

Short communication

## SHP-2 AND PTP-PEST INDUCTION DURING Rb-E2F ASSOCIATED APOPTOSIS

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**Abstract:** Apoptosis is intimately connected to cell cycle regulation *via* the Retinoblastoma (Rb)-E2F pathway and thereby serves an essential role in tumor suppression by eliminating aberrant hyperproliferative cells. Upon loss of Rb activity, an apoptotic response can be elicited through both p53-dependent and p53-independent mechanisms. While much of this apoptotic response has been attributed to the p19ARF/ p53 pathway, increasing evidence has supported the role of protein tyrosine phosphatases (PTPs) in contributing to the initiation of the Rb-E2F-associated apoptotic response. One protein tyrosine phosphatase, PTP-1B, which is induced by the Rb-E2F pathway, has been shown to contribute to a p53-independent apoptotic pathway by inactivating focal adhesion kinase. This report identifies two additional PTPs, SHP-2 and PTP-PEST, that are also directly activated by the Rb-E2F pathway and which can contribute to signal transduction during p53-independent apoptosis.

**Key words:** Apoptosis, Focal adhesion kinase, Rb, E2F-1, Phosphatase, PTP-1B, SHP-2, PTP-PEST, PTPN1, PTPN11, PTPN12, Tumor suppressor

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Abbreviations used: 4OHT – 4-hydroxytamoxifen; dnE2F – dominant negative mutant of E2F-1; ER – estrogen receptor; FAK – focal adhesion kinase; PTP – protein tyrosine phosphatase; PTP-PEST – protein tyrosine phosphatase rich in proline, glutamic acid/aspartic acid, and serine/threonine residues; Rb – retinoblastoma protein; SHP-2 – SH2 domain-containing tyrosine phosphatase

## INTRODUCTION

Cancer is a complex genetic disease that is estimated to affect one in four persons [1]. One of the hallmarks of human cancers is uncontrolled proliferation. Oncogenic cells originate from normal cells that have been transformed by successive genetic mutations to become hyperproliferative [2]. A fundamental target for genetic mutation and deregulation is the Rb pathway [3]. Retinoblastoma tumor suppression protein, or Rb, is a critical cell cycle regulator that interacts with the E2F family of transcription factors to control the progression of the cell cycle from the G<sub>1</sub> to S phase. There are eight known members of the E2F family: E2F1-3a, which function as transcriptional activators, and E2F3b-8, which function as transcriptional repressors [4-8]. Upon activation by phosphorylation, Rb interacts with the E2F activators to negatively regulate the transcriptional expression of E2F-responsive genes. To maintain tissue homeostasis and suppress tumor formation, programmed cell death, or apoptosis, is functionally linked to the cell cycle *via* the Rb-E2F complex. Apoptosis is a vital process by which aberrant or hyperproliferative cells are eliminated. To date, E2F-1 is the only confirmed E2F family member to have the capacity to initiate apoptosis [3-8]. E2F-3 has been shown to promote apoptosis; however its function is dependent on E2F-1 activity, indicating that E2F-1 is the critical activator [8, 9]. E2F-1 contains a transactivation domain that is required for the activation of genes critical for cell cycle progression. E2F-1 forms complexes with Rb through a site within its transactivation domain, which leads to epigenetic remodeling and active repression of pro-proliferative and pro-apoptotic genes *via* interaction with histone deacetylases (HDACs) [10]. Still, the relative contribution of transcriptional activation and/or repression by E2F-1 to the apoptotic response is still largely unresolved. What is known is that loss of Rb activity frees E2F-1, permitting the initiation of an apoptotic response. In this way, E2F-1 can act as an important tumor suppressor should Rb become mutated or functionally inactivated.

