2007

Analysis of E2F1 target genes involved in cell cycle and apoptosis

Scott N. Freeman
University of South Florida

Analysis of E2F1 Target Genes Involved in Cell Cycle and Apoptosis

by

Scott N. Freeman

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Graduate Studies
University of South Florida

Major Professor: W. Douglas Cress, Ph.D.
Srikumar P. Chellappan, Ph.D.
Eric B. Haura, M.D.
Kenneth L. Wright, Ph.D.

Date of Approval:
October 15, 2007

Keywords: Cancer, Mitosis, Transcription, Mcl-1, Rb

DEDICATION

To my parents Thomas N. and Linda L. Freeman, my brother Michael T. Freeman, and my future wife Alyson K. Fay.

ACKNOWLEDGEMENTS

I would like to thank my dissertation advisor and mentor W. Douglas Cress, Ph.D. for the years of guidance, assistance, and training that he contributed to and invested in my development as a scientist, my dissertation committee members Srikumar P. Chellappan, Ph.D., Eric B. Haura, M.D., and Kenneth L. Wright, Ph.D. for their guidance and direction throughout my doctoral training, David G. Johnson, Ph.D. for being so kind as to serve as my outside chair, Eric B. Haura M.D., Rebecca Sutphen, Ph.D, Yihong Ma, Ph.D., and Gerold Bepler, M.D, Ph.D., and the core facilities of the H. Lee Moffitt Cancer Center and Research Institute for their respective contributions to this manuscript, and finally the Cancer Biology Ph.D. Program, the University of South Florida, and the H. Lee Moffitt Cancer Center and Research Center for providing me with the opportunity to accomplish this goal.

NOTE TO READER

The original of this document contains color that is necessary for understanding the data.

The original dissertation is on file with the USF library in Tampa, Florida

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
ALL	Acute lymphoblastic leukemia
Apaf-1	Apoptosis activating factor-1
ATM	Ataxia telangiectasia mutated
Bcl-2	B-cell lymphoma/leukemia-2
BH1-4	Bcl-2 homology 1-4
BTB/POZ	Broad-complex bric-a-brac/poxvirus zinc finger
Cdc2	Cell division control 2
Cdk	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CHX	Cyclohexamide
CKI	Cyclin-dependent kinase inhibitor
CLL	Chronic lymphocytic leukemia
CREB	cAMP response element-binding
CRE	cAMP response element
Cul3	Cullin 3
DBC2	Deleted in breast cancer 2
DP	DRTF1-polypeptide
E2F	Early 2 factor

ER	Estrogen receptor
FBS	Fetal bovine serum
GFP	Green fluorescent protein
hTERT	Human telomerase reverse transcriptase
IFM	Immunofluorescenct microscopy
IRES	Internal ribosome entry site
K5	Keratin 5
Mcl-1	Myeloid cell leukemia-1
Mdm2	Murine double minute 2
NES	Nuclear exclusion sequence
NLS	Nuclear localization sequence
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
P/S	Penicillin/streptomycin
Rb	Retinblastoma
RYBP	RING1 and YY1-binding protein
shRNA	Short hairpin inhibitory RNA
SIE	Serum-inducible element
siRNA	Small inhibitory RNA
Skp2	S-phase kinase-associated protein 2
TM	Transmembrane
TRAF2	TNF Receptor-Associated Factor 2

ANALYSIS OF E2F1 TARGET GENES INVOLVED IN CELL CYCLE AND APOPTOSIS

Scott N. Freeman

ABSTRACT

One of the main results of Rb-E2F pathway disruption is deregulation of the E2F family of transcription factors, which can lead to inappropriate proliferation, oncogenic transformation, or the induction of apoptosis. Given the potential negative biological effects associated with deregulated E2F activity, it is of great importance to study E2F targets that mediate these effects. In Part I of this manuscript, we identify the RhoBTB2 putative tumor suppressor gene as a direct physiological target of the E2F1 transcription factor. We find that RhoBTB2 is highly upregulated during mitosis due in part to E2F1, and that overexpression of RhoBTB2 increases the S-phase fraction and slows the rate of proliferation. We also find RhoBTB2 similarly upregulated during drug-induced apoptosis due primarily to E2F1 and that knockdown of RhoBTB2 expression via siRNA slows drug-induced apoptosis. Taken together, we describe RhoBTB2 as a novel direct target of E2F1 with roles in cell cycle and apoptosis.

In Part II, we independently identify from cancer cell lines two novel variants from the promoter of E2F1 target MCL-1—MCL-1 +6 and +18—as initially published by Moshynska et al (1). In contrast to Moshynska et al., we find the variant promoters identically present in both cancerous and adjacent noncancerous clinical lung samples,

suggesting that the variants are germ-line encoded. We also find the variant promoters prevalent in genomic DNA derived from healthy control samples and present at frequencies similar to that observed in cancerous cell lines. In further contrast, we find the activity of the MCL-1 +6 and +18 promoters approximately 50% less than the common MCL-1 +0 promoter—both during normal cellular homeostasis and under conditions that actively induce Mcl-1 transcription. Given our results and those of others, we conclude that the MCL-1 +6 and +18 promoters are likely benign polymorphisms and do no represent a reliable prognostic marker for CLL as reported by Moshynska et al.

