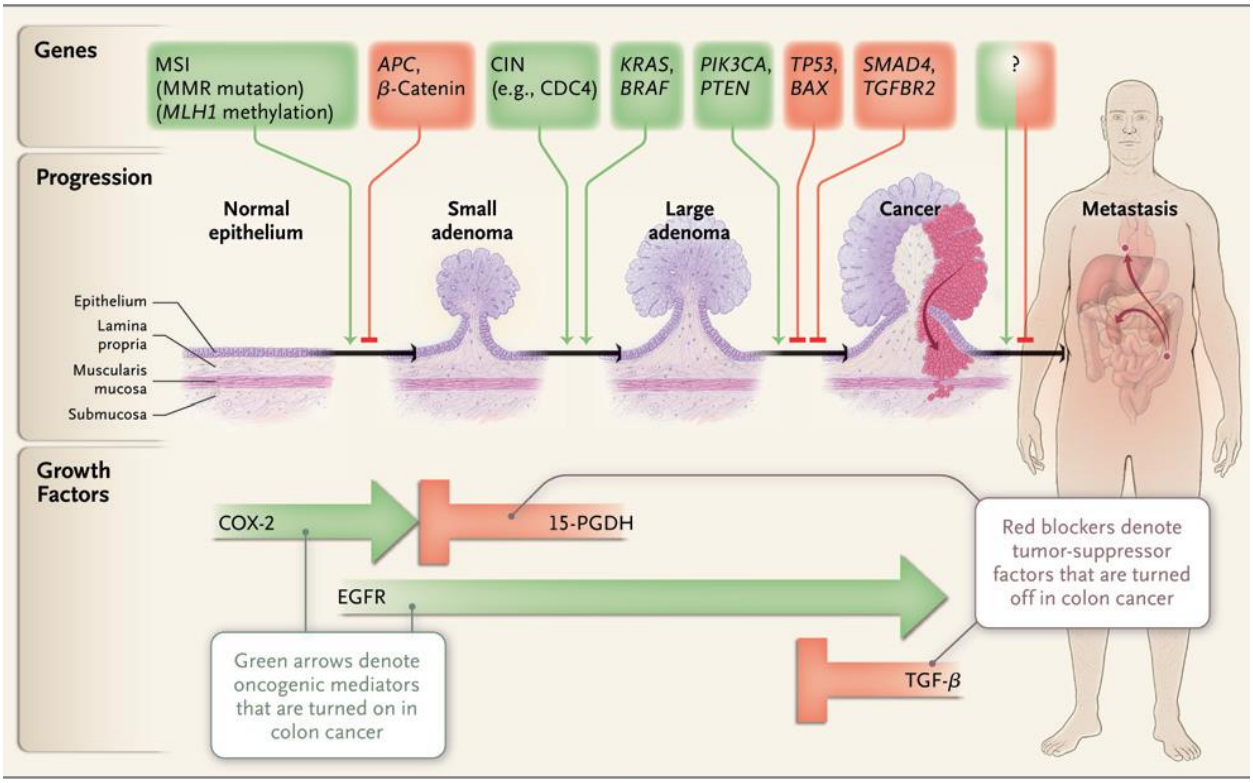

Genes
MSI
(MMR mutation)
(MLH1 methylation)
APC,
β-Catenin
CIN
(e.g., CDC4)
KRAS,
BRAF
PIK3CA,
PTEN
TP53,
BAX
SMAD4,
TGFBR2
?
Progression
Normal epithelium
Small adenoma
Large adenoma
Cancer
Metastasis
Epithelium
Lamina propria
Muscularis mucosa
Submucosa
Growth Factors
COX-2
15-PGDH
EGFR
TGF-β
Red blockers denote tumor-suppressor factors that are turned off in colon cancer
Green arrows denote oncogenic mediators that are turned on in colon cancer

**Figure 1.3:** Illustrates the inefficiency of metastasis. Most of the steps of metastasis are inefficient, with metastatic colonization being the rate-limiting step. This figure depicts the fraction of cells that have died along the metastatic cascade. There is about a 99% attrition of cells in the process of formation of metastatic spots. This figure has been adapted from Valastyan and Weinberg (2011) Cell 2011: 147 (2): 275-292 with permission.

**Figure 1.3:**

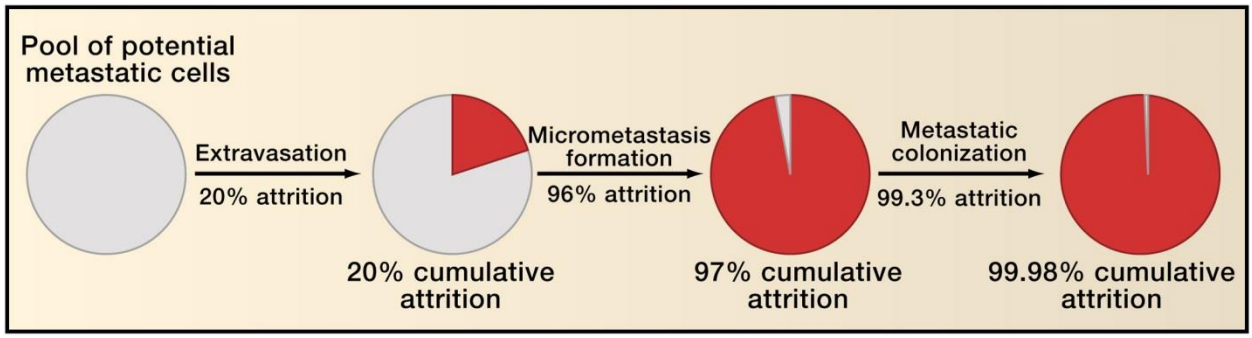

Pool of potential metastatic cells
Extravasation
20% attrition
Micrometastasis formation
96% attrition
Metastatic colonization
99.3% attrition
20% cumulative attrition
97% cumulative attrition
99.98% cumulative attrition

**Figure 1.4:** Illustration of the various steps of metastasis and some of the important signaling molecules that help cells to undergo metastasis. This figure has been adapted from Valastyan and Weinberg (2011) Cell 2011: 147 (2): 275-292 with permission.

**Figure 1.4:**

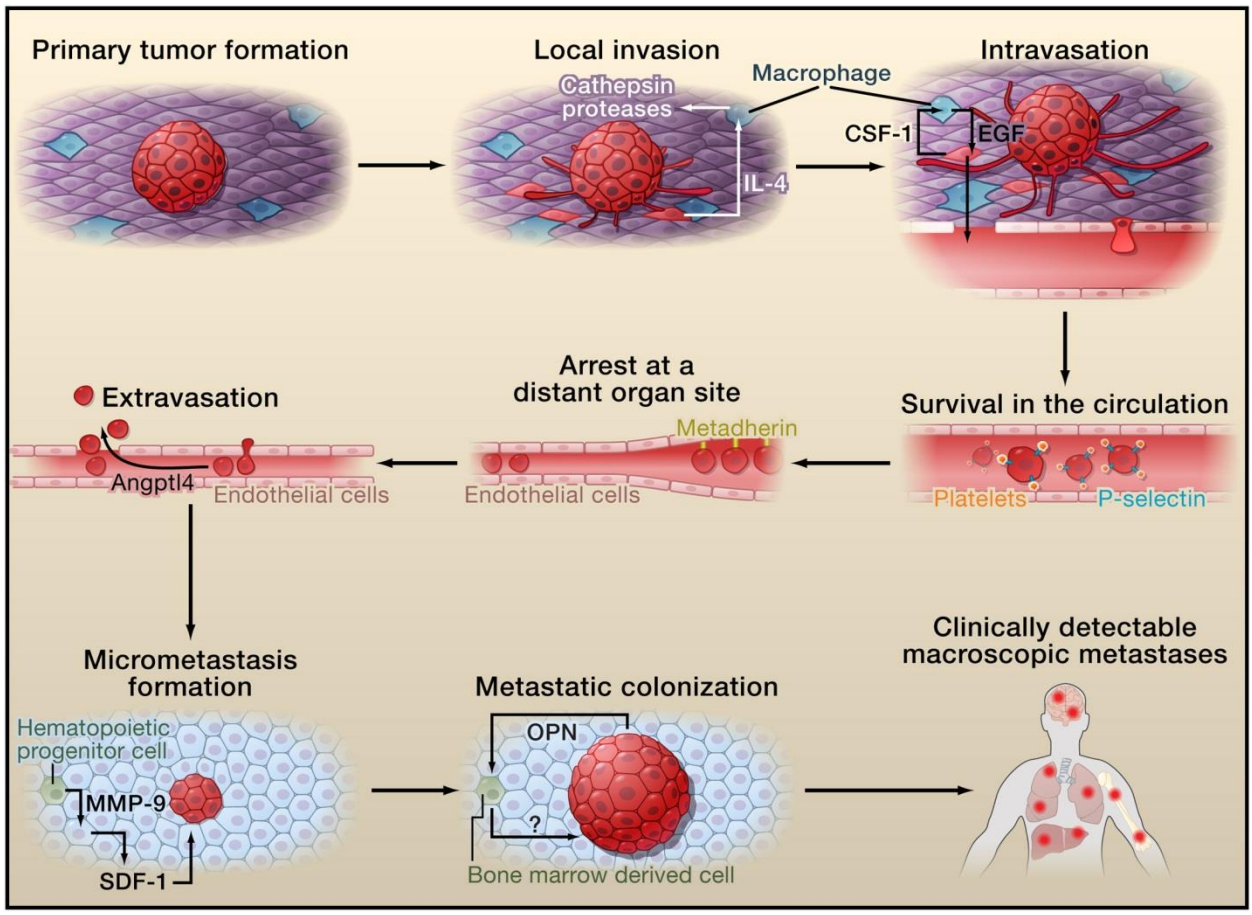

Primary tumor formation
Local invasion
Cathepsin proteases
Macrophage
IL-4
Intravasation
CSF-1
EGF
Survival in the circulation
Platelets
P-selectin
Arrest at a distant organ site
Metadherin
Endothelial cells
Extravasation
Angptl4
Endothelial cells
Micrometastasis formation
Hematopoietic progenitor cell
MMP-9
SDF-1
Metastatic colonization
OPN
?
Bone marrow derived cell
Clinically detectable macroscopic metastases

**Figure 1.5:** Representation of the domain structure of IAP family members. This figure has been adapted from Ribe and Troy (2008) Biochemical journal 2008: 415 (2) 156-182 with permission.

**Figure 1.5:**

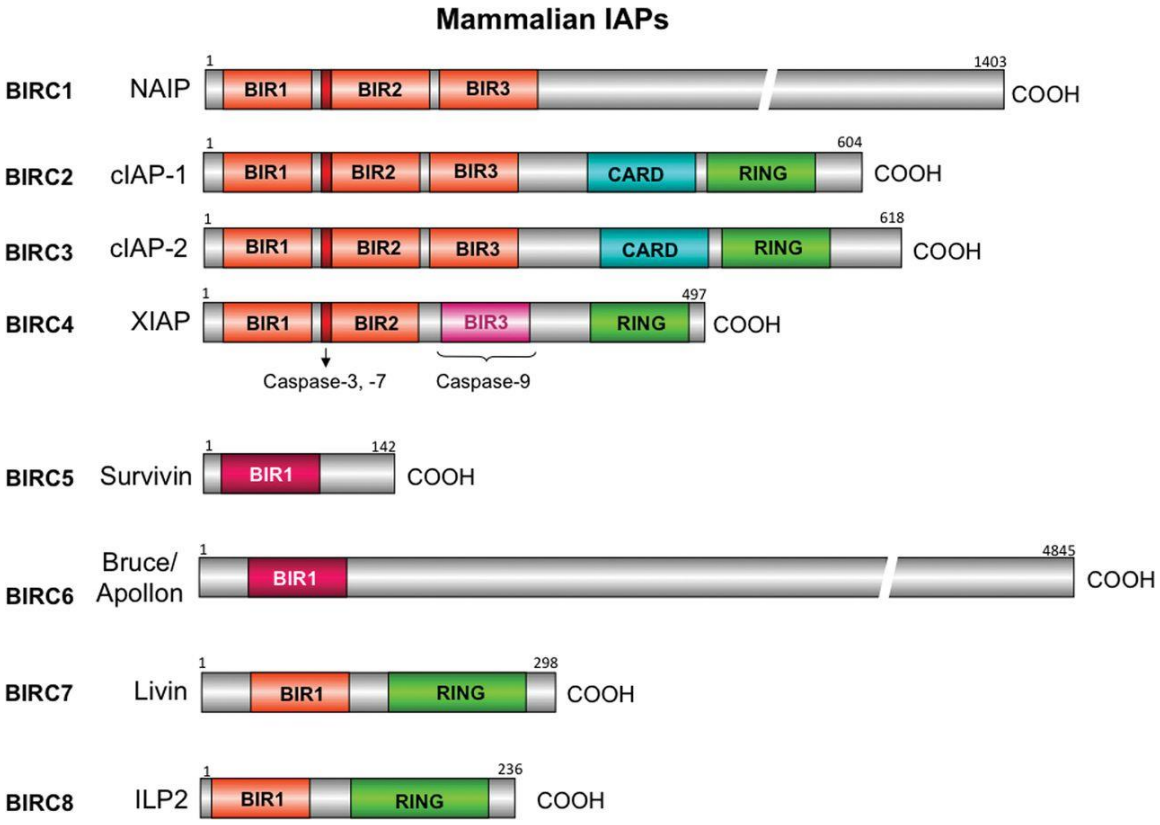

Mammalian IAPs
BIRC1
NAIP
1
1403
BIR1
BIR2
BIR3
COOH
BIRC2
cIAP-1
1
604
BIR1
BIR2
BIR3
CARD
RING
COOH
BIRC3
cIAP-2
1
618
BIR1
BIR2
BIR3
CARD
RING
COOH
BIRC4
XIAP
1
497
BIR1
BIR2
BIR3
RING
COOH
Caspase-3, -7
Caspase-9
BIRC5
Survivin
1
142
BIR1
COOH
BIRC6
Bruce/
Apollon
1
4845
BIR1
COOH
BIRC7
Livin
1
298
BIR1
RING
COOH
BIRC8
ILP2
1
236
BIR1
RING
COOH

**Figure 1.6**: Depiction of XIAP signaling to regulate cell death. The BIR domains of XIAP inhibit the caspases leading to increase in apoptosis. Ubiquitination of XIAP leads to a reduction in the expression of XIAP and an increase in the expression of AIF. This figure has been adapted from Fillipovich and Marsh (2010) Blood 2010:116 (8) : 3398-3408 with permission.

**Figure 1.6:**

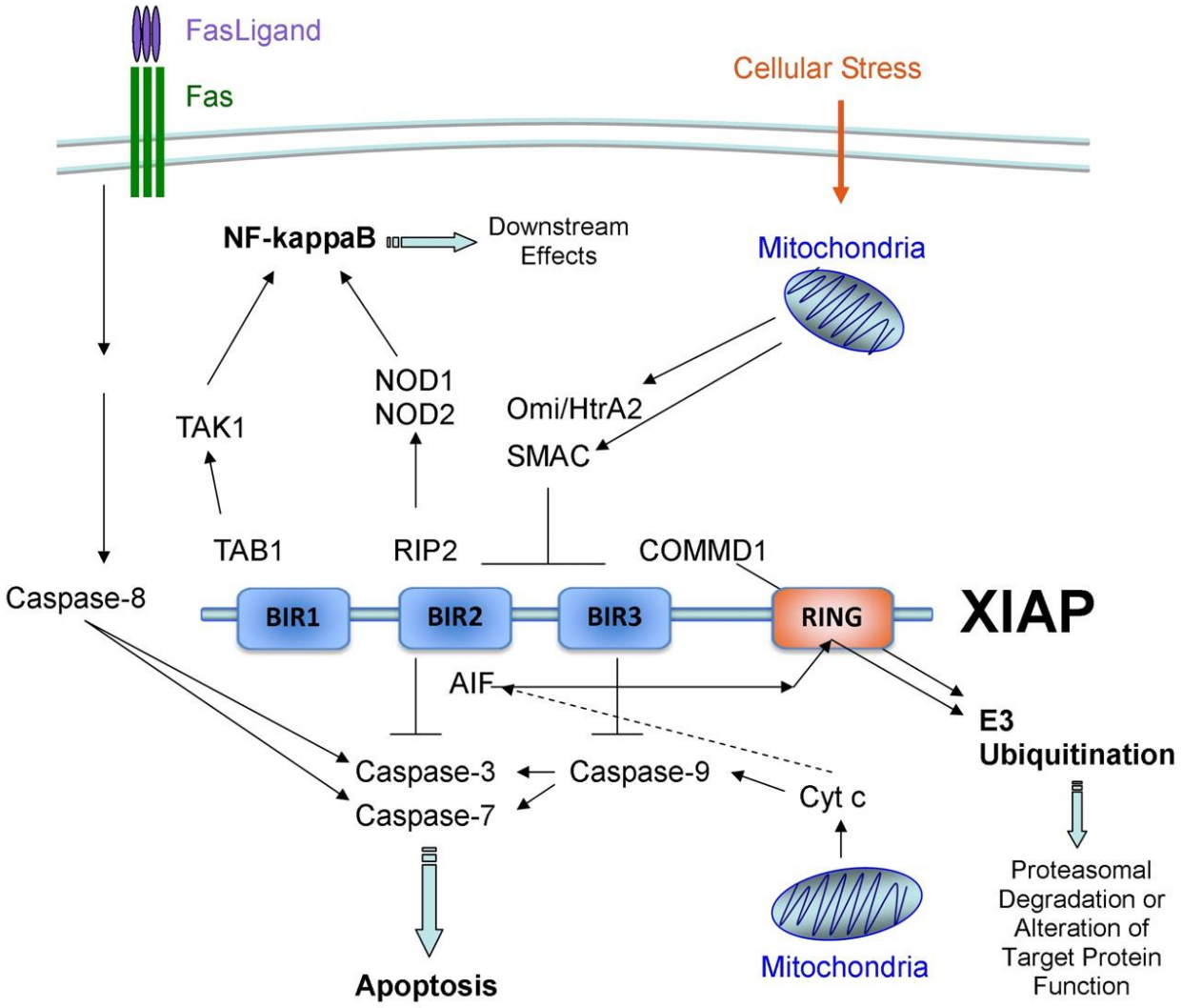

FasLigand
Fas
Cellular Stress
NF-kappaB
Downstream Effects
Mitochondria
NOD1
NOD2
TAK1
Omi/HtrA2
SMAC
TAB1
RIP2
COMMD1
Caspase-8
BIR1
BIR2
BIR3
RING
XIAP
AIF
E3 Ubiquitination
Caspase-3
Caspase-7
Caspase-9
Cyt c
Proteasomal Degradation or Alteration of Target Protein Function
Apoptosis
Mitochondria

**Figure 1.7:** Illustrates the functions of survivin. This IAP family member regulates mitosis through CPCs. It forms a complex with XIAP thus leading to inhibition of apoptosis; additionally, via alpha 5 integrin, survivin also regulates cell motility. This figure has been adapted from McKenzie and Grossman (2012) Anticancer Res. (2012) 32 (2) : 397-404 with permission.

**Figure 1.7:**

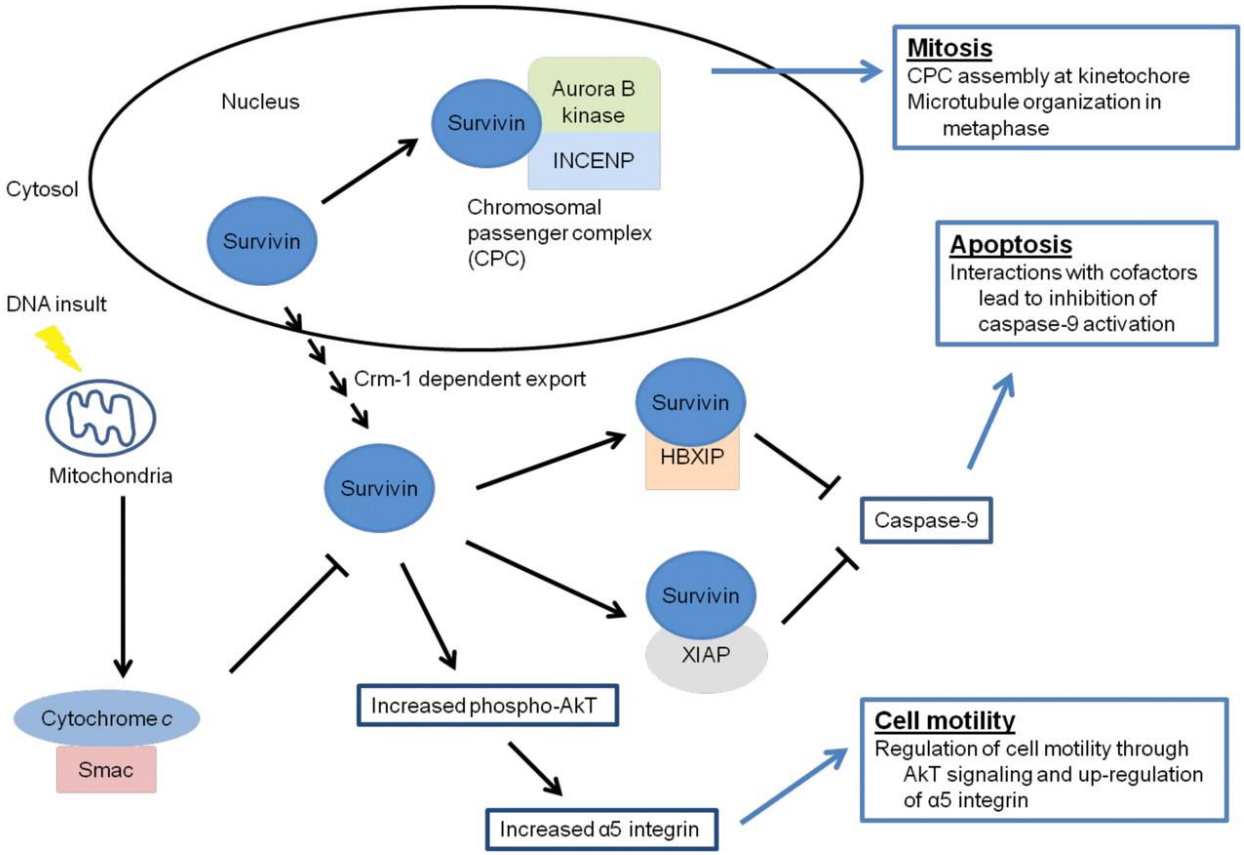

Nucleus
Survivin
Aurora B kinase
INCENP
Chromosomal passenger complex (CPC)
Survivin
Cytosol
DNA insult
Mitochondria
Crm-1 dependent export
Survivin
Survivin
HBXIP
Survivin
XIAP
Caspase-9
Cytochrome c
Smac
Increased phospho-AkT
Increased α5 integrin
Mitosis
CPC assembly at kinetochore
Microtubule organization in metaphase
Apoptosis
Interactions with cofactors lead to inhibition of caspase-9 activation
Cell motility
Regulation of cell motility through AkT signaling and up-regulation of α5 integrin

**Figure 1.8:** Represents that survivin can lead to both caspase-dependent and caspase-independent cell death. Caspase-independent cell death is mediated via AIF. This figure has been adapted from Grossman (2004) Oncogene 2004: 23 (1): 39-48 with permission.

**Figure1.8:**

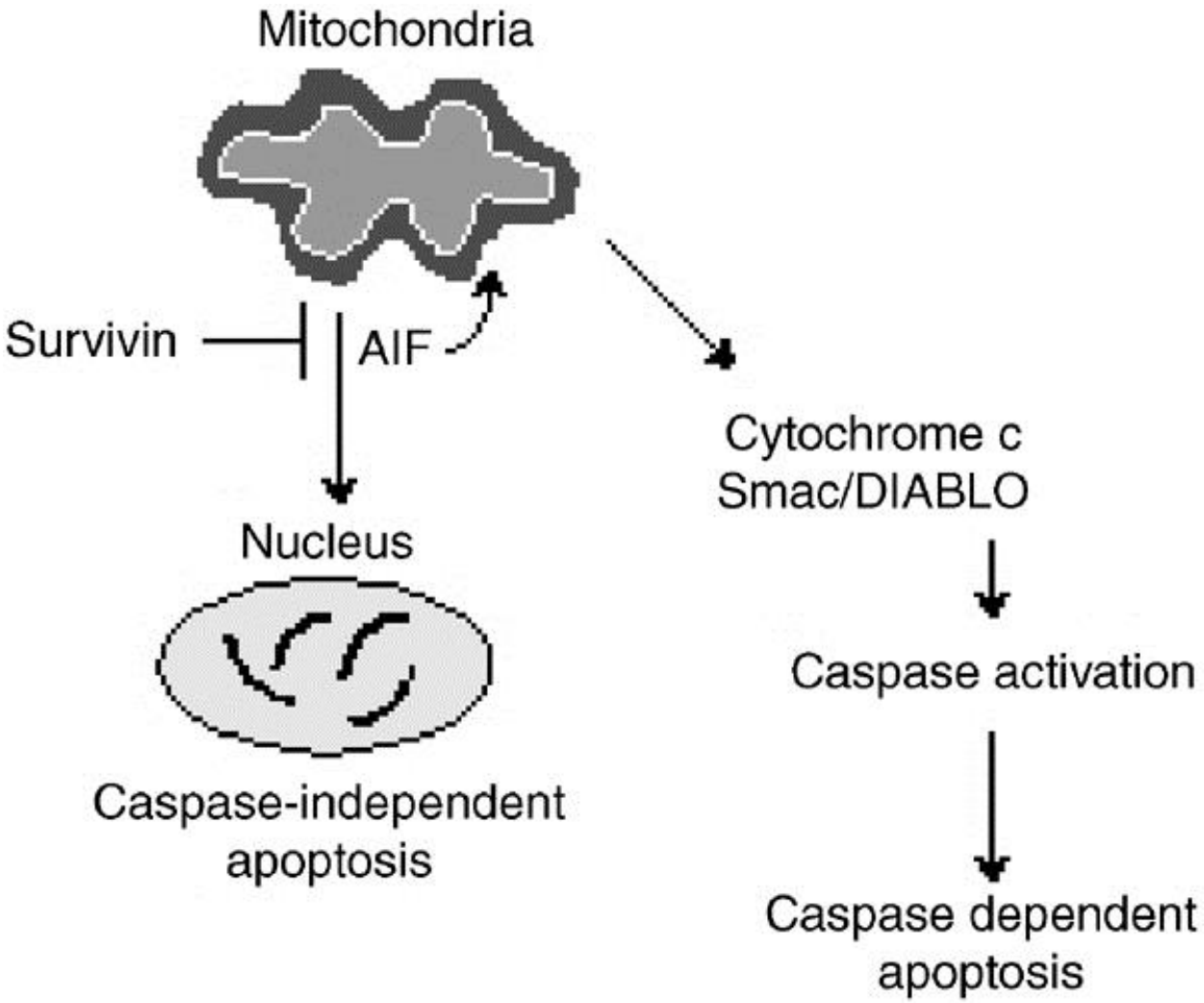

Mitochondria
Survivin
AIF
Nucleus
Caspase-independent apoptosis
Cytochrome c
Smac/DIABLO
Caspase activation
Caspase dependent apoptosis

**Figure 1.9:** Domain structure of Apoptosis Inducing Factor. This figure has been adapted from Mader and Bleackely 2009 Mol Cancer Res (2009): 7 (5): 689-702 with permission.

**Figure 1.10:** Represents the mechanism of action of Apoptosis Inducing Factor. Stress causes the release of AIF from the mitochondria to the nucleus, where it leads to DNA fragmentation and then leading to cell death. This figure has been adapted from Mader and Bleackely 2009 Mol Cancer Res (2009): 7 (5): 689-702 with permission.

**Figure 1.9:**


**Figure 1.10:**

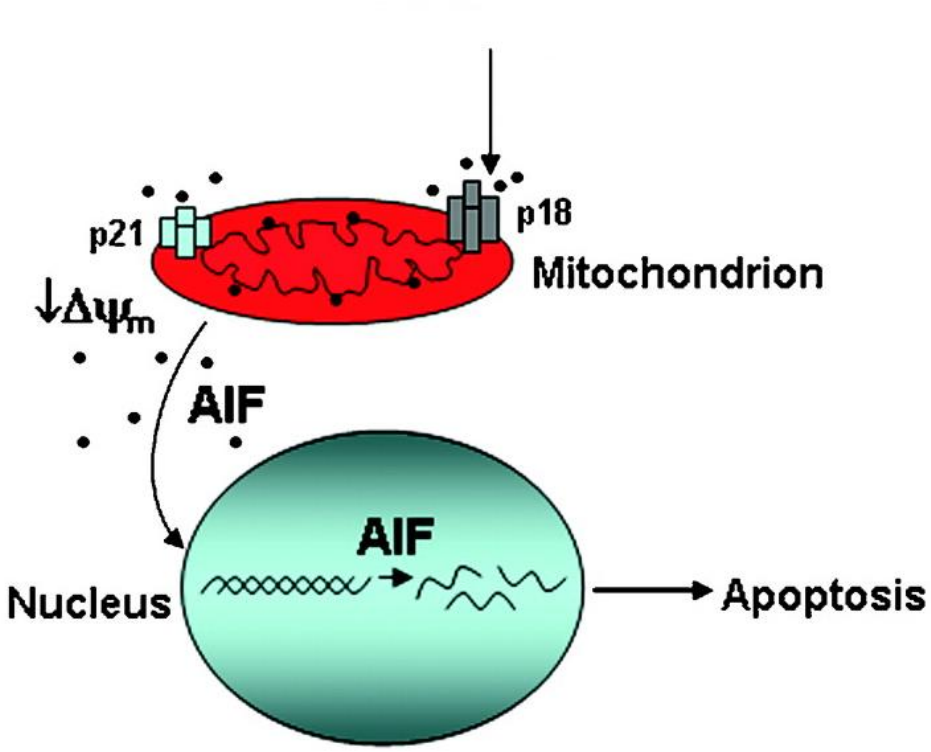


**Figure 1.11:** Domain structure of MTSS1. It has an IMD domain, a serine-rich domain and proline-rich domain. The figure also depicts the binding partners of MTSS1. This figure has been adapted from Machesky and Johnston (2007) J Mol Med (2007): 85: 569-576 with permission.

**Figure1.11:**

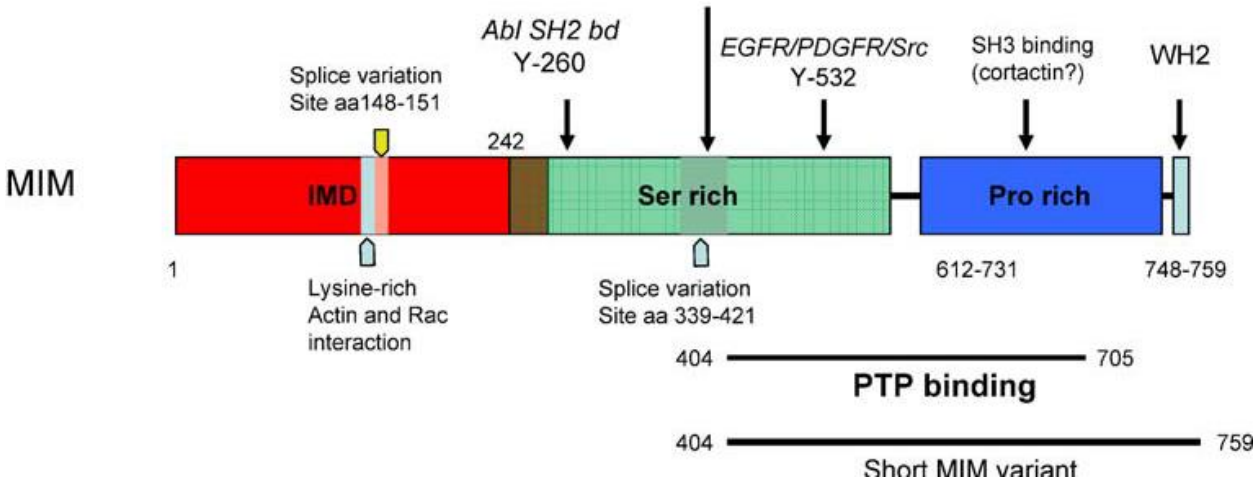

MIM
Splice variation
Site aa148-151
Abl SH2 bd
Y-260
EGFR/PDGFR/Src
Y-532
SH3 binding
(cortactin?)
WH2
242
IMD
Ser rich
Pro rich
1
Lysine-rich
Actin and Rac
interaction
Splice variation
Site aa 339-421
612-731
748-759
404
705
PTP binding
404
759
Short MIM variant

**Figure 1.12:** (A) Domain structure and the binding partners of Cortactin. CTTN has a NTA domain at the N-terminus and a SH3 binding domain at the C-terminus. There are binding sites for several kinases which then lead to phosphorylation and activation of CTTN. (B) CTTN binds to Arp2/3 comple, leading to actin polymerization. This figure has been adapted from Binker and Kapus (2006) Physiology 2006: 21: 352-361 with permission.

**Figure 1.12:**

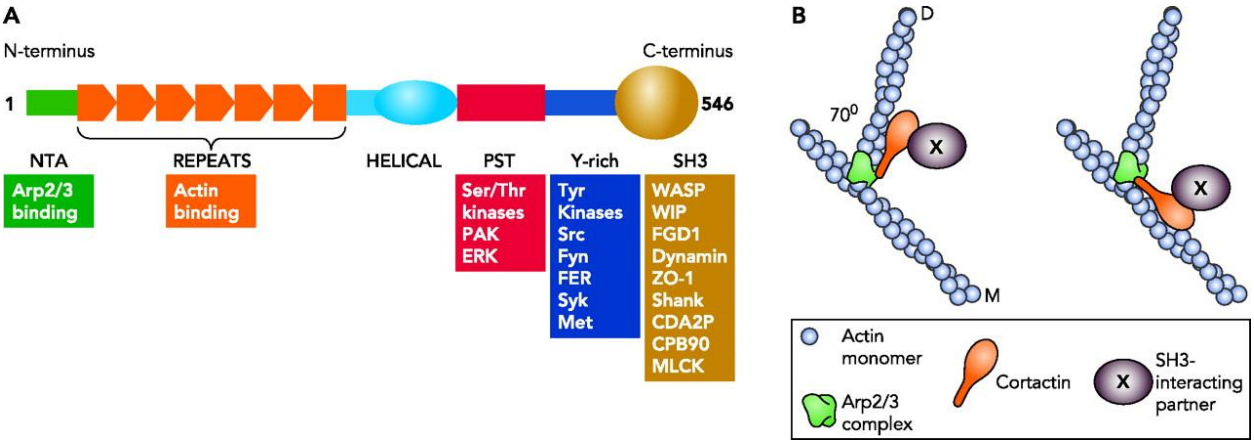
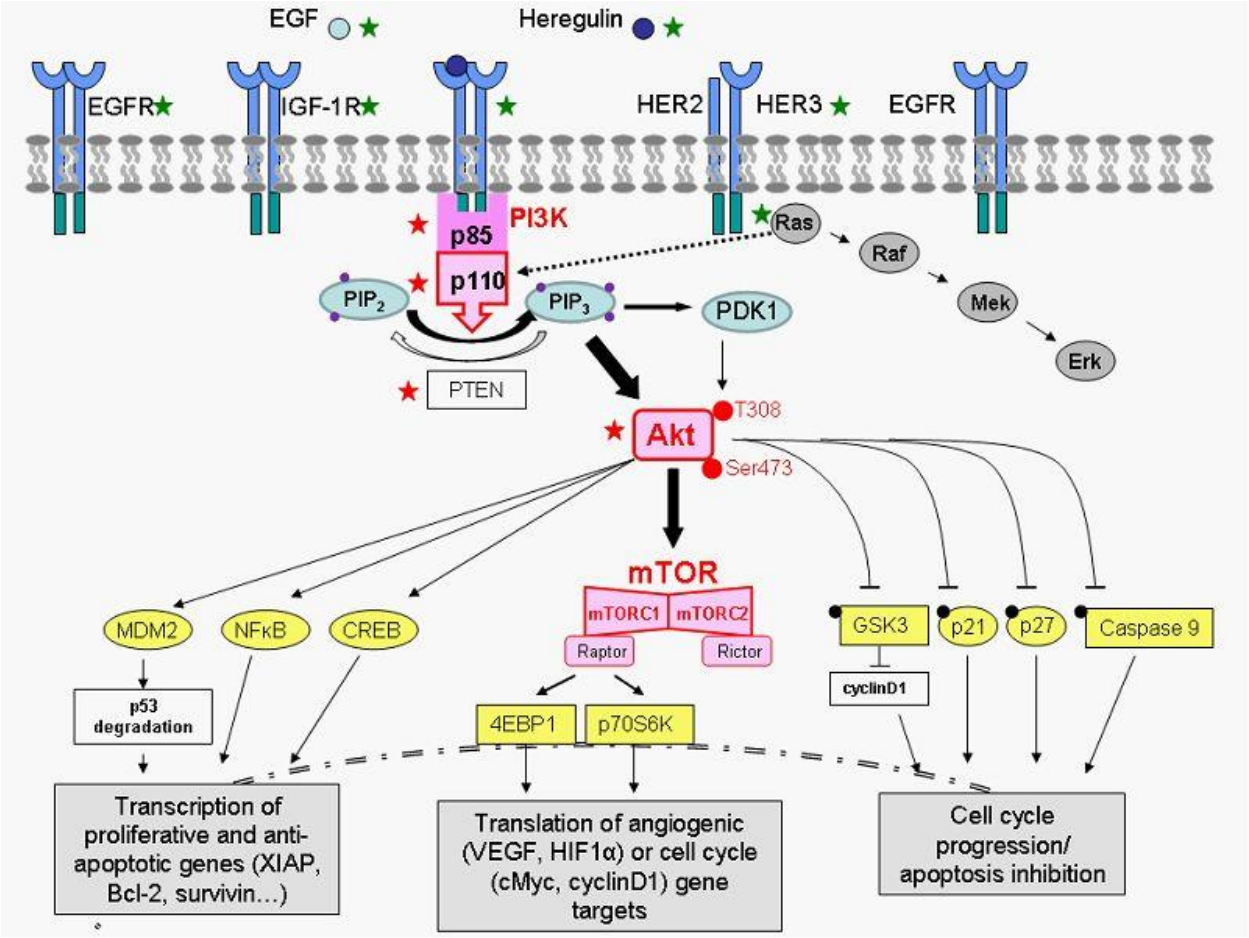

A
N-terminus
C-terminus
1
546
NTA
REPEATS
HELICAL
PST
Y-rich
SH3
Arp2/3 binding
Actin binding
Ser/Thr kinases
PAK
ERK
Tyr Kinases
Src
Fyn
FER
Syk
Met
WASP
WIP
FGD1
Dynamin
ZO-1
Shank
CDA2P
CPB90
MLCK
B
D
70⁰
X
M
Actin monomer
Arp2/3 complex
Cortactin
SH3-interacting partner

**Figure 1.13:** PI3K/Akt signaling. This figure has been adapted from Cheaib and Leary (2015) Chin J Cancer 2015 : 34 (1) : 4-16 with permission.

**Figure 1.13:**


EGF
Heregulin
EGFR
IGF-1R
HER2
HER3
EGFR
p85
PI3K
p110
Ras
Raf
Mek
Erk
PIP2
PIP3
PDK1
PTEN
Akt
T308
Ser473
mTOR
mTORC1
mTORC2
Raptor
Rictor
MDM2
NFκB
CREB
GSK3
p21
p27
Caspase 9
cyclinD1
p53 degradation
4EBP1
p70S6K
Transcription of proliferative and anti-apoptotic genes (XIAP, Bcl-2, survivin…)
Translation of angiogenic (VEGF, HIF1α) or cell cycle (cMyc, cyclinD1) gene targets
Cell cycle progression/ apoptosis inhibition
Upstream activation of PI3K pathway: directly via membrane RTKs or their ligands, or indirectly via Ras
Intrinsic activation of PI3K pathway via mutations in the p110 (PIK3CA) or p85 (PIK3R) subunits of PI3K, Akt mutations/amplifications, or PTEN loss.
Cross-talk

# CHAPTER II

## Cell Survival and Metastasis Regulation by Akt Signalling in Colorectal Cancer

Ekta Agarwal[1,2], Michael G. Brattain[1,2] and Sanjib Chowdhury[2]*

[1] Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA

[2] Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA

*Corresponding author

**The following chapter has been published in Cellular Signalling (2013). Aug; 25(8): 1711-9**

## 2. Cell Survival and Metastasis Regulation by Akt Signalling in Colorectal Cancer

### 2.1 Abstract

Dissemination of cancer cells to distant organ sites is the leading cause of death due to treatment failure in different types of cancer. Mehlen and Puisieux have reviewed the importance of the development of inappropriate cell survival signalling for various steps in the metastatic process and have noted the particular importance of aberrant cell survival to successful colonization at the metastatic site. Therefore, the understanding of mechanisms that govern cell survival fate of these metastatic cells could lead to the understanding of a new paradigm for control of metastatic potential and could provide the basis for developing novel strategies for the treatment of metastases. Numerous studies have documented the widespread role of Akt in cell survival and metastasis in colorectal cancer, as well as many other types of cancer. Akt acts as a key signalling node that bridges the link between oncogenic receptors to many essential pro-survival cellular functions, and is perhaps the most commonly activated signalling pathway in human cancer. In recent years, Akt2 and Akt3 have emerged as significant contributors to malignancy alongside the well-characterized Akt1 isoform, with distinct non-overlapping functions. This chapter is aimed at gaining a better understanding of the Akt-driven cell survival mechanisms that contribute to cancer progression and metastasis and the pharmacological inhibitors in clinical trials designed to counter the Akt-driven cell survival responses in cancer.

### 2.2 Introduction

Colorectal cancer (CRC) is one of the most common malignancies with high incidence rates globally (Stein, Walther et al. 2009). It develops due to various genetic, epigenetic and environmental factors. The stage I CRC is localized to the mucosal and submucosal layers of the colon wall. Surgery and multimodal treatments are the preferred strategies following early stage diagnosis of CRC with a 5-year survival rate of approximately 100%. This rate in 5-year survival

is significantly decreased during stage III CRC which is characterized by lymph node metastasis (Johnson, Gulhati et al. 2010). However, metastasis to distant organ sites (stage IV) is the most frequent cause of cancer-related deaths with a 5-year survival rate of <5% (Christofori 2006, Stein and Schlag 2007). CRCs progress slowly to an invasive stage, but the progression from invasive carcinoma to metastatic phase occurs rapidly (Nguyen, Bos et al. 2009). The disseminating CRC cells acquire enhanced cell survival capabilities to counteract apoptosis initiated by the multi-step metastatic cascade including anoikis (Weigelt, Peterse et al. 2005), migration through the basement membrane and intravasation into the blood and/or lymphatic vessels. The CRC cells develop the ability to proliferate and colonize secondary organ sites with unrelated microenvironments (Weigelt, Peterse et al. 2005). There has been limited success in the early identification of patients at high risk for developing metastasis through the tissue-based biomarkers currently available for such purposes (Vansant, Johnson et al. 1992, Duffy, van Dalen et al. 2007). The mechanisms involved in regulating the early stages of the metastatic cascade that are crucial for diagnosis are currently not fully understood (Fearon and Vogelstein 1990, Bernards and Weinberg 2002, Vogelstein and Kinzler 2004, Takayama, Miyanishi et al. 2006). In recent years, several aberrant cell survival mechanisms have been linked to successful metastatic colonization (Mehlen and Puisieux 2006) including the deregulation of pro-survival Bcl-2 family proteins (Cory and Adams 2002), inhibitor-of-apoptosis (IAP) family proteins (Srinivasula and Ashwell 2008) and the PI3K/AKT signalling. The Bcl-2 family members are either pro-apoptotic or anti-apoptotic in nature (Youle and Strasser 2008). The pro-apoptotic proteins such as BID, BAX and BAK translocate in the mitochondria leading to caspase activation thus leading to cell death. The Bcl-2 family members have a BH3 domain that inhibits the anti-apoptotic members of the Bcl-2 family thus leading to apoptosis. Bcl-$X_L$ protein inhibits the activity of BAX and BAK by binding to them and inhibiting apoptosis (Grad, Zeng et al. 2000). BAX and BAK are responsible for caspase activation thus downregulating cell survival. Several lines of evidence

have implicated the role of Bcl-2 overexpression in promoting cell survival and metastasis (Del Bufalo, Biroccio et al. 1997, Pinkas, Martin et al. 2004). However, contrasting reports from Subhawong *et al.* concluded that Bcl-2 is infrequently upregulated in metastatic breast carcinoma and hormone therapy resistance may lead to downregulation of Bcl-2 (Subhawong, Nassar et al. 2010). IAPs are also responsible for regulating apoptosis by inhibiting caspases. Some IAPs have been shown to inhibit caspases by inhibiting their enzyme activity and/or by targeting them for proteasomal degradation (Vaux and Silke 2005). Caspases 3,7 and 9 bind to the BIR (Baculoviral IAP repeat) domains of IAPs leading to their inhibition and increased cell survival (Deveraux, Leo et al. 1999). Recently, the Altieri laboratory has shown that IAP family members survivin and XIAP are playing an essential role in metastasis (Mehrotra, Languino et al. 2010). Overexpression of survivin has been observed in colorectal tumorigenesis. Survivin plays a role in the progression of adenomas from the mild dysplasia to the more advanced highly dysplastic lesions (Kawasaki, Toyoda et al. 2001). Several survivin inhibitors are currently in phase I or II clinical trials in solid tumors and non-Hodgkins lymphoma (Kelly, Lopez-Chavez et al. 2011). YM155 (1-(2-Methoxyethyl)-2-methyl-4, 9-dioxo-3- (pyrazin-2-ylmethyl)-4,9-ihydro-H-naphtho [2,3]-dimidazolium bromide) (Astellas Pharma Inc) is a transcriptional inhibitor of survivin. It has been demonstrated to have a potent anti-tumor activity and has been used as a radio-sensitizing agent to potentiate the anti-cancer functions of various chemotherapeutic agents (Nakahara, Kita et al. 2007, Iwasa, Okamoto et al. 2008, Kelly, Lopez-Chavez et al. 2011). The phosphoinositide 3‑ kinase (PI3K) – Akt signalling pathway, which transmits anti-apoptotic signals, is involved in a significant fraction of human tumors promoting cancer cell growth, metabolism, survival and has been implicated in EMT, angiogenesis and metastasis (see reference (Fresno Vara, Casado et al. 2004, Altomare and Testa 2005, Hennessy, Smith et al. 2005, Manning and Cantley 2007, Sheng, Qiao et al. 2009) for extensive on PI3K/Akt signalling in cancer. A better understanding of the molecular signalling pathways involved in the process of

metastasis will help in effectively targeting these aggressive cancer cells using novel therapeutic strategies.

