Fall 12-18-2015

EPACs: epigenetic regulators that affect cell survival in cancer.

Catherine Murari
University of Nebraska Medical Center

EPACs – epigenetic regulators that affect cell survival in cancer

By

Catherine Murari-Kanti

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in partial fulfilment of the requirements
for the Degree of Doctor of Philosophy

Cancer Research Graduate Program

Under the supervision of Professor Michael G. Brattain

University of Nebraska Medical Center
Omaha, Nebraska

December, 2015

Supervisory Committee
Jing (Jenny) Wang, Ph.D.
Xu Luo, Ph.D.
Jennifer Black, Ph.D.

Acknowledgements

I would like to thank my mentor and advisor, Dr. Michael G. Brattain for his support and unwavering faith in me. He provided opportunities to work independently, but was available when needed to traverse through obstacles.

I am thankful for a very supportive committee, Dr. Jenny Wang, Dr. Xu Luo, and Dr. Jennifer Black. Thank you for your comments and feedback and expertise through my scientific journey.

I would also like to thank, Dr. Gillian Howell with whom I would work on a daily basis. Her critical eye, as well as constant support is what has brought me thus far in my graduate career.

Of course, no graduate career is ever complete without the support of fellow lab members. The friendship and support goes a long way to succeed in the lab. Many thanks to Meghan Mendick, Katie Bailey, Ekta Agarwal, Shane Laschanzky, Brittany Poelart, Caroline Robb and Premila L. Also former members, Carol Teggart, Neka Simms, Wang Wang, Michelle, Jon Person and Hannah Weber who have played a role in guiding me through my career. Special mention goes out to Meghan and Brittany, who have helped me the last few months on this project and kept it going.

Many thanks to the Hammonds – my American family and the love and constant support of the Omaha Christian Fellowship folks who made living in Omaha enjoyable.

Ofcourse, I wouldn't have made it thus far without the support of my husband, who has been my rock and who pushed me to succeed when I thought I couldn't. Thank you to my parents, my siblings and my in-laws who have been there for me through some hard times and are standing with me today as I cross the finish line.

Thank you Amanda Lakamp, Meghan Mendick, Elizabeth Blowers, Sarah Driskell and Alyssa Yeik for being such an awesome group of friends, supporters and cheerleaders. Thank you for being there for me through the tears, laughter and the many cups of coffee that were consumed contemplating the intricacies of Grad school.

I thank my Almighty Father and Friend – the Lord Jesus Christ without whom my life would be incomplete.

EPACs – epigenetic regulators that affect cell survival in cancer

Catherine Murari-Kanti, Ph.D.

University of Nebraska Medical Center, 2015

Supervisor: Michael G. Brattain, Ph.D

Cyclic adenosine monophosphate (cAMP) is a second messenger responsive to many external stimuli, playing an important role in cellular gene expression, metabolism, migration, differentiation, hypertrophy, apoptosis and secretion. All of these cellular functions are important in many diseases including cancer. Most of its effects were initially attributed to the classical protein kinase A (PKA) protein, but cellular functions such as proliferation and migration were found to be PKA independent and dependent on the newly discovered exchange proteins directly activated by cAMP (EPACs). EPACs are single polypeptides that primarily function as guanine exchange factors (GEFs) for Rap proteins that allow the replacement of guanine diphosphate (GDP) with the more abundant guanosine triphosphate (GTP), under cAMP stimulation.

EPAC has been reported to promote cancer cell growth and activate phosphhatidylinositol 3-kinase (PI3K) in direct opposition to the effects of PKA. This was confirmed in the Brattain Laboratory, treating colorectal cancer (CRC)

cell lines with the EPAC specific activator 8CPT (8-pCPT-2-O-Me-cAMP) induced AKT expression along with increased expression of inhibitor of apoptosis protein (IAP) survivin and X-linked IAP (XIAP); which are implicated with poor prognosis in cancer. However, there are 2 isoforms of EPAC – EPAC1 and 2 and to dissect which EPAC is pro-tumorigenic we used EPAC specific inhibitors (ESI) – ESI09 (inhibits EPAC1 and 2), ESI05 (inhibits EPAC2) and CE3F4 (inhibits EPAC1) and confirmed that EPAC1 is associated with cell survival in CRC cell line as well as in pancreatic cancer (PaCa) cell lines.

Inhibition of EPACs decreased histone deacetylase (HDAC) 4 and 5 that are overexpressed in many cancers. Inhibition of HDAC4 and 5 with LMK235 decreased survivin and XIAP allowing us to hypothesize that the cell survival effects observed on EPAC inhibition must be due to the repression of HDACs 4 and 5 making EPACs epigenetic regulators.

Table of Contents

Chapter 1

EPACs – epigenetic regulators that affect cell survival

Catherine Murari-Kanti, Ph.D.

University of Nebraska Medical Center, 2015

Introduction:

3',5' -cyclic adenosine mono-phosphate (cAMP) is a second messenger produced intracellularly by the action of adenylyl cyclase on adenosine triphosphate (ATP) as an output of G-protein coupled receptor signaling. cAMP is involved in multiple cell processes such as cell proliferation, metabolism, adhesion, apoptosis, gene expression and differentiation, all of which are pivotal in numerous diseases, including cancer [1].

The cell functions of cAMP were first thought to be solely mediated by Protein kinase A (PKA) but it was later discovered that Exchange factor activated by cAMP (EPAC) was also involved [2-4]. EPACs are most commonly involved as the guanine exchange factor (GEF) for Rap proteins. Upon binding cAMP, EPAC undergoes a conformational change that allows Rap to bind to EPAC and replace guanosine diphosphate (GDP) with the more abundant guanine triphosphate (GTP) [5]. In mammals, Rap guanine nucleotide exchange factors 3 and 4 (RAPGEF3 and 4) encode for EPAC1 and EPAC2 respectively. EPAC1 is

conserved in chimpanzee, Rhesus monkey, dog, cow, rat, chicken and mouse while EPAC2 is additionally conserved across zebra fish, fruit fly, C.elegans and mosquito.

RAPGEF3 (EPAC1) is located on chromosome 12 and has 2 isoforms: EPAC1a (923 amino acids) and EPAC1b (881 amino acids). For the purpose of this document, we will be considering EPAC1a as EPAC1. RAPGEF4 (EPAC2) is located on Chromosome 2 and has 3 isoforms – EPAC2a, EPAC2b and EPAC2c [6, 7].

Structure of EPACs:

The N-terminus of EPAC is the regulatory domain while the C-terminus is the catalytic domain. The cyclic nucleotide binding domain(s) (CNB) and the disheveled-Egl-10-Pleckstrin (DEP) domain make up the regulatory domain while the catalytic domains consist of the Ras-exchange motif (REM), Ras association domain (RA) and the CDC25 homology domain (CDC25HD). All isoforms of EPAC except EPAC2a possess one CNB domain. The extra CNB domain in EPAC2A has no other effect but binds to cAMP with a 20-fold lower affinity than the shared CNB. The DEP domains are essential in compartmentalization of EPACs within the cell through protein-protein or protein-membrane interactions [8, 9]. (Figure 1)

The catalytic domains are highly homologous between EPAC1 and EPAC2. The REM domain is essential for stabilizing EPAC while the RA domain in EPAC2 is able to bind to activated Ras and allow for the transfer of EPAC2 to Ras containing membranes in proximity to Rap [10]. This association is not observed in EPAC1. The CDC25HD is responsible for performing the catalytic GEF activity for Ras-like GTPases.

The regulatory region associates with the catalytic region in such fashion to confer an auto-inhibitory configuration and thus prevent GEF activation [8]. When cAMP binds to the CNB domain of EPACs it causes the EPAC structure to open up allowing binding of Rap-GTP. (Figure 2.)

Although EPAC has been associated with GTPase activity, some activity of this cAMP-dependent protein appears to be independent of GTPase activity. These effects are dependent on cellular localization.

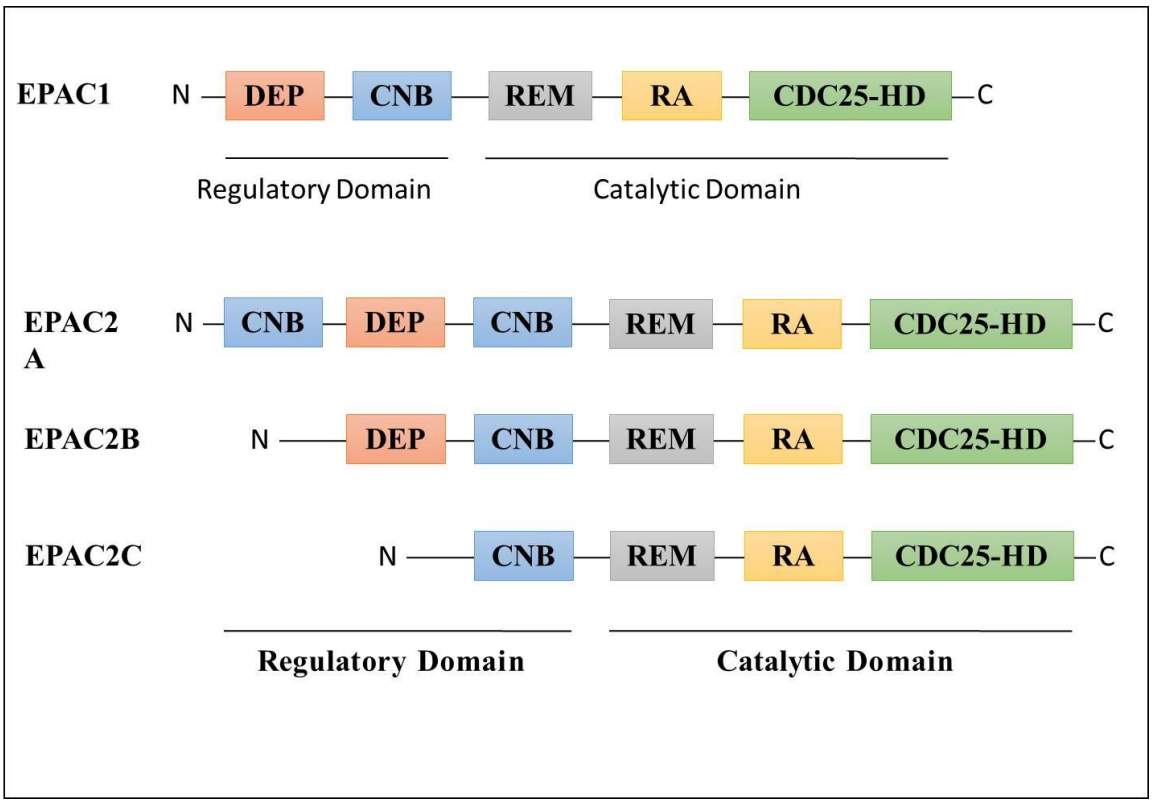


Figure 1. The DEP (Dishevelled, Egl-10 and Pleckstrin), CNB (cyclic nucleotide binding domain), REM (Ras exchange motif domain), RA (Ras association domain) and the CDC25HD (CDC25 homology domain) make up the domain structure of EPACS. cAMP binds to the CNB domain activating EPACs.

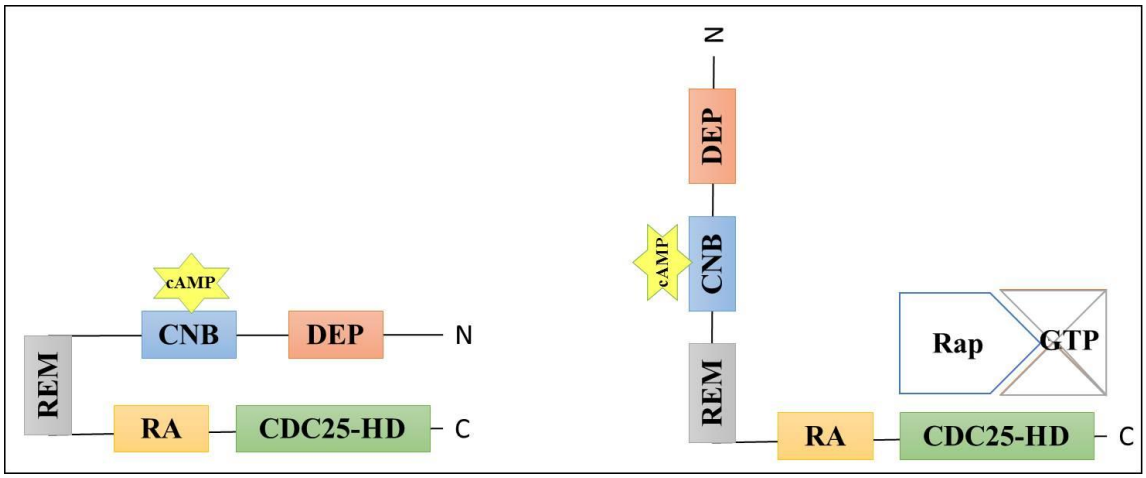


Figure 2: Activation of EPACs. Inactive EPAC is folded upon itself and upon cAMP binding to the CNB domain, the structure opens up at right angles to allow Rap-GDP to be replaced by the more ubiquitous GTP.

Location and Functions of EPAC:

EPAC1 is more ubiquitously distributed in the body than EPAC2. There is higher prevalence of EPAC1 in the kidney, ovary, skeletal muscle and thyroid [11]. EPAC1 levels change during development and are at their peak at about 3 weeks after birth [12]. During interphase, EPAC1 is found on the nuclear membrane and mitochondria and later is localized to the spindle, centrosome and contractile ring during mitosis, in COS7 cells [9]. Varying levels of cAMP in the cell affect the localization of EPAC1. High levels of intracellular cAMP allows for EPAC1 to bind to phosphatidic acid via the DEP domain and bring it to the plasma membrane, while low levels direct EPAC1 to the microtubule cytoskeleton [9, 13, 14].

EPAC2 has specific localization in the human body – in the brain, in endocrine glands such as the pituitary and pancreas and the heart [6]. The EPAC2C isoform is specifically observed in the liver, where it may control bile acid stimulated canalicular formation [7, 15].

Localization and compartmentalization of EPAC depends on the availability and the removal of cAMP. Phosphodiesterases are responsible for the degradation for cAMP and cGMP while A-kinase anchoring proteins (AKAPs) are scaffolding proteins responsible for retaining EPAC and allowing for downstream signaling

to occur.[16, 17] The complex of EPAC1, PKA, mAKAP, PDE4D3 and ERK5 was found in neonatal cardiomyocytes [18].

Physiological functions of EPAC

- **Cardiac Function:**

  - PKA is involved in cardiac contractility, relaxation and automaticity leading to studies on the role of EPAC in cardiac function [19, 20].

  - Mice in which Phospholipase C (PLC) ε was knocked out showed a decrease in β-adrenergic receptor (βAR)-dependent cardiac contraction. PLCε is downstream of EPAC and is the Rap effector [21].

  - EPAC inhibits TGFβ and adenosine-2 receptor induced collagen synthesis required for profibrotic response in cardiac fibroblasts [22, 23].

  - Connexin43 that is required for gap junction assembly is recruited by EPAC via Rap1 [24]. Stressed mice expressed higher levels of EPAC1 leading to cardiac hypertrophy, confirming the role of EPAC in cardiac function and in disease [25].

- **Insulin Secretion:**

  - Glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) are released from gastrointestinal cells in response to glucose, which stimulates cAMP production from pancreatic beta cells. This process was

first thought to be mediated by cAMP on PKA but it was later found that EPAC2 was required for the complete induction of insulin under the GLP-1/GIP stimulation [26, 27].

- Rim2 (a $Ca2^+$ tether) and Piccolo (a Rim2 interacting protein) interact with EPAC2 to prime vesicles required for insulin secretion [27-29].

- EPAC2 and sulfonyl urea receptor 1 interact leading to the exocytosis of insulin through the inhibition of K^+ATP channels causing membrane polarization and influx of $Ca2^+$[30].

Due its role in insulin production, EPAC is implicated in the etiology of diabetes and other metabolic disorders.

Cell adhesion:

Through interaction with Rap, EPAC activates the lymphocyte function associated antigen 1, the very late activation antigen 4 and the macrophage integrin 1 that are required for integrin-mediated cell adhesion and E-cadherin mediated cell-cell junction formation [31-40].

8-pCPT-2'-O-Me-cAMP (8CPT) an EPAC specific activator was synthesized by the Bos laboratory in 2002. This activator activated EPAC and not PKA, thus providing an extremely useful pharmacological tool that could be used to the study PKA-indepent effects of cAMP [41].

8CPT allowed determination of the specific role of EPAC1 along with Rap1 in β1-integrin-mediated adhesion. This process was cAMP dependent and PKA independent.

Cell permeability induced by 8CPT was decreased by the knockdown of EPAC1 or inhibition of Rap [42]. Similar results were also observed in vascular endothelial cadherin null cells confirming that the EPAC activator is unable to bring about cell permeability in the absence of vascular endothelial cadherin [43]. EPAC stimulation has the ability to increase cortical actin through the down regulation of Rho [42, 43].

Role of EPAC in Cancer:

EPAC was discovered in 1998, and the potential role of this cAMP activated protein is being studied in cancer. EPAC has been implicated in cancer angiogenesis, DNA damage repair, invasion and migration. cAMP is heavily involved in myriad cancer processes, thus implying the importance of EPAC in cancer.

- **Lung Cancer**

  Cho and colleagues determined that stimulation of EPAC1 led to a decrease in the DNA damage repair protein - x-ray repair cross complementing 1 and the related DNA damage apoptosis in lung cancer

[44]. However, Pullamsetti and colleagues demonstrated that phosphodiesterase 4 (PDE4) – which hydrolyzes cAMP, has the ability to decrease hypoxia inducible factor (HIF) in lung cancer cell lines. This was further confirmed by activation of EPAC [45].

Thus, we see pro- and anti- tumor effects of EPAC in lung cancer, demonstrating the need for further studies of the EPAC isoforms and their roles in tumorigenesis and metastasis.

- **Melanoma**

Narita et al., demonstrated that both the inhibition of PDE4 and stimulation of EPAC promoted melanoma cell growth [46]. The Baljinnyam laboratory has done extensive work on the functions of EPAC in melanoma and have shown that EPAC promotes translocation of syndecan-2, a cell surface heparin sulphate (HS) proteoglycan, which causes an increase in melanoma cell migration. Higher levels of EPAC were found in metastatic melanoma than in the primary melanoma and thus an increased effect on HS in metastatic versus primary tumor [47]. They have also shown that EPAC stimulation increases melanoma migration by increasing intracellular Ca2+ via the phospholipase-C/inositol 3 (PIP3) receptor pathway that leads to increased actin assembly [48]. The laboratory also showed that melanoma cells with higher

expression of EPAC1 control the melanoma/melanoma as well melanoma/endothelial cell communication through fibroblast growth factor-HS interaction [49].

- **Ovarian Cancer**

  Studies on EPAC in ovarian cancer are at the nascent stage. EPAC activation of gonadotropin stimulated human ovarian surface epithelial cells resulted in an increase of epidermal growth factor receptor signaling through ERK1/2 and AKT [50]. However, in ovarian cancer ES2 cells, treatment with norepinephrine decreased migration activity through EPAC activation of phospholipase C enzymes [51] Although there is conflicting data regarding EPACs in ovarian cancer, its role cannot be overlooked.

- **Breast Cancer**

  In breast cancer, EPAC activation leads to the inhibition of leptin (an important mediator of obesity). This resulted in a decreased leptin induced migration in highly invasive, MDA-MB-231 breast cancer cells [52].

- **Prostate Cancer**

  EPACs inhibit the proliferative and migratory characteristics of prostate cancer cells. These effects are medited through mitogen activated protein (MAP) kinase and RhoA pathways [53]. EPAC upregulates the B-Raf (isoform of Raf1)/ERK and mammalian target of rapamycin (mTOR) pathways to promote prostate cancer cell proliferation [54]. Though both studies contradict each other, EPACs appear to play a role in the progression of prostate cancer.

- **Pancreatic Cancer**

  Pro- and anti-tumor effects of EPAC have been reported in pancreatic cancer. In one study, elevated levels of cAMP, through EPAC activation caused a decrease in migration [55]. However, other studies suggest that inhibition of EPAC through selective inhibitors decreases migration and eventual progression of the disease.

  EPAC1 is overexpressed in pancreatic cancer [56]. Epac specific inhibitor (ESI) – 09, that selectively inhibits EPACs 1 and 2, decreased pancreatic cancer cell invasion and migration. This was possible through the activation and regulation of integrin β1. This drug exhibited in-vivo effects by inhibiting pancreatic cancer cell metastasis. Mice with tumors

from pancreatic cancer cell lines, when treated with ESI09, exhibited a decrease in metastasis to the liver as compared to the controls [57, 58].

- **Colorectal Cancer (CRC)**

  So far there have been no reported studies examining the role of EPAC in CRC. However, the oncomine database study indicates that EPAC expression increases as CRC progresses from adenoma to carcinoma. (Figure 3)

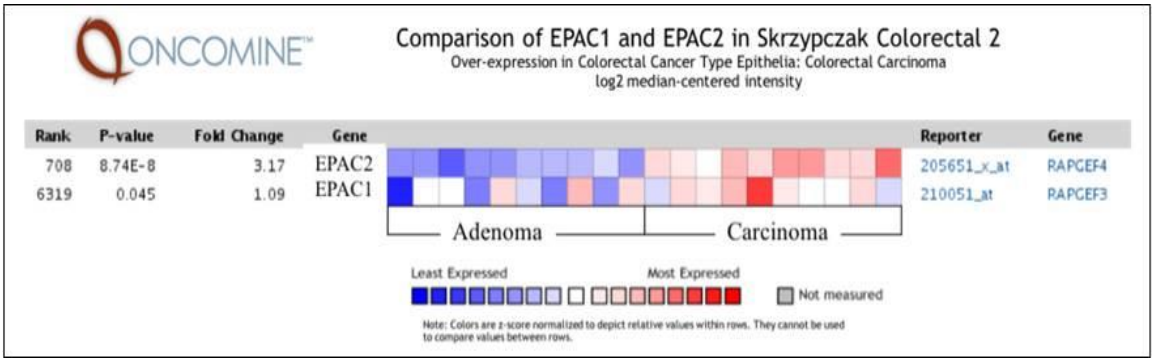


Figure 3: Oncomine data indicating the increase of EPAC expression as CRC progresses from adenoma to carcinoma.

Transforming Growth Factor (TGF) β Signaling Pathway:

CRC is the third most common cancer in the United States and the third most fatal in both men and women. In 2014, there were 136,830 new cases of CRC and 50,310 resulted in death. Individuals who have adenomatous polyps diagnosed in their life have a higher risk of developing CRC and one-third of those diagnosed with CRC will develop metastatic disease. The death rate from CRC has decreased over the years because of regular screenings [59]. However, there are no effective treatments for metastatic disease. Morever, patients who present with advanced disease are in danger of relapsing with disseminated disease.

A number of genetic changes occur in the colon for the development of full-blown cancer from a polyp and the development of metastatic disease. Changes in genes such as adenomatous polyposis coli (APC), V-Ki-ras2 kerstin rat sarcoma oncogene homolog (Kras), Smad4 and TGFβ receptor II (TGFβRII), phosphatidyl inositol-4,5-bisphosphate 3-kinase alpha (PIK3Ca), phosphatase and tensin homolog (PTEN), tumor protein 53 (TP53) and Bax are all documented to play a role in the development and progression of CRC [60].

Activins, inhibins, bone morphogenetic proteins, growth differentiation factors, anti-mullerian hormone, glial cell line-derived neutrotrophic factors and the TGFβ subfamily are part of the TGFβ superfamily [61]. TGFβ has 3 isoforms - TGFβ1, 2 and 3 and their function is predominantly growth inhibitory in

epithelial cells. The TGFβ family also plays an important role in wound healing, angiogenesis, cell proliferation, fibrosis and cancer progression [61, 62]. The TGFβ1 isoform is the most expressed and is usually referred to as TGFβ.

TGFβ binds to the TGFβ receptor II (TGFβRII) which autophosphorylates itself. This causes the recruitment and transphosphorylation of TGFβRI. TGFβRI then phosphorylates receptor-activated Smads (Smads 2 and 3) that then associates with the Co-Smads - Smad4. The Smad complex then translocates into the nucleus where it activates downstream signaling by interacting with promoter regions of target genes. This TGFβ signaling pathway is called the canonical signaling pathway [60]. (Figure 4)

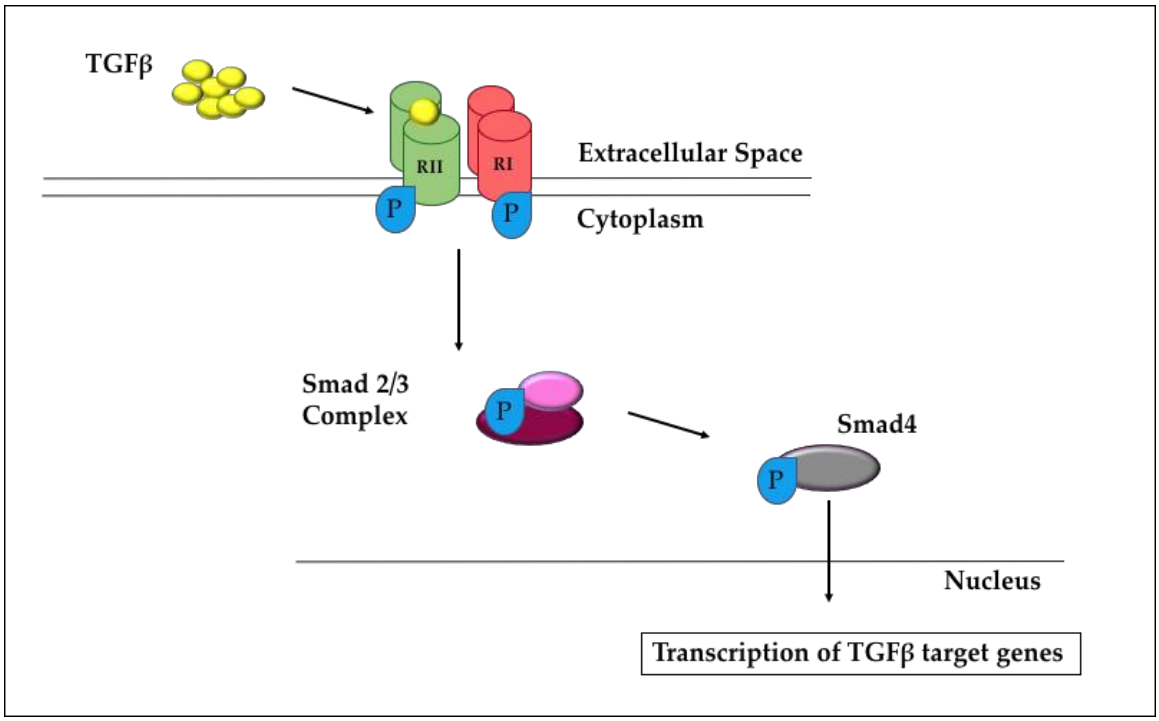


Figure 4: Canonical TGFβ signaling pathway.

TGFβ/PKA Signaling:

The Brattain laboratory, in 2011, discovered a non-canonical, TGFβ mediated, cAMP independent, Smad3 dependent pathway that was able to regulate cell survival in CRC [63]. In this pathway, TGFβ activated Smad3 through TGFβ receptor binding. After its activation, Smad3 would bind to PKA, anchored by A-kinase anchoring protein (AKAP) 149 scaffolding protein. PKA activation by Smad3 mediates TGFβ repression of survivin and XIAP. Upon cellular stress, survivin and XIAP are released from the mitochondria and in the cytosol, they heterodimerize to inhibit caspase activation. Activated PKA phosphorylates survivin on Ser^{20} that leads to loss of formation of XIAP and survivin complex that protects these IAPs from proteosome degradation and leads to loss of caspase inhibition. The resulting caspase activation leads to apoptosis.

Smad3 activated PKA also phosphorylates protein phosphatase 2A (PP2A) resulting in increased dephosphorylation of protein kinase B (AKT) on Ser^{473}. This inactivates AKT and prevents XIAP phosphorylation on Ser^{87}, required for binding to survivin again leading to ubiquination and degradation by the proteosome. (Figure. 5)

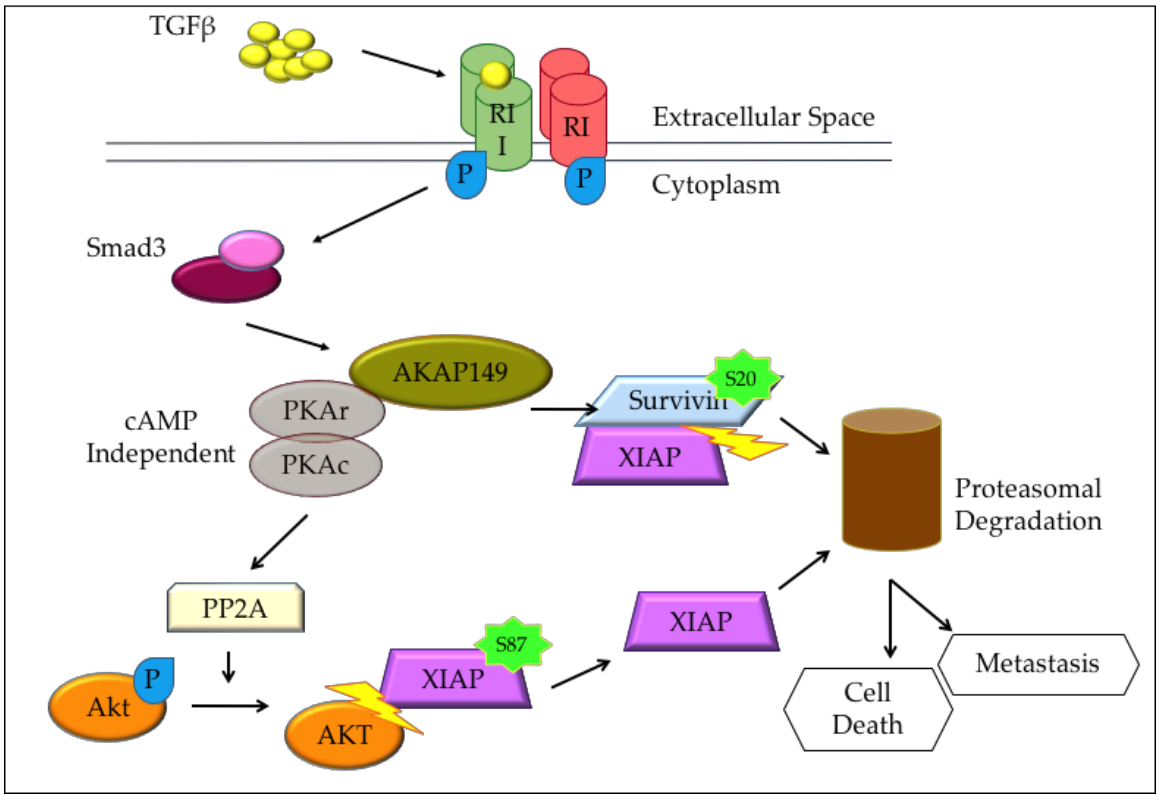


Figure 5: TGFβ/PKA signaling pathway.

Protein kinase A (PKA)

As noted above, PKA is one of the main effectors of cAMP and plays a central role in the TGFβ non-canonical pathway. PKA is an inactive tetramer of 4 subunits – 2 regulatory (R) and 2 catalytic (C). When cAMP binds to the 2 catalytic units, the 2 regulatory units are released and PKA can phosphorylate various molecules. Four different genes encode the regulatory subunits. These are PKARIα, PKARIβ, PKARIIα and PKARIIβ. Each of these units vary in cellular distribution as well as in functionality.

PKARIα is ubiquitous while PKARIβ is found in the brain, testis and the B- & T-lymphocytes. Both of these subunits are found in the cytoplasm and are associated with cell growth and proliferation. PKARIIα is ubiquitously distributed but PKARIIβ is found in the brain, adipose and in endocrine tissues. They vary in their expression in the cell being distributed within different subcellular organelles as well in the cytoplasm. The RII subunits are responsible for a marked increase in cell differentiation and a decrease in proliferation [64]. Differential binding to AKAP accounts for the differential distribution of the subunits in the cell [65].

In cancer, an overexpression of PKARI isoforms has been observed compared to the PKARII isoforms. PKARI is usually associated with increased proliferation and tumor formation while PKARII is found in growth-arrested cells [66-68].

They are involved in apoptosis through the MDM2/Bcl2 pathway and cause cell growth arrest through the Ras/MAPK & Shh/Gli pathways and have the ability to remodel the cytoskeleton of the cancer cell [69].

The Brattain laboratory has demonstrated that PKA can be activated through cAMP independent, Smad3 dependent means [63]. cAMP independent activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) – where PKACα associates with inhibitor of κB (I-κB) to form an NF-κB-I-κB-PKACα complex in the inactive state, has also been observed. Upon degradation of I-κB, PKACα is released and NFκB is activated [70].

Protein kinase B (AKT)

AKT is a serine-threonine kinase that has 3 isoforms - AKT1, AKT2 and AKT3. All three isoforms are activated by phosphorylation at their highly conserved Thr308 and Ser473 by PDK1 and mTORC1, respectively [71].

AKT1 (PKBα) is more ubiquitous found in the brain, heart and lungs and is essential for cell growth and survival [72, 73]. It is found in the cytoplasm and is overexpressed in many cancers, playing a role in cell proliferation in gastric cancer [74].

AKT2 (PKBβ) is found primarily in the skeletal muscles and plays a role in insulin secretion [75, 76]. It plays a role in cell migration and recently has been implicated in metastasis of breast, ovarian and colon cancer [72, 77, 78].

AKT3 (PKBγ) is mostly found in the brain, kidney and embryonic heart and is localized on the nuclear membrane within the cell [79, 80]. It has been implicated to play a role in DNA repair in breast and prostate cancer [76].

The different isoforms of AKT have their docking sites on phosphatidylinositol 3-kinase (PI3K) that constitutes a major pro-survival pathway that is frequently activated in cancer. PI3K converts to phosphatidylinositol-4,5-bis phosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane. mTORC2 and PI3K dependent protein kinases (PDK)1 also dock on PI3K, the former being able to phosphorylate AKT on Thr^{308} and Ser^{473}, activating it.

XIAP

XIAP is an IAP found on the X-chromosome and contains 3 BIR domains, 1 of which binds to caspase 3 & 7 and the other to caspase 9. It also has the (ubiquitin associated) UBA domain and a RING finger domain through which XIAP is able to ubiquinate and degrade caspase 3 & 7 [81]. The cells' normal function of XIAP is associated with its release from the mitochondria to the cytosol but it also has a nuclear function where it inhibits apoptosis by binding to tumor necrosis factor α

(TNFα) receptor associated factor (TRAF) 1 & 2[82]. XIAP is known to inhibit the intrinsic Bcl2 pathway too [83]. It has the ability to induce NF-κB and thus inhibit cell death. XIAP has the ability to form a complex for another IAP – survivin leading to poor tumor outcomes by inhibiting caspases.

Overexpression of XIAP is associated with enhanced chemoresistance [84], while whole mouse XIAP knockout was non-toxic and restored chemosensititivity [85, 86]. Smac memetic peptides [87, 88], Embelin [89-91] and AEG35156 (antisense oligonucleotide) [92, 93] are some of the recent strategies used to inhibit XIAP in different cancer, none of which have been effectively translated into treatment therapies. (Figure 6)

Survivin

Survivin is a unique molecule found mostly in the proliferating cells of intestinal crypts and is otherwise barely detectable in normal or benign tissues but is overexpressed in many cancers including lung cancer [94], osteosarcoma [95], oral squamous cell carcinoma [96], breast cancer [97] and thyroid cancer [98].

Survivin is the smallest member of the IAP family and is encoded by the baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) gene and has three splice variants [99, 100]. Unique to IAPs, it contains one baculoviral IAP domain and requires interaction with XIAP to inhibit casapases.

There is a direct relationship between an increased presence of survivin (usually during the G2/M phase of the cell cycle) and cancer related death because it has the ability to inhibit apoptosis in the both the intrinsic and extrinsic pathways [101, 102]. This increased expression is also associated with poor prognosis, high risk of relapse and resistance to therapy [103-105]. [106]Survivin is also known to suppress radiation induced apoptosis [106-108].

Survivin forms a complex with XIAP which inhibits caspases and this complex is responsible for promoting survival and metastasis [109-111]. Since its presence is high in tumor cells versus normal, survivin appears to be a promising therapeutic target [112].

Drugs such as YM155 and FL118 are small molecule inhibitors that are capable of suppressing survivin promoter activity [113-115]. Gene therapy was a newly developed method to inhibit survivin which included using a dominant negative survivin where certain amino-acid mutant substitutions in survivin (Cys84 to Ala in the BIR domain) led to the development of a survivin molecule that acts as a competitive antagonist to the survivin found normally in the tumor cells. This therapy has led to decreased tumor growth through increased cell death in breast, thymic lymphoma and gastric cancer cells [116-118]. Development of survivin antisense oligonucleotides such as LY2181308 and SPC3042 has allowed for the transcriptional repression of survivin [119-121]. (Figure 6)

XIAP-Survivin complex

Survivin is usually localized in the mitochondria and upon cellular stress, is released from the mitochondria into the cytosol where it complexes with XIAP [109, 122]. Survivin is able to bind to all 3 BIR domains of XIAP and stabilize it. PKA has the ability to prevent this association by phosphorylating survivin on serine[20]. The complex prevents XIAP from getting ubiquinated and destroyed allowing XIAP to promote cell survival by inhibiting caspases. This complex formation also protects survivin, since XAF-1 (XIAP associated molecule) has the ability to polyubiquitinate survivin, targeting it for degradation by the proteosome. (Figure 7)

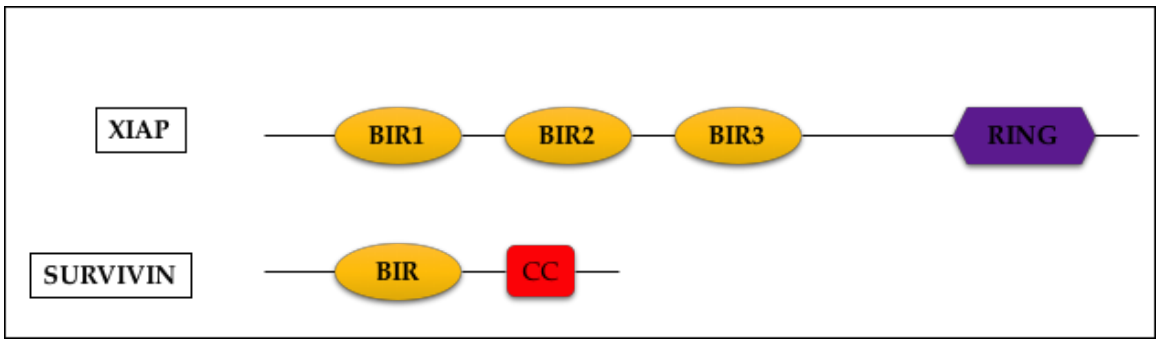


Figure 6: Domain structure of inhibitor of apoptosis (IAP) proteins – X-linked IAP (XIAP) and survivin.

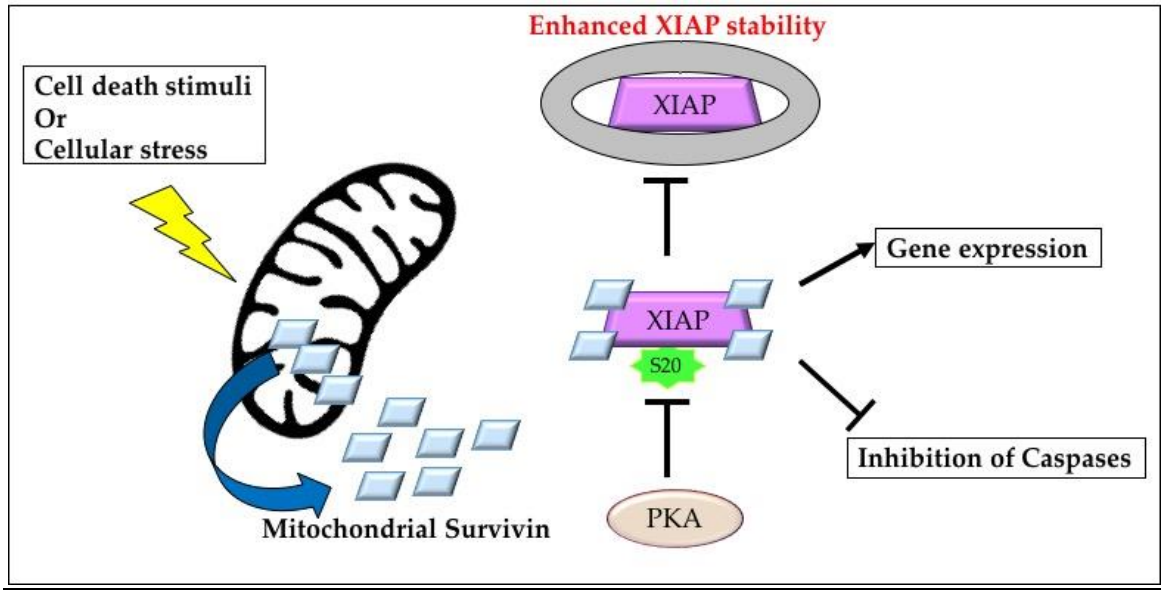


Figure 7: XIAP and survivin form a complex in the cytoplasm that is cytoprotective to the cancer cells. The complex prevents XIAP from getting ubiquinated therefore promoting cell survival by inhibiting caspases. The XIAP-survivin complex also functions to protect survivin from degradation.