PART I

RHOBTB2 (DBC2) IS A MITOTIC E2F1 TARGET WITH A NOVEL ROLE IN APOPTOSIS

Abstract

We have identified the RhoBTB2 putative tumor suppressor gene as a direct target of the E2F1 transcription factor. Overexpression of E2F1 leads to upregulation of RhoBTB2 at the levels of mRNA and protein. This also occurs during the induction of an estrogen receptor-fused E2F1 construct by 4-hydroxytamoxifen in the presence of cyclohexamide, thus indicating that RhoBTB2 is a direct target. RNAi-mediated knockdown of E2F1 expression decreases RhoBTB2 protein expression, demonstrating that RhoBTB2 is a physiological target of E2F1. Since E2F1 primarily serves to transcribe genes involved in cell cycle progression and apoptosis, we explored whether RhoBTB2 played roles in either of these processes. We find RhoBTB2 expression highly upregulated during mitosis, which is partially dependent on the presence of E2F1. Furthermore, overexpression of RhoBTB2 leads to an increase in the S-phase fraction of asynchronously growing cells and also slows the rate of proliferation. We similarly find RhoBTB2 upregulated during drug-induced apoptosis, and that this is primarily dependent on E2F1. Finally, we demonstrate that knockdown of RhoBTB2 levels via siRNA slows the rate of drug-induced apoptosis. Taken together, we describe RhoBTB2 as a novel direct target of E2F1 with roles in cell cycle and apoptosis.

Introduction

The Rb-E2F pathway

The Retinoblastoma (Rb)-Early 2 Factor (E2F) pathway is a critical regulator of molecular mechanisms governing various aspects of cell proliferation, differentiation, and survival (for review, see refs. (2-6)). It regulates these biological effects by integrating both positive and negative signals to ultimately control the transcriptional repression or activation of genes involved in the aforementioned processes. Given the importance of tight regulation of proliferation, differentiation, and survival in the avoidance of human malignancy, it is not surprising to find that this pathway is aberrantly regulated by various means in almost every instance of human malignancy (7). One of the results of deregulation of the Rb-E2F pathway is unrestrained transcriptional activation by certain members of the E2F family of proteins, which can contribute to oncogenic transformation (4). Indeed, many identified E2F target genes play direct roles in the biological effects associated with deregulation of the Rb-E2F pathway (8,9). Yet while many crucial E2F targets associated with the biological phenotype of deregulated Rb-E2F have been identified, many more remain to be characterized. Given the prevalence of Rb-E2F pathway deregulation in human malignancy and the role of E2F

targets in mediating the biological effects, characterizing E2F target genes involved in this process is of great importance.

At the center of a cell's decision to divide is the Rb-E2F pathway, and as such one of its major roles is to regulate the G_1/S-phase transition. While the function of the pathway encompasses more than regulation of this transition, its model of activity is best explained under that context. In this model, the Rb-E2F pathway responds to both pro- and anti-proliferative signals to either activate or repress the transcription of genes involved in further cell cycle progression and DNA synthesis. Many reviews have thoroughly documented this functional paradigm (6,10,11), and the reader is encouraged to reference these for greater detail. As such, only a brief description of the current paradigm is provided.

As illustrated in figure 1, in cells that are in a resting or quiescent state, the pRb protein resides hypophosphorylated, which allows it to restrain the transcriptional activity of E2F proteins. Mitogenic signaling in early G_1 or G_0 serves to upregulate the expression of D-type cyclins—the regulatory subunit of the cyclin D/cyclin-dependent kinase (cdk) 4/6 complex. Cyclin D binds to cdk4/6 to create the active kinase complex, which along with the reported activity of Raf-1, places the initial phosphorylation events on pRb family proteins (12-14). Phosphorylation of pRb family proteins decreases their ability to inhibit E2F family members, thus freeing some transcriptionally active DRTF1-polypeptide (DP)/E2F complex. This free complex sets in motion a feed-forward mechanism that results in increased expression of E2F target genes such as E2F1, E2F2, and E2F3a, as well as cyclin E, the regulatory subunit of the cyclin E/cdk2 complex