In this chapter, we have focused on the emerging roles of Akt isoforms in various types of cancer, including colorectal, breast, lung and pancreatic cancer, and have discussed the TGFβ/PKA metastatic suppressor pathway that negatively regulates Akt-driven aberrant cell survival mechanisms that contribute to metastatic progression in CRC. Additionally, we have provided updates on various Akt inhibitors that are in clinical trials.

**2.3 PI3K – Akt signalling in cancer**

The PI3K signalling pathway is a major link between oncogenic receptors and downstream pro-survival molecules and is one of the most frequently activated signalling pathways in human cancers (Vivanco and Sawyers 2002, Bader, Kang et al. 2005, Engelman, Luo et al. 2006). It is known that multiple small GTPases that belong to Ras and Rho-kinase family of GTPases activate PI3K (Yang, Shin et al. 2012). It has also been shown that Rho GTPases are downstream activators of PI3K. These Rho GTPases amplify the PI3K activity by feedback mechanisms (Weiner, Neilsen et al. 2002). The stimulation by growth factors leads to the activation of receptor tyrosine kinases (RTKs), that in turn recruits the class IA PI3Ks, consisting of p110α–p85 subunits, to the membrane due to the direct protein-protein interaction between p85 and activated RTKs (for example, IGFR and EGFR). Alternatively, the p85 subunit can also interact with specific adaptor proteins, such as Insulin Receptor Substrate 1/2 (IRS1/2) associated with IGFR (Figure 2.1).

The activated p110α catalytic subunit converts phosphatidylinositol4, 5bis phosphate (PIP2) to phosphatidylinositol3, 4, 5trisphosphate (PIP3) at the membrane. The conversion of PIP2 to PIP3 provides docking sites for pleckstrin homology (PH) domain-containing proteins, including 3phosphoinositide-dependent protein kinase 1 (PDPK1) and serine–threonine protein kinase Akt

(also known as protein kinase B). PDPK1 phosphorylates Akt at Ser473 and PDK2 phosphorylates Akt at Thr308. The dual activation of Akt at Ser473 and Thr308 phosphorylation sites elicits a broad range of downstream signalling events as shown in Figure 2.2. The tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) inhibits PI3K activation and downstream signalling by dephosphorylating PIP3 (Vivanco, Rohle et al. 2010). Deletion, mutation and hypermethylation of PTEN are observed in various cancer conditions thus resulting in elevated Akt activity (Georgescu 2010). A study in rhabdomyosarcoma cells has shown that PTEN is responsible for dephosphorylation specifically at the Ser473 site of Akt (Wan and Helman 2003). PHLPP (PH domain leucine-rich repeat protein phosphatase) specifically dephosphorylates Akt at Ser473 thus attenuating Akt signalling (Gao, Furnari et al. 2005). It has two isoforms PHLPP1 and PHLPP2, which are very specific in their mode of action. PHLPP1 and PHLPP2 specifically dephosphorylate Akt2 and Akt1 respectively (Nitsche, Edderkaoui et al. 2012). The expression of these phosphatases decreases in many cancer types, including colon cancer and glioblastoma, thus resulting in increased Akt activation and its downstream oncogenic signalling pathways (Liu, Weiss et al. 2009). PP2A (protein phosphatase 2A) is a serine threonine phosphatase, which is ubiquitously expressed in the tissues. It regulates Akt by inhibiting its phosphorylation at Thr308 (Kuo, Huang et al. 2008). It is a tumor suppressor gene since its inhibition by okadaic acid results in increase in cell survival thus increasing tumor growth (Suganuma, Fujiki et al. 1988). It has been shown that there is an increase in the expression of PP2A inhibitors in various cancer conditions (Li, Chen et al.). We have recently demonstrated a novel pathway of cell death in colon cancer, mediated by PP2A where it inhibits the phosphorylation of Akt, thus disrupting the XIAP/Survivin complex resulting in increased cell death (Chowdhury, Howell et al. 2011). The overall mechanism of cell death mediated by this pathway has been explained in detail in the later part of the chapter.

Genomic analyses have revealed that many components of the PI3K/Akt pathway are frequently mutated or altered in common human cancers underscoring the importance of this pathway in cancer (Liu, Cheng et al. 2009). The genes encoding the PI3K catalytic subunits PI3KCA, PI3KCB, regulatory subunit of PI3K, p85, PDK1, PTEN undergo loss of function or overexpression in various tumor conditions thus leading to cancer.

**2.4 Distinct roles for Akt isoforms in cancer progression and metastasis**

Akt is an evolutionarily conserved serine/threonine kinase consisting of 3 members, i.e., Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). The Akt isoforms have distinct non-redundant functional characteristics despite sharing a high level of structural homology and similar mechanisms of activation (Cantley 2002, Chin and Toker 2009). The Akt isoforms are ubiquitously expressed in all cell types and tissues. However, the pattern of Akt3 expression is restricted (Toker and Yoeli-Lerner 2006). Akt1 is widely expressed in brain, heart and lungs; however, Akt2 is mostly expressed in skeletal muscles(Suganuma, Fujiki et al. 1988, Brodbeck, Cron et al. 1999). Akt3 is expressed in brain, kidney and embryonic heart (Nakatani, Thompson et al. 1999). These Akt isoforms also show difference in their cellular localization. Akt1 is known to localize in the cytoplasm; Akt2 localizes in the nucleus, cytoplasm and mitochondria (Andjelkovic, Alessi et al. 1997, Calera, Martinez et al. 1998, Kupriyanova and Kandror 1999, Cantley 2002, Borgatti, Martelli et al. 2003). However, Akt3 is known to localize in the nuclear membrane (Kupriyanova and Kandror 1999, Yang, Tschopp et al. 2003). Knockdown of individual Akt isoforms does not affect the localization pattern of other Akt isoforms and also has been shown to have no compensatory effects on the level of other isoforms (Santi and Lee 2009). Numerous studies have been done in genetically engineered mice deficient for Akt1, Akt2 or Akt3 to confirm that these isoforms have different physiological functions (Peng, Xu et al. 2003). Variable phenotypic differences are observed following the genetic inactivation and/or removal of the Akt isoforms. Akt1 knockout mice exhibit retardation in growth and an increase in

perinatal lethality (Okano, Gaslightwala et al. 2000, Yang, Tschopp et al. 2003). Removal of Akt2 in mice results in insulin resistance and diabetes mellitus (Cho, Mu et al. 2001) whereas Akt3 knockout results in reduction in brain size and development (Easton, Cho et al. 2005). Double knockout mice harboring deficiency of both Akt1 and Akt2 isoforms die immediately after birth (Dummler and Hemmings 2007). However there are no studies available related to double knockdown of Akt3 in combination with Akt1 or Akt2.

Akt isoforms are aberrantly expressed in tumor conditions but these isoforms have tumor specific expression. Akt1 amplification is commonly observed in gastric cancer cells and knockdown of Akt1 increases the sensitivity of gastric cancer cells to chemotherapy as determined by treatment with cisplatin. Akt1 knockdown in gastric cancer cells increases the expression of BAX and reduces the expression of Bcl-2 thus increasing cell death *in vitro* and *in vivo* (Zhou, Fu et al. 2012). Akt2 is abnormally expressed in breast, ovarian and colon cancers (Suganuma, Fujiki et al. 1988, Chau and Ashcroft 2004, Easton, Cho et al. 2005). Akt3 undergoes amplification in breast and prostate cancer (Nakatani, Thompson et al. 1999, Bacus, Altomare et al. 2002, Zhou, Fu et al. 2012). In the following section, we have discussed the role of Akt isoforms in colorectal, breast, lung and pancreatic cancer. Numerous cross-talk and feedback mechanisms regulate PI3K/Akt signalling. Recently, Heron-Milhanet *et al.* has extensively reviewed the differential action of Akt1 and Akt2 in cancer and pointed towards the significance of targeting isoform-specific downstream events for the development of effective anti-cancer therapies involving Akt kinases (Heron-Milhavet, Khouya et al. 2011).

### 2.4.1 Colorectal cancer

Several studies have documented the inhibition of apoptosis as a critical event in the development of colorectal malignancies. The overexpression of anti-apoptotic proteins or the inactivation of pro-apoptotic proteins is a common event in colorectal carcinogenesis that is usually dependent on the genetic background of the tumor (Roy, Olusola et al. 2002). Sporadic colon cancers are

associated with inhibition of apoptosis in association with the loss of function of tumor suppressor adenomatous polyposis coli (APC), along with overexpression of anti-apoptotic Bcl-2 proteins (Chung 2000). However, CRC that originates due to DNA mismatch repair deficiency resulting in microsatellite instability (MSI) are usually associated with inactivating mutations of the pro-apoptotic BAX protein. About 6-15% of sporadic (non-hereditary) colon cancers are MSI positive, while the majority of tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC) are MSI positive (Lynch and Smyrk 1998). Roy *et al*. demonstrated for the first time that Akt overexpression is an early event during sporadic colon carcinogenesis (Roy, Olusola et al. 2002). This increased Akt expression was specific to sporadic colon carcinogenesis as opposed to the human MSI-high tumors represented by a series of HNPCC tumors. It was shown that 57% of all colorectal cancer specimens tested were positively immunostained for Akt. However, normal colonic mucosa and hyperplastic polyps expressed negligible levels of Akt. Roy *et al*. also reported for the first time that 57% of all adenomas overexpressed Akt indicating that overexpression of Akt is an early event in colon carcinogenesis. Interestingly, th Akt2 isoform was indicated as a predominant player in colon carcinogenesis. Additionally, Akt phosphorylation at Ser473 was detected in colon carcinomas but not in normal epithelium. This study concluded that Akt overexpression (especially Akt2) may be important in the early inhibition of apoptosis during colon carcinogenesis (Roy, Olusola et al. 2002).

The independent roles for Akt isoforms in regulating malignant progression in colorectal and breast cancer was recently reported by Yoeli-Lerner *etal*. (Toker and Yoeli-Lerner 2006, Yoeli-Lerner and Toker 2006) demonstrating that Akt1 overexpression inhibits the transcriptional activity of NFAT (nuclear factor of activated T cells) thus resulting in the blockade of invasion and migration. It is postulated that NFAT is targeted through Akt1-HDM2 (Human analogue of MDM2) pathway. HDM2 is an E3 ubiquitin ligase, that ubiquitinates its target proteins, such as NFAT, for proteasomal degradation. It was observed that NFAT degradation was rescued in the

presence of HDM2 siRNA (Yoeli-Lerner, Yiu et al. 2005). Feng *et al*. has shown that Akt1 stabilizes MDM2 by phosphorylating Ser166 and Ser188 residues, thus inhibiting its self-ubiquitination and increasing its stability. (Feng, Tamaskovic et al. 2004). In contrast, Akt2 overexpression up-regulates $\beta_1$-integrin (a component of collagen IV- binding receptor; referred to here as $\beta_1$) expression both *in vivo* and *in vitro* (Arboleda, Lyons et al. 2003). Increased $\beta_1$ expression is responsible for increased metastasis (Coffer and Woodgett 1992, Morini, Mottolese et al. 2000). This capability of Akt2 has been attributed to the fact that Akt2, in addition to regulating cell migration and invasion, also inhibits apoptosis. Thus Akt1 inhibits metastasis and invasion by degrading NFAT, however, Akt2 up-regulates $\beta_1$-integrin expression resulting in increased cell migration and invasion, depicting their contradictory roles in cancer. Amplification and overexpression of Akt2 has been shown to play a critical role in CRC metastatic colonization (Rychahou, Kang et al. 2008). Akt2 is a proto-oncogene, and is highly expressed in metastatic colon carcinoma as compared to primary colon cancer (Yuan, Sun et al. 2000). Genetic inactivation of Akt2 has been shown to result in reduced ability of colon carcinoma cells to metastasize thus confirming that Akt2 is required for the establishment of colon cancer metastasis (Ericson, Gan et al. 2010).However, the exact mechanism of Akt2-driven metastasis is poorly understood. Genetic inactivation of Akt1 and Akt2 results in reduction in clonal growth of colon cancer cells *in vitro*, but this reduction was much more significant when the cells were cultured in media lacking growth factors (Ericson, Gan et al. 2010).This led to the conclusion that tumor microenvironment plays a significant role in regulating the effects of gene inactivation (Vogelstein and Kinzler 2004). Inactivation of Akt1 and Akt2 also results in reduced metastasis to liver and reduced tumor burden (Ericson, Gan et al. 2010). Activated Akt regulates the expression of Bcl-2 and FAK (focal adhesion kinase) proteins mediating CRC metastasis (Lee, Kim et al. 2011, Wang and Basson 2011). In response to stress, Akt1 binds directly to FAK thus phosphorylating it at three serine residues (Ser 517, 601, 695) (Berwick, Hers et al. 2002).

Phosphorylation of the 3 serine residues in turn phosphorylate the tyrosine residue (Tyr397) thus activating it. This in turn induces cell adhesion by increasing the binding of integrins to matrix thus leading to increased metastasis (Thamilselvan, Craig et al. 2007). In addition to the overexpression of Akt isoforms in different cancer types, Akt phosphorylation in human CRCs has been shown to correlate with cell proliferation and apoptosis inhibition, and has also been demonstrated to increase with advancement of CRC (Itoh, Semba et al. 2002, Khaleghpour, Li et al. 2004). It is well documented that mTOR (mammalian target of rapamycin), which promotes growth, protein translation and metabolism is regulated by Akt kinase. The Sabatini laboratory has made the seminal discovery that mTOR is a direct substrate of Akt and identified Ser2448 residue as the Akt phosphorylation site on mTOR (Guertin and Sabatini 2007). The Evers laboratory has recently demonstrated that the mTOR1/mTOR2 proteins Raptor and Rictor are highly expressed in CRC tissues (Gulhati, Cai et al. 2009). Importantly, Rictor expression was correlated with pAKT Ser473 expression in CRCs derived from same patient. Johnson *et al.* showed that p85α, Akt1, Akt2 and pmTOR Ser2448 were all overexpressed in CRC compared to control and additionally reported that the expression of Akt1 and Akt2 was more pronounced in the left-sided CRCs compared to the right-sided CRCs (Johnson, Gulhati et al. 2010).

The molecular networks and pathways affected by the predicted mRNA targets of Akt2 in context of CRC metastasis signalling were depicted using the Ingenuity Pathway Analysis (IPA) tool and are shown in Figure 2.3. The IPA analysis of Akt2 revealed that Akt2 targets multiple genes frequently deregulated in cancer. Some of these target genes have been identified previously as directs targets for Akt2 and have been implicated in invasion and metastasis (including the MMP-9, EGF and SRC). Additionally, the predicted mRNA targets of Akt2 also included apoptosis regulators such as BAX, JNK/p38, TP53, NF-κB and Caspase3. Yuan et.al. demonstrated that Akt2 altered the cisplatin-induced apoptosis in ovarian cancer cells by regulating the ASK1/JNK/p38 pathway (Yuan, Feldman et al. 2003). ASK1 (apoptosis signal-

regulating kinase 1) is a member of the mitogen-activated protein kinase kinase kinase family that mediates cell death in response to various stresses including chemotherapeutic drugs such as cisplatin and paclitaxel (Yuan, Feldman et al. 2003). Stimulation of JNK/p38 activity has also been shown to be critical for cisplatin-induced apoptosis in certain cancer cell types. It was shown that AKT2 interacts with and phosphorylates ASK1 (apoptosis signal-regulating kinase 1) at Ser-83 residue. This leads to the inhibition of ASK1 kinase activity, blockade of JNK/p38 activation and activation of BAX. This work provided a new highlight on the role of Akt2 in regulating the ASK1/JNK/p38/BAX pathway and provided a new mechanistic insight into the anti-apoptotic effects of Akt2 (Yuan, Feldman et al. 2003).

### 2.4.2 Breast cancer

In breast cancer, Akt upregulates matrix metalloproteases (MMPs)-mediated matrix degradation resulting in increased metastasis (Jung, Park et al. 2010). In estrogen receptor-positive (ER+) breast cancer cells, Akt2 activation is triggered by ER-α. Akt2 expression is higher in Her2/neu breast cancer compared to Her2 negative tumors with increased resistance to stress-mediated cell death and less susceptibility to chemotherapeutics (Bacus, Altomare et al. 2002). Due to the intrinsic tyrosine kinase activity of Her2/neu, the receptor undergoes dimerization and activation, even in the absence of ligand, which in turn activates PI3K (Newby, Johnston et al. 1997, Zhou, Hu et al. 2000). Akt1 is upregulated in breast cancer samples although there is no significant correlation between Her2/neu and Akt1 expression (Bacus, Altomare et al. 2002). In contrast, ER-β causes reduction in Akt signalling in ERα positive breast cancer cells, thus affecting cell proliferation and survival along with increasing the sensitivity of cells for tamoxifen (ER antagonist) treatment (Lindberg, Helguero et al. 2011). Increased expression of Akt3 has been observed in hormone-unresponsive breast tumors thus contributing to their aggressive and metastatic phenotype. Chung *et al.*(Chung, Yao et al. 2012) showed that N-cadherin specifically suppressed Akt3 activity and promoted tumor cell migration in breast cancer cells, however, N-

cadherin had no effects on Akt1 and Akt2 isoforms, thereby associating Akt3 with epithelial-to-mesenchymal transition (EMT), a crucial step in metastasis. These studies were further confirmed by knockdown of Akt3, which resulted in increased cell motility with no effect on cell proliferation (Chung, Yao et al. 2012). The mechanism of reduction in Akt3 activity by N-cadherin is yet to be determined; however, it was observed that the expression of Akt3 mRNA was affected by N-cadherin but the protein expression remains unchanged, thus the transcription of Akt3 is reduced by N-cadherin.

### 2.4.3 Lung cancer

Grabinski *et al.* reported a high activity of Akt3 isoform in lung cancer cells that regulates the expression of cyclin D3, thus promoting G1-S transition of cell cycle (Cristiano, Chan et al. 2006). Akt3 siRNA knockdown reduced proliferation, survival and migration of lung cancer cells. In contrast, Akt1 siRNA knockdown resulted in a reduction in MEK/ERK1/2 activity (Rommel, Clarke et al. 1999) and IkB protein expression leading to cell death (Romashkova and Makarov 1999). However, reduction in Akt2 expression induces MCL-1 cleavage cell death through mitochondrial membrane potential loss and release of cytochrome c in the cytosol (Lee, Kim et al. 2011). Akt1 undergoes a mutation at the $17^{th}$ position (Glutamic acid is mutated to Lysine E17K) and this mutation is generally observed in breast, colon and ovarian cancers (Carpten, Faber et al. 2007). Recently, it was reported that non-small cell lung carcinoma (NSCLC) patients also possess a E17K mutation in Akt1 although the frequency is much less (Sung, Park et al. 2012). Kim *et al.* has confirmed that Akt1 polymorphism might be used as a prognostic marker for NSCLS (Kim, Kim et al. 2006). However, Sung et al reported that polymorphism in Akt2 and Akt3 is not associated with NSCLC (Sung, Han et al. 2008).

### 2.4.4 Pancreatic cancer

Akt activation due to mutation of the Kras or PTEN pathway is a potent survival signal in pancreatic cancer cell lines (Stambolic, Suzuki et al. 1998) and correlates with the aggressiveness of tumor. Specifically, Akt2 is amplified in 60% of pancreatic cancer (Schlieman, Fahy et al. 2003) and has been associated with increased growth and invasiveness in human ductal pancreatic cancer. There is a significant increase in the expression of Akt2 and pAkt ($p<0.01$) in pancreatic ductal adenocarcinoma (PDAC) (Yamamoto, Tomita et al. 2004). Akt2 is considered a prognostic marker for PDAC. Inhibition of Akt2 signaling by PI3K inhibitor results in the reduction in growth of pancreatic cancer cells *in vitro* and *in vivo*, by induction of apoptosis. Therefore Akt2 can be an important therapeutic target for treatment of pancreatic cancer (Ng, Tsao et al. 2000). It has been reported that Akt isoforms show differential roles in pancreatic cancer. Whereas Akt2 is responsible for poor prognosis, Akt1 is associated with favorable prognosis of pancreatic cancer (Yamamoto, Tomita et al. 2004). As mentioned above PHLPPs are responsible for dephosphorylation of Akt thus inactivating it. Nitschie *et al*. studied the expression of PHLPP1 and PHLPP2 in pancreatic cancer and observed a reduction in the expression of these phosphatases in PDAC as compared to normal pancreas. It was also reported that increased expression of PHLPP1 results in reduction in tumor growth confirming that it is the loss of phosphorylation of Akt2 that causes reduced growth of cancer cells (Nitsche, Edderkaoui et al. 2012).

**2.5 TGFβ-Akt signalling crosstalk regulates aberrant cell survival in CRC metastasis**

TGFβ signalling plays a critical role in cell survival and metastasis. TGFβ inhibitory/tumor suppressor responses are decreased with increasing malignant progression and in advanced-stages, TGFβ promotes invasion and metastasis. Substantial reports from our laboratory have established a loss or epigenetic silencing of TGFβ receptor expression in a wide range of cancer (Ammanamanchi, Freeman et al. 2003, Ammanamanchi and Brattain 2004, Huang, Zhao et al. 2005, Chowdhury, Howell et al. 2011). The mechanisms of epigenetic loss of TGFβ receptors

and their therapeutic implications have been reviewed in detail (Chowdhury, Ammanamanchi et al. 2009). Recently, we have demonstrated that reconstitution of TGFβ receptor expression in CRC cell lines with attenuated TGFβ receptor signalling resulted in the inhibition of metastatic colonization from primary colon tumors to lungs and liver. Significantly, invasion at the primary colon tumor was not prevented indicating that the effect of receptor reconstitution was on the ability to form progressively growing metastatic deposits at liver and/or lungs rather than preventing the initial steps in metastasis (Chowdhury, Howell et al. 2011). It has been shown that TGFβ-mediated apoptosis can be inhibited by insulin in hepatocytes and that the PI3K/Akt pathway (which is activated by insulin stimulation) is involved in this protective effect conferred by insulin (Tanaka and Wands 1996, Chen, Su et al. 1998, Buenemann, Willy et al. 2001). Although additional signalling pathways might contribute to TGFβ-induced responses, Smads are the only direct effectors known to act as transcription factors and therefore represent the most direct mediators for the transmission of TGFβ signalling from the cell surface to the nucleus (Kretschmer, Moepert et al. 2003). In systemic screening for Akt-associated proteins, Smad3 was identified as a binding partner (Conery, Cao et al. 2004, Remy, Montmarquette et al. 2004). We have shown previously that endogenous cellular TGFβ signalling increases apoptosis during growth factor deprivation stress (GFDS) in CRC cells (Wang, Yang et al. 2008). Further, the loss of autocrine TGFβ in colon cancer cells with attenuated TGFβ signalling resulted in increased PI3K/Akt activation and survivin expression, as well as resistance to GFDS-induced apoptosis (Wang, Yang et al. 2008). Interestingly, poorly metastatic FET colon cancer cells transfected with the neomycin vector (designated FETNeo) were compared with the highly metastatic FETDNRII cells that have been stably transfected with a dominant negative RII construct to knock out TGFβ signalling (Ongchin, Sharratt et al. 2009). In Figure 2.4 (unpublished data), the upper panel shows that relatively little Smad3 binds to Akt when TGFβ signalling is intact or in the absence of GFDS (“0” time for FETDNRII). When stress as reflected by GFDS is initiated there is rapid

induction of complex formation. In Figure 2.4, the lower panel shows that when GFDS is initiated in TGFβ autocrine competent cells there is a rapid loss of survivin expression. In contrast, loss of autocrine activity and Smad3/Akt complex formation results in the induction of survivin expression. The linkage of this apoptotic mechanism in human colon cancer cell lines to repression of survivin expression may be of significance, because survivin overexpression is strongly associated with poor prognosis in colon cancer (Wang, Yang et al. 2008). Survivin/XIAP complexes that mediate caspase inhibition have been shown to be a key cell survival mechanism enabling metastasis process (Dohi, Xia et al. 2007, Mehrotra, Languino et al. 2010). The complex is critical for stabilization of XIAP to inhibit caspases. Survivin and XIAP are in separate mitochondrial compartments, but are released by mitochondria into the cytoplasm in response to stress (such as metastatic growth in a foreign microenvironment) to promote cell survival. We recently identified a novel TGFβ/PKA signalling transduceome by which TGFβ-mediated cyclic AMP-independent PKA activation leads to disruption and subsequent destabilization of the survivin/XIAP complex to enable cell death due to PP2A mediated inhibition of Akt phosphorylation of a stabilizing XIAP site (S87) and direct phosphorylation of survivin at S20 (Figure 2.5). Moreover, we have shown that Akt phosphorylation was higher in the highly metastatic CRC cells with attenuated TGFβ receptor. However, reconstitution of TGFβ receptors in these CRC cells led to a reduction in Akt phosphorylation followed by a reduction in metastatic colonization (Simms, Rajput et al. 2012).

### 2.6 Clinical advancement of Akt as a therapeutic target

#### 2.6.1 MK-2206

MK-2206 is an orally active allosteric kinase inhibitor of Akt. The chemical name of MK-2206 is 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-*f*] [1,6]naphthyridin-3(2*H*)-one hydrochloride [1:1]. MK-2206 is a highly potent and selective Akt kinase inhibitor (Hirai, Sootome et al. 2010). MK-2206 binds to the pleckstrin homology (PH) domain of Akt and thus

inhibits its phosphorylation and translocation to the plasma membrane thus inactivating Akt. It is presently in phase II clinical trial in colon cancer and breast cancer. Hirai *et al*. (2010) have demonstrated similar levels of MK-2206-mediated inhibition of the enzymatic activity of different Akt isoforms using purified recombinant human Akt1 ($IC_{50}$, 5 nmol/L) and Akt2 enzyme ($IC_{50}$, 12 nmol/L) and approximately 5-fold less effectiveness against human Akt3 ($IC_{50}$, 65 nmol/L) (Hirai, Sootome et al. 2010). The authors showed for the first time the effectiveness of MK-2206 as a sensitizing agent for use in combination with various chemotherapeutic agents including docetaxel, carboplatin, gemcitabine, 5-fluorouracil (5-FU), doxorubicin, camptothecin, and RTK inhibitors such as lapatinib and erlotinib (Hirai, Sootome et al. 2010). In recent years, MK-2206 is being used in combination with EGFR and IGFR inhibitors in leukemia, breast, colon cancer and malignant gliomas (Cheng, Zhang et al. 2012). A recent study showed that MK-2206 has antitumor role in breast cancer *in vivo*, which was further augmented in the presence of paclitaxel (Sangai, Akcakanat et al. 2012). This combination of MK-2206 and paclitaxel is important since paclitaxel is used in the treatment of breast cancer. A report on nasopharyngeal carcinoma (NPC) reveals that MK-2206 inhibits growth of NPC cells by inhibiting Akt and mTOR signaling. MK-2206 treatment results in reduction in survival of pediatric cancer cell lines *in vitro* and *in vivo* (Gorlick, Maris et al. 2011). In T cell acute lymphoblastic leukemia it has been demonstrated that MK-2206 results in induction of apoptosis, autophagy and cell cycle arrest (de Frias, Iglesias-Serret et al. 2009). The treatment of MK-2206 in various cancer conditions as a single agent or in combination with other inhibitors has been effective in diminishing the tumor growth and reducing cell survival of cancer cells. Thus MK-2206 has been demonstrated as a promising anti-cancer agent that potentially augments the effectiveness of the current front line cancer therapeutic drugs.

**2.6.2 Perifosine**

Perifosine is an allosteric inhibitor that binds the PH domain of Akt, thus preventing the translocation to plasma membrane (Pinton, Manente et al. 2012). It inhibits the activation of Akt signaling, thus reducing cell survival in many cancer conditions like multiple myeloma, T-cell acute lymphoblastic leukemia, NSCLC, prostate and ovarian carcinoma (Ma, Gibson et al. 2006, Yang, Fraser et al. 2006, Floryk and Thompson 2008, Richardson, Wolf et al. 2011) . It has been reported that perifosine in combination with curcumin results in increased cell death and cell proliferation of CRC cells *in vivo* and *in vitro*. It is believed that this combination inhibits Akt and NF-kB signalling and increases endoplasmic stress and thus plays an anti-tumorigenic role (Chen, Wu et al. 2012). The role of perifosine was studied in hepatocellular carcinoma and it was found that this Akt inhibitor results in increased apoptosis by affecting the phosphorylation of ERK and JNK (Chiarini, Del Sole et al. 2008). In a study in hepatocellular carcinoma it was observed that perifosine in combination with cisplatin increases apoptosis by increasing the expression of BAX and by reduction in the expression of Bcl-2 (Chiarini, Del Sole et al. 2008). A recent report by Richardson *et al*. has revealed that periforsine in combination with bortezomib shows promising results in patients suffering from multiple myeloma with a response rate of 40% (Richardson, Wolf et al. 2011).

### 2.6.3 Deguelin

Deguelin is a plant product derived from rotenone, which is used as an important chemotherapeutic agent. It plays anti-tumorigenic role by affecting multiple pathways, such as the inhibition of PI3K/Akt pathway, suppressing the expression of cyclooxygenase 2 (COX2) and cyclin D1 expression (Nair, Shishodia et al. 2006) . Deguelin causes an increase in apoptosis both *in vivo* and *in vitro* in a dose and time-dependent manner by inhibiting phosphorylation of Akt in lung cancer. It is a heat shock protein 90 (Hsp90) inhibitor with potent anti-angiogenic activity (Kim, Chang et al. 2008). This drug inhibits Akt activity both via PI3K dependent and PI3K independent pathways (Chun, Kosmeder et al. 2003). Deguelin suppresses the growth of colon

cancer cells in mice by modulating the NF-kB and Wnt signalling pathway. However, it is a potent inhibitor of complex-1 of electron transport chain and its long-term use can result in Parkinson's disease thus making it incompetent for clinical trials (Kim, Chang et al. 2008). Li *et al.* studied the role of deguelin in murine myeloma cell proliferation and observed that it plays a pro-apoptotic role by upregulation of BAX, down-regulation of Bcl-2 and activation of caspase3 (Li, Wu et al. 2012).

### 2.6.4 A443654 and Akti1/2

A443654 is a potent ATP competitive inhibitor of Akt activity. It inhibits all isoforms of Akt with equal potency. Another Akt inhibitor Akti1/2, which is an ATP non-competitive inhibitor, inhibits Akt1 and 2 but not Akt3 (Gilot, Giudicelli et al. 2010). Both these inhibitors are being studied in chronic lymphocytic leukemia (CLL). Treatment of CLL cells with these inhibitors results in reduced expression of MCL-1, which is a critical survival protein in CLL cells (de Frias, Iglesias-Serret et al. 2009). It has also been shown that A443654 increases phosphorylation of Akt at Ser473 thus resulting in increase in Akt activity. This increase in Akt activity is considered as a feedback response of the cell to maintain activation of Akt (Han, Leverson et al. 2007).

### 2.6.5 GSK690693

GSK690693 is a potent ATP competitive Akt inhibitor that inhibits phosphorylation of downstream targets. It is given intravenously to patients with hematological malignancy (Levy, Kahana et al. 2009). Altmore *et al.* has studied the effect of this inhibitor on mouse model that develop spontaneous athymic lymphoma, endometrial carcinoma or ovarian cancer. Since all these mouse models had activated Akt, treatment with GSK690693 results in reduction in tumor progression, phosphorylation of Akt substrates, cell proliferation and increased apoptosis (Altomare, Zhang et al.). This Akt kinase inhibitor has been shown to reduce tumor growth in

xenograft models for ovarian, prostate and breast tumors (Rhodes, Heerding et al. 2008). It has been reported that long-term use of this GSK690693 inhibits glycogen synthesis and activates glycogenolysis thus resulting in hyperglycemia as one of the major side effects and making this drug unfit for further clinical trials (Crouthamel, Kahana et al. 2009). It is being used in combination with other inhibitors, such as mTOR inhibitor and have shown to induce cell death in non-small lung cancer cells (Jeong, Choi et al. 2012).

### 2.6.6 HSP90 inhibitors

HSP90 is a molecular chaperone which plays an important role in the stability and post-translational maturation and activation of some of the proteins which are implicated in oncogenesis (Mahalingam, Swords et al. 2009). HSP90 is responsible for maturation of certain cell signalling proteins such as Raf Serine kinases, CDK4 and receptor tyrosine kinases (Xu, Marcu et al. 2002). It has been reported that HSP90 is overexpressed in number of cancers and in virally-transformed cells (Flandrin, Guyotat et al. 2008). HSP90 is a dimer and contains an ATP binding pocket, which is required for its optimal activity. It was reported that amino acid residues (229-309) of Akt are responsible for binding to HSP90. The formation of Akt –HSP90 complex stabilizes Akt activity by preventing its dephosphorylation from PP2A. Stabilization of Akt in phosphorylated state prevents apoptosis in the normal cells making them tumorigenic (Sato, Fujita et al. 2000). Thus, HSP90 has now become an attractive target in anti-cancer therapy. Multiple small molecule inhibitors are being used to inhibit HSP90-client protein interaction that leads to client protein degradation (Neckers 2002). Whitesell *et al.* reported that benaoquinone anamycins like geldanamycin inhibit HSP90 by binding at the ATP binding pocket and thus release the client protein (such as Akt) and directs it to proteasome for degradation, thus resulting in shortening of half-life of Akt from 36 h to 12 h (Whitesell, Mimnaugh et al. 1994). Geldanamycin is a naturally occurring drug, which is produced by microorganisms and is an HSP90 inhibitor, that is being used in combination with chemotherapy and radiotherapy for tumor

suppression in preclinical models. In human neuroblastoma and multiple myeloma studies, HSP90 inhibitor was combined with Akt inhibitor perifosine demonstrating increased anti-tumor activity (Kim, Kang et al. 2003, Mitsiades, Mitsiades et al. 2006). It is presently in phase II clinical trial for Her2-positive breast cancer in combination with Herceptin (Modi, Stopeck et al. 2007).

## 2.7 Conclusions

As discussed in this chapter, Akt acts a nodal onco-protein critical for regulating cell survival and metastasis of cancer cells. Thus, targeting Akt as a potential anti-metastatic therapeutic strategy either as a single agent or in combination holds significant promise.

## 2.8 Figures

**Figure 2.1: PI3K/Akt signalling in cancer**

**Figure 2.1:**

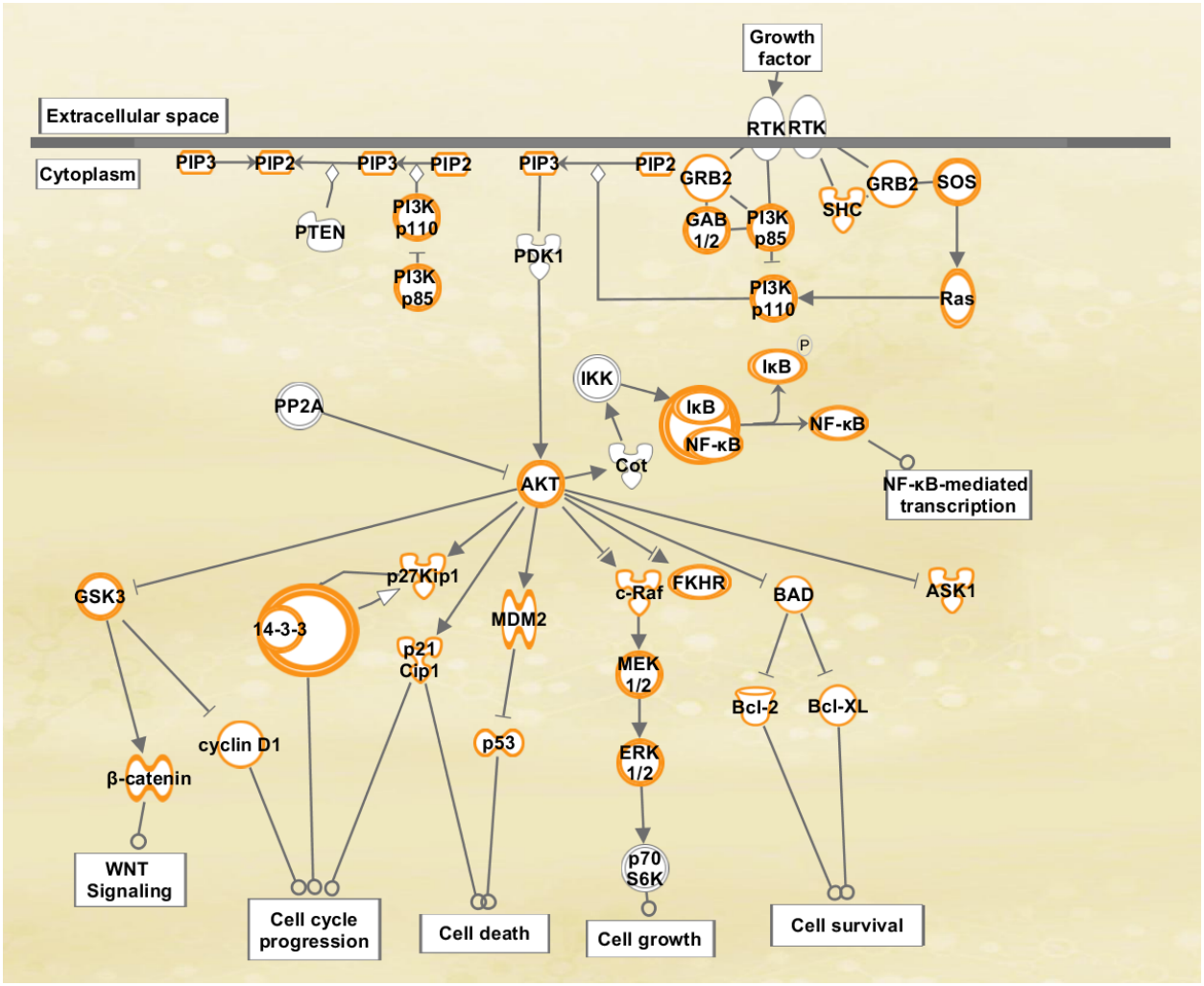

Growth factor
Extracellular space
Cytoplasm
RTK
RTK
PIP3
PIP2
PIP3
PIP2
PIP3
PIP2
GRB2
GRB2
SOS
SHC
PTEN
PI3K p110
PI3K p85
GAB 1/2
PI3K p85
PDK1
PI3K p110
Ras
IκB
P
IKK
IκB
NF-κB
NF-κB
PP2A
Cot
AKT
NF-κB-mediated transcription
p27Kip1
GSK3
14-3-3
MDM2
c-Raf
FKHR
BAD
ASK1
p21 Cip1
MEK 1/2
Bcl-2
Bcl-XL
cyclin D1
p53
ERK 1/2
β-catenin
p70 S6K
WNT Signaling
Cell cycle progression
Cell death
Cell growth
Cell survival

**Figure 2.2: Akt isoform functions in cancer**

**Figure 2.2:**

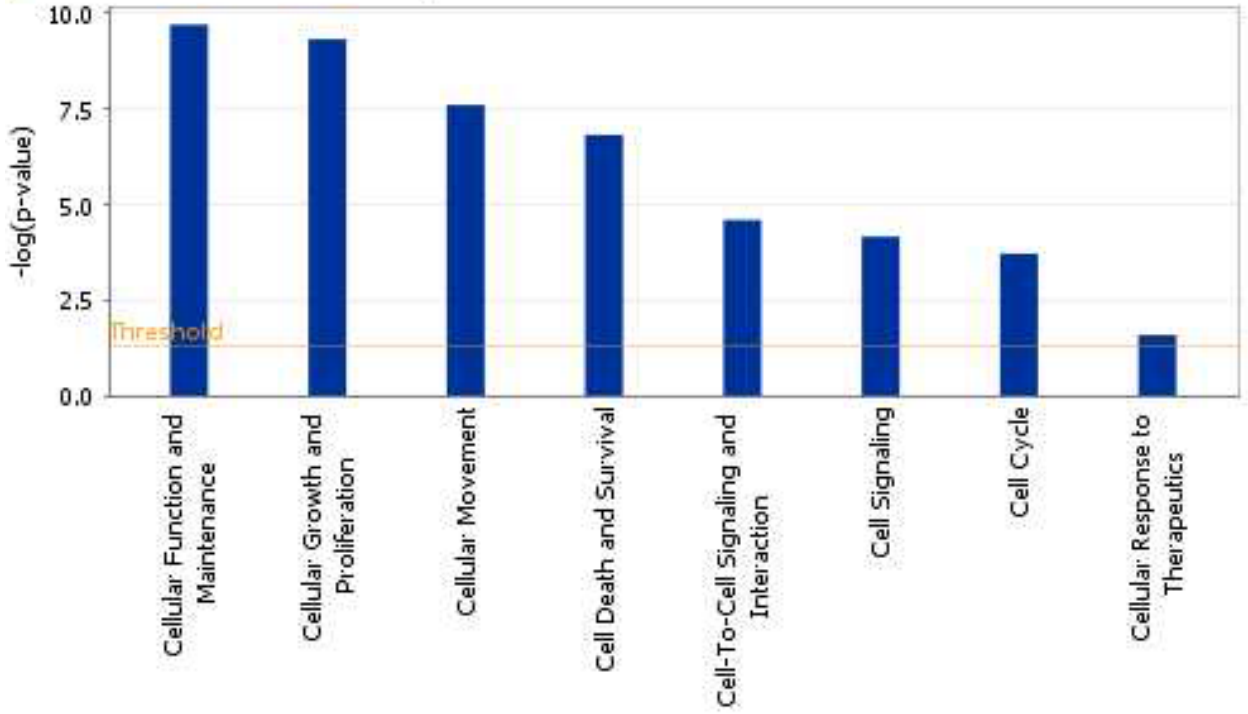

10.0
7.5
5.0
2.5
0.0
-log(p-value)
Threshold
Cellular Function and Maintenance
Cellular Growth and Proliferation
Cellular Movement
Cell Death and Survival
Cell-To-Cell Signaling and Interaction
Cell Signaling
Cell Cycle
Cellular Response to Therapeutics

**Figure 2.3: Molecular interactions of Akt2 in colorectal cancer metastasis signalling**

**Figure 2.3:**

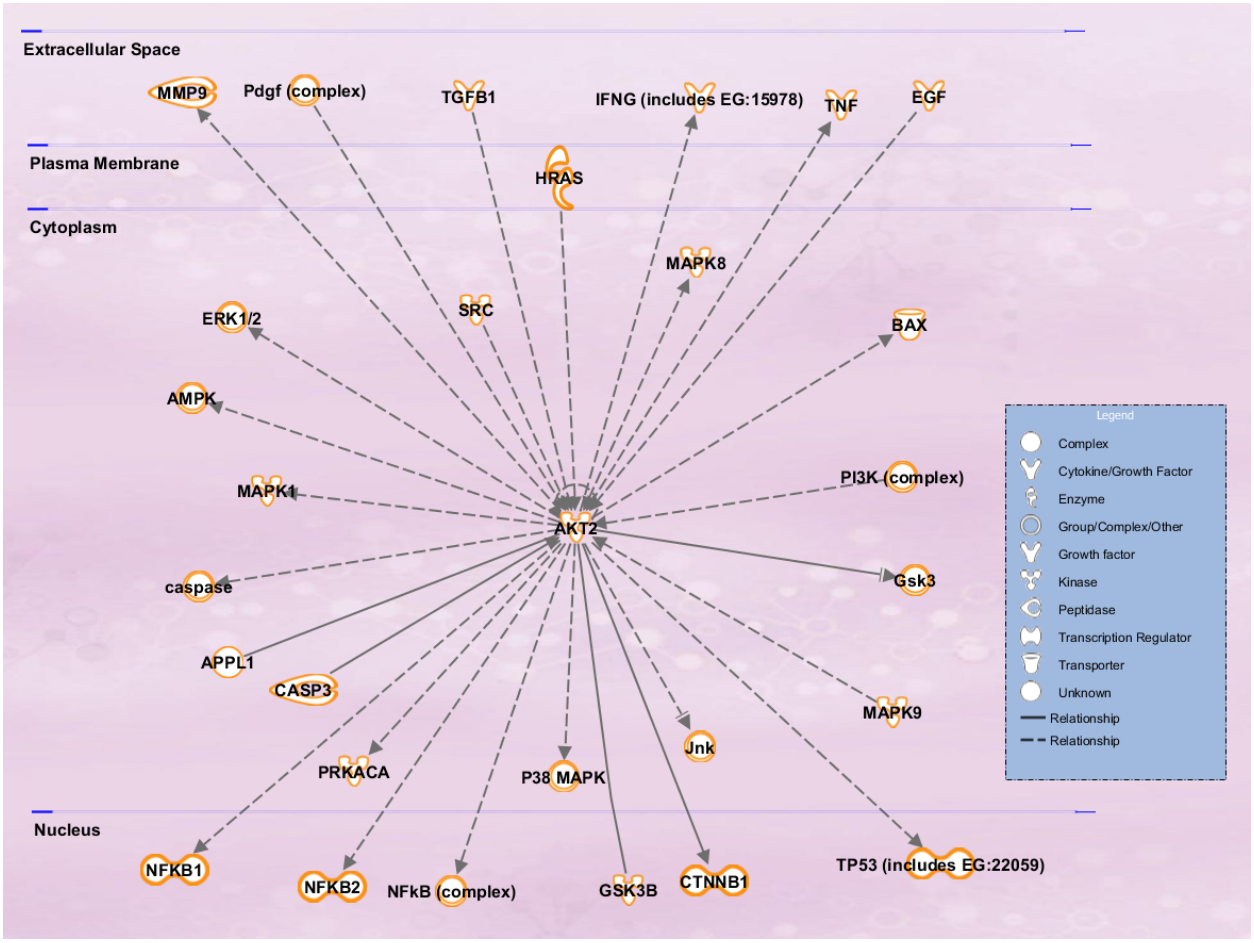

Extracellular Space
Plasma Membrane
Cytoplasm
Nucleus
MMP9
Pdgf (complex)
TGFB1
IFNG (includes EG:15978)
TNF
EGF
HRAS
MAPK8
ERK1/2
SRC
BAX
AMPK
MAPK1
PI3K (complex)
AKT2
caspase
Gsk3
APPL1
CASP3
MAPK9
Jnk
PRKACA
P38 MAPK
NFKB1
NFKB2
NFkB (complex)
GSK3B
CTNNB1
TP53 (includes EG:22059)
Legend
Complex
Cytokine/Growth Factor
Enzyme
Group/Complex/Other
Growth factor
Kinase
Peptidase
Transcription Regulator
Transporter
Unknown
Relationship
Relationship