Figure was adapted from [109]

Rationale for our study

cAMP plays an essential role in a of myriad cell functions like proliferation, differentiation and apoptosis. These functions were initially thought to be carried out solely by PKA. However, it became clear that various functions of cAMP were contradictory and PKA independent. With the discovery of EPAC, some of these contradictions were solved because EPAC can work antagonistically or agonistically with PKA [4].

Previous work in our lab on the TGFβ/PKA transduceome has demonstrated that the cAMP independent, Smad3 dependent activation of PKA led to a decrease in XIAP and survivin expression and an overall decrease in cell survival in CRC (apoptosis). However, there are no studies on the function of EPAC in CRC [63].

Oncomine data reveals that expression of EPAC1 and EPAC2 increases with progression from adenoma to carcinoma. This lead us to hypothesize that EPAC may antagonize PKA function in CRC and maybe responsible for the pro-tumor effects of cAMP in this disease.

Work done in the Brattain Laboratory with the EPAC specific activator (8CPT) showed an increase in the phosphorylation of AKT, and an increase in XIAP and survivin expression. (unpublished data) Thus, we confirmed that EPAC and PKA are acting antagonistically and the pro-survival effect observed on EPAC

activation is specifically through EPAC and not PKA [123-125]. This implicates EPAC as a potentially important therapeutic target in cancer.

CRC is the third most common and fatal cancer in the United States. The statistics are even worse for pancreatic cancer (PaCa) because of its late detection [59]. Therefore, there is a need to develop new therapies that provide benefits in the treatment of advanced stage cancer and we hypothesize that EPAC may represent such a target.

Inhibitors of EPAC are useful because EPAC has the ability to interact with the TGFβ pathway and its inhibition would repress XIAP and survivin as well as enhance the inhibitory effects of cAMP activated PKA on cell proliferation.

Despite the research done on EPACs over the last few years, contributions of the specific EPAC isoforms to cancer are only now being addressed. To understand these roles, EPAC specific inhibitors (ESI) 05, ESI09 and CE3F4 are some of the drugs that have recently been developed.

EPAC as an epigenetic regulator:

A recent study demonstrated that cAMP signaling, through an EPAC1-mediated inhibition of p38 MAPK, decreased the levels of histone acetyltransferase, p300 by ubiquinating it in the proteasome in lung cancer cells [126]. p300 HAT acetylates histones opening up the chromatin structure allowing for gene

transcription. This led us to question if EPAC represses p300 and, if so, is this accompanied by an increased expression of HDACs that deacetylate, acetylated histones. In both CRC and PaCa, an overexpression of histone deacetylases (HDACs) is associated with poor prognosis and death. We will therefore test the **hypothesis that EPAC is an epigenetic regulator affecting cell survival in cancer.**

EPAC activation caused an efflux of HDAC4 and HDAC5 from the nucleus confirming an epigenetic relationship between them [127, 128]. This nuclear efflux is EPAC specific and was not observed with PKA activation confirming a relationship between EPAC and HDACs [129]. Previous work in our laboratoy has shown that the pan-HDAC inhibitor, Belinostat, causes an induction of tumor suppressor gene - TGFβRII through survivin repression [60]. We believe that EPAC inhibition would lead to an eventual abrogation of HDAC activity as seen with the pan-HDACi's - causing a decrease in cancer cell survival.

Histone Deacetylases (HDACs)

Histone modifications determine chromatin structure. Acetylation of the ε-amino of lysines confers negative charge opening up the DNA allowing for transcription. In contrast, deacetylation results in closed chromatin configuration and transcriptional repression [130, 131]. HDACs are classified into 4 main

classes – Classes I, II, III and IV. Of these, the class III HDACs are called sirtuins and require Nad+ for their activity while the other classes require a Zn^{2+} cation for their function. There are a total of 11 HDACs plus sirtuins that comprise the HDAC family [132, 133]. (Figure. 8)

Of particular interest to our study are Class II HDACs that are further divided into Class IIa and IIb. Class IIa includes HDAC4, 5, 7 and 9 while class IIb includes HDAC6 and 10. Class IIa HDACs are unique in that they have a conserved binding site for transcription factor myocyte enhancing factor (MEF) 2 as well as 14-3-3 binding sites where there are multiple serines that get phosphorylated and allow the HDACs to shuttle between the nucleus and cytoplasm. 14-3-3 has a deacetylase domain for its catalytic activity towards the C-terminal [134].

Histone Deacetylase4 (HDAC4)

HDAC4 is a Class II HDAC that is found exclusively in the brain and growing areas of the skeleton in normal cells [135, 136]. HDAC4 is mutated in breast cancer and melanoma and its inhibition reduces cancer cell survival [137-141]. HDAC4 is overexpressed in several cancers [142, 143]. HDAC4 is also able to bind to HIF1-α thus protecting it from degradation and favoring tumor progression under hypoxic conditions [144, 145]. Wilson and colleagues

confirmed that HDAC4 promotes colon cancer cell growth through the repression of p21 [146]. (Figure 9)

Histone Deacetylase5 (HDAC5)

HDAC5 is found exclusively in the muscles, heart and brain in normal tissue [135, 147, 148]. The role of HDAC5 cancer hasn't been studied in-depth yet but there is increased expression in medullablastomas [149]. HDAC5 inherently plays an important role in normal angiogenesis but this role in cancer progression has not been studied. In pancreatic cancer, oxysterol binding protein-related protein 5 (associated with poor prognosis) is indirectly stimulated by HDAC5 [150]. (Figure 9)

Relationship between EPACs and HDACs 4 and 5

PKA is known to cause the influx of HDAC4 into the nucleus while EPACs cause an efflux of HDAC4 into the cytoplasm [129]. EPAC activation caused HDAC4 to efflux from the nucleus into the cytoplasm (in a Ras-dependent signaling pathway) while HDAC5 did not. However, in the presence of HDAC4, HDAC5 was more responsive to EPAC stimulation [127, 128, 151, 152]. This confirms a closer relationship between EPAC and HDAC4 than HDAC5.

The EPAC1/Rap1/CamKI/HDAC5 complex is involved in the placental cell fusion, further supporting a relationship between EPAC1 and HDAC5 [153, 154].

Pereira and colleagues, in 2015, showed that EPAC1 was more localized in the nuclear envelope of cardiomyocytes while EPAC2 was present in abundance in the Z-lines of these cells. On knocking out EPAC1, the nuclear export of HDAC5 was abrogated [155].

These studies point to a relationship between EPAC activation and HDAC 4 & 5 regulation but it has yet to be determined whether this relationship exists in cancer cells and whether this interaction plays a role in cancer progression.

Class	HDAC	Localization	Role in cancer	Type of cancer
I	HDAC1	Nucleus	Over/under expression	Colon, pancreatic, prostate etc.
	HDAC2	Nucleus	Overexpresssion/mutation	Prostate, colon, gastric
	HDAC3	Nucleus	Overexpression	Endometrial
	HDAC8	Nucleus	Overexpression	Colon
IIA	HDAC4	Nucleus/Cytoplasm	Over & underexpression	Colon, pancreatic, prostate, breast
	HDAC5	Nucleus/Cytoplasm	Overexpression/mutation	Colon, pancreatic
	HDAC7	Nucleus/Cytoplasm	Overexpression	Colon, pancreatic
	HDAC9	Nucleus/Cytoplasm	Over and under expression	Mixed
IIB	HDAC6	Mostly Cytoplasm	Overexpression	Breast, AML
	HDAC10	Mostly Cytoplasm	Over and underexpression	Hepatic, colon, multiple myeloma
IV	HDAC11	Nucleus/Cytoplasm	Overexpression	Neuroblastoma, renal cancer

Figure 8: Classification of HDACs and roles in different types of cancer.

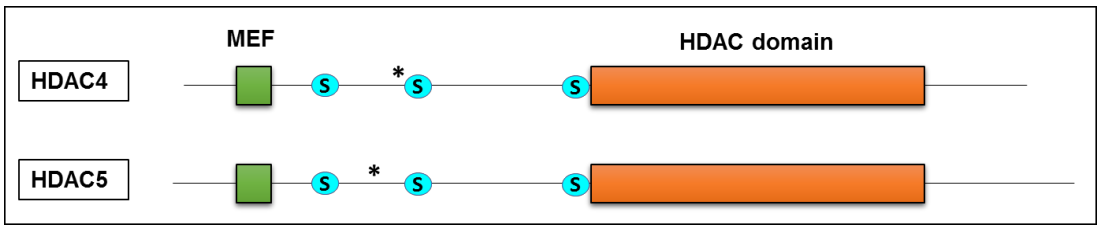


Figure 9: HDACs 4 and 5 belong to Class IIA and have 1084 and 1122 amino acids, respectively. They contain a long N-terminal adaptor domain – green rectangles are for the myocyte enhance factor (MEF) binding site; turquoise circles indicate where chaperone protein 14-3-3 binds and is labeled with S – serine phosphorylation; while the asterisks indicate nuclear localization signals. The orange rectangles indicate the conserved catalytic (deacetylase) domain.

Challenges in studying EPACs:

- Unlike PKA that has a direct assay to measure its activation there is no direct assay to measure EPACs. The only assay that exists measures Rap-GTP levels which precludes analysis of any functions of EPAC that are independent of Rap.
- There is a lack of specific antibodies to EPAC1 and EPAC2 and this makes quantification of protein expression difficult. We have a developed an IP protocol to help circumvent these difficulties. EPAC mRNA is readily quantifiable but this does not directly provide information of protein expression or post-translational modifications that may affect activity.

The best available tool is an EPAC specific activator - 8CPT- that was only developed in 2003, after the discovery of EPACs in 1998. This activator is specific to EPAC because it contains a methyl group near the catalytic binding site that makes it too large for interaction with PKA's cAMP binding site. Previous work done in our laboratory has shown that EPAC activation with 8CPT causes an increase in cancer cell survival through an increase in XIAP, survivin and phosphorylation of AKT. Other EPAC activator analogues have recently been reported, these are the sulfonylureas – Tolbutamide, Glibendamide and Glicazide. These activators are specific for EPAC2 and are able to bind to the CNB domains [41, 147, 156-159]. However, they are not commercially available.

Tsalkova and colleagues in 2012 developed an EPAC specific inhibitor (ESI). ESI05 selectively inhibits EPAC2 by binding to its CNB domain [160]. ESI09 inhibits both EPAC1 & 2 and it does this by competing with cAMP for the CNB domain on EPAC as well as inhibiting the GEF activity in both isoforms [57, 58, 159].

A new inhibitor, CE3F4, has recently been developed that inhibits EPAC1 specifically. This racemic drug exerts its inhibition allosterically – by binding to cAMP bound EPAC1 which is in the open conformation [161]. The Courilleau laboratory also confirmed in 2013 that the R-enantiomer has 10-fold more selectivity towards EPAC1 than the racemic mixture [162].

For the purpose of my study, we will be examining the roles played by ESI09 (EPAC1 and 2 inhibitor), ESI05 (EPAC2 inhibitor), and CE3F4 (EPAC1 inhibitor) in decreasing XIAP and survivin and overall cell survival in both colon and pancreatic cancers.

Materials and Methods

Cell culture and Reagents:

CRC cell lines were derived from patient tumors and grown in serum free (SF) medium that contains Supplemental McCoy's 5A (Sigma Aldrich), 5ng/ml epidermal growth factor (EGF) (R&D Systems), 20μg/ml insulin (Sigma) and 4μg/ml transferrin (Sigma).

The FET cell line is non-tumorigenic and retains low levels of TGFβ signaling. The CBS cell line retains metastatic capability. The CBSRII colon cancer cell line was made in our laboratory using a stable transfection of the overexpression vector of TGFβRII gene with neomycin selection vector into parental CBS cells. These same parental CBS cells without the TGFβRII gene inserted were named CBSNeo. Due to epigenetic silencing of TGFβRII in the parental CBS cell lines, the CBSNeo cell line is resistant to growth inhibition by TGFβ [163, 164].

The pancreatic cancer cell lines Miapaca, Capan, CFPAC and T3M4 were all cultured in 10F medium which contained Supplemental McCoy's 5A supplemented with 10% FBS. Miapaca and T3M4 retain wild-type Smad4, Capan has mutant Smad4 and CFPAC is Smad4 null.

All cell lines were grown and incubated at 37°C in a humidified atmosphere of 6% CO2.

8CPT was obtained from Sigma Aldrich. ESI09 and ESI-05 were purchased from Biolog. LMK235 was purchased from Selleck. The racemic mixture of CE3F4 was first obtained from Dr. Courilleau's lab in France and since then the racemic mixture, and the R & S enantiomers individually were synthesized by the Amar Natarajan Lab at the University of Nebraska Medical Center, Omaha, NE.

Western Blotting and Immunoprecipitation:

Colon cancer as well pancreatic cancer cells were plated and treated on day 3 and day 2, respectively. Cells were washed 3 times with cold phosphate buffer saline (PBS) that contains 0.1% EDTA. Washed cells were scraped and lysed with TNESV buffer [50mmol/L Tris (Ph 7.5), 150mmol/L NaCl, 1% NP40, 50mmol/L NaF, 1mmol/L Na_3VO_4, 25μg/ml β-glycerophosphate, 1mmol/L phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche,Indianapolis, IN)]. Cells were lysed using a syringe, after 30min incubation with the lysis buffer and centrifuged at 14000g's for 20min at 4°C. The supernantant was extracted and protein in it was quantified using the bicinchoninic acid(BCA; Pierce). The protein was diluted in SDS sample buffer (50mM Tris, ph 6.8, 1% SDS, 10% glycerol, 0.03% bromophenol blue and 1% β-mercaptoethanol) and separated by SDS-PAGE (7.5-15% gels). The gels were then transferred by electroblotting (100V for 90min or overnight at 10V at 4°C) onto a nitrocellulose membrane (GE Amersham). Post transfer, the membrane

was blocked with 5% non-fat dry milk in TBS-T (150mmol/L NaCl, 10mmol/L Tris-HCl pH7.4, 0.05% Tween 20) for 1hr at room temperature or overnight at 4°C. The membrane was cut at requisite molecular weights and primary antibody was added in either 5% non-fat dry milk or 5% bovine serum albumin (BSA) in TBS-T for 2hr at room temperature or overnight at 4°C according to the antibody data sheets. The membrane was washed three times for 10min in TBS-T and the appropriate secondary was added in 5% non-fat dry milk for 1hr at room temperature. The membrane was washed three more times with TBS-T for 10min at room temperature and the proteins were detected by the enhanced chemiluminescence system (ECL; GE Amersham). Immunoprecipitation was performed with 500μg of protein samples using agarose beads (Santa Cruz) according to manufacturer's protocol and previously established method. (Howell 2011) Survivin (#2808), XIAP (#14334), and HDAC4 (#5392) were purchased from Cell Signaling. EPAC1 (sc-28366) was purchased from Santa Cruz. GAPDH (G8795) was acquired from Sigma.

MTT and DNA Fragmentation:

The colon cancer cell lines, FET, CBSNeo and CBSRII were seeded at 5,000 cells per well and pancreatic cancer cell lines - Miapaca (4000 cells/well), Capan (5000 cells/well), CFPAC (4000 cells/well) and T3M4 (4000 cells/well) in 96 well plates for MTT and DNA Fragmentation. Three days following plating, colon cancer cells were treated for the specified times and MTT/DNA Fragmentation assays were performed on day 5. Pancreatic cancer cells were treated 2 days after plating and assays began on day 4. 50µl of the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution from Sigma was added to each well to the existing media and incubated at 37°C for 2hr. The solution from each cell was aspirated – which stopped the reaction and 100µl of di-methy sulfoxide (DMSO) was added to each well and followed by shaking for 15mins at room temperature, covered in foil. Absorbance was read at 570nm using a 96-well plate reader.

The second 96-well plate was used to assess apoptosis. DNA fragmentation was measured using the Cell Death Detection ELISA Plus kit from Roche according to the manufacturer's instructions. DNA Fragmentation results were normalized to the MTT findings.

Transient transfection:

On-TARGETplus SMARTpool HDAC4 siRNA was purchased from Dharmacon (Thermo Fisher Scientific Inc., USA) and knockdown was performed according to the manufacturer's protocol.

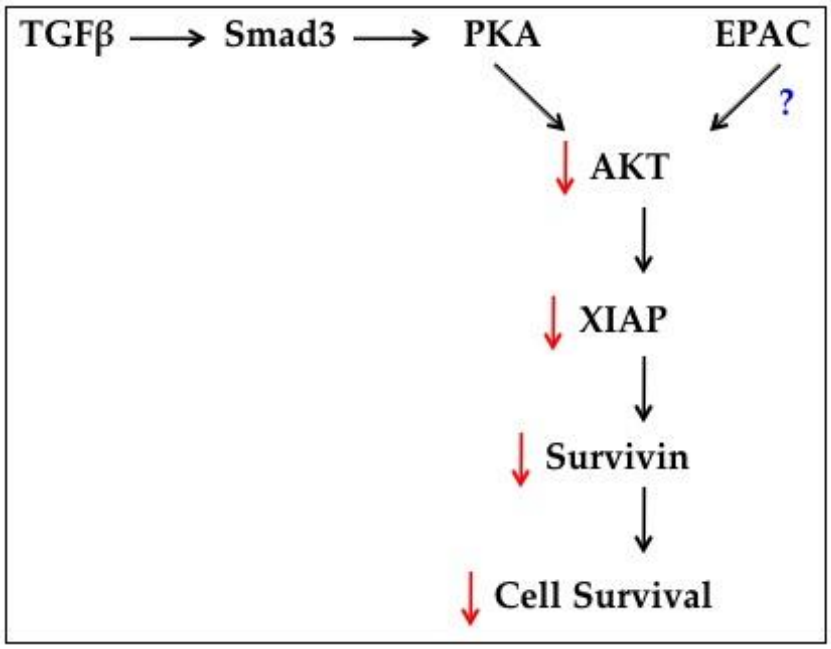
RNA isolation and quantitative real time PCR:

RNA was collected from treated cells using the High Pure RNA Isolation kit (Roche Applied Science) according to the manufacturer's protocol. The two-step quantitative PCR using TaqMan reagent was performed according to the manufacturer's instructions (Applied Biosystems). The mRNA expression was normalized to GAPDH. All probes were purchased from Applied Biosystems.

Results:

1. **EPAC activation by 8CPT increases cancer cell survival.**

   To determine the role of EPAC in CRC we first used 8CPT – EPAC specific activator – and examined the effect on cell survival. We hypothesized that EPAC would mediate pro-tumorigenic effects of cAMP in opposition to the effects of PKA as depicted in the schematic below.



We treated the FET CRC cell line with 8CPT and observed an increased of XIAP and survivin proteins by Western blot. (Figure 1.1) FET is a non-tumorigenic CRC cell line with low TGFβ signaling. On treatment with 8CPT, there was no change in cell proliferation but DNA fragmentation decreased confirming that EPAC activation was behaving antagonistically to PKA.

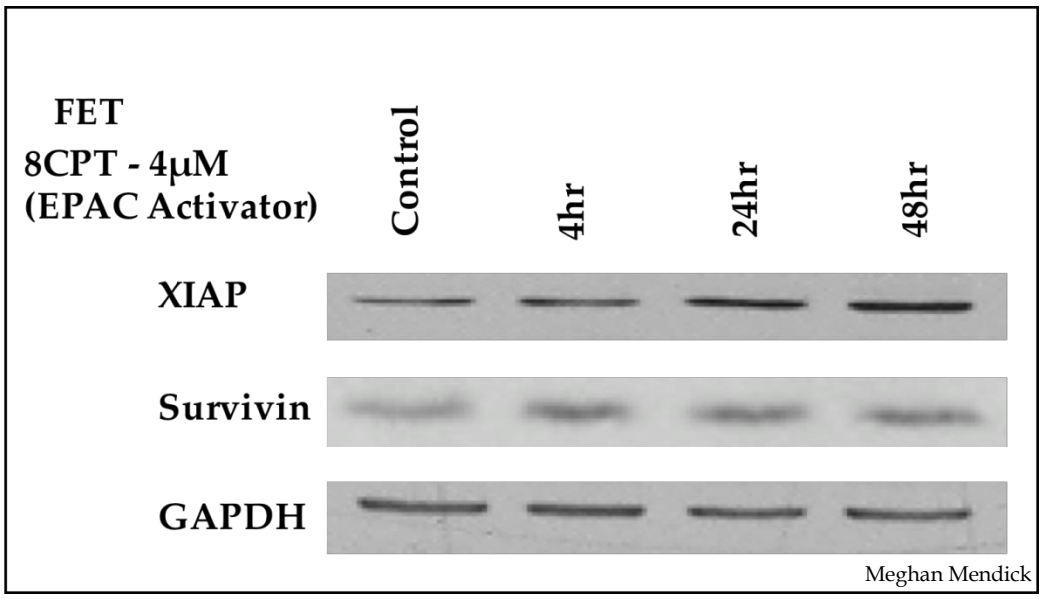
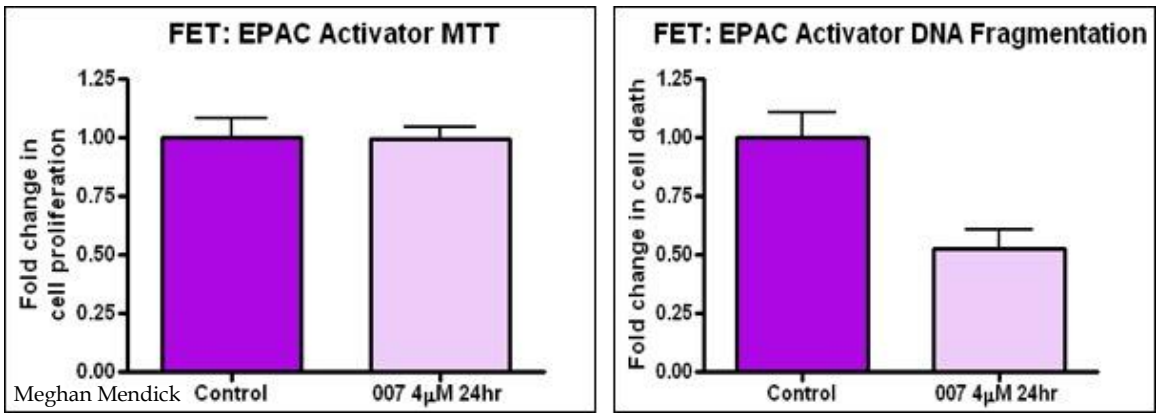


Figure1.1: Treatment of CRC cell line FET with EPAC activator increases cell survival associated proteins in a time-dependent manner. Data shown are representative of three independent experiments.



Meghan Mendick

Figure 1.2: EPAC activation has no effect on cell proliferation but decreases DNA fragmentation therefore increasing cell survival in CRC cell line – FET. Data shown are representative of three independent experiments.

2. Inhibition of EPAC1 and EPAC2 by ESI09 decreases cell survival.

The EPAC activator activates both EPAC1 and 2 isoforms. In order to begin to dissect out the roles of the specific EPACs, we utilized the newly developed EPAC specific inhibitors.

As EPAC activation results in an increased expression of survivin and XIAP, we hypothesized that EPAC inhibition would inhibit cell suvival and therefore represent a potential therapy for cancer.

We treated the CRC cell lines - FET (weak TGFβ signaling; non-metastatic), CBSNeo (no TGFβ signaling; highly metastatic) and CBSRII (functional TGFβ signaling, poorly metastatic) as well as PaCa cell lines – Miapaca (Smad4 wildtype), Capan (Smad4 mutant), CFPAC (Smad4 null) and T3M4 (Smad4 wildtype) with ESI09 – selective inhibitor of EPAC1 and 2 which resulted in a decrease in cell proliferation as observed in the MTT assay as well as an increase in cell death as observed in the DNA Fragmentation Assay. Western Blot analysis of IAP proteins XIAP and Survivin showed a decrease as well.

Q-RTPCR results of XIAP and survivin on treatment with ESI09 showed a decrease too.

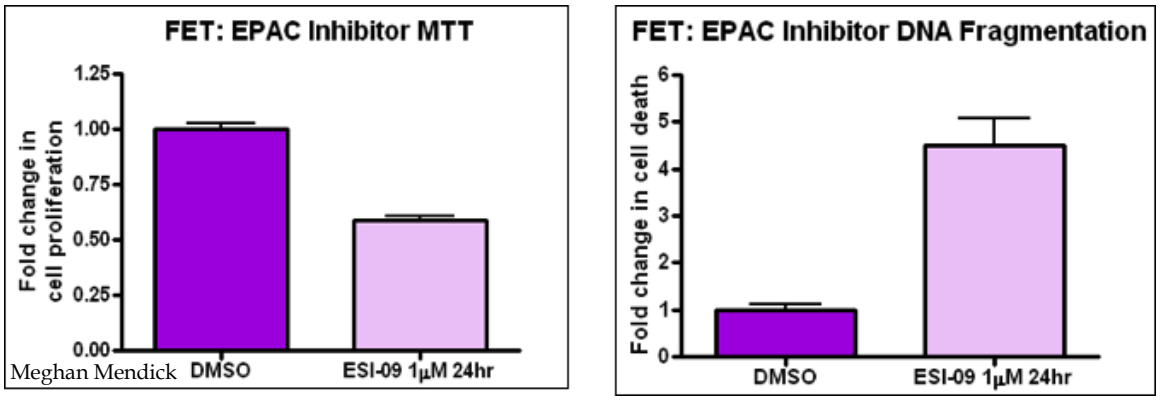


Figure 2.1: ESI09 (1um) decreased cell proliferation and increased DNA fragmentation in the FET CRC cell line. Data shown are representative of three independent experiments.

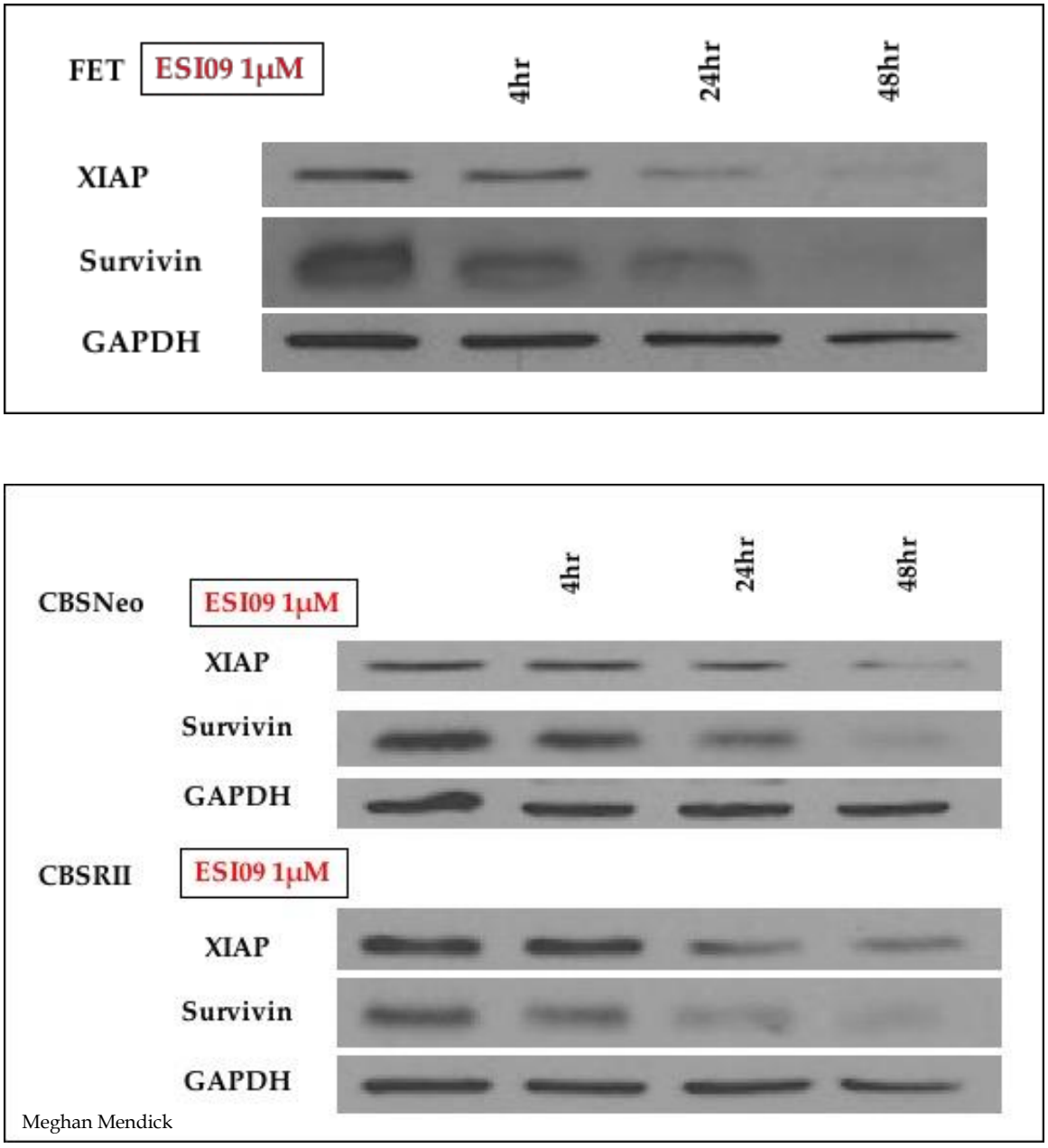


Figure 2.2: Treatment of the 3 CRC cell lines with 1um of ESI09 caused a time-dependent decrease in IAP proteins confirming that EPAC inhibition causes a decrease in cancer cell survival. Data shown are representative of three independent experiments.

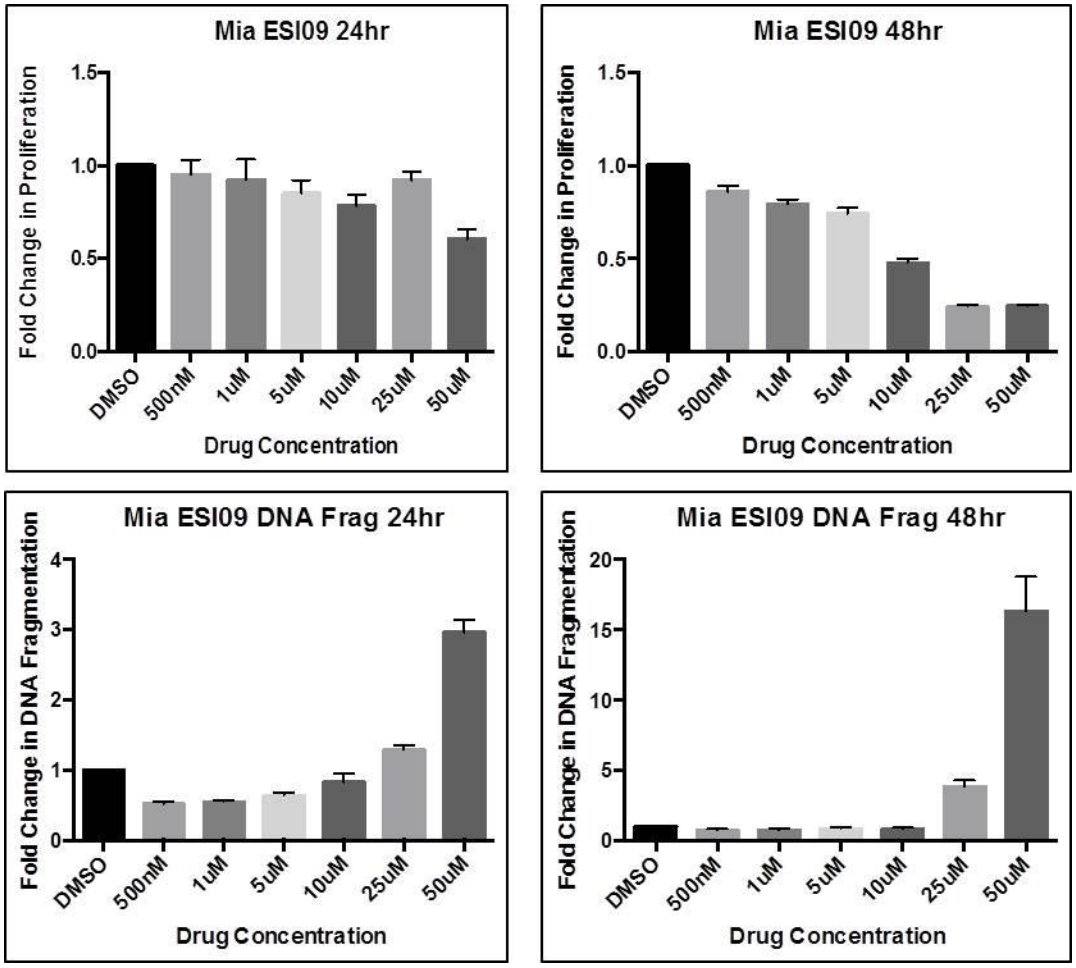


Figure 2.3a: Treatment of Miapaca cell line with ESI09 decreased cell proliferation and increased DNA fragmentation. Data shown are representative of three independent experiments.

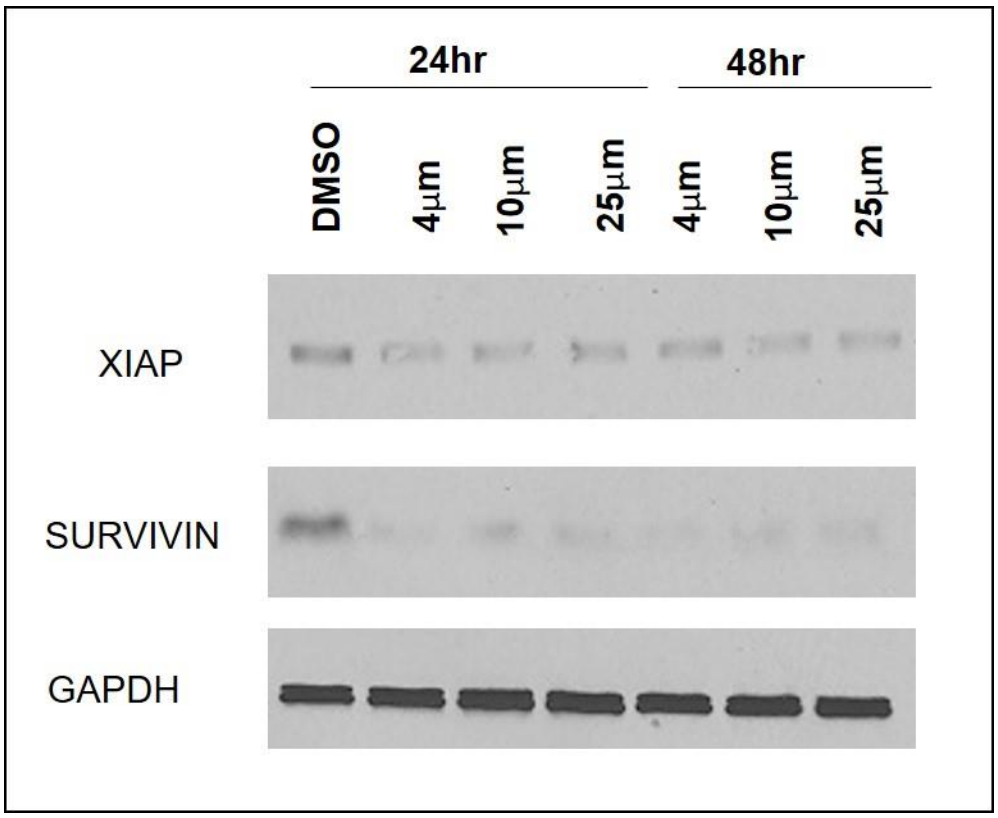


Figure 2.3b: Western blot analysis of the treatment of Miapaca with ESI09 demonstrated a decrease in IAP proteins thus decreasing cell survival. Data shown are representative of three independent experiments.

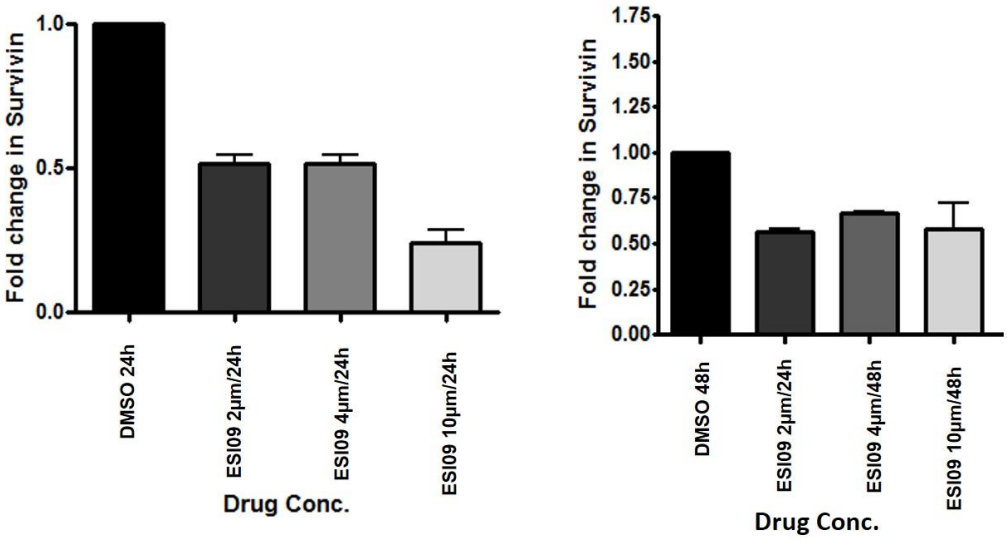


Figure 2.3c: Treatment of Miapaca with ESI09 decreased IAP molecule survivin at the mRNA level both at 24 and 48hrs. Data shown are representative of three independent experiments.

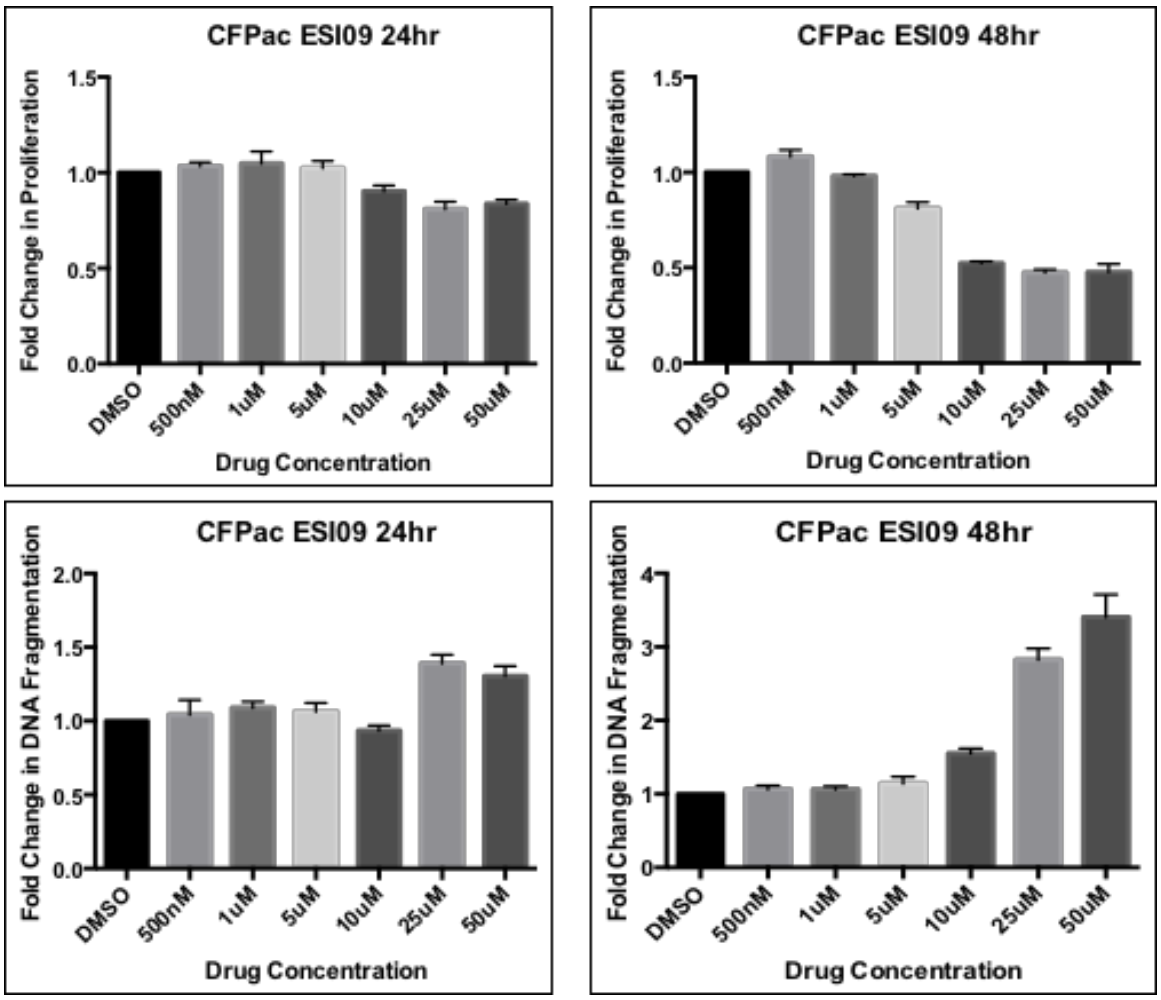


Figure 3.a: Treatment of CFPAC cell line with ESI09 decreased cell proliferation and increased DNA fragmentation. Data shown are representative of three independent experiments.