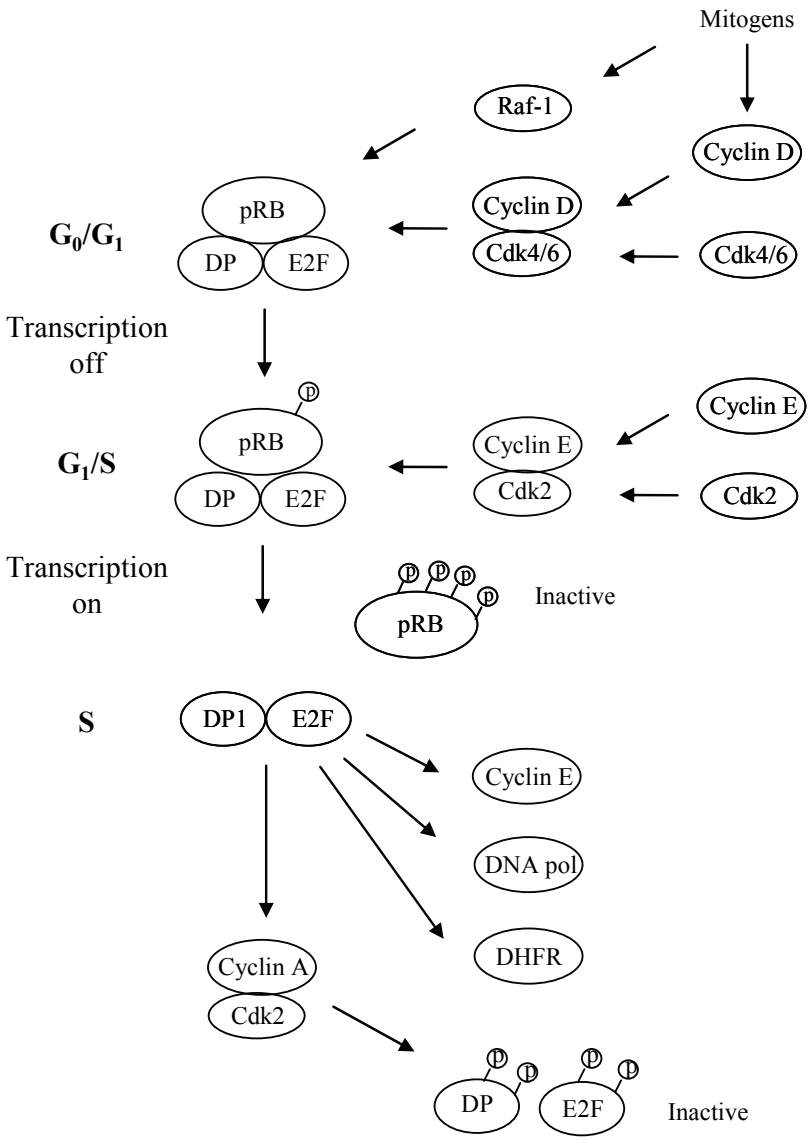


Figure 1. The Rb-E2F pathway. Mitogenic signaling in G_0/G_1 upregulates cyclin D1 and Raf-1, which contributes to phosphorylation of pRb family proteins—thus relieving some inhibition of E2F/DP complex. Further E2F-mediated upregulation of cyclin E at the G_1/S-phase transition leads to additional phosphorylation of pRb by cyclin E/cdk2 complex, leading to full inactivating of pRb. This initiates S-phase entry and allows E2F/DP complex to activate the transcription of genes involved in DNA replication, further cell cycle progression, and genes that subsequently deactivate E2F and DP.

(15-18). Cyclin E binds to cdk2, the catalytic subunit, to create the active kinase complex, and the main target of the cyclin E/cdk2 complex is again the pRb protein (19). Cyclin E/cdk2 complex fully phosphorylates pRb, thus allowing for the full induction of E2F target genes.

Among the many genes induced by E2F family proteins at this stage of the cell cycle include those involved in further cell cycle progression, DNA replication, and nucleotide biosynthesis. Subsequent sections discuss the full range of E2F target genes in greater detail; however, it should be noted that two important targets of E2F at this stage of the cell cycle are cyclin A and S-phase kinase-associated protein 2 (Skp2), which are responsible for down-regulating E2F activity through two separate mechanisms (20,21). Cyclin A is another regulatory subunit for cdk2 which, along with promoting further cell cycle progression, phosphorylates E2F and DP family proteins when in complex with cdk2—resulting in a decreased ability to bind DNA (19,22). Skp2 activity also decreases E2F activity through ubquitination, thus targeting it for proteasomal degradation (23).

Mechanisms of Rb-E2F pathway disruption in human malignancy

One of the defining features of malignancy is uncontrolled cellular proliferation, and given the pivotal role that the Rb-E2F pathway plays in regulating this process, it is not surprising to find that disruption of the Rb-E2F pathway is a unifying factor in virtually every instance of human malignancy (7). An examination of figure 1 reveals multiple potential points for deregulation, and indeed, most have been described in the literature. Figure 2 provides examples of various methods employed by malignant cells to

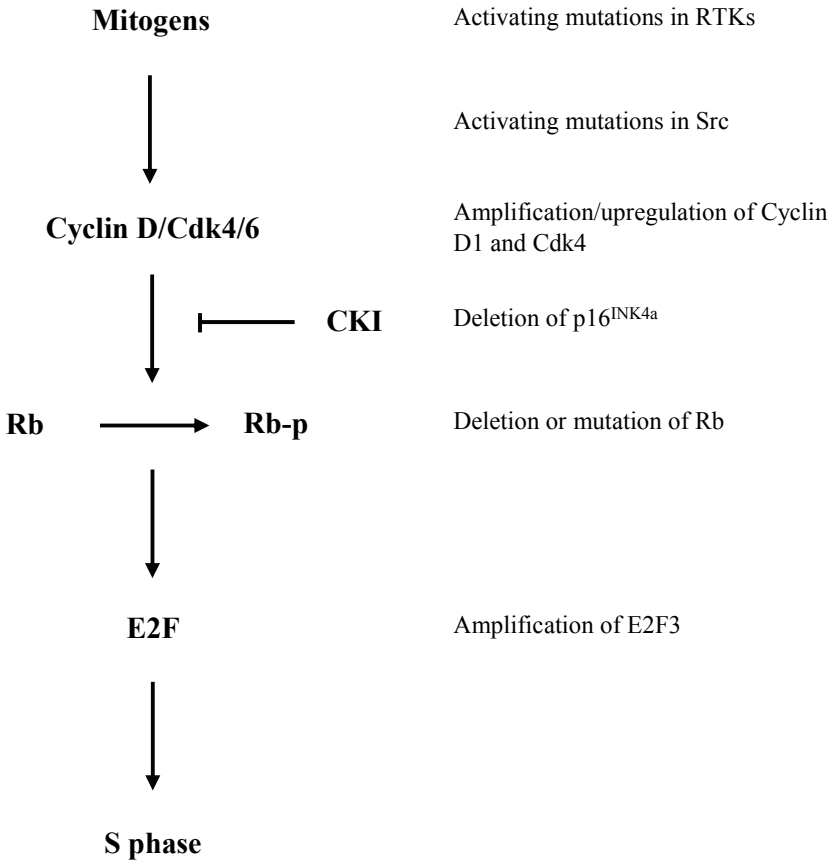


Figure 2. Examples of Rb-E2F pathway disruptions in human malignancy. The Rb-E2F pathway is subject to various regulatory mechanisms that prevent inappropriate proliferation, however malignant cells override these controls through various oncogenic mutations. Some examples described in human malignancy include activating mutations in receptor tyrosine kinases, activating mutations in signaling molecules such as Src, amplification or upregulation of cyclin D or cdk4, deletion of CKI $p16^{INK4a}$, deletion or mutation of Rb, and amplification of E2F.

circumvent control mechanisms preventing inappropriate entry into the cell cycle, yet this is by no means a comprehensive list of all reported mechanism utilized to deregulate Rb-E2F pathway in human malignancy.

The most obvious and most prominent point of deregulation lies with the *RB1* gene itself. Indeed, the *RB1* gene was first described in its namesake retinoblastoma as being the inherited genetic component behind this childhood familial malignancy of the eye (24,25). Interestingly, *RB1* has the notorious distinction of being the first identified tumor suppressor gene. While identified as an inherited genetic component contributing to malignancy, it has become clear that somatically arising disruptions of the *RB1* gene by means of deletion or mutation are more common in malignancy than inherited germ-line mutations (7).