**Figure 2.4: Loss of autocrine TGFβ leads to complex formation between Akt & Smad3 during stress**

**Figure 2.4:**

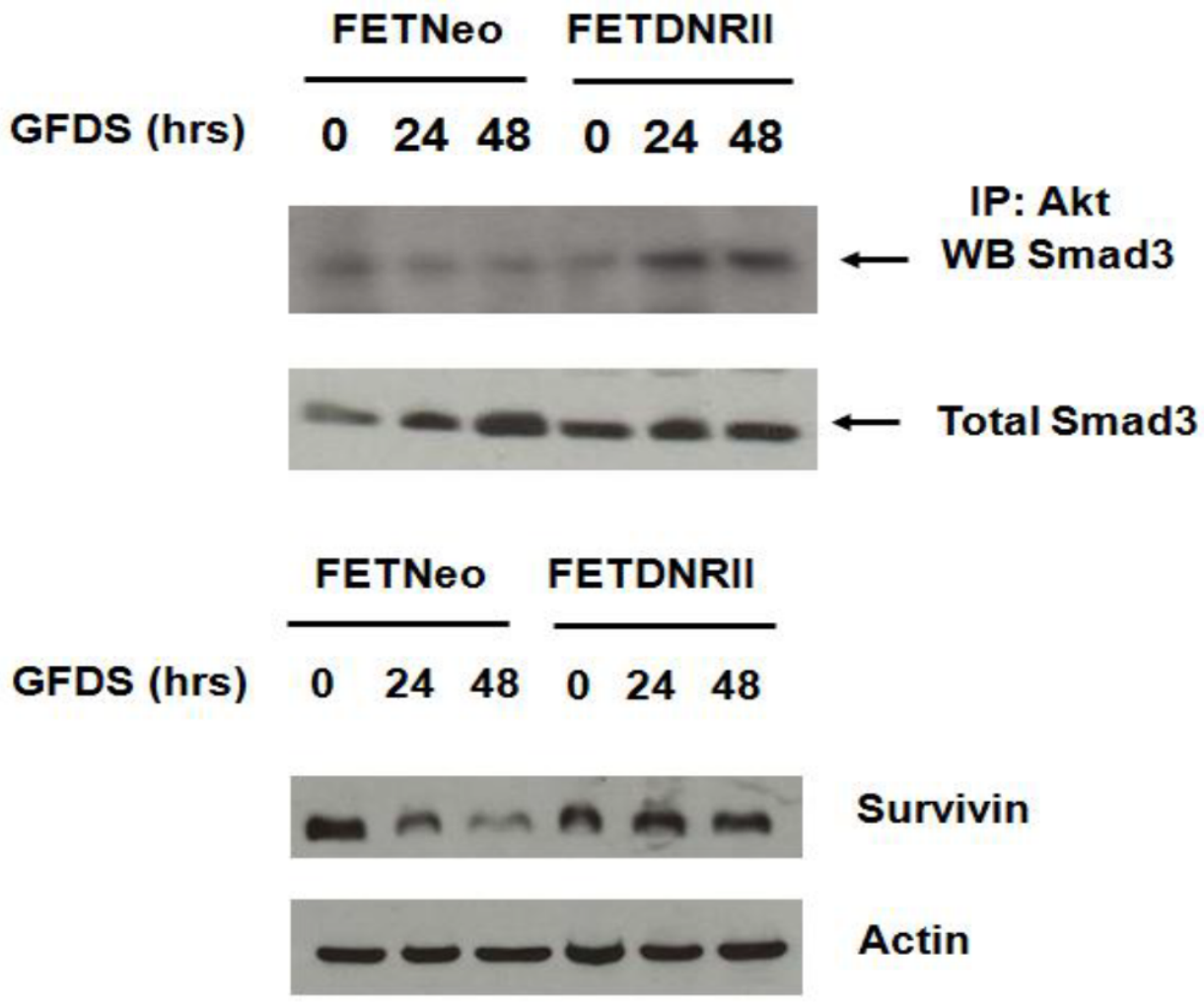
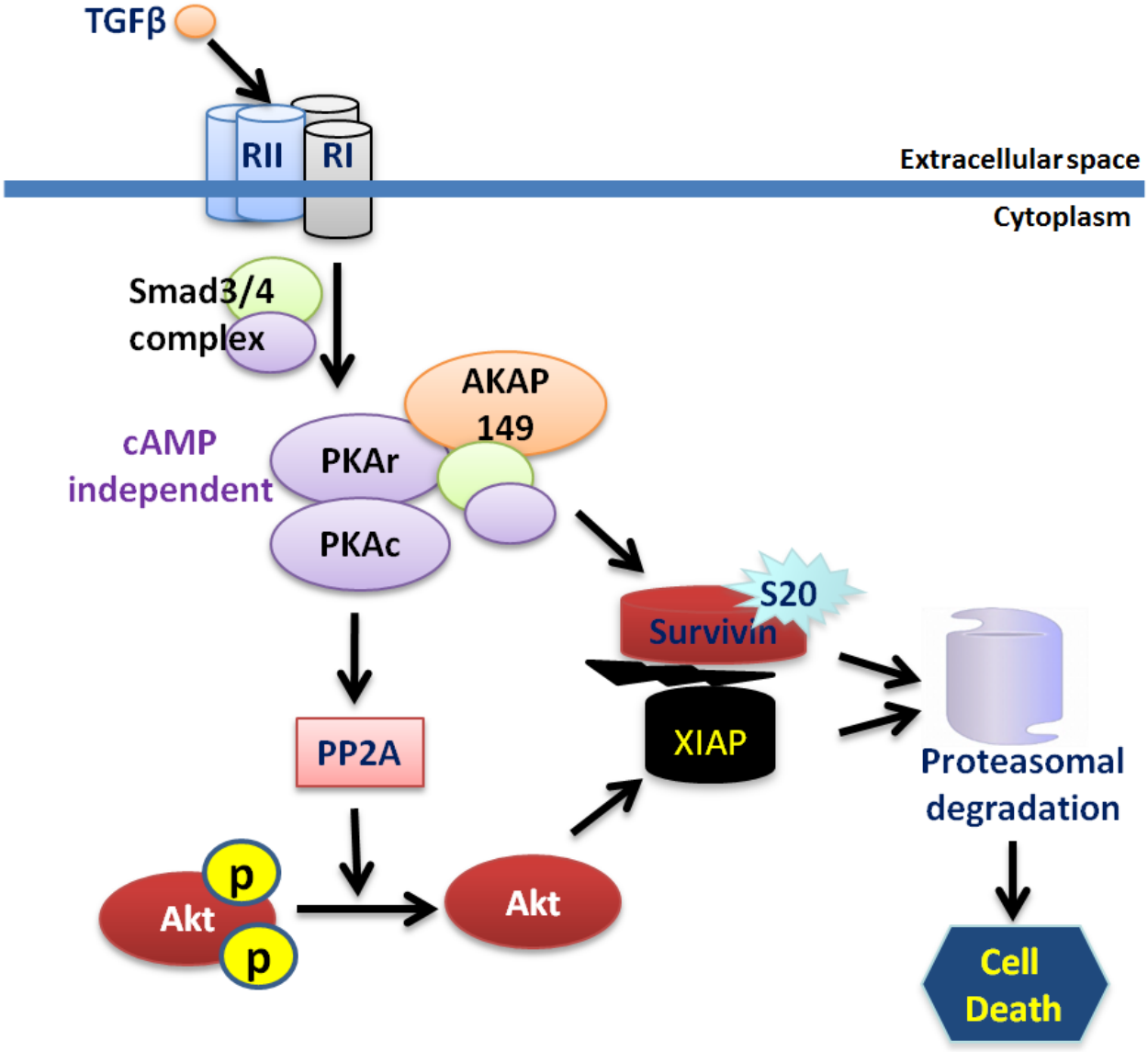

FETNeo
FETDNRII
GFDS (hrs)
0 24 48 0 24 48
IP: Akt
WB Smad3
Total Smad3
FETNeo
FETDNRII
GFDS (hrs)
0 24 48 0 24 48
Survivin
Actin

**Figure 2.5: TGFβ/PKA mediated Akt regulation leading to cell death in CRC**

**Figure 2.5:**


TGFβ
RII
RI
Extracellular space
Cytoplasm
Smad3/4
complex
AKAP
149
cAMP
independent
PKAr
PKAc
S20
Survivin
XIAP
PP2A
Proteasomal
degradation
p
Akt
p
Akt
Cell
Death

# CHAPTER III

## Materials and Methods

## 3. Materials and Methods

### 3.1 Cell lines and reagents

GEO (Wang, Han et al. 1996) and CBS (Ye, Foster et al. 1999) colon carcinoma cells were cultured in serum-free (SF) medium (McCoy's 5A with pyruvate, vitamins, amino acids and antibiotics) supplemented with 10 ng/ml epidermal growth factor, 20 μg/ml insulin and 4 μg/ml transferrin at 37°C in a humidified atmosphere of 5% $CO_2$ (Chowdhury, Howell et al. 2011, Chowdhury, Howell et al. 2011). When the cells were under GFDS (growth factor deprivation stress) (Wang, Yang et al. 2008), they were cultured in Supplemental McCoy's (SM) medium in the absence of growth factor or serum supplements for the indicated times as described previously (Wang, Yang et al. 2008). HCT 116 (IGF1R- independent colon cancer cell lines) and MiaPaCa (pancreatic cancer cells with constitutive activation of IGF-1R) cells were used as control to demonstrate the specificity of the dose of the kinase inhibitor. The *in vivo* experiments were carried out with GEO cells stably transfected with a GFP vector to visualize the tumor size. MK-2206 was provided by Merck and Co., Inc. MK-2206 was dissolved in DMSO for *in vitro* experiments. However, for *in vivo* experiments 30% Captisol (Cydex Pharmaceuticals) was used as a vehicle for the drug. In *in vitro* experiments the control cells were treated with DMSO. The control animals also received 30% Captisol. AIF inhibitor, N- Phenylmaleimide was purchased from Sigma.

### 3.2 Inducible knockdown of Akt isoforms

Three different shRNA in pTripZ Doxycycline (Dox) inducible vector for each Akt isoform were purchased from Thermoscientific Open Biosystems (Clone ID for Akt1: V2THS_192049:V3THS_358718:V3THS_358720) (Clone ID for Akt2: V2THS_237948: V3THS_325553: V3THS_325558) (Clone ID for Akt3: V2THS_68871: V2THS_68872: V2THS_68874) (Clone ID for Non-targeting: RHS4743). The pTripZ vector has Red Florescent

Protein (RFP), which is induced on addition of Dox, thus the cells infected with this vector show red florescence under immunofluorescence microscope on treatment with Dox. HEK293T cells were transfected with the vector. The lentivirus formed was infected in GEO and CBS GFP-tagged cells. The cells were selected and maintained in puromycin (4ug/ml) (Geng, Chaudhuri et al. 2013). The shRNA was induced by adding Dox (1ug/ml) for five-days.

**3.3 shRNA mediated knockdown of MTSS1**

MTSS1 shRNA and Non Silencing shRNA in GIBz vector was purchased from Open Biosystems (Clone ID for MTSS1: V2THS_229344 Clone ID for Non-targeting: RHS4346). Transfections were done in HEK293T cells followed by infection in GEO GFP Akt2 knockdown cells using manufacturer's instructions.

**3.4 Retroviral knockdown of Akt1 and Akt2**

Small hairpin RNA sequence for Akt1si, Akt2si, Akt3si and scramble si were cloned and expressed in a retroviral expression vector pSUPER.Retro.Puro (Oligoengine). 293T derived Phi-NX cells were used for transfection. A 19-nucleotide sequence for Akt1, Akt2 and Akt3 were designed from Dharmacon si design center. The target sequence for Akt1 5'GAGACTGACACCAGGTATT 3' was 1634 bases while that for Akt2 5'TGAATGAGGTGTCTGTCAT 3' selected was 301 bases downstream of 5'UTR. Akt3 target sequences selected were 5'GCAAAATGCCAGTTAATGA 3'. Another non-targeting small hairpin siRNA was used as an experimental control. The GEO cells were stably transfected with siRNA to reduce the expression of Akt1, Akt2 and Akt3. The cells were selected with Puromycin (4 μg/ml) and the resistant cells were pooled. Stable cell lines with Akt1, Akt2 and Akt3 knockdown were maintained in serum free medium with puromycin (4 μg/ml).

**3.5 RNA interference studies**

XIAP siRNA (ON-TARGET plus Human XIAP (331) siRNA smart pool) , AIF si RNA (ON-TARGET plus Human AIF siRNA smart pool) were purchased from Thermo scientific and transient transfections were done as per manufacturer's protocol.

**3.6 Proliferation Assay**

GEO and CBS cells were plated at a density of $8X10^3$ cells per well in a 96 well plate. After 72 h the cells were treated with increasing concentrations of MK-2206. Cell proliferation was measured after 48 h by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay as described previously (Hu, Patil et al. 2008).

**3.7 DNA Fragmentation Assay**

Cells were seeded in 96 well plates at the same density as for proliferation assays. MK-2206 was treated 72 h after plating the CRC cells. DNA fragmentation assays were performed after 48 h of treatment using a Cell Death Detection ELISA plus kit (Roche) according to the manufacturer's protocol as described previously (Wang, Yang et al. 2008). To confirm AIF mediated cell death, DNA fragmentation was performed by pretreating the cells with AIF inhibitor (50μM/L) for 1 h prior to treatment with MK-2206 for 48 h. Additionally a DNA fragmentation assay was performed after siRNA-mediated knockdown of AIF followed by treatment with MK-2206 for 48 h. GEO cells were treated with XIAP siRNA for 48 h and then DNA fragmentation was performed to confirm the effect of XIAP on cell death.

**3.8 Transwell Migration Assay**

Transwell migration inserts with 1.2u pores were purchased from BD Biosciences (Cat#P1XP01250). 80,000 cells were plated in SM media in the transwell. 0.7 ul of serum-rich media was kept in the bottom chamber. The transwell inserts with the cell suspension was carefully placed into the chamber with serum-rich media. The plate was incubated for 32 hrs. The cells were fixed using 4% paraformaldehyde followed by staining with crystal violet.

### 3.9 RNA Isolation and Quantitative Real Time PCR

RNA were collected using the High Pure RNA Isolation kit (Roche Applied Science)(Wang, Yang et al. 2008). The two-step quantitative PCR using TaqMan reagents were performed according to the manufacturer's instructions (Applied Biosystems). The mRNA expression was normalized to GAPDH.

### 3.10 $RT^2$ Profiler PCR Array Analysis

Real-time PCR was performed on cDNA samples from Non-targeting shRNA and Akt isoform knockdowns using the Human Tumor Metastasis $RT^2$ Profiler PCR array (Super Array Bioscience, Frederick, MD, USA) in a C1000 Thermal Cycler from BioRad according to manufacturer's instructions. Data were normalized for GAPDH levels by the ΔΔCt method.

### 3.11 Subcellular fractionation

Cells were washed with ice-cold phosphate buffer saline (PBS) twice. The cells were suspended in 1ml of PBS and centrifuged for 1 min at 4°C. The supernatant was removed, the pellet was dissolved in 1ml of CE buffer, and samples were vortexed for about 15 sec. The samples were kept on ice for an hour, passed through a syringe every 20 minutes and centrifuged for 1 min. The supernatant was collected and the pellet was left out to isolate nuclear extract. The supernatant was centrifuged again for 1 min to get rid of any debris. The supernatant isolated now was designated as the cytoplasmic extract and was stored at -80°C. Nuclear extract buffer was added to the pellet and the sample was vortexed for 20 seconds. The samples were kept on ice for an hour and sonicated twice for 10 seconds at 60% amplitude. The samples were centrifuged for 20 min at 4°C and supernatant collected was stored at -80°C.

### 3.12 Western Blot Analysis and Immunoprecipitation

Cells were lysed in a buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L NaVO3, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 25 μg/mL aprotinin, 25 μg/mL trypsin inhibitor, and 25 μg/mL leupeptin. The supernatants were cleared by centrifugation at 4°C. Protein concentration was measured by bicinchoninic acid assay (Pierce) using a Biotek 96 well plate reader. Protein (30–100 μg) was fractionated by electrophoresis on a 10% acrylamide denaturing gel and transferred onto a nitrocellulose membrane (Life Science, Amersham) by electroblotting. The transfer on the nitrocellulose membrane was routinely confirmed by Ponceau S staining. The membrane was blocked with 5% nonfat dry milk in TBST [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.05% Tween 20] for 1h at room temperature or overnight at 4°C and washed in TBST. The membrane was then incubated with primary antibodies at 1:200-1:1000 in TBST overnight at 4°C. After washing with TBST for 15 min, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody (Life Science, Amersham) at 1:1000 dilutions for 1h at room temperature. The proteins were detected by the enhanced chemiluminescence system (Amersham). Immunoprecipitation was performed with 500 μg of protein samples using magnetic beads (Millipore) according to manufacturer's protocol. Antibodies were purchased from Cell Signaling for tAkt, pAkt (S473), pAkt(T308), AIF (Apoptosis Inducing Factor), pEzrin (Thr567), Akt1, Akt2, Akt3 survivin, Bad and pBad (S136). Ezrin antibody was purchased from Santa Cruz. XIAP antibody was purchased from Abcam.

**3.13 Immunofluorescence**

The translocation of AIF from mitochondria to nucleus was determined by an immunofluorescence assay. GEO cells were plated on a cover slip in a six well plate. When the cells were 60-70% confluent, culture medium with 400nm of Mitotracker (CMX Ros, Invitrogen) was added to the cells. The cells were checked for red fluorescence under the microscope after

one hour. The cells were stained, washed with growth medium and fixed by placing in ice-cold methanol for 5 minutes. The cells were washed with PBS, permeabilized by incubating with PBS containing 0.1% Triton X-100 for 15 minutes and subsequently blocked with 10% normal goat serum. After one hour of blocking, the cells were incubated with primary antibody for AIF (1:100) for 2h. Fluorescein isothiocyanate- conjugated anti rabbit antibody (FITC) was used as the secondary antibody. Nuclei were counter stained with 4'-6 diamino-2- phenylindole and mounted on glass slide in anti fade vecta shield mounting medium (vector labs). An LSM 510 microscope (Carl Ziess GmbH, Oberkochen, Germany) was used to perform laser confocal microscopy.

**3.14 Xenograft Studies**

All the experiments involving animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. 3-5 week old athymic nude mice (N=16) were purchased from NCI. $7X10^6$ GEO GFP-labeled cells were subcutaneously injected on one side in the right flank pad of mice and allowed to form xenografts. When the tumor size was approximately 100 $mm^3$, 120 mg/kg body weight of MK-2206 was administered orally. Captisol was used as a vehicle for the drug and the control animals were treated with vehicle only. MK-2206 was given orally for 3 weeks on alternate days. The dose and the duration mentioned in the study have been provided by Merck and Co. Tumor growth and body weight were measured every other day. The tumor size was measured manually with calipers, and the tumor volume was calculated using the formula ($l^2$ x h x $\pi/6$). We used Near-IR enhanced Macro Imaging System Plus Cooled with the LT-99D2 with the Dual Tool dual excitation upgrade for viewing the 2D image of the tumor as well as to image the mice. All *in vivo* characterizations were confirmed in at least 3 independent control and MK-2206 treated animals.

**3.15 Orthotopic Implantation Studies**

All the experiments involving animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. 3-5 week old athymic nude mice (N=75, 25 animals in each group) were purchased from NCI. Orthotopic implantation was performed as previously described (Wang, Yang et al. 2008, Geng, Chaudhuri et al. 2013). Briefly, green fluorescent protein (GFP)-labeled GEO Non-targeting sh, Akt1 sh and Akt2 shcells ($7 \times 10^6$) were subcutaneously injected onto the dorsal surfaces of separate athymic nude male mice and allowed to grow to 500 $mm^3$. Once xenografts were established, they were excised and minced into 1-mm3 pieces. Two of these pieces were then orthotopically implanted into the colon of other athymic nude mice. For operative procedures, animals were anesthetized with isoflurane inhalation. A 1-cm laparotomy was performed and the cecum and ascending colon were exteriorized. Using 7X magnification and microsurgical techniques, the serosa was disrupted in two locations. Pieces of xenograft (1 $mm^3$) were subserosally implanted using an 8–0 nylon suture at the disrupted serosal locations. The bowel was then returned to the peritoneal cavity and the abdomen was closed with 5–0 vicryl suture. Fluorescence imaging was performed weekly on the animals to follow tumor growth (LightTools). After six weeks of orthotopic implantation, Dox (2mg/ml) was introduced in the drinking water of the animals for 3 weeks. Animals were euthanized at 9 weeks after implantation. Organs were explanted, imaged, and immediately placed in buffered 10% formalin. Tissues were then processed and embedded in paraffin. Histological slides were cut for H&E staining. Metastases were determined by histological evaluation of each liver lobe and both lungs as described in detail (Guo, Rajput et al. 2007, Wang, Yang et al. 2008).

### 3.16 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Sections (4 μM) from paraffin-embedded blocks were stained according to the Apotag terminal nucleotidyl transferase mediated nick end labeling (TUNEL) assay kit. The apoptotic rates were

determined by counting the number of positively stained apoptotic bodies at 40X magnification. Ten different fields were randomly selected per slide for analysis. The ratio of the average number of apoptotic cells to the total number of cells counted was used to determine apoptotic rates (Wang, Yang et al. 2008, Geng, Chaudhuri et al. 2013).

**3.17 Hematoxylin and Eosin staining and Ki67 staining**

Sections (4 μM) from paraffin-embedded blocks were used for H and E staining and for Ki67 IHC using antibody for Ki67 from BD biosciences. Ki67 is a non-histone nuclear antigen present in late G1, G2 and S phase of cell cycle but absent in $G_0$. The dilution of Ki67 antibody used was 1:100. The proliferation rate was determined quantitatively by utilizing Definience software (public domain software). Ten different, but histologically similar fields per sample were selected for analysis (Wang, Yang et al. 2008, Geng, Chaudhuri et al. 2013).

**3.18 Immunohistochemistry**

The slides were deparafinized by keeping them at 60°C for 1 h and then rehydrated using graded alcohol for 5 min each. Subsequently the slides were treated with 0.3% $H_2O_2$/methanol for 10 min and then submerged in 95°C citrate buffer (pH=7.8) for 15 min. Blocking was performed in 5% normal goat serum for 1h at room temperature and then the slides were incubated with primary antibody for Akt1, Akt2, MTSS1 and XIAP at 4°C overnight. The slides were treated with biotinylated secondary antibody for 30 min at RT, followed by incubation with streptavidin peroxidase complex (Invitrogen). Reaction products were developed using diaminobenzidine containing 0.3% $H_2O_2$ as a substrate for peroxidase (Dako). Nuclei were counterstained with hematoxylin (Protocol)(Wang, Yang et al. 2008, Geng, Chaudhuri et al. 2013). To determine the difference in staining intensity of Akt1, Akt2, MTSS1 and XIAP, 10 different but histologically similar fields were selected per sample and the slides were analyzed Definience Tissue Studies 64

(Dual 4.1) software. The staining intensity measured by the software was plotted using Graph pad 5.0.

**3.19 Statistical analysis**

Statistical analysis was performed using Graph pad 5.0 software for student's t test. A p value of less than 0.05 was considered significant.

# CHAPTER IV

## Akt inhibitor MK-2206 promotes anti-tumor activity and cell death by modulation of AIF and Ezrin in colorectal cancer.

Ekta Agarwal[1,2], Anathbandhu Chaudhuri, Premila D. Leiphrakpam[2], Katie L. Haferbier[2], Michael G. Brattain[2*], Sanjib Chowdhury [2*]

[1] Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA

[2] Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA

*Corresponding author

**This chapter has been published in BMC Cancer (2014) Mar 1; 14:145.**

## 4. Akt inhibitor MK-2206 promotes anti-tumor activity and cell death by modulation of AIF and Ezrin in colorectal cancer.

### 4.1 Abstract

There is extensive evidence for the role of aberrant cell survival signaling mechanisms in cancer progression and metastasis. Akt is a major component of cell survival-signaling mechanisms in several types of cancer. It has been shown that activated Akt stabilizes XIAP by S87 phosphorylation leading to survivin/XIAP complex formation, caspase inhibition and cytoprotection of cancer cells. We have reported that TGFβ/PKA/PP2A-mediated tumor suppressor signaling regulates Akt phosphorylation in association with the dissociation of survivin/XIAP complexes leading to inhibition of stress-dependent induction of cell survival. IGF1R-dependent colon cancer cells (GEO and CBS) were used for the study. Effects on cell proliferation and cell death were determined in the presence of MK-2206. Xenograft studies were performed to determine the effect of MK-2206 on tumor volume. The effect on various cell death markers such as XIAP, survivin, AIF, Ezrin, pEzrin was determined by western blot analysis. We characterized the mechanisms by which a novel Akt kinase inhibitor MK-2206 induced cell death in IGF1R-dependent colorectal cancer (CRC) cells with upregulated PI3K/Akt signaling in response to IGF1R activation. MK-2206 treatment generated a significant reduction in tumor growth *in vivo* and promoted cell death through two mechanisms. This is the first report demonstrating that Akt inactivation by MK-2206 leads to induction of and mitochondria-to-nuclear localization of the Apoptosis Inducing Factor (AIF), which is involved in caspase-independent cell death. We also observed that exposure to MK-2206 dephosphorylated Ezrin at the T567 site leading to the disruption of Akt-pEzrin-XIAP cell survival signaling. Ezrin phosphorylation at this site has been associated with malignant progression in solid tumors. The identification of these two novel mechanisms leading to induction of cell death indicates MK-

2206 might be a potential clinical candidate for therapeutic targeting of the subset of IGF1R-dependent cancers in CRC**.**

**4.2 Introduction**

The interplay between oncogenic signal transduction pathways and their downstream mediators has been extensively characterized over the past two decades. These signaling events are transmitted by protein-protein interactions that are frequently regulated by phosphorylation events (Shtilbans, Wu et al. 2008). PI3K/Akt signaling is a major signal transduction cascade involved in the regulation of a number of cellular processes including cellular proliferation, survival, and metabolism. PI3K/Akt signaling has been implicated in the progression and metastasis of a wide range of cancers (Grabinski, Bartkowiak et al. 2011). The Akt protein kinase, comprised of 3 isoforms (Akt1, 2 and 3), is a direct downstream effector of PI3K, which becomes fully activated by phosphorylation at the T308 and S473 sites (Fayard, Xue et al. 2011, Grabinski, Bartkowiak et al. 2011). Activated Akt is frequently observed in poorly differentiated tumors where it bridges the link between various oncogenic receptors and pro-survival cellular functions making the tumor cells highly invasive and less responsive to chemotherapeutic drugs (Agarwal, Brattain et al. , Grabinski, Bartkowiak et al. 2011).

The Akt effects on aberrant cell survival are mediated by the regulation of a number of critical downstream proteins that have been implicated in apoptosis and anoikis including Bad, Caspase9, IKK, Mdm2 and FHKR (Janes and Watt 2004, Zhan, Zhao et al. 2004, Shtilbans, Wu et al. 2008). Akt is also involved in cell cycle regulation by phosphorylation and inactivation of the cyclin dependent kinase inhibitors p21 and p27/kip1 (Nicholson and Anderson 2002, Shtilbans, Wu et al. 2008). Constitutively activated Akt has been linked to epithelial-to-mesenchymal transition (EMT) by regulating MMPs resulting in reduced cell-to-cell adhesion, increased motility and invasion. It has also been reported that Akt–driven EMT may confer the motility required for malignant progression and dissemination of cancer cells to distant organs

(Grille, Bellacosa et al. 2003, Larue and Bellacosa 2005). Recently, we identified a new pathway by which TGFβ/PKA/PP2A signaling deactivates Akt phosphorylation leading to downregulation of IAPs, XIAP and survivin in colorectal cancer (CRC) cells (Chowdhury, Howell et al. 2011, Chowdhury, Howell et al. 2011).

The broad roles of this enzyme in cancer have established Akt as an attractive therapeutic candidate in cancer. Small molecule inhibitors of the PI3K/Akt pathway are being developed for clinical use. Several Akt inhibitors have been synthesized, including MK-2206, a novel allosteric kinase inhibitor of Akt (Cheng, Zhang et al. 2012, Lai, Liu et al. 2012, Liu, Liu et al. 2012). MK-2206 binds to the pleckstrin-homology (PH) domain of Akt and thereby inhibits PDK1 binding and activation of Akt. This results in change in confirmation of Akt and its inability to localize to the plasma membrane (Cheng, Zhang et al. 2012, Lai, Liu et al. 2012, Liu, Liu et al. 2012). MK-2206 has shown promising preclinical activity and is currently undergoing phase II clinical evaluation. Although the specific mechanisms underlying the anti-cancer activity of MK-2206 remain to be fully elucidated, MK-2206 has been shown to induce cell cycle arrest and apoptosis (Cheng, Zhang et al. 2012, Lai, Liu et al. 2012, Liu, Liu et al. 2012).

We now report that MK-2206 induces anti-tumor activity in a subset of human CRC cell lines characterized by their dependence on IGF1R signaling which leads to PI3K/Akt upregulation for cell survival (Hu, Patil et al. 2008). Strikingly, exposure to MK-2206 resulted in the generation of 2 mechanisms of cell death, which have not previously been documented for this drug. The MK-2206-dependent death of IGF1R-dependent CRC cells *in vitro* and *in vivo* was characterized by Apoptosis Inducing Factor (AIF) induction and its mitochondria-to-nuclear translocation, which is known to induce caspase-independent cell death (Daugas, Nochy et al. 2000, Wang, Zhang et al. 2007). Additionally, MK-2206-dependent cell death was also characterized by the inactivation of the cytoskeletal organizing protein Ezrin at T567 leading to the loss of Inhibitor of Apoptosis (IAP) family protein XIAP. It has been reported that aberrant

increase of Ezrin phosphorylation at the T567 site generates increased cell survival and metastatic capabilities of cancer cells (Wu, Khan et al. 2004, Li, Wu et al. 2008, Chen, Wang et al. 2011, Nakabayashi and Shimizu 2011). In summary, our results indicate that MK-2206 is a promising therapeutic candidate for treatment of IGF1R-dependent CRC characterized by PI3K/Akt signaling upregulation.

### 4.3 Results

**4.3.1 Effect of MK-2206 on apoptosis of CRC cells -**MK-2206 inhibits the phosphorylation of Akt at both Ser473 and Thr308 in two IGF1R-dependent GEO and CBS colon cancer cell lines. However the total Akt protein levels remain unchanged (Figure 4.1A, 4.1B). HCT116 showed a marginal loss of pAkt (S473) however MiaPaCa cells showed a robust loss of pAkt with MK-2206 treatment (Figure S1). We performed MTT assays to study the effect of MK-2206 on proliferation of IGF1R-dependent colon cancer cells. MK-2206 treatment for 48h showed a concentration-dependent reduction in cell proliferation (Figure 4.2A). The $IC_{50}$ value of MK-2206 for GEO cells was observed to be 350 nM. Treatment with 500 nM of MK-2206 reduced cell proliferation by approximately 75%. DNA fragmentation assays were performed to determine the effect of MK-2206 treatment on cell death. It was observed that cell death increased in a concentration dependent manner on treatment with MK-2206 as shown in Figure 4.2B. Treatment with 500nM of MK-2206 increased cell death by approximately 85% as compared to control. Western blot analysis of various apoptotic markers revealed a decrease in Bad phosphorylation at Ser136 following treatment with MK-2206. (Figure 4.2C). Bad can undergo phosphorylation at two sites (Ser112 and Ser136). Akt preferentially phosphorylates Bad at Ser136 (Datta, Dudek et al. 1997). Phosphorylated Bad at Ser136 associates with cytoplasmic14-3-3 proteins. Treatment with MK-2206 results in reduced interaction of pBad with 14-3-3 due to increased cell death (Figure 4.2D). On the other hand dephosphorylated Bad interacts with Bcl-$x_L$ a pro-survival molecule, and inactivates it to generate cell death (Datta, Dudek et al. 1997). We observed that

there was an increase in the interaction of Bcl-xL with total Bad on treatment with MK-2206 which results in more inactivation of Bcl-$x_L$ thus leading to increased cell death (Figure 4.2E). Furthermore, we observed a reduction in the interaction of Bad with 14-3-3 on treatment of MK-2206 (Figure 4.2D). It has been determined previously that there is an increase in the expression of IAPs (Survivin and XIAP) in colon, lung and breast cancer. There was an increase in cell death on transient knockdown of XIAP as determined by DNA fragmentation, which confirms that XIAP is responsible for increased survival of cells by inhibiting caspase mediated cell death (Figure S2). We observed a reduction in the expression of survivin and XIAP on treatment with MK-2206 *in vitro and in vivo* (Figure 4.2C, S3). Therefore, MK-2206 regulates aberrant cell survival of CRC cells by down regulating IAPs in CRC cells.

**4.3.2 MK-2206 inhibits colon tumor xenograft growth** - The antitumor activity of MK-2206 on GEO colon cancer xenografts was determined by injection of GEO-GFP cells subcutaneously into the flank of athymic nude mice. One week after implanting the cells, MK-2206 was administered at 120 mg/kg body weight by oral gavage for three weeks on alternate days. As shown in Figure 4.3A, MK-2206 significantly inhibits tumor growth. The tumor volume was found to be significantly reduced in MK-2206 treated animals ($P<0.01$) as compared to control animals (Figure 4.3B, 3C). The excised tumors from control animals showed an average weight of 2.5g compared to treated animal tumors weighing approximately 0.8g. (Figure 4.3D). Importantly, there was no significant decrease in the body weight in treated animals compared to control (Figure S4).

The expression of pAkt S473 was found to be reduced by treatment with MK-2206 *in vivo* by IHC(Figure 4.4A). Densitometry of the IHC images showed a significant reduction in the expression of pAkt S473 in treated animals as compared to control animals ($p<0.02$) as shown in Figure 4.4B. The loss of phosphorylation of Akt was further confirmed by western blot analysis of MK-2206-treated tumor tissue lysates showing a reduction in pAkt at both S473 and T308

sites, in comparison to the control xenograft tumors (Figure 4.4C). However the change in total Akt (termed here as tAkt) was not statistically significant (Figure S5, S6).

**4.3.3 MK-2206 inhibits cell proliferation and cell death in vivo** - H&E staining indicated that MK-2206 treatment induced an increase in necrosis that was observed by scanning the entire tissue section using an image scanner and visually inspecting the necrotic areas (Figure S7). Cell death (quantified by TUNEL assay) was also observed to be significantly increased following MK-2206 treatment (Figure 4.5A, 4.5B). MK-2206 treatment also resulted in reduced cell proliferation as measured by Ki67 staining (Figure 4.5C, 4.5D). Figure S8 shows the images of control and treated mice just before euthanizing.

**4.3.4 Mechanisms of cell death by MK-2206** - MK-2206 treatment promotes cell death both *in vitro* and *in vivo*. We characterized the molecular effects underlying MK-2206 mediated cell death in colon cancer cells. Western blot analysis showed that there was an increase in the expression of AIF protein after treatment with MK-2206 (Figure 4.6A). The mechanism by which the loss of pAkt might be related to this induction is not known. Cregan et al. (2002) previously reported that AIF is responsible for caspase-independent apoptosis (Cregan, Fortin et al. 2002) by undergoing translocation from the mitochondria to nucleus. To determine the migration of AIF, we prepared nuclear and cytoplasmic extracts of untreated cells and cells treated with MK-2206 at 500nM (since there was a higher increase in expression of AIF at 500nM). Immunoblot analysis indicated higher AIF expression in nuclear extracts of cells treated with MK-2206 as compared to nuclear extracts of untreated cells (Figure 4.6C), thus confirming that treatment by MK-2206 stimulates translocation of AIF to the nucleus. Translocation of AIF was further confirmed by immunofluorescence using confocal microscopy (Figure 4.6B). AIF mediated cell death was further confirmed by AIF inhibitor N-Phenylmaleimide (Daugas, Nochy et al. 2000, Wang, Zhang et al. 2007). Treatment with the AIF inhibitor at a concentration of 50 μM/L for 1 h prior to treatment with MK-2206 for 48 h shows a reduction in cell death thus confirming MK-

2206 mediated cell death is through stimulation of AIF (Figure 4.6D). Additionally loss of AIF by siRNA-mediated knock down results in reduction in cell death in presence of MK-2206 as determined by DNA fragmentation assay (Figure S9)

In addition to caspase-independent cell death, we also observed caspase-dependent cell death through XIAP downregulation following treatment with MK-2206 (Figure 4.2D). It has been shown that Akt2 regulates phosphorylation of Ezrin at T567 leading to the translocation and activation of the $Na^+$-$H^+$ exchanger (NHE3) (Shiue, Musch et al. 2005) and NHE regulatory factor 1 (NHERF1) supports Akt-dependent cell survival (Wu, Khan et al. 2004). We observed that MK-2206 might inactivate Ezrin by affecting its phosphorylation at the T567 site ( Figure 4.7A, 4.7B) *in vitro* as well as *in vivo*. The loss of Ezrin phosphorylation is known to affect cellular survival and proliferation (Wu, Khan et al. 2004). Stable retroviral knockdown of Akt2 also results in reduction in Ezrin phosphorylation at T567. However there was no change in expression of total Ezrin on knockdown of Akt2 as shown in (Figure 4.7C). Interestingly no such loss of phospho Ezrin T567 was observed with Akt1 and Akt3 knockdown (Figure 4.7D, S10). Furthermore, Ezrin knock down resulted in complete loss of XIAP and survivin (Figure S11). Therefore, it appears that Akt2 plays an important role in regulating cell survival mediated by the Akt2-pEzrinT567-XIAP axis. MK-2206 treatment caused AIF activation and Ezrin dephosphorylation at the T567 site and, ultimately, this leads to loss of survivin/XIAP-mediated aberrant cell survival and increased cell death.

**4.4 Discussion**

Extensive drug development efforts and clinical evaluations are underway targeting the aberrant cell survival properties associated with PI3K/Akt signaling in regulating cancer progression and metastasis (Shtilbans, Wu et al. 2008). Inhibition of Akt activation by small molecule kinase inhibitors is an attractive candidate for targeting aberrant cell survival associated with malignant progression and metastasis and could be effective in the treatment of CRC. MK-2206 is a novel

Akt allosteric kinase inhibitor, which is currently in clinical evaluation (Cheng, Zhang et al. 2012, Lai, Liu et al. 2012, Liu, Liu et al. 2012).

Several studies have described MK-2206 effects as a single agent or in combination with other inhibitors (e.g. PI3K or mTOR inhibitors) on cell proliferation and/or cell death. Gorlick et.al. (2011) demonstrated a significant reduction in tumor volume *in vivo* and decreased cell survival *in vitro* in pediatric cancer cell lines following MK-2206 treatment (Gorlick, Maris et al. 2011). Simoni et.al. (2012) studied the effect of MK-2206 in T cell acute lymphoblastic leukemia, demonstrating cell cycle arrest in G0/G1 phase, apoptosis and autophagy (Simioni, Neri et al.). Ma et.al. (2012) showed that MK-2206 treatment in nasopharyngeal carcinoma cells (NPC) induced cell cycle arrest and apoptosis (Ma, Lui et al.). Similarly, we observed that MK-2206 treatment in the IGF1R-dependent GEO cells reduced cell proliferation and increased cell death in a concentration-dependent manner (Figure 4.5.2A, 2B), while MK-2206 has been shown to be effective in causing cell death in different types of cancer. However, specific mechanisms associated with MK-2206-mediated cell death have not been characterized. This study identifies molecular mechanisms involved in MK-2206-mediated cell death in IGF1R- dependent CRC cells in response to Akt inhibition. Identification of specific mechanisms may generate new therapeutic targets that offer potential for enhancing death of CRC cells. The mechanistic novelty of this study is our identification of two pathways whereby MK-2206 treatment leads to control of aberrant cell survival and induction of cell death *in vitro* and *in vivo*.

We studied the expression of various apoptosis regulators following exposure to MK-2206. As expected, a reduction in phospho-Bad (pBad) at the Ser 136 site was observed (Figure 4.2D), which is known to be regulated by Akt signaling (Datta, Dudek et al. 1997). It is known that pBad interacts with 14-3-3, a major mediator of cell survival providing an anti-apoptotic milieu to the cellular environment (Muslin, Tanner et al. 1996). We observed that treatment with MK-2206 results in reduced 14-3-3 interaction with pBad (Ser136) indicating that MK-2206

results in reduction in cell survival through this mechanism. The protein expression of Bad remained unchanged following MK-2206 treatment; however, there was an increase in the interaction of Bad with Bcl-xL. Bad inactivates Bcl-xL thus leading to increases in cell death. Additionally we observed a decrease in interaction of Bad with 14-3-3 on treatment with MK-2206 thus suggesting that Bad remains activated thus leading to apoptosis.

Strikingly, we made the observation that MK-2206 exposure led to an induction of pro-apoptotic protein AIF and its translocation from mitochondria to the nucleus of the GEO cells (Figure 4.6 A-C). It has been reported that AIF is responsible for caspase-independent death in ovarian cancer cells (Yang, Fraser et al. 2008),(Cregan, Fortin et al. 2002, Cheung, Melanson-Drapeau et al. 2005). AIF is localized in the mitochondria but upon activation it translocates to the nucleus and causes DNA fragmentation (Susin, Lorenzo et al. 1999). However, the mechanism that regulates AIF induction leading to its caspase-independent apoptotic functions is not well understood. Treatment with AIF inhibitor resulted in reduced cell death thus indicating that AIF is responsible for cell death mediated by MK-2206.

MK-2206 treatment of GEO cells reduced survivin and XIAP levels both *in vivo* and *in vitro* (Figure 4.2D, S3). Survivin and XIAP are key cell survival-associated proteins that have been characterized as having an important role in metastasis (Mehrotra, Languino et al. 2010). XIAP binds to caspases3, 7 and 9, thereby inhibiting their pro-apoptotic activity (Dohi, Xia et al. 2007, Mehrotra, Languino et al. 2010). During stress conditions, mitochondrial XIAP and survivin migrate to the cytosol forming a survivin/XIAP complex, which inhibits caspases and promotes cytoprotection (Dohi, Xia et al. 2007). Dan et.al (2004) made the novel finding that Akt phosphorylates XIAP at a stabilizing Ser87 site (Dan, Sun et al. 2004). We demonstrated that TGFβ/PKA signaling regulates aberrant cell survival in IGF1R-dependent CRC cells by disengaging survivin/XIAP complex formation thus causing caspase activation and inducing cell death. We sought to determine the mechanism by which MK-2206 increased XIAP loss and cell

death. It was observed that MK-2206 treatment dephosphorylates Ezrin at the Thr567 site (Figure 4.7A, 4.7B). However, no change in total Ezrin protein expression was observed. Ezrin is a member of Ezrin-radixin-moesin (ERM) protein family that plays a key role in cancer progression and metastasis in a wide range of cancers, including CRC (Turunen, Wahlstrom et al. 1994). Ezrin is found in a closed conformation in the cytosol. Ezrin phosphorylation at Thr567 leads to its activation and conformational change to an open conformation, resulting in its localization to the plasma membrane for its oncogenic-associated functions (Fievet, Gautreau et al. 2004). Several kinases are known to phosphorylate Ezrin at T567 including Rho kinase and PI3K/Akt (Ng, Parsons et al. 2001). We performed siRNA knockdown of Ezrin and observed a complete loss of XIAP and survivin (Figure S11). Thus, we have found that MK-2206 treatment inhibits the Akt-pEzrinT567-XIAP cell survival-signaling axis leading to a caspase-dependent cell death in the IGF1R-dependent CRC cells, in addition to caspase independent cell death accompanying AIF translocation from the mitochondria to the nucleus.

Stable knockdown of Akt2 in the IGF1R-dependent and highly metastatic colon cancer cell line GEO was performed to give a better understanding of the mechanism of cell death mediated by loss of pEzrin. Loss of Akt2 resulted in decreased the activation of Ezrin since there was a loss of phosphorylation of Ezrin at the T567 site. Besides loss of pEzrin we also observed a reduction in the expression of XIAP on knockdown of Akt2. However, there was no such loss of pEzrin on knockdown of Akt1 and Akt3 in GEO cells. Thus we can conclude that loss of the Akt2 isoform is responsible for Akt-pEzrin-XIAP mediated cell death.

**4.5 Conclusion**

We provided novel mechanistic insights on MK-2206-mediated cell death. Importantly, this work provides a new paradigm for MK-2206-mediated control of aberrant cell survival associated with IGF1R-dependent CRC that may offer new targets for enhancing cell death in cancer cells.

## 4.6 Figures

**Figure 4.1: MK-2206 inhibits Akt signaling in IGF1R-dependent CRC cells.**A) & B) Loss of pAkt at Ser473 and T308 on treatment with increasing concentration of MK-2206 for 72 hours in GEO and CBS cells respectively. GAPDH is used as a loading control.

**Figure 4.1:**

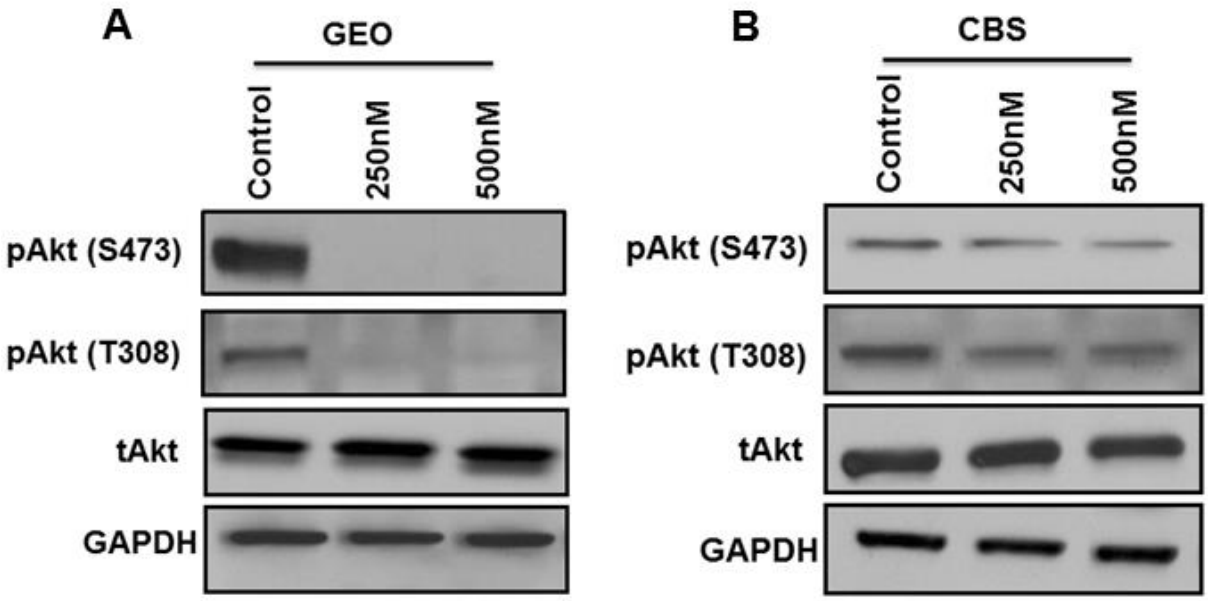

A
GEO
Control
250nM
500nM
pAkt (S473)
pAkt (T308)
tAkt
GAPDH
B
CBS
Control
250nM
500nM
pAkt (S473)
pAkt (T308)
tAkt
GAPDH

**Figure 4.2: MK-2206 affects cell proliferation and cell death *in vitro*.** A) MTT analysis shows reduction in cell proliferation on treatment with MK-2206. B) DNA fragmentation showing an increase in cell death with increasing concentration of MK-2206. C) Western blot analysis of various apoptotic members as pBad (Ser136), XIAP and Survivin. D) IP for 14-3-3 to determine the interaction with pBad (Ser136) and Bad showing a loss in the interaction on treatment with MK-2206. E) IP for Bad to determine the interaction with anti-apoptotic protein Bcl-$x_L$.. (*=P<0.01 and **=P<0.001).