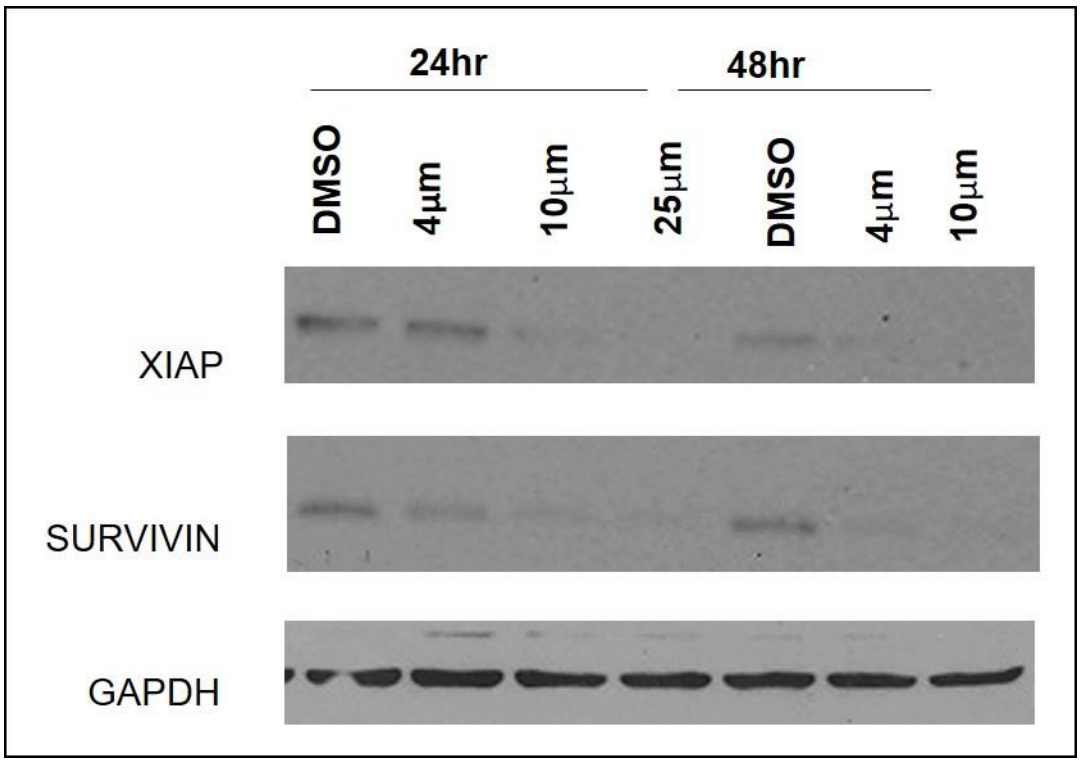


Figure 3.b: Western blot analysis of the treatment of CFPAC with ESI09 demonstrated a decrease in IAP proteins thus decreasing cell survival. Data shown are representative of three independent experiments.

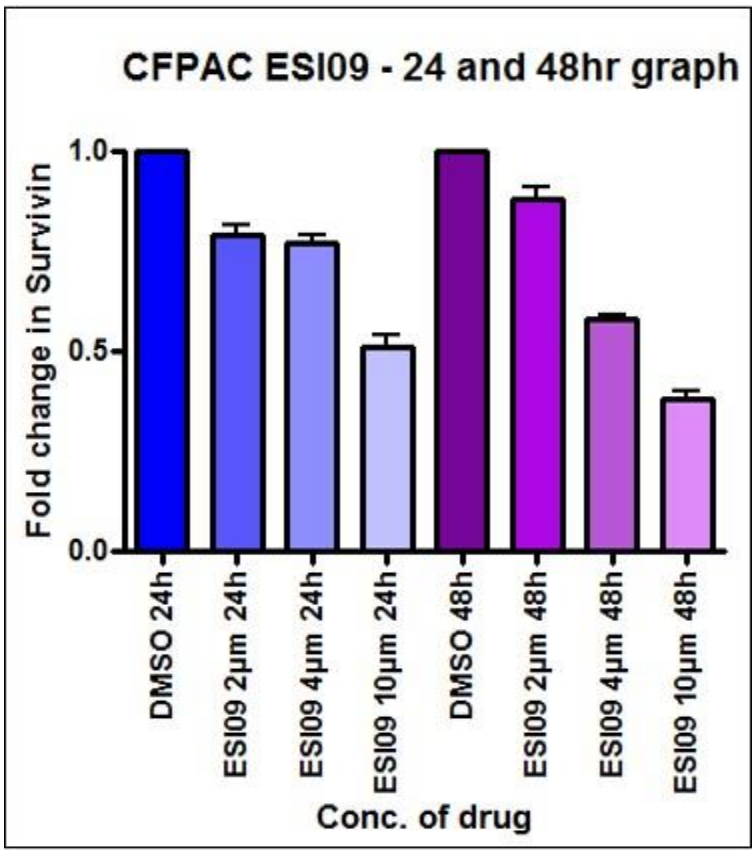


Figure 3.c: Treatment of CFPAC with ESI09 decreased IAP protein Survivin at the mRNA level at 24hrs. Data shown are representative of three independent experiments.

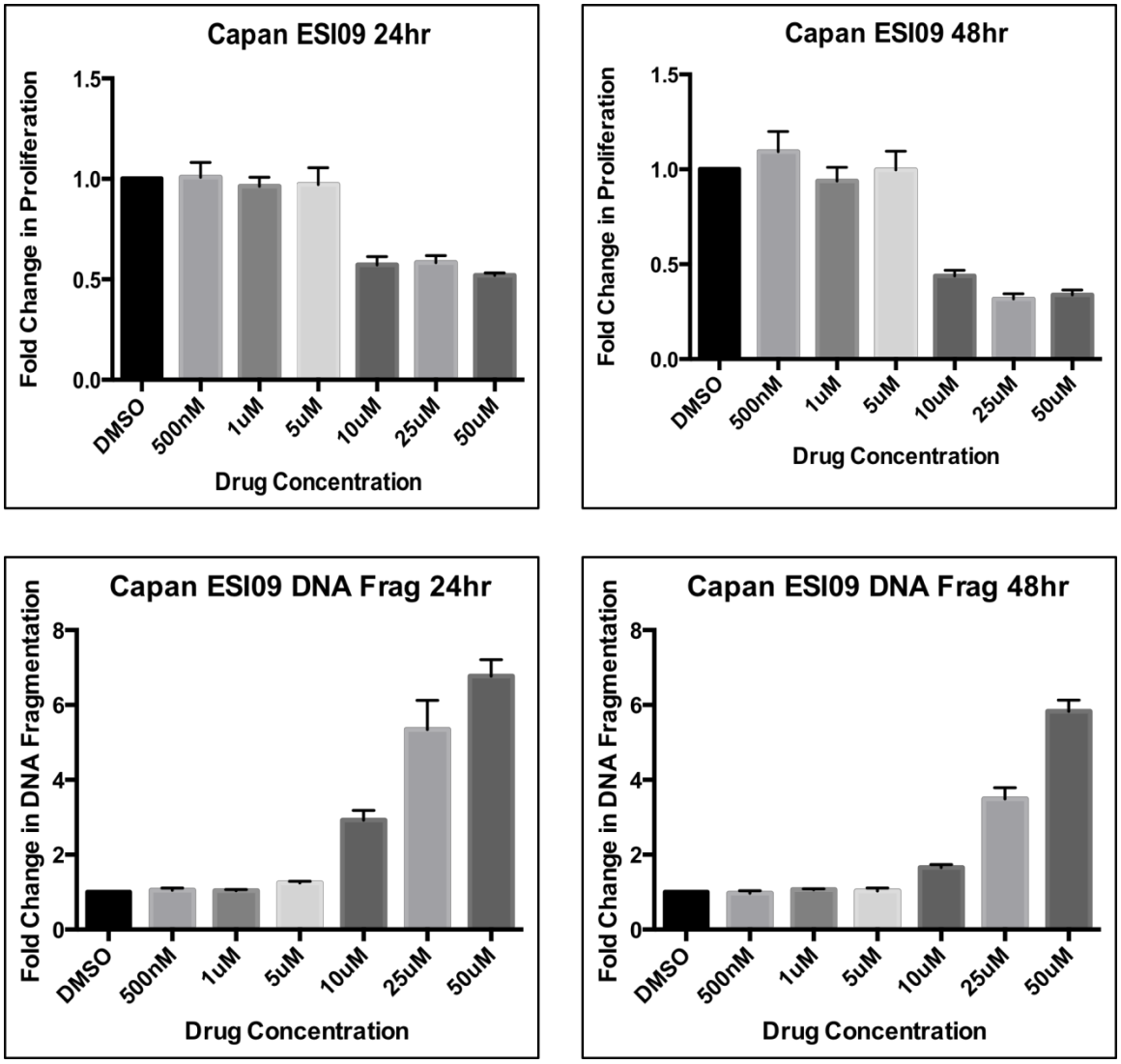


Figure 4.a: Treatment of Capan cell line with ESI09 decreased cell proliferation and increased DNA fragmentation. Data shown are representative of three independent experiments.

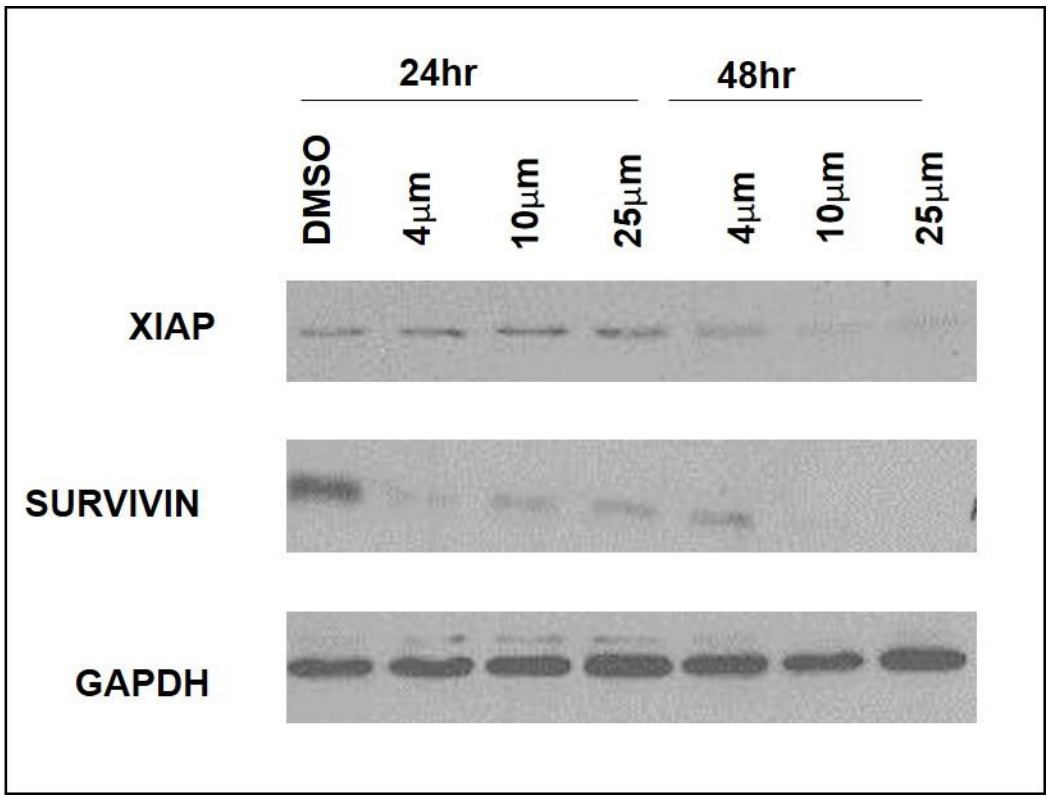


Figure 4.b: Western blot analysis of the treatment of CAPAN with ESI09 demonstrated a decrease in IAP proteins thus decreasing cell survival. Data shown are representative of three independent experiments.

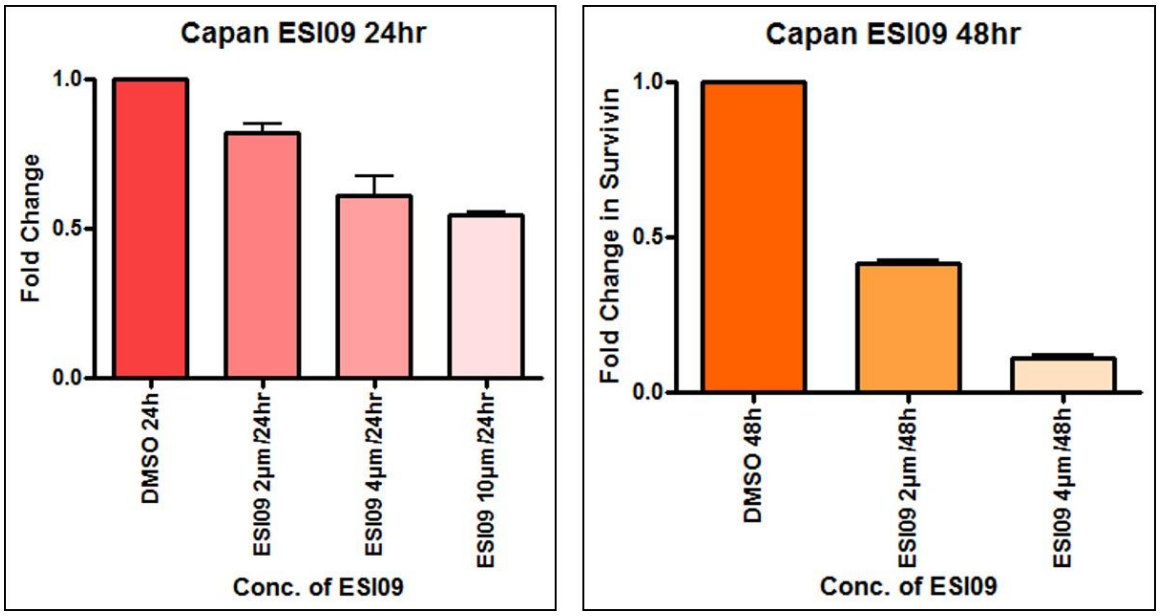


Figure 4.c: Treatment of Capan with ESI09 decreased IAP molecule survivin at the mRNA level both at 24 and 48hrs. Data shown are representative of three independent experiments.

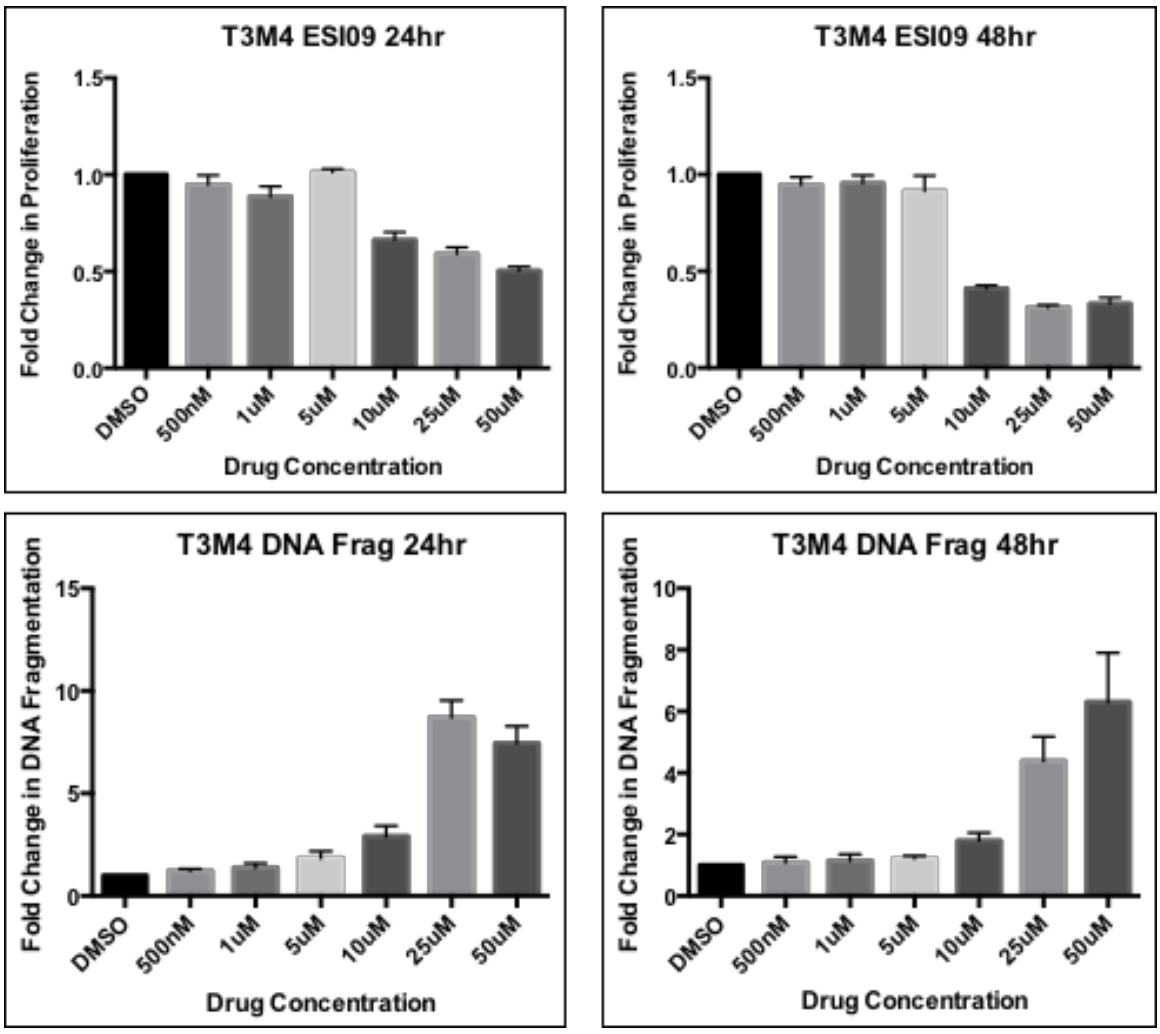


Figure 5.a: Treatment of T3M4 cell line with ESI09 decreased cell proliferation and increased DNA fragmentation. Data shown are representative of three independent experiments.

.

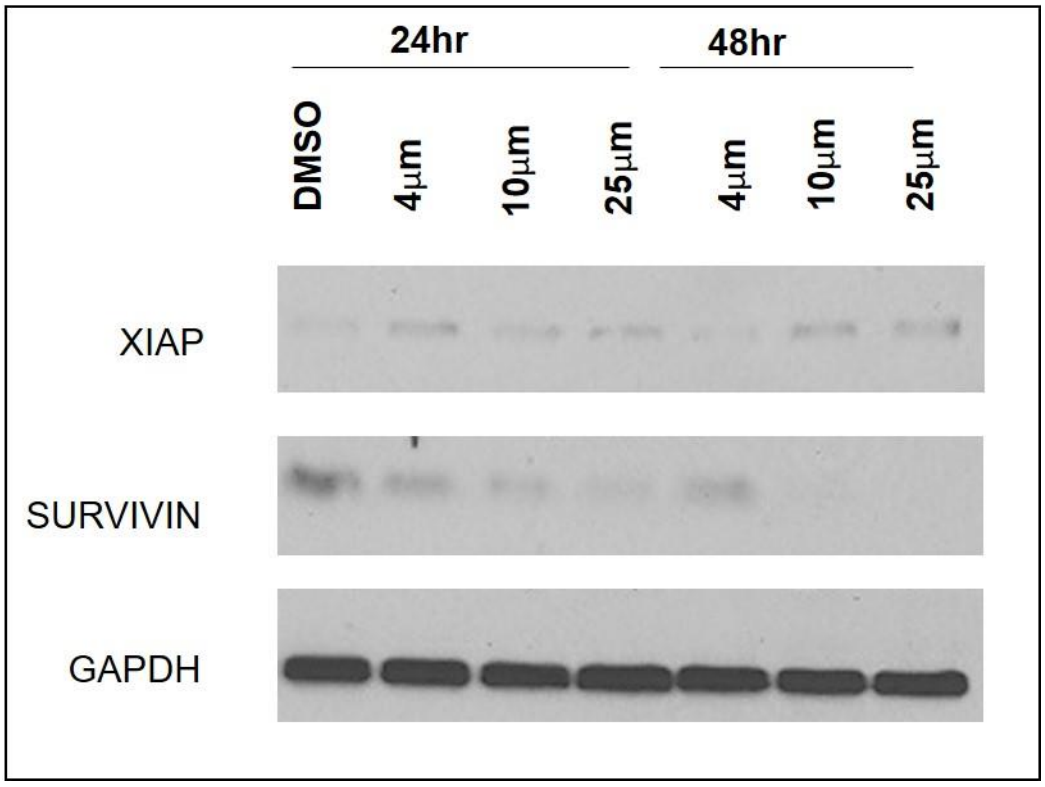


Figure 5.b: Western blot analysis of the treatment of T3M4 with ESI09 demonstrated a decrease in IAP proteins thus decreasing cell survival. Data shown are representative of three independent experiments.

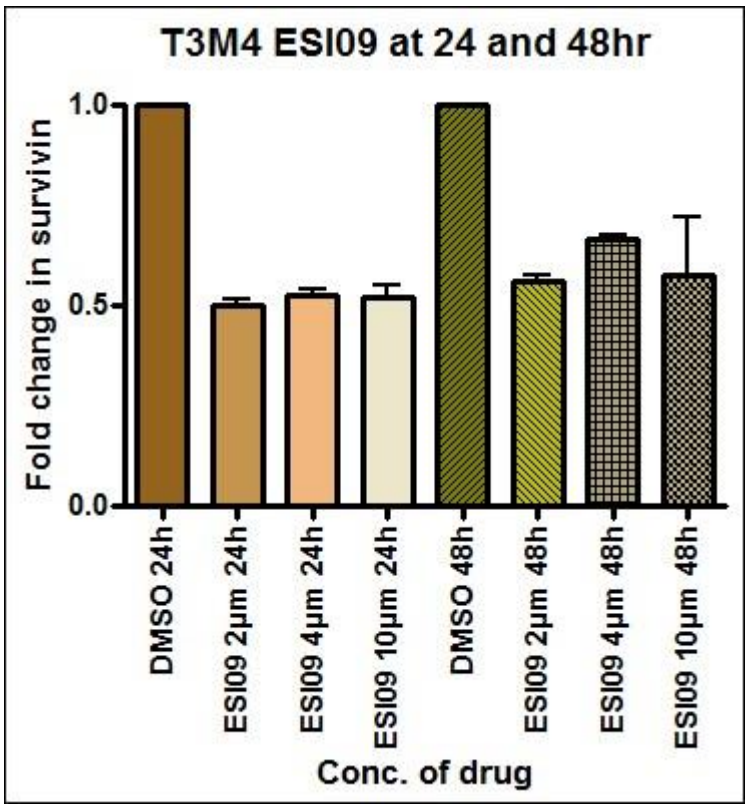


Figure 5c: Treatment of T3M4 with ESI09 decreased IAP molecule-survivin at the mRNA level both at 24 and 48hrs. Data shown are representative of three independent experiments.

3. EPAC2 selective inhibition cannot inhibit XIAP and survivin.

ESI09 inhibits both EPAC isoforms. To begin to dissect which isoform is involved in cell survival, we utilized ESI05, which inhibits EPAC2 selectively. ESI05 binds the hinge region of EPAC2 and prevents cAMP binding to the CNB domain. We treated the CRC cell lines - FET (weak TGFβ signaling; non-metastatic), CBSNeo (no TGFβ signaling; highly metastatic) and CBSRII (functional TGFβ signaling, poorly metastatic) as well as PaCa cell lines – Miapaca (Smad4 wildtype), Capan (Smad4 mutant), CFPAC (Smad4 null) and T3M4 (Smad4 wildtype) with ESI05 and observed no change in XIAP and survivin in both the protein and mRNA levels. Changes in mRNA levels of ESI05 were compared to that of ESI09. No changes in cell proliferation and cell death were observed.

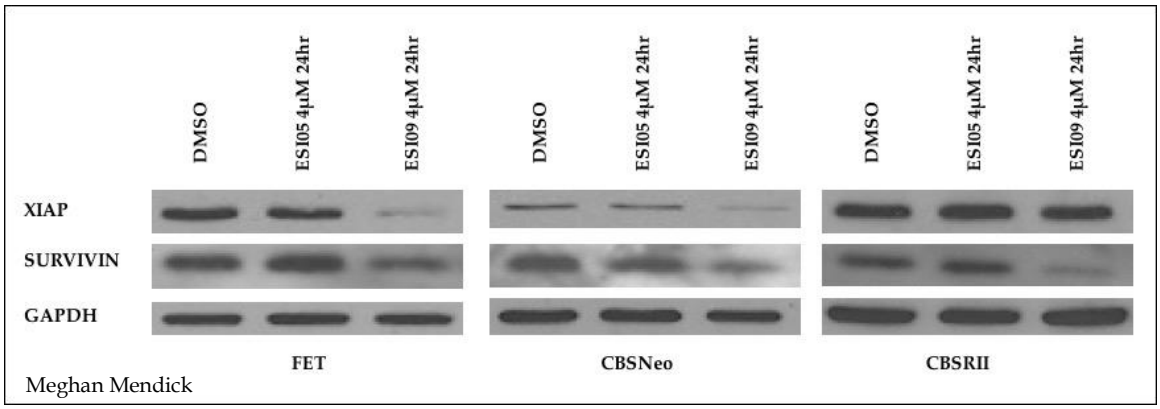


Figure 6: Expression of survivin and XIAP were unaffected in CRC cell lines – FET, CBS and CBSRII was treated with ESI05 (EPAC2 selective inhibitor). However, when treated with the same concentration of ESI09 (inhibits both isoforms) there was a decrease, indicating that EPAC1 could be involved in the cell survival effects. Data shown are representative of three independent experiments.

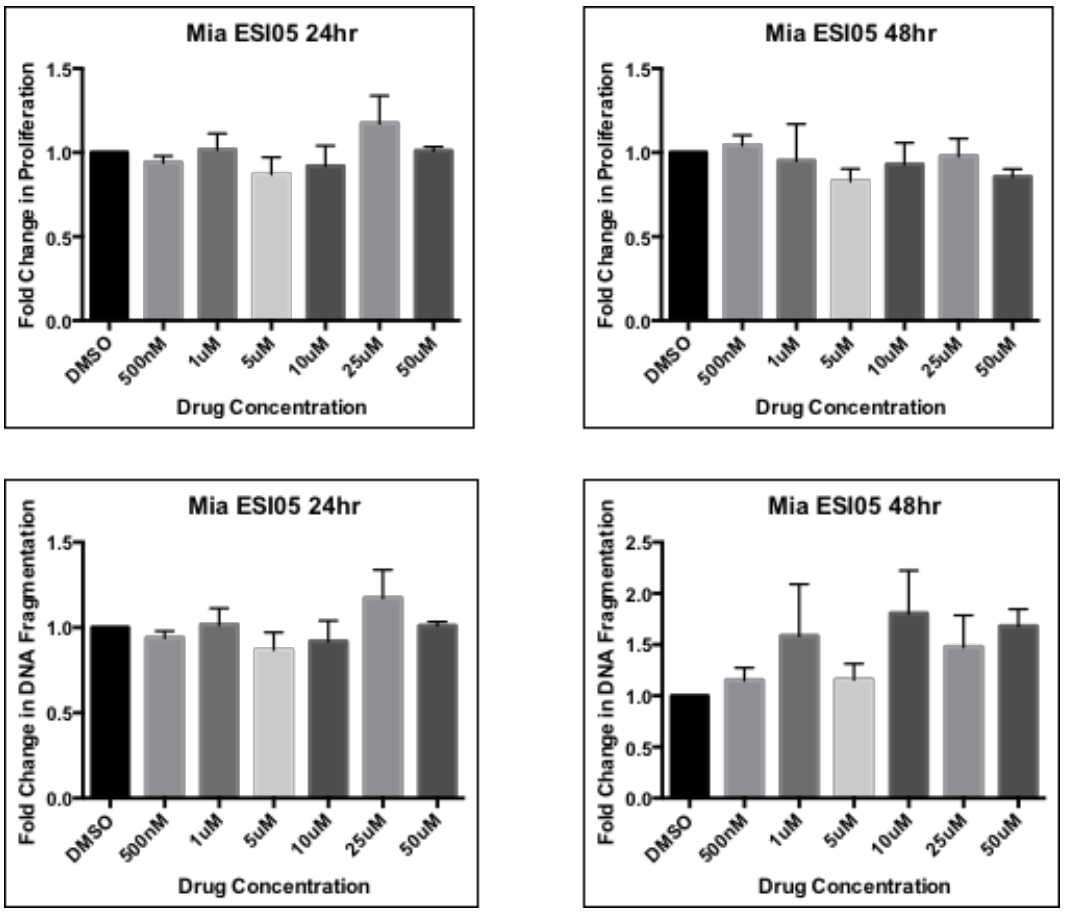


Figure 7.a: Treatment of Miapaca cell line with ESI05 did not significantly change cell proliferation or DNA fragmentation. Data shown are representative of three independent experiments.

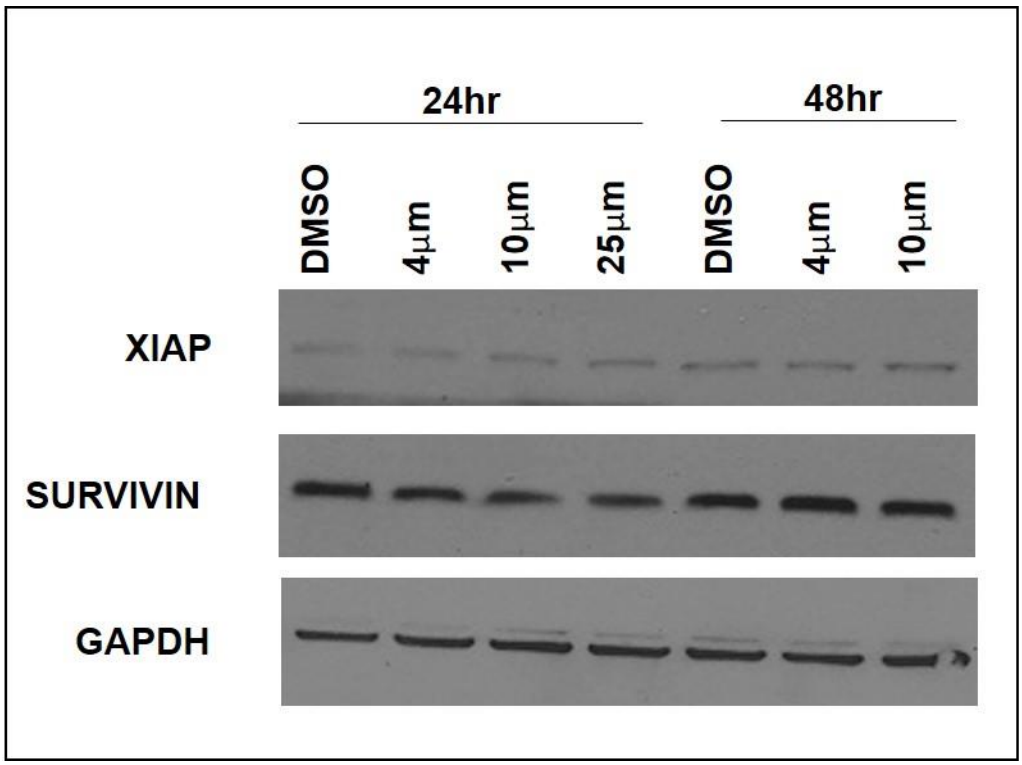


Figure 7.b: Western blot analysis of the treatment of Miapaca with ESI05 demonstrated no change in IAP proteins. Data shown are representative of three independent experiments.

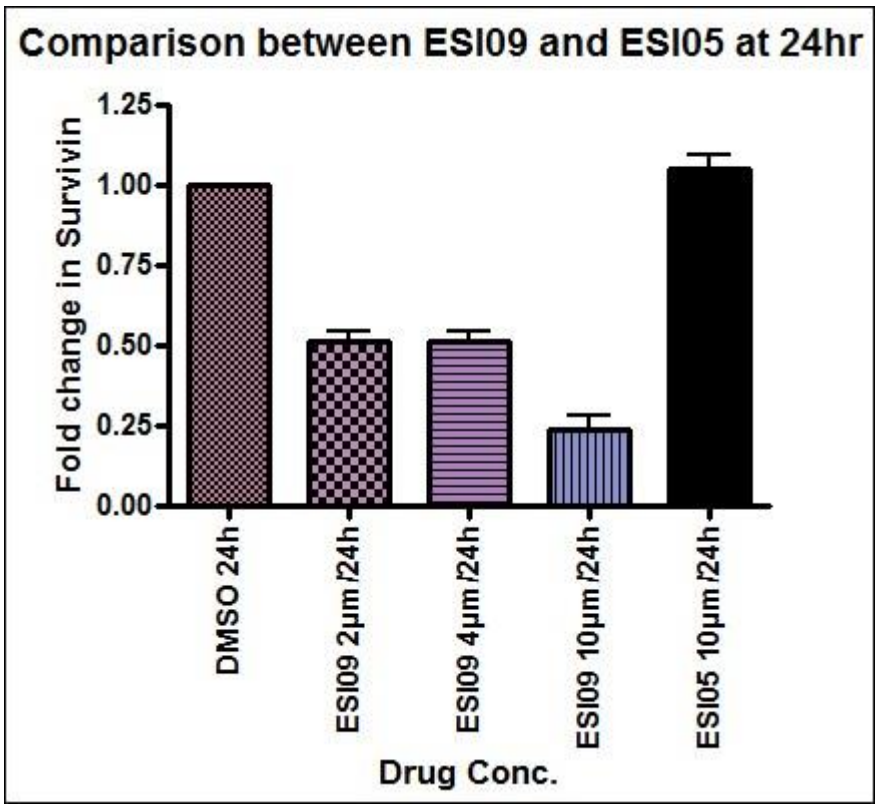


Figure 7c: The Miapaca cell line was treated with ESI05 at 10um at 24 hours and survivin levels were compared to the ESI09 treatment at the same concentration. Compared to ESI09 treated at 10um at 24 hours, there is no decrease in survivin in the ESI05 treated under similar conditions. Data shown are representative of three independent experiments.

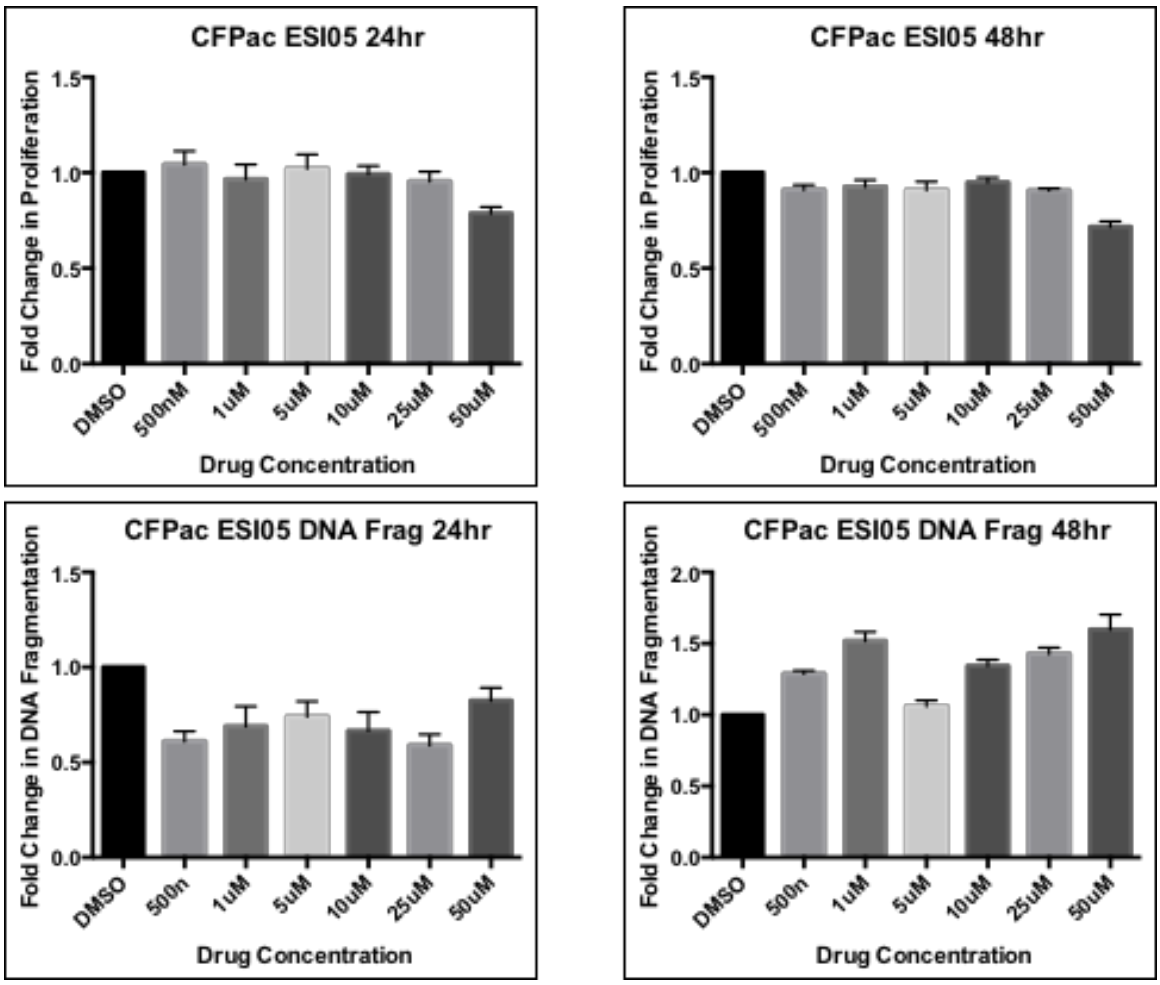


Figure 7.a: Treatment of CFPAC cell line with ESI05 exhibited no significant change in cell proliferation or DNA fragmentation. Data shown are representative of three independent experiments.