Until recently, it had been assumed that the apoptotic response triggered by the loss of Rb activity occurred solely through E2F-1 transactivation of pro-apoptotic signal transduction mechanisms. One well-characterized signaling pathway is initiated by E2F-1 transactivation of p19ARF which leads to the activation of p53 and induction of an associated apoptotic response [5]. However, the apoptotic response is not solely mediated by E2F-1 transactivation; a previous study demonstrated that loss of constitutive repression by Rb-E2F-1 complexes is capable of eliciting a p53-independent apoptotic response [11]. This Rb-E2F-1-associated apoptotic pathway can be examined using a dominant negative E2F-1 (dnE2F1) which lacks the C-terminal transactivation domain that contains the Rb binding site. The dnE2F1 is able to displace active Rb-E2F-1 complexes from gene promoter binding sites to simulate loss of Rb-E2F-1 repression, which induces apoptotic signaling without triggering the signaling pathway(s) that is dependent on transactivation by E2F-1 [11, 12]. Expression of dnE2F1 results in a robust apoptotic response, illustrating the fact that constitutive repression of pro-

apoptotic genes by Rb-E2F-1 complexes does indeed play a key role in preventing apoptosis.

Our lab previously identified one target of the Rb-E2F-associated apoptotic pathway, focal adhesion kinase (FAK); FAK is dephosphorylated (i.e. inactivated) in this pathway, leading to the initiation of a p53-independent apoptotic pathway [11]. FAK is a tyrosine kinase found at focal adhesions that relays signals from the extracellular matrix, and it plays a pivotal role in many cellular processes such as cell attachment, cell migration, and cell cycle regulation [13, 14]. FAK activity is also important for prevention of an apoptotic response resulting from loss of adhesion to the extracellular matrix, known as anoikis [15, 16]. FAK activity is largely dependent on phosphorylation of its tyrosine residues. We previously demonstrated that induction of apoptosis by Rb-E2F-1 de-repression resulted in the dephosphorylation of FAK by protein tyrosine phosphatases (PTPs), and the subsequent apoptotic response was mediated by caspase-8 activity [11]. Protein tyrosine phosphatase 1B (PTP-1B), which is encoded by *PTPN1*, was identified as one PTP that was induced by Rb-E2F-1 de-repression to dephosphorylate FAK [11].

Another hallmark of tumors is loss of Rb function. It frequently occurs during tumor initiation; therefore the negative regulation of PTPs by the Rb-E2F-1 complex may serve as a safeguard to trigger apoptosis through FAK upon loss of Rb function. For a tumor to progress it must acquire subsequent mutations that allow it to circumvent this apoptotic mechanism. FAK is often overexpressed in several types of tumors, beginning in the early stages of tumorigenesis, and it is associated with invasion, metastasis, and angiogenesis [13, 17]. In this study, we demonstrate that E2F-1 specifically binds to the gene promoters of PTP-1B, SHP-2 (encoded by *PTPN11*) and PTP-PEST (encoded by *PTPN12*) *in vivo* and that the loss of Rb-E2F-1 repression in association with Rb-E2F-associated apoptosis is sufficient for activation of these genes. These findings are significant because, like PTP-1B, SHP-2 and PTP-PEST have been implicated in FAK regulation [2, 18-24]. Furthermore, SHP-2 is an established oncogene, and PTP-PEST demonstrates tumor suppressive capabilities in breast cancer and kidney tumors [25]. Our results suggest that Rb-E2F-1 normally suppresses PTP gene expression, while FAK can be activated and function as usual. Upon loss of Rb function, the resulting de-repression of PTP transcription could facilitate the inactivation of FAK which triggers Rb-E2F-associated apoptosis. This signal transduction mechanism serves an important function as a guard against cellular transformation and tumor initiation by eliminating hyperproliferative cells that have acquired mutations in the Rb-E2F pathway.

## MATERIALS AND METHODS

### Cell line and culture

ER-dnE2F cells (gift of D. Dean) were maintained in DMEM with 10% FBS, 400  $\mu\text{g/ml}$  zeocin, 150  $\mu\text{g/ml}$  hygromycin B and 300  $\mu\text{g/ml}$  G418 [12].