In addition to the *RB1* gene itself, genetic alterations in regulators of pRb phosphorylation status are also very prevalent. The $p16^{INK4A}$ protein is a member of a family of cyclin-dependent kinase inhibitors (CKIs) that directly oppose the action of cyclin/cdk complexes. $p16^{INK4A}$ specifically inhibits the activity of cyclin D/cdk4/6 complexes, thus inhibiting pRb phosphorylation (26). Not withstanding, disruption of $p16^{INK4A}$ activity by means of deletion, mutation, or promoter methylation is also well documented. Similarly, the $p16^{INK4A}$ target cyclin D/cdk4/6 is frequently altered in cancer by means of amplification or translocation of either cyclin D or cdk4/6. The end result of both of these aberrances is unwarranted inactivation of pRb (7).

It was long thought that genetic aberrances in E2F genes themselves were not a common occurrence in malignancy, yet recent reports have identified a handful of genetic alterations in E2F. Amplification of *E2F3* is present in some retinoblastomas and urinary

bladder carcinomas (27-31), and amplification of *E2F1* has been reported in melanoma, colorectal, esophageal, and ovarian cancers (32-37). While upregulation of the activating E2Fs is a common occurrence in malignancy, it is not understood why genetic aberrances in the *E2F* gene itself are not more prevalent.

Deregulated E2F activity

One of the main results of Rb-E2F pathway disruption is deregulation of the E2F family of transcription factors. This can manifest itself through both the loss of ability to repress E2F target genes—mediated primarily by the repressive E2Fs in complex with pRb, and the loss of ability to restrain gene transactivation, which is primarily a function of the activating E2Fs. Since the subsequent experiments concentrate on the consequences of deregulated E2F-mediated gene transactivation in malignancy, mechanisms relating to the loss of ability to repress E2F target genes are not discussed. Likewise, studies utilizing loss-of-function techniques to determine physiological functions of E2F are also not discussed. Instead, the subsequent sections describe the various members and subgroups within the E2F family and the biological effects associated with deregulated E2F transactivation—primarily being the promotion of proliferation and oncogenesis and the induction of apoptosis. It should be noted that under some contexts, deregulated E2F can paradoxically promote survival, induce growth arrest, or contribute to tumor suppression (38-45). While the mechanisms and contexts of these biological effects are not as well-defined, in many instances they are dependent on the presence of one or more tumor suppressors such as $p19^{ARF}$, p53, p21, $p16^{INK4A}$, or Rb,

which is often not the case in cancer (42-45). However, studies examining this seemingly contradictory role of deregulated E2F are relevant to the present study and are also addressed.

The E2F family of transcription factors

Nine E2F family members have been identified to date (E2F1-8, with E2F3 having two variants: E2F3a and E2F3b) and have traditionally been divided into three subgroups based on both structure and function (46-62). However, emerging data illustrating the highly complex nature of function within the E2F family has rendered this view overly simplistic (3). It is clear though that in general terms, certain subgroups of E2Fs are more associated with either target gene transactivation or target gene repression, and in the interest of presenting an overview of members within the E2F family, the traditional model will be utilized.

E2F1, E2F2, and E2F3a constitute the first subgroup of E2Fs and are commonly referred to as the 'activating' E2Fs by virtue of their ability to potently activate the transcription of genes from model promoters. Structurally, these E2Fs contain an N-terminal nuclear localization sequence (NLS) and cyclin A/cdk2-binding domain followed by a DNA-binding domain, a DP dimerization domain and a C-terminal transactivation/pRb-binding domain (Fig. 3, top). These E2Fs associate exclusively with pRb and not p107 or p130. In normal cells, the expression of these E2Fs is tightly coupled to cell cycle, with expression increasing transcriptionally upon mitogenic stimulation in G_1 (15,16,63), and decreasing in part due to post-translational modification