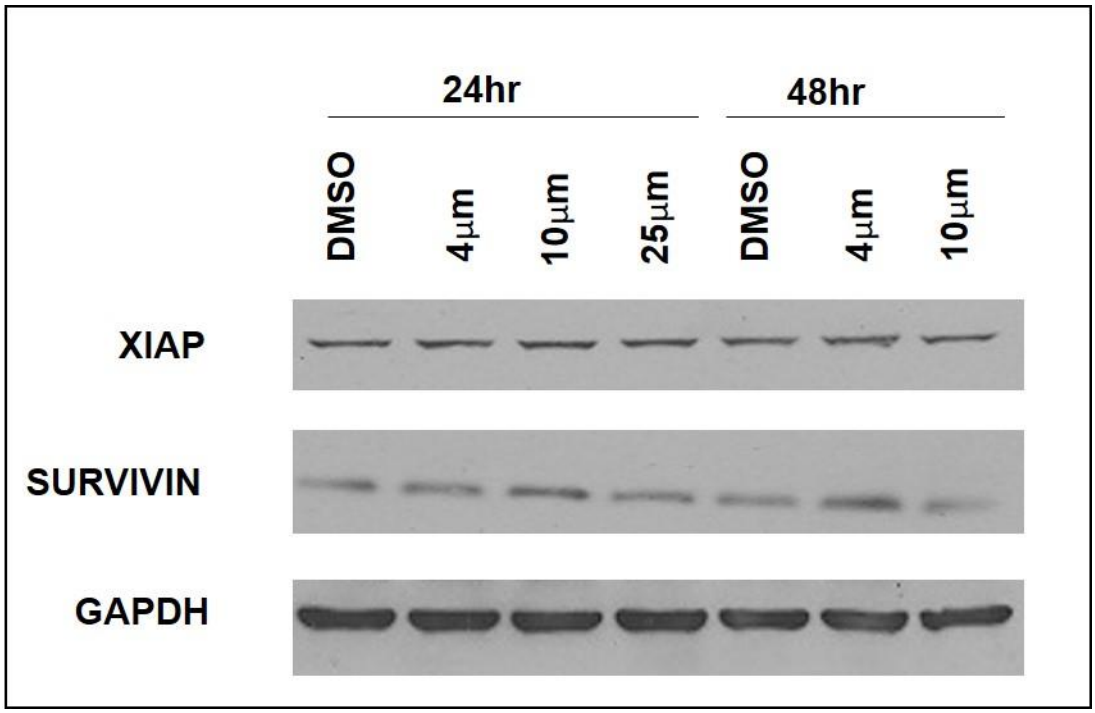


Figure 7.b: Western blot analysis of the treatment of CFPAC with ESI05 demonstrated no change in IAP proteins. Data shown are representative of three independent experiments.

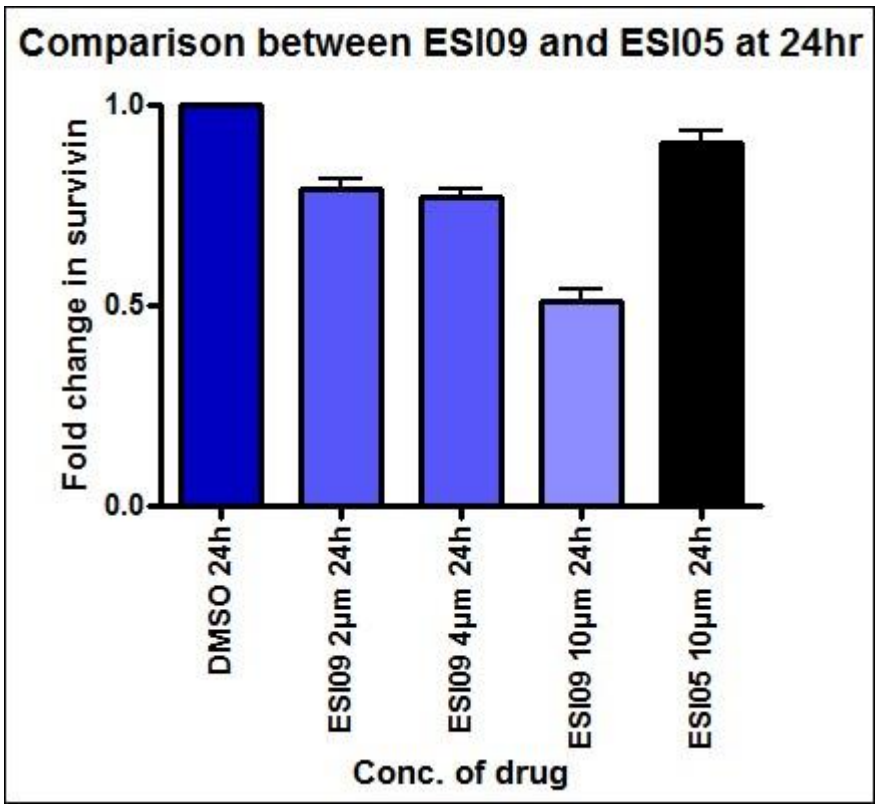


Figure 8c: The CFPAC cell line was treated with ESI05 at 10um at 24 hours and survivin levels were compared to the ESI09 treatment at the same concentration. Compared to ESI09 treated at 10um at 24 hours, there is no decrease in survivin in the ESI05 treated under similar conditions. Data shown are representative of three independent experiments.

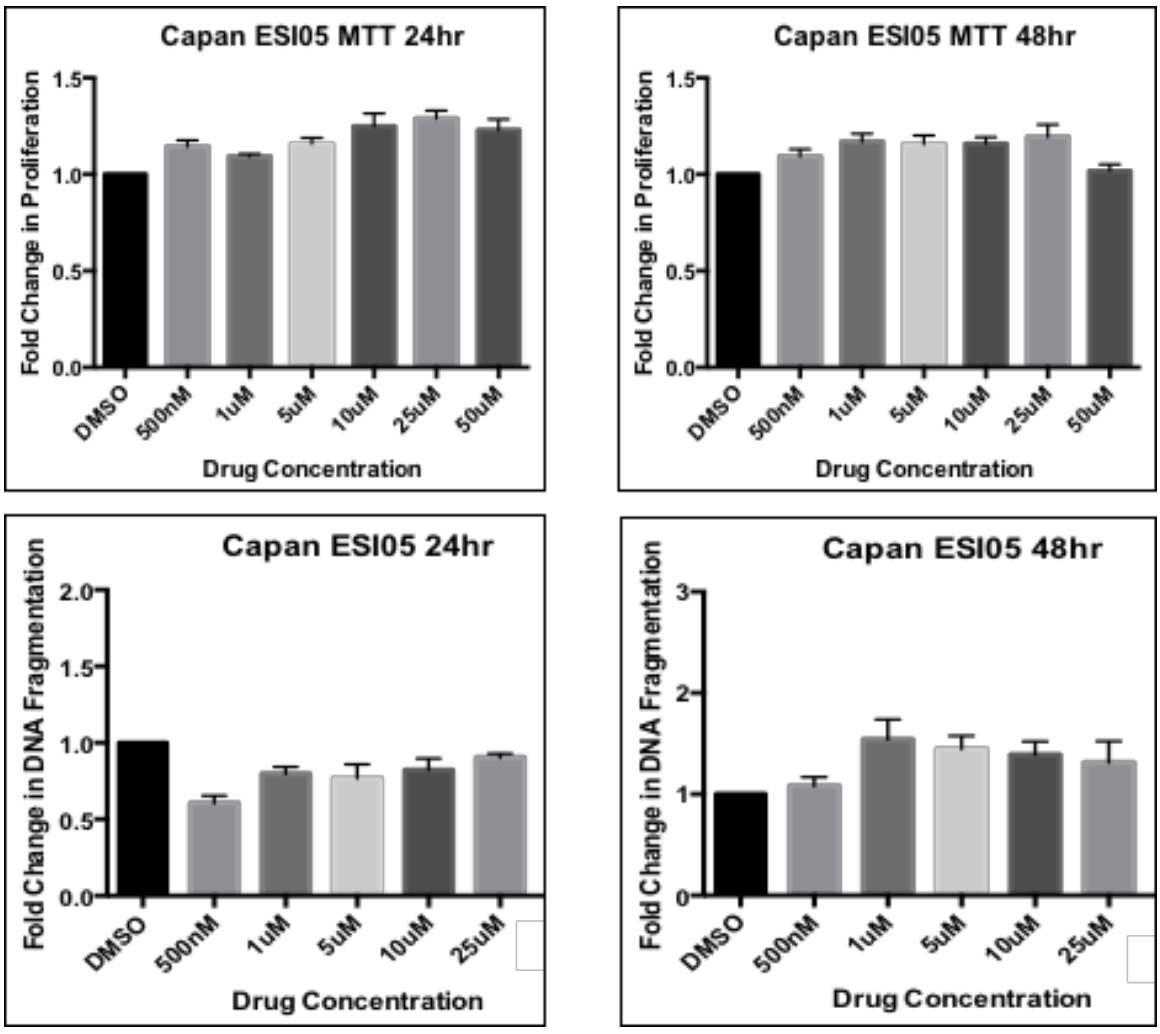


Figure 9.a: Treatment of Capan cell line with ESI05 exhibited no significant change in cell proliferation or DNA fragmentation. Data shown are representative of three independent experiments.

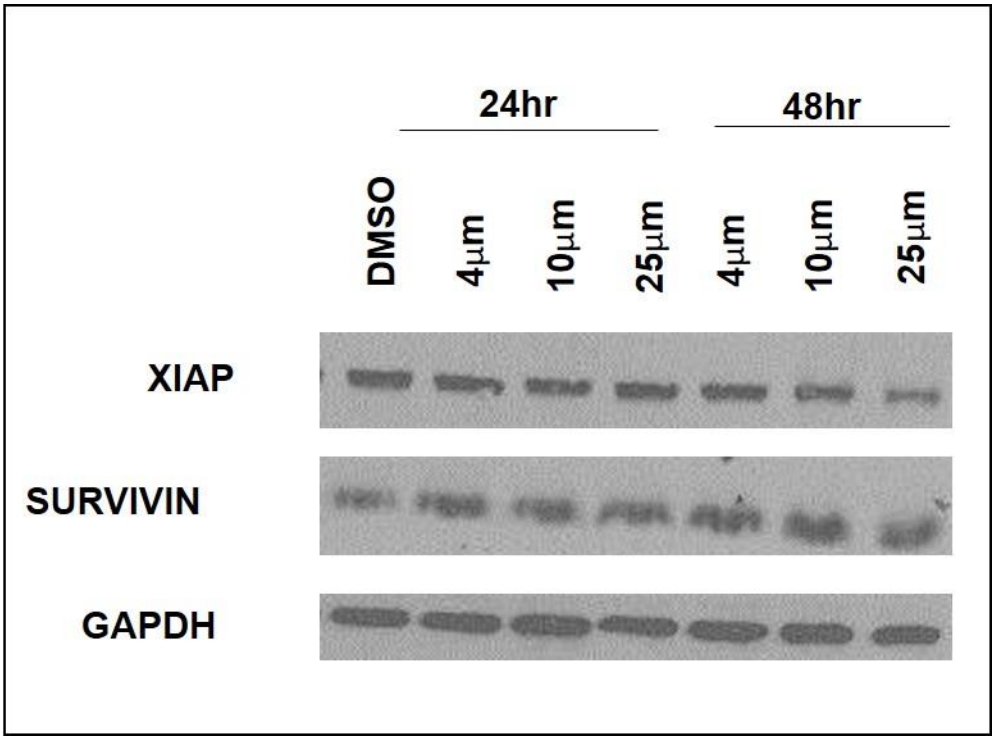


Figure 9.b: Western blot analysis of the treatment of Capan cell line with ESI05 demonstrated no change in IAP proteins. Data shown are representative of three independent experiments.

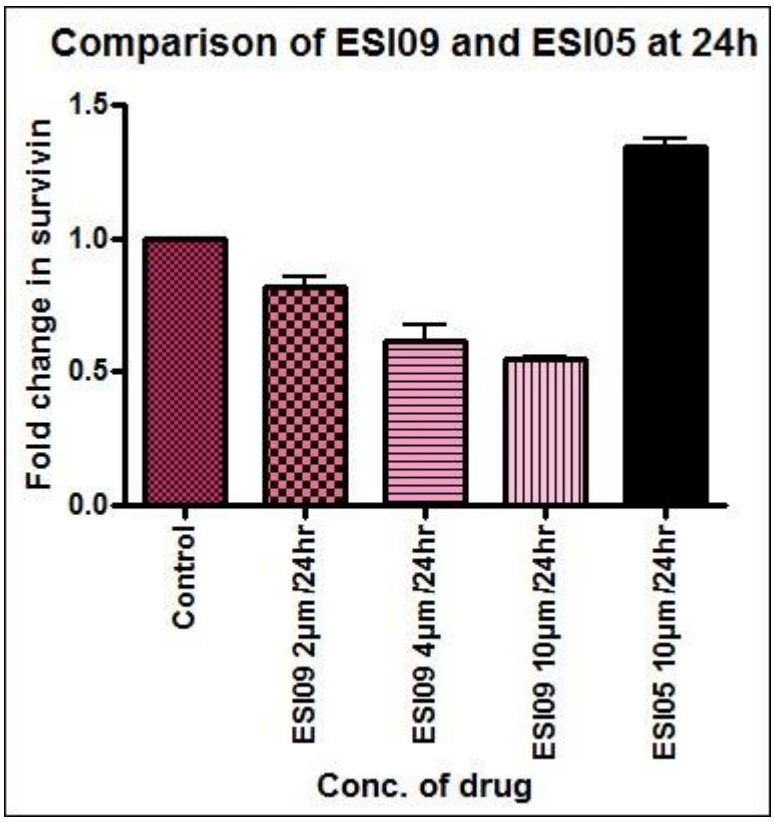


Figure 9c: The Capan cell line was treated with ESI05 at 10um at 24 hours and survivin levels were compared to the ESI09 treatment at the same concentration. Compared to ESI09 treated at 10um at 24 and 48 hours, there is no decrease in survivin in the ESI05 treated under similar conditions. Data shown are representative of three independent experiments.

4. EPAC1 selective inhibition represses XIAP and survivin.

Selective inhibition of EPAC2 produced no detectable effects on cell survival in either CRC or PaCa cell lines. Therefore, we hypothesized that EPAC1 was the key isoform involved in cell survival. In order to test this hypothesis, we utilized the selective EPAC1 inhibitor, CE3F4, that was obtained from the Corilleau laboratory in France. They provided us with the racemic mixture which was used to treat CRC as well as PaCa cell lines. CE3F4 is an allosteric inhibitor of EPAC1.

Cell proliferation assays were performed and a maximal effect was observed at 50uM. Treatment of the CRC cell lines -FET & CBS with CE3F4 produced a decrease in XIAP and survivin at the protein level.

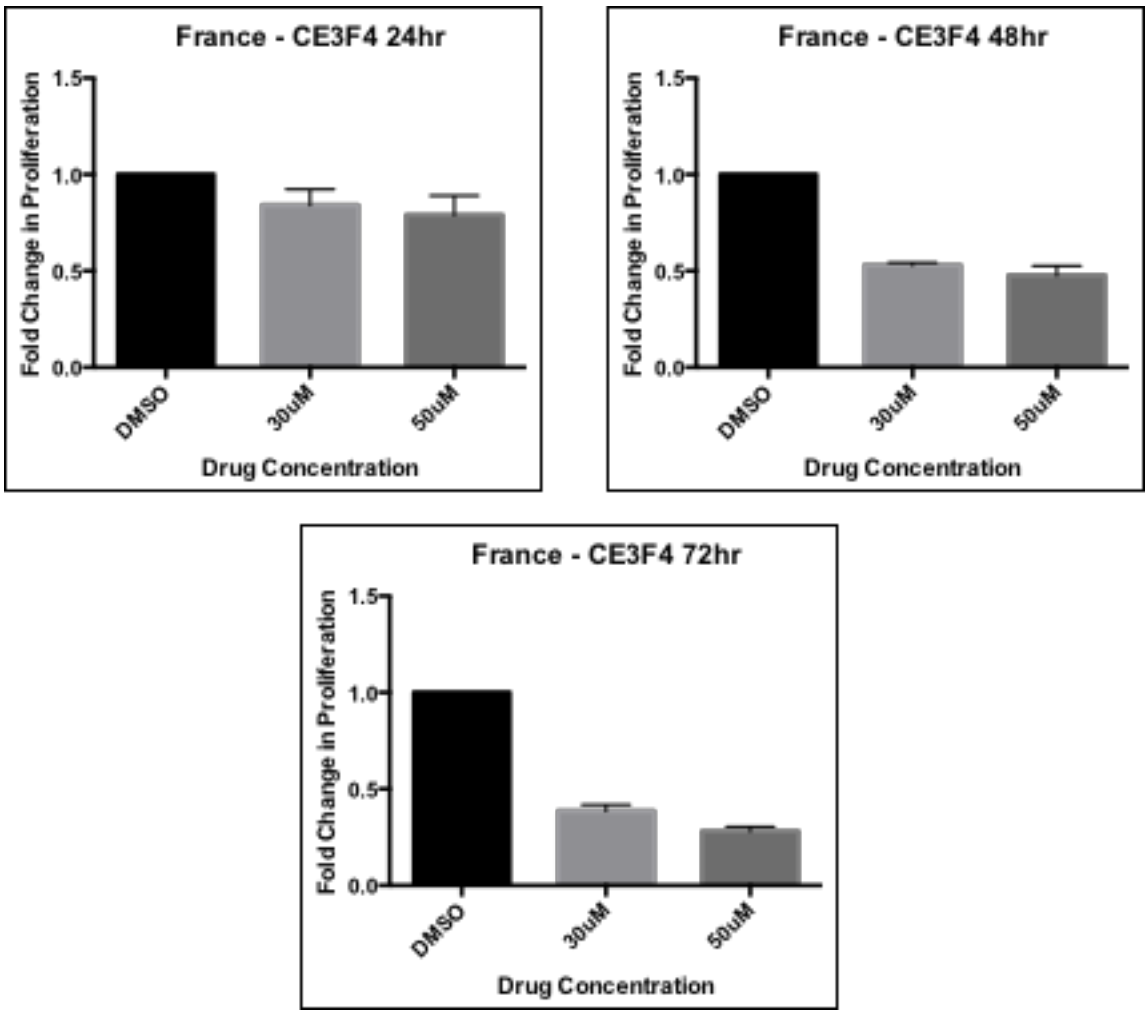


Figure 10.1: Treatment of FET CRC cell line with CE3F4 -EPAC1 selective inhibitor resulted in a decrease in cell proliferation. CE3F4 is a racemic mixture and therefore, responses are observed at higher concentrations. The R-enantiomer exhibits activity at much lower concentrations. Data shown are representative of two independent experiments.

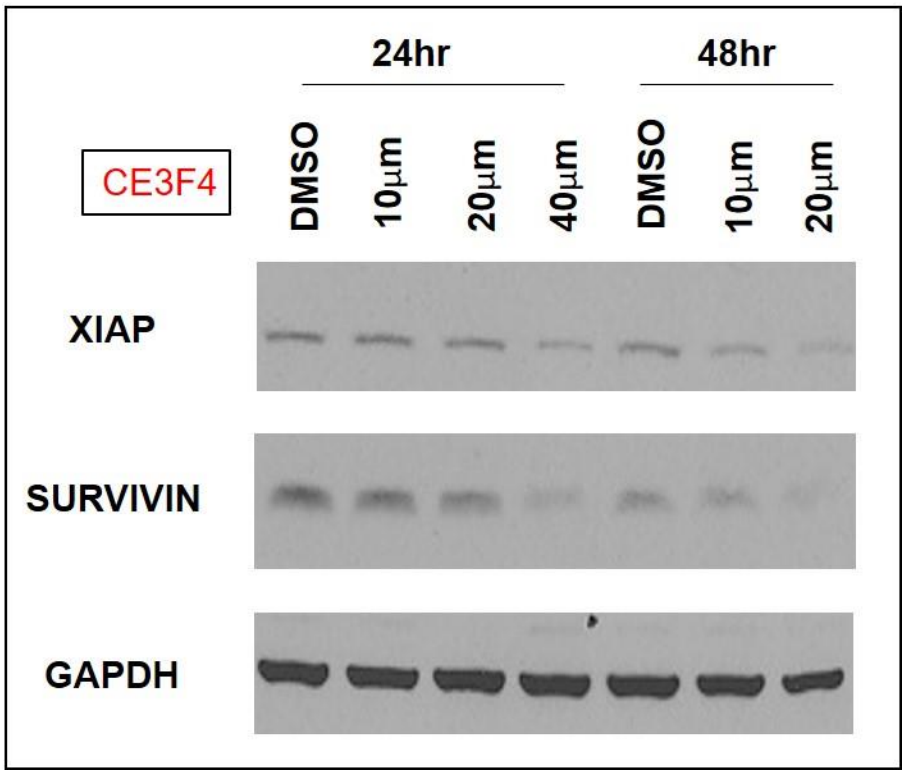


Figure 10.2: The FET cell line when treated with CE3F4 (EPAC1 selective inhibitor) reduces XIAP and survivin expression at 40μm which is the IC_{50} of the racemic mixture of the drug. Data shown are representative of two independent experiments.

5. EPAC inhibition decreases HDACs 4 and 5.

Jeong et al demonstrated that EPAC activation causes a repression in HAT p300. This HAT acetylates histones so we hypothesized that in addition to decreasing the acetylating enzyme, EPACs would induce HDACs.

Therefore, we studied the effect of EPAC inhibition on HDAC expression in our CRC cell lines. If EPACs are able to induce HDAC expression, then inhibition should decrease expression and potentially be regarded as an epigenetic regulator.

Treatment of our CRC cell lines with ESI09 produced a decrease in HDACs 4 and 5. In contrast, the inhibitor produced no effect on HDAC3 expression and only a slight decrease in HDAC1 expression. These results demonstrate that the EPACs exhibit specificity with regards to their effects on HDAC expression.

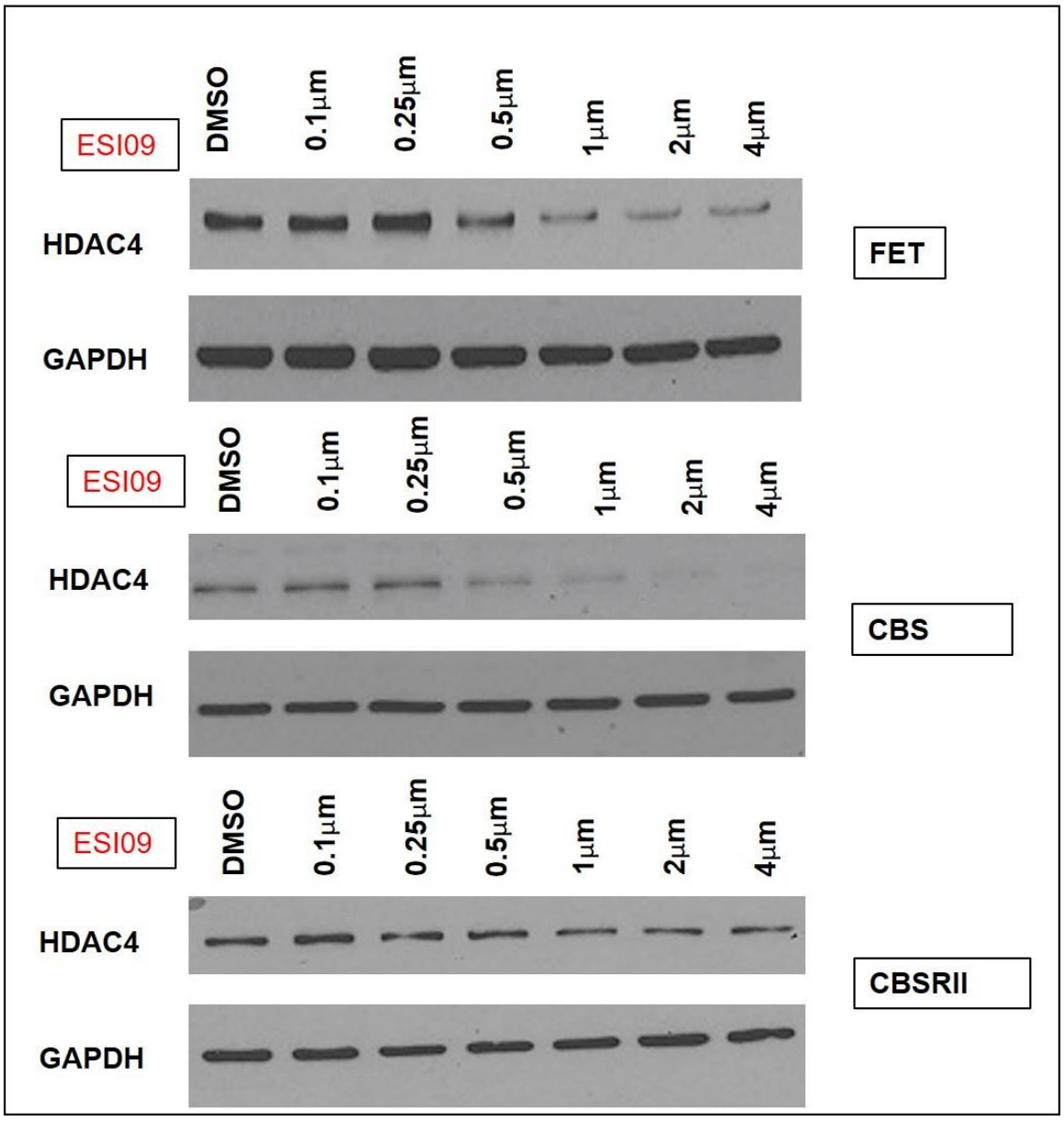


Figure 12a: Treatment of CRC cell lines with ESI09 decreased HDAC4 in a dose-dependent manner. Data shown are representative of three independent experiments.

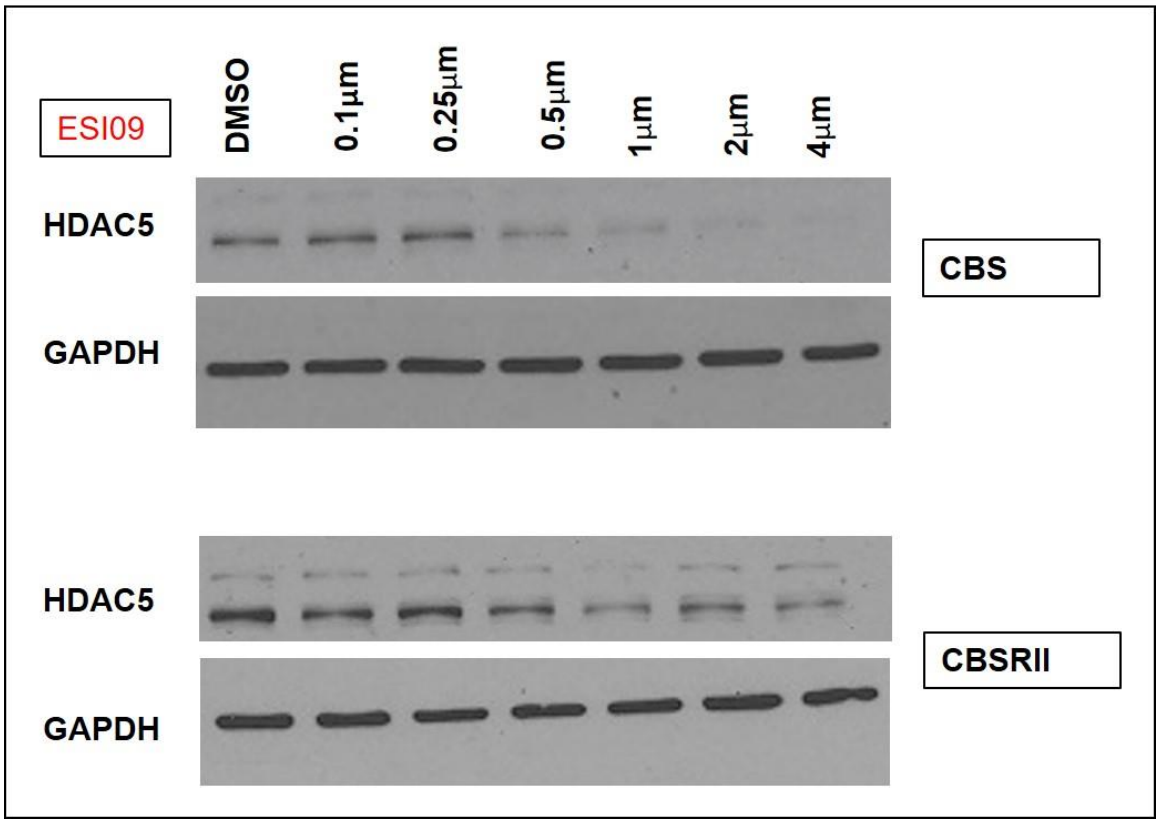


Figure 12b: Treatment of CRC cell lines with ESI09 decreased HDAC5 in a dose-dependent manner. Data shown are representative of three independent experiments.

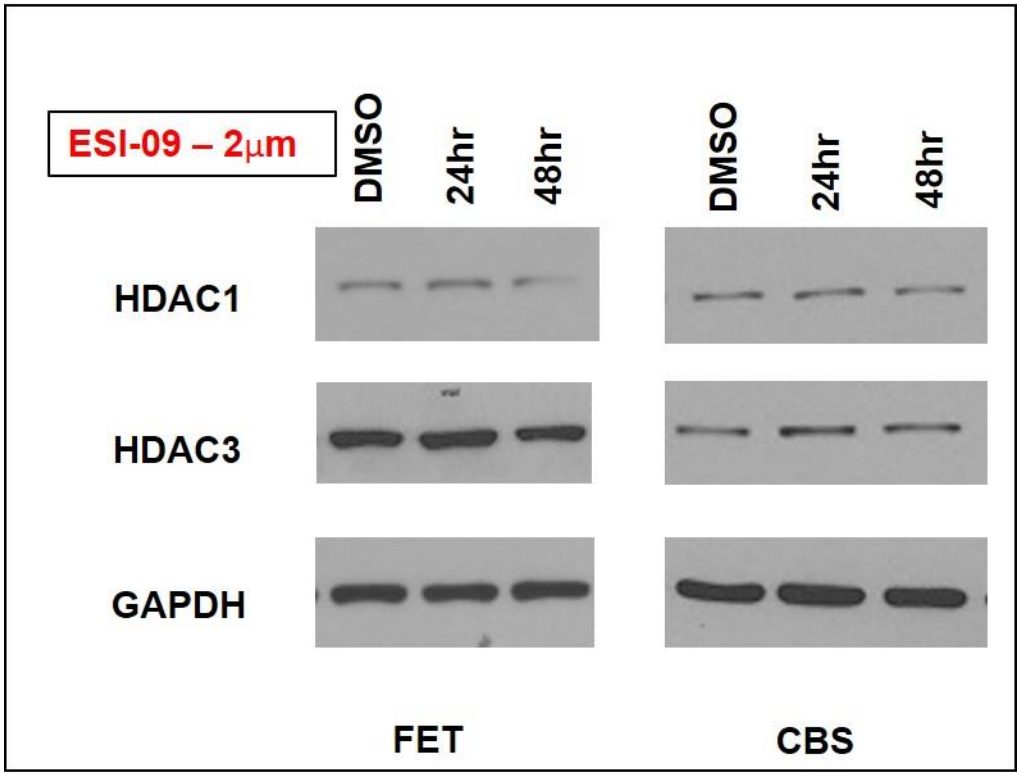


Figure 12.c: Inhibition of the CRC cell lines with ESI09 produced only a slight decrease in HDAC1 but no decrease in HDAC3. Data shown are representative of three independent experiments.

6. Inhibition of HDAC4 and 5 by LMK235 (selective inhibitor of HDAC4 and 5) causes a decrease in cell survival.

The previous studies provide evidence that HDAC4 and 5 expression is regulated by EPACs. This raised the question whether the regulation of HDAC 4 and 5 was directly involved in the cell survival effects mediated by EPAC.

We therefore hypothesized that if HDAC4 (and 5) are involved in mediating downstream signaling of EPACs then HDAC 4 and 5 inhibition should recapitulate the effects of EPAC inhibition on cell survival.

In order to test this, we first used the HDAC4 and 5 inhibitor, LMK235. It is selective towards HDAC 4 and 5 in low nanomolecular levels because of the way it is able to dock and inhibit the Zn^{2+} cation required for both of these HDACs to function [165]. This drug decreased XIAP and survivin expression in both Smad4 wild type and Smad4 mutant (Capan) cells, in a concentration dependent manner. This was confirmed through western blot as well q-RTPCR.

We also examined the effect of specific HDAC4 knockdown (KD) on XIAP expression using siRNA.

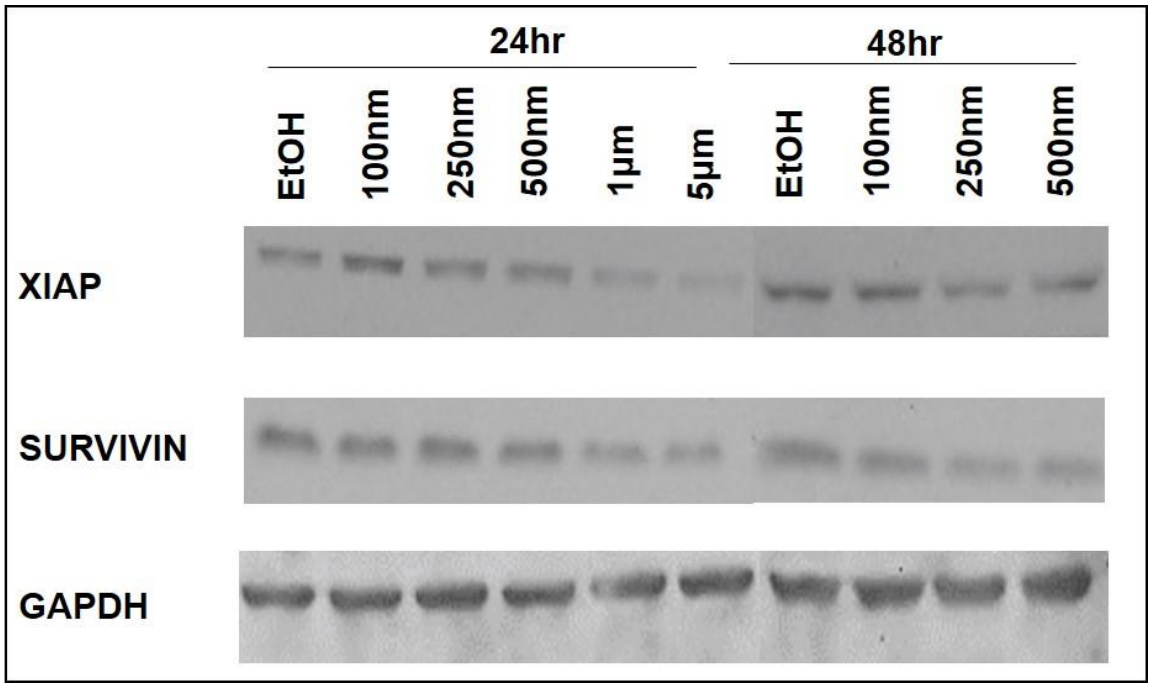


Figure 13a: Treatment of the Miapaca cell line with LMK235 decreases XIAP and survivin in a dose dependent manner at both 24 and 48hours. This cell survival effect through HDAC4 and 5, points to a role of EPACs as epigenetic regulators. Data shown are representative of three independent experiments.

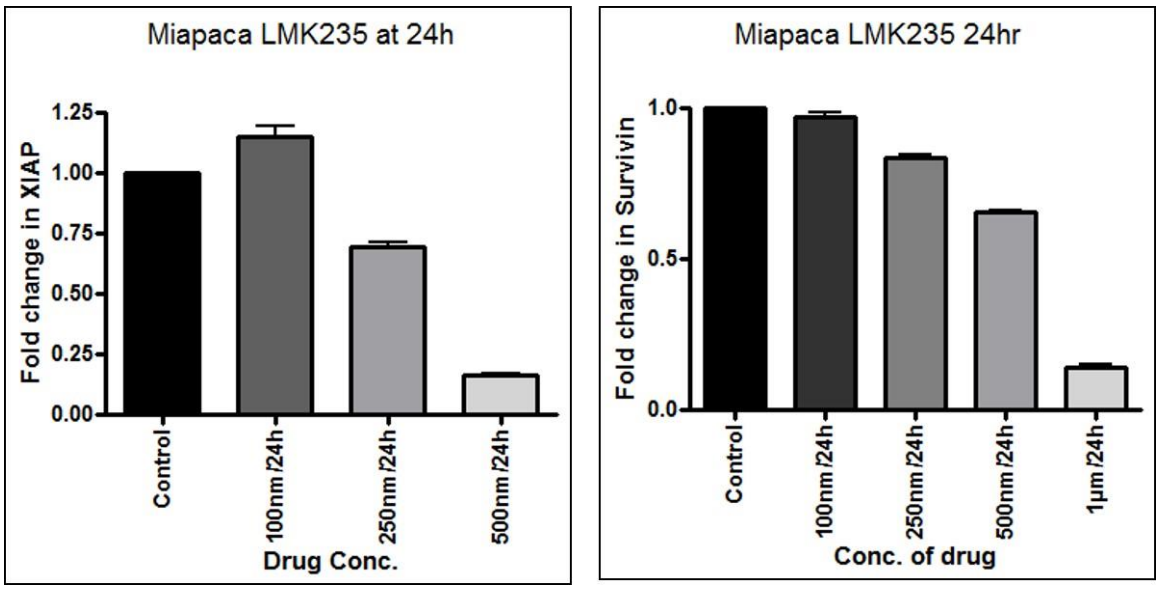


Figure 13b: Treatment of the Miapaca cell line with LMK235 decreases survivin at the RNA level at 24hours. Data shown are representative of three independent experiments.

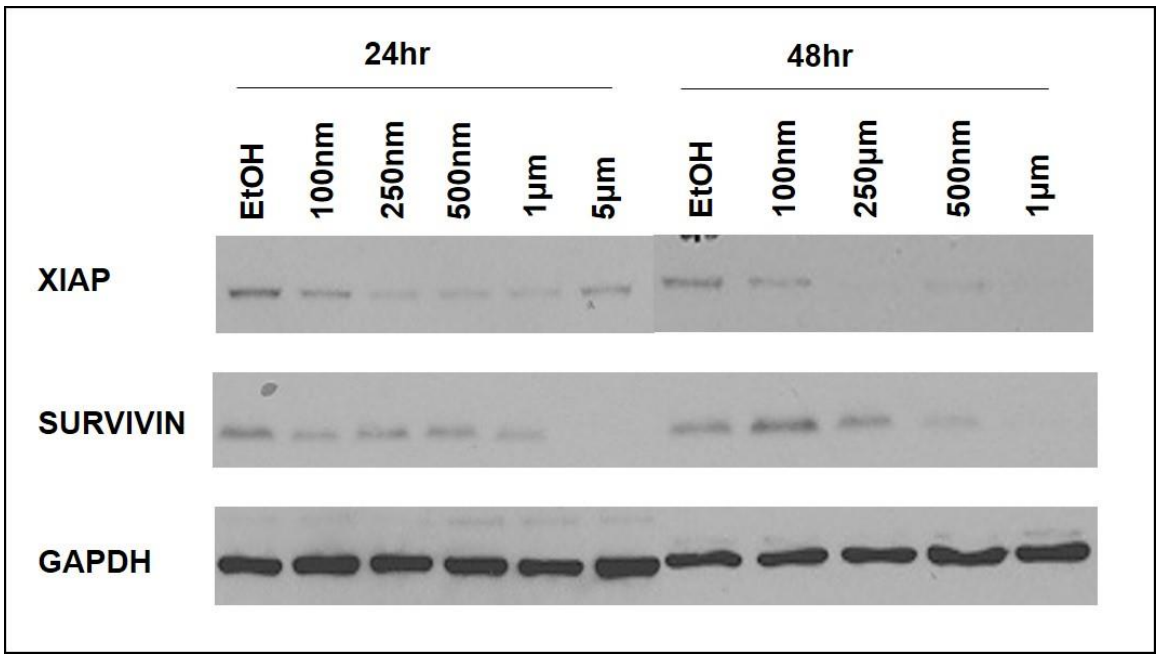


Figure 14a: Treatment of the Capan cell line with LMK235 decreases XIAP and survivin in a dose dependent manner at both 24 and 48hours. This cell survival effect through HDAC4 and 5, points to a role of EPACs as epigenetic regulators. Data shown are representative of three independent experiments.

