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Identification of Phosphatases Involved in E2F-Associated Apoptosis

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IDENTIFICATION OF PHOSPHATASES INVOLVED IN E2F-ASSOCIATED APOPTOSIS

A Thesis

by

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ABSTRACT

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Retinoblastoma (Rb) is a tumor suppressor protein that controls a critical checkpoint between the G_1 phase and the S phase of the cell cycle. Rb is able to suppress cell proliferation by binding to the E2F family of transcription factors, inhibiting its ability to activate transcription of genes necessary for cell cycle progression. Mutations in proteins involved with the Rb/E2F pathway can result in hyper-proliferative cells that overtime can acquire and accumulate additional mutations, which could lead to tumorigenesis. To prevent hyper-proliferation, aberrant cells can be eliminated through apoptosis. E2F can induce apoptosis through two pathways: through transactivation of the tumor suppressor protein p53 or through Rb de-repression. This investigation provides evidence for E2F regulation of the phosphatases PTEN, Shp-2 and PTP-1B during E2F-associated apoptosis.

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CHAPTER I

INTRODUCTION

Statement of the Problem

Cancer is a complex genetic disease that is estimated to affect one in four persons [Ghavami et. al, 2009]. For a cell to become cancerous it must undergo a number of successive mutations that systematically de-regulate the extensive and highly efficient regulatory systems that determine cell fate. At the center of this intricate network is the Rb/E2F pathway. Retinoblastoma tumor suppression protein, or Rb, is a key cell cycle regulator that interacts with the E2F family of transcription factors to either promote cell proliferation or to initiate apoptosis, or programmed cell death, the mechanism by which unessential or abnormal cells are eliminated in a judicious and timely manner.

Nearly all tumors have mutations that de-regulate the Rb/E2F pathway, which transforms cells so that they become susceptible to secondary mutations over time; the accumulation of secondary mutations may yield cancer cells that exhibit one or more of the following characteristics: 1) can proliferate without stimulation by exogenous growth factors; 2) are invulnerable to anti-growth signals; 3) can overcome apoptosis; 4) can undergo an unlimited number of replications, or doublings; 5) can invade tissue and metastasize; or 6) can undergo angiogenesis [Hanahan and Weinberg, 2000].

One of the most commonly mutated genes in tumors is the p53 tumor suppressor gene; as a result, a great deal of work has been done to delineate p53-dependent apoptotic pathways in an effort to discover the causes of cancer and to derive effective treatments. However, in recent years novel alternative apoptotic pathways have been identified that do not utilize p53. The redundant duties of these pathways create a fail-safe mechanism that ensures the proper disposal of aberrant cells so that genomic integrity is maintained. One so-called p53-independent pathway, known as the E2F-associated apoptotic pathway, is induced by the loss of transcriptional repression imposed by the Rb/E2F complex. This pathway was characterized using an inducible cell line that is capable of over-expressing a dominant-negative form of E2F (dnE2F) that displaces Rb/E2F from pro-apoptotic gene promoters to trigger an apoptotic response. One gene whose expression is induced is *PTPN1* which encodes for protein tyrosine phosphatase 1B, or PTP1B. PTP1B is responsible for inactivating the signaling factor focal adhesion kinase (FAK) by dephosphorylation in order to initiate apoptosis [Lieman et. al, 2005].

Statement of the Purpose

Continued investigation into how the E2F-associated apoptotic pathway is regulated would help identify novel targets for the development of anti-cancer drugs. It would also provide information on how FAK is regulated in the Rb/E2F network and how it promotes cell survival. It is already known that FAK is activated and inactivated by phosphorylation and dephosphorylation, respectively. Phosphatases other than PTP-1B that have been implicated in regulating FAK include PTEN (phosphatase and tensin homolog), PTP-PEST (protein tyrosine phosphatase rich in proline, glutamic

acid/aspartic acid and serine/threonine residues), and SHP-2 (SH2 domain-containing tyrosine phosphatase). Study of the E2F-associated apoptotic pathway would identify which of these phosphatases are induced by loss of Rb/E2F transcriptional repression. Moreover, identification of phosphatases regulated by the Rb/E2F pathway would provide evidence for their role in the regulation of cell death (through their interaction with FAK), and it would identify potential targets for the creation of more specific, more effective, and possibly less toxic anti-cancer treatments.

CHAPTER II

REVIEW OF LITERATURE

Retinoblastoma and Cell Cycle Regulation

Retinoblastoma is a hereditary cancer of the eye that predominately affects children under five years of age. Investigation into the causes of retinoblastoma led to the isolation of the first known tumor suppressor gene, RB, and it is the loss of heterozygosity at the RB locus that predisposes individuals to retinoblastoma and other cancers [Kaelin Jr., 1999; Harbour and Dean, 2000; Giancinti and Giordano, 2006]. RB encodes the retinoblastoma nuclear phosphoprotein Rb which is involved in cell cycle regulation. It is comprised of an A box domain and a B box domain that are separated by a spacer, and these domains form a binding region shaped like a pocket when Rb is hypophosphorylated, or active (Fig. 1) [Kaelin, 1999; Giacinti and Giordano 2006; Sun et. al, 2007]. The bond at the Ser^{567} residue holds the shape of the "pocket." This region allows Rb to bind to other cellular proteins containing a Leucine-X-Cysteine-X-Glutamic acid (LXCXE) motif, a motif also found in the B box domain [Kaelin, 1999; Chan et. al, 2000; Giacinti and Giordano 2006].

When it is bound, Rb inhibits cell cycle progression at the critical checkpoint between the G_1 phase and the S phase of the cell cycle. It the transition from the G_1

phase to the S phase by repressing the transcription of genes required for entry into the S phase. For the cell cycle to proceed, Rb must be inactivated through phosphorylation by cyclin/cyclin dependent kinase (cdk) complexes, specifically cyclin D/cdk 4 (or cdk 6) and cyclin E/cdk 2 [Kaelin, 1999; Giacinti and Giordano 2006]. As the name suggests, the levels of cyclin expression fluctuate, or cycle, throughout the cell cycle progression to regulate it; the waxing and waning of the cyclin levels is determined by signals received from internal and external growth signaling pathways [Harbour and Dean, 2000; Giacinti and Giordano 2006]. Phosphorylation of serine residues, particularly Ser^{567}, disrupts the structural conformation of RB's "pocket" so that RB releases its key substrate, the E2F family of transcription factors [Harbour and Dean, 2000; Giacinti and Giordano 2006]. The S phase can then begin.

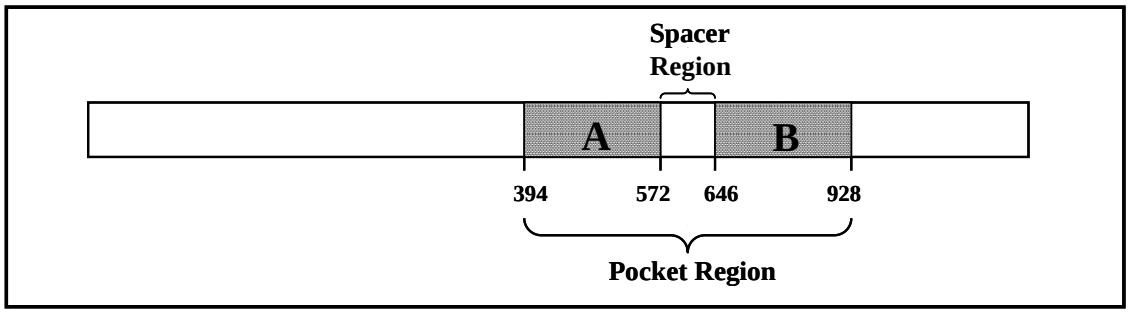

Figure 1. Structure of The Retinoblastoma Protein The interaction between the A box domain and the B box domain shape the catalytic domain into a pocket that associates with substrates through a LXCXE motif.