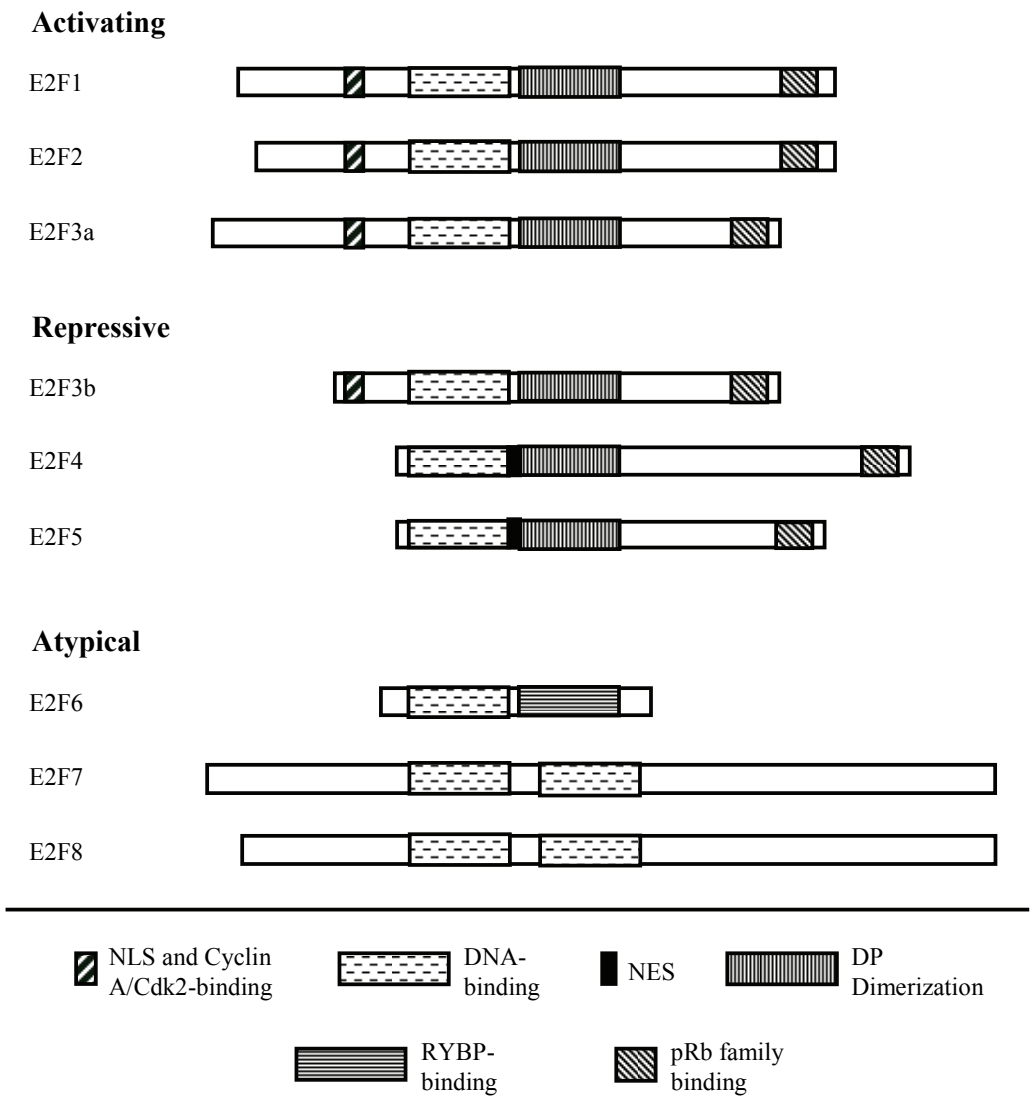


Figure 3. The E2F family of transcription factors. The E2F family of transcription factors is commonly divided into the activating, repressive and atypical subgroups. The activating E2Fs consist of E2F1, E2F2, and E2F3a and contain a NLS and cyclin A/cdk2 binding domain, DNA-binding domain, DP dimerization domain, and a pRb family member-binding domain. The repressive E2Fs are E2F3b, E2F4, and E2F5 and contain a DNA-binding domain, DP-dimerization domain, and pRb family member-binding domain. While E2F3b contains a NLS, E2F4 and E2F5 harbor a NES. The atypical E2Fs are E2F6, E2F7, and E2F8. E2F6 contains a DNA-binding domain and RYBP-binding domain, and E2F7 and E2F8 contain a tandem of two DNA-binding domains.

imposed by the activity of Skp2 in late S-phase, which targets E2F1 for proteasomal degradation (23). These E2Fs have been implicated in promoting the transcription of a multitude of genes with various cellular functions, which is discussed in greater detail in further sections.

The second subgroup of E2Fs is made up of E2F3b, E2F4, and E2F5 and is commonly referred to as the 'repressive' E2Fs due to their poor ability to activate transcription, as well as their potent ability to repress transcription when in complex with pRb family members. While E2F3b contains an N-terminal NLS and cyclin A/cdk2-binding domain, this sequence is absent from E2F4 and E2F5, which instead have a nuclear exclusion sequence (NES) following the DNA-binding domain (Fig. 3, middle). These E2Fs also contain a DP dimerization domain and a C-terminal pRb family member-binding domain. While E2F3b associates exclusively to pRb, E2F4 can associate with pRb, p107, or p130, and E2F5 only associates with p130. In contrast to the activating E2Fs, expression of the repressive E2Fs is relatively static throughout the cell cycle. Given the constant nature of expression of E2F4 and E2F5, it stands to reason that other mechanisms are in place to regulate their activity. Indeed, these E2Fs are regulated by localization—with inactive E2F4 and E2F5 being cytoplasmic and association with pRb or DP family members being required for nuclear import (11). It appears that the primary role of E2F3b, E2F4, and E2F5 is to repress the transcription of E2F target genes through the recruitment of repressive complexes containing pRb family members.

The final subgroup of E2Fs will be referred to as the 'atypical' E2Fs due to their divergence from E2F1-5. These E2Fs have been identified more recently, and therefore less is known about their cellular functions. E2F6 was the first identified atypical E2F

and contains only one E2F family conserved sequence: the DNA-binding domain (Fig. 3, bottom). Given the absence of a pRb-binding domain, E2F6 does not bind to pRb family members, but instead recruits components of the mammalian polycomb group complex through a RING1 and YY1-binding protein (RYBP) domain to repress the transcription of E2F target genes (64). E2F7 and E2F8 represent an entirely new class of E2Fs whose homology to other E2F family members is limited to a tandem of two DNA-binding domains (Fig. 3, bottom). Given the lack of pRb-binding or dimerization domains, these E2Fs are thought to bind DNA independent of DP or pRb. The limited amount of studies examining the functions of E2F7 and E2F8 suggest that these proteins act as repressors of transcription through as yet uncharacterized mechanisms (58,59,62,65).

Promotion of proliferation and oncogenesis

One of the most pronounced biological effects of unrestrained transactivation by the activating E2Fs is the promotion of cell cycle progression, which is typically manifested as inappropriate S-phase entry. In cell culture-based assays utilizing rodent fibroblasts, overexpression of E2F1, E2F2 or E2F3a is capable of inducing S-phase entry from quiescence (66-69), and in the case of E2F1, can override anti-proliferation signals imposed by the expression of CKIs p16, p21, p27 or treatment with TGF-β (70-72). This potent ability to promote cell cycle progression can also manifest in the transformation of primary cells, where overexpression of E2F1, E2F2, or E2F3 can induce transformation either alone or in combination with oncogenic *ras* (73-76).