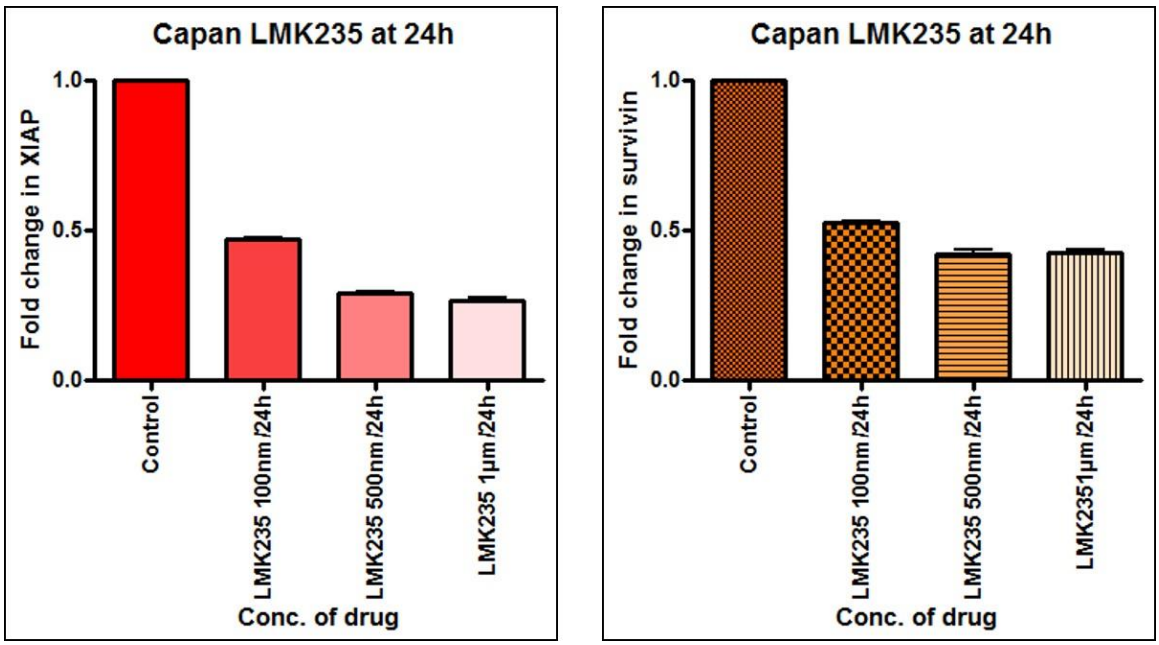


Figure 14b: Treatment of the Capan cell line with LMK235 decreases XIAP and survivin at the RNA level at 24hours. Data shown are representative of three independent experiments.

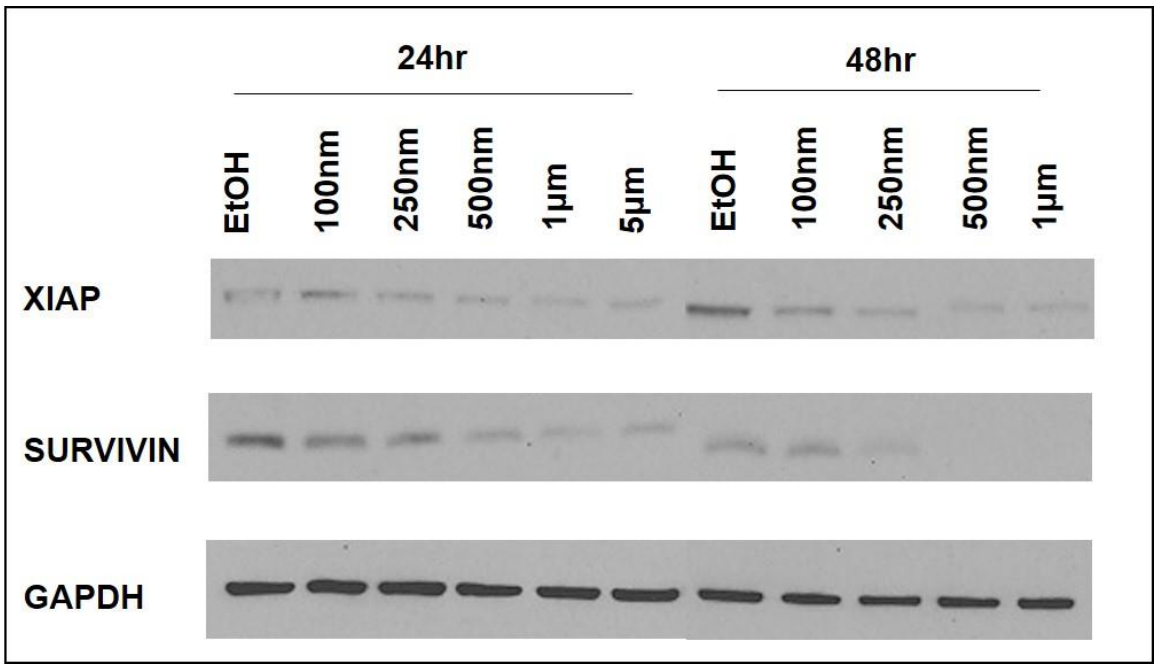


Figure 15a: Treatment of the CFPAC cell line with LMK235 decreases XIAP and survivin in a dose dependent manner at both 24 and 48hours. This cell survival effect through HDAC4 and 5, points to a role of EPACs as epigenetic regulators. Data shown are representative of three independent experiments.

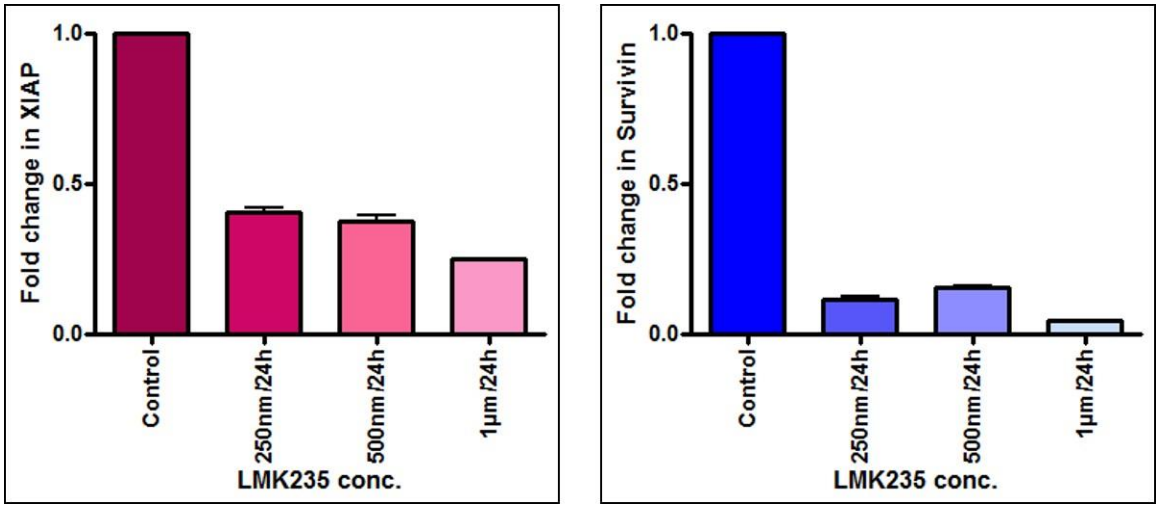


Figure 15b Treatment of the CFPAC cell line with LMK235 decreases XIAP and survivin at the RNA level at 24hours. Data shown are representative of three independent experiments.

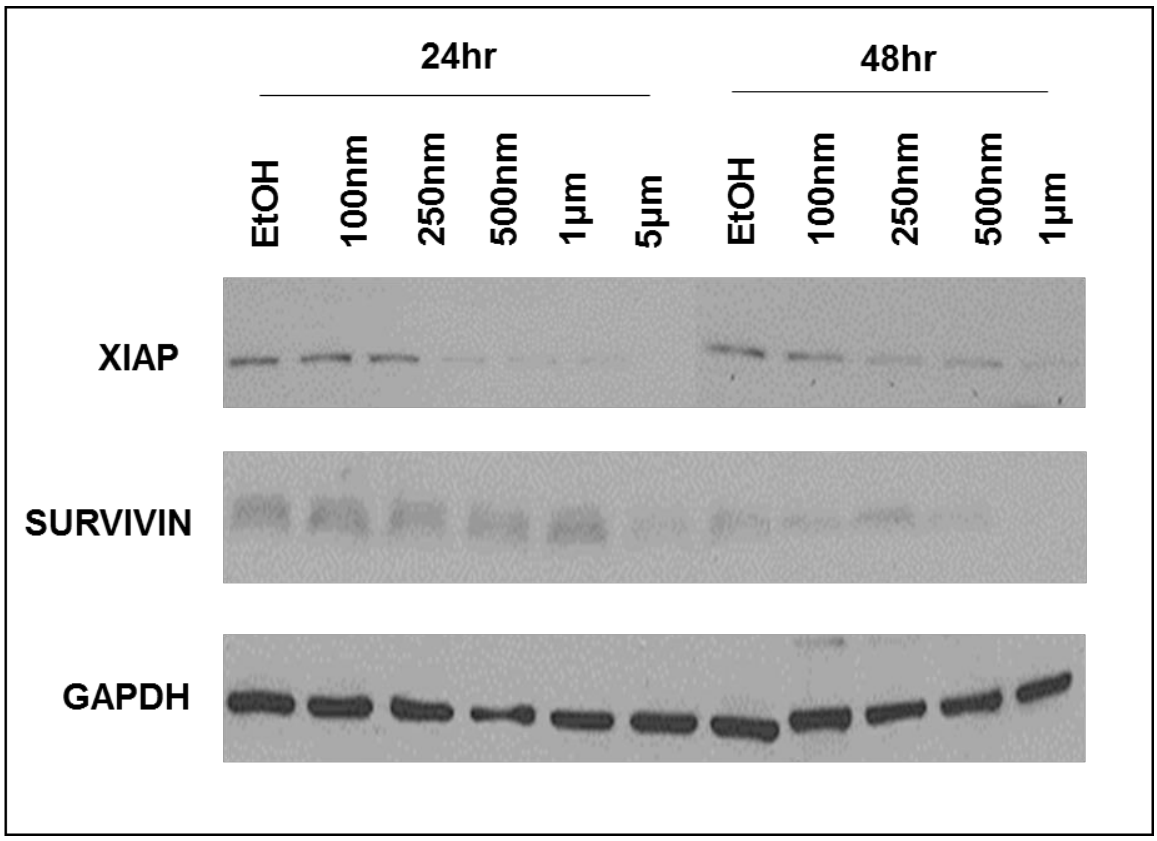


Figure 16a: Treatment of the T3M4 cell line with LMK235 decreases XIAP and survivin in a concentration dependent manner at both 24 and 48hours., suggesting the role of EPACs as epigenetic regulators. Data shown are representative of three independent experiments.

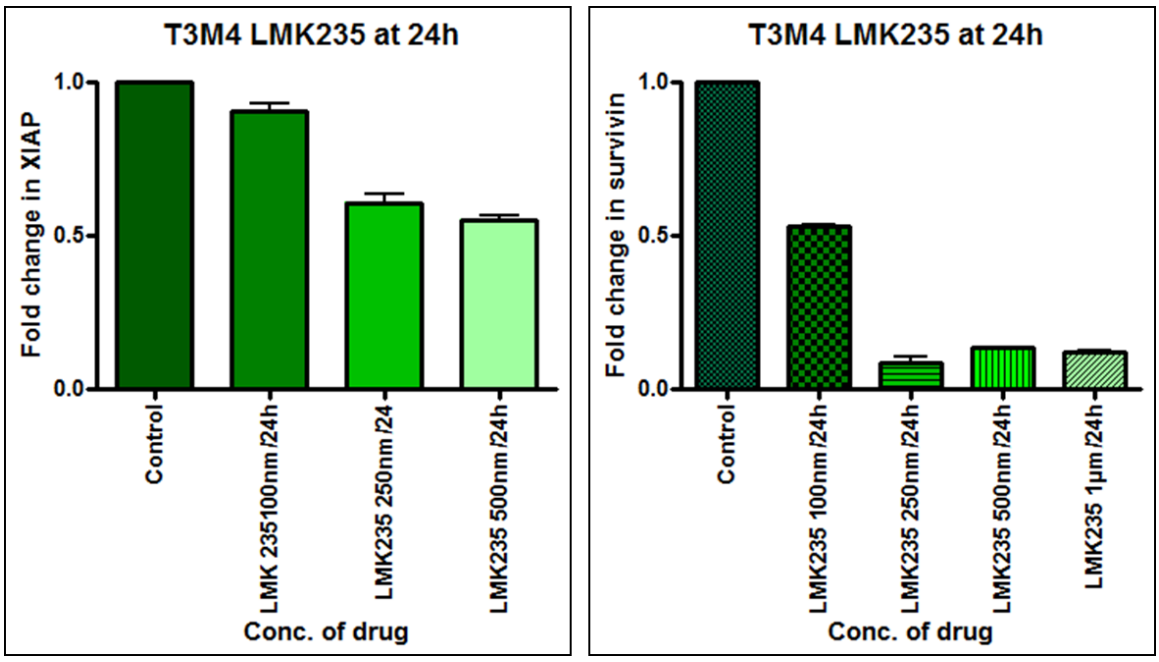


Figure 16.c: Treatment of the T3M4 cell line with LMK235 decreases XIAP and survivin at the RNA level at 24hours. Data shown are representative of three independent experiments.

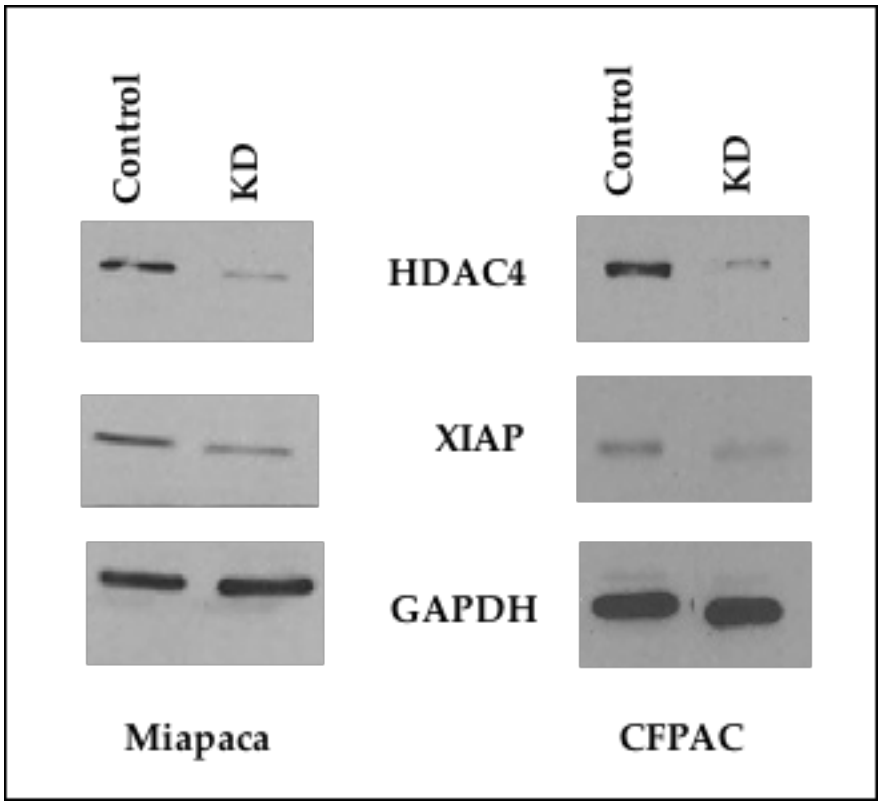


Figure 17: siRNA mediated HDAC4 KD decreased XIAP expression in the Miapaca and CFPAC cells. Data shown are representative of two independent experiments.

Discussion:

PKA is the traditional effector of cAMP but it has since been discovered that many cellular functions like proliferation, migration exocytosis and secretion are PKA independent [1, 2, 166]. This opened the field to other cAMP effectors, notably the EPACs. Misra and Pizzo in 2009 reported that PKA and EPAC functioned antagonistically [54]. Of particular importance was the regulation of phosphorylation of AKT – a key survival molecule in cancer that is required for the stabilization of the IAP proteins – XIAP and survivin - PKA and EPAC function antagonistically in the phosphorylation of AKT.

Previous work in our laboratory identified a unique TGFβ signaling transduceome which exerted its anti-tumorigenic effects in a non-canonical TGFβ pathway thorugh the deactivation of AKT and destabilization of XIAP and survivin [63]. It does this by activating PKA in a cAMP independent, Smad3 dependent manner that leads to AKT dephosphorylation through PKA mediated PP2A activation and PKA dependent survivin phosphorylation that causes destabilization of the XIAP/survivin complex leading to the proteosomal degradation of the IAPs.

EPACs however, promote AKT mediated cancer proliferation in an antagonistic fashion as compared to the anti-tumor effects of PKA [54]. EPACs have been

implicated in cancer cell migration, invasion and invadopodia formation and form part of the TGFβRI interactome [57, 167, 168]. Thus EPACs function antagonistically to the inhibitory TGFβ signaling effects observed in cancer cells. Previous studies done in our lab using RAPGEF inhibitor, Brefeldin A led to a decrease in pAKT, XIAP and survivin in CRC. When treated together with TGFβ there was a further decrease in survivin expression.

Based on these previous findings we hypothesized that EPAC would exert pro-tumorigenic effects through enhancement of cell survival in opposition to the effects of TGFβ and PKA. Initially to test this hypothesis, we utilized the EPAC specific activator, 8CPT which could not interact with PKA due to the presence of a methyl group. EPAC activation led to increased cell proliferation, decreased apoptosis and increased expression of the cell survival proteins survivin and XIAP. The EPAC activator does not distinguish between the two EPAC isoforms. Therefore in order to determine which isoforms(s) was mediating the cell survival effects, we utilized newly available selective EPAC inhibitors in both CRC and PaCa cell lines.

ESI09 (inhibits EPAC1 and 2), ESI05 (inhibits only EPAC2) and CE3F4 (inhibits only EPAC1). We discovered that inhibition of EPACs by ESI09 led to a decrease in XIAP and survivin but this effect was not observed in cells treated with ESI05 – allowing us to hypothesize that EPAC1 is responsible for the pro-survival

effects found in cancer. We used the racemic mixture of CE3F4 in CRC cells and observed a decrease in XIAP and survivin confirming that EPAC1 indeed was connected to the pro-survival role in cancer. We were then able to synthesize the different enantiomers of CE3F4 and cell proliferation studies with these enantiomers showed a concentration dependent decrease with the R-enantiomer compared to the S-enantiomer and the racemic mixture. Identification of EPAC1 as the isoform responsible for pro-survival effects observed in CRC and PaCa cancer was one of the major results from this study. These results identify EPAC1 as a potential therapeutic target for CRC and PaCa.

We examined the effect of ESI09 on HDACs. ESI09 treatment led to a decrease in HDACs 4 and 5 which led us to hypothesize that EPACs act as an epigenetic regulator and cause a decrease in cell survival through the inhibition of these HDACs.

HDACs are overexpressed in many different kinds of cancers. HDAC4 is on chromosome 2q37.3 and is involved in neuronal stabilization, bone growth and skeletal muscle development. HDAC4 and 5 are Class II HDACs that shuttle between the cytoplasm and nucleus. Certain signals will allow HDAC4 to interact with 14-3-3 and enter the cytoplasm [169]. Importin is another protein that HDAC4 interacts with to efflux from the nucleus into the cytoplasm [170]. HDAC4 along with HDAC5 when overexpressed decreased the βcells (produces

insulin) and the δcells (produces somatostatin) of the pancreas indicating their role in diabetes [171]. HDAC4 directly interacts with tumor suppressor micro-RNA22 and downregulates it in hepatocellular cancer and inhibiting HDAC4 increases micro-RNA22 decreasing cell proliferation of hepatocellular cancer cells [172]. Platinum chemoresistant ovarian tumors had an increased level of HDAC4 compared to the non-chemoresistant tumors [173]. Compared to the normal bladder cells, HDAC4 was found at higher levels in the tumor cells [174]. HDAC4 is mutated in breast cancer and melanoma [141]. HDAC5 also is important in cancer but its role so far appears to be mostly in angiogenesis [175]. HDAC5 also regulates PTEN in PaCa and plays a role in cancer cell proliferation [149, 150].

EPAC activation is known to cause an efflux of HDAC4 from the nucleus into the cytoplasm. HDAC5 was not responsive to this activation [127]. Activation of EPAC also causes a decrease in HAT p300 levels allowing us to hypothesize that if EPAC activation is causing a decrease in HAT levels and an increase in HDAC levels (efflux from nucleus) then EPAC inhibition must cause a decrease in HDACs, therefore EPAC behaves as an epigenetic regulator.

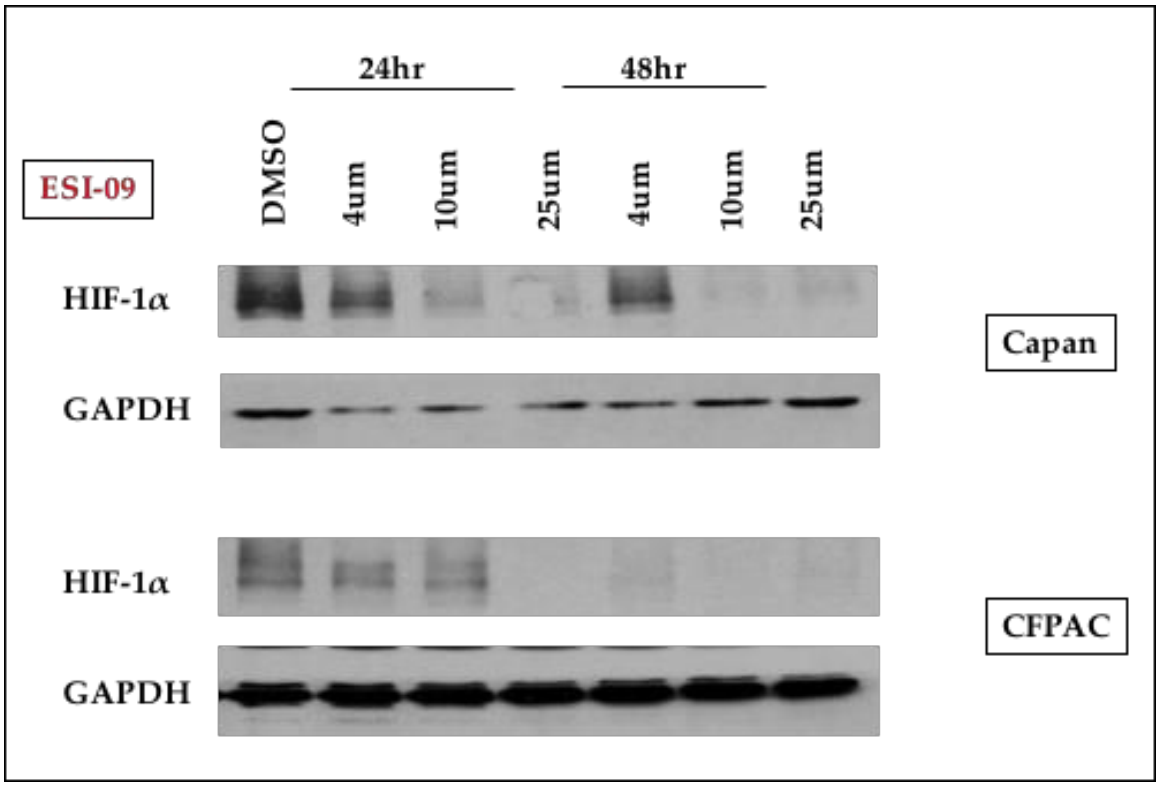
Inhibition of EPACs by ESI09 caused a decrease in the levels of HDAC4 and 5. Of importance is the relationship between HDAC4 and hypoxia inducible factor 1-α (HIF1-α). HIF1-α is part of the hypoxic response that is found in most solid

tumors and is responsible for the generation of survival pathways by upregulating protein responsible for angiogenesis and anaerobic metabolism [176-178].

Inhibitors of HIF-1α or circumstances that can mitigate it may be beneficial in many solid tumors. ESI09 causes a decrease in HDAC4 and 5 – of importance to this study is the decrease in HDAC4.

HDAC4 regulates HIF1-α's acetylation and stability because it directly interacts with HIF1-α through its multiple lysines. When HDAC4 and 5 bind to HIF1-α, binding of FIH-1 (factor inhibiting HIF1-α) is prevented and association with p300 (HAT) is increased – causing increased stability of HIF1-α. Inhibition of HDAC4 leads to deacetylation of HIF1-α and eventual degradation because of lack of stability in non-small cell lung cancer. Panobinostat (pan-HDAC inhibitor) when given in combination with cisplatin exhibited higher levels of apoptosis and reduced tumor growth in non-small cell lung cancer. HDAC4 inhibition was also responsible in the reduction of glycolysis (hypoxia related) and chemoresistance to docetaxel [144, 179, 180]. Thus, HDAC4 presents itself as an exciting therapeutic target for the treatment of cancer.

PaCa cell lines when treated with ESI09 (inhibits EPAC1 and 2) showed a decrease in HIF1-α.



We inhibited HDAC4 and 5 using the drug LMK235 and observed a decrease in XIAP and survivin. Knocking down HDAC4 with siRNA also showed a similar effect.

Thus the inhibition of EPAC plays an important role in cell apoptosis and tumor progression. EPAC when inhibited will decrease the levels of HDAC4 and 5 and this in turn will cause the destabilization and degradation of HIF1-α. EPAC1 may represent a therapeutic target and CE3F4 represents a prototype drug to develop for therapy.

Future Directions:

1. Synthesize different enantiomers of CE3F4 and treat CRC cells and PaCa cells to confirm the role of EPAC1 in the progression of cancer.
2. Develop stable inducible knockdowns of EPAC1 and 2 and confirm their role in cell survival.
3. Confirm the relationship between HDAC4 and HIF1-α. Perform hypoxic studies with a combination of CE3F4 and LMK235.
4. Generate stable knockdowns of HDACs 4 and 5.

Conclusions:

EPAC1 enhances pro-survival signaling in CRC or PaCa. My research in this part of the dissertation has shown that EPACs, particularly EPAC1 behaves as an epigenetic regulator and is able to regulate cancer cell survival. Published literature has thus far not shown any relationship between epigenetics and EPAC making this a novel finding.

References:

1. Kleppe, R., et al., *The cAMP-dependent protein kinase pathway as therapeutic target: possibilities and pitfalls.* Curr Top Med Chem. **11**(11): p. 1393-405.
2. Naviglio, S., et al., *Protein kinase A as a biological target in cancer therapy.* Expert Opin Ther Targets, 2009. **13**(1): p. 83-92.
3. Cheng, I., et al., *Pleiotropic effects of genetic risk variants for other cancers on colorectal cancer risk: PAGE, GECCO and CCFR consortia.* Gut, 2014. **63**(5): p. 800-7.
4. Cheng, X., et al., *Epac and PKA: a tale of two intracellular cAMP receptors.* Acta Biochim Biophys Sin (Shanghai), 2008. **40**(7): p. 651-62.
5. Tsalkova, T., et al., *Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities.* J Biol Chem, 2009. **284**(35): p. 23644-51.
6. Niimura, M., et al., *Critical role of the N-terminal cyclic AMP-binding domain of Epac2 in its subcellular localization and function.* J Cell Physiol, 2009. **219**(3): p. 652-8.
7. Ueno, H., et al., *Characterization of the gene EPAC2: structure, chromosomal localization, tissue expression, and identification of the liver-specific isoform.* Genomics, 2001. **78**(1-2): p. 91-8.
8. de Rooij, J., et al., *Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs.* J Biol Chem, 2000. **275**(27): p. 20829-36.
9. Qiao, J., et al., *Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP.* J Biol Chem, 2002. **277**(29): p. 26581-6.
10. Li, Y., et al., *The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane.* J Biol Chem, 2006. **281**(5): p. 2506-14.
11. Kawasaki, H., et al., *A family of cAMP-binding proteins that directly activate Rap1.* Science, 1998. **282**(5397): p. 2275-9.
12. Ulucan, C., et al., *Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy.* Am J Physiol Heart Circ Physiol, 2007. **293**(3): p. H1662-72.
13. Ponsioen, B., et al., *Direct spatial control of Epac1 by cyclic AMP.* Mol Cell Biol, 2009. **29**(10): p. 2521-31.
14. Consonni, S.V., et al., *cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane.* Proc Natl Acad Sci U S A. **109**(10): p. 3814-9.
15. Fu, D., et al., *Bile acid stimulates hepatocyte polarization through a cAMP-Epac-MEK-LKB1-AMPK pathway.* Proc Natl Acad Sci U S A. **108**(4): p. 1403-8.

16. Stangherlin, A. and M. Zaccolo, *Phosphodiesterases and subcellular compartmentalized cAMP signaling in the cardiovascular system.* Am J Physiol Heart Circ Physiol. **302**(2): p. H379-90.
17. Dodge-Kafka, K.L., A. Bauman, and M.S. Kapiloff, *A-kinase anchoring proteins as the basis for cAMP signaling.* Handb Exp Pharmacol, 2008(186): p. 3-14.
18. Dodge-Kafka, K.L., et al., *The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways.* Nature, 2005. **437**(7058): p. 574-8.
19. Bers, D.M., *Calcium cycling and signaling in cardiac myocytes.* Annu Rev Physiol, 2008. **70**: p. 23-49.
20. Metrich, M., et al., *Functional characterization of the cAMP-binding proteins Epac in cardiac myocytes.* Pharmacol Rep, 2009. **61**(1): p. 146-53.
21. Wang, H., et al., *Phospholipase C epsilon modulates beta-adrenergic receptor-dependent cardiac contraction and inhibits cardiac hypertrophy.* Circ Res, 2005. **97**(12): p. 1305-13.
22. Yokoyama, U., et al., *Prostaglandin E2-activated Epac promotes neointimal formation of the rat ductus arteriosus by a process distinct from that of cAMP-dependent protein kinase A.* J Biol Chem, 2008. **283**(42): p. 28702-9.
23. Villarreal, F., et al., *Regulation of cardiac fibroblast collagen synthesis by adenosine: roles for Epac and PI3K.* Am J Physiol Cell Physiol, 2009. **296**(5): p. C1178-84.
24. Somekawa, S., et al., *Enhanced functional gap junction neoformation by protein kinase A-dependent and Epac-dependent signals downstream of cAMP in cardiac myocytes.* Circ Res, 2005. **97**(7): p. 655-62.
25. Metrich, M., et al., *Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy.* Circ Res, 2008. **102**(8): p. 959-65.
26. Kang, G., et al., *A cAMP and Ca2+ coincidence detector in support of Ca2+-induced Ca2+ release in mouse pancreatic beta cells.* J Physiol, 2005. **566**(Pt 1): p. 173-88.
27. Kashima, Y., et al., *Critical role of cAMP-GEFII--Rim2 complex in incretin-potentiated insulin secretion.* J Biol Chem, 2001. **276**(49): p. 46046-53.
28. Ozaki, N., et al., *cAMP-GEFII is a direct target of cAMP in regulated exocytosis.* Nat Cell Biol, 2000. **2**(11): p. 805-11.
29. Fujimoto, K., et al., *Piccolo, a Ca2+ sensor in pancreatic beta-cells. Involvement of cAMP-GEFII.Rim2. Piccolo complex in cAMP-dependent exocytosis.* J Biol Chem, 2002. **277**(52): p. 50497-502.
30. Kang, G., et al., *Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca2+-induced Ca2+ release and exocytosis in pancreatic beta-cells.* J Biol Chem, 2003. **278**(10): p. 8279-85.

31. Reedquist, K.A., et al., *The small GTPase, Rap1, mediates CD31-induced integrin adhesion.* J Cell Biol, 2000. **148**(6): p. 1151-8.
32. Sebzda, E., et al., *Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling.* Nat Immunol, 2002. **3**(3): p. 251-8.
33. Shimonaka, M., et al., *Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow.* J Cell Biol, 2003. **161**(2): p. 417-27.
34. Katagiri, K., M. Shimonaka, and T. Kinashi, *Rap1-mediated lymphocyte function-associated antigen-1 activation by the T cell antigen receptor is dependent on phospholipase C-gamma1.* J Biol Chem, 2004. **279**(12): p. 11875-81.
35. Ghandour, H., et al., *Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion.* Blood, 2007. **110**(10): p. 3682-90.
36. Arai, A., et al., *Rap1 is activated by erythropoietin or interleukin-3 and is involved in regulation of beta1 integrin-mediated hematopoietic cell adhesion.* J Biol Chem, 2001. **276**(13): p. 10453-62.
37. Caron, E., A.J. Self, and A. Hall, *The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators.* Curr Biol, 2000. **10**(16): p. 974-8.
38. Liu, L., et al., *Requirement for RhoA kinase activation in leukocyte de-adhesion.* J Immunol, 2002. **169**(5): p. 2330-6.
39. de Bruyn, K.M., et al., *The small GTPase Rap1 is activated by turbulence and is involved in integrin [alpha]IIb[beta]3-mediated cell adhesion in human megakaryocytes.* J Biol Chem, 2003. **278**(25): p. 22412-7.
40. Bos, J.L., et al., *The role of Rap1 in integrin-mediated cell adhesion.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 83-6.
41. Enserink, J.M., et al., *A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK.* Nat Cell Biol, 2002. **4**(11): p. 901-6.
42. Fukuhara, S., et al., *Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway.* Mol Cell Biol, 2005. **25**(1): p. 136-46.
43. Kooistra, M.R., et al., *Epac1 regulates integrity of endothelial cell junctions through VE-cadherin.* FEBS Lett, 2005. **579**(22): p. 4966-72.
44. Cho, E.A. and Y.S. Juhnn, *The cAMP signaling system inhibits the repair of gamma-ray-induced DNA damage by promoting Epac1-mediated proteasomal degradation of XRCC1 protein in human lung cancer cells.* Biochem Biophys Res Commun. **422**(2): p. 256-62.

45. Pullamsetti, S.S., et al., *Phosphodiesterase-4 promotes proliferation and angiogenesis of lung cancer by crosstalk with HIF.* Oncogene. **32**(9): p. 1121-34.
46. Narita, M., et al., *A role for cyclic nucleotide phosphodiesterase 4 in regulation of the growth of human malignant melanoma cells.* Oncol Rep, 2007. **17**(5): p. 1133-9.
47. Baljinnyam, E., et al., *Epac increases melanoma cell migration by a heparan sulfate-related mechanism.* Am J Physiol Cell Physiol, 2009. **297**(4): p. C802-13.
48. Baljinnyam, E., et al., *Exchange protein directly activated by cyclic AMP increases melanoma cell migration by a Ca2+-dependent mechanism.* Cancer Res. **70**(13): p. 5607-17.
49. Baljinnyam, E., et al., *Epac1 promotes melanoma metastasis via modification of heparan sulfate.* Pigment Cell Melanoma Res. **24**(4): p. 680-7.
50. Choi, J.H., et al., *Gonadotropin-stimulated epidermal growth factor receptor expression in human ovarian surface epithelial cells: involvement of cyclic AMP-dependent exchange protein activated by cAMP pathway.* Endocr Relat Cancer, 2009. **16**(1): p. 179-88.
51. Bastian, P., et al., *The inhibitory effect of norepinephrine on the migration of ES-2 ovarian carcinoma cells involves a Rap1-dependent pathway.* Cancer Lett, 2009. **274**(2): p. 218-24.
52. Spina, A., et al., *cAMP Elevation Down-Regulates beta3 Integrin and Focal Adhesion Kinase and Inhibits Leptin-Induced Migration of MDA-MB-231 Breast Cancer Cells.* Biores Open Access. **1**(6): p. 324-32.
53. Grandoch, M., et al., *Epac inhibits migration and proliferation of human prostate carcinoma cells.* Br J Cancer, 2009. **101**(12): p. 2038-42.
54. Misra, U.K. and S.V. Pizzo, *Epac1-induced cellular proliferation in prostate cancer cells is mediated by B-Raf/ERK and mTOR signaling cascades.* J Cell Biochem, 2009. **108**(4): p. 998-1011.
55. Burdyga, A., et al., *cAMP inhibits migration, ruffling and paxillin accumulation in focal adhesions of pancreatic ductal adenocarcinoma cells: effects of PKA and EPAC.* Biochim Biophys Acta. **1833**(12): p. 2664-72.
56. Lorenz, R., et al., *The cAMP/Epac1/Rap1 pathway in pancreatic carcinoma.* Pancreas, 2008. **37**(1): p. 102-3.
57. Almahariq, M., et al., *A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion.* Mol Pharmacol. **83**(1): p. 122-8.
58. Almahariq, M., et al., *Pharmacological inhibition and genetic knockdown of exchange protein directly activated by cAMP 1 reduce pancreatic cancer metastasis in vivo.* Mol Pharmacol. **87**(2): p. 142-9.
59. Laken, S.J., et al., *Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC.* Nat Genet, 1997. **17**(1): p. 79-83.

60. Chowdhury, S., et al., *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): p. 30937-48.
61. Piek, E., C.H. Heldin, and P. Ten Dijke, *Specificity, diversity, and regulation in TGF-beta superfamily signaling.* FASEB J, 1999. **13**(15): p. 2105-24.
62. Derynck, R. and X.H. Feng, *TGF-beta receptor signaling.* Biochim Biophys Acta, 1997. **1333**(2): p. F105-50.
63. Chowdhury, S., et al., *Identification of a novel TGFbeta/PKA signaling transduceome in mediating control of cell survival and metastasis in colon cancer.* PLoS One. **6**(5): p. e19335.
64. Skalhegg, B.S. and K. Tasken, *Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA.* Front Biosci, 2000. **5**: p. D678-93.
65. Carlisle Michel, J.J., et al., *PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex.* Biochem J, 2004. **381**(Pt 3): p. 587-92.
66. Nesterova, M. and Y.S. Cho-Chung, *Oligonucleotide sequence-specific inhibition of gene expression, tumor growth inhibition, and modulation of cAMP signaling by an RNA-DNA hybrid antisense targeted to protein kinase A RIalpha subunit.* Antisense Nucleic Acid Drug Dev, 2000. **10**(6): p. 423-33.
67. Neary, C.L., et al., *Protein kinase A isozyme switching: eliciting differential cAMP signaling and tumor reversion.* Oncogene, 2004. **23**(54): p. 8847-56.
68. Cho-Chung, Y.S. and M.V. Nesterova, *Tumor reversion: protein kinase A isozyme switching.* Ann N Y Acad Sci, 2005. **1058**: p. 76-86.
69. Caretta, A. and C. Mucignat-Caretta, *Protein kinase a in cancer.* Cancers (Basel). **3**(1): p. 913-26.
70. Zhong, H., et al., *The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism.* Cell, 1997. **89**(3): p. 413-24.
71. Chin, Y.R. and A. Toker, *Function of Akt/PKB signaling to cell motility, invasion and the tumor stroma in cancer.* Cell Signal, 2009. **21**(4): p. 470-6.
72. Suganuma, M., et al., *Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter.* Proc Natl Acad Sci U S A, 1988. **85**(6): p. 1768-71.
73. Chen, X., et al., *Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer.* Oncogene, 2001. **20**(42): p. 6073-83.
74. Zhou, X.D., et al., *Protein kinase B phosphorylation correlates with vascular endothelial growth factor A and microvessel density in gastric adenocarcinoma.* J Int Med Res. **40**(6): p. 2124-34.

75. Brodbeck, D., P. Cron, and B.A. Hemmings, *A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain.* J Biol Chem, 1999. **274**(14): p. 9133-6.
76. Nakatani, K., et al., *Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines.* J Biol Chem, 1999. **274**(31): p. 21528-32.
77. Easton, R.M., et al., *Role for Akt3/protein kinase Bgamma in attainment of normal brain size.* Mol Cell Biol, 2005. **25**(5): p. 1869-78.
78. Chau, N.M. and M. Ashcroft, *Akt2: a role in breast cancer metastasis.* Breast Cancer Res, 2004. **6**(1): p. 55-7.
79. Kupriyanova, T.A. and K.V. Kandror, *Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin.* J Biol Chem, 1999. **274**(3): p. 1458-64.
80. Yang, Z.Z., et al., *Protein kinase B alpha/Akt1 regulates placental development and fetal growth.* J Biol Chem, 2003. **278**(34): p. 32124-31.
81. Deveraux, Q.L., et al., *Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases.* EMBO J, 1999. **18**(19): p. 5242-51.
82. Saleem, M., et al., *Inhibitors of apoptotic proteins: new targets for anticancer therapy.* Chem Biol Drug Des. **82**(3): p. 243-51.
83. Silke, J. and D. Vucic, *IAP family of cell death and signaling regulators.* Methods Enzymol. **545**: p. 35-65.
84. Tong, Q.S., et al., *Downregulation of XIAP expression induces apoptosis and enhances chemotherapeutic sensitivity in human gastric cancer cells.* Cancer Gene Ther, 2005. **12**(5): p. 509-14.
85. McManus, D.C., et al., *Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics.* Oncogene, 2004. **23**(49): p. 8105-17.
86. Harlin, H., et al., *Characterization of XIAP-deficient mice.* Mol Cell Biol, 2001. **21**(10): p. 3604-8.
87. Fakler, M., et al., *Small molecule XIAP inhibitors cooperate with TRAIL to induce apoptosis in childhood acute leukemia cells and overcome Bcl-2-mediated resistance.* Blood, 2009. **113**(8): p. 1710-22.
88. Vogler, M., et al., *Targeting XIAP bypasses Bcl-2-mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth in vitro and in vivo.* Cancer Res, 2008. **68**(19): p. 7956-65.
89. Ahn, K.S., G. Sethi, and B.B. Aggarwal, *Embelin, an inhibitor of X chromosome-linked inhibitor-of-apoptosis protein, blocks nuclear factor-kappaB (NF-kappaB) signaling pathway leading to suppression of NF-kappaB-regulated*

antiapoptotic and metastatic gene products. Mol Pharmacol, 2007. **71**(1): p. 209-19.