Treatment with IPTG (1 mM, Sigma) resulted in activation of p16Ink4a, and treatment with 4-hydroxytamoxifen (4OHT) (100 nM, Sigma) resulted in activation of the dominant-negative E2F-1 mutant (dnE2F1), as previously described [11, 12].

#### **Flow cytometry**

As previously described [26], cells were fixed overnight in cold 70% ethanol, and stained with a solution containing 50 µg/ml propidium iodide and 2 mg/ml RNase A in PBS, and analyzed for DNA content on a FACS Caliber (Becton Dickinson).

#### **Real-time polymerase chain reaction (qPCR)**

Total RNA was collected and isolated from cell cultures treated with IPTG (uninduced) or IPTG and 4OHT (induced) at 0 h, 1.5 h, 6 h, 12 h, 24 h, and 48 h using RNeasy® kit (Qiagen). The concentrations of the RNA samples were determined using the NanoDrop ND-1000 spectrophotometer. 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 *PTPNI* (PTP-1B), *PTPN11* (SHP-2), *PTPN12* (PTP-PEST), and human GAPD (GAPDH) Endogenous Control (FAM/MGB probe) according to manufacturer's instructions.

#### **Data analysis**

The threshold  $C_T$  values were generated by the Applied Biosystems RQ Manager software version 1.2, and the experimental  $\Delta C_T$  values were normalized to the  $\Delta C_T$  values of the GAPDH endogenous control. Relative expression was calculated using the comparative  $C_T$  method ( $2^{-\Delta\Delta C_T}$ ). Experiments were repeated in triplicate and data are reported as mean  $\pm$  standard deviation of the mean. Statistical analysis by T-test for Equality of Means (SPSS Statistics version 20) was performed on samples collected at 1.5 h to determine the significant difference between uninduced and induced mRNA expression levels at that time point.

#### **Bioinformatics**

The TRANSFAC® Professional Software (<http://www.biobase-international.com/>) was used to identify putative E2F-1 binding sites in human *PTPNI* (PTP-1B), *PTPN11* (SHP-2), and *PTPN12* (PTP-PEST) gene promoters.

#### **Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed using an E2F-1 specific antibody (Anti E2F-1 clones KH20 and KH95, Millipore) [11, 12] with the ChIP-IT Express Kit (Active Motif) according to manufacturer's instructions. Briefly, induced cells from one 15 cm plate were fixed with 37% formaldehyde for 8 min. Cells were dounced

on ice, and the nuclear lysate was sonicated 10 x 20 s at 100% power using a sonicator to shear chromatin to an average size of 800 bp. IP's were carried out with 100  $\mu$ l of the sheared chromatin and 3  $\mu$ g of antibody overnight at 4°C. Nuclease-free water was substituted for chromatin or antibody during parallel IPs as negative controls. PCR was performed with 0.2  $\mu$ l chromatin per reaction using GoTaq® Green Master Mix (Promega) according to manufacturer's instructions. Sequence-specific primers were generated using DNASTAR® Lasergene Version 8 Sequence Builder software (Table 1).

Table 1. Primers identifying E2F binding sites.

Phosphatase	Gene	Promoter region	Primer sequence	Putative E2F site (orientation)	Match score
PTP-1B	<i>PTPNI</i>	-7940...-	(F) 5'-ATGGTAGCAGTGCAGCTGATGTG-3'	cGCGCC (-)	1.000
		8180	(R) 5'-AAGTCACTCTTAATGTCAGGC-3'		
SHP-2	<i>PTPNI1</i>	-571...-	(F) 5'-AGCAAGGAGCGGGTCCGTC-3'	GGCGCg (+)	1.000
		851	(R) 5'-GAACTCGGGGAGGCAGGAAAATG-3'	cGCGCC (-)	1.000
PTP-PEST	<i>PTPNI2</i>	-921...-	(F) 5'-AGCGCGAGCCGCGCATCTG-3'	gtTGGCGc (+)	0.980
		1341	(R) 5'-CTTCCCCAGCTCGTTCCCC-3'		