The E2F Family of Transcription Factors

The E2F family of transcription factors coordinate the expression of genes involved in cell proliferation, differentiation, development, and apoptosis [Young et. al, 2003; Korenjak and Brehm, 2005; Polager and Ginsberg, 2008; Wu et. al, 2009]. There are eight known members of the E2F family: E2F1-3a, which function as transcriptional activators, and E2F3b-8, which function as transcriptional repressors [Pützer, 2007]. All

of these proteins possess two highly conserved DNA binding domains (DBD) that allow E2F to bind specific sequences within the promoters of many genes that are required for S phase entry, DNA replication, mitosis, DNA repair, and apoptosis (Fig.2) [Young et. al, 2003; Sun et. al, 2007; Iaquinta and Lees, 2007; Müller et. al, 2000; Polager and Ginsberg, 2008; Wu et. al, 2009].

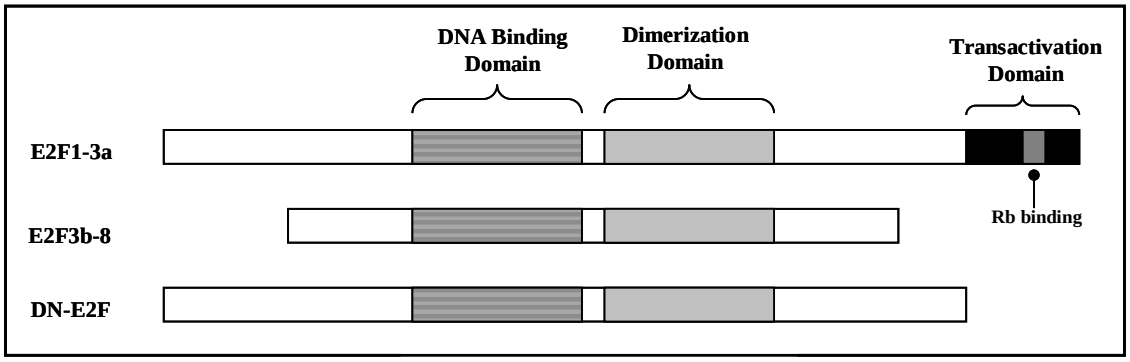

Figure 2. Structure of The E2F Family of Transcription Factors The E2F family can be subdivided into two groups: transcriptional activators and transcriptional repressors. The transcriptional activators possess a transactivation domain that the transcriptional repressors lack, and this domain is the site for Rb binding. All E2F family members contain a DNA binding domain and a dimerization domain that allows E2F to associate with the DP family of proteins.

E2F1-3a also have a C-terminal transactivation domain that allows activation of E2F-associated genes; within this domain is the RB binding site [Polager and Ginsberg, 2008; Wu et. al, 2009]. E2F1-6 are capable of dimerizing with a member of the family of DP (DRFT1-polypeptide, or dimerization-partner) proteins: DP1-4 [Pützer, 2007]. E2F and DP associate through the dimerization domain that lies downstream of the DBD. The DBD's of these two proteins are homologous; consequently they recognize the same DNA sequences, which boosts E2F activity and E2F DNA binding activity [Hitchens and Robbins, 2003; Milton et. al, 2006; Wu et. al, 2009].

Since Rb and E2F/DP are the central regulators of cell cycle progression and cell proliferation their interactions are carefully choreographed. When DP dimerizes with E2F, it not only enhances DNA binding, but it also promotes the recruitment of RB to

E2F. During nonproliferation or under unfavorable growth conditions, Rb associates with activator E2Fs through its "pocket" and carboxyl terminal region in order to silence genes containing E2F binding sites. The association between Rb and E2F silences the genes by concealing residues in E2F that are required for transactivation and by recruiting co-repressors, such as chromatin remodeling factors, to the gene promoter regions [Kaelin, 1999; Harbour and Dean, 2000; Stevens and La Thangue, 2003; Young et. al, 2003; Laquinta and Lees, 2007; Sun et. al, 2007; Polager and Ginsberg, 2008; Wu et. al, 2009]. Under normal conditions, cell cycle-dependent phosphorylation of Rb by G_1 phase cyclin/cdk complexes frees E2F/DP to interact with its responsive genes so the cell cycle can progress to the S phase.

Impact of the Rb/E2F Pathway

Apoptosis

In order to ensure proper development and to maintain tissue homeostasis, cell proliferation is closely connected to programmed cell death, or apoptosis, through the Rb/E2F pathway. Apoptosis is a strictly regulated biological process by which unessential, damaged or aberrant cells are eliminated. It can be initiated by one of two possible pathways: an extrinsic pathway or an intrinsic pathway. External or internal stimuli are received by the extrinsic or intrinsic pathways, respectively, to trigger a specific, coordinated series of intracellular signals. These signals facilitate the initiation of a signaling cascade that activates the caspases. Caspases are cysteine proteases that target and destroy cellular proteins during cell death. Initiator caspases, which include caspase-2, -8, -9 and -10, are activated first because they activate the executioner

caspases, which include caspase-3, -6 and -7 [Nahle et. al, 2002; Iaquinta and Lees, 2007; Pützer, 2007; Kurokawa and Kornbluth, 2009]. During apoptosis dying cells demonstrate distinct morphological changes including: cell detachment, membrane blebbing, chromatin condensation, nuclear fragmentation, cell rounding, and cell shrinkage [Zimmerman et. al, 2001; Hallé et. al, 2007].

E2F-1 is the only confirmed member of the E2F family of transcription factors to have the capacity to initiate apoptosis [Nahle et. al, 2002; Pützer, 2007; Steven and La Thangue, 2003; Denchi and Helin, 2005; Halstrom and Nevens, 2009; Wu et. al, 2009]. E2F-associated apoptosis can proceed through two mechanisms: through the E2F transactivation of pro-apoptotic genes and through loss of Rb/E2F repression, otherwise known as de-repression [Stevens and La Thangue, 2003; Pützer, 2007; Wu et. al, 2009].

The best understood apoptotic pathway involving E2F transactivation is the ARF/MDM2/p53 pathway which triggers an intrinsic apoptotic response. p53 is a tumor suppressor and a vital transcription factor that activates numerous genes involved in cell cycle arrest, senescence, apoptosis and differentiation. Its activities prevent the accumulation of abnormalities during successive cell divisions; therefore it is known as the guardian of the genome [Xu, 2008]. Under normal conditions p53 is ubiquitinized and thereby targeted for degradation by the E3 ubiquitin ligase MDM2. However, E2F-1 can induce expression of ARF, an alternate reading frame protein that inhibits MDM2, allowing stable p53 to accumulate and to concurrently turn on pro-apoptotic genes (including the caspases) and turn off cell survival genes [Pützer, 2007]. E2F-1 can also stimulate expression of p53 apoptotic co-factors to enhance and direct p53 activity in alternative p53-dependent apoptotic pathways [Wu et. al, 2009]. Even in the absence of

p53, E2F-1 can trigger apoptosis directly through the up-regulation of genes encoding proteins such as the p53 family member p73, the apoptosis protease activating factor Apaf1, BH3 only proteins (which regulate cell death) and the caspases [Stevens and La Thangue, 2003; Iaquinta and Lees, 2007; Pützer, 2007; Wu et. al, 2009; Halstrom and Nevens, 2009]. E2F association with Rb during non-proliferation conceals the E2F transactivation domain, effectively prohibiting the expression of all these genes.