**Figure 4.2:**

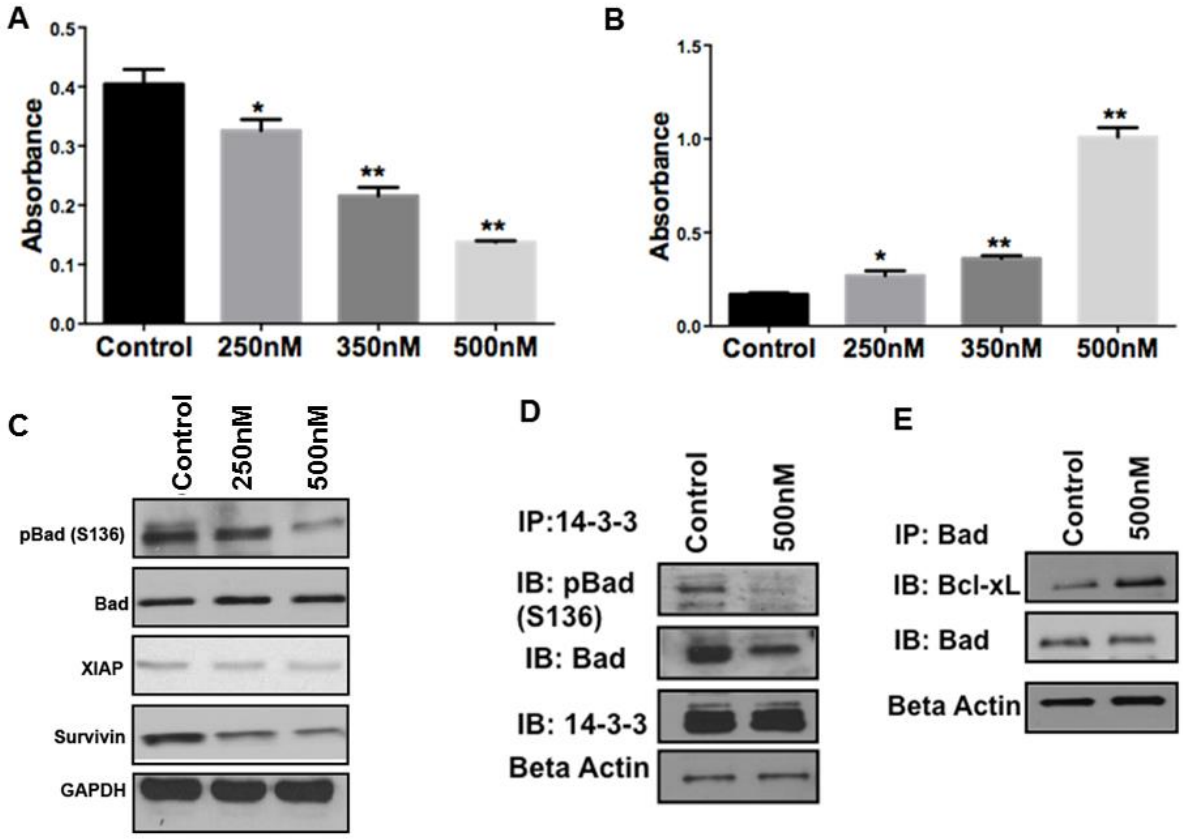

A
Absorbance
0.5
0.4
0.3
0.2
0.1
0.0
*
**
**
Control
250nM
350nM
500nM
B
Absorbance
1.5
1.0
0.5
0.0
*
**
**
Control
250nM
350nM
500nM
C
Control
250nM
500nM
pBad (S136)
Bad
XIAP
Survivin
GAPDH
D
IP:14-3-3
Control
500nM
IB: pBad (S136)
IB: Bad
IB: 14-3-3
Beta Actin
E
IP: Bad
Control
500nM
IB: Bcl-xL
IB: Bad
Beta Actin

**Figure 4.3: MK-2206 inhibits the growth of colon tumor xenograft.** A) Reduction in tumor size on treatment with MK-2206. B) Reduction in tumor volume in treated animals as compared to control animals. C) Reduction in the average tumor volume in animals treated with MK-2206 as compared to control animals. D) Reduction in tumor weight on treatment with Akt kinase inhibitor. (*=$P<0.01$ and **=$P<0.001$)

**Figure 4.3:**

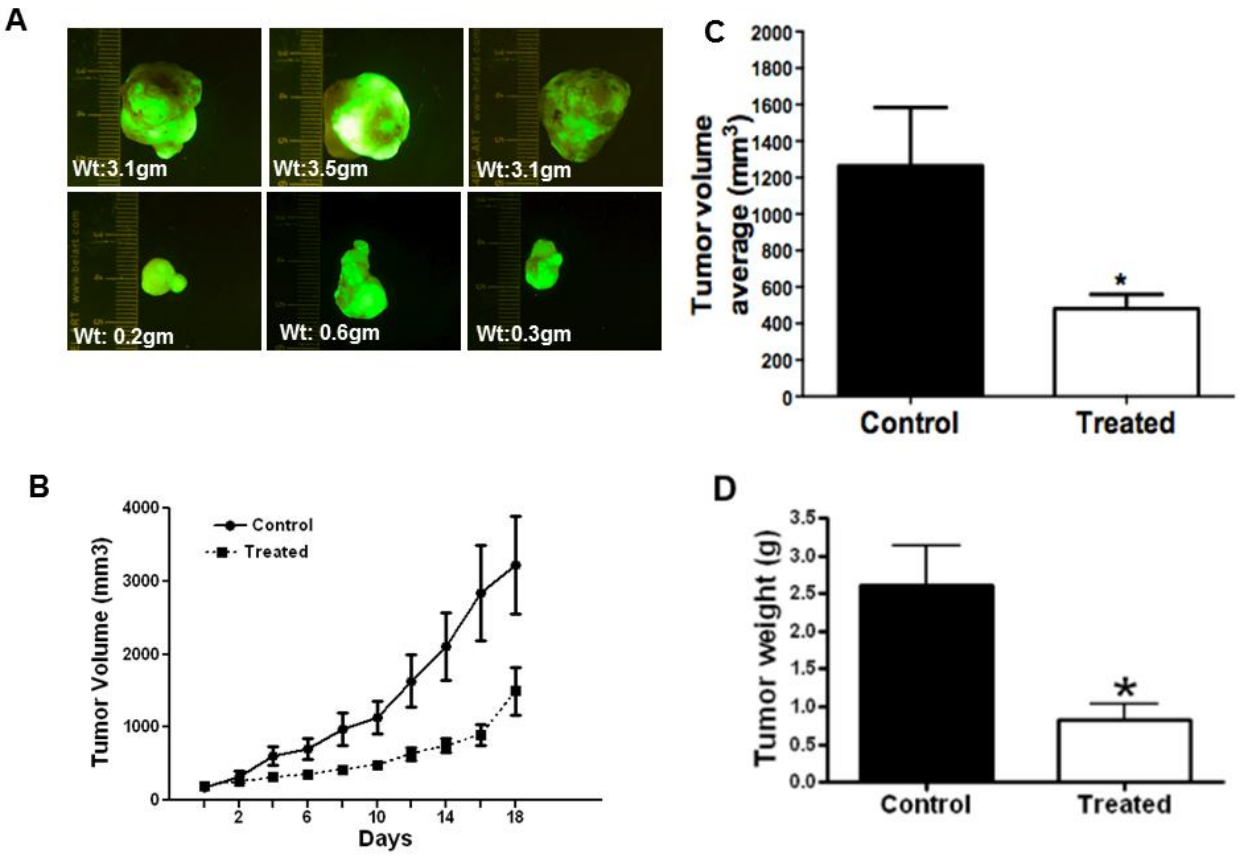

A
Wt:3.1gm
Wt:3.5gm
Wt:3.1gm
Wt: 0.2gm
Wt: 0.6gm
Wt:0.3gm
C
Tumor volume average (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
Control
Treated
B
Tumor Volume (mm3)
4000
3000
2000
1000
0
Control
Treated
2
6
10
14
18
Days
D
Tumor weight (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Treated

**Figure 4.4: MK-2206 inhibits Akt signaling *in vivo*:** A) IHC images showing a reduction in pAkt at Ser473. B) Relative quantification was performed, followed by statistical analysis to determine the decrease in phosphorylation of Akt at Ser473 on treatment with MK-2206. C) Western blot analysis to confirm the loss of pAkt at Ser473 and Thr 308 in treated animals. (*=P<0.01 and **=P<0.001)

**Figure 4.4:**

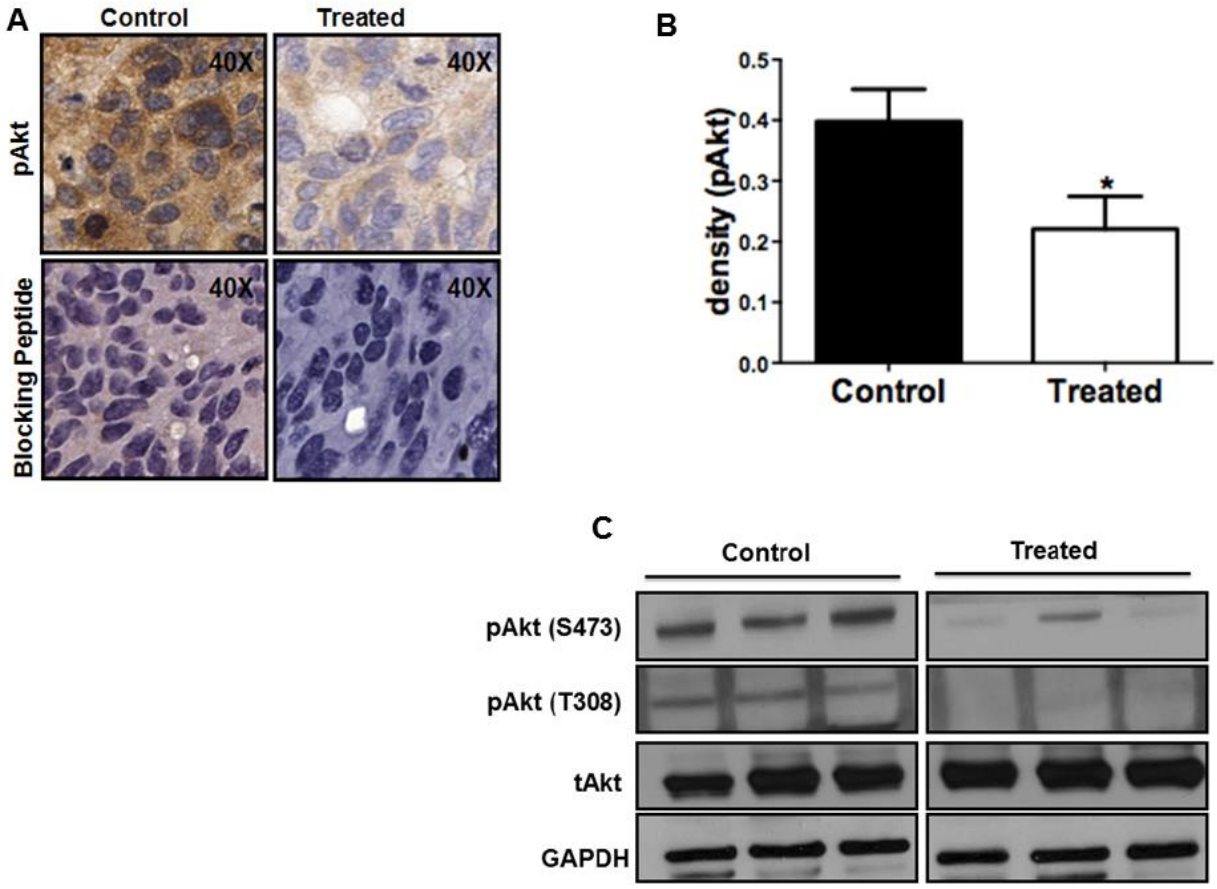

A
Control
Treated
pAkt
Blocking Peptide
40X
40X
40X
40X
B
density (pAkt)
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
Treated
C
Control
Treated
pAkt (S473)
pAkt (T308)
tAkt
GAPDH

**Figure 4.5: Increased cell death and decreased cell proliferation on treatment with the allosteric Akt kinase inhibitor:** TUNEL and Ki67 IHC was performed on control and treated samples A) Increased cell death on treatment with the inhibitor. B) Relative quantification was performed followed by statistical analysis to quantify the increase in death. There was a significant increase in cell death in treated animals. C) Shows a loss in cell proliferation on treatment with MK-2206. D) Bar graphs representing a highly significant loss in Ki67 staining in treated animals.

**Figure 4.5:**

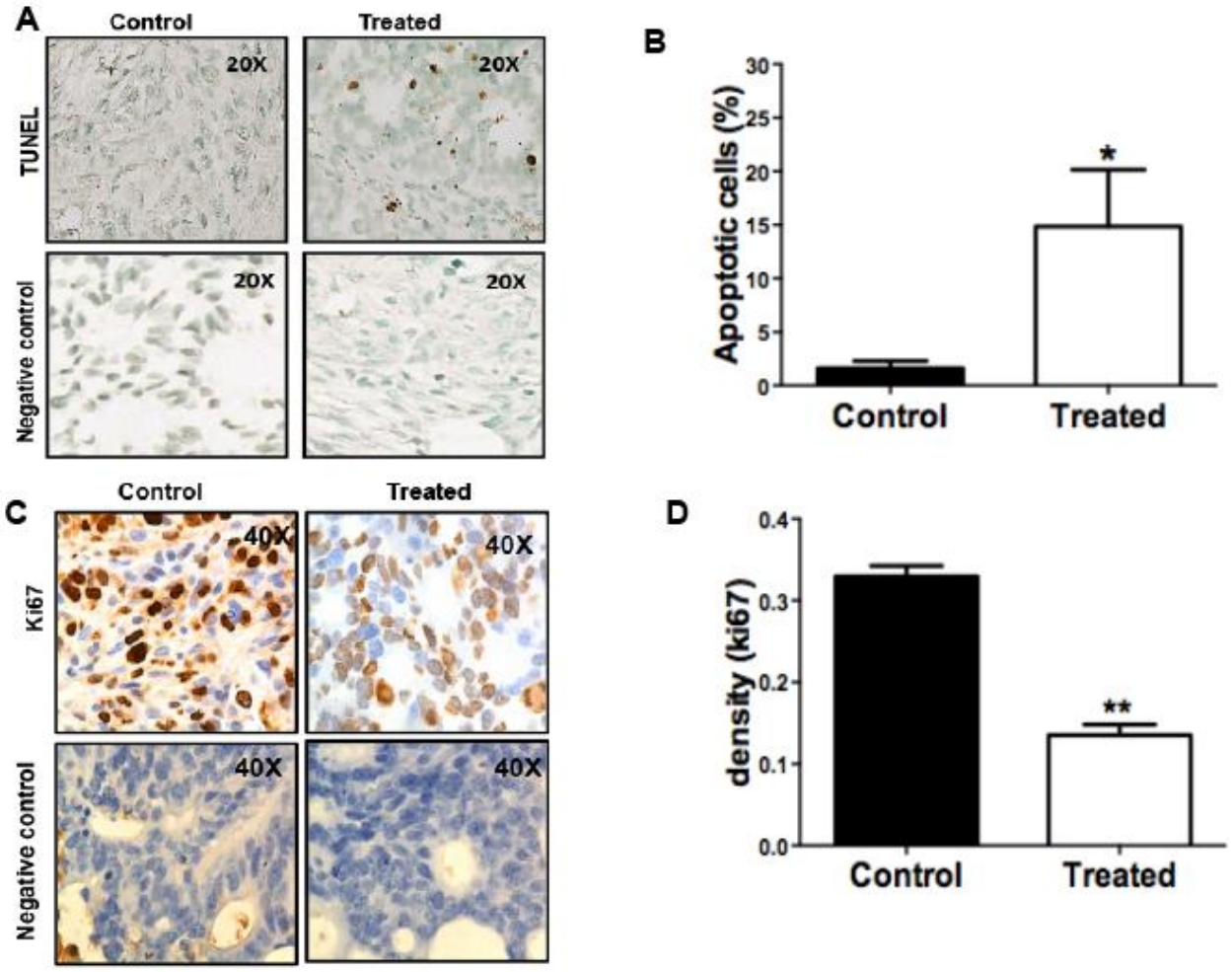

A
Control
Treated
TUNEL
Negative control
20X
B
Apoptotic cells (%)
30
25
20
15
10
5
0
*
Control
Treated
C
Control
Treated
Ki67
Negative control
40X
D
density (ki67)
0.4
0.3
0.2
0.1
0.0
**
Control
Treated

**Figure 4.6: Increase in the expression of AIF on treatment with MK-2206 mediates cell death:** A) western blot analysis showing an increase in the expression of AIF on treatment with MK-2206. Immunofluorescence was performed to study the translocation of AIF from mitochondria to the nucleus during cell death. B) Confocal images showing a reduced co-localization of mitotracker (red) and AIF (green) in treated as compared to control cells. . C)Cellular fractionation to separate nucleus from cytosol was performed followed by western blot analysis for AIF. HDAC1 and GAPDH were used as compartmentalization control for nucleus and cytosol respectively. D) DNA fragmentation after treatment with AIF inhibitor results in reduction in cell death in presence and absence of MK-2206 thus confirming that MK-2206 causes AIF mediated cell death.

**Figure 4.6:**

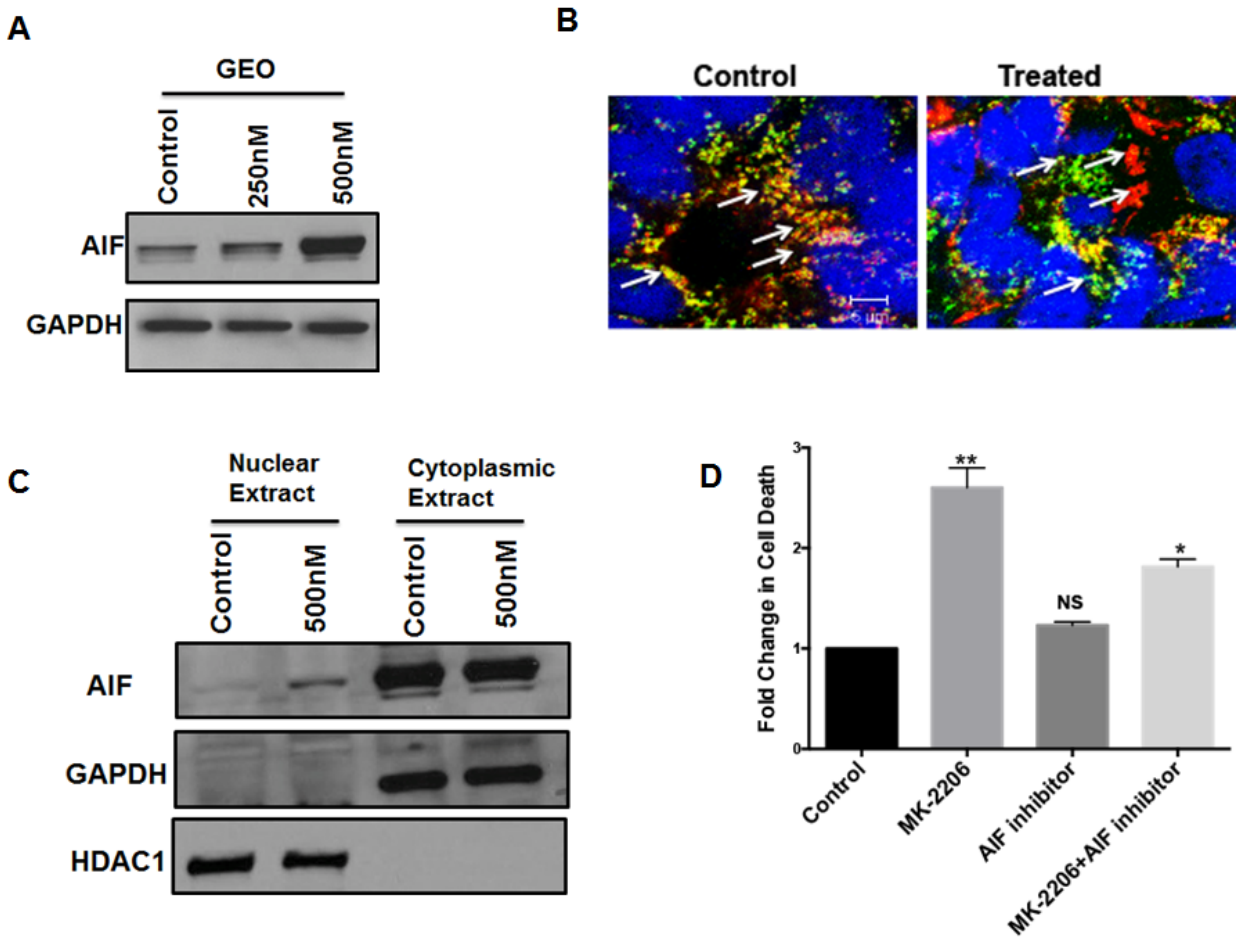
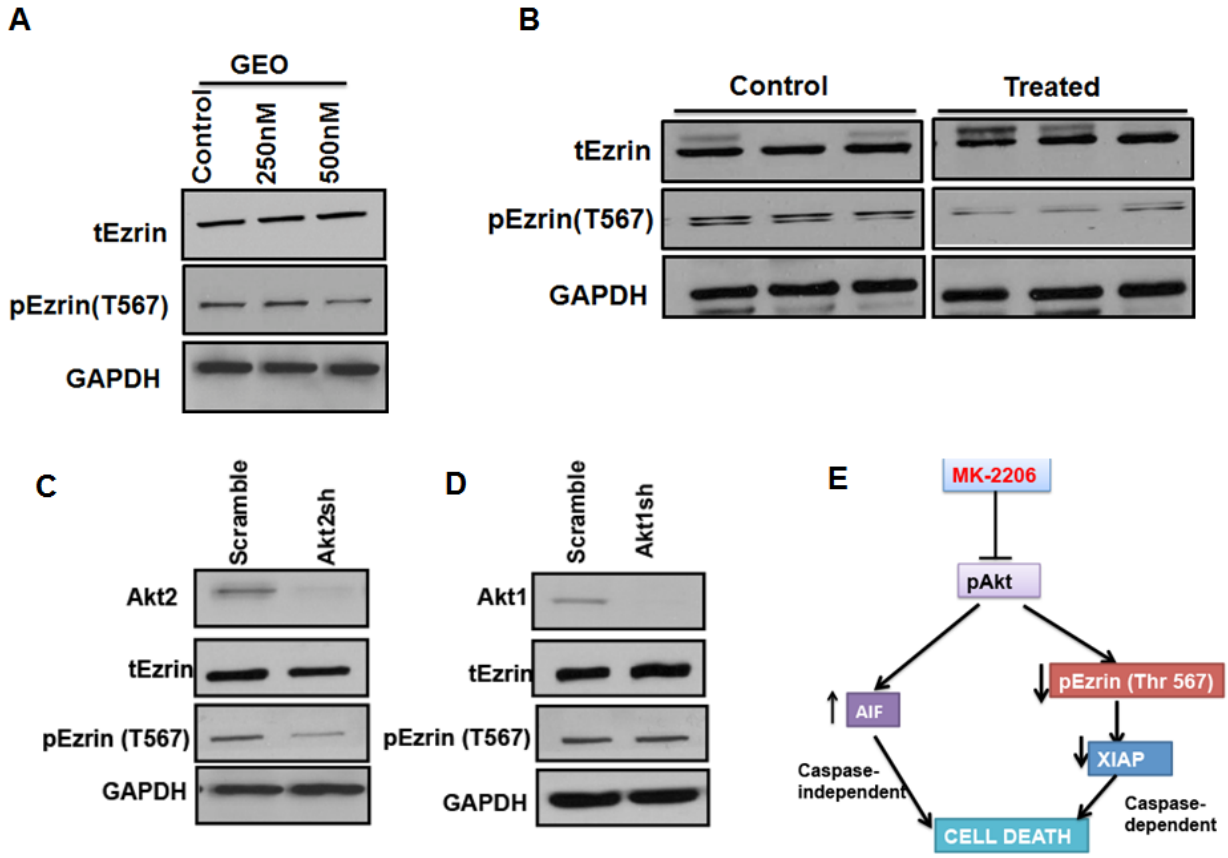

A
GEO
Control
250nM
500nM
AIF
GAPDH
B
Control
Treated
C
Nuclear Extract
Cytoplasmic Extract
Control
500nM
Control
500nM
AIF
GAPDH
HDAC1
D
Fold Change in Cell Death
**
NS
*
Control
MK-2206
AIF inhibitor
MK-2206+AIF inhibitor

**Figure 4.7:** A) Western blot analysis showing a reduction in the expression pEzrin (T567) on treatment with MK-2206 *in vitro*. B) Treatment with MK-2206 reduces the expression of pEzrin (T567) *in vivo.*Stable knockdown of Akt2 was performed in GEO cells. C) Western blot analysis showing a loss of pEzrin (T567) on knockdown of Akt2. No change in total Ezrin was observed on loss of Akt2. D) Western blot analysis showing loss of Akt1 does not affects the expression of pEzrin (T567). GAPDH is used as a loading control. E) Overall mechanism for induction of cell death by MK-2206. Akt kinase inhibitor MK-2206 mediates cell death by two different mechanisms. Loss of phosphorylation of Akt results in induction and translocation of AIF from the mitochondria to the nucleus, where it results in DNA fragmentation. On the other hand treatment with MK-2206 results in loss of pEzrin (T567), which results in loss of XIAP thus mediating cell death.

**Figure 4.7:**


A
GEO
Control
250nM
500nM
tEzrin
pEzrin(T567)
GAPDH
B
Control
Treated
tEzrin
pEzrin(T567)
GAPDH
C
Scramble
Akt2sh
Akt2
tEzrin
pEzrin (T567)
GAPDH
D
Scramble
Akt1sh
Akt1
tEzrin
pEzrin (T567)
GAPDH
E
MK-2206
pAkt
AIF
pEzrin (Thr 567)
XIAP
Caspase-independent
Caspase-dependent
CELL DEATH

**Supplementary Figures**

**Figure S1:** Western blot analysis showing a loss of pAkt (S473) after treatment with MK-2206 in HCT116 and MiaPaCa cells.

**Figure S1**

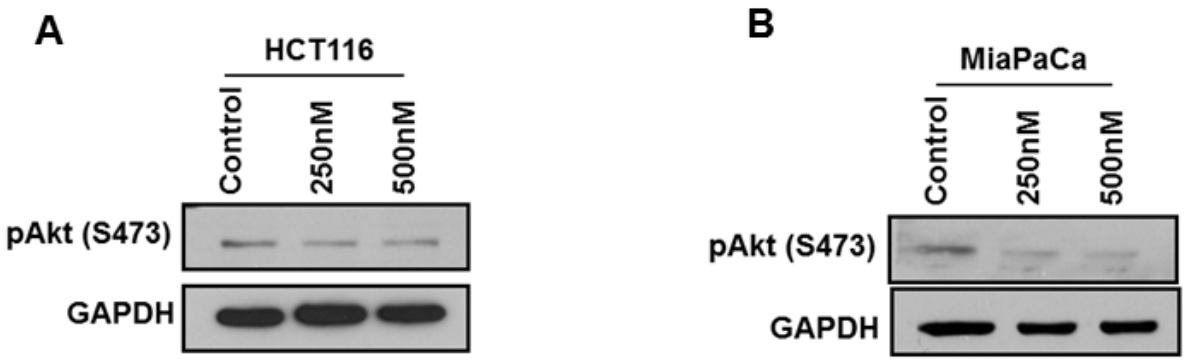


**Figure S2:** Transfection with siRNA for XIAP results in increase in cell death as determined by DNA fragmentation.

**Figure S2**

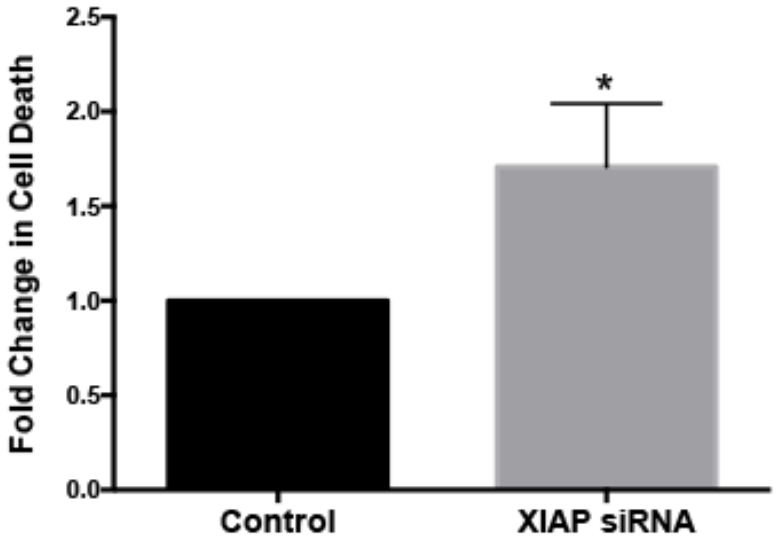


**Figure S3:** Western blot analysis to determine the loss of survivin and XIAP in animals treated with MK-2206.

**Figure S3**

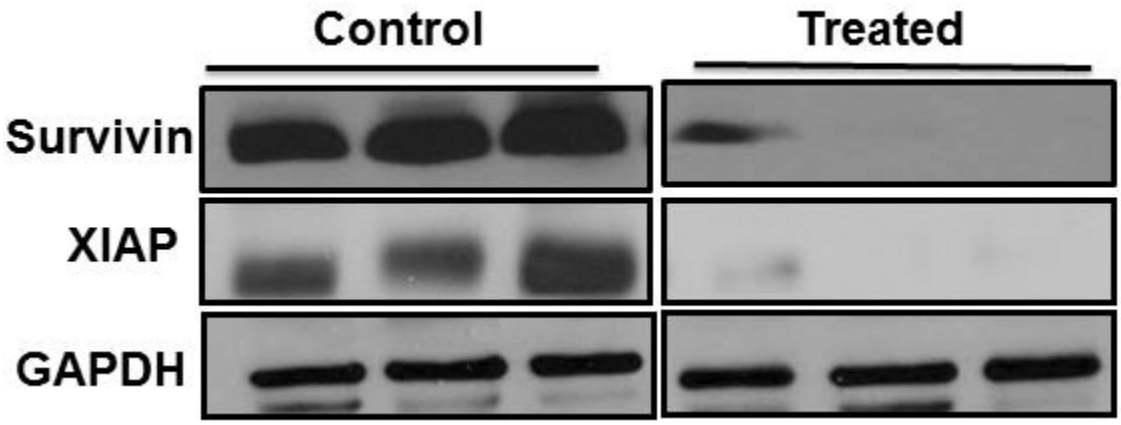


**Figure S4:** There was no significant loss of body weight in mice on treatment with MK-2206.

**Figure S4**

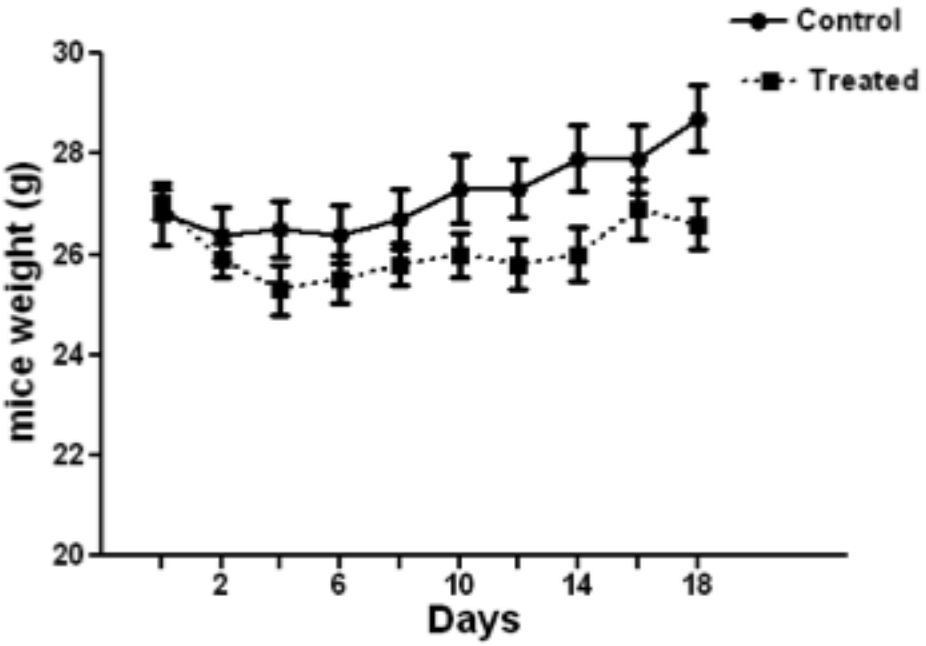


**Figure S5:** IHC images showing no change in the expression of total Akt in treated animals as compared to control.

**Figure S5**

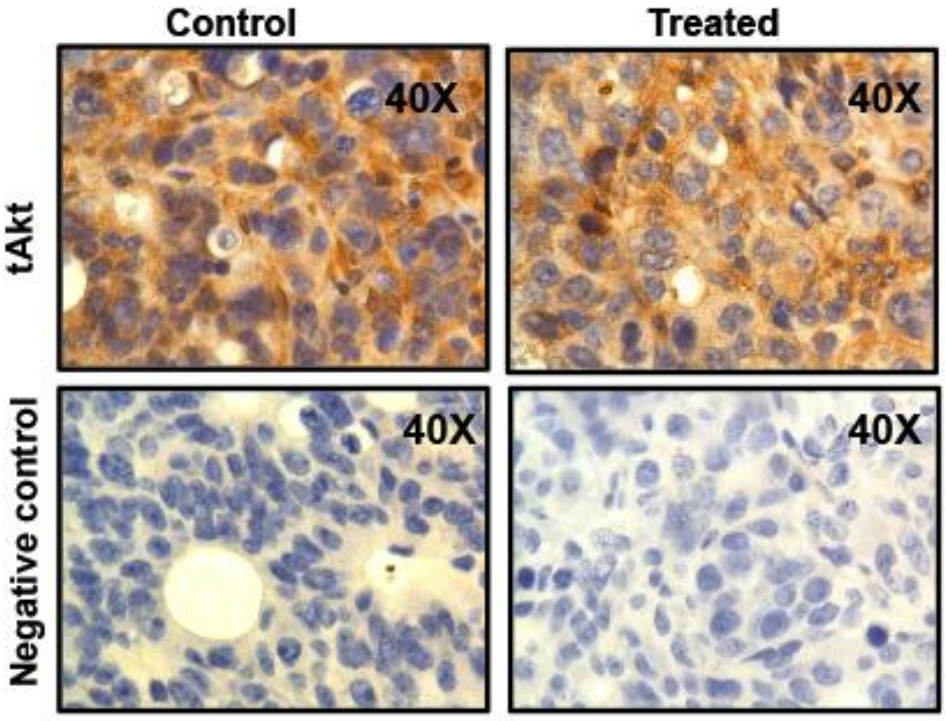


**Figure S6:** Relative quantification followed by statistical analysis was performed to determine the change in expression of total Akt. There was no significant change in the expression of total Akt.

**Figure S6**

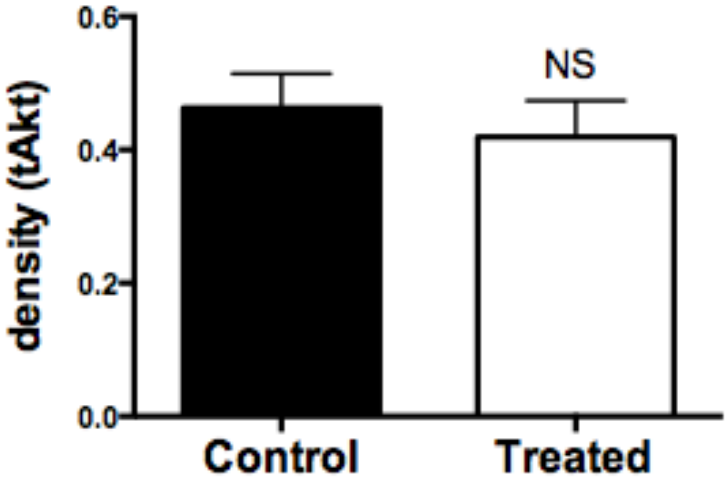


**Figure S7:** Eosin and Hematoxylin staining of control and treated xenograft tumors showing more necrosis on treatment with MK-2206.

**Figure S7**

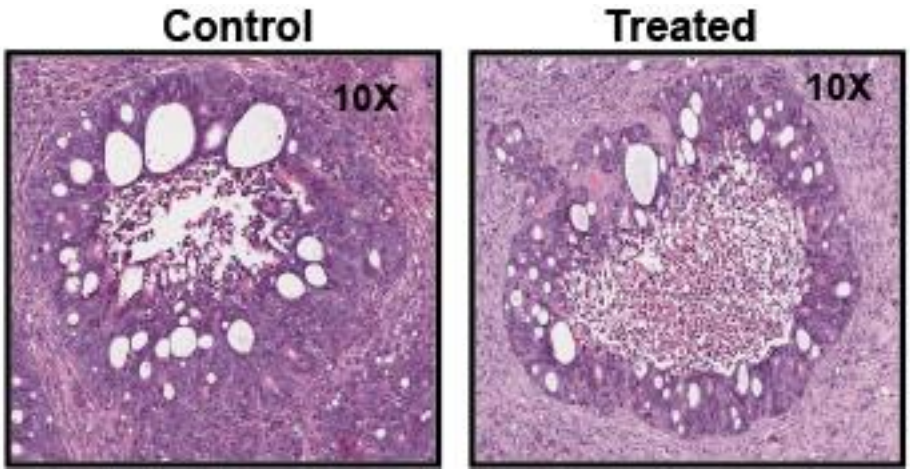


**Figure S8:** Images of control and treated animals before euthanizing.

**Figure S8**

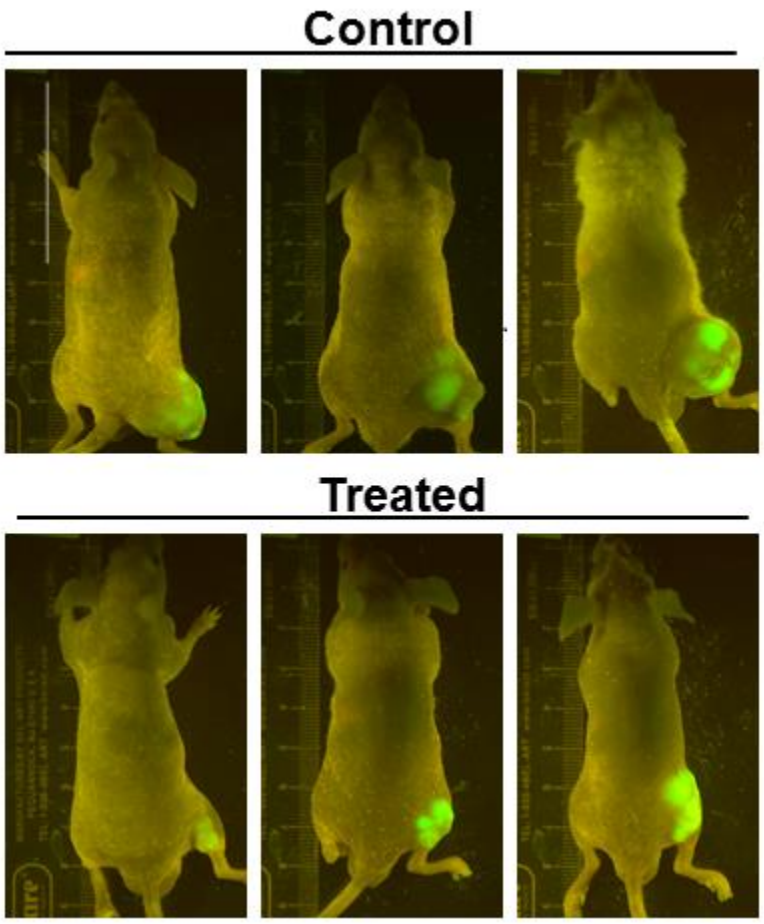


**Figure S9:** A) Western blot showing a knockdown of AIF in presence of siRNA. B) DNA fragmentation after knockdown of AIF shows reduction in cell death in presence and absence of MK-2206.

**Figure S9**

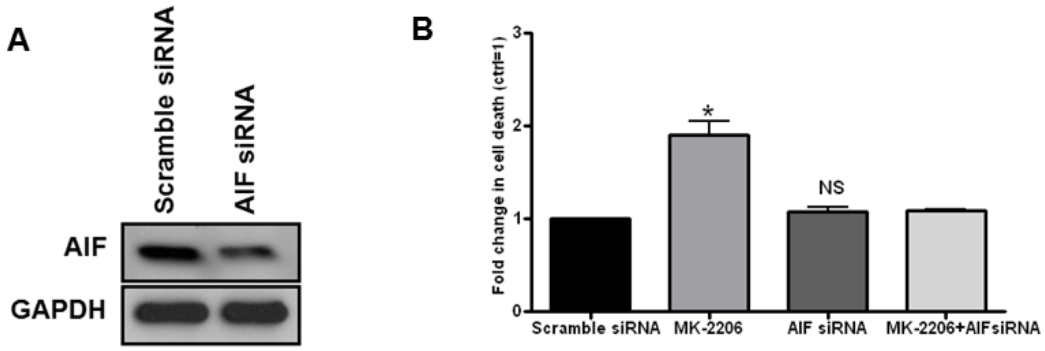


**Figure S10:** No change in pEzrin (T567) and total Ezrin on knockdown of Akt3.

**Figure S10**

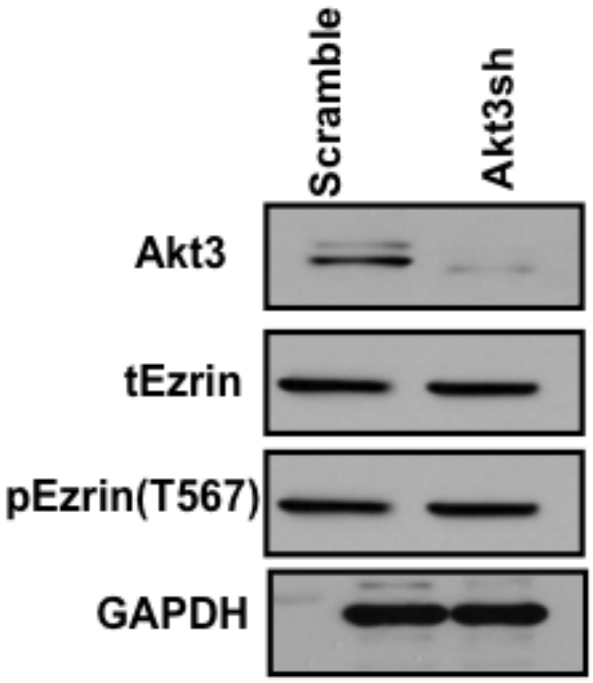
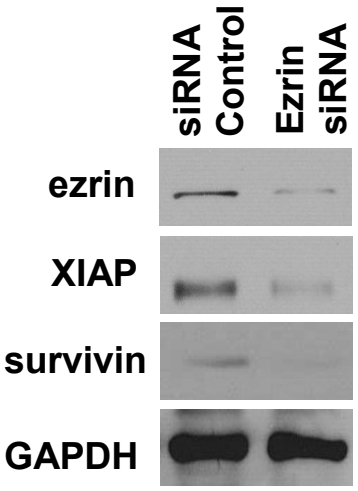


**Figure S11:** siRNA-mediated knockdown of Ezrin showing a loss in XIAP expression.

**Figure S11**



# Chapter V

## Role of Akt2 in cell survival, establishment and/or maintenance of colorectal cancer metastasis

Ekta Agarwal[1,2], Caroline Robb[2], Lynette M. Smith[3], Michael G. Brattain[1,2*], Sanjib Chowdhury[1,4*]

[1] Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA

[2] Fred and Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA

[3]Department of Biostatistics, University of Nebraska Medical Center, Omaha, NE, USA

[4] Department of Surgical Oncology, University of Nebraska Medical Center, Omaha, NE, USA

*Corresponding author

**This chapter is under preparation for publication.**

## 5. Role of Akt2 in cell survival, establishment and/or maintenance of colorectal cancer metastasis

### 5.1 Abstract

Akt is critical to survival signaling in various cancer types. Although it is known that Akt2 is responsible for establishment of CRC metastasis, the mechanism of Akt2-driven metastases is still not known. We show in this study that only Akt2 among Akt isoforms is required for metastasis. The shRNA mediated knockdown of Akt2, not Akt1, causes reduction in the metastatic burden in CRC. This study shows that loss of Akt2 upregulates MTSS1 (metastasis suppressor 1) and in parallel inhibits the expression of anti-apoptotic genes XIAP and survivin thus inhibiting cell survival which in turn could lead to reduction in metastatic potential of the cells. This study might help in understanding if loss of Akt2 could be a mechanism of increasing cell death thus leading to reduction in metastasis.

### 5.2 Significance

Loss of Akt2 leads to a reduction in metastasis, identifying Akt2 as a potentially important therapeutic target for metastases. Strategies to inhibit Akt2 as opposed to other two isoforms may provide a therapeutic approach for treatment of metastases. A negative correlation between Akt2 and MTSS1 in human primary CRC samples might be useful in identification of metastatic patients.