(F) forward primer, (R) reverse primer

## RESULTS AND DISCUSSION

To investigate the potential role of SHP-2 and PTP-PEST in Rb-E2F-associated apoptosis, a U2OS cell line that conditionally expresses an ER-fused dnE2F1 was employed. Upon activation of the dnE2F1 with 4-hydroxytamoxifen (4OHT or tamoxifen), dnE2F1 is localized to the nucleus where it displaces endogenous Rb/E2F complexes. (Fig. 1) [11, 12]. Induced cells undergo a rapid p53-independent apoptotic response, demonstrated by the accumulation of cells with a < 2n DNA content, with most cell death occurring within the first 24-48 hours (Fig. 2). It has been shown much of this apoptotic response is facilitated by PTPs, given that addition of phosphatase inhibitors effectively rescues all the cells from apoptosis [11]. Quantitative real-time PCR was performed on uninduced control cells (Fig. 3A) and induced cells in which ER-dnE2F1 fusion protein was expressed over a 48 hour time frame (Fig. 3B). Consistent with prior studies, there was a significant ( $p < 0.001$ ) concomitant increase of PTP-1B mRNA expression in induced cells relative to uninduced, with a 20 fold increase evident at 1.5 hours after initiation of Rb-E2F-associated apoptosis. Expression of both SHP-2 and PTP-PEST mRNA was also significantly ( $p < 0.001$  and  $p = 0.001$ , respectively) increased in induced cells within 1.5 hours relative to uninduced: SHP-2 exhibited a 13-fold increase while PTP-PEST expression increased 9-fold. Transcript expression levels then declined to baseline levels over the 48 hour period. GAPDH mRNA expression remained unchanged in both uninduced and induced cells (data not shown).

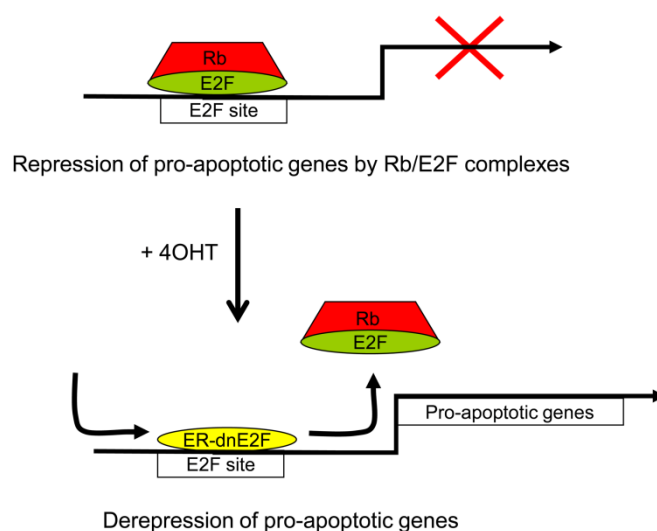


Fig. 1. Addition of tamoxifen to EH-1 cells expressing an ER-dnE2F1 fusion protein results in the nuclear localization, and subsequent de-repression of pro-apoptotic phosphatases – The ER-dnE2F1 protein displaces Rb-E2F-1 complexes which are bound to E2F-1 sites in the promoters of pro-apoptotic genes.

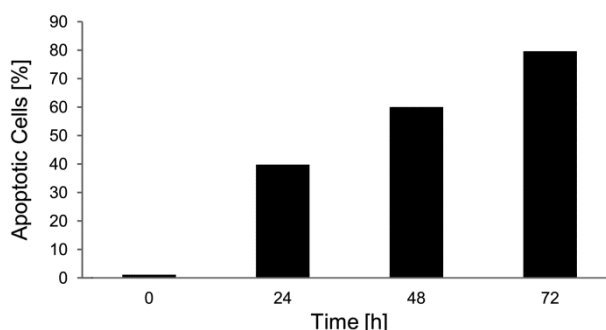


Fig. 2. Derepression of apoptotic genes inhibited by Rb-E2F-1 complexes induce apoptosis – ER-dnE2F cells were activated by addition of 4OHT and the accumulation of cells with < 2N DNA content, consistent with apoptosis, was measured by flow cytometry.