In addition to transactivation, E2F-1 is capable of influencing an extrinsic apoptotic pathway through its active repression of pro-apoptotic factors, such as the tumor necrosis factors FAS and TNF-α, when it is in complex with Rb. As previously mentioned, Rb/E2F can repress transcription by recruiting co-repressors such as chromatin remodeling enzymes to the promoter regions of E2F-responsive targets; complexes block promoter enhancers and prevent the transcription machinery from assembling [Harbour and Dean, 2000]. These targets include additional pro-apoptotic factors that only will be expressed when the Rb/E2F complex is displaced from their promoters. This de-repression event does not require the E2F transactivation domain [Young et. al, 2003; Lieman et. al, 2005; Pützer, 2007]. To facilitate the general sensitization of an atypical cell to the apoptotic response, E2F-1 is also capable of inhibiting survival factors and anti-apoptotic signals such as NF-κB – a nuclear factor involved in inflammation and the immune response – and the Bcl-2 family of cell death regulators [Lieman et. al, 2005; Pützer, 2007; Halstrom and Nevens, 2009; Wu et. al, 2009].

Tumorigenesis

Given the importance of the Rb/E2F pathway in regulating cell proliferation and cell death, it is easy to understand why disruption of this pathway can lead to disease. Nearly all tumors contain genetic and epigenetic mutations that de-regulate the Rb/E2F pathway. Tumor cells develop from mutated, or transformed, cells that are incapable of regulating the cell cycle and that continue to proliferate and accumulate mutations until eventually they become tumorigenic and ultimately mestastasize. One common target for mutation is Rb. As previously described, the association of Rb with E2F prevents the transcription of several cell proliferative and pro-apoptotic genes. Consequently, mutation(s) that would result in loss of Rb (loss of Rb regulation of E2F or loss of Rb/E2F repression) would lead to hyperactivation of these genes. Activation of apoptosis under a condition of uncontrolled proliferation provides a selective pressure by which transformed cells can acquire additional mutations that may allow them to avoid elimination. Failure to undergo apoptosis allows the aberrant cells to accumulate more mutations over the next several cell divisions which in time can lead to cancer. Two events can lead to the loss of Rb activity: 1) mutation(s) within the RB gene that inactivate it or 2) mutation(s) in the Rb/E2F pathway which results in functional inactivation of Rb. For example, cyclin D is over-expressed in several types of tumors, and this protein promotes the inactivation of Rb [van Nimwegan and van de Water, 2007].

Another common target for mutation is p53. Mutations in p53 are found in over 50% of all cancers [Pützer, 2007; Xu, 2008; Cance and Golubovskaya, 2008; Golubovskaya et. al, 2008]. During times of cell damage and/or stress, p53 is responsible

for promoting the transcription of genes involved in growth arrest and apoptosis, and it is responsible for inhibiting the transcription of anti-apoptotic genes, such as the genes that encode focal adhesion kinase (FAK) and the Bcl-2 family of proteins [Pützer, 2007; Golubovskaya et. al, 2008]. Generally, mutations in *p53* either disrupt its protein's activity by making it non-functional or they disrupt p53 binding to its associated gene promoters so that pro-apoptotic genes are not turned on and survival genes are not turned off [Xu, 2008]. As a result, cells that would normally be targeted for elimination are allowed to survive and proliferate, promoting cellular transformation through the accumulation of mutation and in the end tumorigenesis. Cancers containing mutations in p53 are a good indicator of poor prognosis because they tend to be resistant to the radiation and chemotherapeutics used to fight the cancer, due to the de-regulation of the DNA damage repair system that p53 is a part of as well and due to the loss of apoptosis [Xu, 2008].

Focal Adhesion Kinase

FAK is a highly conserved non-receptor tyrosine kinase that functions as a scaffolding protein that is able to recruit various cytoskeletal proteins and signaling proteins to the focal adhesions for turnover at the focal adhesions [Parsons, 2003; Cox et. al, 2006; Siesser and Hanks, 2006]. Focal adhesions are the physical points of contact between the cell cytoskeleton and the extracellular matrix (ECM) where a vast amount of cellular signaling occurs [Cance and Golubovskaya, 2008]. FAK is also involved in many other cellular processes including cell migration, cell cycle regulation, and

apoptosis [Parsons, 2003; Golubovskaya et. al, 2005]. As a result, it is expressed in almost all tissues and cell types [van Nimwegan and van de Water, 2007; Parsons, 2008].

FAK is comprised of three domains: a large N-terminal domain, a centrally located kinase domain, and a large C-terminal domain (Fig. 3) [Parsons, 2003; Golubovskaya et. al, 2005]. The N-terminal domain possesses a proline-rich (PR) region that enables FAK to interact with different proteins [Cance and Golubovskaya, 2008]. It also contains a region known as the FERM (band 4.1-ezrin-radixin-moesin) domain; the FERM domain inhibits FAK kinase activity by physically interacting with the kinase domain [Cox et. al, 2006; Siesser and Hanks, 2006]. Furthermore, it is the location of a critical autophosphorylation site, Tyr^{387}. FAK is activated in response to phophosphorylation of this residue during the clustering of integrins at the focal adhesions [Parsons, 2003; Siesser and Hanks, 2006; van Nimwegan and van de Water, 2007]. Integrins are transmembrane proteins that not only help form the physical links between a cell and the ECM and/or another cell but that can act as receptors for cell-cell signaling. Once FAK is activated it is able to directly bind to SH-2 (Src homology 2) domain proteins through the N-terminal domain. In particular it binds to phosphatidylinositol 3-kinase (PI3-K) and sarcoma (Src) tyrosine kinase [Golubovskaya et. al, 2005; Cox et. al, 2006; van Nimwegan and van de Water, 2007; Cance and Golubovskaya, 2008]. Association with the latter promotes disassociation of the FERM domain from the kinase domain, and it triggers a kinase cascade that results in the hyperphosphorylation of FAK at residues: Tyr^{407}, $Tyr^{576/577}$, Tyr^{861}, and Tyr^{925} [Zhang et. al, 2006; Cance and Golubovskaya, 2008]. $Tyr^{576/577}$ and Tyr^{925} reside in the kinase domain and are required for maximal enzymatic activation [Parsons, 2003; Golubovskaya

et. al, 2005; Cox et. al, 2006].

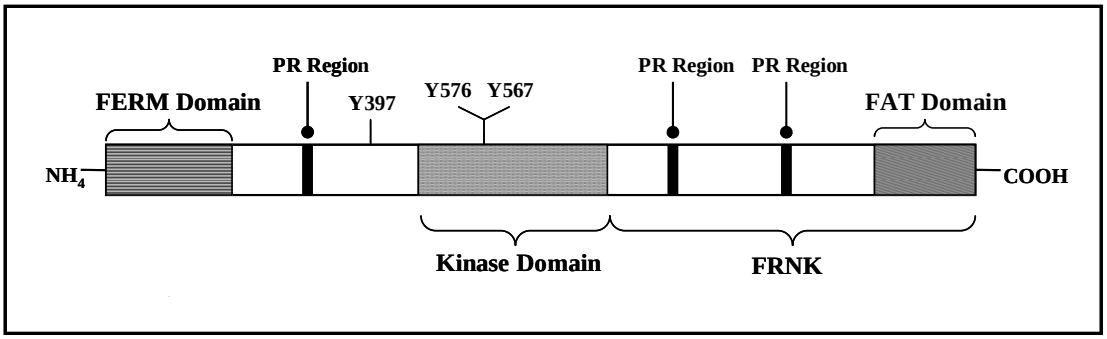

Figure 3. Structure of Focal Adhesion Kinase FAK is composed of three major domains: the N-terminal domain, the kinase domain, and the C-terminal domain. The N-terminal domain and the C-terminal domain possess various structural elements that allow the protein to participate in a number of protein-protein interactions.