### 5.3 Introduction

The metastatic colonization of the cancer cells is the major cause of mortality in CRC patients (Jin, Gao et al. 2012). Significant advancements in cancer research have explained some mechanisms for establishment of metastasis; however, the molecular mechanisms underlying the maintenance of metastatic colonization are not well understood. Cancer cells have to overcome several barriers (matrix detachment, shear forces and resistance from the immune system) before

they can colonize as metastases in distant organs, thus making metastasis an inefficient process (Gupta and Massague 2006). Less than 1% cells of the primary tumor leave their site and survive in the blood circulation to reach distant organs to form metastatic colonies (Valastyan and Weinberg 2011). Recent studies have shown that increased cell survival is required in circulation as well as at the extravascular sites for metastasis of cancer cells (Eccles and Welch 2007); however very small populations of the cells survive and proliferate to form distant metastatic spots thus making "survival" a rate limiting step for metastatic colonization (Kienast, von Baumgarten et al. 2009). Once metastasis has occurred, there is an increase in resistance to apoptosis to maintain the established metastasis (Eccles and Welch 2007). It is well known that apoptosis is a barrier for development of cancer (Lowe, Cepero et al. 2004). Physiological stress stimulates a portion of cancer cells to evade apoptosis thus allowing for progression to higher grade of malignancy and metastasis (Adams and Cory 2007). Cancer cells limit apoptosis by triggering the anti-apoptotic machinery of the cell i.e. IAPs (Inhibitor of Apoptosis Protein family), members of Bcl-2 family (Bcl-xl and Mcl-1) or by inhibiting the pro-apoptotic proteins (Bax and Bak) (Lowe, Cepero et al. 2004), by activation of survival pathways as PI3K-Akt , or by upregulating MMPs or by overexpression of FAK (Townson, Naumov et al. 2003, Liotta and Kohn 2004, Mehlen and Puisieux 2006). According to SEER's report for the year 2016, 20% of the CRC cases are diagnosed at a stage when the cancer has undergone metastasis, thus reducing the 5-year survival rate to 13% as compared to 91% (in case of stage I and stage II). These statistics suggest that it is important to identify the survival mechanisms that lead to increased metastasis so that they can be targeted therapeutically.

The PI3K-Akt signaling pathway is a downstream effector of growth factor receptors and is often deregulated in cancer (Fruman and Rommel 2014). Auto phosphorylation of the growth factor receptors leads to the synthesis of phosphotidyl inositol triphosphate (PIP3) (Vivanco and Sawyers 2002). Akt is a serine/threonine kinase consisting of 3 isoforms, Akt1 Akt2 and Akt3.

These isoforms of Akt have a high level of structural homology and share similar mechanisms of activation (Clark and Toker 2014). Activation of Akt is mediated by its interaction with PIP3, which causes a change in conformation of Akt, and its recruitment to the plasma membrane (Blume-Jensen and Hunter 2001), where it comes in close proximity with PDK-1 and mTORC2 leading to phosphorylation of Akt at Thr308 and Ser473 respectively (Stokoe, Stephens et al. 1997, Sarbassov, Guertin et al. 2005, Salmena, Carracedo et al. 2008). Akt promotes cell proliferation, cell survival, growth and various other signaling pathways by phosphorylating its downstream targets (Manning and Cantley 2007). Termination of Akt signaling is accomplished by multiple phosphatases including PTEN, PP2A, and PHLPP1/2 (Brognard, Sierecki et al. 2007, Salmena, Carracedo et al. 2008, Gewinner, Wang et al. 2009).

Although Akt isoforms share structural homology, phenotypes of knockout mice of individual Akt isoforms suggest that they have non-redundant functions (Stokoe, Stephens et al. 1997, Cho, Mu et al. 2001, Easton, Cho et al. 2005) . Several studies have shown contradictory role of Akt isoforms in same cancer type as in breast cancer (Irie, Pearline et al. 2005). Additionally the isoforms have different roles in different cancer types (Agarwal, Brattain et al. 2013). In breast cancer Akt1 and 2 show opposing role as Akt1 inhibits cell motility and invasion whereas Akt2 enhances these processes. (Irie, Pearline et al. 2005, Yoeli-Lerner, Yiu et al. 2005). Akt isoforms are aberrantly expressed in cancer showing heterogeneity in their expression pattern with overexpression of Akt1 in gastric and colon cancer. Akt2 is highly expressed in breast, ovarian and colon cancers and Akt3 expression increases in breast and prostate cancer. The role of Akt isoforms in various cancers have been discussed in detail in a review article (Agarwal, Brattain et al. 2013).

An analysis on human CRC samples showed increased expression of Akt2 in 40% of the tumors (Parsons, Wang et al. 2005). Previous work has shown that Akt2 is highly expressed during late stages of CRC and metastatic tumors. It has been demonstrated that suppression of

Akt2 reduces the metastasis of CRC cells which cannot be restored by overexpressing Akt1 (Rychahou, Kang et al. 2008). Additionally, it has been reported that there is a correlation between PTEN deficiency, Akt2 overexpression and increased metastatic potential in CRC (Rychahou, Kang et al. 2008). Recent studies have shown that PTEN-deficient prostate cancer cells are dependent on only Akt2 for survival (Chin, Yuan et al. 2014). Genetic inactivation of Akt1 and Akt2 leads to reduction in proliferation *in vitro* and significantly reduced metastasis *in vivo*(Ericson, Gan et al. 2010). Although reports indicate the role of Akt2 in establishment of CRC metastasis, the mechanism of Akt2-driven metastasis is still unknown. Ingenuity pathway analysis (IPA) shows that there are several signaling pathways (Src, p38MAPK, Bax, ERK1/2) which are specifically affected by Akt2 (Agarwal, Brattain et al. 2013) thus affecting survival of cancer cells. Aberrant Akt2 activation may be playing an important role in sustaining processes important for metastasis.

Metastasis Tumor Suppressor Gene 1 (MTSS1) also known as Missing in Metastasis (MIM) which was initially considered important for early organ development has recently been identified as a suppressor of metastasis of cancer cells. It is known to affect actin assembly thus inhibiting cell growth and cell migration. It has been reported that MTSS1 expression decreases in various cancers such as breast, esophageal, bladder, kidney, gastric and pancreatic cancer (Du, Ye et al. 2011, Wang, Xu et al. 2011, Xie, Ye et al. 2011, Du, Ye et al. 2013, Zhou, Li et al. 2015). MTSS1 interacts with an actin-binding protein, Cortactin, to mediate its action. Cortactin is a nucleation-promoting factor, which gets activated on binding with MIM-B (Lin, Liu et al. 2005). It is responsible for formation of lamellipodia by enhancing the formation of branched actin network.

Although it is known that Akt2 overexpression is required for establishment of metastasis, not much is known about the role of Akt2 in maintenance of established metastasis in colorectal cancer. Moreover, it is not known whether Akt2 provides a survival advantage to the cells after

establishment of metastasis. To investigate the role of Akt isoforms in maintenance of established metastasis we have used lentiviral doxycycline inducible knockdown of Akt isoforms (*in vitro*), an orthotopic implantation model for *in vivo* studies, and data from The Cancer Genome Atlas for information about CRC patients. We have demonstrated an increase in the expression of MTSS1 on loss of Akt2; however, loss of Akt1 and Akt3 did not show any such effect. Furthermore, for *in vivo* studies, the effect of loss of Akt2 on established metastases was determined by introducing Dox in the drinking water after metastases was established. This was done to determine whether Akt2 loss impacts the maintenance of established metastases. It was observed that loss of only Akt2 efficiently reduced the metastatic burden. These data help us to postulate that specific reduction of Akt2 could be a means of enhancing cell death, thus leading to reduced survival in established metastasis.

## 5.4 Results

### 5.4.1 Knockdown of Akt isoforms affect cell proliferation and cell death in highly metastatic colon cancer cells

To understand the role of Akt isoform on cell proliferation and cell death, we have established doxycycline (Dox)-inducible knockdown of the isoforms in GEO and CBS cells which express high levels of all the three Akt isoforms (Fig.5.1 A) . Non-targeting sh was used as the control. We observed no change in the expression of Akt isoforms on treatment with Dox in non-targeting sh (Fig.S1 A, B). GEO and CBS cells are IGF1R (Insulin like growth factor 1 receptor) dependent cell lines. It is known that 30-40% cases of CRC are IFG1R-dependent, thus making these cells a suitable model for this study. Figure5.1 (B, C, D and E) show that addition of Dox leads to loss of Akt2 both at the protein and RNA levels. The shRNA for Akt2 is specific to the respective gene as there is no change in the expression of Akt1 and Akt3 on knockdown of Akt2. To determine the effect of Akt2 on cell growth, MTT assays were performed after treatment with Dox. There was a significant ($p<0.001$) reduction in cell proliferation on loss of Akt2 in

comparison to non-targeting sh. (Fig.5.1 F, G). Additionally we observed that Akt2 knockdown results in about a 6-fold increase in cell death as determined by DNA fragmentation assay (Fig.5.1 H, I) in comparison to non-targeting sh +Dox. Fig. S2,S3 (E, F) show that there was no change in cell proliferation on loss of Akt1 and Akt3 respectively, however a two fold increase in cell death was observed on loss of Akt1 and Akt3 (Fig.S2,S3 (G, H)) respectively.

### 5.4.2 Effect of Akt isoforms on motility of colon cancer cells

We performed transwell migration assays on GEO GFP Akt isoform knockdown cells to determine the effect of Akt isoforms on motility. We observed that there is a highly significant (about 75% reduction) reduction in motility after knockdown of Akt2 (Fig.5.1 J, K). A reduction in cell motility on loss of Akt1 and Akt3 was also observed, although to a much lesser extent (about 35% reduction) (Fig. S2,S3 (I, J)). Non-targeting sh was used as a control.

### 5.4.3 Regulation of cell survival by Akt isoforms

The Inhibitor of Apoptosis Protein (IAP) family members are aberrantly expressed in various cancers (Berezovskaya, Schimmer et al. 2005). The members of IAP family, XIAP (X-linked IAP) and survivin are usually present in mitochondria. Under stress conditions, XIAP and survivin move into the cytoplasm where they form a complex which then stabilizes the IAP members leading to increased cell survival (Dohi, Okada et al. 2004). Additionally, Akt phosphorylates XIAP at serine 87 resulting in its stabilization (Dan, Sun et al. 2004), thereby leading to increased survival. Loss of Akt isoforms leads to reduction in the expression of both XIAP and survivin (Fig.5.2 A, B: Fig. S4,S5 (A, B)). Additionally we observed an increase in the expression of Apoptosis Inducing Factor (AIF) on loss of Akt1 and Akt2 (Fig.5.2 A, Fig.S4 (A, B)). Previous studies have shown that loss of pAkt in the presence of an Akt kinase inhibitor, MK-2206, leads to an increase in the expression of AIF (Agarwal, Chaudhuri et al. 2014). AIF is normally present in the mitochondria but upon induction of apoptosis this protein translocates

from mitochondria to nucleus and causes DNA fragmentation. Treatment with MK-2206 leads to translocation of AIF into the nucleus thus leading to increased cell death (Agarwal, Chaudhuri et al. 2014). Additionally loss of Akt2 reduces the phosphorylation of the cytoskeletal protein ezrin (T567), which then inhibits cell survival. Loss of Akt1 and Akt3 do not affect the phosphorylation of ezrin. We observed no change in the expression of XIAP, survivin, AIF, ezrin and pEzrin (T567) on treatment with Dox of GEO and CBS cells expressing non-targeting sh (Fig.S1 C, D)

### 5.4.4 Differential regulation of MTSS1 by Akt isoforms

The differential effect of Akt isoforms on metastatic genes was determined by performing a $RT^2$ Profiler PCR array analysis on human metastatic genes. We observed that there was differential expression of several metastatic genes on loss of the Akt isoforms (Fig.5.3 A). Loss of Akt2 led to an increase in the expression of MCAMs, Kiss1 and E-Cadherin. The role of these proteins on metastasis has been well studied (Cheng, Chan et al. 2007, Navenot, Fujii et al. 2009, Williams, Schneider et al. 2014). However, we identified a novel protein, Metastasis Suppressor Gene 1 (MTSS1), which was differentially expressed on loss of Akt isoforms. We observed that there was a robust increase in the expression of MTSS1 on loss of Akt2 (Fig.5.3 B, C), however, loss of Akt1 and Akt3 did not affect its expression (Fig.S4,S5 (C, D)). MTSS1 has been identified as a potential anti-metastatic protein as its expression is less in metastatic cells compared to non-metastatic cancer cells in several types of malignancies, including prostate, stomach, bladder and liver cancer (Parr and Jiang 2009), (Lee, Macoska et al. 2002). Contradictory reports suggest that MTSS1 over expression increases the metastatic potential of the melanoma cells (Mertz, Pathria et al. 2014). MTSS1 is still understudied in CRC. Bompard et.al. have shown that MTSS1 affects cytoskeleton dynamics by interacting with cortactin (CTTN), a substrate of Src kinase and major activator of actin- branching and polymerization (Bompard, Sharp et al. 2005). We made the observation that only Akt2 depletion increases the expression of MTSS1 and reduces the

expression of pSrc (Y416) and pCortactin (Y421) (Fig.5.3 B, C). MTSS1 has been shown to interact with protein tyrosine receptor phosphatase delta, and leads to its increased expression, which in turn inhibits the phosphorylation of Src. We observed a loss in the expression of pSrc (Y416) and pCortactin (Y421) on knockdown of Akt2. However, no change in the expression of pSrc and pCortactin was observed on loss of Akt1 and Akt3 (Fig.S3, S4 (C, D)).

The expression of MTSS1 is regulated by DNA promoter methylation (Fan, Chen et al. 2011), ubiquitination (Zhong, Shaik et al. 2013) and/or by miRNAs (Guo, Ren et al. 2015). Methylation pyrosequencing was performed (-141 to -236 upstream of the transcription start site) and CpG methylation percentage was aligned with Human Genome build Dec. 2013 (GRCh38/hg38) to determine the percentage of methylation as indicated in Supplementary table1. We observed no change in the percentage of MTSS1 promoter methylation on loss of any of the isoforms (Supplementary table1). β-TRCP is an E3 ubiquitin ligase, which causes the ubiquitination of MTSS1 thus leading to its proteasomal degradation (Zhong, Shaik et al. 2013). Interestingly, we observed a robust loss in the expression of β-TRCP on loss of Akt2 with no change in the expression on loss of Akt1 and Akt3 (Fig.5.3 G, Fig.S4,S5 (E)) , thus suggesting that loss of Akt2 leads to less ubiquitination and less degradation of MTSS1, resulting in a increase in its expression.

MTSS1 affects the motility of cells by affecting the actin cytoskeleton (Bompard, Sharp et al. 2005). To confirm that the reduction in motility of CRC cells by Akt2 loss is driven by MTSS1, we knocked down MTSS1 in GEO GFP Akt2 knockdown cells. We observed that loss in MTSS1 generates increased expression of pSrc and pCortactin (Fig.5.3 E). Transwell migration assay showed that there was a 55% increase in the motility of the cells on loss of MTSS1 as compared to Akt2 sh +Dox (Fig.5.3 F). Additionally, we determined the effect of MTSS1 loss on the actin cytoskeleton by performing immunofluorescence staining with Phalloidin antibody.

Fig.S6 shows that there is more actin polymerization (as can be seen by higher phalloidin staining) on loss of MTSS1 thus leading to increase in the motility of the cells.

### 5.4.5 Regulation of metastasis by Akt isoforms *in vivo*

Orthotopic implantation studies were performed with Akt1 sh and Akt2 sh to determine the effect of Akt isoform on metastasis. Akt isoforms were knocked down by adding Dox to the drinking water. The sh was induced after six weeks of caecal implantation of the tumor, thus allowing establishment of primary tumor and metastases. This strategy is designed to determine the effect of Akt isoforms on maintenance of established metastasis. Non-targeting sh was used as a control. We observed that 59% of animals had liver metastasis in non-targeting sh +Dox, 52% of animals had metastasis in Akt1 sh +Dox (Fig.S7 B), however only 35% of animals in Akt2 sh +Dox had metastasis (Fig.5.4 B). Importantly, no change in the weight of primary tumor was observed on loss of Akt1 (Fig.S7 C) and Akt2 (Fig.5.4 C) indicating that primary tumor burden remains unchanged. Expression of human GAPDH was determined in the mice liver on loss of Akt1 and Akt2 to determine the metastatic tumor burden. There was a significant reduction in metastatic burden on loss of Akt2 as compared to non-targeting sh +Dox (Fig.5.4 D), however the metastatic burden on loss of Akt1 did not change significantly in comparison to non-targeting sh +Dox (Fig.S7 D). Furthermore hematoxylin and eosin staining shows less metastatic areas in the liver after loss of Akt2 (Fig.5.5 A) as compared to non-targeting sh +Dox and Akt1 sh +Dox (Fig.S8 A). To quantify metastatic spots we counted the RFP spots on each lobe of the liver under immunofluorescence microscope. Fig.5.5 B shows the representative images of liver metastatic spots as observed in Akt2sh +Dox and non-targeting sh +Dox. Fig.5.5 C represents the quantification of the metastatic spots. (Each dot on the graph is a representation of one mouse). There was a significant reduction in the number of metastatic spots in Akt2 sh +Dox as compared to non-targeting sh +Dox. However loss of Akt1 did not result in significant change in liver metastatic spots as shown in Fig.S8 C.

### 5.4.6 Regulation of cell survival by Akt isoforms *in vivo*

TUNEL staining of primary tumors showed that there was an increase in the number of apoptotic cells after knockdown of Akt isoforms. We observed a six-fold increase in the % of apoptotic cells on loss of Akt2 (Fig.5.6 A, B) and a two-fold increase on loss of Akt1 (Fig.S9 A, B) in comparison to non-targeting sh +Dox. Additionally there was strong nuclear staining for Ki67 in non-targeting sh +Dox. Quantitative analysis by Definience Tissue Studies 64 (Dual 4.1) software revealed that there was a significant reduction ($p<0.001$) in Ki67 staining intensity on loss of Akt2 (Fig.5.6 C, D). Akt1 knockdown showed significant reduction ($p<0.05$) in Ki67 staining intensity as compared to non-targeting sh +Dox (Fig.S9 (C, D)). Additionally, IHC analysis reveals loss of Akt1 and Akt2 in the primary tumor as shown in Fig.S9 E, F and Fig.5.6 E,F respectively. These data suggest that introduction of Dox led to a reduction in expression of Akt 1 and Akt2 in the primary tumor leading to an increase in apoptosis as shown by increased TUNEL staining. However, due to inter-tumor and intra-tumor heterogeneity there was no significant change in the overall primary tumor burden, which is represented by no significant change in the weight of the primary tumor.

Next we performed IHC analysis for MTSS1 on primary tumor samples. We observed strong staining of MTSS1 in Akt2 knockdown primary tumor samples as compared to Akt1sh +Dox and non-targeting sh +Dox. Quantitative analysis reveals, that, in comparison to non-targeting sh +Dox, Akt2 knockdown results in a significant ($p<0.001$) increase in the staining intensity of MTSS1 (Fig.5.6 G, H). There was no significant difference in the staining intensity of MTSS1 in Akt1 shRNA primary tumor samples (Fig.S9 (G, H). Additionally it was observed that there was a loss in the expression of the IAP family member XIAP on loss of Akt 1 and Akt2. Fig.5.6 I and Fig.S9 I showed strong nuclear and cytoplasmic staining of XIAP in non-targeting sh +Dox. Quantitative analysis revealed that there was a significant reduction ($p<0.001$) in the staining intensity for XIAP on loss of Akt1 and Akt2 (Fig.S9 J and Fig.5.6 J).

Western blot analysis was performed on the primary tumor samples to confirm the IHC results. We observed a decrease in the expression of β-TRCP on loss of Akt2 (Fig.5.6 K) in the primary tumor, with no change on loss of Akt1 (Fig.S9 K), which suggests less ubiquitin-mediated degradation of MTSS1 on loss of Akt2, corroborating our *in vitro* data.

### 5.4.7 Expression of Akt isoforms and MTSS1 in human CRC samples

The Cancer Genome Atlas data sets showed that there was a significant increase ($p<0.0001$) in Akt2 in tumor samples in comparison to normal samples, however the expression levels of Akt1, Akt3 and MTSS1 were reduced significantly in tumor samples (Fig.5.7 A). Additionally we observed that there was a negative correlation between Akt2 and MTSS1 in primary human tumor samples as seen in the scatter plot (Fig.5.7 B). Furthermore, Oncomine data also showed that Akt2 and MTSS1 are negatively correlated. There was an increase in the expression of Akt2 as colon cancer progresses in comparison to normal colon although a decrease in the expression of MTSS1 was observed in the matched samples.

## 5.5 Discussion

CRC is the second leading cause of cancer related deaths in the United States. Advances in CRC screening, early detection, prevention and research during past few decades have significantly increased the 5-year survival rate. However, the survival rate has continued to show dismal progress in the metastatic stage of the disease. The higher survival rate at early stage is attributed to surgical resection of primary tumor followed by adjuvant chemotherapy, however metastasis is untreatable (Jin and Mu 2015) thus emphasizing the urgent need to develop anti-metastatic therapies . There are several molecular pathways that lead to increased proliferation/survival at primary as well as metastatic sites (Valastyan and Weinberg 2011). Anti-metastatic therapies might be more effective if they inhibit the survival of the cancer cells which have already spread out from the primary tumor instead of targeting the escape of cells from the primary tumor

(Valastyan and Weinberg 2011). This is important because by the time the patient is diagnosed with tumor, the cancer cells have already left the primary tumor (Valastyan and Weinberg 2011) and are either at the step of intravasation, circulation or extravasation of metastasis. It has been reported that drugs targeting metastasis have to be used for long term, thus making them incompetent in the clinic due to their known side effects (Valastyan and Weinberg 2011). Thus, anti-metastatic therapies need to be designed in such a way that they elicit regression of established metastasis so that they can qualify for clinical practice.

To determine the effect of a critical survival node "Akt" in metastasis, various studies have been performed. Rychahou et.al.have shown that suppression of Akt2 reduces the metastatic potential of CRC cells both *in vitro* and *in vivo*. Furthermore, they have also reported a link between PTEN loss, Akt2 overexpression and increased metastatic efficiency of CRC cells (Rychahou, Kang et al. 2008). Ericson et.al.reported that genetic loss of Akt1, Akt2 and PDPK1 inhibit the growth of CRC cells (Ericson, Gan et al. 2010). These studies confirmed that loss of Akt2 leads to reduction in the establishment of metastasis however mechanism for reduction in metastasis on loss of Akt2 were not well characterized. Splenic injections of CRC cells were performed to determine the effect of Akt2 on metastasis in these studies. The limitation of this model is that it does not mimic the natural pattern of CRC metastasis. In this method, the earlier steps of metastasis (invasion and intravasation) are bypassed and only later stages of metastasis (circulation and extravasation) can be studied. The orthotopic implantation model used in the present study recapitulates the pattern of human CRC primary and metastatic deposits as it addresses both the early stages i.e. local invasion at the primary site as well as the later stages of metastasis. Although no change in primary tumor burden was observed on loss of Akt2, we observed 40% reduction (6 animals out of 17) in the number of animals with liver metastasis on loss of Akt2, demonstrating that loss of Akt2 might play a role in regression of established CRC metastasis. It is known that 20% of the cases of CRC are diagnosed at stage 3 or stage 4 i.e. when

cancer has already undergone metastasis. Identification and genetic validation of the role of Akt2 in the maintenance of established metastasis provides insight for inhibiting Akt2 for CRC patients with metastasis.

Mechanistically we identified that loss of Akt2 leads to decrease in the expression of IAPs such as XIAP and survivin, leading to increase in cell death. Shiue et.al.have shown that Akt2 is responsible for the phosphorylation of ezrin at T567, thus leading to its activation, which in turn leads to increase in metastasis (Shiue, Musch et al. 2005). Additionally, it has been shown that phosphorylated ezrin (T567) increases cell survival by increasing the expression of XIAP. We observed a decrease in the phosphorylation of ezrin (T567) on loss of Akt2. Loss of Akt2 led to an increase in the expression of Apoptosis Inducing Factor (AIF). AIF is responsible for DNA fragmentation thus leading to cell death (Agarwal, Chaudhuri et al. 2014).

Importantly an increase in the expression of MTSS1 was observed on loss of Akt2. It has been reported that loss of MTSS1 promotes tumor growth by enhancing cell proliferation, invasion and/or cell migration (Fan, Chen et al.), (Parr and Jiang 2009), (Loberg, Neeley et al. 2005). MTSS1 leads to a reduction in the expression of pSrc at Y416 by activating PTP δ (Protein Tyrosine Phosphatase δ), which in turn leads to loss in the phosphorylation of Cortactin at Tyrosine 421, thus affecting the actin cytoskeleton (Bershteyn, Atwood et al. 2010). Interestingly, we observed that loss of Akt2 leads to the activation of MTSS1→pSrc→pCortactin axis leading to reduction in metastasis (Fig.5.7 C). MTSS1 is regulated by methylation (Utikal, Gratchev et al. 2006) and/or ubiquitination (Zhong, Shaik et al. 2013) . β-TRCP is a ubiquitin ligase enzyme that ubiquitinates MTSS1 thus leading to its degradation via 26S proteasome (Fuchs, Spiegelman et al. 2004). MTSS1 expression is inhibited by methylation of the CpG Island in its native promoter region (Utikal, Gratchev et al. 2006). We observed that loss of Akt2 does not affect the methylation status of MTSS1 (Supplementary table 1). However there was a decrease in the

expression of β-TRCP on loss of Akt2. This might indicate less ubiquitin-mediated degradation of MTSS1 leading to an increase in its expression. Loss of Akt1 and Akt3 does not show any change in the expression of β-TRCP leading to no reduction in the expression of MTSS1.

TCGA data analysis pointed out Akt2 as the only isoform which is significantly increased ($p<0.0001$) in CRC tumor samples as compared to normal samples, whereas Akt1, Akt3 and were significantly reduced. Interestingly, it was also observed that in primary tumor, Akt2 and MTSS1 are negatively correlated ($r= -0.12$). These data help us to hypothesize that Akt2/MTSS1 negative correlation might be useful in identification of patients with metastasis. This would need to be confirmed by performing analysis on larger sets of human samples. This study as well as the earlier reports on the role of Akt2 in metastasis emphasizes that Akt2-specific inhibitors can be used as a targeted therapy for metastatic patients.

Overall we have demonstrated that out of the three isoforms of Akt, Akt2 and its downstream signaling plays a critical role in mediating cell survival and maintenance of CRC metastasis, thus identifying Akt2 as an important therapeutic target. The translational importance of determining the effect of Akt2 on established metastasis needs to be clinically evaluated in the future.

**5.6 Figures**

**Figure 5.1:** (A) Western blot analysis to show the endogenous level of Akt1, 2 and 3 in colon cancer cells. (B) and (C) Immunoblot showing knockdown of Akt2 in presence of Dox in GEO and CBS cells respectively. The blot represents that the shRNA is specific to Akt2 as there is no change in the expression of Akt1 and Akt3 in the presence of Dox. GAPDH is used as a loading control. (D) and (E) qPCR analysis indicates that there is a significant loss of Akt2 on treatment with Dox in both GEO and CBS cells respectively. (F) and (G) demonstrate that there is a significant loss in cell proliferation on loss of Akt2 in comparison to non-targeting sh+Dox as determined by MTT assay. (H) and (I) DNA fragmentation assay reveal that there is 5-6 fold increase in cell death on removal of Akt2 in both GEO and CBS cell lines. (J) Representative images depicting less migration of cells on loss of Akt2. (K) Quantification of transwell migration assay showing a significant reduction in motility of cells on loss of Akt2. (***=$p<0.0001$)

**Figure 5.1**

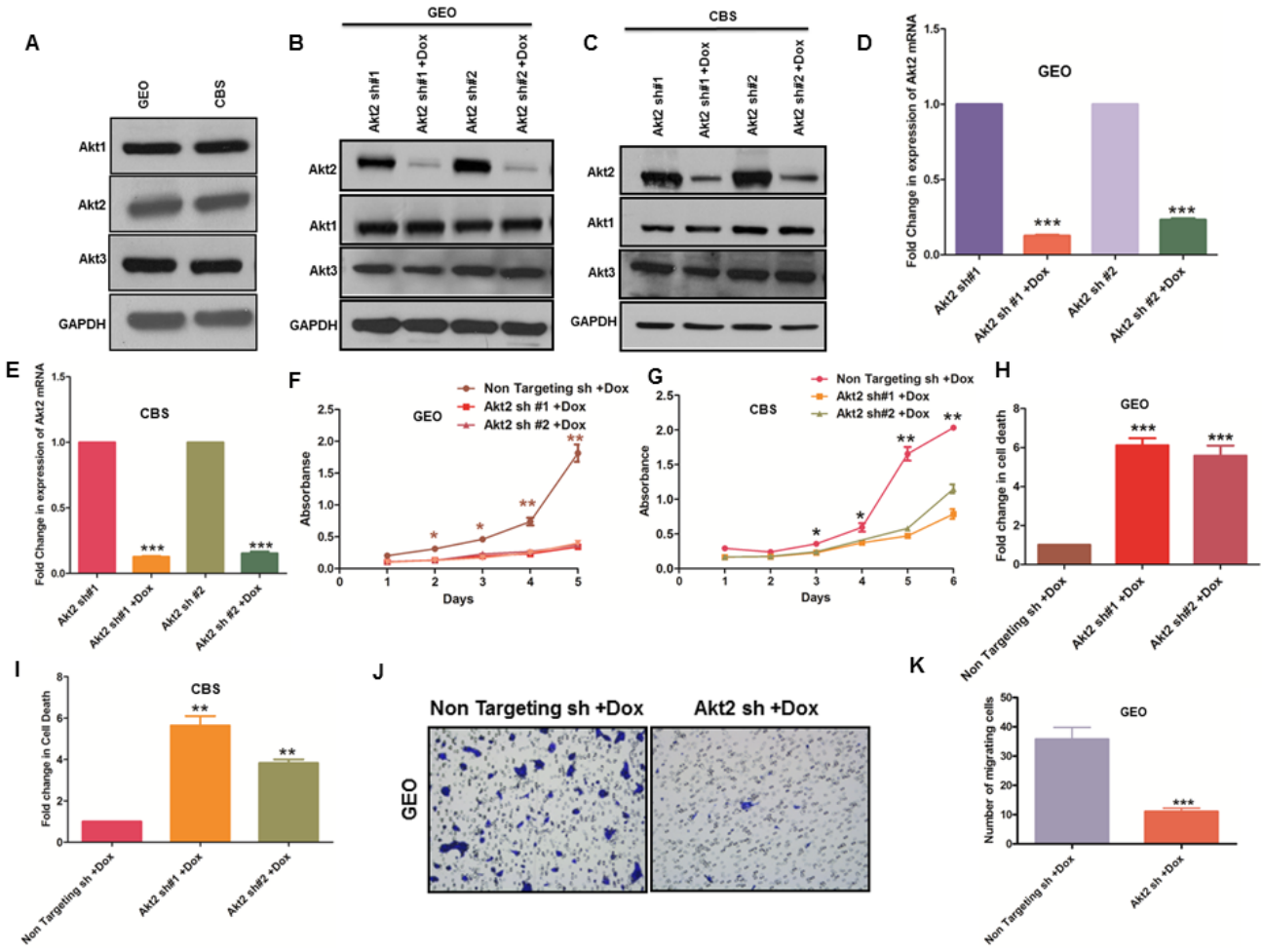

A
GEO
CBS
Akt1
Akt2
Akt3
GAPDH
B
GEO
Akt2 sh#1
Akt2 sh#1 +Dox
Akt2 sh#2
Akt2 sh#2 +Dox
C
CBS
D
GEO
Fold Change in expression of Akt2 mRNA
E
CBS
F
GEO
Non Targeting sh +Dox
Akt2 sh #1 +Dox
Akt2 sh #2 +Dox
Absorbanse
Days
G
CBS
Absorbance
H
GEO
Fold change in cell death
Non Targeting sh +Dox
I
CBS
Fold change in Cell Death
J
Non Targeting sh +Dox
Akt2 sh +Dox
GEO
K
GEO
Number of migrating cells

**Figure 5.2:** (A) and (B) Western blot analysis shows a robust loss in the expression of XIAP and Survivin on loss of Akt2. There is an increase in the expression of AIF and a decrease in the expression of pEzrin (T567) on knockdown of Akt2 in GEO and CBS respectively. However there is no change in the expression of total ezrin. GAPDH has been used as a loading control.

**Figure 5.2:**

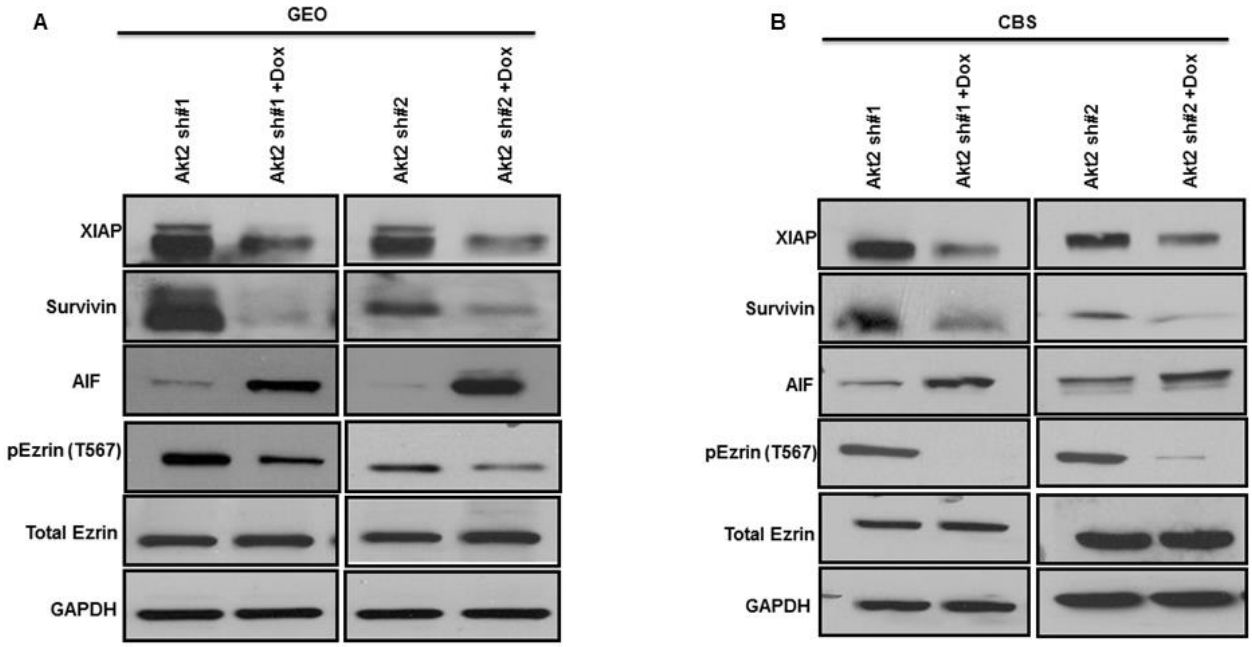

A
GEO
Akt2 sh#1
Akt2 sh#1 +Dox
Akt2 sh#2
Akt2 sh#2 +Dox
XIAP
Survivin
AIF
pEzrin (T567)
Total Ezrin
GAPDH
B
CBS
Akt2 sh#1
Akt2 sh#1 +Dox
Akt2 sh#2
Akt2 sh#2 +Dox
XIAP
Survivin
AIF
pEzrin (T567)
Total Ezrin
GAPDH

**Figure 5.3:** (A) Venn diagram showing the genes which were differentially expressed on knockdown of the three Akt isoforms. The genes in Red have higher expression as compared to non-targeting sh and the genes in green color have reduced expression. (B) and (C) Western blot showing an increase in the expression of MTSS1 and a loss in the expression of pSrc (Y416) and pCortactin (Y421) on removal of Akt2 in GEO and CBS cells respectively. The expression of total Src remains unchanged. (D) Immunoblot showing a knockdown on MTSS1 in Akt2 knockdown cells. (E) Demonstrates a loss in the expression of pSrc (Y416) and pCortactin (Y421) on loss of Akt2, this loss is rescued when MTSS1 was knocked down in Akt2 sh +Dox cells. (F) Representative images of transwell migration assay showing a reduction in migration of cells on loss of Akt2, however MTSS1 knockdown leads to increase in the motility of the cells. (G) Quantification of migration assay shows a significant reduction in cell motility on loss of Akt2, which is rescued on loss of MTSS1. (H) Western blot analysis shows that there is a loss in the expression of βTRCP on loss of Akt2.

**Figure 5.3:**

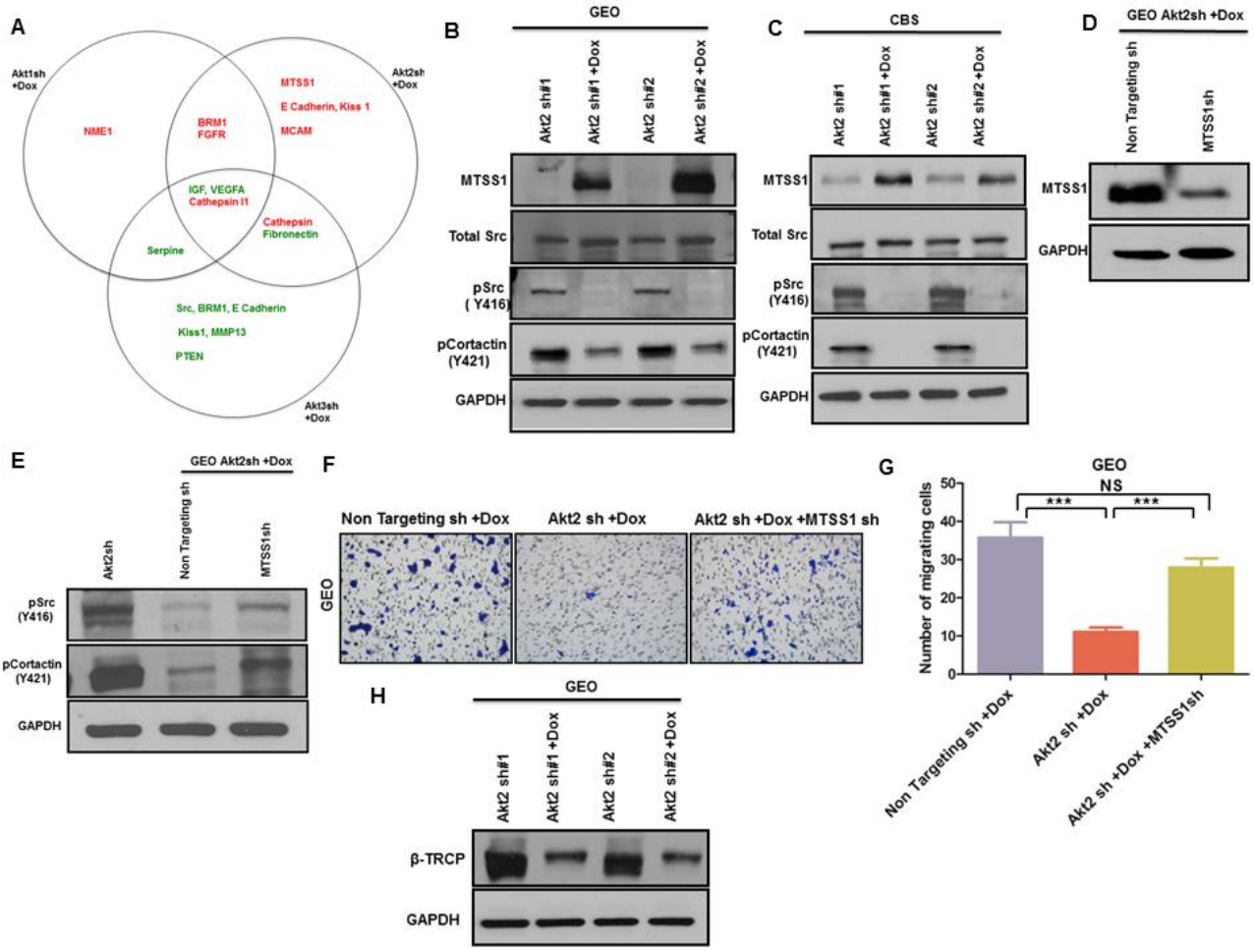
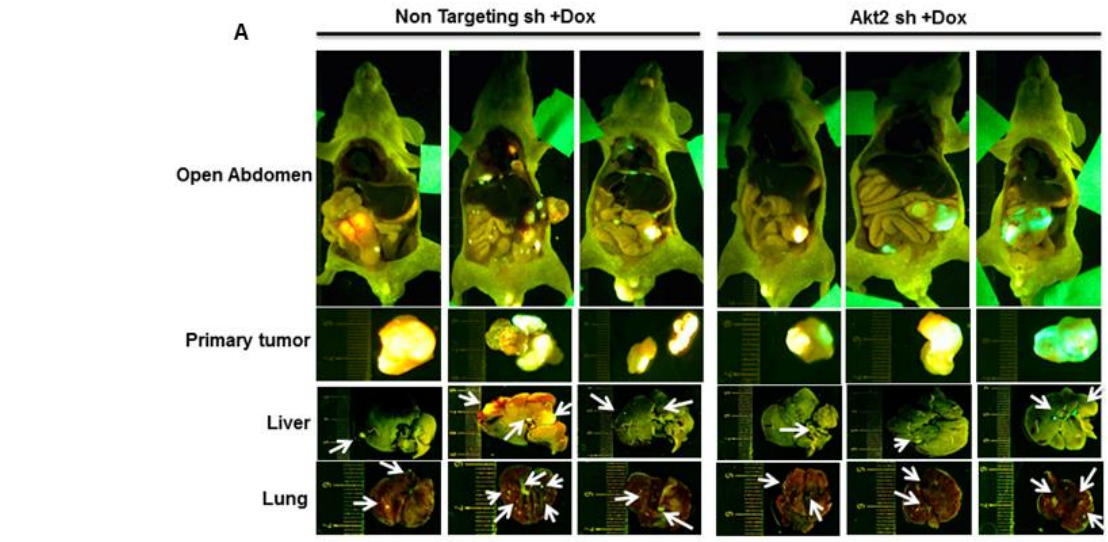

A
Akt1sh +Dox
Akt2sh +Dox
Akt3sh +Dox
NME1
BRM1
FGFR
MTSS1
E Cadherin, Kiss 1
MCAM
IGF, VEGFA
Cathepsin l1
Cathepsin
Fibronectin
Serpine
Src, BRM1, E Cadherin
Kiss1, MMP13
PTEN
B
GEO
Akt2 sh#1
Akt2 sh#1 +Dox
Akt2 sh#2
Akt2 sh#2 +Dox
MTSS1
Total Src
pSrc ( Y416)
pCortactin (Y421)
GAPDH
C
CBS
Akt2 sh#1
Akt2 sh#1 +Dox
Akt2 sh#2
Akt2 sh#2 +Dox
MTSS1
Total Src
pSrc (Y416)
pCortactin (Y421)
GAPDH
D
GEO Akt2sh +Dox
Non Targeting sh
MTSS1sh
MTSS1
GAPDH
E
GEO Akt2sh +Dox
Akt2sh
Non Targeting sh
MTSS1sh
pSrc (Y416)
pCortactin (Y421)
GAPDH
F
Non Targeting sh +Dox
Akt2 sh +Dox
Akt2 sh +Dox +MTSS1 sh
GEO
G
GEO
NS
***
***
Number of migrating cells
50
40
30
20
10
0
Non Targeting sh +Dox
Akt2 sh +Dox
Akt2 sh +Dox +MTSS1sh
H
GEO
Akt2 sh#1
Akt2 sh#1 +Dox
Akt2 sh#2
Akt2 sh#2 +Dox
β-TRCP
GAPDH

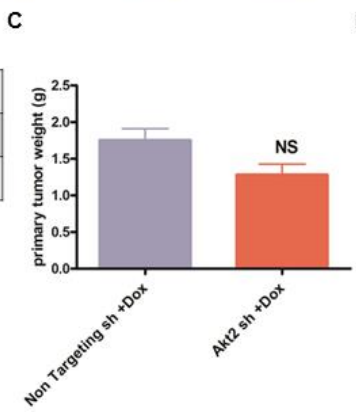
**Figure 5.4:** (A)Fluorescent image of whole mice, primary tumor, liver, lung from orthotopic implantation of non-targeting sh and Akt2 sh xenograft tumor respectively. (B) showing that there is a 40% reduction in the number of animals with metastasis on loss of Akt2 in comparison to non-targeting sh. (C) Bar graph showing no change in the weight of primary tumor on loss of Akt2. (D) qPCR analysis shows a reduction in the expression of human GAPDH in mice liver with Akt2 sh tumor , thus showing a reduction in metastatic tumor burden.

**Figure 5.4:**




B

| | Non Targeting sh +Dox | Akt2 sh +Dox |
|---|---|---|
| No. of animals with Primary tumor | 22/22 (100%) | 17/17 (100%) |
| No. of animals with Metastasis | 13/22 (59%) | 6/17 (35.2%) |



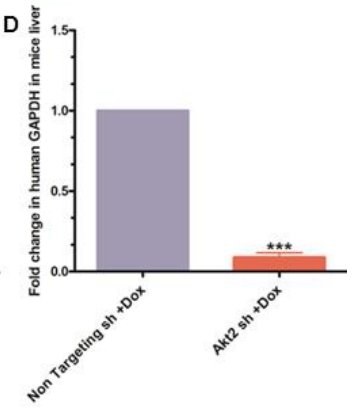



**Figure 5.5:** (A) H and E staining of the primary tumor and liver metastasis samples. (B) Representative images of liver metastatic spots as seen under immunofluorescence microscope. (C) Quantification of the number of liver metastatic spots, showing a significant ($p<0.05$) reduction in the number of metastatic spots on loss of Akt2.