90. Nikolovska-Coleska, Z., et al., *Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization.* Anal Biochem, 2004. **332**(2): p. 261-73.
91. Nikolovska-Coleska, Z., et al., *Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database.* J Med Chem, 2004. **47**(10): p. 2430-40.
92. Agrawal, S. and E.R. Kandimalla, *Role of Toll-like receptors in antisense and siRNA [corrected].* Nat Biotechnol, 2004. **22**(12): p. 1533-7.
93. Agrawal, S., et al., *Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: in vitro and in vivo studies.* Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2620-5.
94. Mohamed, S., et al., *Nuclear survivin in pN2 nonsmall cell lung cancer: prognostic and clinical implications.* Eur Respir J, 2009. **33**(1): p. 127-33.
95. Soleimanpour, E. and E. Babaei, *Survivin as a Potential Target for Cancer Therapy.* Asian Pac J Cancer Prev. **16**(15): p. 6187-91.
96. Poomsawat, S., J. Punyasingh, and P. Vejchapipat, *Overexpression of survivin and caspase 3 in oral carcinogenesis.* Appl Immunohistochem Mol Morphol. **22**(1): p. 65-71.
97. Lv, Y.G., et al., *The role of survivin in diagnosis, prognosis and treatment of breast cancer.* J Thorac Dis. **2**(2): p. 100-10.
98. Vandghanooni, S., et al., *Survivin-deltaEx3: a novel biomarker for diagnosis of papillary thyroid carcinoma.* J Cancer Res Ther. **7**(3): p. 325-30.
99. Mahotka, C., et al., *Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas.* Int J Cancer, 2002. **100**(1): p. 30-6.
100. Mahotka, C., et al., *Differential subcellular localization of functionally divergent survivin splice variants.* Cell Death Differ, 2002. **9**(12): p. 1334-42.
101. Altieri, D.C., *Survivin in apoptosis control and cell cycle regulation in cancer.* Prog Cell Cycle Res, 2003. **5**: p. 447-52.
102. Altieri, D.C., *Validating survivin as a cancer therapeutic target.* Nat Rev Cancer, 2003. **3**(1): p. 46-54.
103. Kawasaki, H., et al., *Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer.* Cancer Res, 1998. **58**(22): p. 5071-4.
104. Swana, H.S., et al., *Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer.* N Engl J Med, 1999. **341**(6): p. 452-3.
105. Hausladen, D.A., et al., *Effect of intravesical treatment of transitional cell carcinoma with bacillus Calmette-Guerin and mitomycin C on urinary survivin levels and outcome.* J Urol, 2003. **170**(1): p. 230-4.

106. Rodel, C., et al., *Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor.* Int J Radiat Oncol Biol Phys, 2003. **55**(5): p. 1341-7.
107. Asanuma, K., et al., *Survivin as a radioresistance factor in pancreatic cancer.* Jpn J Cancer Res, 2000. **91**(11): p. 1204-9.
108. Pennati, M., et al., *Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression.* J Invest Dermatol, 2003. **120**(4): p. 648-54.
109. Dohi, T., et al., *An IAP-IAP complex inhibits apoptosis.* J Biol Chem, 2004. **279**(33): p. 34087-90.
110. Eckelman, B.P., G.S. Salvesen, and F.L. Scott, *Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family.* EMBO Rep, 2006. **7**(10): p. 988-94.
111. Mehrotra, S., et al., *IAP regulation of metastasis.* Cancer Cell. **17**(1): p. 53-64.
112. Salvesen, G.S. and C.S. Duckett, *IAP proteins: blocking the road to death's door.* Nat Rev Mol Cell Biol, 2002. **3**(6): p. 401-10.
113. Coumar, M.S., et al., *Treat cancers by targeting survivin: just a dream or future reality?* Cancer Treat Rev. **39**(7): p. 802-11.
114. Kelly, R.J., et al., *Impacting tumor cell-fate by targeting the inhibitor of apoptosis protein survivin.* Mol Cancer. **10**: p. 35.
115. Nakahara, T., et al., *YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts.* Cancer Res, 2007. **67**(17): p. 8014-21.
116. Mesri, M., et al., *Cancer gene therapy using a survivin mutant adenovirus.* J Clin Invest, 2001. **108**(7): p. 981-90.
117. Kanwar, J.R., S.K. Kamalapuram, and R.K. Kanwar, *Targeting survivin in cancer: the cell-signalling perspective.* Drug Discov Today. **16**(11-12): p. 485-94.
118. Tu, S.P., et al., *Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer.* Cancer Res, 2003. **63**(22): p. 7724-32.
119. Tanioka, M., et al., *Phase I study of LY2181308, an antisense oligonucleotide against survivin, in patients with advanced solid tumors.* Cancer Chemother Pharmacol. **68**(2): p. 505-11.
120. Hansen, J.B., et al., *SPC3042: a proapoptotic survivin inhibitor.* Mol Cancer Ther, 2008. **7**(9): p. 2736-45.
121. Carrasco, R.A., et al., *Antisense inhibition of survivin expression as a cancer therapeutic.* Mol Cancer Ther. **10**(2): p. 221-32.
122. Altieri, D.C., *Survivin and IAP proteins in cell-death mechanisms.* Biochem J. **430**(2): p. 199-205.

123. Hochbaum, D., et al., *Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis.* J Biol Chem, 2008. **283**(8): p. 4464-8.
124. Mei, F.C. and X. Cheng, *Interplay between exchange protein directly activated by cAMP (Epac) and microtubule cytoskeleton.* Mol Biosyst, 2005. **1**(4): p. 325-31.
125. Misra, U.K. and S.V. Pizzo, *Evidence for a pro-proliferative feedback loop in prostate cancer: the role of Epac1 and COX-2-dependent pathways.* PLoS One. **8**(4): p. e63150.
126. Jeong, M.J., et al., *cAMP signalling decreases p300 protein levels by promoting its ubiquitin/proteasome dependent degradation via Epac and p38 MAPK in lung cancer cells.* FEBS Lett. **587**(9): p. 1373-8.
127. Metrich, M., et al., *Epac activation induces histone deacetylase nuclear export via a Ras-dependent signalling pathway.* Cell Signal. **22**(10): p. 1459-68.
128. Ruiz-Hurtado, G., et al., *Epac in cardiac calcium signaling.* J Mol Cell Cardiol. **58**: p. 162-71.
129. Liu, Y. and M.F. Schneider, *Opposing HDAC4 nuclear fluxes due to phosphorylation by beta-adrenergic activated protein kinase A or by activity or Epac activated CaMKII in skeletal muscle fibres.* J Physiol. **591**(Pt 14): p. 3605-23.
130. Grunstein, M., *Histone acetylation in chromatin structure and transcription.* Nature, 1997. **389**(6649): p. 349-52.
131. Mai, A., et al., *Histone deacetylation in epigenetics: an attractive target for anticancer therapy.* Med Res Rev, 2005. **25**(3): p. 261-309.
132. Thiagalingam, S., et al., *Histone deacetylases: unique players in shaping the epigenetic histone code.* Ann N Y Acad Sci, 2003. **983**: p. 84-100.
133. Jenuwein, T., et al., *SET domain proteins modulate chromatin domains in eu- and heterochromatin.* Cell Mol Life Sci, 1998. **54**(1): p. 80-93.
134. Haberland, M., R.L. Montgomery, and E.N. Olson, *The many roles of histone deacetylases in development and physiology: implications for disease and therapy.* Nat Rev Genet, 2009. **10**(1): p. 32-42.
135. Clocchiatti, A., C. Florean, and C. Brancolini, *Class IIa HDACs: from important roles in differentiation to possible implications in tumourigenesis.* J Cell Mol Med. **15**(9): p. 1833-46.
136. Vega, R.B., et al., *Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis.* Cell, 2004. **119**(4): p. 555-66.
137. Ozdag, H., et al., *Differential expression of selected histone modifier genes in human solid cancers.* BMC Genomics, 2006. **7**: p. 90.
138. Kao, G.D., et al., *Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response.* J Cell Biol, 2003. **160**(7): p. 1017-27.

139. Geng, L., et al., *Histone deacetylase (HDAC) inhibitor LBH589 increases duration of gamma-H2AX foci and confines HDAC4 to the cytoplasm in irradiated non-small cell lung cancer.* Cancer Res, 2006. **66**(23): p. 11298-304.
140. Stark, M. and N. Hayward, *Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays.* Cancer Res, 2007. **67**(6): p. 2632-42.
141. Sjoblom, T., et al., *The consensus coding sequences of human breast and colorectal cancers.* Science, 2006. **314**(5797): p. 268-74.
142. Datta, J., et al., *Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis.* Cancer Res, 2008. **68**(13): p. 5049-58.
143. Nasser, M.W., et al., *Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1.* J Biol Chem, 2008. **283**(48): p. 33394-405.
144. Qian, D.Z., et al., *Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589.* Clin Cancer Res, 2006. **12**(2): p. 634-42.
145. Bodily, J.M., K.P. Mehta, and L.A. Laimins, *Human papillomavirus E7 enhances hypoxia-inducible factor 1-mediated transcription by inhibiting binding of histone deacetylases.* Cancer Res. **71**(3): p. 1187-95.
146. Wilson, A.J., et al., *HDAC4 promotes growth of colon cancer cells via repression of p21.* Mol Biol Cell, 2008. **19**(10): p. 4062-75.
147. Zhang, C.L., T.A. McKinsey, and E.N. Olson, *Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation.* Mol Cell Biol, 2002. **22**(20): p. 7302-12.
148. Chang, S., et al., *Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development.* Mol Cell Biol, 2004. **24**(19): p. 8467-76.
149. Milde, T., et al., *HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth.* Clin Cancer Res. **16**(12): p. 3240-52.
150. Ishikawa, S., et al., *The role of oxysterol binding protein-related protein 5 in pancreatic cancer.* Cancer Sci. **101**(4): p. 898-905.
151. Berthouze-Duquesnes, M., et al., *Specific interactions between Epac1, beta-arrestin2 and PDE4D5 regulate beta-adrenergic receptor subtype differential effects on cardiac hypertrophic signaling.* Cell Signal. **25**(4): p. 970-80.
152. Schmidt, M., F.J. Dekker, and H. Maarsingh, *Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions.* Pharmacol Rev. **65**(2): p. 670-709.

153. Chang, C.W., et al., *Involvement of Epac1/Rap1/CaMKI/HDAC5 signaling cascade in the regulation of placental cell fusion.* Mol Hum Reprod. **19**(11): p. 745-55.
154. Ohnuki, Y., et al., *Role of cyclic AMP sensor Epac1 in masseter muscle hypertrophy and myosin heavy chain transition induced by beta2-adrenoceptor stimulation.* J Physiol. **592**(Pt 24): p. 5461-75.
155. Pereira, L., et al., *Novel Epac fluorescent ligand reveals distinct Epac1 vs. Epac2 distribution and function in cardiomyocytes.* Proc Natl Acad Sci U S A. **112**(13): p. 3991-6.
156. Rehmann, H., et al., *Communication between the regulatory and the catalytic region of the cAMP-responsive guanine nucleotide exchange factor Epac.* J Biol Chem, 2003. **278**(26): p. 23508-14.
157. Rehmann, H., et al., *Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac.* J Biol Chem, 2003. **278**(40): p. 38548-56.
158. Rehmann, H., *Epac2: a sulfonylurea receptor?* Biochem Soc Trans. **40**(1): p. 6-10.
159. Herbst, K.J., et al., *Direct activation of Epac by sulfonylurea is isoform selective.* Chem Biol. **18**(2): p. 243-51.
160. Tsalkova, T., F.C. Mei, and X. Cheng, *A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists.* PLoS One. **7**(1): p. e30441.
161. Courilleau, D., et al., *Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac.* J Biol Chem. **287**(53): p. 44192-202.
162. Courilleau, D., et al., *The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1).* Biochem Biophys Res Commun. **440**(3): p. 443-8.
163. Brattain, M.G., et al., *Characterization of human colon carcinoma cell lines isolated from a single primary tumour.* Br J Cancer, 1983. **47**(3): p. 373-81.
164. Brattain, M.G., et al., *Heterogeneity of human colon carcinoma.* Cancer Metastasis Rev, 1984. **3**(3): p. 177-91.
165. Marek, L., et al., *Histone deacetylase (HDAC) inhibitors with a novel connecting unit linker region reveal a selectivity profile for HDAC4 and HDAC5 with improved activity against chemoresistant cancer cells.* J Med Chem. **56**(2): p. 427-36.
166. Gloerich, M. and J.L. Bos, *Epac: defining a new mechanism for cAMP action.* Annu Rev Pharmacol Toxicol. **50**: p. 355-75.

167. Harper, K., et al., *Autotaxin promotes cancer invasion via the lysophosphatidic acid receptor 4: participation of the cyclic AMP/EPAC/Rac1 signaling pathway in invadopodia formation.* Cancer Res. **70**(11): p. 4634-43.
168. Conrotto, P., et al., *Interactome of transforming growth factor-beta type I receptor (TbetaRI): inhibition of TGFbeta signaling by Epac1.* J Proteome Res, 2007. **6**(1): p. 287-97.
169. Grozinger, C.M. and S.L. Schreiber, *Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization.* Proc Natl Acad Sci U S A, 2000. **97**(14): p. 7835-40.
170. Wang, A.H. and X.J. Yang, *Histone deacetylase 4 possesses intrinsic nuclear import and export signals.* Mol Cell Biol, 2001. **21**(17): p. 5992-6005.
171. Lenoir, O., et al., *Specific control of pancreatic endocrine beta- and delta-cell mass by class IIa histone deacetylases HDAC4, HDAC5, and HDAC9.* Diabetes. **60**(11): p. 2861-71.
172. Candia, A.F., et al., *Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads.* Development, 1997. **124**(22): p. 4467-80.
173. Stronach, E.A., et al., *HDAC4-regulated STAT1 activation mediates platinum resistance in ovarian cancer.* Cancer Res. **71**(13): p. 4412-22.
174. Xu, X.S., et al., *Histone deacetylases (HDACs) in XPC gene silencing and bladder cancer.* J Hematol Oncol. **4**: p. 17.
175. Urbich, C., et al., *HDAC5 is a repressor of angiogenesis and determines the angiogenic gene expression pattern of endothelial cells.* Blood, 2009. **113**(22): p. 5669-79.
176. Koh, M.Y., T.R. Spivak-Kroizman, and G. Powis, *HIF-1alpha and cancer therapy.* Recent Results Cancer Res. **180**: p. 15-34.
177. Suwaki, N., et al., *A HIF-regulated VHL-PTP1B-Src signaling axis identifies a therapeutic target in renal cell carcinoma.* Sci Transl Med. **3**(85): p. 85ra47.
178. Qian, D.Z., et al., *Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha.* Cancer Res, 2006. **66**(17): p. 8814-21.
179. Seo, H.W., et al., *Transcriptional activation of hypoxia-inducible factor-1alpha by HDAC4 and HDAC5 involves differential recruitment of p300 and FIH-1.* FEBS Lett, 2009. **583**(1): p. 55-60.
180. Geng, H., et al., *HDAC4 protein regulates HIF1alpha protein lysine acetylation and cancer cell response to hypoxia.* J Biol Chem. **286**(44): p. 38095-102.

Chapter 2

Identification of epigenetic targets responsible in silencing tumor suppressor gene - TGFβRII in Colorectal Cancer

Abstract

Identification of epigenetic targets responsible in silencing tumor suppressor gene - TGFβRII in Colorectal Cancer

Catherine Murari-Kanti, Ph.D.

University of Nebraska Medical Center, 2015

Advisor: Michael G. Brattain, Ph.D

Colorectal cancer is the 3rd leading cause of cancer deaths in the United States. Activation of oncogenes and silencing of tumor suppressor genes (TSG) contribute to the development and progression of colorectal cancer. One such TSG is the transforming growth factor β receptor II (TGFβRII) which results in loss of growth inhibitory TGFβ signaling and is a common event in cancer progression. Although mutation of this TSG is common, especially in tumors which exhibit microsatellite instability there is increasing evidence that TGFβRII is epigenetically silenced in many different cancers. Restoration of TGFβ signaling by re-introduction of TGFβRII results in anti-cancer effects in colon and other cancer cell lines.

Histone deacetylases (HDACs) are involved in the mechanism of epigenetic silencing. These enzymes remove the acetyl groups from lysine tails of histones usually resulting in gene repression. HDACs are classified into 4 groups (Classes

I, IIa, IIb, III and IV) and, except for Class III, need the zinc cation for their function. Deacetylation by Class III HDACs (Sirtuins) is NAD^+ mediated. Histone deacetylase inhibitors (HDACi) have recently entered clinical trials and are effective in inhibiting growth and inducing apoptosis in many hematological malignancies but results against solid tumors as single therapies has been disappointing. A significant effect of these drugs is reactivation of TSGs via histone deacetylation inhibition which results in alterations in the chromatin permitting transcription of these silenced genes.

Our laboratory has previously demonstrated that the pan HDACi Belinostat, which inhibits Class I and II HDACs, effectively induced TGFβRII expression in cancer cell lines with epigenetically silenced receptor restoring TGFβ downstream signaling effects including the TGFβ dependent decrease in survivin. Therefore, HDACi provide a potential therapy to restore the growth inhibitory and apoptotic effects of the TGFβ inhibitory pathway. We hypothesize that the identification of the specific HDACs involved in reactivation of epigenetically silenced TGFβRII would allow the use of more specific HDACi's which would increase the therapeutic index of these drugs, decrease side effects and permit more effective use in combination therapies.

We are using both genetic and pharmacological approaches to identify the specific HDACs involved in reactivation of TGFβRII. We have performed

lentiviral shRNA knockdown of HDAC1, 2 and 3 and observed that KD of HDACs 1 and 3 caused the induction of TGFβRII. However knock down of HDAC2 had no effect on TGFβRII expression. Treatment of colon cancer cells which exhibit epigenetically silenced TGFβRII with the HDACi Mocetinostat, specific for the Class I HDACs1, 2, 3 and 11, resulted in robust TGFβRII expression. However, treatment with Droxinostat, specific for HDACs 6 and 8, and at higher concentrations HDAC3, did not result in TGFβRII induction until concentrations effective in inhibiting HDAC3 were achieved. Treatment of colon cancer cells with histone methyltransferase inhibitors – UNC0638 and DZNEP – G9a and EZH2 inhibitors respective induced TGFβRII. HDAC inhibitors are able to induce TGFβRII but also cause a decrease in histone methyl transferases G9a and EZH2 confirming their role in the silencing of TSG TGFβRII.

Introduction:

Colorectal Cancer (CRC)

CRC is the the 3rd most common cancer and the 3rd most fatal cancer in the United States. According to the American Cancer Society, 136,830 men and women were diagnosed with CRC in 2014, out of which 50,310 died from the disease. This cancer develops slowly over a period of 5-10years [181]. Contributing factors include sedentary lifestyle, obesity and a diet rich in red meat but lacking in fresh fruit, vegetables and fiber[182-185]. Regular colonoscopies (screening) have improved outcomes with early detection. However, 1/3rd of patients diagnosed with CRC have advanced disease with metastases or will relapse with metastatic disease. The 5-year survival rate for patients with distant metastasis is 12% [186]. There is a need to understand the progression of the disease from normal to carcinomas to metastatic disease as well as develop therapies that will combat advanced CRC at each step.

Multiple genetic mutations are responsible for normal colon epithelium to develop into a cancerous tissue [187]. (Figure1) Early studies on CRC have determined the genetic targets in CRC with the help of 2 inherited syndromes – Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HPNCC) also known as the Lynch syndrome [188-191]. The

adenomatous polyposis coli (APC) protein is involved in cell cycle regulation, cell death and proliferation and plays a central role in FAP where benign polyps develop from the normal colon. Mutation of the APC gene allows for the aberrant proliferation of the colon cells. Benign polyps then progress to form adenomas and eventually into full-blown carcinomas [192, 193].

Lynch syndrome occurs when an inherited mutation occurs in the mismatch repair genes (MMR) which include – MutL homolog 1 (MLH1), MutS protein homolog 2 (MSH2), MutS homolog 6 (MSH6) or postmeiotic segregation increased 2 (PMS2) [194]. Insertions or deletion mutations that occur in any one of these genes at the nucleotide repeat sequences also known as microsatellites cause microsatellite instability (MSI) and this is common in many tumor suppressor genes (TSG) like transforming growth factor receptor (TGFβRII) [195-197]. Around 30% of all tumors in CRC have TGFβRII mutations [198].

CRC progresses from a benign polyp to an adenomatous polyp. If detected at this stage and surgically excised, the patient is rescued from cancer development. If not removed, the adenomatous polyp may progress into an adenoma (surgery + chemotherapy), to carcinoma (radiation, surgery and chemotherapy) and finally to metastatic disease to other organs in the body. Genes such as APC, K-ras, p53, Smad4 and TGFβRII are mutated in CRC [199-203].

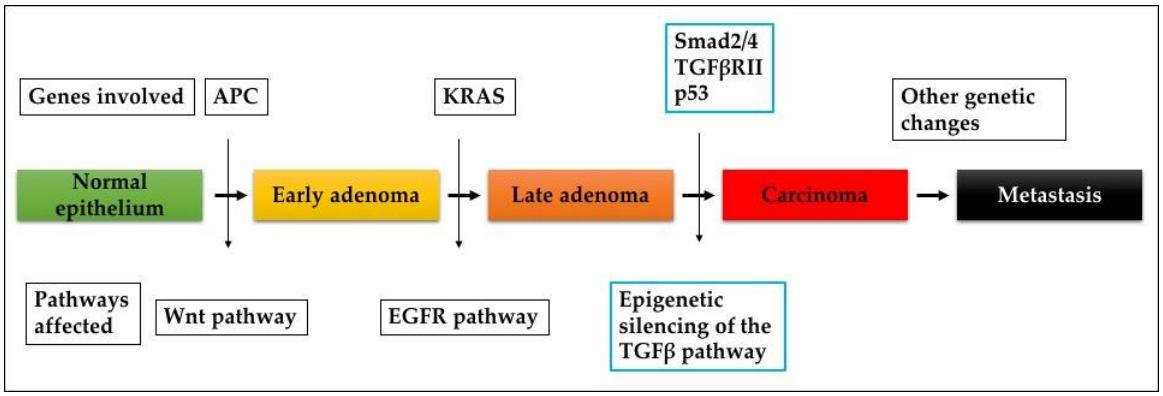


Figure 1. Genetic changes in different genes involved in tumorigenesis and progression of CRC.

TGFβ signaling pathway

TGFβ signaling generates a growth inhibitory pathway that is pivotal in tumor suppression in CRC. It has been reported that TGFβ signaling can promote tumorigenesis in advanced stages of other cancers, particularly breast cancer [204]. However, the Brattain Laboratory has demonstrated that reconstitution of TGFβ receptors into cell lines lacking receptor expression inhibits progression and metastasis [205].

The TGFβ family comprises 3 isoforms – 1, 2 and 3, that possess a high degree of homology and are part of a family of structurally related proteins such as the activins, inhibins, bone morphogenetic factors, growth differentiation factors, glial cell line-derived neurotrophic factors and anti-mullerian hormones [62, 206-209]. The TGFβ1 isoform is the most abundant and is often referred to TGFβ.

TGFβ functions through the TGFβ receptors I and II (TGFβRI and TGFβRII) that are serine threonine kinases [210]. TGFβ binds to TGFβRII and causes it to autophosphorylate itself leading to activation of the TGFβ canonical pathway. Phosphorylated TGFβRII sequesters TGFβRI and trans-phosphorylates it. TGFβRI in turn phosphorylates the receptor-Smads (R-Smads which are Smads 2 and 3) that associate with the Co-Smads (Smad4). This entire Smad complex then translocates to the nucleus where it interacts with promoter regions of different downstream genes [211].

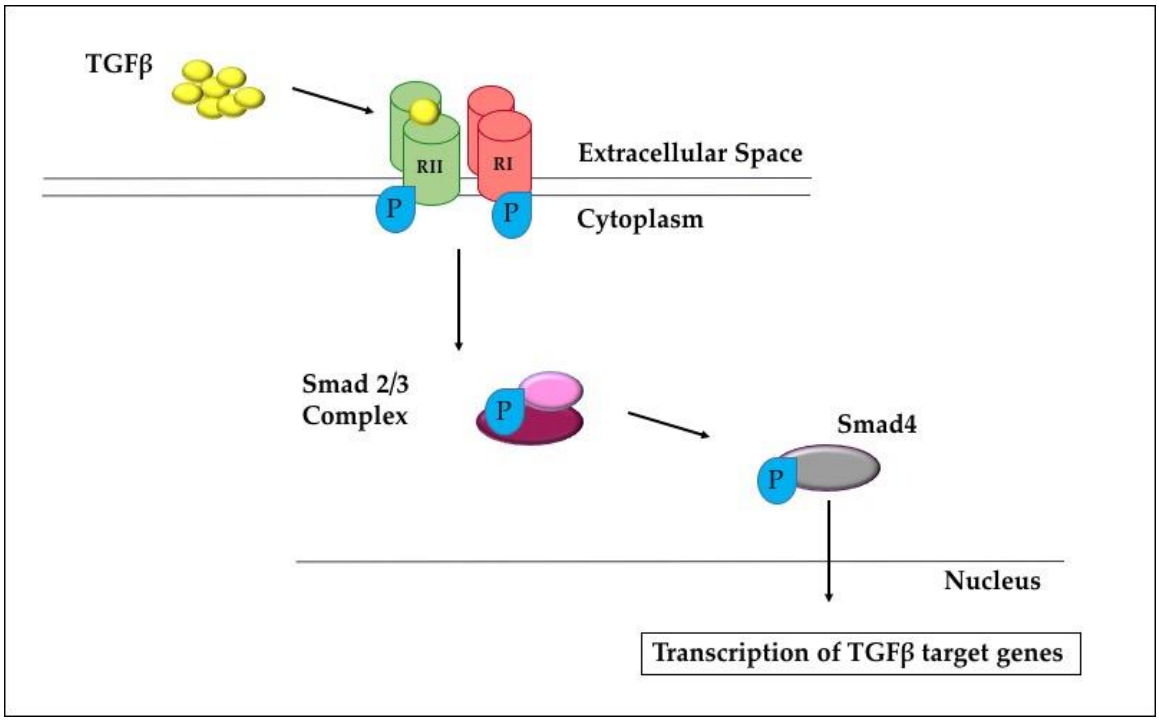


Figure 2: The canonical TGFβ signaling pathway.

Epigenetic Silencing of TGFβRII

Mutation of TGFβRII is common microsatellite instability CRC. However, TGFβRII is commonly lost in microsatellite stable CRC. This is achieved through epigenetic silencing. Many studies have shown that neither promoter mutation nor DNA methylation is responsible for the silencing of TGFβRII. Chromatin immunoprecipitation (ChIP) revealed that at the TGFβRII promoter there was a decreased histone 3 (H3) acetylation and an increased histone3lysine9 trimethylation (H3K9) – suggesting that epigenetic changes (silencing) of the TGFβRII promoter are responsible for its loss in cancer. These epigenetic changes are mediated by histone deactylases (HDACs) or histone methytransferases (HMTs) [212, 213].

Histone Deacetylases

The acetylation status of the ε-amino of lysines on histones renders chromatin active or inactive. The addition of the negative charge opens up the chromatin structure for active transcription Hyperacetylation is associated with active transcription while hypoacetylation is responsible for gene repression [130, 131]. (HDACs are classified into 4 main classes – Classes I, II, III and IV. Of these, the class III HDACs are called sirtuins and require Nad+ for their activity while the other three classes require a Zn^{2+} cation for their function. There are a total of 11 HDACs plus sirtuins that fall in the HDAC category [132, 133].

HDACs are overexpressed in many kinds of cancers including CRC and are usually associated with poor prognosis [214-221]. (Figure 3) Osada and co-workers studied the epigenetic silencing of TGFβRII in lung cancer cell lines and showed increased H3K9 methylation (silencing) and decreased H3K4 (activation) leading to increased TGFβRII silencing [222, 223].

Class	HDAC	Localization	Role in cancer	Type of cancer
I	HDAC1	Nucleus	Over/under expression	Colon, pancreatic, prostate etc.
	HDAC2	Nucleus	Overexpresssion/mutation	Prostate, colon, gastric
	HDAC3	Nucleus	Overexpression	Endometrial
	HDAC8	Nucleus	Overexpression	Colon
IIA	HDAC4	Nucleus/Cytoplasm	Over & underexpression	Colon, pancreatic, prostate, breast
	HDAC5	Nucleus/Cytoplasm	Overexpression/mutation	Colon, pancreatic
	HDAC7	Nucleus/Cytoplasm	Overexpression	Colon, pancreatic
	HDAC9	Nucleus/Cytoplasm	Over and under expression	Mixed
IIB	HDAC6	Mostly Cytoplasm	Overexpression	Breast, AML
	HDAC10	Mostly Cytoplasm	Over and underexpression	Hepatic, colon, multiple myeloma
IV	HDAC11	Nucleus/Cytoplasm	Overexpression	Neuroblastoma, renal cancer

Figure 3: Classification of HDACs and their status in different cancers.

Histone methytransferases (HMTs)

Histone methylation usually occurs on ε-lysines of histones 3 and 4 and can be associated with both gene activation and silencing [130, 224]. G9a is an HMT that catalyzes the histone-3 lysine-9 methylation (H3K9me) and this reaction is usually associated with euchromatic gene silencing. G9a and G9a-like protein partner with each other and are responsible for the H3K9methylation, which can be either mono- or di-methylation [225, 226].

EZH2 is yet another HMT that is part of the polycomb repressive complex and is responsible for H3K27 methylation that is again associated with gene silencing [227]. The roles of HMTs in the silencing of TGFβRII have not been studied yet but since there is plasticity in epigenetic silencing, it is likely that methylation works in concert with deacetylation to produce the observed transcriptional repression [227, 228].

HDAC inhibitors (HDACi's)

HDACs are aberrantly overexpressed in different cancer and therefore present themselves as excellent therapeutic targets. Development of the different HDACi's was based on their chemical structure and their ability to obstruct the Zn^{2+} cation that is required for HDAC activity [229, 230]. The pan-HDACi's are selective for Class I, II and IV HDACs but do not affect sirtuins [231]. Initial pan-

HDACi's inhibited all HDACs to differing degrees but more class selective HDACi's are now becoming available [232, 233].

The different chemical groups are as follows:

1. Hydroxamic acids (TSA and Vorinostat)
2. Carboxylic acid (Valproate and Butyrate)
3. Aminobenzamides (Etinostat and Mocetinostat)
4. Cyclic peptides (Apicidin, Romidepsin)
5. Epoxyketones (Trapoxin)

- **MGCD0103 (Mocetinostat)**

  This is an aminobenzamide HDAC inhibitor that inhibits HDACs 1, 2, 3 and 11 at nanomolecular ranges and is most effective against HDACs1 and 2. It has the ability to prevent cell proliferation, cause cell cycle arrest with the induction of p21 and is active in-vitro for about 48hrs. The drug is knows to affect only cancer cells and not normal human cells, so providing a therapeutic index [234-238].

- **Droxinostat**

  This HDACi is a hydroxamic acid moiety that inhibits HDACs 3, 6 and 8 and is able to cause cancer cell death by activating the death ligands FAS and tumor necrosis factor related apoptosis inducing ligand. The

hydroxamic acid moiety is important for its function. It inhibits HDAC 6 and 8 at much lower concentrations than it does HDAC3 [239, 240].

HMT inhibitors:

These inhibitors have the ability to prevent the methylation of histones and so permit the transcription of TSGs. Drugs developed against HMTs include UNC0638 (G9a inhibitor), EPZ004777 (DOTL1 inhibitor), AZO5 (SMYD2 inhibitor) and DZNEP (EZH2 inhibitors) [241].

- **UNC0638 (UNC)**

  It is a selective inhibitor of G9a and GLP in the low nanomolar range. It functions by blocking the enzymatic activity of G9a/GLP and therefore does not affect protein or mRNA levels. It inhibits H3K9 methylation with high potency and low toxicity [242, 243].

- **DZNEP**

  This selective inhibitor of EZH2 exhibits only mild toxicity against normal human cells. The drug has proven to be anti-tumorigenic in breast, lung, brain, prostate and liver cancer. It has the ability to inhibit cell migration and invasion. DZNEP when combined with HDACi Panobinostat had better outcomes in Acute myeloid leukemia [244-248].

Rationale and Hypothesis:

The Brattain Laboratory has shown that introduction of TGFβRII through genetic expression into CRC with epigenetically silenced receptor decreases tumorigenecity and metastatic capacity. Conversely, introduction of a dominant negative TGFβRII into the FET CRC cell line, which retains low levels of TGFβ signaling, results in acquisition of tumorigenecity in vivo. Therefore, reactivation of expression of TGFβRII in CRC, where this tumor suppressor gene (TSG) is epigenetically silenced might represent a potential useful therapeutic strategy given the growth inhibitory and anti-metastatic effects of the TGFβ signaling pathway. The development of epigenetic drugs affords an opportunity to utilize this potential therapeutic benefit. However, current HDACi's inhibit all classes of HDACs except the sirtuins. Identification of the epigenetic enzymes (HDACs and HMTs) involved in the epigenetic silencing of TGFβRII would allow for the development of improved therapy, due to fewer off target effects.

Previous work in our lab demonstrated that pan-HDACi Belinostat was able to induce the expression of the TSG TGFβRII in CRC cell lines with epigenetically silenced TGFβRII. Induction of TGFβRII was associated with decrease in inhibitor of apoptosis (IAP) proteins X-linked IAP (XIAP) and survivin [211].

Belinostat is a pan-HDAC and inhibits all classes of HDACs except Class III HDACs (sirtuins) and therefore is associated with higher incidence of side and off-target effects[249]. Therapy would be improved by the development of more selective HDACs. Therefore there is a need to discover the HDACs responsible in the silencing of TGFβRII and develop therapeutic HDACi's to target them. The TGFβRII promoter lacks a specific transcription start site (no TATA box) but contains GC boxes, which bind Sp1 and Sp3. The Brattain laboratory reported that on HDACi treatment, Sp3 becomes acetylated by p300. Sp1 and Sp3 are bound to HDACs that cause TGFβRII to stay in an inactive state. On treatment with HDACi TSA, acetylated Sp3 along with Sp1 acts as a transcriptional activator leading to the reactivation of expression of TGFβRII [250]. (Figure 4) The induction of TGFβRII upon treatment with pan-HDACi's points to an essential role of HDACs in the silencing of TGFβRII. Futher, although histone methylation status changes with TGFβRII induction, the HMTs involved in TGFβRII silencing have not been studied [223].

Therefore we hypothesize that the identification of the specific HDAC/HDACs or HMT/HMTs involved in the reactivation of epigenetically silenced TGFβRII would allow the use of more specific HDACi's/HMT inhibitors which would increase the therapeutic index of these drugs, decrease side effects and permit more effective use in combination therapies.

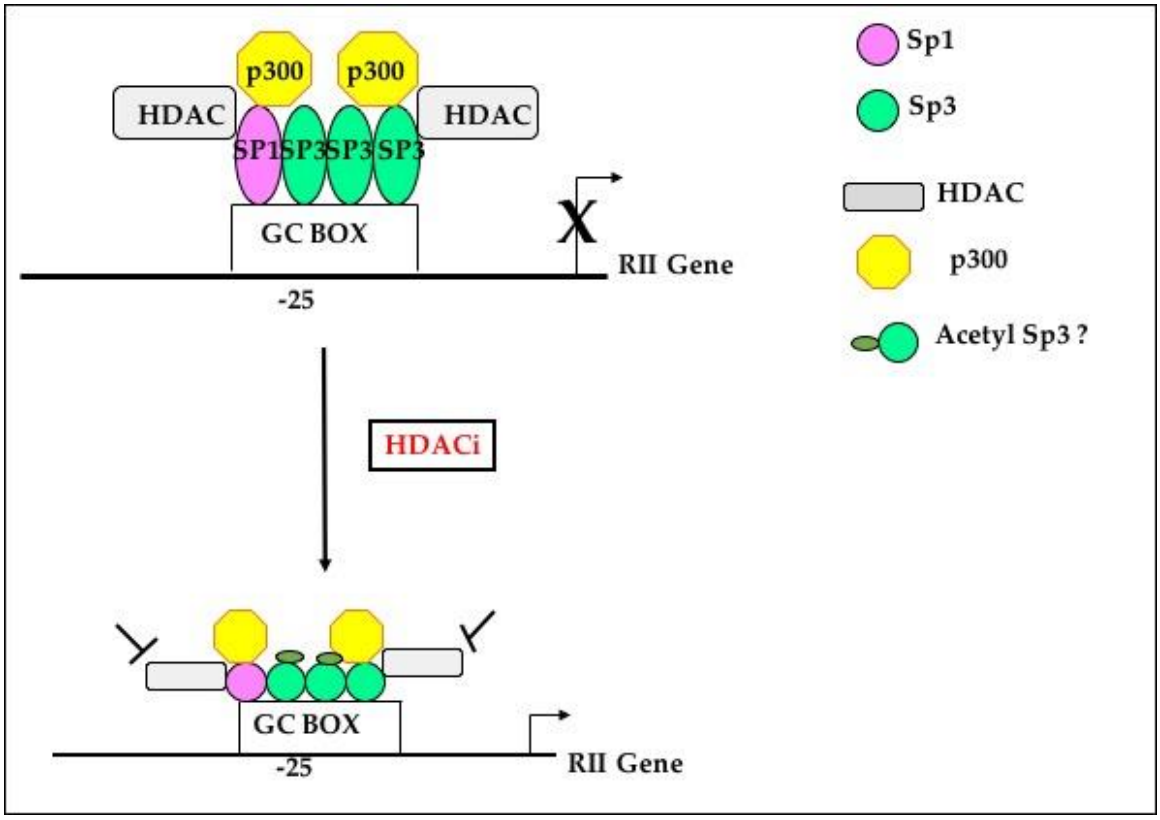


Figure 4: Effect of HDACs on the promoter region of TGFβRII.

Materials and Methods:

Cell culture and Reagents

CRC cell lines were derived from patient tumors and grown in serum free (SF) medium that contains Supplemental McCoy's 5A (Sigma Aldrich), 5ng/ml epidermal growth factor (EGF) (R&D Systems), 20μg/ml insulin (Sigma) and 4μg/ml transferrin (Sigma).

The FET cell line is non-tumorigenic and retains low levels of TGFβ signaling. The CBS cell line retains metastatic capability [163, 164].

All cell lines were grown and incubated at 37°C in a humidified atmosphere of 6% CO2.

MGCD0103 (S1122) and Droxinostant (S1422) were purchased from Selleck, while UNC0638 (U4885) and DZNEP (SML0305) were purchased from Sigma-Aldrich.

Western Blotting and Antibodies:

Colon cancer as well pancreatic cancer cells were grown to the required number days for confluence after drug treatment. Cells were washed 3 times with cold phosphate buffer saline (PBS) that contains 0.1% EDTA. Washed cells were scraped and lysed with TNESV buffer [50mmol/L Tris (Ph 7.5), 150mmol/L NaCl,

1% NP40, 50mmol/L NaF, 1mmol/L Na_3VO_4, 25μg/ml β-glycerophosphate, 1mmol/L phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche,Indianapolis, IN)]. After 30min incubation with the lysis buffer, cells were centrifuged at 14000g's for 20min at 4°C. The supernatant was extracted and protein in it was quantified using the bicinchoninic acid(BCA; Pierce). The protein was diluted in SDS sample buffer (50mM Tris, ph 6.8, 1% SDS, 10% glycerol, 0.03% bromophenol blue and 1% β-mercaptoethanol) and separated by SDS-PAGE (7.5-15% gels). The gels were then transferred by electroblotting (100V for 90min or overnight at 10V) onto a nitrocellulose membrane (GE Amersham). Post transfer, the membrane was blocked with 5% non-fat dry milk in TBS-T (150mmol/L NaCl, 10mmol/L Tris-HCl pH7.4, 0.05% Tween 20) for 1hr at room temperature or overnight at 4°C. The membrane was cut at requisite molecular weights and primary antibody was added in either 5% non-fat dry milk or 5% bovine serum albumin (BSA) in TBS-T for 2hr at room temperature or overnight at 4°C according to the antibody data sheets. The membrane was washed three times for 10min in TBS-T and the appropriate secondary was added in 5% non-fat dry milk for 1hr at room temperature. The membrane was washed three more times with TBS-T for 10min at room temperature and the proteins were detected by the enhanced chemiluminescence system (ECL; GE Amersham). TGFβRII (sc-177799) antibody was purchased from Santa Cruz.

pSmad3 (#9520) HDAC1(#2062), HDAC2(#5113), HDAC3(#3949), Survivin (#2308), G9a (#3306) and EZH2(#5246) antibodies were purchased from Cell Signaling. GAPDH (G8795) was acquired from Sigma.