The C-terminal domain also conducts a number of protein-protein interactions. It possesses two proline-rich regions that are capable of interacting with various proteins including the SH3 domain proteins [Parsons, 2003; Cox et. al, 2006], and it contains a region known as the FAT (focal adhesion targeting) domain. This region enables FAK to localize at the focal adhesions through associations with integrins and integrin-associated proteins, particularly paxillin and talin [Lieman et. al, 2005; Golubovskaya et. al, 2005; Parsons, 2003]. Interestingly, alternative splicing can yield an isoform of FAK that is composed of only the C-terminal domain; it is known as FAK non-related kinase or FRNK. Like the FERM domain, FRNK is capable of inhibiting FAK kinase activity; however it accomplishes this task by competing with full-length FAK for association with the focal adhesions [van Nimwegan and van de Water, 2007]. FAK localization stimulates various downstream signals, signals involved in cell attachment (adhesion), cell survival, cell proliferation, and cell migration [Parsons, 2003]. As a result, FAK is a central element in the regulation of these different cellular processes.

Thanks to its roles in cell survival and cell cycle control, FAK is essential to suppressing apoptosis; in fact, it is overexpressed in several types of tumors, beginning in the early stages of tumorigenesis. It also is associated with invasion, metastasis, and angiogenesis [Parsons, 2003; van Nimwegan and van de Water, 2007]. However, the mechanism(s) by which FAK promotes cellular transformation and tumorigenesis is (are) not completely understood. FAK can promote cell survival in many ways. For example, overexpression of FAK accelerates the transition from the G1 phase to the S phase through its upregulation of cyclin D1 [Cox et. al, 2006; van Nimwegen and van de Water, 2007]. During cell adhesion, FAK activates the ERK transcription pathway which in turn triggers cyclin D1 expression [Cance and Golubovskaya, 2008]. As previously discussed, cyclin D1 forms a complex with cdk4/6 to phosphorylate RB, and thus inactivates it so the cell cycle can proceed unchecked. FAK has also been associated with the up-regulation of cyclin D3 through the protein kinase C (PKC) and PI3-K signaling pathways which also results in Rb inactivation and cell proliferation.

Another way in which FAK promotes survival is through its interaction with p53. p53 can regulate FAK by binding to its promoter to inhibit its transcription, thereby suppressing cellular survival and growth. It also has been shown that FAK can bind to p53 through the FERM domain in a negative feedback loop [Cance and Golubovskaya, 2008; Golubovskaya et. al, 2008]. The FERM domain has three lobes: F1, F2, and F3. The F1 lobe allows FAK to enter the nucleus of the cell where it binds to p53 and MDM2 at F2 and F3, respectively. These interactions facilitate the association of p53 with MDM2 which promotes p53 degradation [Cance and Golubovskaya, 2008; Lim et. al,

2008]. Of course, loss of p53 disables p53-dependent apoptosis that is stimulated by the Rb/E2F pathway, and it leads to the accumulation of FAK.

Additionally, FAK can promote survival is through its inactivation of the PI3-K signaling pathway. As mentioned previously, activated FAK binds to PI3-K, and this interaction activates the kinase. Upon activation PI3-K produces two secondary messengers that recruit the proto-oncogene serine/threonine kinase protein kinase B, or Akt, to the plasma membrane for activation through by PI (3,4,5)-triphosphate dependent kinase (PDK) phosphorylation. Akt can then facilitate survival by down-regulating the activity of pro-apoptotic proteins and by up-regulating the activity of anti-apoptotic proteins [DiCristofano and Pandolifi, 2000; Mayo and Donner, 2002].

FAK can also suppress apoptosis through its association with the death receptor complex interacting protein, RIP. RIP is a serine/threonine kinase that contains a death domain; the death domain is a protein motif shared by a family of proteins that are involved in the signaling pathways leading to apoptosis. RIP is able to either induce apoptosis or facilitate cell survival signaling through its interaction with the death domains of cell surface receptors of the tumor necrosis factor superfamily and through its interactions with death domain adaptor proteins [Kurenova et. al, 2004; Golubovskaya et. al, 2005; Lieman et. al, 2005; Cance and Golubovskaya, 2008]. How it switches between the two processes is not clear. RIP also plays a role in NF-κB activation [Kurenova et. al, 2004] which is a survival factor that can be inhibited by E2F-1. When FAK is bound to RIP, it inhibits the interaction between RIP and the death receptor complex, thereby suppressing the proapoptotic signals normally induced by RIP [Lieman et. al, 2005] that not only trigger apoptosis but that also promote the degradation of FAK [Kurenova et. al,

2004]. During de-repression-mediated apoptosis, FAK is inactivated by dephosphorylation, allowing RIP to facilitate apoptosis.

FAK-Associated Protein Tyrosine Phosphatases

Protein tyrosine phosphorylation is an important event that occurs in numerous signaling pathways as a means of regulating key cellular processes like cell proliferation and apoptosis [Thompson et. al, 2001; Östman et. al, 2006; Jiang and Zhang, 2008; Mohi and Neel, 2008; Stuible et. al, 2008; Lessard et. al, 2010]. The appropriate levels of tyrosine phosphorylation are sustained by the well-balanced counter-activities of the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPs). De-regulation of these activities results in aberrant tyrosine phosphorylation which contributes to the pathogenesis of various human diseases including cancer [Dubé and Tremblay, 2004; Jiang and Zhang, 2008]. Mutations affecting either PTK activity or PTP regulation result in malignant transformation [Dubé and Tremblay, 2004; Östman et. al, 2006; Mohi and Neel, 2007]. Malignant transformation involves major re-organization of the cytoskeleton, leading to decreased cell adhesion *via* de-regulated adhesion-mediated signaling [Östman et. al, 2006].

FAK has several potential phosphorylation sites, and its activity depends on which tyrosines are phosphorylated and consequently which are dephosphorylated. At least four PTPs have been identified that interact with FAK; they are: PTP-1B encoded by *PTPN1*, PTEN (phosphatase and tensin homolog deleted on chromosome 10) encoded by *PTEN*, PTP-PEST (protein tyrosine phosphatase rich in proline, glutamic acid/aspartic

acid, and serine/threonine residues) encoded by *PTPN12*, and Shp-2 (SH-2 domain-containing tyrosine phosphatase) encoded by *PTPN11*.

PTP-1B

PTP-1B was the first of the PTP superfamily to be identified, but since then at least 107 additional enzymes have been characterized [Östman et. al, 2006; Mohi and Neel, 2007; Hallé et. al, 2008; Stuible et. al, 2008]. Members of the PTP superfamily are identified by a highly conserved catalytic domain containing a characteristic (H/V)CXXXXXR(S/T) motif known as the PTP signature motif [Thompson et. al, 2001; Jiang and Zhang, 2008]. Thirty eight of the 107 phosphatases can be sub-categorized as classical PTPs because they are exclusively tyrosine specific [Mohi and Neel, 2007; Stuible et. al, 2008; Lessard et. al, 2010]. PTP-1B is a ubiquitously expressed classical PTP that acts as a key regulator of body weight, glucose homeostasis, and energy expenditure due to its ability to down-regulate insulin and leptin signaling [Jiang and Zhang, 2008]. Because of its regulatory role in insulin and leptin signaling, PTP-1B's involvement in metabolic disorders such as diabetes and obesity has been well-studied. Investigation of PTP-1B also has provided evidence indicating that it is involved in the development of cancer; its expression is increased in human breast, ovarian and epithelial carcinomas, and it is decreased in oesophageal carcinomas [Dubé and Tremblay, 2004]. PTP-1B can either promote or suppress apoptosis – acting as either a tumor suppressor or tumor promoter, respectively – depending on its substrate which could vary depending on cell type and cell context [Jiang and Zhang, 2008]. For example, PTP-1B can facilitate cell death by inhibiting the PI3-K/Akt pro-survival signal transduction pathway. The

exact regulatory mechanism is unknown, but it has been clearly demonstrated that loss of PTP-1B expression in *PTPN1*-null hepatocytes leads to resistance from growth factor deprivation induced apoptosis which correlates with increased Akt phosphorylation and decreased nuclear expression of Foxo1, a substrate of Akt that regulates the transcription of several pro-apoptotic genes [Hallé et. al, 2008; Stuible et. al, 2008; Lessard et. al, 2010].