The pro-proliferative and oncogenic effects of E2F overexpression observed in cell culture-based assays are also evident *in vivo* by means of mice transgenic for E2F, where transgenic expression of E2F can promote inappropriate entry into the cell cycle, hyperplasia, and even tumor formation. Consistent with the *in vitro* models, transgenic expression of E2F1, E2F2 or E2F3a targeted to the lens fiber is capable of inducing reentry into the cell cycle in postmitotic cells (77,78). Transgenic E2F4 can also induce cell cycle reentry in this model, albeit to a lesser extent (77). When expressed under control of the megakaryocyte-specific platelet factor 4 promoter, E2F1 blocks terminal differentiation and induces proliferation in megakaryocytes, and the differentiation block imposed cannot be rescued by administration of platelet growth factors (79). Furthermore, short-term induction of an E2F3 transgene in the pituitary gland induces proliferation of quiescent melanotrophs (45)—indicating that long-term expression of deregulated E2F is not necessary to observe a biologically relevant effect.

While short-term induction of E2F3 in the pituitary gland induces the proliferation of quiescent cells, long-term induction leads to the development of hyperplasia (45), and targeting transgenic expression of E2F1 or E2F3a to the epidermis and squamous epithelial tissues via the keratin 5 (K5) promoter also results in hyperplasia (80,81). Similarly, targeting transgenic E2F2 to the thymic epithelium results in hyperplasia (82). When targeted to the liver, transgenic E2F1 leads to pericentral large cell dysplasia (83), and conditional expression of E2F1 in the testes from an inducible promoter induces dyplasia that mimics carcinoma *in situ*—indicating that short-term E2F expression is sufficient to drive aberrant tissue proliferation *in vivo* (84).

In addition to the promotion of aberrant non-malignant tissue proliferation, transgenic expression of E2F can also lead to tumor development—either alone or in combination with other oncogenic mutations. In the presence of oncogenic *ras* or the absence of one or both *p53* alleles, transgenic expression of E2F1 in K5 tissues leads to the development of skin tumors (80,85). Furthermore, K5 E2F1 transgenic mice are also prone to the spontaneous development of tumors in K5-expressing tissues as they age (40). In addition to dysplasia, transgenic expression of E2F1 in the liver also induces spontaneous tumor development (83), and targeting of E2F2 to the thymus epithelium can similarly induce tumor development in addition to hyperplasia (82). In the case of E2F3a, transgenic expression to K5 tissues increases the rate of spontaneous tumor development by 20% and additionally enhances tumor development in response to treatment with chemical carcinogens (81). Taken together, these studies demonstrate the ability *in vitro* and *in vivo* of deregulated E2F activity to promote cell proliferation in presence of antiproliferative signals, promote aberrant non-malignant tissue growth, and in some contexts, to promote tumorigenesis alone or in combination with other oncogenic mutations.

Promotion of apoptosis

In addition to promoting cell cycle progression and oncogenic transformation, E2F1, E2F2 and E2F3a also have the ability to induce apoptosis, although there is significant disagreement as to the apoptosis-inducing ability of E2F2 and E2F3a (45,68,77,78,81,82,86,87). This is thought to act as a failsafe mechanism to counteract

the potential tumorigenicity associated with unrestrained E2F-mediated proliferation, and can occur in both p53 family-dependent and –independent mechanisms, which are described in detail in the section on E2F1 target genes.

In cell culture-based experiments, ectopic overexpression of E2F1 in quiescent rodent fibroblasts by means of cDNA or adenovirus results in both S-phase entry and apoptosis (67,74,88,89). While the ability of E2F1 to induce apoptosis *in vitro* is quite clear, cell culture-based studies examining a role of E2F2 and E2F3a in E2F-induced apoptosis have yielded conflicting results. While one study reports no increase in apoptosis upon E2F2 or E2F3a overexpression (68), others have reported the contrary (86,87). Given this apparent contradiction, it is likely that the ability of E2F2 and E2F3a to induce apoptosis is highly context-dependent, whereas the ability of E2F1 is more ubiquitous.

The ability of E2F overexpression to induce apoptosis as observed in cell culture-based assays is also evident *in vivo* by means of mice transgenic for E2F. In addition to E2F1 blocking differentiation and inducing proliferation when transgenicly targeted to megacaryocytes, significant megakaryocyte apoptosis is also observed (79). Likewise, when targeted to the liver or lens fiber, transgenic E2F1 induces proliferation as well as apoptosis (78), and an inducible E2F1 transgene targeted to the testes also promotes proliferation and apoptosis (84)—indicating that short-term deregulation of E2F1 is sufficient to drive apoptosis *in vivo*. Targeting of E2F1 to the K5 expressing epidermal tissues induces follicular apoptosis, and when crossed to a $p53^{+/-}$ or $p53^{-/-}$ background, E2F1-induced keratinocyte apoptosis is reduced (85)—indicating a role for the p53 tumor suppressor gene in E2F1-induced apoptosis. Oddly, when expressed under a non tissue-

specific promoter, transgenic expression is only observed in the testicles and results in atrophy and sterility by means of increased apoptosis in the germinal epithelium (90). This however is independent of p53, as crossing these mice to a $p53^{-/+}$ or $p53^{-/-}$ background does not result in decreased apoptosis (90).

Similar results have also been obtained in mice transgenic for E2F2 or E2F3a. Trangenic expresion of E2F2 or E2F3a in the lens fiber promotes cell cycle reentry with subsequent apoptosis in postmitotic cells (77,78), yet there is no evident increase in apoptosis when E2F2 is targeted to the thymic epithelium (82), or when E2F3a is targeted to the pituitary gland (45). However, targeted expression of E2F3a to K5 tissues results in increased p53-independent apoptosis, as indicated by no decrease in the proportion of apoptotic cells when crossed to a p53-null background (81). As with *in vitro*-based studies examining a role for E2F2 and E2F3a in apoptosis induction, it is likely that their ability to induce apoptosis *in vivo* is also highly context dependent. It should also be noted that a recent study demonstrates that apoptosis induced by transgenic expression of E2F3a is dependent on E2F1 (91). In summary, under some contexts deregulated E2F, primarily E2F1, is capable of promoting apoptosis in addition to cell cycle progression though both p53-dependent and –independent pathways.