Transient transfection:

HDAC2 (sc-29345) and HDAC3 (sc-35538) siRNA were purchased from Santa Cruz Biotechnology Inc., and knockdown was performed according to the manufacturer's protocol.

Stable transfection:

HDAC1 shRNA (sc-29343) was purchased from Santa Cruz Biotechnology Inc. FET and CBS cell lines were plated in 10cm plates in serum free medium which was changed to Opti-MEM media (Invitrogen), when they were 40% confluent. The cells were transfected with a pool of 3 shRNA's directed against HDAC1. Two stable clones were selected and used in this study.

Results:

1. **MGCD0103 induces TGFβRII (RII) in colon cancer cells and reduces cell survival.**

   In order to begin to dissect the specific HDACs involved in the epigenetic silencing of TGFβRII, we used some of the newer generation HDACi's which exhibit a more limited range of HDAC selectivity. Mocetinostat (MGCD0103) inhibits HDACs 1, 2, 3 and 11. We first tested the effect of this drug on the CBS cell line that has an epigenetically silenced TGFβRII, and FET cells which retain a low level of TGFβ signaling. MGCD0103 induced increased TGFβRII expression by 48hrs. (Figure 1a) The induction was dose dependent. This induction of TGFβRII was accompanied by a dose-dependent decrease in IAP protein, survivin.

   We next determined whether MGCD0103 would induce TGFβRII expression in the CBS CRC cell line that has an epigenetically silenced TGFβRII and lacks functional TGFβ signaling. Again MGCD0103 induced TGFβRII expression by 24hours, accompanied by decreased survivin.

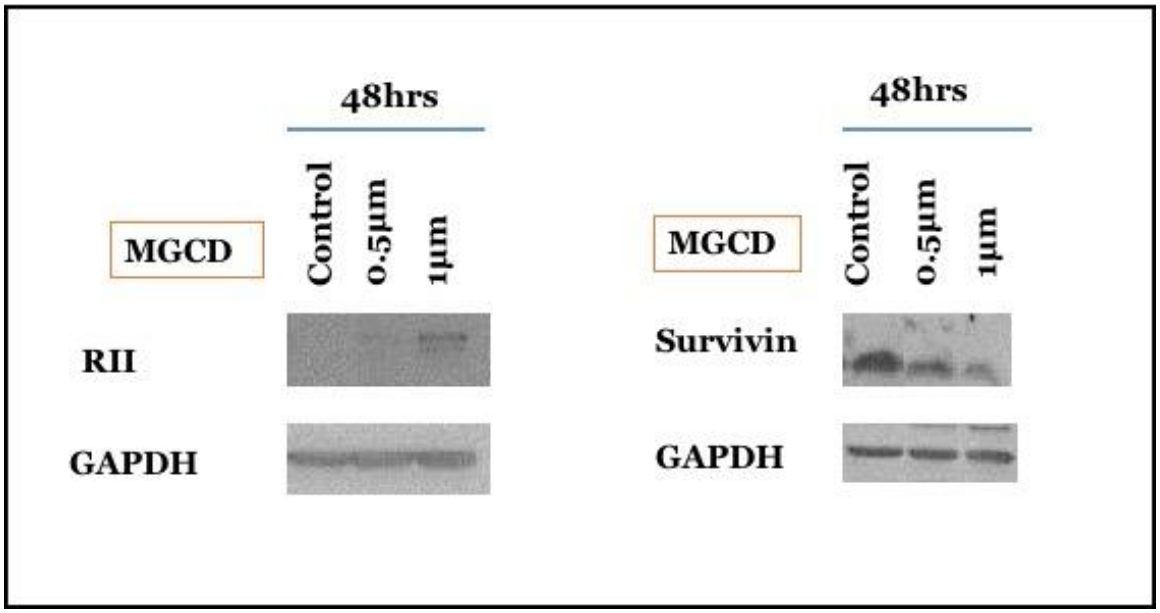


Figure 1a: Mocetinostat (MGCD) inhibits HDACs 1, 2, 3 and 11 and induces expression of TGFβRII at 48hours in a dose dependent manner, accompanied by a decrease in IAP protein, survivin; in the FET CRC cell line. Data shown are representative of three independent experiments.

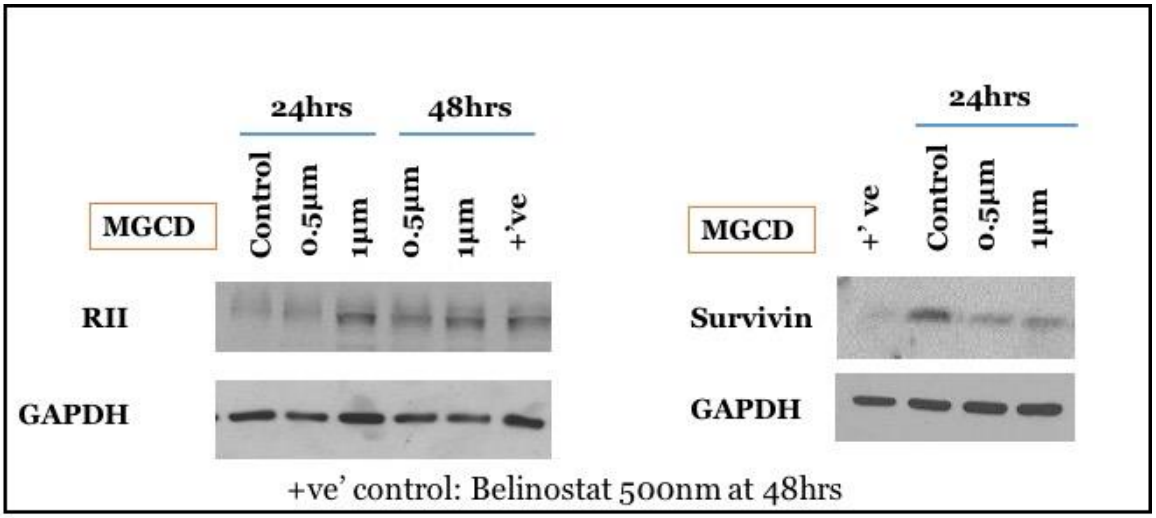
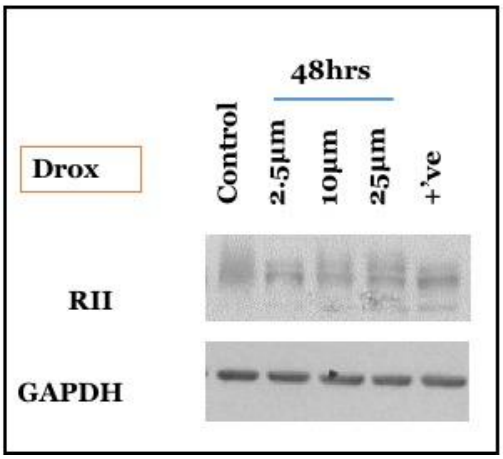


Figure 1b: Mocetinostat (MGCD) inhibits HDACs 1, 2, 3 and 11 and induces expression of TGFβRII at 48hours in a dose dependent manner, accompanied by a decrease in IAP protein, survivin; in the CBS CRC cell line which has epigenetically silenced TGFβRII. CBS cells were treated with Belinostat, a pan-HDACi at 500nm for 48hrs as a positive control. Data shown are representative of three independent experiments.

2. Inhibition of HDACs 3, 6 and 8 by Droxinostat does not induce TGFβRII in CRC cell lines.

Droxinostat inhibits the ClassII HDACs 6 and 8 with IC50 of 2.47 and 1.46 μm, respectively, with inhibition of HDAC3 at around 10-fold higher concentrations. Treatment of the CBS CRC cell line, which has epigenetically silenced TGFβRII, with Droxinostat did not induce TGFβRII at 10um. TGFβRII expression was restored to control level at 25μm which would correspond to the same concentration at which HDAC3 inhibited.



+ve' control: Belinostat 500nm at 48hrs

Drox: Inhibits predominantly HDACs 6 and 8 (higher dose inhibits HDAC3)

Figure 2. Treatment of the CBS CRC cell line with Droxinostat saw no induction of TGFβRII at the lower concentrations but levels equivalent to the control at higher concentrations, when HDAC3 would get inhibited. Data shown are representative of three independent experiments.

3. Genetic approaches to confirm that HDAC1 and HDAC3 are involved in the epigenetic silencing of TGFβRII.

The drug studies indicate that HDAC1 and HDAC3 may be key players in the epigenetic silencing of TGFβRII. Therefore, we performed stable knockdown (KD) of HDAC1, generating clones 7 and 9 in the FET CRC cell line and clones 6 and 17 in the CBS CRC cell line. siRNA was used to knock down HDACs 2 and 3 and TGFβRII expression was determined. KD of HDAC2 was performed as a control to confirm that only the KD of HDAC1 and HDAC3 resulted in increased TGFβRII expression.

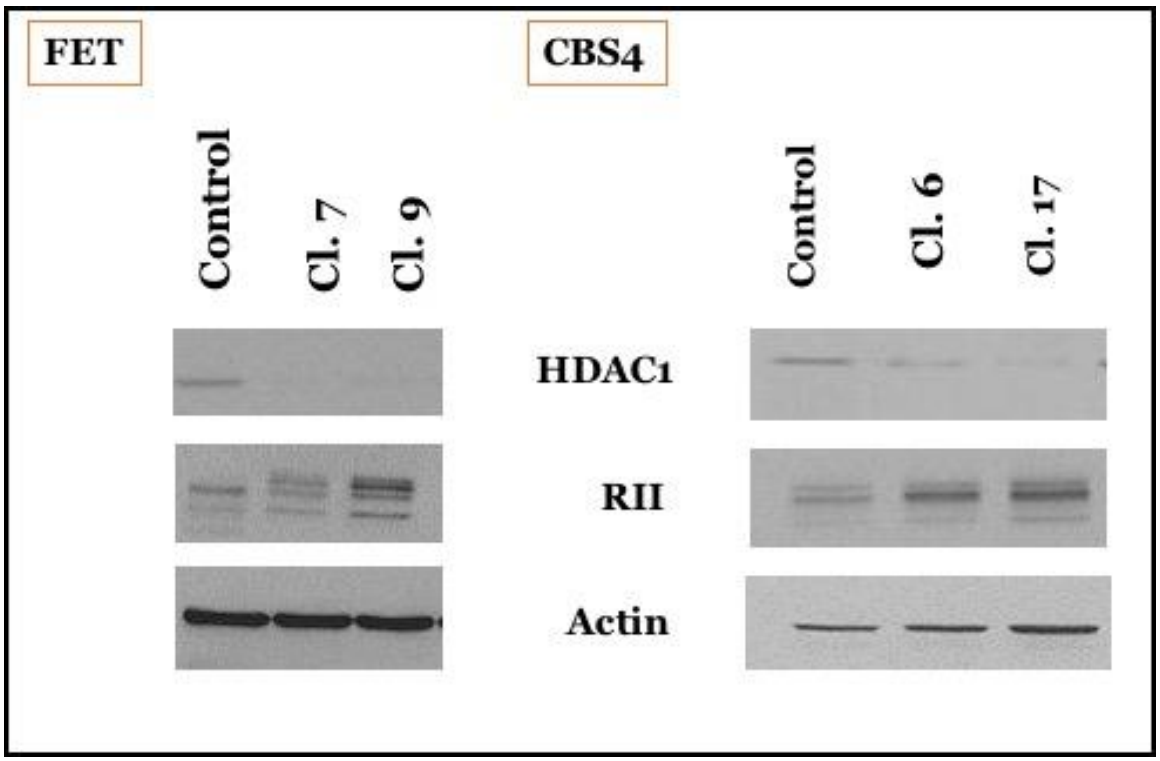


Figure 3a: KD of HDAC1 in both the FET and CBS CRC cell lines caused the re-expression of TGFβRII.

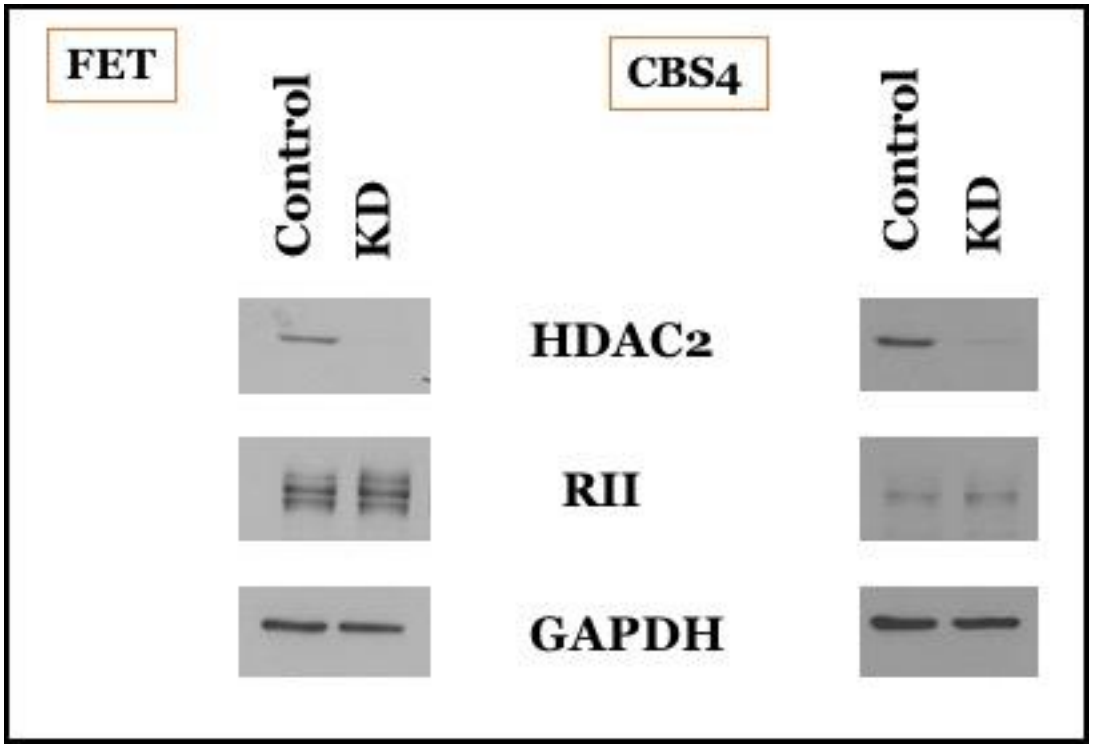


Figure 3b: KD of HDAC2 in both the FET and CBS CRC cell lines did not induce TGFβRII expression. Data shown are representative of three independent experiments.

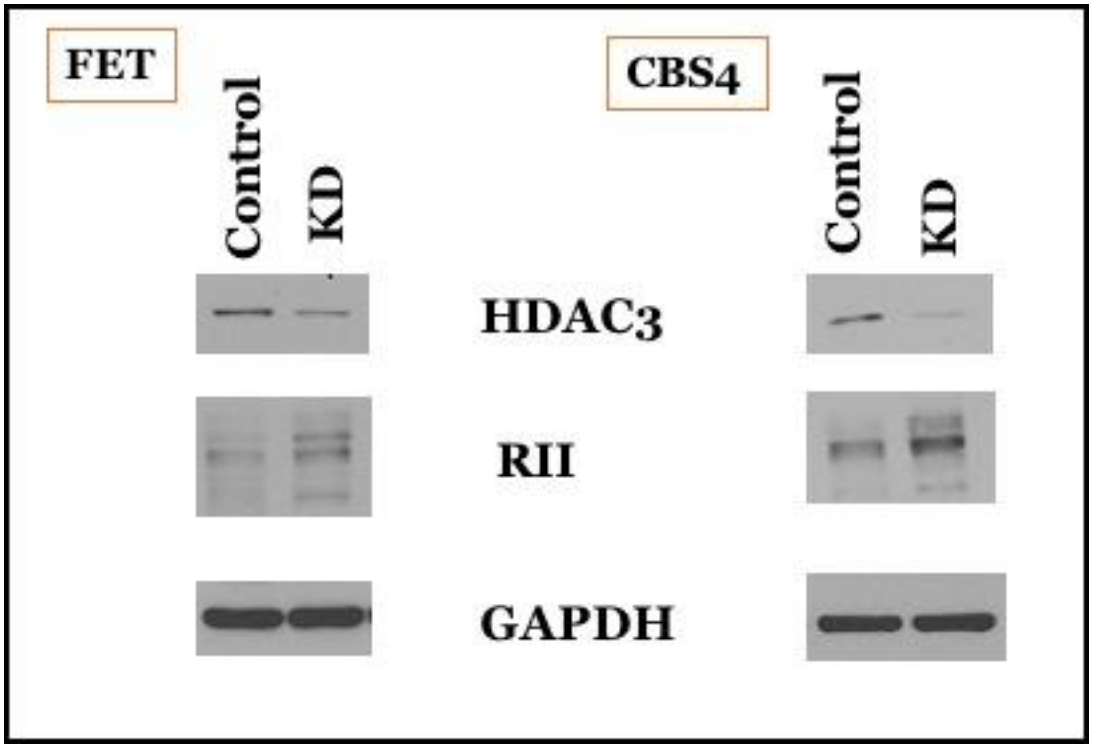


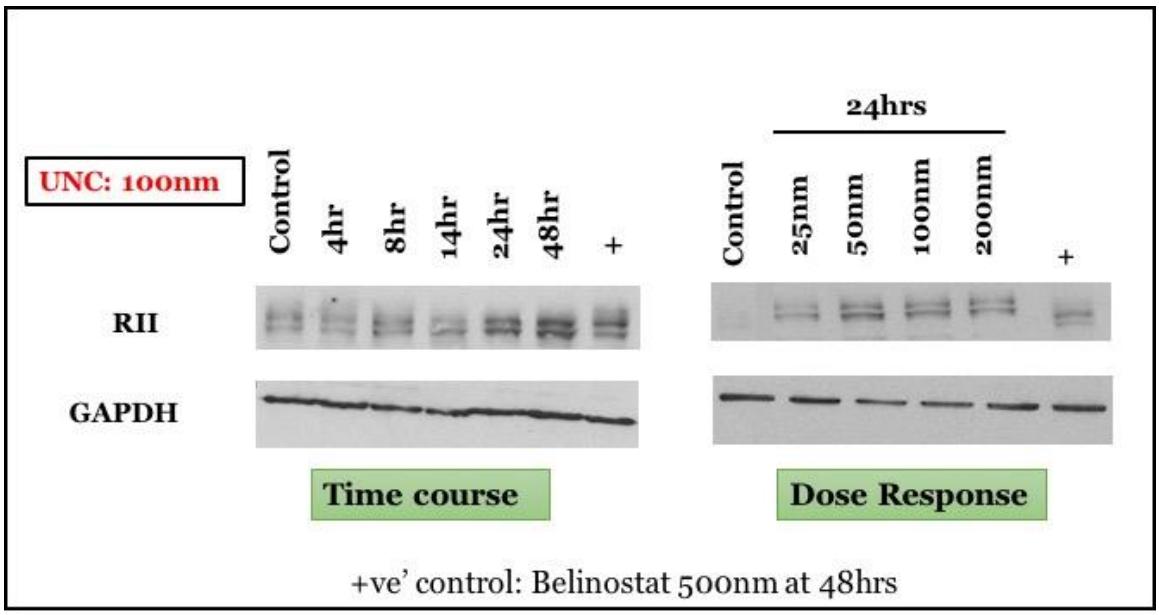
Figure 3c: KD of HDAC3 in both the FET and CBS CRC cell lines caused the re-expression of TGFβRII. Data shown are representative of three independent experiments.

4. The role of HMTs G9a and EZH2 in the epigenetic silencing of TGFβRII.

The role of HDACs in the epigenetic silencing of TGFβRII has been well established. However, epigenetic silencing also involves histone methylation. We hypothesized that HMTs activity might be involved in the epigenetic silencing of TGFβRII. Therefore in order to address whether HMTs are involved in the silencing of TGFβRII we used specific G9a and EZH2 inhibitors, UNC0638 and DzNEP, respectively.

Treatment of CRC cell lines CBS with UNC0638 allowed for the re-expression of TGFβRII in a time dependent manner- with maximal effect at 48 hours and in a dose dependent manner. Whole cell lysates from FET cells treated with Belinostat were run as positive control.

To confirm that the inhibition of G9a was inducing the expression of TGFβRII and reactivating the TGFβ inhibitory pathway, we examined the phosphorylation status of Smad3 upon activation. Smad3 is phosphorylated when the canonical TGFβ pathway is activated. Smad3 was activated following drug treatment of the cells.



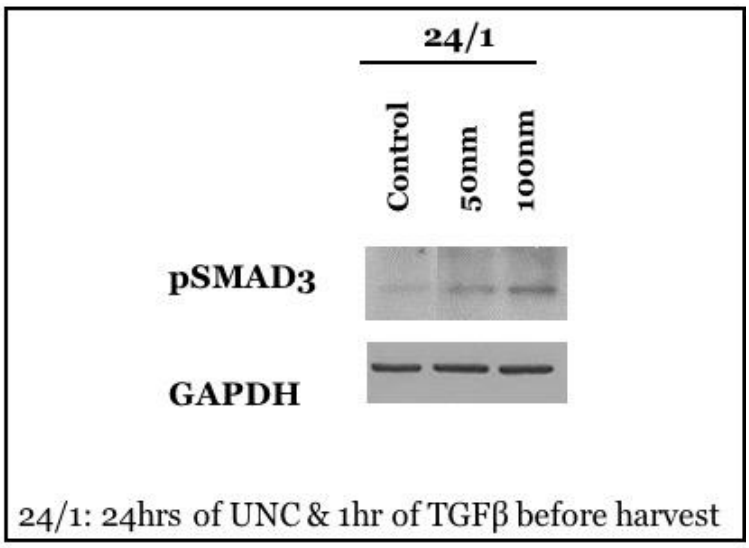
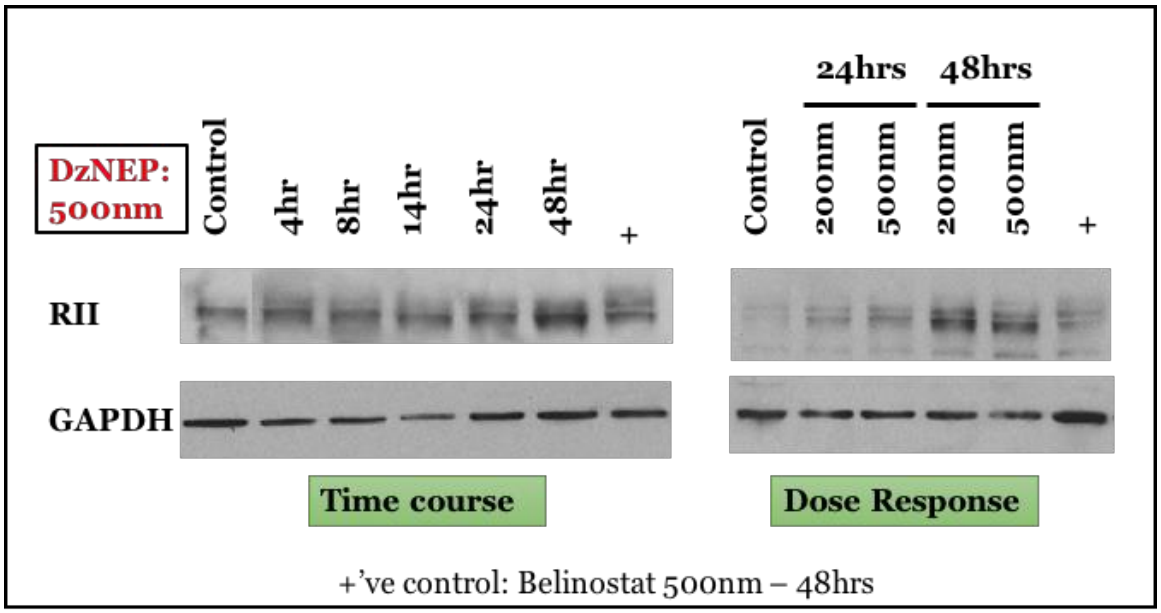


Figure 4: Treatment of CBS cell line that has epigenetically silenced TGFβRII, with UNC0638 (UNC), induced TGFβRII in a time and dose-dependent manner. pSmad3 induction confirmed the activation of the canonical TGFβ signaling pathway. Data shown are representative of three independent experiments.

5. Inhibition of HMT DzNEP induces expression of TGFβRII in a time and dose-dependent manner.

Treatment of CRC cell line CBS with DzNEP allowed for the re-expression of TGFβRII in a time-dependent manner with a maximal effect at 48 hours and in a dose-dependent manner at both 24 and 48 hours. Whole cell FET lysates treated with Belinostat were used a positive control.

To confirm the re-expression of TGFβRII, through the inhibition of EZH2, was reactivating the TGFβ inhibitory pathway, we investigated the effect of DzNEP on Smad3 phosphorylation.by adding TGFβ to the DzNEP treated cells, 1 hour before harvest and as observed in CBS cells, DzNEP treatment was associated with Smad3 activation.



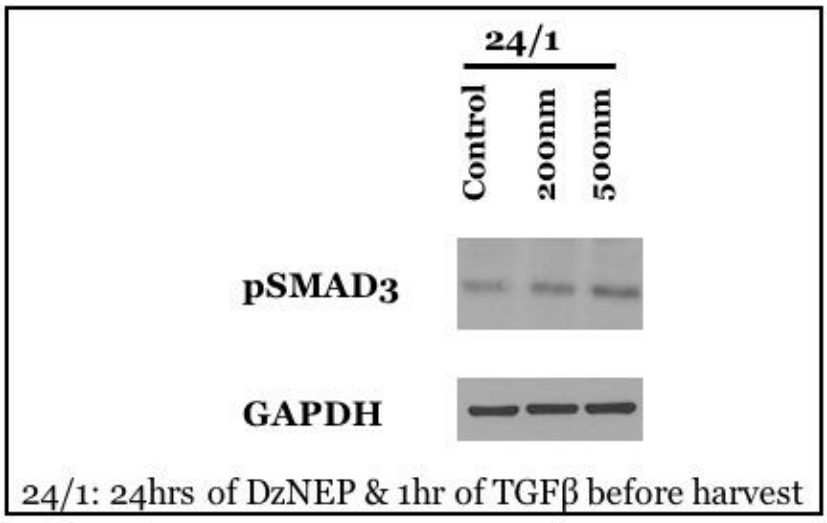


Figure 5: Treatment of CBS cell line that has epigenetically silenced TGFβRII, with DzNEP, induced TGFβRII in a time and dose-dependent manner. pSmad3 induction confirmed the activation of the canonical TGFβ signaling pathway. Data shown are representative of three independent experiments.

6. Interaction between HDACs and HMTs in the epigenetic silencing of TGFβRII.

Our data indicate that HMTs are involved in the epigenetic silencing of TGFβRII as inhibition of those enzymes results in the induction of TGFβRII expression. However, HDAC inhibition alone is sufficient to induce TGFβRII expression without additional HMT inhibition. Therefore, we hypothesized that HDAC inhibition might decrease HMT activity allowing for HDAC inhibition alone to induce expression of TGFβRII.

Conversely, we examined the effect of the HDACi Belinostat on the expression of G9a and EZH2. Belinostat treatment of FET CRC cells resulted in a dose-dependent decrease in G9a and EZH2 24 hours. Previous work done in the Brattain laboratory has confirmed that pan-HDACi, Belinostat, is able to reactivate the TGFβ signaling pathway

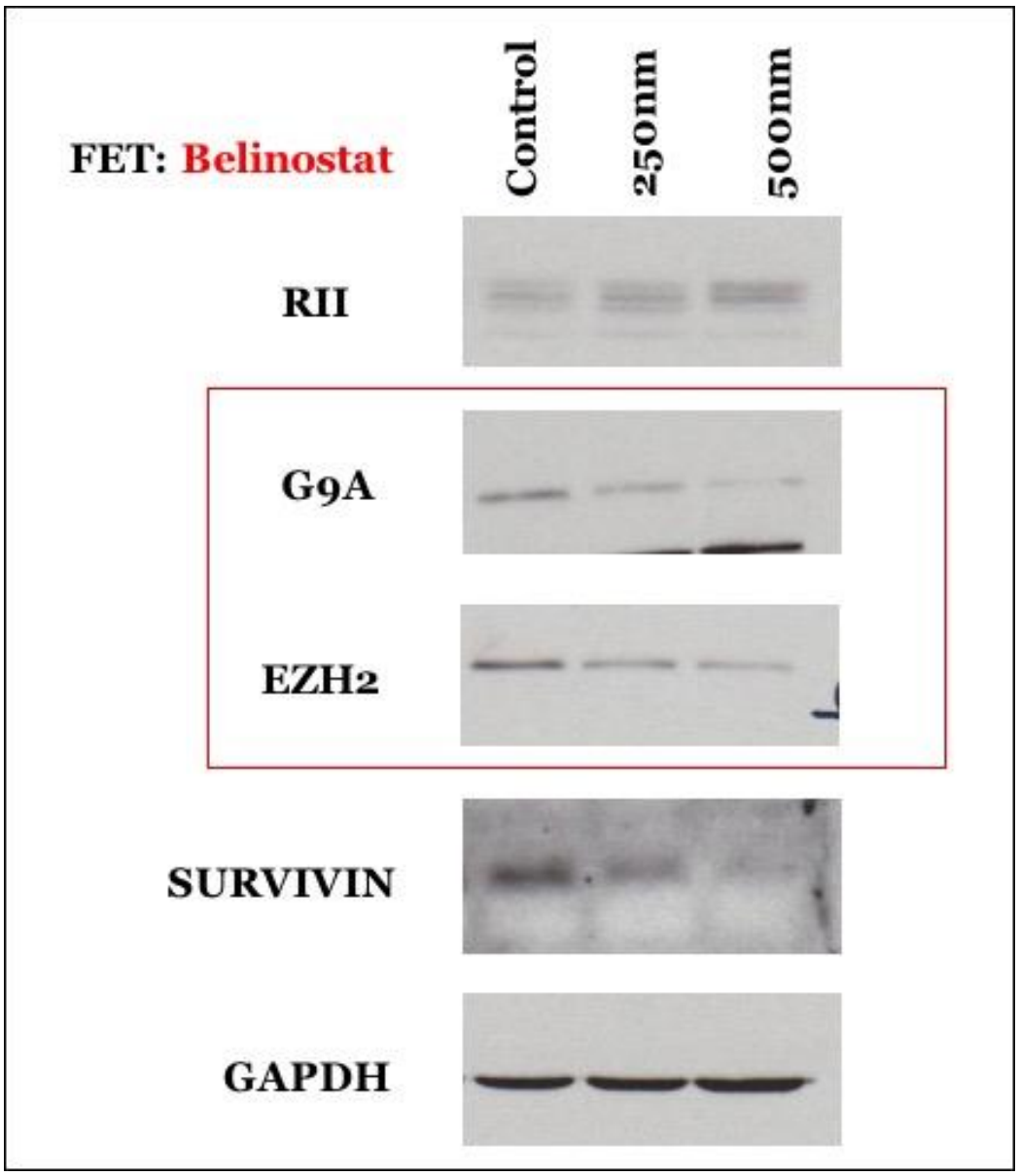


Figure 7: Treatment of FET CRC cell line with Belinostat decreases HMTs, G9a and EZH2 confirming the dual role of HDACi's of inhibiting HDACs as well as regulating HMT status at gene promoters. Data shown are representative of three independent experiments.

Discussion:

The mutational changes underlying the development and progression of CRC have been well documented [192]. Mutations in TSG's or oncogenes result in TSG silencing or oncogene activation. These mutational changes are common in the microsatellite instability phentoype of CRC [251, 252]. However, recently the importance of epigenetic changes, which lead to TSG silencing or oncogene activation, have been recognized [253-255]. Epigenetic regulation involves modification of DNA histones or other DNA binding proteins that affects chromatin conformation that either activates or represses transcription of genes at the altered site.

DNA methylation and histone modifications are two such epigenetic events that occur in the cell. Histone modifications include acetylation and/or methylation of the lysine tails of the histones around which DNA wraps. Histone acetylation is brought about by HATs and is usually associated with gene activation where histone deacetylation is brought on by HDACs and is associated with gene repression. These changes, unlike DNA sequence mutation, are potentially reversible if the specific enzymes facilitating the modifications are targeted. Therefore, epigenetic therapy has the potential to target cancer through silencing of oncogenes or activation of silenced TSG's [256].

The Brattain laboratory has extensively documented the anti-tumorigenic abilities of the TSG TGFβRII. Previous work confirmed that the HDACi Belinostat induces expression of TGFβRII [211]. However two aspects of the silencing of TGFβRII were not understood: – (i) the role of specific HDACs involved in the silencing, and (ii) the potential role of HMTs in the silencing of TGFβRII.

We hypothesized that identification of the specific HDACs and/or HMTs involved in the silencing of TGFβRII will allow us to develop drugs specific to those HDACs and/or HMTs, so reducing off-target effects resulting in reduced toxicities. We employed both pharmacological and genetic approaches to indentify the specific epigenetic regulators involved in the epigenetic silencing of TGFβRII.

We treated CRC cell lines with MGCD0103, which selectively inhibits HDACs 1, 2, 3 and 11 at nanomolar values [235]. Inhibition of these HDACs resulted in an induction of TGFβRII in FET cells at 48 hrs and in CBS cells at 24 hrs as well as 48 hrs. FET is a non-metastatic cell line that expresses low-level autocrine TGFβ signaling. In contrast CBS is a metastatic cell line with silenced TGFβRII expression and so lacks inhibitory TGFβ signaling. MGCD0103 caused a robust re-expression of TGFβRII confirming that HDAC1, 2, 3 and/or 11 are probably involved in its silencing. In contrast, treatment with Droxinostat, which inhibits

HDAC 6 and 8 (IC50 2.47μm and 1.46μm, respectively) and HDAC3 at high micromolar concentration (IC50 = 20μm) did not induce TGFβRII [239, 240]. There appeared to be slight induction of the TGFβRII expression at the 25μm dose at which HDAC3 would be inhibited. These pharmacological studies pointed to the involvement of of HDACs 1 and 3 in the epigenetic silencing of TGFβRII.

In order to confirm the roles of HDACs 1 and 3 , we developed stable HDAC1 knockdown clones in both FET and CBS cell lines. We successfully developed the HDAC2 KD as a control. The KD of HDAC1 resulted in re-expression of TGFβRII. The same result was observed upon KD of HDAC3 but HDAC2 had no effect on TGFβRII expression. This confirmed that HDACs 1 and 3 are the major HDACs involved in the epigenetic silencing of TGFβRII. Based on the work done, we were able to confirm the roles of HDACs 1 and 3 in the silencing of the TSG TGFβRII.

Histone methylation is also involved in epigenetic silencing. G9a is a H3K9 methyltransferase while EZH2 is a H3K9 as well as H3K27 methyltransferase. These methytransferases are capable of di- and tri-methylation and can be associated with gene activation or repression. There is no literature published confirming a relationship between G9a and the epigenetic silencing of TGFβRII. While the relationship between the cytokine TGFβ and EZH2 has been studied,

the potential role of EZH2 in the epigenetic silencing of TGFβRII has not been investigated. We therefore examined the role of G9a and EZH2 in the silencing of TGFβRII.

UNC0638 inhibits both G9a and its partner GLP at low nanomolecular levels [242]. On treating the CRC cell lines with this drug we observed an induction of TGFβRII in a time and dose-dependent manner. Reactivation of the TGFβ inhibitory pathway was confirmed by the phosphorylation of Smad3. We were able to obtain similar results by the inhibition of EZH2 by DZNEP [245]. These preliminary studies confirmed that G9a as well as EZH2 play a role in the silencing of TGFβRII and contribute to tumorigenesis as well progression of CRC.

These findings also raised an inherent question. HDACi's such as Belinostat and MGCD0103 are sufficient to induce re-expression of TGFβRII but clearly histone methylation is involved in the silencing too. We therefore hypothesize that HDACi's might regulate the HMTs, G9a and EZH2. Panobinostat, a pan-HDACi decreased EZH2 levels in acute leukemia cells and enhanced cancer cell survival [257]. Belinostat treatment of the FET CRC cells decreased G9a and EZH2. Therefore, though HDACi's predominantly regulate histone deacetylation at gene promoters, they can also play a role in histone methylation status through down regulation of HMTs.

Future Directions:

- Chromatin Immunoprecipitation studies of the TGFβRII promoter will give insight of the on the actual interaction between the HDACs 1 and 3 and the promoter region.

  Confirm that the knockdown of HDACs 1 and 3 allows for active inhibitory TGFβ signaling by examining the phosphorylation status of Smad3. Confirm that re-expression of TGFβRII decreases survivin and XIAP through western blot analysis.

- Knockdown of G9a and EZH2 using siRNA and confirmation of their association with the TGFβRII promoter using chromatin immunoprecipitation.

  Confirm that knockdown of G9a and EZH2 activates the inhibitory TGFβ signaling pathway by examining the phosphorylation status of Smad3.

  Confirm that re-expression of TGFβRII decreases survivin and XIAP through western blot analysis .

Conclusions:

My study has confirmed the role of HDACs 1 and 3 in the silencing of TSG TGFβRII in CRC. We also confirmed that HDACi's have the ability not only to inhibit HDACs but also to regulate HMTs to effect silencing of TSG's.

References:

1. Kleppe, R., et al., *The cAMP-dependent protein kinase pathway as therapeutic target: possibilities and pitfalls.* Curr Top Med Chem. **11**(11): p. 1393-405.
2. Naviglio, S., et al., *Protein kinase A as a biological target in cancer therapy.* Expert Opin Ther Targets, 2009. **13**(1): p. 83-92.
3. Cheng, I., et al., *Pleiotropic effects of genetic risk variants for other cancers on colorectal cancer risk: PAGE, GECCO and CCFR consortia.* Gut, 2014. **63**(5): p. 800-7.
4. Cheng, X., et al., *Epac and PKA: a tale of two intracellular cAMP receptors.* Acta Biochim Biophys Sin (Shanghai), 2008. **40**(7): p. 651-62.
5. Tsalkova, T., et al., *Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities.* J Biol Chem, 2009. **284**(35): p. 23644-51.
6. Niimura, M., et al., *Critical role of the N-terminal cyclic AMP-binding domain of Epac2 in its subcellular localization and function.* J Cell Physiol, 2009. **219**(3): p. 652-8.
7. Ueno, H., et al., *Characterization of the gene EPAC2: structure, chromosomal localization, tissue expression, and identification of the liver-specific isoform.* Genomics, 2001. **78**(1-2): p. 91-8.
8. de Rooij, J., et al., *Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs.* J Biol Chem, 2000. **275**(27): p. 20829-36.
9. Qiao, J., et al., *Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP.* J Biol Chem, 2002. **277**(29): p. 26581-6.
10. Li, Y., et al., *The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane.* J Biol Chem, 2006. **281**(5): p. 2506-14.
11. Kawasaki, H., et al., *A family of cAMP-binding proteins that directly activate Rap1.* Science, 1998. **282**(5397): p. 2275-9.
12. Ulucan, C., et al., *Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy.* Am J Physiol Heart Circ Physiol, 2007. **293**(3): p. H1662-72.
13. Ponsioen, B., et al., *Direct spatial control of Epac1 by cyclic AMP.* Mol Cell Biol, 2009. **29**(10): p. 2521-31.
14. Consonni, S.V., et al., *cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane.* Proc Natl Acad Sci U S A. **109**(10): p. 3814-9.
15. Fu, D., et al., *Bile acid stimulates hepatocyte polarization through a cAMP-Epac-MEK-LKB1-AMPK pathway.* Proc Natl Acad Sci U S A. **108**(4): p. 1403-8.
16. Stangherlin, A. and M. Zaccolo, *Phosphodiesterases and subcellular compartmentalized cAMP signaling in the cardiovascular system.* Am J Physiol Heart Circ Physiol. **302**(2): p. H379-90.