Also, PTP-1B can promote apoptosis by enhancing the activities of caspase 8 and caspase 9 as seen in human glioma cells treated with the PPAR-γ (peroxisome proliferator-activated receptor) agonist, troglitazone [Hallé et. al, 2008; Stuible et. al, 2008; Lessard et. al, 2010]. Troglitazone induces apoptosis through its effect on the death ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). In this case, PTP-1B mediates the desphophorylation of Jak2 (Janus kinase 2) which subsequently leads to the dephosphorylation (i.e. down-regulation) of the STAT3 (signal transducer and activator of transcription 3) pro-survival signaling pathway [Hallé et. al, 2008]. As a result, anti-apoptotic proteins FLIP (FLICE inhibitory protein) and Bcl-2 are inhibited so cells become susceptible to caspase-dependent apoptosis induced by TRAIL [Stuible et. al, 2008; Lessard et. al, 2010].

On the other hand, PTP-1B mediation of integrin signaling can promote tumorigenesis. In breast cancer and colon cancer cells, PTP-1B increases c-Src kinase activity by dephosphorylating it at its tyrosine residue Tyr^{530} which normally, when phosphorylated, inhibits Src activity [Stuible et. al, 2008; Lessard et. al, 2010]. PTP-1B also can inactivate CrkII and $p130^{Cas}$, effector proteins downstream of c-Src and FAK. Integrin activation is significant in tumorigenesis given that it promotes growth and

metastasis of cancer cells. Inhibitors of integrin activation have been investigated for their potential as anticancer drugs [Stuible et. al, 2008].

PTEN

PTEN is a lipid and protein phosphatase that can dephosphorylate serine, threonine, and tyrosine residues. Its primary function is to regulate the PI3-K/Akt pro-survival signaling cascade [Shen et. al, 2007; Yin and Shen, 2008]. It is encoded by a tumor suppressor gene that, after p53, is the second most frequently mutated (or deleted) gene in human cancer [Mohi and Neel, 2007; Yin and Shen, 2008]; these mutations most often occur in glioblastomas, malignant melanomas, endometrial cancer, prostate cancer [Tamura et. al, 1999; Yin and Shen, 2008].

PTEN is well-established as a tumor suppressor. It has been strongly associated with maintaining genomic stability and, like p53, has been dubbed a guardian of the genome [Shen et. al, 2007; Yin and Shen, 2008]. To prevent cellular transformation, PTEN regulates cell death upon loss of cell adhesion by negatively regulating the PI3-K/Akt signaling pathway [DiCristofano and Pandolfi, 2000]. It dephosphorylates phosphatidylinositol (3,4,5) triphosphate (PIP-3), one of the secondary messengers produced by PI3-K [Tamura et. al, 1999; Mayo and Donner, 2002; Yin and Shen, 2008]. Accumulation of PIP-3 at the plasma membrane triggers the recruitment of Akt [DiCristofano and Pandolfi, 2000]. Akt interacts with several substrates to enhance cell survival. For example, Akt phosphorylation of MDM2 allows the protein to enter the nucleus where it interacts with p53 to promote p53 degradation and inhibit apoptosis.

Also, Akt can inhibit CDK inhibitors, activating cyclin D [Mayo and Donner, 2002]. As a result, Rb remains turned off and E2F is free to initiate cell proliferation.

Like PTP-1B, PTEN can also inactivate FAK and $p130^{Cas}$ to inhibit cell migration and invasion [Yin and Shen, 2008]. It dephosphorylates FAK at the Tyr^{397} residue, its key autophosphorylation site [Tamura et. al, 1999]. Inactivation of FAK impacts a number of cellular pathways including the PI3-K/Akt signaling pathway since FAK interacts with PI3-K at Tyr^{397} upon integrin-mediated activation [Tamura et. al, 1999]. PTEN-deficient cells demonstrate increased FAK activity correlated with increased FAK/PI3-K association and increased PI3-K activity. Subsequently, these cells also demonstrate enhanced Akt activation [Parise et. al, 2000].

Loss of PTEN would result in enhanced cell survival due to hyperactivation of Akt through increased PIP-3 activity [DiCristofano and Pandolfi, 2000] and due to overexpression of FAK because of increased p53 degradation. PTEN itself directly down-regulates the promoter activity of MDM2 [Yin and Shen, 2008], consequently loss of PTEN would have an even greater affect in promoting cell survival. Studies have shown that *PTEN*-deficient cells demonstrate increased proliferation, reduced apoptosis, and increased migration [Yin and Shen, 2008].

Shp-2 and PTP-PEST

Shp-2. Shp-2 is a ubiquitously expressed classical, non-receptor PTP. It plays a key regulatory role in signaling cell growth, transformation, differentiation, spreading, migration, and cytoskeleton organization [Yu et. al, 1998; Bentires-Alj et. al, 2004; Mohi and Neel, 2007]. It enhances signal transduction through its interactions with different

growth factors, scaffolding adaptors, cytokines, and ECM receptors that possess a SH2 domain [Yu et. al, 1998; Mohi and Neel, 2007]. In particular, Shp-2 is required for the activation of the Ras GTPase/extracellular signal-regulated kinase (ERK) signaling cascade [Bentires-Alj et. al, 2004; Östman et. al, 2006; Hallé et. al, 2007; Mohi and Neel, 2007]. The exact mechanism of activation remains unclear; however it is known that ERK activation results in the inactivation of the pro-apoptotic proteins Bim and BAD – members of the Bcl-2 family of proteins. It also has been implicated in the regulation of the PI3-K/Akt pathway; it can activate PI3-K to stimulate Akt, resulting in the promotion of cell survival through suppression of caspase 3-mediated apoptosis [Zito et. al, 2004; Hallé et. al, 2007].

Shp-2 has become established as an oncogenic protein given that it is mutated in different types of leukemia and that it is a target for other oncogenes [Mohi and Neel, 2007]. Germline mutations of *PTPN11* cause ~50% of the cases of Noonan Syndrome, a disease associated with increased risk of malignancy and leukemia, especially juvenile myelomonocytic leukemia (JMML) [Bentires-Alj et. al, 2004; Östman et. al, 2006; Mohi and Neel, 2007]. *PTPN11* mutations have also been identified in lung and colon cancer, neuroblastoma, and melanoma [Östman et. al, 2006].

PTP-PEST. PTP-PEST is a ubiquitously expressed non-receptor PTP that plays a role in focal adhesion turnover and, subsequently, cell adhesion, cell migration and cytoskeleton organization [Shen et. al, 1998; Angers-Loustau et. al, 1999; Hallé et. al, 2007; Hallé et. al, 2007b]. PTP-PEST functions in focal adhesion turnover through its ability to dephosphorylate paxillin and $p130^{Cas}$; association with paxillin allows PTP-PEST to

indirectly regulate FAK [Shen et. al, 1998; Anger-Loustau et. al, 1999; Östman et. al, 2007; Hallé et. al, 2007b].

Previous work has provided evidence indicating that PTP-PEST may play a role in apoptosis. Caspase 3 has the ability to cleave PTP-PEST during apoptosis, and the result is alteration of downstream signaling pathways that influence cytoskeleton organization [Hallé et. al, 2007b]. Apoptotic cells demonstrate cell detachment, cell rounding, and cell shrinkage since cell adhesion is required for proper cell survival signaling [Hallé et. al, 2007]. Malignant transformation also involves cytoskeleton reorganization.