Contradictory roles: promotion of growth arrest, tumor suppression, and survival

The previous sections discuss the ability of deregulated E2F to promote cell cycle progression, apoptosis, and oncogenesis, however it should be noted that under some contexts deregulated E2F can promote somewhat contradictory biological effects such as

growth arrest, tumor suppression, and cell survival (38-45). In cell culture-based assays, overexpression of E2F1 in primary fibroblasts can induce a growth arrest or checkpoint response that in some instances resembles a senescent-like state, which is dependent on the presence of one or more potent tumor suppressor genes such as $p19^{ARF}$, p53, p21, $p16^{INK4A}$, or Rb (42-45). Similarly, when transgenically targeted to the pituitary gland, E2F3 can induce an irreversible senescent-like state upon long-term exposure (45). However, reports of E2F-mediated growth arrest are sparse, and under most published contexts deregulated E2F induces proliferation.

In the case of tumor suppression, overexpression of E2F1 in transformed mouse fibroblasts or normal human foreskin fibroblasts can reduce colony formation, and in the context of mouse fibroblasts, can abrogate focus formation induced by ras (38,39). The necessity of a functional tumor suppressor in this process is exemplified by the ability of dominant-negative p53 to abrogate the ability of E2F1 to suppress focus formation (39). While transgenic expression of E2F1 in K5 expressing tissues can lead to hyperplasia and the development of spontaneous tumors, it paradoxically suppresses tumor formation induced by treatment with a two-stage chemical carcinogenesis protocol (40). In agreement with studies describing the ability of E2F1 to induce growth arrest, tumor suppressors p53 and $p19^{ARF}$ are necessary for deregulated E2F1 to inhibit tumor formation in this context (41).

In line with deregulated E2F having contradictory biological effects in the regulation of cell cycle progression and tumor development, deregulated E2F can also inhibit the induction of apoptosis under some contexts. As of yet this ability appears to be exclusive to instances of radiation-induced apoptosis, and is thought to facilitate DNA

repair (92,93). Transgenic expression of E2F1 to K5 expressing tissues suppresses epidermal apoptosis induced by UVB-irradiation in a p53-independent manner (92,93). Furthermore, K5 E2F1 transgenic mice display accelerated repair of UVB-induced DNA damage, indicating a role for E2F1 in promoting this type of DNA repair (92). While it is clear that deregulated E2F can promote growth arrest, tumor suppression, or survival under some contexts, it appears as though the requisite context is a normal cell absent of any losses of tumor suppressor function. Studies utilizing E2F loss-of-function models better describe these effects and lend further support to the idea that these contradictory biological effects are indeed important to normal physiology (94-99). However, the ability of deregulated E2F to inhibit cell growth, suppress tumor formation, and promote survival outside of the published contexts remains unclear.

E2F target genes: connecting the biology of deregulated E2F to mechanisms

E2F family proteins have been implicated in controlling the expression of genes involved in functions as diverse as DNA replication, the G_1/S-phase transition, mitosis, DNA damage and repair, differentiation and development, and apoptosis (8,9,100). Some target genes have been thoroughly characterized by means of a comprehensive promoter analysis of E2F-mediated transactivation, or by inducing E2F activity in the presence of cyclohexamide, while others have been implicated in large-scale array-based analysis of E2F-induced transcripts or E2F-immunoprecipitated DNA. While a comprehensive review of all published E2F target genes involved in the many biological functions

attributed to E2F is beyond the scope of this manuscript, target genes relevant to the subsequent experimental data are discussed in detail.

Mitotic targets of E2F

In addition to the well-characterized role of E2F-mediated transactivation of genes involved in the G_1/S-phase transition and DNA replication, E2F has also been implicated in regulating the expression of cell cycle-associated genes with mitotic functions. Based on mircoarray analysis of transcripts, adenovirus-mediated overexpression of E2F1 or E2F2 in quiescent fibroblasts leads to the induction of a large subset of genes with mitotic functions, such as *kifC1, cdc2, cyclin B* and *cdc20* (101). Strikingly, a comparison of E2F1 and E2F2 induced transcripts to temporal regulation of whole genome transcripts during the cell cycle reveals targets of E2F1 and E2F2 to be physiologically induced primarily at either the G_1/S transition or during G_2—suggesting a physiological role for E2F in the regulation of mitotic genes (101). While this study does not address whether the mitotic genes induced by E2F are direct or indirect targets, chromatin immunoprecipitation (ChIP) of E2F coupled to DNA microarray analysis reveals E2F present at the promoters of genes involved in chromatin assembly, condensation, and segregation, as well as the mitotic spindle checkpoint (102,103).

A promoter based analysis of mitotic genes cell division control 2 (cdc2) and cyclin B1 reveals the presence of both positive and negative acting E2F elements, and that both E2F1 and E2F4 bind to the cdc2 and cyclin B1 promoters *in vivo* (104). Interestingly, E2F1 is only found at the cdc2 and cyclin B1 promoters during the G_1/S-

phase transition and S-phase, with E2F1 being completely disassociated by G_2 (104). In addition to cdc2 and cyclin B1, the mitotic checkpoint protein mad2 is also a direct E2F1 target gene, which couples deregulated E2F activity with the promotion of genomic instability (105). While a number of E2F targets with mitotic functions have been identified, only a handful have been characterized. Yet given the presence of E2F at the promoters of genes with mitotic functions, it would appear that E2F-mediated regulation is at least in part a direct mechanism. This presents as somewhat of a paradox, as E2F is thought to be no longer active when these genes are induced, and furthermore ChIP assays reveal E2F to be fully disassociated by G_2 as well (104). While the precise mechanism by which E2F regulates the expression of genes with mitotic functions is yet to be determined, it is clear that E2F indeed plays a role that is in some instances direct.