**Figure 5.5:**

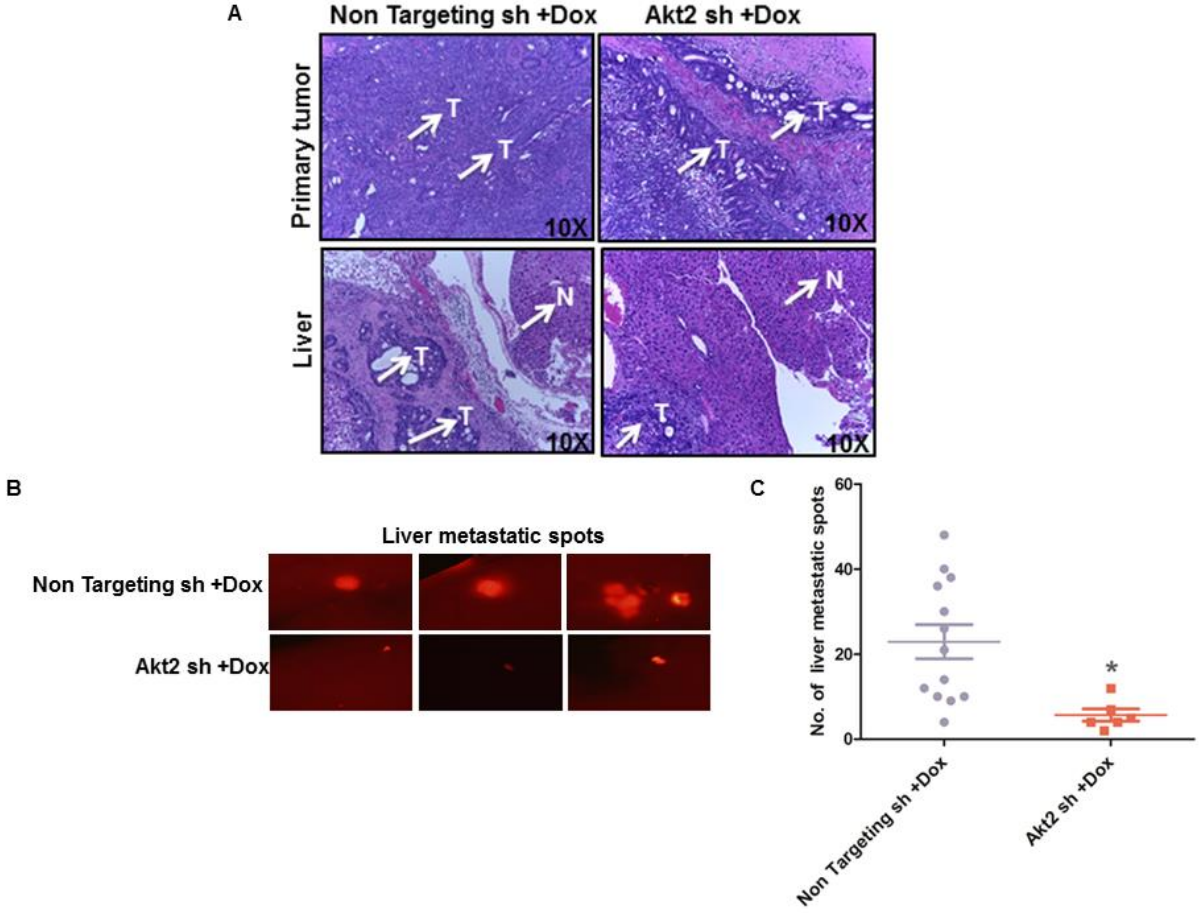

A
Non Targeting sh +Dox
Akt2 sh +Dox
Primary tumor
Liver
T
N
10X
B
Liver metastatic spots
Non Targeting sh +Dox
Akt2 sh +Dox
C
No. of liver metastatic spots
60
40
20
0
*
Non Targeting sh +Dox
Akt2 sh +Dox

**Figure 5.6:**(A) TUNEL staining of the primary tumor. The brown staining represents the apoptotic cells. (B) Statistical analysis shows that there is a highly significant ($p<0.001$) increase in the % of apoptotic cells on loss of Akt2. (C) Ki67 staining for Akt2 sh +Dox and non-targeting sh +Dox. (D) Bar graphs show that there is a highly significant ($p<0.001$) decrease in the staining intensity of Ki67 on loss of Akt2. **(**E) IHC analysis showing a loss in expression of Akt2 in the primary tumor in presence of Akt2sh. (F) Quantitative representation shows that there is a significant reduction ($P<0.001$) in the staining intensity of Akt2 in Akt2sh tumors as compared to tumors with non-targeting sh. (G) IHC staining shows that there is a robust increase in the expression of MTSS1 on loss of Akt2 as compared to non-targeting sh +Dox. (H) Bar Graphs show that there is a significant increase ($P<0.001$) in the expression of MTSS1 on loss of Akt2. (I) demonstrates the expression of XIAP on loss of Akt2. (J) Bar graphs show a significant reduction ($p<0.001$) in the staining intensity of XIAP on loss of Akt2 as compared to non-targeting sh. (K) Western blot analysis of primary tumor showing a reduction in the expression of XIAP and β-TRCP and an increase in the expression of MTSS1 on loss of Akt2.

**Figure 5.6:**

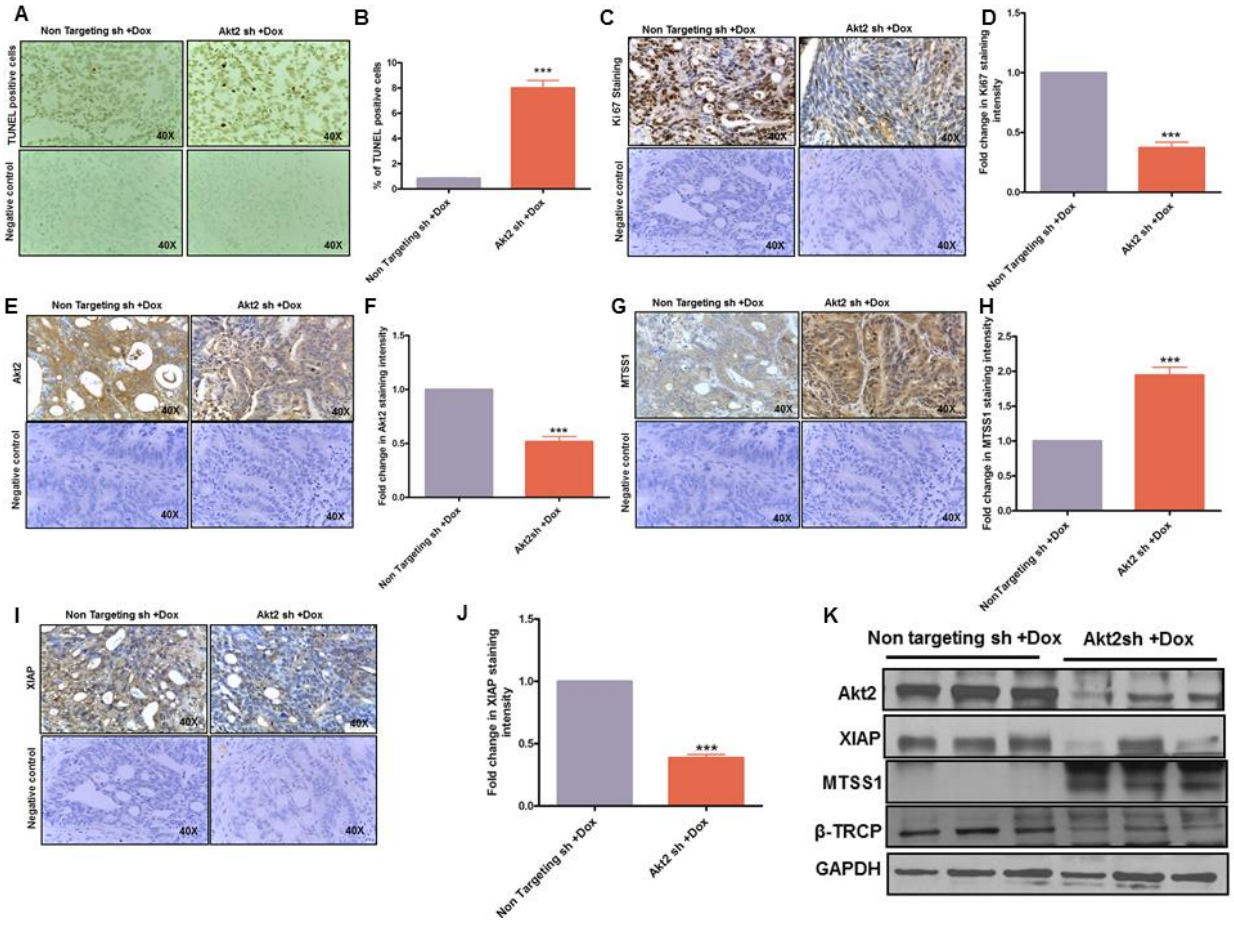

A
Non Targeting sh +Dox
Akt2 sh +Dox
TUNEL positive cells
Negative control
40X
B
% of TUNEL positive cells
***
Non Targeting sh +Dox
Akt2 sh +Dox
C
Non Targeting sh +Dox
Akt2 sh +Dox
Ki 67 Staining
Negative control
40X
D
Fold change in Ki67 staining intensity
***
Non Targeting sh +Dox
Akt2 sh +Dox
E
Non Targeting sh +Dox
Akt2 sh +Dox
Akt2
Negative control
40X
F
Fold change in Akt2 staining intensity
***
Non Targeting sh +Dox
Akt2sh +Dox
G
Non Targeting sh +Dox
Akt2 sh +Dox
MTSS1
Negative control
40X
H
Fold change in MTSS1 staining intensity
***
NonTargeting sh +Dox
Akt2 sh +Dox
I
Non Targeting sh +Dox
Akt2 sh +Dox
XIAP
Negative control
40X
J
Fold change in XIAP staining intensity
***
Non Targeting sh +Dox
Akt2 sh +Dox
K
Non targeting sh +Dox
Akt2sh +Dox
Akt2
XIAP
MTSS1
β-TRCP
GAPDH

**Figure 5.7:**(A)shows the differential expression of Akt1, 2, 3 and MTSS1 in normal and CRC tumor samples. There is a significant increase in the expression of Akt2 in tumor samples as compared to normal. (B)Scatter plot showing a negative correlation between Akt2 and MTSS1. The Pearson correlation coefficient (r= -0.12). The negative correlation is significant (p=0.04).

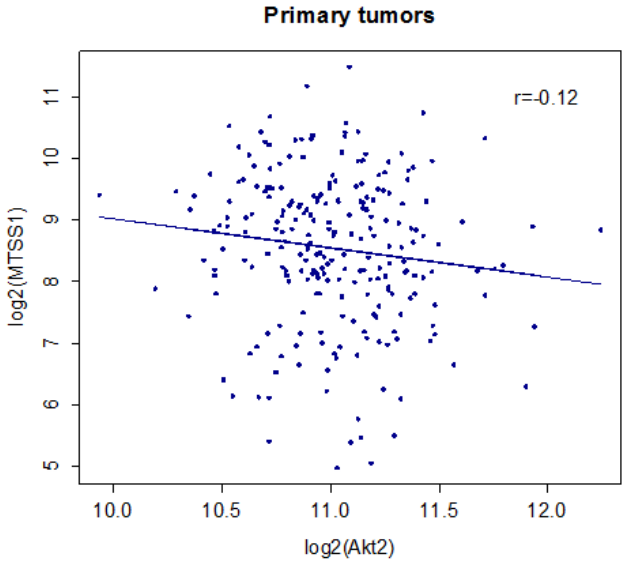
(C) Proposed mechanism for reduction in cell survival and cell motility on loss of Akt2 in CRC cells.

**Figure 5.7:**

A

| | Normal (n=41) | | Tumor (n=287) | | Fold change | |
|---|---|---|---|---|---|---|
| | Log2 expression | | Log2 expression | | tumor/normal | |
| Variable | Mean | SD | Mean | SD | Original scale | p-value |
| Akt1_Log2Expr | 11.845 | 0.192 | 11.751 | 0.356 | 0.937 | 0.012 |
| Akt2_Log2Expr | 10.900 | 0.169 | 11.025 | 0.344 | 1.091 | 0.0003 |
| Akt3_Log2Expr | 8.225 | 0.897 | 7.060 | 1.337 | 0.446 | <0.0001 |
| MTSS1_Log2Expr | 9.388 | 0.524 | 8.565 | 1.203 | 0.565 | <0.0001 |

B




C

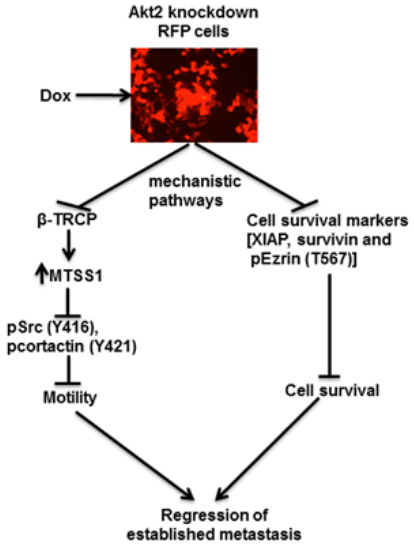


**Figure S1:**(A) and (B) Western blot analysis shows no change in the expression of Akt1, 2 and Akt3 on treatment with Dox in non-targeting sh in GEO and CBS cells respectively. (C) and (D) demonstrates no change in the expression of XIAP, survivin, AIF, ezrin and p ezrin (T567) in non-targeting sh on treatment with Dox in GEO and CBS cells respectively.

**Figure S1:**

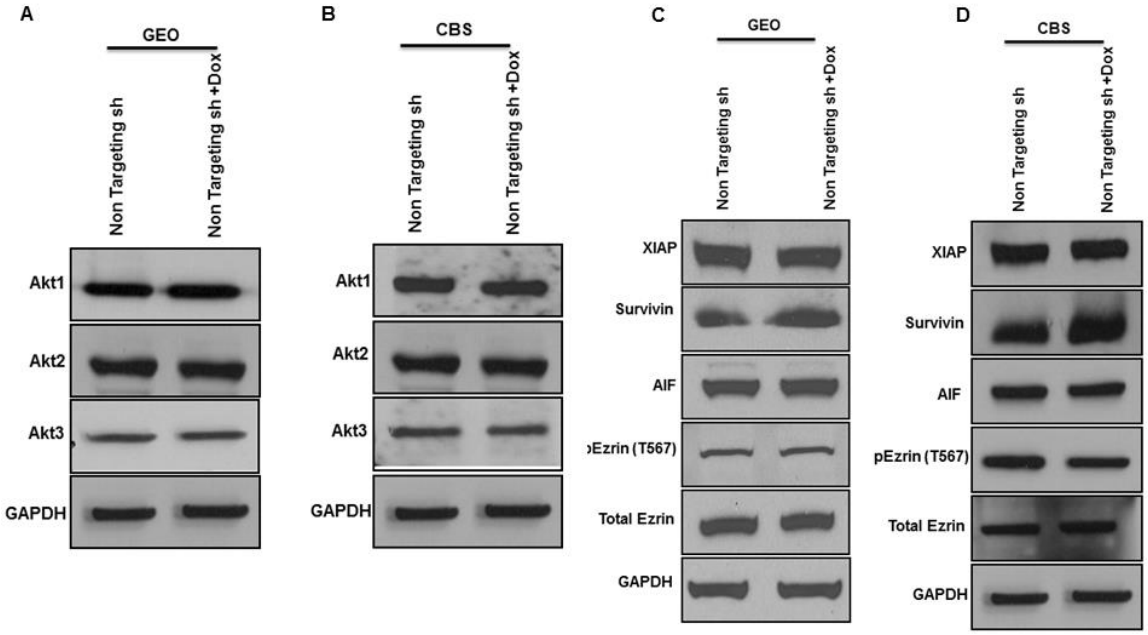


**Figure S2:** (A) and (B) Western blot analysis showing knockdown of Akt1 in presence of Dox in GEO and CBS cells respectively. There is no change in the expression of Akt2 and Akt3 on loss of Akt1, suggesting that the shRNA is specific to Akt1. GAPDH is used as a loading control. (C) and (D) qPCR analysis demonstrates a significant ($p<0.001$) of Akt1 when the cells are treated with Dox in both GEO and CBS cells respectively. (E) and (F) MTT assay shows that there is no significant change in cell proliferation on loss of Akt1 in comparison to non-targeting sh +Dox. (G) and (H) show that there is about 2-3 fold increase in cell death on loss of Akt1 as determined by DNA fragmentation assay in GEO and CBS cells.(I) Representative images showing reduction in motility of the cells on loss of Akt1. (J) Bar graphs show that there is about a 50% reduction in cell motility on loss of Akt1.

**Figure S2:**

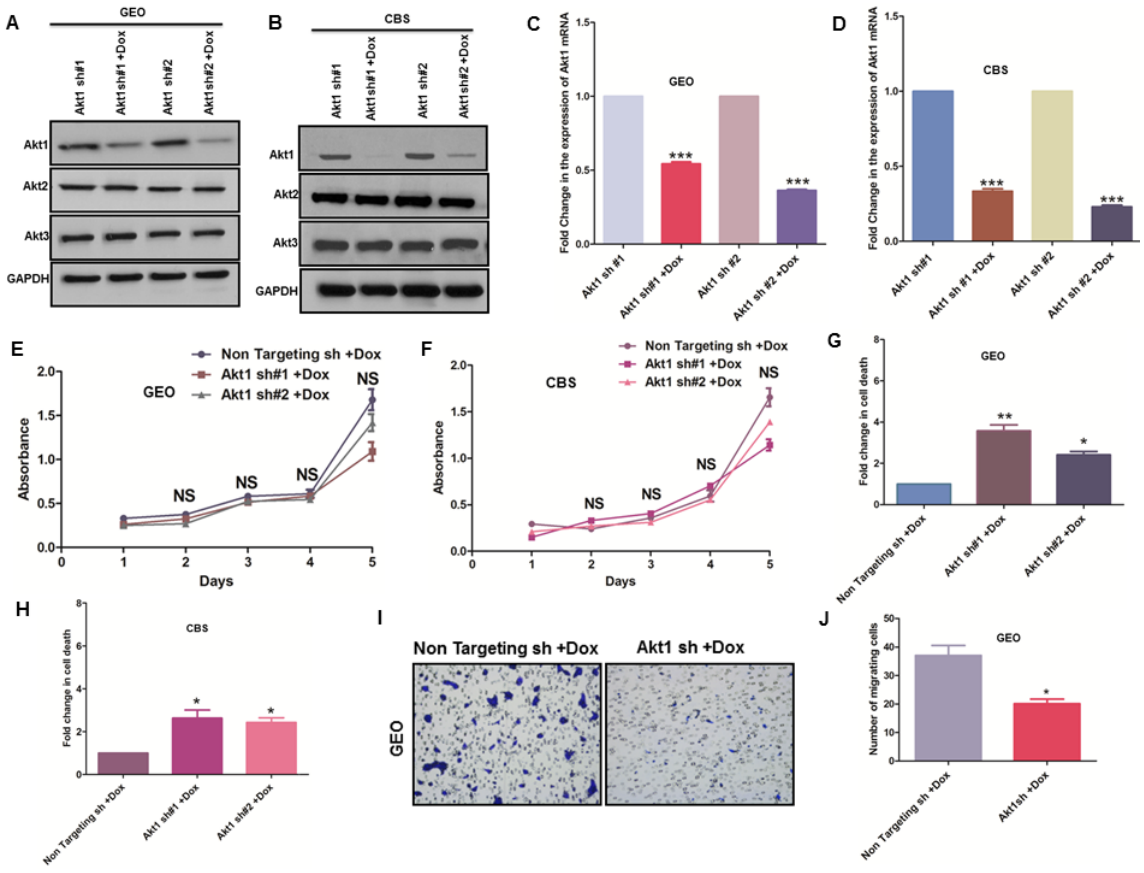


**Figure S3:** (A) and (B) Western blot analysis shows that there is a loss of Akt3 in the cells transfected with Akt3 sh and treated with Dox in GEO and CBS cells respectively. The shRNA is specific to Akt3 as there is no change in the expression of Akt1 and Akt2 when Akt3 is knocked down. (C) and (D) shows that there is a significant ($p<0.01$) loss in the expression of Akt3 at RNA level on treatment with Dox in both GEO and CBS respectively. (E) and (F) MTT assay shows that there is no change in the proliferation of cells on loss of Akt3 in comparison to non-targeting sh +Dox in GEO and CBS cells respectively. (G) and (H) Loss of Akt3 leads to about a two fold increase in cell death as determined by DNA fragmentation assay in GEO and CBS cells respectively. (I) Representative images of transwell migration assay showing a reduction in cell motility on loss of Akt3. (J) Bar graph shows that there is a 50% reduction in the motility of the cells on loss of Akt3.

**Figure S3:**

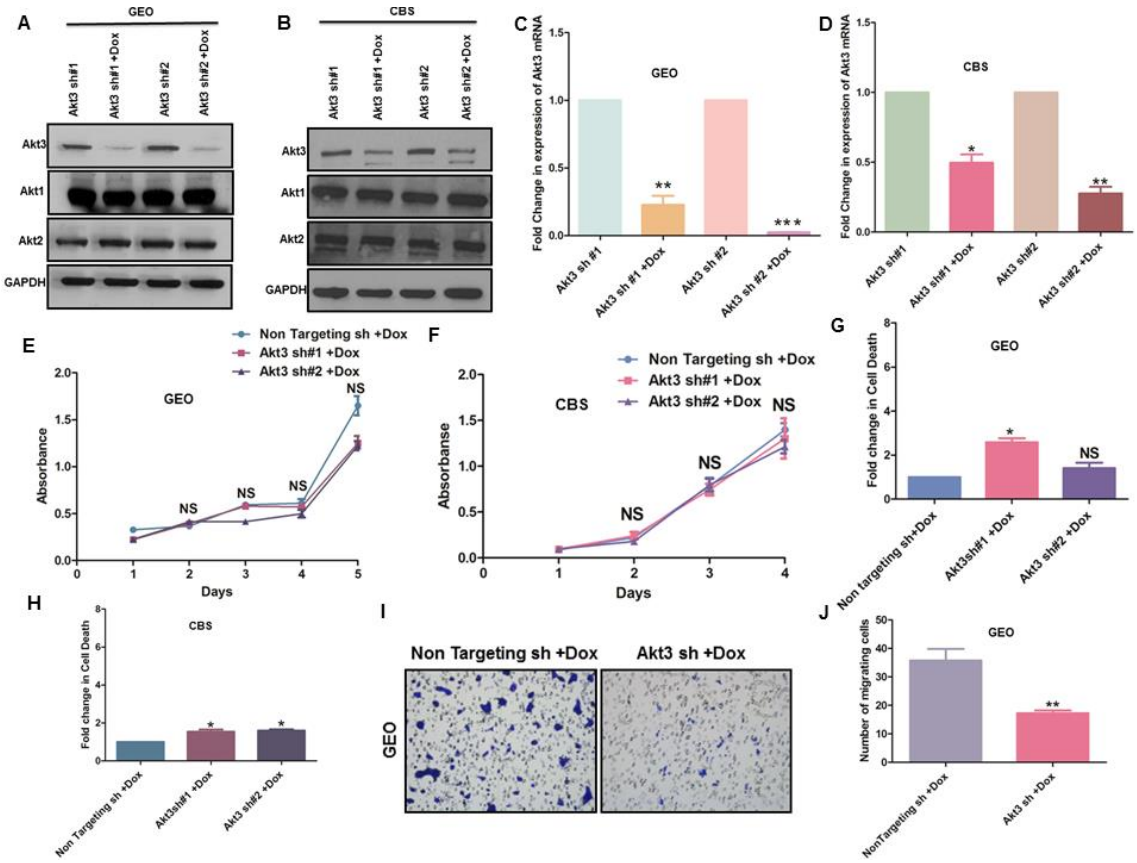


**Figure S4:** (A) and (B) Western blot analysis shows that there is a loss in the expression of XIAP and survivin on loss of Akt1 in both GEO and CBS cells respectively. Removal of Akt1 leads to an increase in the expression AIF, however there is no change in the expression of ezrin and p ezrin (T567). (C) and (D) A loss in the expression of MTSS1 on knockdown of Akt1 in GEO and CBS respectively. There is no change in the expression of total Src and pCortactin (Y421), however there is an increase in the expression of pSrc (Y416) on loss of Akt1 in GEO and CBS cells respectively. (E) Shows no change in the expression of β-TRCP on loss of Akt1.

**Figure S4:**

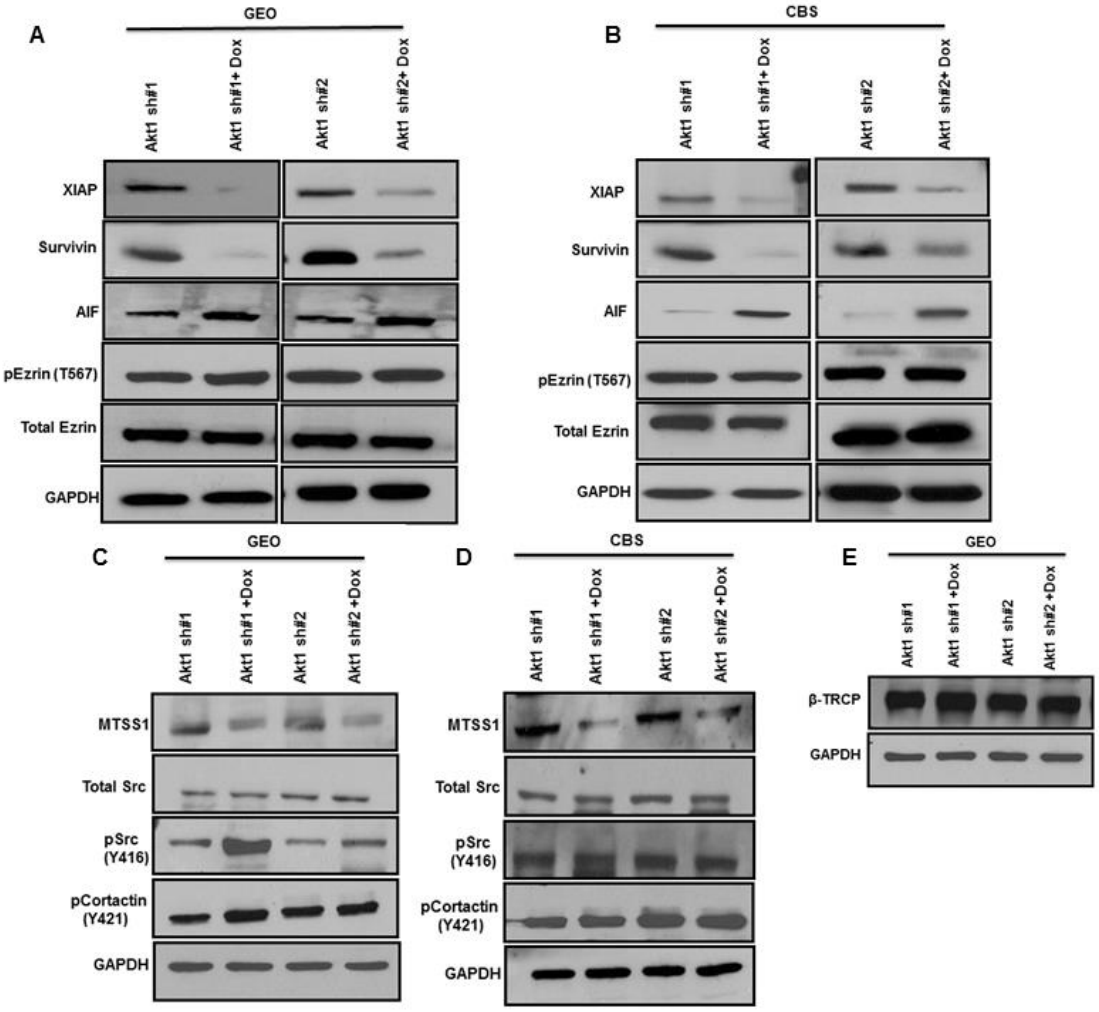


**Figure S5:** (A) and (B) shows loss in XIAP, survivin and AIF on knockdown of Akt3 in both GEO and CBS cells. (C) and (D) represents reduction in the expression of MTSS1 on loss of Akt3 in GEO and CBS respectively. There is no change in the expression of total Src, pSrc (Y416) and pCortactin (Y421) on loss of Akt3 in GEO and CBS cells respectively. (E) Demonstrates no change in the expression of βTRCP on loss of Akt3.

**Figure S5:**

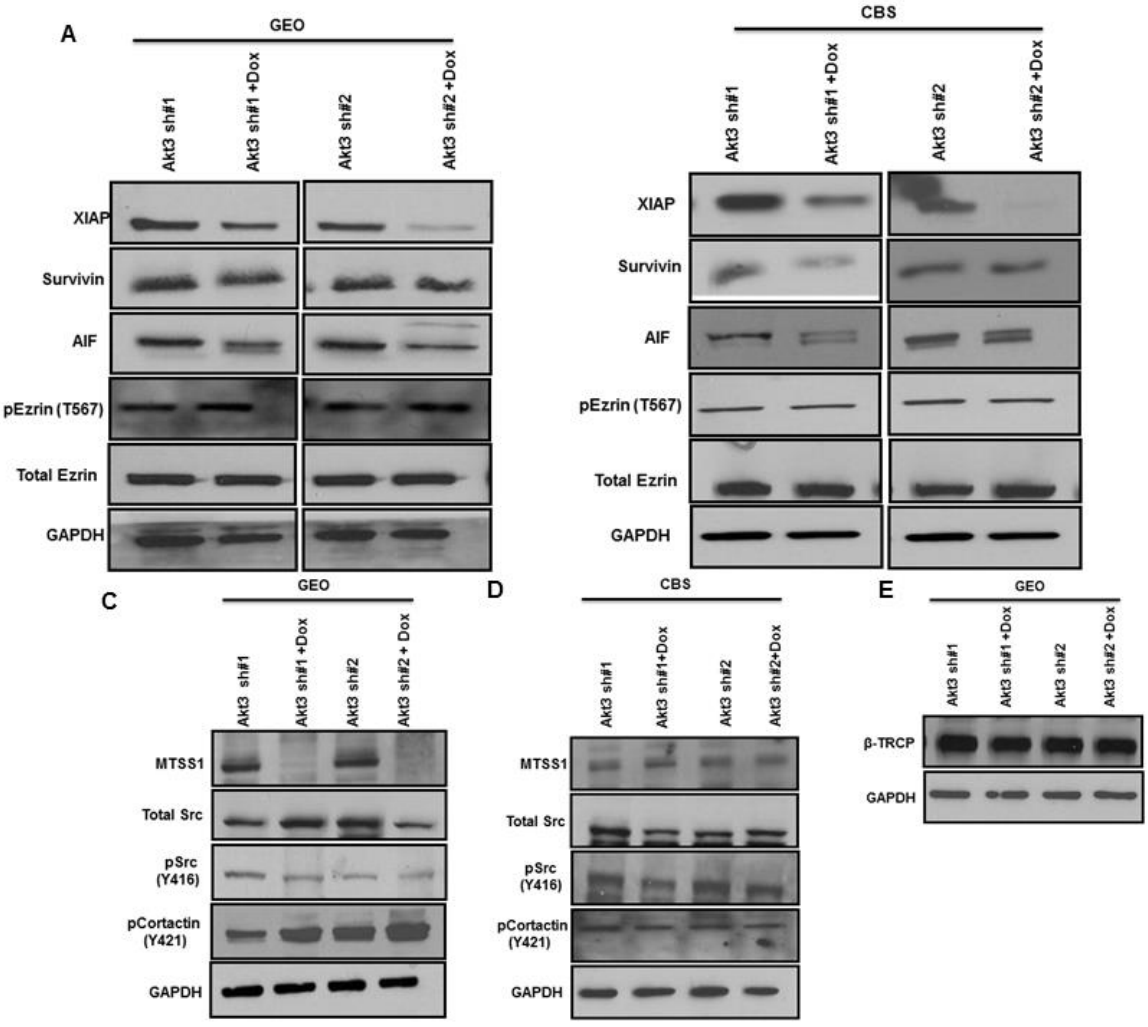


**Figure S6:** (A) Representative images of immunofluorescence with antibody for phalloidin (far red) and DNA (blue). Scale bar is 10μm.

**Figure S6:**

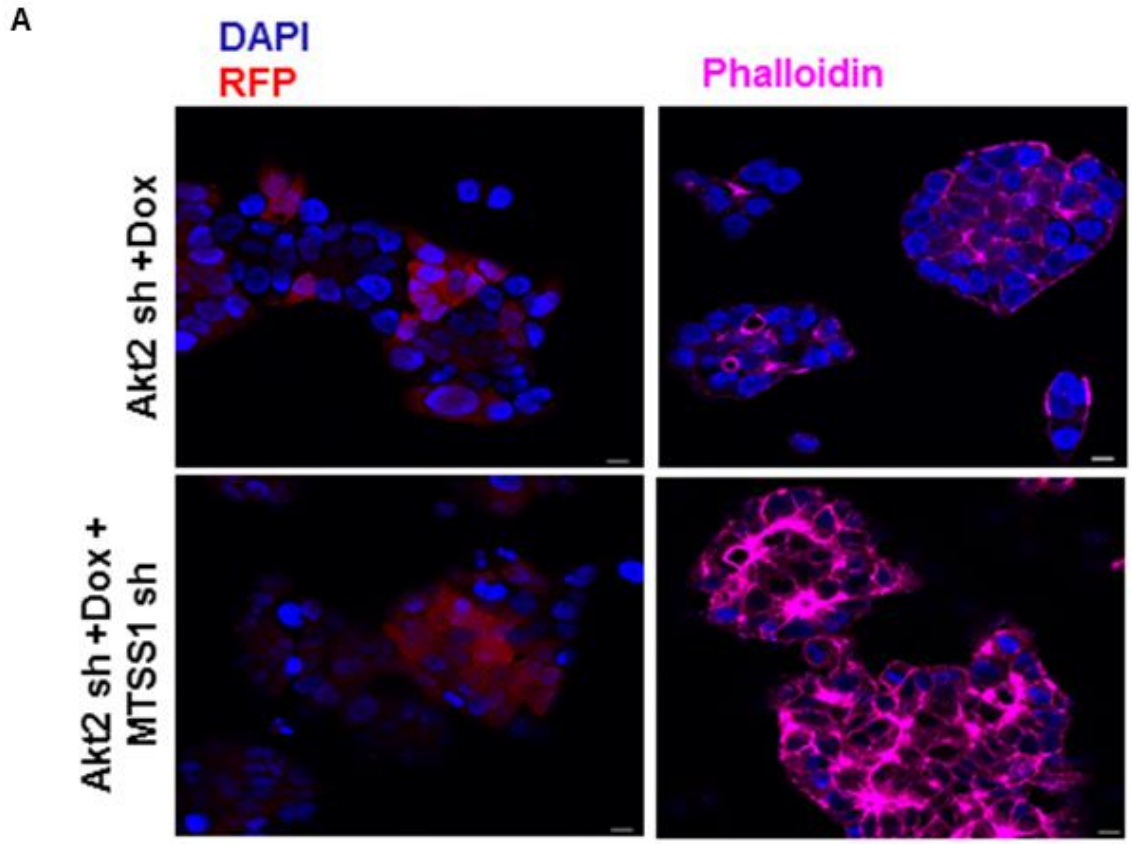
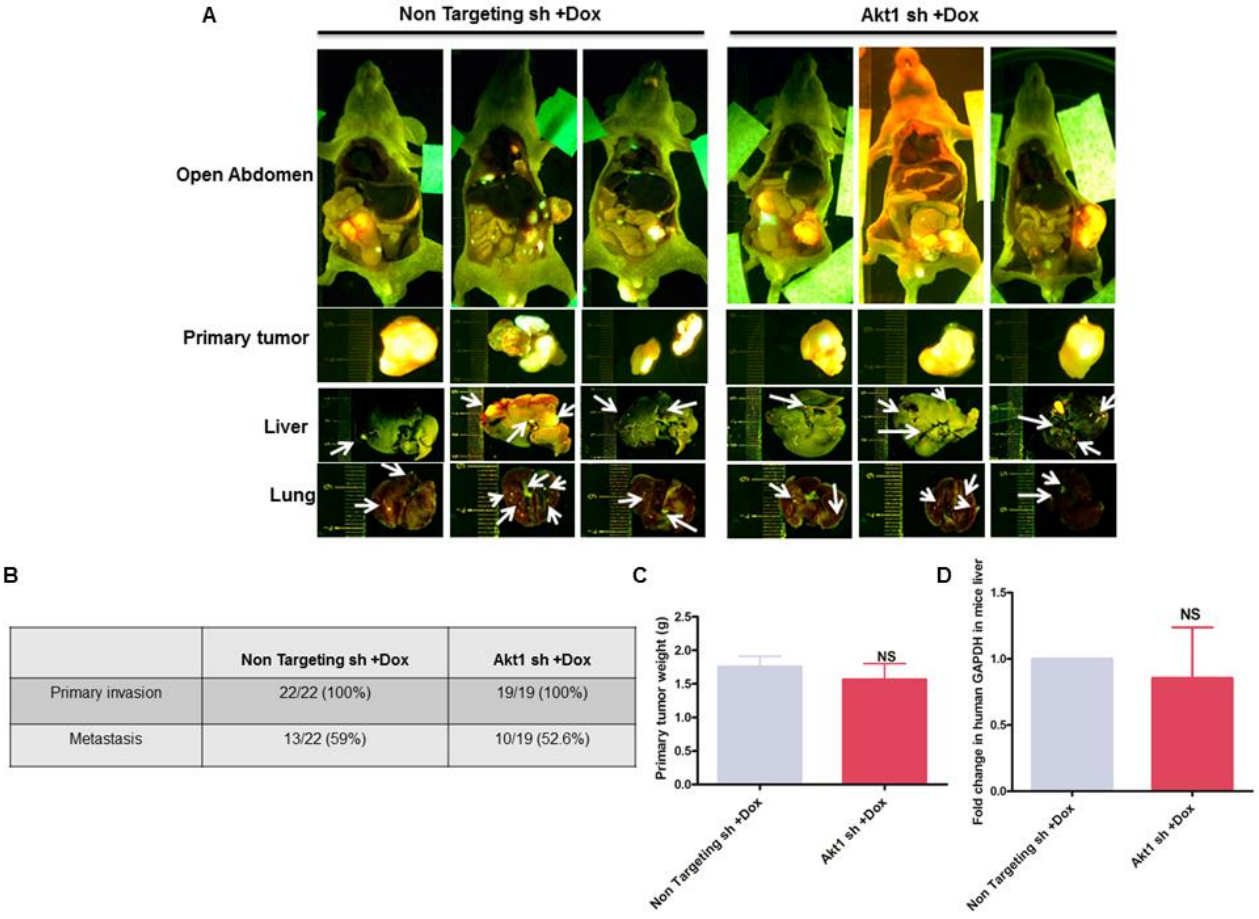


**Figure S7:** (A) Fluorescent image of whole mice, primary tumor, liver, lung from orthotopic implantation of non-targeting sh and Akt1 sh xenograft tumor respectively. (B) Shows no significant change in the number of animals with metastasis on loss of Akt1 in comparison to non-targeting sh +Dox. (C) Bar graph shows that there is no significant change in the weight of primary tumor. (D) Bar graphs show that there is no significant change in the expression of human GAPDH in the mice liver on loss of Akt1.

**Figure S7:**




| | Non Targeting sh +Dox | Akt1 sh +Dox |
|---|---|---|
| Primary invasion | 22/22 (100%) | 19/19 (100%) |
| Metastasis | 13/22 (59%) | 10/19 (52.6%) |

**Figure S8:** (A)H and E staining of the primary tumor and liver metastasis samples on loss of Akt1.(B) Representative images of liver metastatic spots as seen under immunofluorescence microscope. (C) Quantification of the number of liver metastatic spots, showing no change in the number of metastatic spots on loss of Akt1.

**Figure S8:**

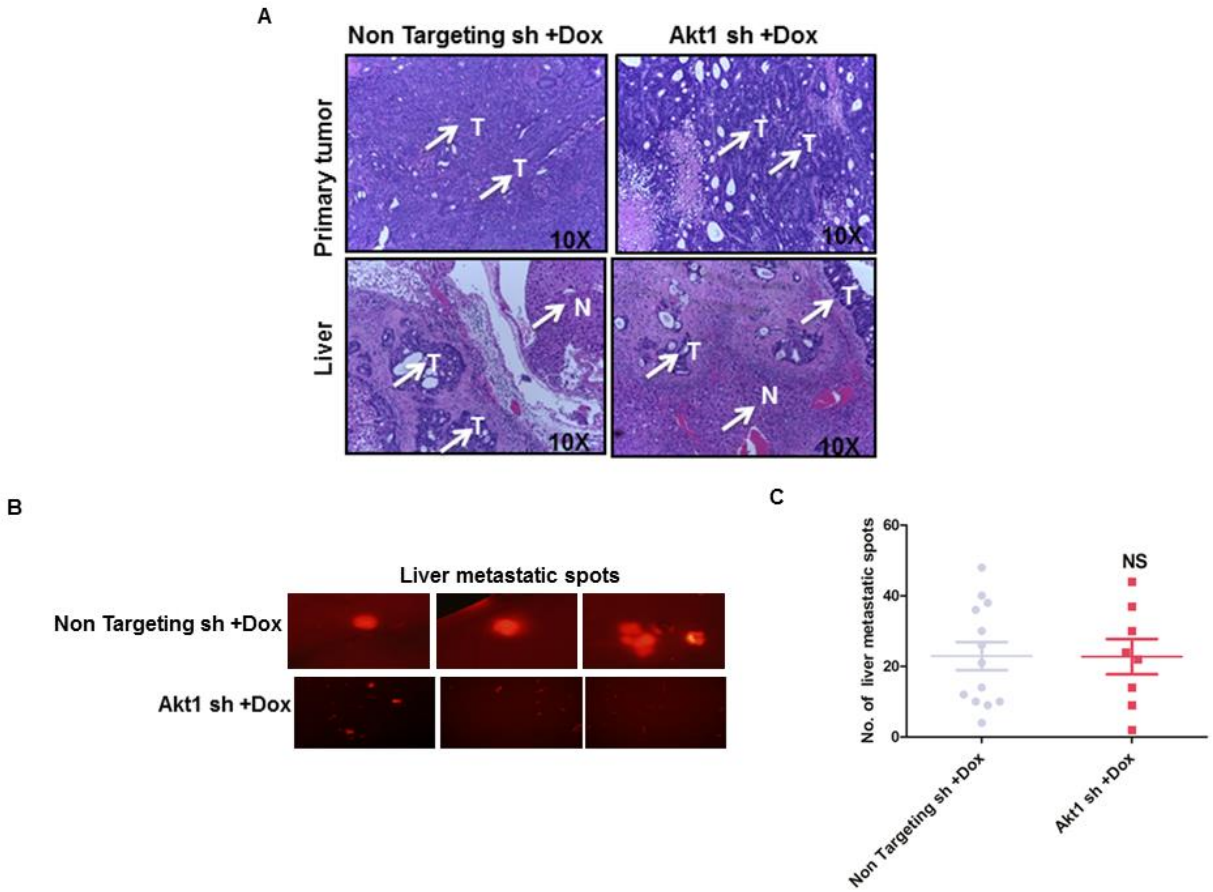
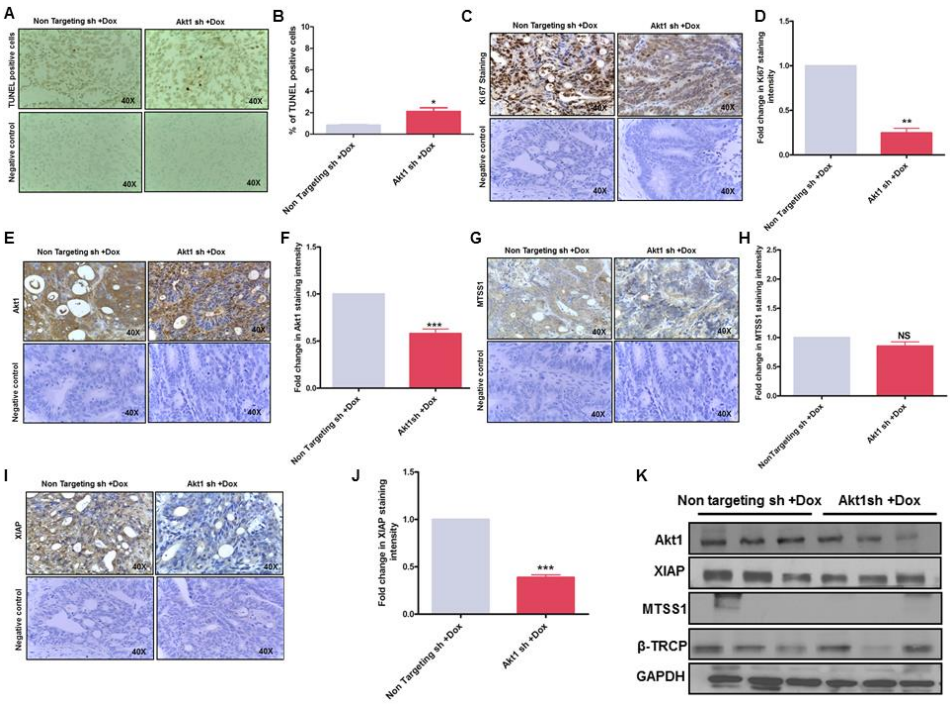


**Figure.S9:**(A) TUNEL staining of the primary tumor. The brown staining represents the apoptotic cells. (B) Statistical analysis shows that there is a significant ($p<0.01$) increase in the % of apoptotic cells on loss of Akt1. (C) Ki67 staining for Akt1 sh +Dox and non-targeting sh +Dox. (D) Bar graphs show that there is a significant ($p<0.01$) decrease in the staining intensity of Ki67 on loss of Akt1.(E) IHC analysis showing a loss in expression of Akt1 in the primary tumor in presence of Akt1sh. (F) Quantitative representation shows that there is a significant reduction ($p<0.001$) in the staining intensity of Akt1 in Akt1sh +Dox tumors as compared to tumors with non-targeting sh +Dox. (G) IHC staining shows that there is no change in the expression of MTSS1 on loss of Akt1 as compared to non-targeting sh +Dox. (H) Bar Graphs show there is no significant change in the expression of MTSS1 on loss of Akt1. (I) demonstrates expression of XIAP on loss of Akt1. (J) Bar graphs show a significant reduction ($p<0.001$) in the staining intensity of XIAP on loss of Akt1 as compared to non-Targeting sh +Dox. (K) Western blot analysis on primary tumor for XIAP, MTSS1 and β-TRCP on loss of Akt1.

**Figure.S9:**



**Supplementarytable 1**shows the pyrosequencing data to determine the status of methylation of MTSS1 on loss of Akt isoforms.The percent methylation values that hover around 40-50% are suggestive of one allele being methylated (not expressed) and the other allele being of low methylation (possibly expressed).