To verify that PTP-1B, SHP-2, and PTP-PEST are direct targets of the Rb-E2F-1 complex, the promoters of these genes were evaluated for putative E2F-1 binding sites using TRANSFAC® professional software (Table 1). For each phosphatase, those promoter sequences with the highest match score were used to generate sequence-specific probes for chromatin immunoprecipitation (ChIP) assays. The results confirmed E2F-1 specifically binds only to the sites identified within the three PTP promoters, indicating E2F-1 directly regulates PTP-1B, SHP-2 and PTP-PEST transcription (Fig. 4). The E2F-1 antibody that was used is able to detect both endogenous E2F-1 in complex with Rb and exogenous dnE2F1 by

the N-terminus [11, 12]. Together, these data suggest loss of Rb-E2F-1 repression directly promotes the transcription of PTP-1B, SHP-2 and PTP-PEST in association with Rb-E2F-associated cell death. These PTPs may serve an important role in the signaling pathway. Since PTP-1B, SHP-2, and PTP-PEST have been implicated in the regulation of FAK, it is possible that the function of these three PTPs is to act in concert to inactivate FAK, thereby signaling the p53-independent apoptotic response. Further investigation will be needed to confirm the physiological role of PTP-1B, SHP-2, and PTP-PEST in Rb-E2F-associated apoptosis.

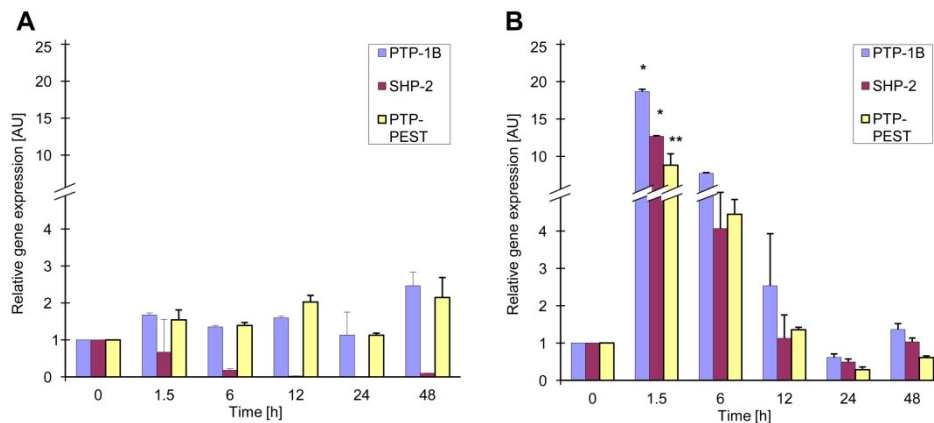


Fig 3. Phosphatase mRNA transcription is induced during Rb-E2F-associated apoptosis – ER-dnE2F1 cells (A) were treated with tamoxifen (B) to induce expression of dnE2F1 and initiate the apoptotic response. Total RNA was isolated at the indicated time points following induction of apoptosis and used for qPCR analysis which was performed in triplicate. (AU) arbitrary units, (\*)  $p < 0.001$  control vs. treated, (\*\*)  $p = 0.001$  control vs. treated.

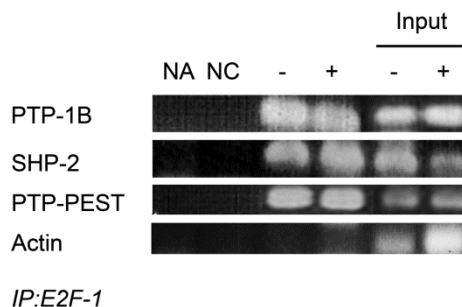


Fig 4. PTP1B, SHP-2 and PTP-PEST transcription is directly regulated by E2F-1 – tamoxifen was added to ER-dnE2F cells to initiate apoptosis (+). Control cells were left untreated with tamoxifen (-). Chromatin was isolated from the cells, sheared and immunoprecipitated with E2F-1. The promoters amplified by PCR are indicated to the left. Parallel ChIP assays were also performed without antibody (NA) and without chromatin (NC) as negative controls.