17. Dodge-Kafka, K.L., A. Bauman, and M.S. Kapiloff, *A-kinase anchoring proteins as the basis for cAMP signaling.* Handb Exp Pharmacol, 2008(186): p. 3-14.
18. Dodge-Kafka, K.L., et al., *The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways.* Nature, 2005. **437**(7058): p. 574-8.
19. Bers, D.M., *Calcium cycling and signaling in cardiac myocytes.* Annu Rev Physiol, 2008. **70**: p. 23-49.
20. Metrich, M., et al., *Functional characterization of the cAMP-binding proteins Epac in cardiac myocytes.* Pharmacol Rep, 2009. **61**(1): p. 146-53.
21. Wang, H., et al., *Phospholipase C epsilon modulates beta-adrenergic receptor-dependent cardiac contraction and inhibits cardiac hypertrophy.* Circ Res, 2005. **97**(12): p. 1305-13.
22. Yokoyama, U., et al., *Prostaglandin E2-activated Epac promotes neointimal formation of the rat ductus arteriosus by a process distinct from that of cAMP-dependent protein kinase A.* J Biol Chem, 2008. **283**(42): p. 28702-9.
23. Villarreal, F., et al., *Regulation of cardiac fibroblast collagen synthesis by adenosine: roles for Epac and PI3K.* Am J Physiol Cell Physiol, 2009. **296**(5): p. C1178-84.
24. Somekawa, S., et al., *Enhanced functional gap junction neoformation by protein kinase A-dependent and Epac-dependent signals downstream of cAMP in cardiac myocytes.* Circ Res, 2005. **97**(7): p. 655-62.
25. Metrich, M., et al., *Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy.* Circ Res, 2008. **102**(8): p. 959-65.
26. Kang, G., et al., *A cAMP and Ca2+ coincidence detector in support of Ca2+-induced Ca2+ release in mouse pancreatic beta cells.* J Physiol, 2005. **566**(Pt 1): p. 173-88.
27. Kashima, Y., et al., *Critical role of cAMP-GEFII--Rim2 complex in incretin-potentiated insulin secretion.* J Biol Chem, 2001. **276**(49): p. 46046-53.
28. Ozaki, N., et al., *cAMP-GEFII is a direct target of cAMP in regulated exocytosis.* Nat Cell Biol, 2000. **2**(11): p. 805-11.
29. Fujimoto, K., et al., *Piccolo, a Ca2+ sensor in pancreatic beta-cells. Involvement of cAMP-GEFII.Rim2. Piccolo complex in cAMP-dependent exocytosis.* J Biol Chem, 2002. **277**(52): p. 50497-502.
30. Kang, G., et al., *Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca2+-induced Ca2+ release and exocytosis in pancreatic beta-cells.* J Biol Chem, 2003. **278**(10): p. 8279-85.
31. Reedquist, K.A., et al., *The small GTPase, Rap1, mediates CD31-induced integrin adhesion.* J Cell Biol, 2000. **148**(6): p. 1151-8.
32. Sebzda, E., et al., *Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling.* Nat Immunol, 2002. **3**(3): p. 251-8.
33. Shimonaka, M., et al., *Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow.* J Cell Biol, 2003. **161**(2): p. 417-27.

34. Katagiri, K., M. Shimonaka, and T. Kinashi, *Rap1-mediated lymphocyte function-associated antigen-1 activation by the T cell antigen receptor is dependent on phospholipase C-gamma1.* J Biol Chem, 2004. **279**(12): p. 11875-81.
35. Ghandour, H., et al., *Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion.* Blood, 2007. **110**(10): p. 3682-90.
36. Arai, A., et al., *Rap1 is activated by erythropoietin or interleukin-3 and is involved in regulation of beta1 integrin-mediated hematopoietic cell adhesion.* J Biol Chem, 2001. **276**(13): p. 10453-62.
37. Caron, E., A.J. Self, and A. Hall, *The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators.* Curr Biol, 2000. **10**(16): p. 974-8.
38. Liu, L., et al., *Requirement for RhoA kinase activation in leukocyte de-adhesion.* J Immunol, 2002. **169**(5): p. 2330-6.
39. de Bruyn, K.M., et al., *The small GTPase Rap1 is activated by turbulence and is involved in integrin [alpha]IIb[beta]3-mediated cell adhesion in human megakaryocytes.* J Biol Chem, 2003. **278**(25): p. 22412-7.
40. Bos, J.L., et al., *The role of Rap1 in integrin-mediated cell adhesion.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 83-6.
41. Enserink, J.M., et al., *A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK.* Nat Cell Biol, 2002. **4**(11): p. 901-6.
42. Fukuhara, S., et al., *Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway.* Mol Cell Biol, 2005. **25**(1): p. 136-46.
43. Kooistra, M.R., et al., *Epac1 regulates integrity of endothelial cell junctions through VE-cadherin.* FEBS Lett, 2005. **579**(22): p. 4966-72.
44. Cho, E.A. and Y.S. Juhnn, *The cAMP signaling system inhibits the repair of gamma-ray-induced DNA damage by promoting Epac1-mediated proteasomal degradation of XRCC1 protein in human lung cancer cells.* Biochem Biophys Res Commun. **422**(2): p. 256-62.
45. Pullamsetti, S.S., et al., *Phosphodiesterase-4 promotes proliferation and angiogenesis of lung cancer by crosstalk with HIF.* Oncogene. **32**(9): p. 1121-34.
46. Narita, M., et al., *A role for cyclic nucleotide phosphodiesterase 4 in regulation of the growth of human malignant melanoma cells.* Oncol Rep, 2007. **17**(5): p. 1133-9.
47. Baljinnyam, E., et al., *Epac increases melanoma cell migration by a heparan sulfate-related mechanism.* Am J Physiol Cell Physiol, 2009. **297**(4): p. C802-13.
48. Baljinnyam, E., et al., *Exchange protein directly activated by cyclic AMP increases melanoma cell migration by a Ca2+-dependent mechanism.* Cancer Res. **70**(13): p. 5607-17.
49. Baljinnyam, E., et al., *Epac1 promotes melanoma metastasis via modification of heparan sulfate.* Pigment Cell Melanoma Res. **24**(4): p. 680-7.
50. Choi, J.H., et al., *Gonadotropin-stimulated epidermal growth factor receptor expression in human ovarian surface epithelial cells: involvement of cyclic AMP-*

dependent exchange protein activated by cAMP pathway. Endocr Relat Cancer, 2009. **16**(1): p. 179-88.

51. Bastian, P., et al., *The inhibitory effect of norepinephrine on the migration of ES-2 ovarian carcinoma cells involves a Rap1-dependent pathway.* Cancer Lett, 2009. **274**(2): p. 218-24.
52. Spina, A., et al., *cAMP Elevation Down-Regulates beta3 Integrin and Focal Adhesion Kinase and Inhibits Leptin-Induced Migration of MDA-MB-231 Breast Cancer Cells.* Biores Open Access. **1**(6): p. 324-32.
53. Grandoch, M., et al., *Epac inhibits migration and proliferation of human prostate carcinoma cells.* Br J Cancer, 2009. **101**(12): p. 2038-42.
54. Misra, U.K. and S.V. Pizzo, *Epac1-induced cellular proliferation in prostate cancer cells is mediated by B-Raf/ERK and mTOR signaling cascades.* J Cell Biochem, 2009. **108**(4): p. 998-1011.
55. Burdyga, A., et al., *cAMP inhibits migration, ruffling and paxillin accumulation in focal adhesions of pancreatic ductal adenocarcinoma cells: effects of PKA and EPAC.* Biochim Biophys Acta. **1833**(12): p. 2664-72.
56. Lorenz, R., et al., *The cAMP/Epac1/Rap1 pathway in pancreatic carcinoma.* Pancreas, 2008. **37**(1): p. 102-3.
57. Almahariq, M., et al., *A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion.* Mol Pharmacol. **83**(1): p. 122-8.
58. Almahariq, M., et al., *Pharmacological inhibition and genetic knockdown of exchange protein directly activated by cAMP 1 reduce pancreatic cancer metastasis in vivo.* Mol Pharmacol. **87**(2): p. 142-9.
59. Laken, S.J., et al., *Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC.* Nat Genet, 1997. **17**(1): p. 79-83.
60. Chowdhury, S., et al., *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): p. 30937-48.
61. Piek, E., C.H. Heldin, and P. Ten Dijke, *Specificity, diversity, and regulation in TGF-beta superfamily signaling.* FASEB J, 1999. **13**(15): p. 2105-24.
62. Derynck, R. and X.H. Feng, *TGF-beta receptor signaling.* Biochim Biophys Acta, 1997. **1333**(2): p. F105-50.
63. Chowdhury, S., et al., *Identification of a novel TGFbeta/PKA signaling transduceome in mediating control of cell survival and metastasis in colon cancer.* PLoS One. **6**(5): p. e19335.
64. Skalhegg, B.S. and K. Tasken, *Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA.* Front Biosci, 2000. **5**: p. D678-93.
65. Carlisle Michel, J.J., et al., *PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex.* Biochem J, 2004. **381**(Pt 3): p. 587-92.
66. Nesterova, M. and Y.S. Cho-Chung, *Oligonucleotide sequence-specific inhibition of gene expression, tumor growth inhibition, and modulation of cAMP signaling by an RNA-DNA hybrid antisense targeted to protein kinase A RIalpha subunit.* Antisense Nucleic Acid Drug Dev, 2000. **10**(6): p. 423-33.

67. Neary, C.L., et al., *Protein kinase A isozyme switching: eliciting differential cAMP signaling and tumor reversion.* Oncogene, 2004. **23**(54): p. 8847-56.
68. Cho-Chung, Y.S. and M.V. Nesterova, *Tumor reversion: protein kinase A isozyme switching.* Ann N Y Acad Sci, 2005. **1058**: p. 76-86.
69. Caretta, A. and C. Mucignat-Caretta, *Protein kinase a in cancer.* Cancers (Basel). **3**(1): p. 913-26.
70. Zhong, H., et al., *The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism.* Cell, 1997. **89**(3): p. 413-24.
71. Chin, Y.R. and A. Toker, *Function of Akt/PKB signaling to cell motility, invasion and the tumor stroma in cancer.* Cell Signal, 2009. **21**(4): p. 470-6.
72. Suganuma, M., et al., *Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter.* Proc Natl Acad Sci U S A, 1988. **85**(6): p. 1768-71.
73. Chen, X., et al., *Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer.* Oncogene, 2001. **20**(42): p. 6073-83.
74. Zhou, X.D., et al., *Protein kinase B phosphorylation correlates with vascular endothelial growth factor A and microvessel density in gastric adenocarcinoma.* J Int Med Res. **40**(6): p. 2124-34.
75. Brodbeck, D., P. Cron, and B.A. Hemmings, *A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain.* J Biol Chem, 1999. **274**(14): p. 9133-6.
76. Nakatani, K., et al., *Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines.* J Biol Chem, 1999. **274**(31): p. 21528-32.
77. Easton, R.M., et al., *Role for Akt3/protein kinase Bgamma in attainment of normal brain size.* Mol Cell Biol, 2005. **25**(5): p. 1869-78.
78. Chau, N.M. and M. Ashcroft, *Akt2: a role in breast cancer metastasis.* Breast Cancer Res, 2004. **6**(1): p. 55-7.
79. Kupriyanova, T.A. and K.V. Kandror, *Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin.* J Biol Chem, 1999. **274**(3): p. 1458-64.
80. Yang, Z.Z., et al., *Protein kinase B alpha/Akt1 regulates placental development and fetal growth.* J Biol Chem, 2003. **278**(34): p. 32124-31.
81. Deveraux, Q.L., et al., *Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases.* EMBO J, 1999. **18**(19): p. 5242-51.
82. Saleem, M., et al., *Inhibitors of apoptotic proteins: new targets for anticancer therapy.* Chem Biol Drug Des. **82**(3): p. 243-51.
83. Silke, J. and D. Vucic, *IAP family of cell death and signaling regulators.* Methods Enzymol. **545**: p. 35-65.
84. Tong, Q.S., et al., *Downregulation of XIAP expression induces apoptosis and enhances chemotherapeutic sensitivity in human gastric cancer cells.* Cancer Gene Ther, 2005. **12**(5): p. 509-14.

85. McManus, D.C., et al., *Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics.* Oncogene, 2004. **23**(49): p. 8105-17.
86. Harlin, H., et al., *Characterization of XIAP-deficient mice.* Mol Cell Biol, 2001. **21**(10): p. 3604-8.
87. Fakler, M., et al., *Small molecule XIAP inhibitors cooperate with TRAIL to induce apoptosis in childhood acute leukemia cells and overcome Bcl-2-mediated resistance.* Blood, 2009. **113**(8): p. 1710-22.
88. Vogler, M., et al., *Targeting XIAP bypasses Bcl-2-mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth in vitro and in vivo.* Cancer Res, 2008. **68**(19): p. 7956-65.
89. Ahn, K.S., G. Sethi, and B.B. Aggarwal, *Embelin, an inhibitor of X chromosome-linked inhibitor-of-apoptosis protein, blocks nuclear factor-kappaB (NF-kappaB) signaling pathway leading to suppression of NF-kappaB-regulated antiapoptotic and metastatic gene products.* Mol Pharmacol, 2007. **71**(1): p. 209-19.
90. Nikolovska-Coleska, Z., et al., *Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization.* Anal Biochem, 2004. **332**(2): p. 261-73.
91. Nikolovska-Coleska, Z., et al., *Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database.* J Med Chem, 2004. **47**(10): p. 2430-40.
92. Agrawal, S. and E.R. Kandimalla, *Role of Toll-like receptors in antisense and siRNA [corrected].* Nat Biotechnol, 2004. **22**(12): p. 1533-7.
93. Agrawal, S., et al., *Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: in vitro and in vivo studies.* Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2620-5.
94. Mohamed, S., et al., *Nuclear survivin in pN2 nonsmall cell lung cancer: prognostic and clinical implications.* Eur Respir J, 2009. **33**(1): p. 127-33.
95. Soleimanpour, E. and E. Babaei, *Survivin as a Potential Target for Cancer Therapy.* Asian Pac J Cancer Prev. **16**(15): p. 6187-91.
96. Poomsawat, S., J. Punyasingh, and P. Vejchapipat, *Overexpression of survivin and caspase 3 in oral carcinogenesis.* Appl Immunohistochem Mol Morphol. **22**(1): p. 65-71.
97. Lv, Y.G., et al., *The role of survivin in diagnosis, prognosis and treatment of breast cancer.* J Thorac Dis. **2**(2): p. 100-10.
98. Vandghanooni, S., et al., *Survivin-deltaEx3: a novel biomarker for diagnosis of papillary thyroid carcinoma.* J Cancer Res Ther. **7**(3): p. 325-30.
99. Mahotka, C., et al., *Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas.* Int J Cancer, 2002. **100**(1): p. 30-6.
100. Mahotka, C., et al., *Differential subcellular localization of functionally divergent survivin splice variants.* Cell Death Differ, 2002. **9**(12): p. 1334-42.
101. Altieri, D.C., *Survivin in apoptosis control and cell cycle regulation in cancer.* Prog Cell Cycle Res, 2003. **5**: p. 447-52.

102. Altieri, D.C., *Validating survivin as a cancer therapeutic target.* Nat Rev Cancer, 2003. **3**(1): p. 46-54.
103. Kawasaki, H., et al., *Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer.* Cancer Res, 1998. **58**(22): p. 5071-4.
104. Swana, H.S., et al., *Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer.* N Engl J Med, 1999. **341**(6): p. 452-3.
105. Hausladen, D.A., et al., *Effect of intravesical treatment of transitional cell carcinoma with bacillus Calmette-Guerin and mitomycin C on urinary survivin levels and outcome.* J Urol, 2003. **170**(1): p. 230-4.
106. Rodel, C., et al., *Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor.* Int J Radiat Oncol Biol Phys, 2003. **55**(5): p. 1341-7.
107. Asanuma, K., et al., *Survivin as a radioresistance factor in pancreatic cancer.* Jpn J Cancer Res, 2000. **91**(11): p. 1204-9.
108. Pennati, M., et al., *Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression.* J Invest Dermatol, 2003. **120**(4): p. 648-54.
109. Dohi, T., et al., *An IAP-IAP complex inhibits apoptosis.* J Biol Chem, 2004. **279**(33): p. 34087-90.
110. Eckelman, B.P., G.S. Salvesen, and F.L. Scott, *Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family.* EMBO Rep, 2006. **7**(10): p. 988-94.
111. Mehrotra, S., et al., *IAP regulation of metastasis.* Cancer Cell. **17**(1): p. 53-64.
112. Salvesen, G.S. and C.S. Duckett, *IAP proteins: blocking the road to death's door.* Nat Rev Mol Cell Biol, 2002. **3**(6): p. 401-10.
113. Coumar, M.S., et al., *Treat cancers by targeting survivin: just a dream or future reality?* Cancer Treat Rev. **39**(7): p. 802-11.
114. Kelly, R.J., et al., *Impacting tumor cell-fate by targeting the inhibitor of apoptosis protein survivin.* Mol Cancer. **10**: p. 35.
115. Nakahara, T., et al., *YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts.* Cancer Res, 2007. **67**(17): p. 8014-21.
116. Mesri, M., et al., *Cancer gene therapy using a survivin mutant adenovirus.* J Clin Invest, 2001. **108**(7): p. 981-90.
117. Kanwar, J.R., S.K. Kamalapuram, and R.K. Kanwar, *Targeting survivin in cancer: the cell-signalling perspective.* Drug Discov Today. **16**(11-12): p. 485-94.
118. Tu, S.P., et al., *Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer.* Cancer Res, 2003. **63**(22): p. 7724-32.
119. Tanioka, M., et al., *Phase I study of LY2181308, an antisense oligonucleotide against survivin, in patients with advanced solid tumors.* Cancer Chemother Pharmacol. **68**(2): p. 505-11.
120. Hansen, J.B., et al., *SPC3042: a proapoptotic survivin inhibitor.* Mol Cancer Ther, 2008. **7**(9): p. 2736-45.

121. Carrasco, R.A., et al., *Antisense inhibition of survivin expression as a cancer therapeutic.* Mol Cancer Ther. **10**(2): p. 221-32.
122. Altieri, D.C., *Survivin and IAP proteins in cell-death mechanisms.* Biochem J. **430**(2): p. 199-205.
123. Hochbaum, D., et al., *Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis.* J Biol Chem, 2008. **283**(8): p. 4464-8.
124. Mei, F.C. and X. Cheng, *Interplay between exchange protein directly activated by cAMP (Epac) and microtubule cytoskeleton.* Mol Biosyst, 2005. **1**(4): p. 325-31.
125. Misra, U.K. and S.V. Pizzo, *Evidence for a pro-proliferative feedback loop in prostate cancer: the role of Epac1 and COX-2-dependent pathways.* PLoS One. **8**(4): p. e63150.
126. Jeong, M.J., et al., *cAMP signalling decreases p300 protein levels by promoting its ubiquitin/proteasome dependent degradation via Epac and p38 MAPK in lung cancer cells.* FEBS Lett. **587**(9): p. 1373-8.
127. Metrich, M., et al., *Epac activation induces histone deacetylase nuclear export via a Ras-dependent signalling pathway.* Cell Signal. **22**(10): p. 1459-68.
128. Ruiz-Hurtado, G., et al., *Epac in cardiac calcium signaling.* J Mol Cell Cardiol. **58**: p. 162-71.
129. Liu, Y. and M.F. Schneider, *Opposing HDAC4 nuclear fluxes due to phosphorylation by beta-adrenergic activated protein kinase A or by activity or Epac activated CaMKII in skeletal muscle fibres.* J Physiol. **591**(Pt 14): p. 3605-23.
130. Grunstein, M., *Histone acetylation in chromatin structure and transcription.* Nature, 1997. **389**(6649): p. 349-52.
131. Mai, A., et al., *Histone deacetylation in epigenetics: an attractive target for anticancer therapy.* Med Res Rev, 2005. **25**(3): p. 261-309.
132. Thiagalingam, S., et al., *Histone deacetylases: unique players in shaping the epigenetic histone code.* Ann N Y Acad Sci, 2003. **983**: p. 84-100.
133. Jenuwein, T., et al., *SET domain proteins modulate chromatin domains in eu- and heterochromatin.* Cell Mol Life Sci, 1998. **54**(1): p. 80-93.
134. Haberland, M., R.L. Montgomery, and E.N. Olson, *The many roles of histone deacetylases in development and physiology: implications for disease and therapy.* Nat Rev Genet, 2009. **10**(1): p. 32-42.
135. Clocchiatti, A., C. Florean, and C. Brancolini, *Class IIa HDACs: from important roles in differentiation to possible implications in tumourigenesis.* J Cell Mol Med. **15**(9): p. 1833-46.
136. Vega, R.B., et al., *Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis.* Cell, 2004. **119**(4): p. 555-66.
137. Ozdag, H., et al., *Differential expression of selected histone modifier genes in human solid cancers.* BMC Genomics, 2006. **7**: p. 90.
138. Kao, G.D., et al., *Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response.* J Cell Biol, 2003. **160**(7): p. 1017-27.

139. Geng, L., et al., *Histone deacetylase (HDAC) inhibitor LBH589 increases duration of gamma-H2AX foci and confines HDAC4 to the cytoplasm in irradiated non-small cell lung cancer.* Cancer Res, 2006. **66**(23): p. 11298-304.
140. Stark, M. and N. Hayward, *Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays.* Cancer Res, 2007. **67**(6): p. 2632-42.
141. Sjoblom, T., et al., *The consensus coding sequences of human breast and colorectal cancers.* Science, 2006. **314**(5797): p. 268-74.
142. Datta, J., et al., *Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis.* Cancer Res, 2008. **68**(13): p. 5049-58.
143. Nasser, M.W., et al., *Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1.* J Biol Chem, 2008. **283**(48): p. 33394-405.
144. Qian, D.Z., et al., *Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589.* Clin Cancer Res, 2006. **12**(2): p. 634-42.
145. Bodily, J.M., K.P. Mehta, and L.A. Laimins, *Human papillomavirus E7 enhances hypoxia-inducible factor 1-mediated transcription by inhibiting binding of histone deacetylases.* Cancer Res. **71**(3): p. 1187-95.
146. Wilson, A.J., et al., *HDAC4 promotes growth of colon cancer cells via repression of p21.* Mol Biol Cell, 2008. **19**(10): p. 4062-75.
147. Zhang, C.L., T.A. McKinsey, and E.N. Olson, *Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation.* Mol Cell Biol, 2002. **22**(20): p. 7302-12.
148. Chang, S., et al., *Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development.* Mol Cell Biol, 2004. **24**(19): p. 8467-76.
149. Milde, T., et al., *HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth.* Clin Cancer Res. **16**(12): p. 3240-52.
150. Ishikawa, S., et al., *The role of oxysterol binding protein-related protein 5 in pancreatic cancer.* Cancer Sci. **101**(4): p. 898-905.
151. Berthouze-Duquesnes, M., et al., *Specific interactions between Epac1, beta-arrestin2 and PDE4D5 regulate beta-adrenergic receptor subtype differential effects on cardiac hypertrophic signaling.* Cell Signal. **25**(4): p. 970-80.
152. Schmidt, M., F.J. Dekker, and H. Maarsingh, *Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions.* Pharmacol Rev. **65**(2): p. 670-709.
153. Chang, C.W., et al., *Involvement of Epac1/Rap1/CaMKI/HDAC5 signaling cascade in the regulation of placental cell fusion.* Mol Hum Reprod. **19**(11): p. 745-55.
154. Ohnuki, Y., et al., *Role of cyclic AMP sensor Epac1 in masseter muscle hypertrophy and myosin heavy chain transition induced by beta2-adrenoceptor stimulation.* J Physiol. **592**(Pt 24): p. 5461-75.

155. Pereira, L., et al., *Novel Epac fluorescent ligand reveals distinct Epac1 vs. Epac2 distribution and function in cardiomyocytes.* Proc Natl Acad Sci U S A. **112**(13): p. 3991-6.
156. Rehmann, H., et al., *Communication between the regulatory and the catalytic region of the cAMP-responsive guanine nucleotide exchange factor Epac.* J Biol Chem, 2003. **278**(26): p. 23508-14.
157. Rehmann, H., et al., *Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac.* J Biol Chem, 2003. **278**(40): p. 38548-56.
158. Rehmann, H., *Epac2: a sulfonylurea receptor?* Biochem Soc Trans. **40**(1): p. 6-10.
159. Herbst, K.J., et al., *Direct activation of Epac by sulfonylurea is isoform selective.* Chem Biol. **18**(2): p. 243-51.
160. Tsalkova, T., F.C. Mei, and X. Cheng, *A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists.* PLoS One. **7**(1): p. e30441.
161. Courilleau, D., et al., *Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac.* J Biol Chem. **287**(53): p. 44192-202.
162. Courilleau, D., et al., *The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1).* Biochem Biophys Res Commun. **440**(3): p. 443-8.
163. Brattain, M.G., et al., *Characterization of human colon carcinoma cell lines isolated from a single primary tumour.* Br J Cancer, 1983. **47**(3): p. 373-81.
164. Brattain, M.G., et al., *Heterogeneity of human colon carcinoma.* Cancer Metastasis Rev, 1984. **3**(3): p. 177-91.
165. Marek, L., et al., *Histone deacetylase (HDAC) inhibitors with a novel connecting unit linker region reveal a selectivity profile for HDAC4 and HDAC5 with improved activity against chemoresistant cancer cells.* J Med Chem. **56**(2): p. 427-36.
166. Gloerich, M. and J.L. Bos, *Epac: defining a new mechanism for cAMP action.* Annu Rev Pharmacol Toxicol. **50**: p. 355-75.
167. Harper, K., et al., *Autotaxin promotes cancer invasion via the lysophosphatidic acid receptor 4: participation of the cyclic AMP/EPAC/Rac1 signaling pathway in invadopodia formation.* Cancer Res. **70**(11): p. 4634-43.
168. Conrotto, P., et al., *Interactome of transforming growth factor-beta type I receptor (TbetaRI): inhibition of TGFbeta signaling by Epac1.* J Proteome Res, 2007. **6**(1): p. 287-97.
169. Grozinger, C.M. and S.L. Schreiber, *Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization.* Proc Natl Acad Sci U S A, 2000. **97**(14): p. 7835-40.
170. Wang, A.H. and X.J. Yang, *Histone deacetylase 4 possesses intrinsic nuclear import and export signals.* Mol Cell Biol, 2001. **21**(17): p. 5992-6005.
171. Lenoir, O., et al., *Specific control of pancreatic endocrine beta- and delta-cell mass by class IIa histone deacetylases HDAC4, HDAC5, and HDAC9.* Diabetes. **60**(11): p. 2861-71.

172. Candia, A.F., et al., *Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads.* Development, 1997. **124**(22): p. 4467-80.
173. Stronach, E.A., et al., *HDAC4-regulated STAT1 activation mediates platinum resistance in ovarian cancer.* Cancer Res. **71**(13): p. 4412-22.
174. Xu, X.S., et al., *Histone deacetylases (HDACs) in XPC gene silencing and bladder cancer.* J Hematol Oncol. **4**: p. 17.
175. Urbich, C., et al., *HDAC5 is a repressor of angiogenesis and determines the angiogenic gene expression pattern of endothelial cells.* Blood, 2009. **113**(22): p. 5669-79.
176. Koh, M.Y., T.R. Spivak-Kroizman, and G. Powis, *HIF-1alpha and cancer therapy.* Recent Results Cancer Res. **180**: p. 15-34.
177. Suwaki, N., et al., *A HIF-regulated VHL-PTP1B-Src signaling axis identifies a therapeutic target in renal cell carcinoma.* Sci Transl Med. **3**(85): p. 85ra47.
178. Qian, D.Z., et al., *Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha.* Cancer Res, 2006. **66**(17): p. 8814-21.
179. Seo, H.W., et al., *Transcriptional activation of hypoxia-inducible factor-1alpha by HDAC4 and HDAC5 involves differential recruitment of p300 and FIH-1.* FEBS Lett, 2009. **583**(1): p. 55-60.
180. Geng, H., et al., *HDAC4 protein regulates HIF1alpha protein lysine acetylation and cancer cell response to hypoxia.* J Biol Chem. **286**(44): p. 38095-102.
181. Winawer, S.J. and A.G. Zauber, *The advanced adenoma as the primary target of screening.* Gastrointest Endosc Clin N Am, 2002. **12**(1): p. 1-9, v.
182. Campbell, P.T., et al., *Associations of recreational physical activity and leisure time spent sitting with colorectal cancer survival.* J Clin Oncol, 2013. **31**(7): p. 876-85.
183. Seenath, M.M., et al., *Reciprocal relationship between expression of hypoxia inducible factor 1alpha (HIF-1alpha) and the pro-apoptotic protein bid in ex vivo colorectal cancer.* Br J Cancer, 2008. **99**(3): p. 459-63.
184. Cross, A.J., et al., *A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association.* Cancer Res, 2010. **70**(6): p. 2406-14.
185. Aune, D., et al., *Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies.* BMJ, 2011. **343**: p. d6617.
186. *American Cancer Society. Colorectal Cancer Facts & Figures 2014-2016. Atlanta: American Cancer Society, 2014.*; Available from: http://www.cancer.org/acs/groups/content/documents/document/acspc-042280.pdf.
187. Markowitz, S.D. and M.M. Bertagnolli, *Molecular origins of cancer: Molecular basis of colorectal cancer.* N Engl J Med, 2009. **361**(25): p. 2449-60.
188. Gryfe, R., *Inherited colorectal cancer syndromes.* Clin Colon Rectal Surg, 2009. **22**(4): p. 198-208.

189. Gryfe, R., *Overview of colorectal cancer genetics.* Surg Oncol Clin N Am, 2009. **18**(4): p. 573-83.
190. Lynch, H.T., et al., *Hereditary factors in cancer. Study of two large midwestern kindreds.* Arch Intern Med, 1966. **117**(2): p. 206-12.
191. Umar, A., et al., *Testing guidelines for hereditary non-polyposis colorectal cancer.* Nat Rev Cancer, 2004. **4**(2): p. 153-8.
192. Vogelstein, B., et al., *Genetic alterations during colorectal-tumor development.* N Engl J Med, 1988. **319**(9): p. 525-32.
193. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-67.
194. Scott, R.J., et al., *Hereditary nonpolyposis colorectal cancer in 95 families: differences and similarities between mutation-positive and mutation-negative kindreds.* Am J Hum Genet, 2001. **68**(1): p. 118-127.
195. Lu, S.L., et al., *HNPCC associated with germline mutation in the TGF-beta type II receptor gene.* Nat Genet, 1998. **19**(1): p. 17-8.
196. Grady, W.M. and S.D. Markowitz, *Genetic and epigenetic alterations in colon cancer.* Annu Rev Genomics Hum Genet, 2002. **3**: p. 101-28.
197. Seshimo, I., et al., *Expression and mutation of SMAD4 in poorly differentiated carcinoma and signet-ring cell carcinoma of the colorectum.* J Exp Clin Cancer Res, 2006. **25**(3): p. 433-42.
198. Xu, Y. and B. Pasche, *TGF-beta signaling alterations and susceptibility to colorectal cancer.* Hum Mol Genet, 2007. **16 Spec No 1**: p. R14-20.
199. Wang, T.L., et al., *Prevalence of somatic alterations in the colorectal cancer cell genome.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 3076-80.
200. Lindblom, A., *Different mechanisms in the tumorigenesis of proximal and distal colon cancers.* Curr Opin Oncol, 2001. **13**(1): p. 63-9.
201. Baker, S.J., et al., *Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas.* Science, 1989. **244**(4901): p. 217-21.
202. Vogelstein, B., et al., *Allelotype of colorectal carcinomas.* Science, 1989. **244**(4901): p. 207-11.
203. Lengauer, C., K.W. Kinzler, and B. Vogelstein, *Genetic instabilities in human cancers.* Nature, 1998. **396**(6712): p. 643-9.
204. Zhang, L., et al., *A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A.* Mol Cell Biol, 2004. **24**(5): p. 2169-80.
205. Simms, N.A., et al., *Transforming growth factor-beta suppresses metastasis in a subset of human colon carcinoma cells.* BMC Cancer, 2012. **12**: p. 221.
206. Piek, E., et al., *Expression of transforming-growth-factor (TGF)-beta receptors and Smad proteins in glioblastoma cell lines with distinct responses to TGF-beta1.* Int J Cancer, 1999. **80**(5): p. 756-63.
207. Mishra, L., R. Derynck, and B. Mishra, *Transforming growth factor-beta signaling in stem cells and cancer.* Science, 2005. **310**(5745): p. 68-71.

208. Tang, Y., et al., *Transforming growth factor-beta suppresses nonmetastatic colon cancer through Smad4 and adaptor protein ELF at an early stage of tumorigenesis.* Cancer Res, 2005. **65**(10): p. 4228-37.
209. Souchelnytskyi, S., A. Moustakas, and C.H. Heldin, *TGF-beta signaling from a three-dimensional perspective: insight into selection of partners.* Trends Cell Biol, 2002. **12**(7): p. 304-7.
210. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-84.
211. Chowdhury, S., et al., *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, 2011. **286**(35): p. 30937-48.
212. Yamashita, S., et al., *Methylation silencing of transforming growth factor-beta receptor type II in rat prostate cancers.* Cancer Res, 2008. **68**(7): p. 2112-21.
213. Hinshelwood, R.A., et al., *Concordant epigenetic silencing of transforming growth factor-beta signaling pathway genes occurs early in breast carcinogenesis.* Cancer Res, 2007. **67**(24): p. 11517-27.
214. Ishihama, K., et al., *Expression of HDAC1 and CBP/p300 in human colorectal carcinomas.* J Clin Pathol, 2007. **60**(11): p. 1205-10.
215. Weichert, W., *HDAC expression and clinical prognosis in human malignancies.* Cancer Lett, 2009. **280**(2): p. 168-76.
216. Fritzsche, F.R., et al., *Class I histone deacetylases 1, 2 and 3 are highly expressed in renal cell cancer.* BMC Cancer, 2008. **8**: p. 381.
217. Weichert, W., et al., *Expression of class I histone deacetylases indicates poor prognosis in endometrioid subtypes of ovarian and endometrial carcinomas.* Neoplasia, 2008. **10**(9): p. 1021-7.
218. Song, J., et al., *Increased expression of histone deacetylase 2 is found in human gastric cancer.* APMIS, 2005. **113**(4): p. 264-8.
219. Weichert, W., et al., *Class I histone deacetylase expression has independent prognostic impact in human colorectal cancer: specific role of class I histone deacetylases in vitro and in vivo.* Clin Cancer Res, 2008. **14**(6): p. 1669-77.
220. Weichert, W., et al., *Histone deacetylases 1, 2 and 3 are highly expressed in prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical prostatectomy.* Br J Cancer, 2008. **98**(3): p. 604-10.
221. Weichert, W., et al., *Association of patterns of class I histone deacetylase expression with patient prognosis in gastric cancer: a retrospective analysis.* Lancet Oncol, 2008. **9**(2): p. 139-48.
222. Anumanthan, G., et al., *Restoration of TGF-beta signalling reduces tumorigenicity in human lung cancer cells.* Br J Cancer, 2005. **93**(10): p. 1157-67.
223. Osada, H., et al., *Histone modification in the TGFbetaRII gene promoter and its significance for responsiveness to HDAC inhibitor in lung cancer cell lines.* Mol Carcinog, 2005. **44**(4): p. 233-41.
224. Rice, J.C. and C.D. Allis, *Histone methylation versus histone acetylation: new insights into epigenetic regulation.* Curr Opin Cell Biol, 2001. **13**(3): p. 263-73.

225. Shinkai, Y. and M. Tachibana, *H3K9 methyltransferase G9a and the related molecule GLP.* Genes Dev, 2011. **25**(8): p. 781-8.
226. Patnaik, D., et al., *Substrate specificity and kinetic mechanism of mammalian G9a histone H3 methyltransferase.* J Biol Chem, 2004. **279**(51): p. 53248-58.
227. Martin, C., R. Cao, and Y. Zhang, *Substrate preferences of the EZH2 histone methyltransferase complex.* J Biol Chem, 2006. **281**(13): p. 8365-70.
228. Behrens, C., et al., *EZH2 protein expression associates with the early pathogenesis, tumor progression, and prognosis of non-small cell lung carcinoma.* Clin Cancer Res, 2013. **19**(23): p. 6556-65.
229. Mai, A., et al., *Class II (IIa)-selective histone deacetylase inhibitors. 1. Synthesis and biological evaluation of novel (aryloxopropenyl)pyrrolyl hydroxyamides.* J Med Chem, 2005. **48**(9): p. 3344-53.
230. West, A.C. and R.W. Johnstone, *New and emerging HDAC inhibitors for cancer treatment.* J Clin Invest, 2014. **124**(1): p. 30-9.
231. Gryder, B.E., Q.H. Sodji, and A.K. Oyelere, *Targeted cancer therapy: giving histone deacetylase inhibitors all they need to succeed.* Future Med Chem, 2012. **4**(4): p. 505-24.
232. Balasubramanian, S., E. Verner, and J.J. Buggy, *Isoform-specific histone deacetylase inhibitors: the next step?* Cancer Lett, 2009. **280**(2): p. 211-21.
233. Kim, H.J. and S.C. Bae, *Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs.* Am J Transl Res, 2011. **3**(2): p. 166-79.
234. Kell, J., *Drug evaluation: MGCD-0103, a histone deacetylase inhibitor for the treatment of cancer.* Curr Opin Investig Drugs, 2007. **8**(6): p. 485-92.
235. Fournel, M., et al., *MGCD0103, a novel isotype-selective histone deacetylase inhibitor, has broad spectrum antitumor activity in vitro and in vivo.* Mol Cancer Ther, 2008. **7**(4): p. 759-68.
236. Bonfils, C., et al., *Evaluation of the pharmacodynamic effects of MGCD0103 from preclinical models to human using a novel HDAC enzyme assay.* Clin Cancer Res, 2008. **14**(11): p. 3441-9.
237. Bonfils, C., et al., *Pharmacological inhibition of histone deacetylases for the treatment of cancer, neurodegenerative disorders and inflammatory diseases.* Expert Opin Drug Discov, 2008. **3**(9): p. 1041-65.
238. Garcia-Manero, G., et al., *Phase 1 study of the oral isotype specific histone deacetylase inhibitor MGCD0103 in leukemia.* Blood, 2008. **112**(4): p. 981-9.
239. Wood, T.E., et al., *Selective inhibition of histone deacetylases sensitizes malignant cells to death receptor ligands.* Mol Cancer Ther, 2010. **9**(1): p. 246-56.
240. McCourt, C., et al., *Elevation of c-FLIP in castrate-resistant prostate cancer antagonizes therapeutic response to androgen receptor-targeted therapy.* Clin Cancer Res, 2012. **18**(14): p. 3822-33.
241. Zagni, C., U. Chiacchio, and A. Rescifina, *Histone methyltransferase inhibitors: novel epigenetic agents for cancer treatment.* Curr Med Chem, 2013. **20**(2): p. 167-85.

242. Vedadi, M., et al., *A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells.* Nat Chem Biol, 2011. **7**(8): p. 566-74.
243. Loh, S.W., et al., *Inhibition of euchromatic histone methyltransferase 1 and 2 sensitizes chronic myeloid leukemia cells to interferon treatment.* PLoS One, 2014. **9**(7): p. e103915.
244. Tan, J., et al., *Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells.* Genes Dev, 2007. **21**(9): p. 1050-63.
245. Miranda, T.B., et al., *DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation.* Mol Cancer Ther, 2009. **8**(6): p. 1579-88.
246. Fiskus, W., et al., *Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells.* Blood, 2009. **114**(13): p. 2733-43.
247. Crea, F., et al., *An EZH2 polymorphism is associated with clinical outcome in metastatic colorectal cancer patients.* Ann Oncol, 2012. **23**(5): p. 1207-13.
248. Crea, F., et al., *EZH2 inhibition: targeting the crossroad of tumor invasion and angiogenesis.* Cancer Metastasis Rev, 2012. **31**(3-4): p. 753-61.
249. Monks, A., et al., *Gene expression-signature of belinostat in cell lines is specific for histone deacetylase inhibitor treatment, with a corresponding signature in xenografts.* Anticancer Drugs, 2009. **20**(8): p. 682-92.
250. Ammanamanchi, S. and M.G. Brattain, *Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells.* J Biol Chem, 2004. **279**(31): p. 32620-5.
251. Boland, C.R. and A. Goel, *Microsatellite instability in colorectal cancer.* Gastroenterology, 2010. **138**(6): p. 2073-2087 e3.
252. Kurzawski, G., et al., *Importance of microsatellite instability (MSI) in colorectal cancer: MSI as a diagnostic tool.* Ann Oncol, 2004. **15 Suppl 4**: p. iv283-4.
253. Kondo, Y. and J.P. Issa, *Epigenetic changes in colorectal cancer.* Cancer Metastasis Rev, 2004. **23**(1-2): p. 29-39.
254. Goel, A. and C.R. Boland, *Epigenetics of colorectal cancer.* Gastroenterology, 2012. **143**(6): p. 1442-1460 e1.
255. Jia, Y. and M. Guo, *Epigenetic changes in colorectal cancer.* Chin J Cancer, 2013. **32**(1): p. 21-30.
256. Ropero, S. and M. Esteller, *The role of histone deacetylases (HDACs) in human cancer.* Mol Oncol, 2007. **1**(1): p. 19-25.
257. Fiskus, W., et al., *Panobinostat treatment depletes EZH2 and DNMT1 levels and enhances decitabine mediated de-repression of JunB and loss of survival of human acute leukemia cells.* Cancer Biol Ther, 2009. **8**(10): p. 939-50.