CHAPTER III

METHODOLOGY AND FINDINGS

Materials and Methods

Cell Line and Culture

ER-dnE2F cells (gift of D. Dean) were maintained in DMEM with 10% FBS, 400 μg/ml zeocin, 150 μg/ml hygromycin B and 300μg/ml G418 [Young et al., 2003]. Treatment with 4-hydroxytamoxifen (4OHT) (100 nM, Sigma) resulted in activation of the dominant-negative E2F mutant (dnE2F), and treatment with IPTG (1 mM, Sigma) resulted in activation of p16Ink4a, as previously described [Young et. al, 2004].

Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was collected and isolated from induced cell cultures (+p16 +dnE2F) at intervals over a 48 hour time period using RNeasy® kit (Qiagen), and the concentrations of the samples were determined using the NanoDrop ND-1000 spectrophotometer. IPTG only induced cell cultures (+p16) were used as control. cDNA was generated with 0.5 ng of RNA using Superscript® III Reverse Transcriptase (Invitrogen) with the RETROscript® kit (Ambion). Amplification was carried out in the 7900HT Fast Real-Time PCR System (Applied Biosystems) using Taqman® Gene Expression Assay (Applied Biosystems) FAM-labeled probes for human genes *PTPN1*, *PTEN*, *PTPN12*, and *PTPN11* according to manufacturer's instructions. Human

glyceraldehydes-3-phosphate dehydrogenase (GAPDH) Endogenous Control (FAM/MGB probe, Applied Biosystems) was used as a control.

Data Analysis

The threshold C_T values were generated by the Applied Biosystems RQ Manager software version 1.2, and the experimental ΔC_T values were normalized to the ΔC_T values of the GAPDH endogenous control. Relative expression was calculated using the comparative C_T method ($2\text{-}\Delta\Delta C_T$) with the +p16 control cells at each specific time point representing uninduced. Experiments were repeated in triplicate and data are reported as mean $\pm$ standard deviation of the mean.

Bioinformatics

The TRANSFAC® Professional Software (http://www.biobase-international.com/) was used to identify putative E2F binding sites in human PTPN1, PTEN, PTPN12, and PTPN11 gene promoters (Appendix A).

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using an E2F-1 specific antibody (Anti E2F-1 clones KH20 and KH95, Millipore) with the ChIP-IT Express Kit (Active Motif) according to manufacturer's instructions. Briefly, induced cells from one 15cm plate were fixed with 37% formaldehyde for 8min. Cells were dounced on ice, and the nuclear lysate was sonicated 10x20s at 100% power using the Sonic Dismembrator (Fisher) to shear chromatin to an average size of 800bp. IP's were carried out with 100µl of the sheared

chromatin and 3µg of antibody overnight at 4ºC. FAS (B-10) antibody (Santa Cruz, sc8009) was used as a negative control antibody.

PCR was performed with 0.2µl chromatin per reaction using GoTaq® Green Master Mix (Promega) according to manufacturer's instructions; however PCR reactions also included 3% DMSO. Sequence-specific primers were generated using DNASTAR® Lasergene Version 8 Sequence Builder software (Appendix B).

Results

Identification of Phosphatases Induced by E2F-associated Apoptosis

To identify FAK-associated phosphatases whose transcriptional expression is induced by the activation of E2F-associated apoptosis, dnE2F-ER cells were used to provide the conditions for E2F-associated apoptosis. First, p16 expression was induced to establish endogenous Rb-mediated G1 arrest, and then 4OHT was added to activate dnE2F to simulate the effect of Rb loss. Overexpression of dnE2F displaces the Rb/E2F complex which triggers the de-repression of E2F sites and intiates E2F-associated apoptosis.

For qPCR analysis the generated ct values for phosphatases PTP-1B, PTEN, Shp-2, and PTP-PEST were normalized to GAPDH ct values at each time point; the ΔΔct values were calculated using the Δct values at each time point of the control cells untreated with 4OHT as the uninduced control. The results demonstrated 1.5 h after induction of E2F-associated apoptosis PTEN and Shp-2 expression was 2-times and 3-times, respectively, greater than the 0 h (Fig. 4). Their expression levels quickly returned to "normal" and leveled off. PTP-1B and PTP-PEST expression appeared to be repressed

at 1.5 h but their expression levels gradually increased. After the cells were undergoing apoptosis for 6 h, PTP-1B expression was also induced. It appeared that PTP-PEST expression was slightly induced at 6 h; however there is no previous evidence to corroborate the result. Overall, the data suggests that phosphatases PTEN, Shp-2, and PTP-1B are regulated by Rb/E2F.

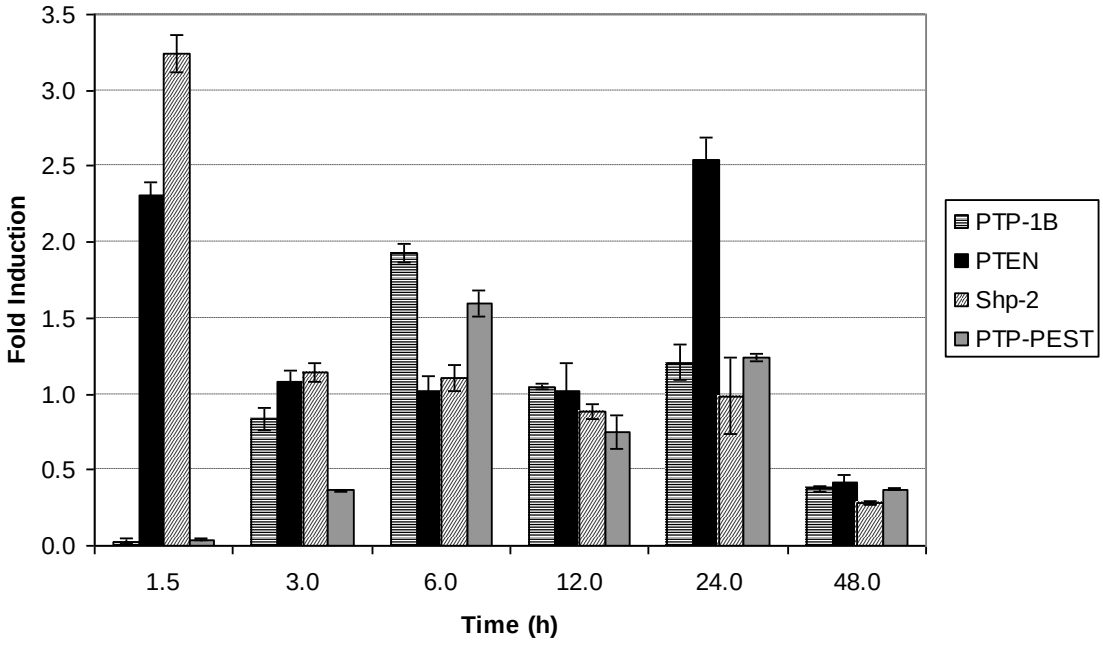

Figure 4. Expression of Phosphatase During E2F-associated Apoptosis ER-dnE2F cells were treated for 24 h with IPTG to induce p16 expression, and then 4OHT was added to initiate E2F-associated apoptosis mediated by dnE2F expression. Control cells were untreated with 4OHT. Total RNA was isolated after 0, 1.5, 3.0, 6.0,12.0, and 48.0 h of treatment with 4OHT and used for real-time quantitative PCR analysis which was performed twice in triplicate.

Phosphatase Promoter Occupancy of E2F Responsive Promoters

Since the phosphatases appeared to be induced after loss of Rb/E2F repression, ChIP analysis was performed to demonstrate that the promoters of the phosphatase genes are directly bound by E2F. The promoter sequences for *PTPN1*, *PTEN*, *PTPN11*, and *PTPN12* were evaluated for putative E2F binding sites, and several highly scoring

matches were identified. Due to time constraints, a few of the highest scoring sequences were randomly selected for each phosphatase in order to generate sequence-specific probes for the ChIP assays. PCR conditions were optimized, resulting in the identification of one probe each for PTP-1B, PTEN, and PTP-PEST (Table 1).