Due to its fundamental role in the regulation of the cell cycle and its tight link to apoptosis, disruption of the Rb-E2F pathway contributes to the pathogenesis of a variety of human diseases including cancer; nearly all tumors develop mutations that inactivate RB protein or functionally inactivate the Rb-E2F pathway. Upon inactivation of Rb, the associated apoptotic response serves a critical role in helping guard against aberrant proliferation. Apoptotic pressures from both the p53-dependent and p53-independent apoptotic pathways need to be overcome in order for tumors to arise. Consequently, the Rb-E2F pathway has been studied extensively in an attempt to discover how cellular transformation and tumorigenesis is initiated and progresses.

This investigation suggests both SHP-2 and PTP-PEST may play a role in tumor suppression through their function in Rb-E2F-associated apoptosis. While neither PTP has been clearly identified as a tumor suppressor, their role in apoptosis, cellular transformation, and tumorigenesis has been documented. Expression of SHP-2 in cardiomyocytes results in decreased phosphorylation of FAK and an apoptotic response [24]. Dephosphorylation of FAK by SHP-2 also provides a means by which SHP-2 can play an important regulatory role in signaling cell growth, transformation, differentiation, spreading, migration, and cytoskeleton organization, all cellular processes that are relevant to cancer [23, 27, 28]. Furthermore, SHP-2 is recognized as playing an oncogenic role in several types of leukemias, and it is frequently a target for other oncogenes [28]. 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) [21, 27, 28]. *PTPN11* mutations have also been identified in lung and colon cancer, neuroblastoma, and melanoma [21].

Like SHP-2, PTP-PEST plays a role in focal adhesion turnover, cell adhesion, cell migration and cytoskeleton organization [5, 18, 19, 22, 29]. For example, caspase-3 cleaves PTP-PEST during apoptosis, and the result is alteration of downstream signaling pathways that influence cytoskeleton organization [19]. Malignant transformation involves major re-organization of the cytoskeleton [21]. Additionally, FAK dephosphorylation by PTP-PEST correlates with increased cell migration and metastasis [30].

Protein phosphorylation at specific tyrosine residues is vital to the regulation of the signaling pathways governing major cellular processes such as cell proliferation, differentiation, survival and apoptosis [21, 28, 31-33]. Tyrosine phosphorylation is catalyzed by protein tyrosine kinases (PTKs), and aberrant phosphotyrosine signaling resulting from mutation and/or overexpression of PTKs has been associated with a number of human diseases including cancer [34, 35]. Consequently, PTKs became a very attractive target for cancer prevention therapies, and several PTK inhibitors have been developed for clinical use [35, 36]. PTPs negatively regulate the rate and duration of phosphotyrosine signaling, and like its counterpart, functional studies have shown PTPs are associated with cancer [21, 37]. During Rb-E2F-associated apoptosis PTP-1B, SHP-2, and PTP-PEST are clearly



activated directly by E2F-1 to facilitate signal transduction. This apoptotic pathway is critical for the maintenance of tissue homeostasis and for the prevention of aberrant proliferation and cellular transformation; therefore it may be that functional inactivation of PTPs is a critical event for tumorigenesis, making PTP-1B, SHP-2, and PTP-PEST potential targets for cancer prevention. Functional studies are in progress to determine the role of these phosphatases in Rb-E2F-associated apoptosis. We are utilizing small interfering RNA (siRNA) and phosphatase "dead" mutations to determine whether silencing these PTPs can reverse FAK dephosphorylation and rescue apoptosis.

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