**Supplementarytable1**

| chr8:12472.... (hg38) | 8742 | 8737 | 8728 | 8726 | 8721 | 8718 | 8714 | 8710 | 8693 | 8686 | 8681 | 8666 | 8658 | 8655 | 8647 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Relative to Transcription Start Site | -236 | -231 | -222 | -220 | -215 | -212 | -208 | -204 | -187 | -180 | -175 | -160 | -152 | -149 | -141 |
| Sample | % methylation | | | | | | | | | | | | | | |
| Non Targeting sh +Dox | 63 | 46 | 58 | 40 | 47 | 33 | 44 | 39 | 38 | 47 | 41 | 42 | 11 | 22 | 32 |
| Akt1 sh +Dox | 51 | 36 | 55 | 34 | 37 | 32 | 38 | 38 | 36 | 33 | 41 | 42 | 10 | 29 | 32 |
| Akt2 sh +Dox | 64 | 52 | 57 | 39 | 42 | 37 | 41 | 40 | 38 | 45 | 43 | 42 | 15 | 27 | 30 |
| Akt3 sh +Dox | 62 | 43 | 59 | 41 | 44 | 27 | 44 | 38 | 40 | 47 | 43 | 46 | 11 | 31 | 33 |
| | | | | | | | | | | | | | | | |
| Lymphocyte DNA | 25 | 26 | 28 | 10 | 12 | 11 | 15 | 13 | 10 | 10 | 10 | 8 | 4 | 5 | 6 |
| Sss1 methylated DNA | 100 | 90 | 92 | 73 | 87 | 94 | 78 | 93 | 90 | 85 | 100 | 100 | 15 | 87 | 100 |

**Supplementary table 2: List of antibodies used**

| Antibody | Source | Cat# | Dilution |
|---|---|---|---|
| Apoptosis Inducing Factor | Cell Signaling | 4642 | 1:1000 |
| Akt1 (Western blot) | Cell Signaling | 2967 | 1:1000 |
| Akt1 (IHC) | Cell Signaling | 2938 | 1:50 |
| Akt2 (Western blot) | Cell Signaling | 5239 | 1:1000 |
| Akt2 (IHC) | Santa Cruz | 5270 | 1:500 |
| Akt3 | Cell Signaling | 4059 | 1:1000 |
| β-TRCP | Cell Signaling | 4394 | 1:1000 |
| Ezrin | Santa Cruz | 71082 | 1:1000 |
| Ki67 (IHC) | BD Biosciences | 550609 | 1:100 |
| MTSS1 (Western blot) | Cell Signaling | 4385 | 1:1000 |
| MTSS1 (IHC) | Santa Cruz | 101204 | 1:50 |
| pCortactin (Y421) | Cell Signaling | 4569 | 1:1000 |
| pEzrin (T567) | Cell Signaling | 3149 | 1:1000 |
| pSrc (Y416) | Cell Signaling | 6943 | 1:1000 |
| Survivin | Cell Signaling | 2803 | 1:1000 |
| Total Src | Cell Signaling | 2108 | 1:1000 |
| XIAP (Western blot) | Cell Signaling | 14334 | 1:1000 |
| XIAP (IHC) | Cell Signaling | 14334 | 1:10 |

# Chapter VI

## Summary and Future Directions

## 6. Summary and future directions

### 6.1 Central theme and therapeutic approach

The central theme of the dissertation is to identify molecules to develop novel therapies directed to target maintenance of established CRC metastasis. It is known that altered expression of Akt isoforms affects the functional characteristics of cancer cells. To clearly understand the effect of Akt activation and Akt isoform expression on colon cancer metastasis, we have utilized kinase inhibitor of Akt for in vitro and in vivo studies. Additionally we have also developed inducible knockdown of Akt isoforms to determine the role the isoforms on CRC cells and specific inhibitors targeted to the isoforms can be designed. Although a lot of work has been done previously to understand the role of Akt isoforms on metastasis, the mechanisms accounting for alteration in metastatic potential of the cells is still unknown.

#### 6.1.1 Current standing

We and others have identified that pAkt can be an important target in multiple cancer types. There are several Akt kinase inhibitors which have used in the past as perifosine, deguelin, A443654 and Akti1/2, GSK690693 and HSP90 inhibitors. These inhibitors have failed in clinical research due to their non-specificity and/or due to their toxic side-effects. We have utilized MK-2206 to inhibit the phosphorylation of Akt. In the current *in vitro* studies we have demonstrated that loss of pAkt on treatment with MK-2206 leads to a reduction in expression of XIAP and survivin. Additionally, it has been also observed that there is an increase in the expression of Apoptosis Inducing Factor (AIF) which is responsible for caspase-independent cell death. Thus loss of pAkt leads to a dual mechanism of cell death in colon cancer cells. We also performed *in vivo* studies to determine the effect of MK-2206 on xenograft tumor formation. Treatment with MK-2206 led to a robust reduction on the formation of xenograft. We observed a significant reduction in tumor volume and tumor weight, however there was no change in the mice weight

suggesting that the drug did not have any side effects on mice. Thus our *in vitro* data corroborated the *in vivo* data. Thus this study led to the identification of MK-2206 as an anti-tumorigenic agent in CRC.

### 6.1.2 Future directions

The next step for this study would be to identify the effect of MK-2206 on metastasis. It would be important to determine whether MK-2206 affects the growth of primary tumor and /or the formation of liver and lung metastases. Additionally we would determine if loss of pAkt affects the establishment of metastasis or maintenance of established metastasis. This would help to determine the stage of CRC at which MK-2206 can be tested in patients.

Previous studies have used MK-2206 in combination with chemotherapeutic agents including docetaxel, paclitaxel, carboplatin, gemcitabine, 5-fluorouracil (5-FU), doxorubicin, camptothecin, RTK inhibitors such as lapatinib and erlotinib, and EGFR and IGFR inhibitors. An *in vitro* study would be carried out with combination of MK-2206 and chemotherapeutics to determine whether these combination studies show syngeneic effect and further increase cell death. If we observe a synergistic effect on our cell models, in vivo studies would be performed with the combination of MK-2206 and chemotherapeutic agents.

## 6.2 Potential therapy against metastasis

The metastatic colonization of tumor cells is the major cause of CRC-related deaths in the United States. Cancer cells overcome several barriers before they can establish and colonize, thus making metastasis an inefficient process. Recently it has been shown that very small population of cells survive and proliferate to form distant metastatic spots, thus making survival a rate-limiting step for metastasis. Once metastasis occurs, there is an increase in resistance to apoptosis to maintain the established metastasis.

Advances in treatment and research in the past few decades have increased the five-year survival rate in the early stages of CRC; however, the survival rate has continued to show dismal progress in the metastatic stage of the disease. The improvement in the survival rate at earlier stages is attributed to surgical resection of the primary tumor followed by adjuvant chemotherapy; however metastasis is untreatable, thus emphasizing the need for developing anti-metastatic therapy. Anti-metastatic therapies might be more effective if they inhibit the survival of the cancer cells that have left the primary tumor site and it undergoing metastasis. Drugs targeting metastasis have to be used for a long time, thus making them incompetent in the clinic due to their known toxic effects. Thus, anti-metastatic therapies need to be designed in such a way that they elicit regression of established metastasis so that they qualify for clinical practice.

### 6.2.1 Current Standing

Various studies have been performed to determine the role of Akt isoforms in regulating metastases. It is known that Akt2 plays an important role in metastases of CRC cells, which are deficient in PTEN. Recent studies have reported a link between PTEN loss, Akt2 overexpression and increased metastatic potential of CRC cells. Additionally, it is known that loss of Akt1 and Akt2 inhibit the growth of CRC cells. However, we still don't know the mechanism of inhibition of establishment of metastasis by Akt2. The important question addressed in this work is the differential role of Akt isoforms in CRC cell survival and the underlying mechanism. Additionally, we have also determined the mechanism of Akt 2 affecting the maintenance of established metastasis.

We have performed orthotopic implantation studies with inducible knockdown of Akt isoforms in our CRC cell models. This model recapitulates the pattern of human CRC primary and metastatic deposits. We observed 40% reduction in the number of animals with metastasis on loss of Akt2. Mechanistically we observed a loss in the expression of XIAP and survivin on loss

of Akt isoforms. Additionally, an increase in the expression of AIF was observed on loss of Akt2 thus leading to increase in cell death.

A key and novel finding in this study was the identification of increase in the expression of a Metastasis Suppressor gene (MTSS1) on loss of Akt2. MTSS1 is known to promote tumor growth by enhancing cell proliferation, invasion and cell migration. MTSS1 has been shown to inhibit the expression of pSrc (Tyr 416), which in turn leads to a loss in the phosphorylation of Cortactin at Tyr 421. Loss of Akt2 leads to the activation of MTSS1→pSrc→pCortactin axis leading to reduction in metastasis. We also identified that loss of Akt2 leads to the loss of β-TRCP, a ubiquitin ligase which ubiquitinates MTSS1 thus leading to its proteasomal degradation.

The Cancer Genome Atlas showed that in CRC patients there is an increase in the expression of Akt2 in comparison to the normal patients, whereas Akt1, Akt3 and MTSS1 expression decreased in tumor samples. Interestingly we also observed that in primary tumors, Akt2 and MTSS1 are negatively correlated. The work presented in this dissertation is novel and unique in its own way but it needs to be studied in detail in larger human patient data sets.

### 6.2.2 Future directions

This dissertation reports a novel mechanism of Akt2-mediated increased maintenance of established CRC metastasis, thus identifying Akt2 as an important therapeutic target. This study emphasizes the need for developing Akt2 specific inhibitors for treating late stage CRC patients, who are diagnosed at a stage when cancer has already undergone metastasis. Additionally, the Cancer Genome Atlas shows that Akt2 and MTSS1 are negatively correlated in the primary tumor samples in CRC. These data help us to postulate that Akt2 high/MTSS1 low expression may be a marker for metastatic disease. We also predict that Akt2-MTSS1 negative correlation may be used to identify patients that might benefit most from Akt2 mediated therapy. This can be confirmed by performing IHC analysis on larger sets of patient sample.

The transwell migration assay shows that loss of Akt1 and Akt3 lead to a reduction in cell motility although it is not as robust as compared to Akt2 loss. The PCR array analysis shows that there is an increase in the expression of NME-1 on loss of Akt2. NME-1 is a metastatic suppressor gene. Currently, there are no studies which show a link between Akt1 and NME-1 expression in any cancer type. We observed an increase in the expression of NME-1 on loss of Akt1 specifically, whereas no change in the expression of this metastatic suppressor gene was observed on loss of Akt1 and Akt3. Future studies are needed to determine the mechanism of effect of Akt1 loss on NME-1. We also need to understand the downstream signaling of NME-1 to determine how this molecule affects metastasis.

PCR array analysis has helped us to determine the differential expression of various metastatic genes on loss of Akt isoforms. We identified a novel mechanism of Akt2 affecting the maintenance of established metastasis via MTSS1, although there were certain genes which were affected by either two or all the three isoforms. We need to determine the effect of these genes and verify their effect on metastasis as well as the underlying mechanism.

**6.3 Final conclusion**

The studies presented in this dissertation have both mechanistic insights and clinical implications. This work has shown that Akt2 can be an important therapeutic target that might prevent the maintenance of established metastasis. Advancement in research in the past few decades have increased the survival rate if CRC is diagnosed at an early stage, however the increase in survival rate at late stages is not very impressive. About 20% of CRC cases are diagnosed when the cancer has already undergone metastasis thus urging the need to identify molecules that might affect be responsible for maintaining metastasis so that they can be targeted therapeutically, thus leading to regression of already established metastasis. This dissertation research has identified that Akt2 as one of the targets that may be responsible for maintaining metastasis via MTSS1.

# Chapter VI

## References

Adams, J. M. and S. Cory (2007). "The Bcl-2 apoptotic switch in cancer development and therapy." Oncogene **26**(9): 1324-1337.

Agarwal, E., M. G. Brattain and S. Chowdhury "Cell Survival and Metastasis Regulation by Akt Signalling in Colorectal Cancer." Cell Signal.

Agarwal, E., M. G. Brattain and S. Chowdhury (2013). "Cell survival and metastasis regulation by Akt signaling in colorectal cancer." Cell Signal **25**(8): 1711-1719.

Agarwal, E., A. Chaudhuri, P. D. Leiphrakpam, K. L. Haferbier, M. G. Brattain and S. Chowdhury (2014). "Akt inhibitor MK-2206 promotes anti-tumor activity and cell death by modulation of AIF and Ezrin in colorectal cancer." BMC Cancer **14**: 145.

Akervall, J. A., Y. Jin, J. P. Wennerberg, U. K. Zatterstrom, E. Kjellen, F. Mertens, R. Willen, N. Mandahl, S. Heim and F. Mitelman (1995). "Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck." Cancer **76**(5): 853-859.

Altomare, D. A. and J. R. Testa (2005). "Perturbations of the AKT signaling pathway in human cancer." Oncogene **24**(50): 7455-7464.

Altomare, D. A., L. Zhang, J. Deng, A. Di Cristofano, A. J. Klein-Szanto, R. Kumar and J. R. Testa (2010). "GSK690693 delays tumor onset and progression in genetically defined mouse models expressing activated Akt." Clin Cancer Res **16**(2): 486-496.

Ambrosini, G., C. Adida and D. C. Altieri (1997). "A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma." Nat Med **3**(8): 917-921.

Ammanamanchi, S. and M. G. Brattain (2004). "Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells." J Biol Chem **279**(31): 32620-32625.

Ammanamanchi, S., J. W. Freeman and M. G. Brattain (2003). "Acetylated sp3 is a transcriptional activator." J Biol Chem **278**(37): 35775-35780.

Andersen, M. H., J. C. Becker and P. Straten (2005). "Regulators of apoptosis: suitable targets for immune therapy of cancer." Nat Rev Drug Discov **4**(5): 399-409.

Andjelkovic, M., D. R. Alessi, R. Meier, A. Fernandez, N. J. Lamb, M. Frech, P. Cron, P. Cohen, J. M. Lucocq and B. A. Hemmings (1997). "Role of translocation in the activation and function of protein kinase B." J Biol Chem **272**(50): 31515-31524.

Arboleda, M. J., J. F. Lyons, F. F. Kabbinavar, M. R. Bray, B. E. Snow, R. Ayala, M. Danino, B. Y. Karlan and D. J. Slamon (2003). "Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells." Cancer Res **63**(1): 196-206.

Auguste, P., L. Fallavollita, N. Wang, J. Burnier, A. Bikfalvi and P. Brodt (2007). "The host inflammatory response promotes liver metastasis by increasing tumor cell arrest and extravasation." Am J Pathol **170**(5): 1781-1792.

Bacus, S. S., D. A. Altomare, L. Lyass, D. M. Chin, M. P. Farrell, K. Gurova, A. Gudkov and J. R. Testa (2002). "AKT2 is frequently upregulated in HER-2/neu-positive

breast cancers and may contribute to tumor aggressiveness by enhancing cell survival." Oncogene **21**(22): 3532-3540.

Bader, A. G., S. Kang, L. Zhao and P. K. Vogt (2005). "Oncogenic PI3K deregulates transcription and translation." Nat Rev Cancer **5**(12): 921-929.

Barrett, R. M., T. P. Osborne and S. P. Wheatley (2009). "Phosphorylation of survivin at threonine 34 inhibits its mitotic function and enhances its cytoprotective activity." Cell Cycle **8**(2): 278-283.

Bellacosa, A., J. R. Testa, S. P. Staal and P. N. Tsichlis (1991). "A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region." Science **254**(5029): 274-277.

Berezovskaya, O., A. D. Schimmer, A. B. Glinskii, C. Pinilla, R. M. Hoffman, J. C. Reed and G. V. Glinsky (2005). "Increased expression of apoptosis inhibitor protein XIAP contributes to anoikis resistance of circulating human prostate cancer metastasis precursor cells." Cancer Res **65**(6): 2378-2386.

Bernards, R. and R. A. Weinberg (2002). "A progression puzzle." Nature **418**(6900): 823.

Bershteyn, M., S. X. Atwood, W. M. Woo, M. Li and A. E. Oro (2010). "MIM and cortactin antagonism regulates ciliogenesis and hedgehog signaling." Dev Cell **19**(2): 270-283.

Berwick, D. C., I. Hers, K. J. Heesom, S. K. Moule and J. M. Tavare (2002). "The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes." J Biol Chem **277**(37): 33895-33900.

Birkey Reffey, S., J. U. Wurthner, W. T. Parks, A. B. Roberts and C. S. Duckett (2001). "X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-beta signaling." J Biol Chem **276**(28): 26542-26549.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**(6835): 355-365.

Bompard, G., S. J. Sharp, G. Freiss and L. M. Machesky (2005). "Involvement of Rac in actin cytoskeleton rearrangements induced by MIM-B." J Cell Sci **118**(Pt 22): 5393-5403.

Borgatti, P., A. M. Martelli, G. Tabellini, A. Bellacosa, S. Capitani and L. M. Neri (2003). "Threonine 308 phosphorylated form of Akt translocates to the nucleus of PC12 cells under nerve growth factor stimulation and associates with the nuclear matrix protein nucleolin." J Cell Physiol **196**(1): 79-88.

Bretscher, A., K. Edwards and R. G. Fehon (2002). "ERM proteins and merlin: integrators at the cell cortex." Nat Rev Mol Cell Biol **3**(8): 586-599.

Brodbeck, D., P. Cron and B. A. Hemmings (1999). "A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain." J Biol Chem **274**(14): 9133-9136.

Brognard, J., E. Sierecki, T. Gao and A. C. Newton (2007). "PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms." Mol Cell **25**(6): 917-931.

Brown, K., S. C. Vial, N. Dedi, J. M. Long, N. J. Dunster and G. M. Cheetham (2005). "Structural basis for the interaction of TAK1 kinase with its activating protein TAB1." J Mol Biol **354**(5): 1013-1020.

Buday, L. and J. Downward (2007). "Roles of cortactin in tumor pathogenesis." Biochim Biophys Acta **1775**(2): 263-273.

Buenemann, C. L., C. Willy, A. Buchmann, A. Schmiechen and M. Schwarz (2001). "Transforming growth factor-beta1-induced Smad signaling, cell-cycle arrest and apoptosis in hepatoma cells." Carcinogenesis **22**(3): 447-452.

Cai, J., J. Yang and D. P. Jones (1998). "Mitochondrial control of apoptosis: the role of cytochrome c." Biochim Biophys Acta **1366**(1-2): 139-149.

Calera, M. R., C. Martinez, H. Liu, A. K. Jack, M. J. Birnbaum and P. F. Pilch (1998). "Insulin increases the association of Akt-2 with Glut4-containing vesicles." J Biol Chem **273**(13): 7201-7204.

Cande, C., F. Cecconi, P. Dessen and G. Kroemer (2002). "Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death?" J Cell Sci **115**(Pt 24): 4727-4734.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-1657.

Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, S. Frisch and J. C. Reed (1998). "Regulation of cell death protease caspase-9 by phosphorylation." Science **282**(5392): 1318-1321.

Carmeliet, P. and R. K. Jain (2011). "Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases." Nat Rev Drug Discov **10**(6): 417-427.

Carpten, J. D., A. L. Faber, C. Horn, G. P. Donoho, S. L. Briggs, C. M. Robbins, G. Hostetter, S. Boguslawski, T. Y. Moses, S. Savage, M. Uhlik, A. Lin, J. Du, Y. W. Qian, D. J. Zeckner, G. Tucker-Kellogg, J. Touchman, K. Patel, S. Mousses, M. Bittner, R. Schevitz, M. H. Lai, K. L. Blanchard and J. E. Thomas (2007). "A transforming mutation in the pleckstrin homology domain of AKT1 in cancer." Nature **448**(7152): 439-444.

Chai, J., E. Shiozaki, S. M. Srinivasula, Q. Wu, P. Datta, E. S. Alnemri and Y. Shi (2001). "Structural basis of caspase-7 inhibition by XIAP." Cell **104**(5): 769-780.

Chambers, A. F., A. C. Groom and I. C. MacDonald (2002). "Dissemination and growth of cancer cells in metastatic sites." Nat Rev Cancer **2**(8): 563-572.

Chau, N. M. and M. Ashcroft (2004). "Akt2: a role in breast cancer metastasis." Breast Cancer Res **6**(1): 55-57.

Chen, M. B., X. Y. Wu, G. Q. Tao, C. Y. Liu, J. Chen, L. Q. Wang and P. H. Lu (2012). "Perifosine sensitizes curcumin-induced anti-colorectal cancer effects by targeting multiple signaling pathways both in vivo and in vitro." Int J Cancer **131**(11): 2487-2498.

Chen, R. H., Y. H. Su, R. L. Chuang and T. Y. Chang (1998). "Suppression of transforming growth factor-beta-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway." Oncogene **17**(15): 1959-1968.

Chen, Y., D. Wang, Z. Guo, J. Zhao, B. Wu, H. Deng, T. Zhou, H. Xiang, F. Gao, X. Yu, J. Liao, T. Ward, P. Xia, C. Emenari, X. Ding, W. Thompson, K. Ma, J. Zhu, F. Aikhionbare, K. Dou, S. Y. Cheng and X. Yao (2011). "Rho kinase phosphorylation promotes ezrin-mediated metastasis in hepatocellular carcinoma." Cancer Res **71**(5): 1721-1729.

Cheng, G. Z., J. Chan, Q. Wang, W. Zhang, C. D. Sun and L. H. Wang (2007). "Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel." Cancer Res **67**(5): 1979-1987.

Cheng, J. Q., X. Jiang, M. Fraser, M. Li, H. C. Dan, M. Sun and B. K. Tsang (2002). "Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway." Drug Resist Updat **5**(3-4): 131-146.

Cheng, Y., Y. Zhang, L. Zhang, X. Ren, K. J. Huber-Keener, X. Liu, L. Zhou, J. Liao, H. Keihack, L. Yan, E. Rubin and J. M. Yang (2012). "MK-2206, a novel allosteric inhibitor of Akt, synergizes with gefitinib against malignant glioma via modulating both autophagy and apoptosis." Mol Cancer Ther **11**(1): 154-164.

Cheung, E. C., L. Melanson-Drapeau, S. P. Cregan, J. L. Vanderluit, K. L. Ferguson, W. C. McIntosh, D. S. Park, S. A. Bennett and R. S. Slack (2005). "Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms." J Neurosci **25**(6): 1324-1334.

Chiang, A. C. and J. Massague (2008). "Molecular basis of metastasis." N Engl J Med **359**(26): 2814-2823.

Chiarini, F., M. Del Sole, S. Mongiorgi, G. C. Gaboardi, A. Cappellini, I. Mantovani, M. Y. Follo, J. A. McCubrey and A. M. Martelli (2008). "The novel Akt inhibitor, perifosine, induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrug-resistant human T-acute leukemia cells by a JNK-dependent mechanism." Leukemia **22**(6): 1106-1116.

Chin, Y. R. and A. Toker (2009). "Function of Akt/PKB signaling to cell motility, invasion and the tumor stroma in cancer." Cell Signal **21**(4): 470-476.

Chin, Y. R., X. Yuan, S. P. Balk and A. Toker (2014). "PTEN-deficient tumors depend on AKT2 for maintenance and survival." Cancer Discov **4**(8): 942-955.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, 3rd, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman and M. J. Birnbaum (2001). "Insulin resistance and a

diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta)." Science **292**(5522): 1728-1731.

Cho, H., J. L. Thorvaldsen, Q. Chu, F. Feng and M. J. Birnbaum (2001). "Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice." J Biol Chem **276**(42): 38349-38352.

Chorna-Ornan, I., V. Tzarfaty, G. Ankri-Eliahoo, T. Joel-Almagor, N. E. Meyer, A. Huber, F. Payre and B. Minke (2005). "Light-regulated interaction of Dmoesin with TRP and TRPL channels is required for maintenance of photoreceptors." J Cell Biol **171**(1): 143-152.

Chowdhury, S., S. Ammanamanchi and G. M. Howell (2009). "Epigenetic Targeting of Transforming Growth Factor beta Receptor II and Implications for Cancer Therapy." Mol Cell Pharmacol **1**(1): 57-70.

Chowdhury, S., G. M. Howell, A. Rajput, C. A. Teggart, L. E. Brattain, H. R. Weber, A. Chowdhury and M. G. Brattain (2011). "Identification of a novel TGFbeta/PKA signaling transduceome in mediating control of cell survival and metastasis in colon cancer." PLoS One **6**(5): e19335.

Chowdhury, S., G. M. Howell, C. A. Teggart, A. Chowdhury, J. J. Person, D. M. Bowers and M. G. Brattain (2011). "Histone deacetylase inhibitor belinostat represses survivin expression through reactivation of transforming growth factor beta (TGFbeta) receptor II leading to cancer cell death." J Biol Chem **286**(35): 30937-30948.

Christofori, G. (2006). "New signals from the invasive front." Nature **441**(7092): 444-450.

Chun, K. H., J. W. Kosmeder, 2nd, S. Sun, J. M. Pezzuto, R. Lotan, W. K. Hong and H. Y. Lee (2003). "Effects of deguelin on the phosphatidylinositol 3-kinase/Akt pathway and apoptosis in premalignant human bronchial epithelial cells." J Natl Cancer Inst **95**(4): 291-302.

Chung, D. C. (2000). "The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis." Gastroenterology **119**(3): 854-865.

Chung, S., J. Yao, K. Suyama, S. Bajaj, X. Qian, O. D. Loudig, E. A. Eugenin, G. R. Phillips and R. B. Hazan (2012). "N-cadherin regulates mammary tumor cell migration through Akt3 suppression." Oncogene.

Clark, A. R. and A. Toker (2014). "Signalling specificity in the Akt pathway in breast cancer." Biochem Soc Trans **42**(5): 1349-1355.

Coffer, P. J. and J. R. Woodgett (1992). "Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families." Eur J Biochem **205**(3): 1217.

Colnaghi, R. and S. P. Wheatley (2010). "Liaisons between survivin and Plk1 during cell division and cell death." J Biol Chem **285**(29): 22592-22604.

Conery, A. R., Y. Cao, E. A. Thompson, C. M. Townsend, Jr., T. C. Ko and K. Luo (2004). "Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis." Nat Cell Biol **6**(4): 366-372.

Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-656.

Cregan, S. P., A. Fortin, J. G. MacLaurin, S. M. Callaghan, F. Cecconi, S. W. Yu, T. M. Dawson, V. L. Dawson, D. S. Park, G. Kroemer and R. S. Slack (2002). "Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death." J Cell Biol **158**(3): 507-517.

Cristiano, B. E., J. C. Chan, K. M. Hannan, N. A. Lundie, N. J. Marmy-Conus, I. G. Campbell, W. A. Phillips, M. Robbie, R. D. Hannan and R. B. Pearson (2006). "A specific role for AKT3 in the genesis of ovarian cancer through modulation of G(2)-M phase transition." Cancer Res **66**(24): 11718-11725.

Crouthamel, M. C., J. A. Kahana, S. Korenchuk, S. Y. Zhang, G. Sundaresan, D. J. Eberwein, K. K. Brown and R. Kumar (2009). "Mechanism and management of AKT inhibitor-induced hyperglycemia." Clin Cancer Res **15**(1): 217-225.

Dan, H. C., M. Sun, S. Kaneko, R. I. Feldman, S. V. Nicosia, H. G. Wang, B. K. Tsang and J. Q. Cheng (2004). "Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP)." J Biol Chem **279**(7): 5405-5412.

Dard, N., S. Louvet-Vallee, A. Santa-Maria and B. Maro (2004). "Phosphorylation of ezrin on threonine T567 plays a crucial role during compaction in the mouse early embryo." Dev Biol **271**(1): 87-97.

Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh and M. E. Greenberg (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery." Cell **91**(2): 231-241.

Datta, S. R., A. Katsov, L. Hu, A. Petros, S. W. Fesik, M. B. Yaffe and M. E. Greenberg (2000). "14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation." Mol Cell **6**(1): 41-51.

Daugas, E., D. Nochy, L. Ravagnan, M. Loeffler, S. A. Susin, N. Zamzami and G. Kroemer (2000). "Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis." FEBS Lett **476**(3): 118-123.

Daugas, E., S. A. Susin, N. Zamzami, K. F. Ferri, T. Irinopoulou, N. Larochette, M. C. Prevost, B. Leber, D. Andrews, J. Penninger and G. Kroemer (2000). "Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis." FASEB J **14**(5): 729-739.

de Frias, M., D. Iglesias-Serret, A. M. Cosialls, L. Coll-Mulet, A. F. Santidrian, D. M. Gonzalez-Girones, E. de la Banda, G. Pons and J. Gil (2009). "Akt inhibitors induce apoptosis in chronic lymphocytic leukemia cells." Haematologica **94**(12): 1698-1707.

Del Bufalo, D., A. Biroccio, C. Leonetti and G. Zupi (1997). "Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line." FASEB J **11**(12): 947-953.

Deveraux, Q. L., E. Leo, H. R. Stennicke, K. Welsh, G. S. Salvesen and J. C. Reed (1999). "Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases." EMBO J **18**(19): 5242-5251.

Deveraux, Q. L. and J. C. Reed (1999). "IAP family proteins--suppressors of apoptosis." Genes Dev **13**(3): 239-252.

Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse and A. M. Zeiher (1999). "Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation." Nature **399**(6736): 601-605.

Dohi, T., K. Okada, F. Xia, C. E. Wilford, T. Samuel, K. Welsh, H. Marusawa, H. Zou, R. Armstrong, S. Matsuzawa, G. S. Salvesen, J. C. Reed and D. C. Altieri (2004). "An IAP-IAP complex inhibits apoptosis." J Biol Chem **279**(33): 34087-34090.

Dohi, T., F. Xia and D. C. Altieri (2007). "Compartmentalized phosphorylation of IAP by protein kinase A regulates cytoprotection." Mol Cell **27**(1): 17-28.

Du, P., L. Ye, H. Li, Y. Yang and W. G. Jiang (2013). "The tumour suppressive role of metastasis suppressor-1, MTSS1, in human kidney cancer, a possible connection with the SHH pathway." J Exp Ther Oncol **10**(2): 91-99.

Du, P., L. Ye, F. Ruge, Y. Yang and W. G. Jiang (2011). "Metastasis suppressor-1, MTSS1, acts as a putative tumour suppressor in human bladder cancer." Anticancer Res **31**(10): 3205-3212.

Duffy, M. J., A. van Dalen, C. Haglund, L. Hansson, E. Holinski-Feder, R. Klapdor, R. Lamerz, P. Peltomaki, C. Sturgeon and O. Topolcan (2007). "Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use." Eur J Cancer **43**(9): 1348-1360.

Dummler, B. and B. A. Hemmings (2007). "Physiological roles of PKB/Akt isoforms in development and disease." Biochem Soc Trans **35**(Pt 2): 231-235.

Easton, R. M., H. Cho, K. Roovers, D. W. Shineman, M. Mizrahi, M. S. Forman, V. M. Lee, M. Szabolcs, R. de Jong, T. Oltersdorf, T. Ludwig, A. Efstratiadis and M. J. Birnbaum (2005). "Role for Akt3/protein kinase Bgamma in attainment of normal brain size." Mol Cell Biol **25**(5): 1869-1878.

Eccles, S. A. and D. R. Welch (2007). "Metastasis: recent discoveries and novel treatment strategies." Lancet **369**(9574): 1742-1757.

Eckelman, B. P., G. S. Salvesen and F. L. Scott (2006). "Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family." EMBO Rep **7**(10): 988-994.

Engelman, J. A., J. Luo and L. C. Cantley (2006). "The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism." Nat Rev Genet **7**(8): 606-619.

Ericson, K., C. Gan, I. Cheong, C. Rago, Y. Samuels, V. E. Velculescu, K. W. Kinzler, D. L. Huso, B. Vogelstein and N. Papadopoulos (2010). "Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation." Proc Natl Acad Sci U S A **107**(6): 2598-2603.

Fan, H., L. Chen, F. Zhang, Y. Quan, X. Su, X. Qiu, Z. Zhao, K. L. Kong, S. Dong, Y. Song, T. H. Chan and X. Y. Guan "MTSS1, a novel target of DNA methyltransferase 3B, functions as a tumor suppressor in hepatocellular carcinoma." Oncogene **31**(18): 2298-2308.

Fan, H., L. Chen, F. Zhang, Y. Quan, X. Su, X. Qiu, Z. Zhao, K. L. Kong, S. Dong, Y. Song, T. H. Chan and X. Y. Guan (2011). "MTSS1, a novel target of DNA methyltransferase 3B, functions as a tumor suppressor in hepatocellular carcinoma." Oncogene **31**(18): 2298-2308.

Fawcett, J., C. L. Holness, L. A. Needham, H. Turley, K. C. Gatter, D. Y. Mason and D. L. Simmons (1992). "Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes." Nature **360**(6403): 481-484.

Fayard, E., G. Xue, A. Parcellier, L. Bozulic and B. A. Hemmings (2011). "Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway." Curr Top Microbiol Immunol **346**: 31-56.

Fearon, E. R. and B. Vogelstein (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-767.

Feng, J., R. Tamaskovic, Z. Yang, D. P. Brazil, A. Merlo, D. Hess and B. A. Hemmings (2004). "Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation." J Biol Chem **279**(34): 35510-35517.

Fidler, I. J. (2003). "The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited." Nat Rev Cancer **3**(6): 453-458.

Fievet, B. T., A. Gautreau, C. Roy, L. Del Maestro, P. Mangeat, D. Louvard and M. Arpin (2004). "Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin." J Cell Biol **164**(5): 653-659.

Flandrin, P., D. Guyotat, A. Duval, J. Cornillon, E. Tavernier, N. Nadal and L. Campos (2008). "Significance of heat-shock protein (HSP) 90 expression in acute myeloid leukemia cells." Cell Stress Chaperones **13**(3): 357-364.

Floryk, D. and T. C. Thompson (2008). "Perifosine induces differentiation and cell death in prostate cancer cells." Cancer Lett **266**(2): 216-226.

Fong, W. G., P. Liston, E. Rajcan-Separovic, M. St Jean, C. Craig and R. G. Korneluk (2000). "Expression and genetic analysis of XIAP-associated factor 1 (XAF1) in cancer cell lines." Genomics **70**(1): 113-122.

Fresno Vara, J. A., E. Casado, J. de Castro, P. Cejas, C. Belda-Iniesta and M. Gonzalez-Baron (2004). "PI3K/Akt signalling pathway and cancer." Cancer Treat Rev **30**(2): 193-204.

Fruman, D. A. and C. Rommel (2014). "PI3K and cancer: lessons, challenges and opportunities." Nat Rev Drug Discov **13**(2): 140-156.

Fu, Y. M., H. Zhang, M. Ding, Y. Q. Li, X. Fu, Z. X. Yu and G. G. Meadows (2006). "Selective amino acid restriction targets mitochondria to induce apoptosis of androgen-independent prostate cancer cells." J Cell Physiol **209**(2): 522-534.

Fu, Y. R., Z. Y. Qiu and Y. R. Yan (2006). "[Effect of hydroxycamptothecin on apoptosis-inducing factor (AIF) expression and on AIF translocation in human hepatocellular cancer cell SMMC-7721]." Zhonghua Gan Zang Bing Za Zhi **14**(4): 285-288.

Fuchs, S. Y., V. S. Spiegelman and K. G. Kumar (2004). "The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer." Oncogene **23**(11): 2028-2036.

Gao, T., F. Furnari and A. C. Newton (2005). "PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth." Mol Cell **18**(1): 13-24.

Garofalo, R. S., S. J. Orena, K. Rafidi, A. J. Torchia, J. L. Stock, A. L. Hildebrandt, T. Coskran, S. C. Black, D. J. Brees, J. R. Wicks, J. D. McNeish and K. G. Coleman (2003). "Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta." J Clin Invest **112**(2): 197-208.

Geller, D. S. and R. Gorlick (2011). "Osteosarcoma: a review of diagnosis, management, and treatment strategies." Clin Adv Hematol Oncol **8**(10): 705-718.

Geng, L., A. Chaudhuri, G. Talmon, J. L. Wisecarver, C. Are, M. Brattain and J. Wang (2013). "MicroRNA-192 suppresses liver metastasis of colon cancer." Oncogene **33**(46): 5332-5340.

Georgescu, M. M. (2010). "PTEN Tumor Suppressor Network in PI3K-Akt Pathway Control." Genes Cancer **1**(12): 1170-1177.

Gewinner, C., Z. C. Wang, A. Richardson, J. Teruya-Feldstein, D. Etemadmoghadam, D. Bowtell, J. Barretina, W. M. Lin, L. Rameh, L. Salmena, P. P. Pandolfi and L. C. Cantley (2009). "Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling." Cancer Cell **16**(2): 115-125.

Giacobbe, A., M. Compagnone, L. Bongiorno-Borbone, A. Antonov, E. K. Markert, J. H. Zhou, M. Annicchiarico-Petruzzelli, G. Melino and A. Peschiaroli (2015). "p63 controls cell migration and invasion by transcriptional regulation of MTSS1." Oncogene.

Gilot, D., F. Giudicelli, D. Lagadic-Gossmann and O. Fardel (2010). "Akti-1/2, an allosteric inhibitor of Akt 1 and 2, efficiently inhibits CaMKIalpha activity and aryl hydrocarbon receptor pathway." Chem Biol Interact **188**(3): 546-552.

Giodini, A., M. J. Kallio, N. R. Wall, G. J. Gorbsky, S. Tognin, P. C. Marchisio, M. Symons and D. C. Altieri (2002). "Regulation of microtubule stability and mitotic progression by survivin." Cancer Res **62**(9): 2462-2467.

Gonzalez-Quevedo, R., M. Shoffer, L. Horng and A. E. Oro (2005). "Receptor tyrosine phosphatase-dependent cytoskeletal remodeling by the hedgehog-responsive gene MIM/BEG4." J Cell Biol **168**(3): 453-463.

Gordan, J. D. and M. C. Simon (2007). "Hypoxia-inducible factors: central regulators of the tumor phenotype." Curr Opin Genet Dev **17**(1): 71-77.

Gordon, G. J., M. Mani, L. Mukhopadhyay, L. Dong, H. R. Edenfield, J. N. Glickman, B. Y. Yeap, D. J. Sugarbaker and R. Bueno (2007). "Expression patterns of inhibitor of apoptosis proteins in malignant pleural mesothelioma." J Pathol **211**(4): 447-454.

Gorlick, R., J. M. Maris, P. J. Houghton, R. Lock, H. Carol, R. T. Kurmasheva, E. A. Kolb, S. T. Keir, C. P. Reynolds, M. H. Kang, C. A. Billups and M. A. Smith (2011). "Testing of the Akt/PKB inhibitor MK-2206 by the Pediatric Preclinical Testing Program." Pediatr Blood Cancer **59**(3): 518-524.

Gould, K. L., A. Bretscher, F. S. Esch and T. Hunter (1989). "cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1." EMBO J **8**(13): 4133-4142.

Grabinski, N., K. Bartkowiak, K. Grupp, B. Brandt, K. Pantel and M. Jucker (2011). "Distinct functional roles of Akt isoforms for proliferation, survival, migration and EGF-mediated signalling in lung cancer derived disseminated tumor cells." Cell Signal **23**(12): 1952-1960.

Grad, J. M., X. R. Zeng and L. H. Boise (2000). "Regulation of Bcl-xL: a little bit of this and a little bit of STAT." Curr Opin Oncol **12**(6): 543-549.

Grille, S. J., A. Bellacosa, J. Upson, A. J. Klein-Szanto, F. van Roy, W. Lee-Kwon, M. Donowitz, P. N. Tsichlis and L. Larue (2003). "The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines." Cancer Res **63**(9): 2172-2178.

Guertin, D. A. and D. M. Sabatini (2007). "Defining the role of mTOR in cancer." Cancer Cell **12**(1): 9-22.

Gulhati, P., Q. Cai, J. Li, J. Liu, P. G. Rychahou, S. Qiu, E. Y. Lee, S. R. Silva, K. A. Bowen, T. Gao and B. M. Evers (2009). "Targeted inhibition of mammalian target of rapamycin signaling inhibits tumorigenesis of colorectal cancer." Clin Cancer Res **15**(23): 7207-7216.

Guo, X. N., A. Rajput, R. Rose, J. Hauser, A. Beko, K. Kuropatwinski, C. LeVea, R. M. Hoffman, M. G. Brattain and J. Wang (2007). "Mutant PIK3CA-bearing colon cancer cells display increased metastasis in an orthotopic model." Cancer Res **67**(12): 5851-5858.

Guo, Y., M. S. Ren, C. Shang, L. Zhu and M. Zhong (2015). "MTSS1 gene regulated by miR-96 inhibits cell proliferation and metastasis in tongue squamous cellular carcinoma Tca8113 cell line." Int J Clin Exp Med **8**(9): 15441-15449.

Gupta, G. P. and J. Massague (2006). "Cancer metastasis: building a framework." Cell **127**(4): 679-695.

Han, E. K., J. D. Leverson, T. McGonigal, O. J. Shah, K. W. Woods, T. Hunter, V. L. Giranda and Y. Luo (2007). "Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent of mTORC1 inhibition." Oncogene **26**(38): 5655-5661.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hawk, E. T. and B. Levin (2005). "Colorectal cancer prevention." J Clin Oncol **23**(2): 378-391.

Heiska, L., K. Alfthan, M. Gronholm, P. Vilja, A. Vaheri and O. Carpen (1998). "Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate." J Biol Chem **273**(34): 21893-21900.

Hennessy, B. T., D. L. Smith, P. T. Ram, Y. Lu and G. B. Mills (2005). "Exploiting the PI3K/AKT pathway for cancer drug discovery." Nat Rev Drug Discov **4**(12): 988-1004.

Heron-Milhavet, L., C. Franckhauser, V. Rana, C. Berthenet, D. Fisher, B. A. Hemmings, A. Fernandez and N. J. Lamb (2006). "Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding." Mol Cell Biol **26**(22): 8267-8280.

Heron-Milhavet, L., N. Khouya, A. Fernandez and N. J. Lamb (2011). "Akt1 and Akt2: differentiating the aktion." Histol Histopathol **26**(5): 651-662.

Hirai, H., H. Sootome, Y. Nakatsuru, K. Miyama, S. Taguchi, K. Tsujioka, Y. Ueno, H. Hatch, P. K. Majumder, B. S. Pan and H. Kotani (2010). "MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo." Mol Cancer Ther **9**(7): 1956-1967.

Hirakawa, H., K. Shibata and T. Nakayama (2009). "Localization of cortactin is associated with colorectal cancer development." Int J Oncol **35**(6): 1271-1276.

Hu, M., J. Yao, D. K. Carroll, S. Weremowicz, H. Chen, D. Carrasco, A. Richardson, S. Violette, T. Nikolskaya, Y. Nikolsky, E. L. Bauerlein, W. C. Hahn, R. S. Gelman, C. Allred, M. J. Bissell, S. Schnitt and K. Polyak (2008). "Regulation of in situ to invasive breast carcinoma transition." Cancer Cell **13**(5): 394-406.

Hu, Y. P., S. B. Patil, M. Panasiewicz, W. Li, J. Hauser, L. E. Humphrey and M. G. Brattain (2008). "Heterogeneity of receptor function in colon carcinoma cells determined by cross-talk between type I insulin-like growth factor receptor and epidermal growth factor receptor." Cancer Res **68**(19): 8004-8013.

Huang, W., S. Zhao, S. Ammanamanchi, M. Brattain, K. Venkatasubbarao and J. W. Freeman (2005). "Trichostatin A induces transforming growth factor beta type II receptor promoter activity and acetylation of Sp1 by recruitment of PCAF/p300 to a Sp1.NF-Y complex." J Biol Chem **280**(11): 10047-10054.

Huerta, S., D. M. Harris, A. Jazirehi, B. Bonavida, D. Elashoff, E. H. Livingston and D. Heber (2003). "Gene expression profile of metastatic colon cancer cells resistant to cisplatin-induced apoptosis." Int J Oncol **22**(3): 663-670.

Irie, H. Y., R. V. Pearline, D. Grueneberg, M. Hsia, P. Ravichandran, N. Kothari, S. Natesan and J. S. Brugge (2005). "Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition." J Cell Biol **171**(6): 1023-1034.

Itoh, N., S. Semba, M. Ito, H. Takeda, S. Kawata and M. Yamakawa (2002). "Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma." Cancer **94**(12): 3127-3134.

Iwasa, T., I. Okamoto, M. Suzuki, T. Nakahara, K. Yamanaka, E. Hatashita, Y. Yamada, M. Fukuoka, K. Ono and K. Nakagawa (2008). "Radiosensitizing effect of YM155, a novel small-molecule survivin suppressant, in non-small cell lung cancer cell lines." Clin Cancer Res **14**(20): 6496-6504.

Jadrich, J. L., M. B. O'Connor and E. Coucouvanis (2003). "Expression of TAK1, a mediator of TGF-beta and BMP signaling, during mouse embryonic development." Gene Expr Patterns **3**(2): 131-134.

Janes, S. M. and F. M. Watt (2004). "Switch from alphavbeta5 to alphavbeta6 integrin expression protects squamous cell carcinomas from anoikis." J Cell Biol **166**(3): 419-431.

Jeong, E. H., H. S. Choi, T. G. Lee, H. R. Kim and C. H. Kim (2012). "Dual Inhibition of PI3K/Akt/mTOR Pathway and Role of Autophagy in Non-Small Cell Lung Cancer Cells." Tuberc Respir Dis (Seoul) **72**(4): 343-351.

Jeyaprakash, A. A., C. Basquin, U. Jayachandran and E. Conti (2011). "Structural basis for the recognition of phosphorylated histone h3 by the survivin subunit of the chromosomal passenger complex." Structure **19**(11): 1625-1634.

Jin, K., W. Gao, Y. Lu, H. Lan, L. Teng and F. Cao (2012). "Mechanisms regulating colorectal cancer cell metastasis into liver (Review)." Oncol Lett **3**(1): 11-15.

Jin, X. and P. Mu (2015). "Targeting Breast Cancer Metastasis." Breast Cancer (Auckl) **9**(Suppl 1): 23-34.

Johnson, S. M., P. Gulhati, B. A. Rampy, Y. Han, P. G. Rychahou, H. Q. Doan, H. L. Weiss and B. M. Evers (2010). "Novel expression patterns of PI3K/Akt/mTOR signaling pathway components in colorectal cancer." J Am Coll Surg **210**(5): 767-776, 776-768.

Joyce, J. A. and J. W. Pollard (2009). "Microenvironmental regulation of metastasis." Nat Rev Cancer **9**(4): 239-252.

Jung, H. H., Y. H. Park, H. J. Jun, J. Kong, J. H. Kim, J. A. Kim, J. Yun, J. M. Sun, Y. W. Won, S. Lee, S. T. Kim, J. S. Ahn and Y. H. Im (2010). "Matrix metalloproteinase-1 expression can be upregulated through mitogen-activated protein kinase pathway under the influence of human epidermal growth factor receptor 2 synergized with estrogen receptor." Mol Cancer Res **8**(7): 1037-1047.

Karin, M. and E. Gallagher (2009). "TNFR signaling: ubiquitin-conjugated TRAFfic signals control stop-and-go for MAPK signaling complexes." Immunol Rev **228**(1): 225-240.

Kawasaki, H., M. Toyoda, H. Shinohara, J. Okuda, I. Watanabe, T. Yamamoto, K. Tanaka, T. Tenjo and N. Tanigawa (2001). "Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis." Cancer **91**(11): 2026-2032.

Kayser, G., A. Csanadi, S. Kakanou, A. Prasse, A. Kassem, E. Stickeler, B. Passlick and A. Zur Hausen (2015). "Downregulation of MTSS1 expression is an independent prognosticator in squamous cell carcinoma of the lung." Br J Cancer **112**(5): 866-873.

Kelley, L. C., K. E. Hayes, A. G. Ammer, K. H. Martin and S. A. Weed (2010). "Cortactin phosphorylated by ERK1/2 localizes to sites of dynamic actin regulation and is required for carcinoma lamellipodia persistence." PLoS One **5**(11): e13847.

Kelly, R. J., A. Lopez-Chavez, D. Citrin, J. E. Janik and J. C. Morris (2011). "Impacting tumor cell-fate by targeting the inhibitor of apoptosis protein survivin." Mol Cancer **10**: 35.