TABLE 1
Primers identifying E2F binding sites

Phosphatase	*Gene*	*Promoter Region*	*Primer Sequence*	*Putative E2F site (Orientation)*
PTP-1B	*PTPN1*	-7940…-8180	(F) 5'-ATGGTAGCAGTGCAGCTGATGTG-3' (R) 5'-AAGTCACTCTTAATGTCAGGC-3'	cGCGCC (-)
PTEN	*PTEN*	-3337…-3068	(F) 5'-TCGCTGGGCTGCAGCTTCCTACC-3' (R) 5'-CTGTGAGTGGGACGCACCCC-3'	ccGCGAAc (-)
PTP-PEST	*PTPN12*	-921…-1341	(F) 5'-AGCGCGAGGCCGCGCATCTG-3' (R) 5'-CTTCCCCAGCTCGTTCCCC-3'	gtTGGCGc (+)

(F) Forward primer
(R) Reverse primer

p16 was induced for 24 h first, and then dnE2F was activated for 24 h. Although there appeared to be differences in the effect of E2F-associated apoptosis on the transcription of the phosphatase genes, E2F-1 was detected at PTP-1B, PTEN and PTP-PEST promoters (Figure 5). In general, the data provides evidence that PTP-1B, PTEN, and PTP-PEST are directly regulated by E2F-1.

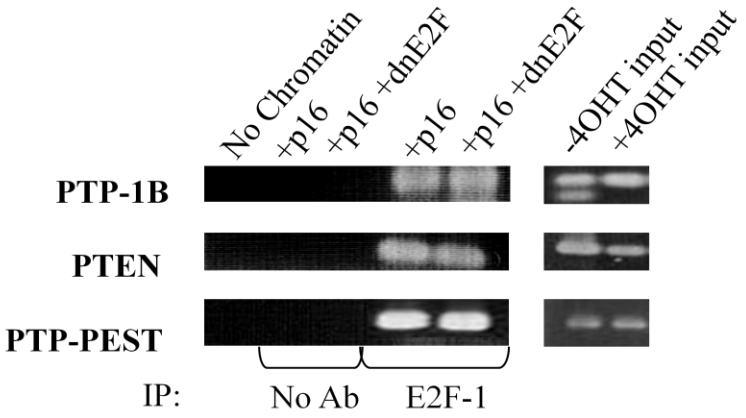

Figure 5. Activation of phosphatase genes by E2F ER-dnE2F cells were treated for 24 h with IPTG to induce p16 expression (+p16) and then treated for 24 h with 4OHT to induce dnE2F expression (+p16 +dnE2F). Control cells were untreated with 4OHT. ChIP assays were performed. Proteins immunoprecipitated are indicated at the bottom, and the promoters amplified are indicated to the left.

CHAPTER IV

SUMMARY AND CONCLUSION

As a mechanism that controls cell proliferation and apoptosis, de-regulation of the Rb/E2F pathway contributes to the pathogenesis of a variety of human diseases including cancer. As a result, the Rb/E2F pathway has been studied extensively in an attempt to discover how cellular transformation and tumorigenesis is initiated and how cancer progesses so that anticancer drugs and therapies can be developed that specifically target the causes.

E2F can initiate apoptosis through transactivatio of the p53 tumor suppressor and through E2F-associated apoptosis. The E2F-associated apoptotic pathway requires the inactivation of FAK to trigger a caspase 8-mediated apoptotic response. Protein tyrosine phosphatase PTP-1B was shown to be directly regulated by E2F-1 in this pathway. PTP-1B is one phosphatase capable of regulating FAK, indicating that PTP-1B plays a functional role in inducing E2F-associated apoptosis by dephosphorylating FAK.

The results from this investigation provide evidence that several phosphatases including PTEN, Shp-2, and PTP-1B are directly regulated by E2F-1 during E2F-associated apoptosis. qPCR data showed that PTEN transcriptional expression was induced ~2-fold, Shp-2 expression was induced ~3-fold, and PTP-1B expression was induced ~2-fold after induction of the E2F-associated apoptotic response. The apoptotic

pathway is initiated upon loss of Rb/E2F repression; therefore, the data imply that under normal conditions, PTEN, Shp-2, and PTP-1B are repressed by Rb/E2F complexes. Interestingly, PTEN expression levels decreased and then appeared to increase again after 24h of E2F-associated apoptosis. This result suggests that at 24h PTEN expression may be indirectly activated. It may be part of a regulatory feedback loop, or its expression may be mediated by other proteins in the pathway that were either activated or inactivated during the apoptotic response.

Since PTEN and Shp-2, as well as PTP-1B, have been implicated in the regulation of FAK, it can be inferred from the results of this investigation that the possible role of these three phosphatases in E2F-associated apoptosis may be to inactivate FAK. Further investigation will be needed to confirm the physiological role of PTEN, Shp-2, and PTP-1B in this pathway. Specific phosphatase inhibitors and siRNAs targeting *PTEN*, *PTPN11*, and *PTPN1* could be utilized to determine the effect of suppressed protein and transcript expression, respectively, on cells undergoing E2F-associated apoptosis. If PTEN, Shp-2, and PTP-1B are required for FAK inactivation and initiation of apoptosis, then the application of the inhibitors or the siRNAs will rescue the cells from cell death. To complement the data, Western blot analysis using phosphorylation-specific antibodies could be performed in order to determine relative FAK phosphorylation in the presence and absence of the inhibitors and siRNAs. PTEN, Shp-2, and PTP-1B could also be overexpressed to determine the effect on E2F-associated apoptosis. Overexpression should result in enhancement of the apoptotic response *via* increased FAK dephosphorylation. Further studies need to be done to not only characterize the role of the phosphatases, but to further delineate the link between FAK and caspase 8.

Previous investigations have implicated PTEN, Shp-2, and PTP1B involvement in the development of cancer. This role appears to stem from the ability of these PTPs to regulate integrin signaling and receptor tyrosine kinase signaling, which mediate cell survival and cell migration and invasiveness. PTEN is a confirmed tumor suppressor; its gene is the second mostly frequently mutated gene found in human tumor cells. Loss of PTEN results in a significant enhancement of cell survival due to hyperactivation of Akt, a protein that mediates p53 degradation and Rb inactivation. Under normal conditions, PTEN negatively regulates the PI3-K/Akt pro-survival signaling pathway by inactivating the PI3-K secondary messenger that facilitates Akt activation.

PTP-1B can act as a tumor suppressor or a tumor promoter depending on its substrate, and mutations in PTP-1B can also be found in different types of tumors. Similar to PTEN, PTP-1B can negatively regulate the PI3-K/Akt pathway, though the mechanism by which it suppresses it is unclear. PTP-1B has also been implicated in enhancing the activities of caspase 8 and caspase 9.

Shp-2 is a known oncogene. That it is induced during E2F-associated apoptosis seems counterintuitive, especially given that, contrary to PTEN and PTP-1B, Shp-2 has been implicated in the activation of the PI-3K/Akt pathway. However, it is well-established that some tumors demonstrate increased apoptotic activity, and they are successful because the rate of cell proliferation is greater than the rate of apoptosis. Future investigation of the E2F-associated apoptotic pathway may provide an explanation as to the role of Shp-2 in this pathway.

In conclusion, understanding of the p53-independent E2F-associated apoptotic pathway is essential as it will provide information on how FAK can mediate apoptosis.

This is a mechanism that all cancers must overcome in order to be successful. Identifying the proteins involved in this pathway identifies potential targets for the development of new specific and highly effective anti-cancer drugs and therapies.