Khaleghpour, K., Y. Li, D. Banville, Z. Yu and S. H. Shen (2004). "Involvement of the PI 3-kinase signaling pathway in progression of colon adenocarcinoma." Carcinogenesis **25**(2): 241-248.

Khanna, C., X. Wan, S. Bose, R. Cassaday, O. Olomu, A. Mendoza, C. Yeung, R. Gorlick, S. M. Hewitt and L. J. Helman (2004). "The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis." Nat Med **10**(2): 182-186.

Kienast, Y., L. von Baumgarten, M. Fuhrmann, W. E. Klinkert, R. Goldbrunner, J. Herms and F. Winkler (2009). "Real-time imaging reveals the single steps of brain metastasis formation." Nat Med **16**(1): 116-122.

Kim, A. H., G. Khursigara, X. Sun, T. F. Franke and M. V. Chao (2001). "Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1." Mol Cell Biol **21**(3): 893-901.

Kim, J. H., H. Kim, K. Y. Lee, K. H. Choe, J. S. Ryu, H. I. Yoon, S. W. Sung, K. Y. Yoo and Y. C. Hong (2006). "Genetic polymorphisms of ataxia telangiectasia mutated affect lung cancer risk." Hum Mol Genet **15**(7): 1181-1186.

Kim, S., J. Kang, W. Hu, B. M. Evers and D. H. Chung (2003). "Geldanamycin decreases Raf-1 and Akt levels and induces apoptosis in neuroblastomas." Int J Cancer **103**(3): 352-359.

Kim, W. Y., D. J. Chang, B. Hennessy, H. J. Kang, J. Yoo, S. H. Han, Y. S. Kim, H. J. Park, S. Y. Seo, G. Mills, K. W. Kim, W. K. Hong, Y. G. Suh and H. Y. Lee (2008). "A novel derivative of the natural agent deguelin for cancer chemoprevention and therapy." Cancer Prev Res (Phila) **1**(7): 577-587.

Kirkbride, K. C., B. H. Sung, S. Sinha and A. M. Weaver (2011). "Cortactin: a multifunctional regulator of cellular invasiveness." Cell Adh Migr **5**(2): 187-198.

Kivela, T., J. Jaaskelainen, A. Vaheri and O. Carpen (2000). "Ezrin, a membrane-organizing protein, as a polarization marker of the retinal pigment epithelium in vertebrates." Cell Tissue Res **301**(2): 217-223.

Kretschmer, A., K. Moepert, S. Dames, M. Sternberger, J. Kaufmann and A. Klippel (2003). "Differential regulation of TGF-beta signaling through Smad2, Smad3 and Smad4." Oncogene **22**(43): 6748-6763.

Kruchten, A. E., E. W. Krueger, Y. Wang and M. A. McNiven (2008). "Distinct phospho-forms of cortactin differentially regulate actin polymerization and focal adhesions." Am J Physiol Cell Physiol **295**(5): C1113-1122.

Kuhar, M., S. Sen and N. Singh (2006). "Role of mitochondria in quercetin-enhanced chemotherapeutic response in human non-small cell lung carcinoma H-520 cells." Anticancer Res **26**(2A): 1297-1303.

Kunda, P., A. E. Pelling, T. Liu and B. Baum (2008). "Moesin controls cortical rigidity, cell rounding, and spindle morphogenesis during mitosis." Curr Biol **18**(2): 91-101.

Kuo, Y. C., K. Y. Huang, C. H. Yang, Y. S. Yang, W. Y. Lee and C. W. Chiang (2008). "Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt." J Biol Chem **283**(4): 1882-1892.

Kupriyanova, T. A. and K. V. Kandror (1999). "Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin." J Biol Chem **274**(3): 1458-1464.

Lai, F. P., M. Szczodrak, J. M. Oelkers, M. Ladwein, F. Acconcia, S. Benesch, S. Auinger, J. Faix, J. V. Small, S. Polo, T. E. Stradal and K. Rottner (2009). "Cortactin promotes migration and platelet-derived growth factor-induced actin reorganization by signaling to Rho-GTPases." Mol Biol Cell **20**(14): 3209-3223.

Lai, Y. C., Y. Liu, R. Jacobs and M. H. Rider (2012). "A novel PKB/Akt inhibitor, MK-2206, effectively inhibits insulin-stimulated glucose metabolism and protein synthesis in isolated rat skeletal muscle." Biochem J **447**(1): 137-147.

Larue, L. and A. Bellacosa (2005). "Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways." Oncogene **24**(50): 7443-7454.

Lee, J. W., E. G. Jeong, Y. H. Soung, S. Y. Kim, S. W. Nam, S. H. Kim, J. Y. Lee, N. J. Yoo and S. H. Lee (2006). "Immunohistochemical analysis of apoptosis-inducing factor (AIF) expression in gastric carcinomas." Pathol Res Pract **202**(7): 497-501.

Lee, M. W., D. S. Kim, J. H. Lee, B. S. Lee, S. H. Lee, H. L. Jung, K. W. Sung, H. T. Kim, K. H. Yoo and H. H. Koo (2011). "Roles of AKT1 and AKT2 in non-small cell lung cancer cell survival, growth, and migration." Cancer Sci **102**(10): 1822-1828.

Lee, Y. G., J. A. Macoska, S. Korenchuk and K. J. Pienta (2002). "MIM, a potential metastasis suppressor gene in bladder cancer." Neoplasia **4**(4): 291-294.

Leiphrakpam, P. D., A. Rajput, M. Mathiesen, E. Agarwal, A. J. Lazenby, C. Are, M. G. Brattain and S. Chowdhury (2014). "Ezrin expression and cell survival regulation in colorectal cancer." Cell Signal **26**(5): 868-879.

Levy, D. S., J. A. Kahana and R. Kumar (2009). "AKT inhibitor, GSK690693, induces growth inhibition and apoptosis in acute lymphoblastic leukemia cell lines." Blood **113**(8): 1723-1729.

Li, F., G. Ambrosini, E. Y. Chu, J. Plescia, S. Tognin, P. C. Marchisio and D. C. Altieri (1998). "Control of apoptosis and mitotic spindle checkpoint by survivin." Nature **396**(6711): 580-584.

Li, Q., M. Wu, H. Wang, G. Xu, T. Zhu, Y. Zhang, P. Liu, A. Song, C. Gang, Z. Han, J. Zhou, L. Meng, Y. Lu, S. Wang and D. Ma (2008). "Ezrin silencing by small hairpin RNA reverses metastatic behaviors of human breast cancer cells." Cancer Lett **261**(1): 55-63.

Li, W., Z. Chen, Y. Zong, F. Gong, Y. Zhu, J. Lv, J. Zhang, L. Xie, Y. Sun, Y. Miao, M. Tao, X. Han and Z. Xu "PP2A inhibitors induce apoptosis in pancreatic cancer cell line PANC-1 through persistent phosphorylation of IKKalpha and sustained activation of the NF-kappaB pathway." Cancer Lett **304**(2): 117-127.

Li, Z., J. Wu, C. Wu, J. Jiang, X. Zheng, B. Xu and M. Li (2012). "Deguelin, a natural rotenoid, inhibits mouse myeloma cell growth in vitro via induction of apoptosis." Oncol Lett **4**(4): 677-681.

Lin, J., J. Liu, Y. Wang, J. Zhu, K. Zhou, N. Smith and X. Zhan (2005). "Differential regulation of cortactin and N-WASP-mediated actin polymerization by missing in metastasis (MIM) protein." Oncogene **24**(12): 2059-2066.

Lindberg, K., L. A. Helguero, Y. Omoto, J. A. Gustafsson and L. A. Haldosen (2011). "Estrogen receptor beta represses Akt signaling in breast cancer cells via downregulation of HER2/HER3 and upregulation of PTEN: implications for tamoxifen sensitivity." Breast Cancer Res **13**(2): R43.

Liotta, L. A. and E. Kohn (2004). "Anoikis: cancer and the homeless cell." Nature **430**(7003): 973-974.

Liu, J., H. L. Weiss, P. Rychahou, L. N. Jackson, B. M. Evers and T. Gao (2009). "Loss of PHLPP expression in colon cancer: role in proliferation and tumorigenesis." Oncogene **28**(7): 994-1004.

Liu, J., D. Zhang, W. Luo, Y. Yu, J. Yu, J. Li, X. Zhang, B. Zhang, J. Chen, X. R. Wu, G. Rosas-Acosta and C. Huang (2011). "X-linked inhibitor of apoptosis protein (XIAP) mediates cancer cell motility via Rho GDP dissociation inhibitor (RhoGDI)-dependent regulation of the cytoskeleton." J Biol Chem **286**(18): 15630-15640.

Liu, P., H. Cheng, T. M. Roberts and J. J. Zhao (2009). "Targeting the phosphoinositide 3-kinase pathway in cancer." Nat Rev Drug Discov **8**(8): 627-644.

Liu, R., D. Liu and M. Xing (2012). "The Akt inhibitor MK2206 synergizes, but perifosine antagonizes, the BRAF(V600E) inhibitor PLX4032 and the MEK1/2 inhibitor AZD6244 in the inhibition of thyroid cancer cells." J Clin Endocrinol Metab **97**(2): E173-182.

Loberg, R. D., C. K. Neeley, L. L. Adam-Day, Y. Fridman, L. N. St John, S. Nixdorf, P. Jackson, L. M. Kalikin and K. J. Pienta (2005). "Differential expression analysis of MIM (MTSS1) splice variants and a functional role of MIM in prostate cancer cell biology." Int J Oncol **26**(6): 1699-1705.

Lowe, S. W., E. Cepero and G. Evan (2004). "Intrinsic tumour suppression." Nature **432**(7015): 307-315.

Lynch, H. T. and T. C. Smyrk (1998). "Identifying hereditary nonpolyposis colorectal cancer." N Engl J Med **338**(21): 1537-1538.

Ma, B. B., V. W. Lui, C. W. Hui, C. P. Lau, C. H. Wong, E. P. Hui, M. H. Ng, S. W. Tsao, Y. Li and A. T. Chan "Preclinical evaluation of the AKT inhibitor MK-2206 in nasopharyngeal carcinoma cell lines." Invest New Drugs.

Ma, S., X. Y. Guan, T. K. Lee and K. W. Chan (2007). "Clinicopathological significance of missing in metastasis B expression in hepatocellular carcinoma." Hum Pathol **38**(8): 1201-1206.

Ma, Z., S. L. Gibson, M. A. Byrne, J. Zhang, M. F. White and L. M. Shaw (2006). "Suppression of insulin receptor substrate 1 (IRS-1) promotes mammary tumor metastasis." Mol Cell Biol **26**(24): 9338-9351.

Mahalingam, D., R. Swords, J. S. Carew, S. T. Nawrocki, K. Bhalla and F. J. Giles (2009). "Targeting HSP90 for cancer therapy." Br J Cancer **100**(10): 1523-1529.

Manning, B. D. and L. C. Cantley (2007). "AKT/PKB signaling: navigating downstream." Cell **129**(7): 1261-1274.

Manning, B. D., A. R. Tee, M. N. Logsdon, J. Blenis and L. C. Cantley (2002). "Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway." Mol Cell **10**(1): 151-162.

Mattila, P. K., M. Salminen, T. Yamashiro and P. Lappalainen (2003). "Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its C-terminal WH2 domain." J Biol Chem **278**(10): 8452-8459.

Maurer, U., C. Charvet, A. S. Wagman, E. Dejardin and D. R. Green (2006). "Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1." Mol Cell **21**(6): 749-760.

Medema, R. H., G. J. Kops, J. L. Bos and B. M. Burgering (2000). "AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1." Nature **404**(6779): 782-787.

Mehlen, P. and A. Puisieux (2006). "Metastasis: a question of life or death." Nat Rev Cancer **6**(6): 449-458.

Mehrotra, S., L. R. Languino, C. M. Raskett, A. M. Mercurio, T. Dohi and D. C. Altieri (2010). "IAP regulation of metastasis." Cancer Cell **17**(1): 53-64.

Meng, S., D. Tripathy, E. P. Frenkel, S. Shete, E. Z. Naftalis, J. F. Huth, P. D. Beitsch, M. Leitch, S. Hoover, D. Euhus, B. Haley, L. Morrison, T. P. Fleming, D. Herlyn, L. W. Terstappen, T. Fehm, T. F. Tucker, N. Lane, J. Wang and J. W. Uhr (2004). "Circulating tumor cells in patients with breast cancer dormancy." Clin Cancer Res **10**(24): 8152-8162.

Mertz, K. D., G. Pathria, C. Wagner, J. Saarikangas, A. Sboner, J. Romanov, M. Gschaider, F. Lenz, F. Neumann, W. Schreiner, M. Nemethova, A. Glassmann, P. Lappalainen, G. Stingl, J. V. Small, D. Fink, L. Chin and S. N. Wagner (2014). "MTSS1 is a metastasis driver in a subset of human melanomas." Nat Commun **5**: 3465.

Millan, A. and S. Huerta (2009). "Apoptosis-inducing factor and colon cancer." J Surg Res **151**(1): 163-170.

Mitsiades, C. S., N. S. Mitsiades, C. J. McMullan, V. Poulaki, A. L. Kung, F. E. Davies, G. Morgan, M. Akiyama, R. Shringarpure, N. C. Munshi, P. G. Richardson, T. Hideshima, D. Chauhan, X. Gu, C. Bailey, M. Joseph, T. A. Libermann, N. S. Rosen and K. C. Anderson (2006). "Antimyeloma activity of heat shock protein-90 inhibition." Blood **107**(3): 1092-1100.

Modi, S., A. T. Stopeck, M. S. Gordon, D. Mendelson, D. B. Solit, R. Bagatell, W. Ma, J. Wheler, N. Rosen, L. Norton, G. F. Cropp, R. G. Johnson, A. L. Hannah and C. A. Hudis (2007). "Combination of trastuzumab and tanespimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2 overexpressing breast cancer: a phase I dose-escalation study." J Clin Oncol **25**(34): 5410-5417.

Modjtahedi, N., F. Giordanetto, F. Madeo and G. Kroemer (2006). "Apoptosis-inducing factor: vital and lethal." Trends Cell Biol **16**(5): 264-272.

Morini, M., M. Mottolese, N. Ferrari, F. Ghiorzo, S. Buglioni, R. Mortarini, D. M. Noonan, P. G. Natali and A. Albini (2000). "The alpha 3 beta 1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity." Int J Cancer **87**(3): 336-342.

Muslin, A. J., J. W. Tanner, P. M. Allen and A. S. Shaw (1996). "Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine." Cell **84**(6): 889-897.

Nagrath, S., L. V. Sequist, S. Maheswaran, D. W. Bell, D. Irimia, L. Ulkus, M. R. Smith, E. L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U. J. Balis, R. G. Tompkins, D. A. Haber and M. Toner (2007). "Isolation of rare circulating tumour cells in cancer patients by microchip technology." Nature **450**(7173): 1235-1239.

Nair, A. S., S. Shishodia, K. S. Ahn, A. B. Kunnumakkara, G. Sethi and B. B. Aggarwal (2006). "Deguelin, an Akt inhibitor, suppresses IkappaBalpha kinase activation

leading to suppression of NF-kappaB-regulated gene expression, potentiation of apoptosis, and inhibition of cellular invasion." J Immunol **177**(8): 5612-5622.

Nakabayashi, H. and K. Shimizu (2011). "HA1077, a Rho kinase inhibitor, suppresses glioma-induced angiogenesis by targeting the Rho-ROCK and the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) signal pathways." Cancer Sci **102**(2): 393-399.

Nakahara, T., A. Kita, K. Yamanaka, M. Mori, N. Amino, M. Takeuchi, F. Tominaga, S. Hatakeyama, I. Kinoyama, A. Matsuhisa, M. Kudoh and M. Sasamata (2007). "YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts." Cancer Res **67**(17): 8014-8021.

Nakatani, K., D. A. Thompson, A. Barthel, H. Sakaue, W. Liu, R. J. Weigel and R. A. Roth (1999). "Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines." J Biol Chem **274**(31): 21528-21532.

Navenot, J. M., N. Fujii and S. C. Peiper (2009). "KiSS1 metastasis suppressor gene product induces suppression of tyrosine kinase receptor signaling to Akt, tumor necrosis factor family ligand expression, and apoptosis." Mol Pharmacol **75**(5): 1074-1083.

Neckers, L. (2002). "Hsp90 inhibitors as novel cancer chemotherapeutic agents." Trends Mol Med **8**(4 Suppl): S55-61.

Newby, J. C., S. R. Johnston, I. E. Smith and M. Dowsett (1997). "Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer." Clin Cancer Res **3**(9): 1643-1651.

Ng, S. S. W., M. S. Tsao, S. Chow and D. W. Hedley (2000). "Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells." Cancer Res **60**(19): 5451-5455.

Ng, T., M. Parsons, W. E. Hughes, J. Monypenny, D. Zicha, A. Gautreau, M. Arpin, S. Gschmeissner, P. J. Verveer, P. I. Bastiaens and P. J. Parker (2001). "Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility." EMBO J **20**(11): 2723-2741.

Nguyen, D. X., P. D. Bos and J. Massague (2009). "Metastasis: from dissemination to organ-specific colonization." Nat Rev Cancer **9**(4): 274-284.

Nicholson, K. M. and N. G. Anderson (2002). "The protein kinase B/Akt signalling pathway in human malignancy." Cell Signal **14**(5): 381-395.

Nitsche, C., M. Edderkaoui, R. M. Moore, G. Eibl, N. Kasahara, J. Treger, P. J. Grippo, J. Mayerle, M. M. Lerch and A. S. Gukovskaya (2012). "The phosphatase PHLPP1 regulates Akt2, promotes pancreatic cancer cell death, and inhibits tumor formation." Gastroenterology **142**(2): 377-387 e371-375.

O'Connor, D. S., D. Grossman, J. Plescia, F. Li, H. Zhang, A. Villa, S. Tognin, P. C. Marchisio and D. C. Altieri (2000). "Regulation of apoptosis at cell division by

p34cdc2 phosphorylation of survivin." Proc Natl Acad Sci U S A **97**(24): 13103-13107.

Obexer, P. and M. J. Ausserlechner (2014). "X-linked inhibitor of apoptosis protein - a critical death resistance regulator and therapeutic target for personalized cancer therapy." Front Oncol **4**: 197.

Ohoka, Y. and Y. Takai (1998). "Isolation and characterization of cortactin isoforms and a novel cortactin-binding protein, CBP90." Genes Cells **3**(9): 603-612.

Okano, J., I. Gaslightwala, M. J. Birnbaum, A. K. Rustgi and H. Nakagawa (2000). "Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation." J Biol Chem **275**(40): 30934-30942.

Okuya, M., H. Kurosawa, J. Kikuchi, Y. Furukawa, H. Matsui, D. Aki, T. Matsunaga, T. Inukai, H. Goto, R. A. Altura, K. Sugita, O. Arisaka, A. T. Look and T. Inaba (2009). "Up-regulation of survivin by the E2A-HLF chimera is indispensable for the survival of t(17;19)-positive leukemia cells." J Biol Chem **285**(3): 1850-1860.

Ongchin, M., E. Sharratt, I. Dominguez, N. Simms, J. Wang, R. Cheney, C. LeVea, M. Brattain and A. Rajput (2009). "The effects of epidermal growth factor receptor activation and attenuation of the TGFbeta pathway in an orthotopic model of colon cancer." J Surg Res **156**(2): 250-256.

Ostrakhovitch, E. A. and M. G. Cherian (2005). "Inhibition of extracellular signal regulated kinase (ERK) leads to apoptosis inducing factor (AIF) mediated apoptosis in epithelial breast cancer cells: the lack of effect of ERK in p53 mediated copper induced apoptosis." J Cell Biochem **95**(6): 1120-1134.

Owen, K. A., F. J. Pixley, K. S. Thomas, M. Vicente-Manzanares, B. J. Ray, A. F. Horwitz, J. T. Parsons, H. E. Beggs, E. R. Stanley and A. H. Bouton (2007). "Regulation of lamellipodial persistence, adhesion turnover, and motility in macrophages by focal adhesion kinase." J Cell Biol **179**(6): 1275-1287.

Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer and D. B. Donner (1999). "NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase." Nature **401**(6748): 82-85.

Pakkanen, R. (1988). "Immunofluorescent and immunochemical evidence for the expression of cytovillin in the microvilli of a wide range of cultured human cells." J Cell Biochem **38**(1): 65-75.

Parr, C. and W. G. Jiang (2009). "Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer." Eur J Cancer **45**(9): 1673-1683.

Parsons, D. W., T. L. Wang, Y. Samuels, A. Bardelli, J. M. Cummins, L. DeLong, N. Silliman, J. Ptak, S. Szabo, J. K. Willson, S. Markowitz, K. W. Kinzler, B. Vogelstein, C. Lengauer and V. E. Velculescu (2005). "Colorectal cancer: mutations in a signalling pathway." Nature **436**(7052): 792.

Pavlyukov, M. S., N. V. Antipova, M. V. Balashova, T. V. Vinogradova, E. P. Kopantzev and M. I. Shakhparonov (2011). "Survivin monomer plays an essential role in apoptosis regulation." J Biol Chem **286**(26): 23296-23307.

Peng, X. D., P. Z. Xu, M. L. Chen, A. Hahn-Windgassen, J. Skeen, J. Jacobs, D. Sundararajan, W. S. Chen, S. E. Crawford, K. G. Coleman and N. Hay (2003). "Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2." Genes Dev **17**(11): 1352-1365.

Perrin, B. J., K. J. Amann and A. Huttenlocher (2006). "Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration." Mol Biol Cell **17**(1): 239-250.

Pestonjamasp, K., M. R. Amieva, C. P. Strassel, W. M. Nauseef, H. Furthmayr and E. J. Luna (1995). "Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes." Mol Biol Cell **6**(3): 247-259.

Pinkas, J., S. S. Martin and P. Leder (2004). "Bcl-2-mediated cell survival promotes metastasis of EpH4 betaMEKDD mammary epithelial cells." Mol Cancer Res **2**(10): 551-556.

Pinton, G., A. G. Manente, G. Angeli, L. Mutti and L. Moro (2012). "Perifosine as a potential novel anti-cancer agent inhibits EGFR/MET-AKT axis in malignant pleural mesothelioma." PLoS One **7**(5): e36856.

Polesello, C. and F. Payre (2004). "Small is beautiful: what flies tell us about ERM protein function in development." Trends Cell Biol **14**(6): 294-302.

Ponta, H., L. Sherman and P. A. Herrlich (2003). "CD44: from adhesion molecules to signalling regulators." Nat Rev Mol Cell Biol **4**(1): 33-45.

Potter, J. D. (1999). "Colorectal cancer: molecules and populations." J Natl Cancer Inst **91**(11): 916-932.

Psaila, B. and D. Lyden (2009). "The metastatic niche: adapting the foreign soil." Nat Rev Cancer **9**(4): 285-293.

Pujuguet, P., L. Del Maestro, A. Gautreau, D. Louvard and M. Arpin (2003). "Ezrin regulates E-cadherin-dependent adherens junction assembly through Rac1 activation." Mol Biol Cell **14**(5): 2181-2191.

Remy, I., A. Montmarquette and S. W. Michnick (2004). "PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3." Nat Cell Biol **6**(4): 358-365.

Ren, L., S. H. Hong, Q. R. Chen, J. Briggs, J. Cassavaugh, S. Srinivasan, M. M. Lizardo, A. Mendoza, A. Y. Xia, N. Avadhani, J. Khan and C. Khanna (2011). "Dysregulation of ezrin phosphorylation prevents metastasis and alters cellular metabolism in osteosarcoma." Cancer Res **72**(4): 1001-1012.

Rhodes, N., D. A. Heerding, D. R. Duckett, D. J. Eberwein, V. B. Knick, T. J. Lansing, R. T. McConnell, T. M. Gilmer, S. Y. Zhang, K. Robell, J. A. Kahana, R. S. Geske, E. V. Kleymenova, A. E. Choudhry, Z. Lai, J. D. Leber, E. A. Minthorn, S. L. Strum, E. R. Wood, P. S. Huang, R. A. Copeland and R. Kumar (2008). "Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity." Cancer Res **68**(7): 2366-2374.

Richardson, P. G., J. Wolf, A. Jakubowiak, J. Zonder, S. Lonial, D. Irwin, J. Densmore, A. Krishnan, N. Raje, M. Bar, T. Martin, R. Schlossman, I. M. Ghobrial, N. Munshi, J. Laubach, J. Allerton, T. Hideshima, K. Colson, E. Poradosu, L. Gardner, P. Sportelli and K. C. Anderson (2011). "Perifosine plus bortezomib and dexamethasone in patients with relapsed/refractory multiple myeloma previously treated with bortezomib: results of a multicenter phase I/II trial." J Clin Oncol **29**(32): 4243-4249.

Romashkova, J. A. and S. S. Makarov (1999). "NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling." Nature **401**(6748): 86-90.

Rommel, C., B. A. Clarke, S. Zimmermann, L. Nunez, R. Rossman, K. Reid, K. Moelling, G. D. Yancopoulos and D. J. Glass (1999). "Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt." Science **286**(5445): 1738-1741.

Roy, H. K., B. F. Olusola, D. L. Clemens, W. J. Karolski, A. Ratashak, H. T. Lynch and T. C. Smyrk (2002). "AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis." Carcinogenesis **23**(1): 201-205.

Roy, N., Q. L. Deveraux, R. Takahashi, G. S. Salvesen and J. C. Reed (1997). "The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases." EMBO J **16**(23): 6914-6925.

Rychahou, P. G., J. Kang, P. Gulhati, H. Q. Doan, L. A. Chen, S. Y. Xiao, D. H. Chung and B. M. Evers (2008). "Akt2 overexpression plays a critical role in the establishment of colorectal cancer metastasis." Proc Natl Acad Sci U S A **105**(51): 20315-20320.

Saarikangas, J., P. K. Mattila, M. Varjosalo, M. Bovellan, J. Hakanen, J. Calzada-Wack, M. Tost, L. Jennen, B. Rathkolb, W. Hans, M. Horsch, M. E. Hyvonen, N. Perala, H. Fuchs, V. Gailus-Durner, I. Esposito, E. Wolf, M. H. de Angelis, M. J. Frilander, H. Savilahti, H. Sariola, K. Sainio, S. Lehtonen, J. Taipale, M. Salminen and P. Lappalainen (2011). "Missing-in-metastasis MIM/MTSS1 promotes actin assembly at intercellular junctions and is required for integrity of kidney epithelia." J Cell Sci **124**(Pt 8): 1245-1255.

Salmena, L., A. Carracedo and P. P. Pandolfi (2008). "Tenets of PTEN tumor suppression." Cell **133**(3): 403-414.

Salvesen, G. S. and V. M. Dixit (1997). "Caspases: intracellular signaling by proteolysis." Cell **91**(4): 443-446.

Sangai, T., A. Akcakanat, H. Chen, E. Tarco, Y. Wu, K. A. Do, T. W. Miller, C. L. Arteaga, G. B. Mills, A. M. Gonzalez-Angulo and F. Meric-Bernstam (2012). "Biomarkers of response to Akt inhibitor MK-2206 in breast cancer." Clin Cancer Res **18**(20): 5816-5828.

Santi, S. A. and H. Lee (2009). "The Akt isoforms are present at distinct subcellular locations." Am J Physiol Cell Physiol **298**(3): C580-591.

Saotome, I., M. Curto and A. I. McClatchey (2004). "Ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine." Dev Cell **6**(6): 855-864.

Sarbassov, D. D., D. A. Guertin, S. M. Ali and D. M. Sabatini (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-1101.

Sato, N., N. Funayama, A. Nagafuchi, S. Yonemura and S. Tsukita (1992). "A gene family consisting of ezrin, radixin and moesin. Its specific localization at actin filament/plasma membrane association sites." J Cell Sci **103 ( Pt 1)**: 131-143.

Sato, S., N. Fujita and T. Tsuruo (2000). "Modulation of Akt kinase activity by binding to Hsp90." Proc Natl Acad Sci U S A **97**(20): 10832-10837.

Schlieman, M. G., B. N. Fahy, R. Ramsamooj, L. Beckett and R. J. Bold (2003). "Incidence, mechanism and prognostic value of activated AKT in pancreas cancer." Br J Cancer **89**(11): 2110-2115.

Scott, F. L., J. B. Denault, S. J. Riedl, H. Shin, M. Renatus and G. S. Salvesen (2005). "XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs." EMBO J **24**(3): 645-655.

Sekulic, A., C. C. Hudson, J. L. Homme, P. Yin, D. M. Otterness, L. M. Karnitz and R. T. Abraham (2000). "A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells." Cancer Res **60**(13): 3504-3513.

Sheng, S., M. Qiao and A. B. Pardee (2009). "Metastasis and AKT activation." J Cell Physiol **218**(3): 451-454.

Shi, Y. H., W. X. Ding, J. Zhou, J. Y. He, Y. Xu, A. A. Gambotto, H. Rabinowich, J. Fan and X. M. Yin (2008). "Expression of X-linked inhibitor-of-apoptosis protein in hepatocellular carcinoma promotes metastasis and tumor recurrence." Hepatology **48**(2): 497-507.

Shiue, H., M. W. Musch, Y. Wang, E. B. Chang and J. R. Turner (2005). "Akt2 phosphorylates ezrin to trigger NHE3 translocation and activation." J Biol Chem **280**(2): 1688-1695.

Shtilbans, V., M. Wu and D. E. Burstein (2008). "Current overview of the role of Akt in cancer studies via applied immunohistochemistry." Ann Diagn Pathol **12**(2): 153-160.

Shvetsov, A., E. Berkane, D. Chereau, R. Dominguez and E. Reisler (2009). "The actin-binding domain of cortactin is dynamic and unstructured and affects lateral and longitudinal contacts in F-actin." Cell Motil Cytoskeleton **66**(2): 90-98.

Simioni, C., L. M. Neri, G. Tabellini, F. Ricci, D. Bressanin, F. Chiarini, C. Evangelisti, A. Cani, P. L. Tazzari, F. Melchionda, P. Pagliaro, A. Pession, J. A. McCubrey, S. Capitani and A. M. Martelli "Cytotoxic activity of the novel Akt inhibitor, MK-2206, in T-cell acute lymphoblastic leukemia." Leukemia **26**(11): 2336-2342.

Simms, N., A. Rajput, E. A. Sharratt, M. Ongchin, C. A. Teggart, J. Wang and M. G. Brattain (2012). "Transforming growth factor-ss suppresses metastasis in a subset of human colon carcinoma cells." BMC Cancer **12**(1): 221.

Slattery, M. L. (2000). "Diet, lifestyle, and colon cancer." Semin Gastrointest Dis **11**(3): 142-146.

Song, J., A. Fadiel, V. Edusa, Z. Chen, J. So, H. Sakamoto, D. A. Fishman and F. Naftolin (2005). "Estradiol-induced ezrin overexpression in ovarian cancer: a new signaling domain for estrogen." Cancer Lett **220**(1): 57-65.

Srinivasula, S. M. and J. D. Ashwell (2008). "IAPs: what's in a name?" Mol Cell **30**(2): 123-135.

Stambolic, V., A. Suzuki, J. L. de la Pompa, G. M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J. M. Penninger, D. P. Siderovski and T. W. Mak (1998). "Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN." Cell **95**(1): 29-39.

Stein, U. and P. M. Schlag (2007). "Clinical, biological, and molecular aspects of metastasis in colorectal cancer." Recent Results Cancer Res **176**: 61-80.

Stein, U., W. Walther, F. Arlt, H. Schwabe, J. Smith, I. Fichtner, W. Birchmeier and P. M. Schlag (2009). "MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis." Nat Med **15**(1): 59-67.

Stokoe, D., L. R. Stephens, T. Copeland, P. R. Gaffney, C. B. Reese, G. F. Painter, A. B. Holmes, F. McCormick and P. T. Hawkins (1997). "Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B." Science **277**(5325): 567-570.

Subhawong, A. P., H. Nassar, M. K. Halushka, P. B. Illei, R. Vang and P. Argani (2010). "Heterogeneity of Bcl-2 expression in metastatic breast carcinoma." Mod Pathol **23**(8): 1089-1096.

Suganuma, M., H. Fujiki, H. Suguri, S. Yoshizawa, M. Hirota, M. Nakayasu, M. Ojika, K. Wakamatsu, K. Yamada and T. Sugimura (1988). "Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter." Proc Natl Acad Sci U S A **85**(6): 1768-1771.

Sun, C., D. Nettesheim, Z. Liu and E. T. Olejniczak (2005). "Solution structure of human survivin and its binding interface with Smac/Diablo." Biochemistry **44**(1): 11-17.

Sung, J. S., S. G. Han, Y. M. Whang, E. S. Shin, J. W. Lee, H. J. Lee, J. S. Ryu, I. K. Choi, K. H. Park, J. S. Kim, S. W. Shin, E. K. Chu and Y. H. Kim (2008). "Putative association of

the single nucleotide polymorphisms in RASSF1A promoter with Korean lung cancer." Lung Cancer **61**(3): 301-308.

Sung, J. S., K. H. Park, S. T. Kim and Y. H. Kim (2012). "Discovery and Evaluation of Polymorphisms in the AKT2 and AKT3 Promoter Regions for Risk of Korean Lung Cancer." Genomics Inform **10**(3): 167-174.

Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger and G. Kroemer (1999). "Molecular characterization of mitochondrial apoptosis-inducing factor." Nature **397**(6718): 441-446.

Takayama, T., K. Miyanishi, T. Hayashi, Y. Sato and Y. Niitsu (2006). "Colorectal cancer: genetics of development and metastasis." J Gastroenterol **41**(3): 185-192.

Tanaka, S. and J. R. Wands (1996). "Insulin receptor substrate 1 overexpression in human hepatocellular carcinoma cells prevents transforming growth factor beta1-induced apoptosis." Cancer Res **56**(15): 3391-3394.

Thamilselvan, V., D. H. Craig and M. D. Basson (2007). "FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway." FASEB J **21**(8): 1730-1741.

Thiery, J. P., H. Acloque, R. Y. Huang and M. A. Nieto (2009). "Epithelial-mesenchymal transitions in development and disease." Cell **139**(5): 871-890.

Toker, A. and M. Yoeli-Lerner (2006). "Akt signaling and cancer: surviving but not moving on." Cancer Res **66**(8): 3963-3966.

Townson, J. L., G. N. Naumov and A. F. Chambers (2003). "The role of apoptosis in tumor progression and metastasis." Curr Mol Med **3**(7): 631-642.

Tran, H., A. Brunet, E. C. Griffith and M. E. Greenberg (2003). "The many forks in FOXO's road." Sci STKE **2003**(172): RE5.

Tsai, Y. C., A. Mendoza, J. M. Mariano, M. Zhou, Z. Kostova, B. Chen, T. Veenstra, S. M. Hewitt, L. J. Helman, C. Khanna and A. M. Weissman (2007). "The ubiquitin ligase gp78 promotes sarcoma metastasis by targeting KAI1 for degradation." Nat Med **13**(12): 1504-1509.

Tsukita, S., K. Oishi, N. Sato, J. Sagara and A. Kawai (1994). "ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons." J Cell Biol **126**(2): 391-401.

Turunen, O., T. Wahlstrom and A. Vaheri (1994). "Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family." J Cell Biol **126**(6): 1445-1453.

Utikal, J., A. Gratchev, I. Muller-Molinet, S. Oerther, J. Kzhyshkowska, N. Arens, R. Grobholz, S. Kannookadan and S. Goerdt (2006). "The expression of metastasis

suppressor MIM/MTSS1 is regulated by DNA methylation." Int J Cancer **119**(10): 2287-2293.

Valastyan, S. and R. A. Weinberg (2011). "Tumor metastasis: molecular insights and evolving paradigms." Cell **147**(2): 275-292.

van Rossum, A. G., J. H. de Graaf, E. Schuuring-Scholtes, P. M. Kluin, Y. X. Fan, X. Zhan, W. H. Moolenaar and E. Schuuring (2003). "Alternative splicing of the actin binding domain of human cortactin affects cell migration." J Biol Chem **278**(46): 45672-45679.

van Rossum, A. G., E. Schuuring-Scholtes, V. van Buuren-van Seggelen, P. M. Kluin and E. Schuuring (2005). "Comparative genome analysis of cortactin and HS1: the significance of the F-actin binding repeat domain." BMC Genomics **6**: 15.

Vansant, J. P., D. H. Johnson, D. M. O'Donnell, J. R. Stewart, A. H. Sonin, B. M. McCook, T. A. Powers, D. J. Salk, W. H. Frist and M. P. Sandler (1992). "Staging lung carcinoma with a Tc-99m labeled monoclonal antibody." Clin Nucl Med **17**(6): 431-438.

Vaux, D. L. and J. Silke (2005). "IAPs, RINGs and ubiquitylation." Nat Rev Mol Cell Biol **6**(4): 287-297.

Vazeux, R., P. A. Hoffman, J. K. Tomita, E. S. Dickinson, R. L. Jasman, T. St John and W. M. Gallatin (1992). "Cloning and characterization of a new intercellular adhesion molecule ICAM-R." Nature **360**(6403): 485-488.

Vivanco, I., D. Rohle, M. Versele, A. Iwanami, D. Kuga, B. Oldrini, K. Tanaka, J. Dang, S. Kubek, N. Palaskas, T. Hsueh, M. Evans, D. Mulholland, D. Wolle, S. Rajasekaran, A. Rajasekaran, L. M. Liau, T. F. Cloughesy, I. Dikic, C. Brennan, H. Wu, P. S. Mischel, T. Perera and I. K. Mellinghoff (2010). "The phosphatase and tensin homolog regulates epidermal growth factor receptor (EGFR) inhibitor response by targeting EGFR for degradation." Proc Natl Acad Sci U S A **107**(14): 6459-6464.

Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." Nat Rev Cancer **2**(7): 489-501.

Vogelstein, B. and K. W. Kinzler (2004). "Cancer genes and the pathways they control." Nat Med **10**(8): 789-799.

Wan, X. and L. J. Helman (2003). "Levels of PTEN protein modulate Akt phosphorylation on serine 473, but not on threonine 308, in IGF-II-overexpressing rhabdomyosarcomas cells." Oncogene **22**(50): 8205-8211.

Wang, D., M. R. Xu, T. Wang, T. Li and J. Zhu (2011). "MTSS1 overexpression correlates with poor prognosis in colorectal cancer." J Gastrointest Surg **15**(7): 1205-1212.

Wang, J., W. Han, E. Zborowska, J. Liang, X. Wang, J. K. Willson, L. Sun and M. G. Brattain (1996). "Reduced expression of transforming growth factor beta type I receptor contributes to the malignancy of human colon carcinoma cells." J Biol Chem **271**(29): 17366-17371.

Wang, J., L. Yang, J. Yang, K. Kuropatwinski, W. Wang, X. Q. Liu, J. Hauser and M. G. Brattain (2008). "Transforming growth factor beta induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells." Cancer Res **68**(9): 3152-3160.

Wang, M., L. Zhang, X. Han, J. Yang, J. Qian, S. Hong, F. Samaniego, J. Romaguera and Q. Yi (2007). "Atiprimod inhibits the growth of mantle cell lymphoma in vitro and in vivo and induces apoptosis via activating the mitochondrial pathways." Blood **109**(12): 5455-5462.

Wang, S. and M. D. Basson (2011). "Akt directly regulates focal adhesion kinase through association and serine phosphorylation: implication for pressure-induced colon cancer metastasis." Am J Physiol Cell Physiol **300**(3): C657-670.

Weigelt, B., J. L. Peterse and L. J. van 't Veer (2005). "Breast cancer metastasis: markers and models." Nat Rev Cancer **5**(8): 591-602.

Weiner, O. D., P. O. Neilsen, G. D. Prestwich, M. W. Kirschner, L. C. Cantley and H. R. Bourne (2002). "A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity." Nat Cell Biol **4**(7): 509-513.

Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers and L. M. Neckers (1994). "Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation." Proc Natl Acad Sci U S A **91**(18): 8324-8328.

Wilkinson, J. C., E. Cepero, L. H. Boise and C. S. Duckett (2004). "Upstream regulatory role for XIAP in receptor-mediated apoptosis." Mol Cell Biol **24**(16): 7003-7014.

Williams, B., R. J. Schneider and S. Jamal (2014). "Akt and PI3K-dependent but CREB-independent upregulation of MCAM by endothelin-3 in human melanocytes." Melanoma Res **24**(4): 404-407.

Wong, C. W., A. Lee, L. Shientag, J. Yu, Y. Dong, G. Kao, A. B. Al-Mehdi, E. J. Bernhard and R. J. Muschel (2001). "Apoptosis: an early event in metastatic inefficiency." Cancer Res **61**(1): 333-338.

Wu, K. L., S. Khan, S. Lakhe-Reddy, G. Jarad, A. Mukherjee, C. A. Obejero-Paz, M. Konieczkowski, J. R. Sedor and J. R. Schelling (2004). "The NHE1 Na+/H+ exchanger recruits ezrin/radixin/moesin proteins to regulate Akt-dependent cell survival." J Biol Chem **279**(25): 26280-26286.

Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.

Wyckoff, J. B., J. G. Jones, J. S. Condeelis and J. E. Segall (2000). "A critical step in metastasis: in vivo analysis of intravasation at the primary tumor." Cancer Res **60**(9): 2504-2511.

Xie, F., L. Ye, J. Chen, N. Wu, Z. Zhang, Y. Yang, L. Zhang and W. G. Jiang (2011). "The impact of Metastasis Suppressor-1, MTSS1, on oesophageal squamous cell carcinoma and its clinical significance." J Transl Med **9**: 95.

Xu, W., M. Marcu, X. Yuan, E. Mimnaugh, C. Patterson and L. Neckers (2002). "Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu." Proc Natl Acad Sci U S A **99**(20): 12847-12852.

Yamamoto, S., Y. Tomita, Y. Hoshida, T. Morooka, H. Nagano, K. Dono, K. Umeshita, M. Sakon, O. Ishikawa, H. Ohigashi, S. Nakamori, M. Monden and K. Aozasa (2004). "Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma." Clin Cancer Res **10**(8): 2846-2850.

Yan, M. D., C. J. Yao, J. M. Chow, C. L. Chang, P. A. Hwang, S. E. Chuang, J. Whang-Peng and G. M. Lai (2015). "Fucoidan Elevates MicroRNA-29b to Regulate DNMT3B-MTSS1 Axis and Inhibit EMT in Human Hepatocellular Carcinoma Cells." Mar Drugs **13**(10): 6099-6116.

Yang, H. W., M. G. Shin, S. Lee, J. R. Kim, W. S. Park, K. H. Cho, T. Meyer and W. Do Heo (2012). "Cooperative activation of PI3K by Ras and Rho family small GTPases." Mol Cell **47**(2): 281-290.

Yang, J. and R. A. Weinberg (2008). "Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis." Dev Cell **14**(6): 818-829.

Yang, X., M. Fraser, M. R. Abedini, T. Bai and B. K. Tsang (2008). "Regulation of apoptosis-inducing factor-mediated, cisplatin-induced apoptosis by Akt." Br J Cancer **98**(4): 803-808.

Yang, X., M. Fraser, U. M. Moll, A. Basak and B. K. Tsang (2006). "Akt-mediated cisplatin resistance in ovarian cancer: modulation of p53 action on caspase-dependent mitochondrial death pathway." Cancer Res **66**(6): 3126-3136.

Yang, Z. Z., O. Tschopp, M. Hemmings-Mieszczak, J. Feng, D. Brodbeck, E. Perentes and B. A. Hemmings (2003). "Protein kinase B alpha/Akt1 regulates placental development and fetal growth." J Biol Chem **278**(34): 32124-32131.

Ye, S. C., J. M. Foster, W. Li, J. Liang, E. Zborowska, S. Venkateswarlu, J. Gong, M. G. Brattain and J. K. Willson (1999). "Contextual effects of transforming growth factor beta on the tumorigenicity of human colon carcinoma cells." Cancer Res **59**(18): 4725-4731.

Yeh, E., M. Cunningham, H. Arnold, D. Chasse, T. Monteith, G. Ivaldi, W. C. Hahn, P. T. Stukenberg, S. Shenolikar, T. Uchida, C. M. Counter, J. R. Nevins, A. R. Means and R. Sears (2004). "A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells." Nat Cell Biol **6**(4): 308-318.

Yoeli-Lerner, M. and A. Toker (2006). "Akt/PKB signaling in cancer: a function in cell motility and invasion." Cell Cycle **5**(6): 603-605.

Yoeli-Lerner, M., G. K. Yiu, I. Rabinovitz, P. Erhardt, S. Jauliac and A. Toker (2005). "Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT." Mol Cell **20**(4): 539-550.

Youle, R. J. and A. Strasser (2008). "The BCL-2 protein family: opposing activities that mediate cell death." Nat Rev Mol Cell Biol **9**(1): 47-59.

Yu, S. W., S. A. Andrabi, H. Wang, N. S. Kim, G. G. Poirier, T. M. Dawson and V. L. Dawson (2006). "Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death." Proc Natl Acad Sci U S A **103**(48): 18314-18319.

Yuan, Z. Q., R. I. Feldman, G. E. Sussman, D. Coppola, S. V. Nicosia and J. Q. Cheng (2003). "AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance." J Biol Chem **278**(26): 23432-23440.

Yuan, Z. Q., M. Sun, R. I. Feldman, G. Wang, X. Ma, C. Jiang, D. Coppola, S. V. Nicosia and J. Q. Cheng (2000). "Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer." Oncogene **19**(19): 2324-2330.

Zhan, M., H. Zhao and Z. C. Han (2004). "Signalling mechanisms of anoikis." Histol Histopathol **19**(3): 973-983.

Zhong, J., S. Shaik, L. Wan, A. E. Tron, Z. Wang, L. Sun, H. Inuzuka and W. Wei (2013). "SCF beta-TRCP targets MTSS1 for ubiquitination-mediated destruction to regulate cancer cell proliferation and migration." Oncotarget **4**(12): 2339-2353.

Zhou, B. P., M. C. Hu, S. A. Miller, Z. Yu, W. Xia, S. Y. Lin and M. C. Hung (2000). "HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway." J Biol Chem **275**(11): 8027-8031.

Zhou, L., J. Li, Q. Q. Shao, J. C. Guo, Z. Y. Liang, W. X. Zhou, T. P. Zhang, L. You and Y. P. Zhao (2015). "Expression and Significances of MTSS1 in Pancreatic Cancer." Pathol Oncol Res.

Zhou, W., X. Q. Fu, J. Liu and H. G. Yu (2012). "RNAi knockdown of the Akt1 gene increases the chemosensitivity of gastric cancer cells to cisplatin both in vitro and in vivo." Regul Pept **176**(1-3): 13-21.

Zimmermann, K. C. and D. R. Green (2001). "How cells die: apoptosis pathways." J Allergy Clin Immunol **108**(4 Suppl): S99-103.