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APPENDIX A

APPENDIX A

IDENTIFIED PUTATIVE E2F SITES FOR FAK-ASSOCIATED PHOSPHATASES

Phosphatase	*Position*	*Orientation*	*Match Score*	*Sequence*
PTP1B	-8171	(-)	1.000	cGCGCC
PTP1B	-6167	(-)	0.802	actTTTTCacgggag
PTP1B	-5450	(+)	0.899	tTTTGCgt
PTP1B	-4142	(+)	1.000	GGCGCg
PTP1B	-3746	(+)	0.797	ttgctggGAAAAagg
PTP1B	-779	(-)	0.800	actTTGTCccgctta
PTP1B	-172	(+)	1.000	GGCGCg

Phosphatase	*Position*	*Orientation*	*Match Score*	*Sequence*
PTEN	-6062	(+)	1.000	GGCGCg
PTEN	-4092	(+)	1.000	GGCGCg
PTEN	-3177	(-)	0.979	ccGCGAAc
PTEN	-1126	(+)	1.000	GGCGCg
PTEN	-1078	(+)	1.000	GGCGCg
PTEN	-986	(+)	1.000	GGCGCg
PTEN	-461	(-)	0.999	ccGCCAAg

Phosphatase	*Position*	*Orientation*	*Match Score*	*Sequence*
PTP-PEST	-6226	(-)	0.908	gccgagatCGCGCcac
PTP-PEST	-6218	(-)	1.000	cGCGCC
PTP-PEST	-3665	(+)	1.000	GGCGCg
PTP-PEST	-3661	(-)	1.000	cGCGCC
PTP-PEST	-3313	(-)	1.000	cGCGCC
PTP-PEST	-3110	(-)	1.000	cGCGCC
PTP-PEST	-2745	(+)	1.000	GGCGCg
PTP-PEST	-2380	(-)	1.000	cGCGCC
PTP-PEST	-1102	(+)	0.980	gtTGGCGc
PTP-PEST	-966	(+)	0.999	cTTGGCgg
PTP-PEST	-632	(+)	1.000	GGCGCg

Phosphatase	*Position*	*Orientation*	*Match Score*	*Sequence*
Shp-2	-10745	(+)	0.894	tTTTGCga
Shp-2	-10514	(-)	0.955	acGCGAAa
Shp-2	-10421	(-)	1.000	cGCGCC
Shp-2	-9801	(+)	1.000	GGCGCg
Shp-2	-9382	(+)	0.942	tTTAGCgg
Shp-2	-8054	(-)	0.893	gcCCCAAa
Shp-2	-3280	(-)	1.000	cGCGCC
Shp-2	-3005	(-)	1.000	cGCGCC
Shp-2	-1994	(+)	0.908	gtgGCGCGatctcggc
Shp-2	-1992	(+)	1.000	GGCGCg
Shp-2	-1908	(+)	1.000	GGCGCg
Shp-2	-1904	(-)	1.000	cGCGCC
Shp-2	-1672	(+)	0.908	gtgGCGCGatctcggc
Shp-2	-1670	(+)	1.000	GGCGCg
Shp-2	-1441	(-)	1.000	cGCGCC
Shp-2	-1175	(-)	1.000	cGCGCC
Shp-2	-931	(+)	0.961	cgTGGCGc
Shp-2	-705	(+)	1.000	GGCGCg
Shp-2	-384	(-)	1.000	cGCGCC

APPENDIX B

APPENDIX B

PRIMERS GENERATED TO DETECT PHOSPHATASE GENES BASED ON IDENTIFIED E2F PUTATIVE SITES

Phosphatase	***Gene***	***Promoter Region***	***Primer Sequence***
PTP-1B	*PTPN1*	-7940…-8180	(F) 5'-ATGGTAGCAGTGCAGCTGATGTG-3'
			(R) 5'-AAGTCACTCTTAATGTCAGGC-3'
		-81…-320	(F) 5'-CCATTCCAAGTCAACACTCTTCT-3'
			(R) 5'-AAAAGCCACTTAATGTGGAGGTC-3'
PTEN	*PTEN*	-6072…-5898	(F) 5'-CCCTGAAGCTTTATATAATTGTC-3'
			(R) 5'-CCTCAGCCTCCCGAGTAGC-3'
		-4087…-3827	(F) 5'-GTCAGCCCAATCGGGGCTGTA-3'
			(R) 5'-AAGGAGCTGGGCGCTAGGG-3'
		-3337…-3068	(F) 5'-TCGCTGGGCTGCAGCTTCCTACC-3'
			(R) 5'-CTGTGAGTGGGACGCACCCC-3'
		-1231…-961	(F) 5'-CCTGCGGCTTGGGGACTCTG-3'
			(R) 5'-TCCGAGAGGAGAGAACTGAGCG-3'
		-303…-583	(F) 5'-GCAGGCCCAGTCGCTGCA-3'
			(R) 5'-ACAGGTCAAGTCTAAGTCGAATC-3'
Shp-2	*PTPN11*	-9466…-9202	(F) 5'-GTCTGGGCTGCAGTGCAGTGAAC-3'
			(R) 5'-TCCATCAAATAATCAAATATGC-3'
		-3332…-2992	(F) 5'-GATTACAGGCGTGAGCCACCG-3'
			(R) 5'-CACATAGTAGCCAGAGTAATCTA-3'
		-2172…-2435	(F) 5'-GTCGCACCATGTTGCCC-3'
			(R) 5'-GGGAACCAGCCCGGGCAAAATAG-3'
		-571…-851	(F) 5'-AGCAAGGAGCGGGTCCGTC-3'
			(R) 5'-GAACTCGGGGAGGCAGGAAAATG-3'
PTP-PEST	*PTPN12*	-6316…-6036	(F) 5'-GCCGGGCGTGGTGGCAG-3'
			(R) 5'-AGCGGCTACCTTTGGCATCTACT-3'
		-3741…-3481	(F) 5'-GGAGACCAGCCCGGCCAACACAG-3'
			(R) 5'-GGAGAATTGCTTGAACCCAG-3'
		-2461…-2241	(F) 5'-GATGTCAGGAAGTCGCTTTATTT-3'
			(R) 5'-TTAAAAATTTATTATTTATTTA-3'
		-921…-1341	(F) 5'-AGCGCGAGGCCGCGCATCTG-3'
			(R) 5'-CTTCCCCAGCTCGTTCCCC-3'

(F) Forward primer
(R) Reverse primer

BIOGRAPHICAL SKETCH

Liza Doreen Morales Smith (1321 E. Crockett Ave., Harlingen, TX, 78550) graduated with two Bachelor of Arts degrees in Biochemistry and Biology from Rice University (Houston, TX) in December of 2001. She completed her Master of Science degree in Biology at the University of Texas-Pan American (Edinburg, TX) in May of 2010.

Mrs. Smith has several years of experience conducting scientific research. From May of 2000 to December of 2002 she worked at Baylor College of Medicine (Houston, TX) as first a project intern and then a research technician in the Department of Molecular Human Genetics. Next, from December of 2002 to September of 2003 she worked as a research assistant at UT-MD Anderson Cancer Center (Houston, TX), Department of Endocrine Neoplasia and Human Disorders. She returned to Baylor College of Medicine to work as a research technician in the Department of Medicine, Thrombosis Research Section from September of 2003 to September 2006. Currently, she is a research area specialist-associate for UT-Health Science Center at San Antonio (San Antonio, TX) at the Edinburg Regional Academic Health Center (Edinburg, TX) campus.

From her work, Mrs. Smith has authored four articles: *J Thromb Haemost* 2007; 5(7):1363-70, *J Thromb Haemost* 2006; 4(2):417-25, *J Bacteriol* 2006; 188(7):2336-42, and *Blood* 2005; 105(5):1986-91.