Apoptotic E2F targets and mechanisms

In addition to promoting cell cycle progression, E2F1 is also a potent inducer of apoptosis, and as such many transcriptional targets of E2F1 have functional roles in various stages of this process. Whereas few of the mitotic targets of E2F are well characterized, much more is known about transcriptional targets and mechanisms of E2F1-induced apoptosis. Indeed, E2F1 is implicated in the regulation of a multitude of genes with apoptotic functions; however the following will concentrate on the best-characterized mechanisms. E2F1-induced apoptosis is generally categorized as occurring through either p53 family-dependent or p53 family-independent pathways by means of

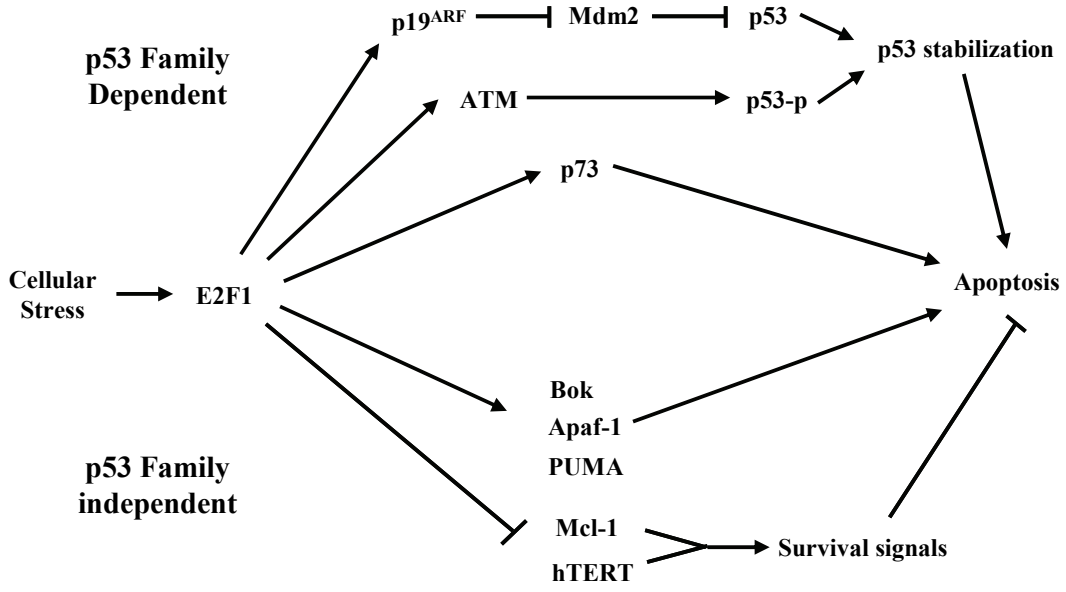


Figure 4. Mechanisms of E2F1-induced apoptosis. E2F1 induces apoptosis through both p53 family-dependent and –independent pathways through both direct and indirect mechanisms. E2F1 indirectly stabilizes p53 by transactivation of $p19^{ARF}$ or ATM. While $p19^{ARF}$ inhibits the activity of negative p53 regulator Mdm2, ATM stabilizes p53 through phosphorylation. E2F1 can also induce the transcription of p53 homologue p73. E2F1 directly induces the transcription of proapoptotic genes, such as Bok, Apaf-1 and PUMA, and can also directly repress the expression of prosurvival genes such as Mcl-1 and hTERT.

three primary mechanisms: the indirect stabilization of p53, the direct tranactivation of proapoptotic genes, and the direct repression of genes that promote cell survival (Fig. 4).

While overexpression of E2F1 induces proapoptotic p53 (106), it does not do so directly and instead indirectly stabilizes p53 protein through two separate mechanisms. In a healthy cell, p53 activity is kept in check primarily at the level of protein stability. In response to cellular stress, E2F1 can directly induce the expression of $p19^{ARF}$ (68,107), which in turn binds to and inhibits the action of murine double minute 2 (Mdm2) (108,109). Mdm2 is an E3 ubiquitin ligase that targets p53 for degradation, and as such the end result of E2F1-mediated transactivation of $p19^{ARF}$ is stabilization of p53 (110,111)—which leads to p53-mediated transactivation of proapoptotic genes. In addition to regulation of p53 protein stability via the ubiquitin-proteasome pathway, p53 is also subject to stabilizing phosphorylations by stress-sensitive kinases. Stabilization of E2F1 in response to DNA damage results in E2F1-mediated direct tranactivation of Ataxia Telangiectasia-Mutated (ATM), which stabilizes p53 protein by phosphoylation at serine 15 (112,113). In addition to indirect stabilization of p53, E2F1 can also directly induce transcription of p53 homologue p73 (114,115), whose activation can induce apoptosis in a manner similar to that of p53.

In addition to p53-family dependent mechanisms of E2F1-induced apoptosis, E2F1 can also contribute to apoptosis through mechanisms independent of p53 family proteins. This can occur through two primary mechanisms: the direct transactivation of proapoptotic genes, and the direct or indirect repression of prosurvival genes. The use of microarray analysis of genes induced upon E2F1 overexpression, as well as array-based analysis of E2F1-bound DNA by ChIP, has implicated a multitude of potential apoptotic

targets of E2F1. However, what is unclear from these analyses is the relevance of these genes in E2F1-induced apoptosis. For this reason, the following will concentrate on those genes that are well characterized targets of E2F1. E2F1 directly induces the expression of multiple Bcl-2 homology (BH)3-only proteins including PUMA, Noxa, Bim, Bik, and Hrk/DP5 (116,117), proapoptotic B-cell lymphoma/leukemia-2 (Bcl-2) family members that function in the intrinsically-mediated apoptotic pathway to promote the release of cytochrome c and other mitotic factors from the mitochondria. In addition to BH3-only Bcl-2 family targets, E2F1 also directly transactivates the expression of proapoptotic Bcl-2 family member Bok (118), which also functions to compromise mitochondrial membrane integrity. Other notable direct targets of E2F1 include Apoptosis activating factor-1 (Apaf-1) and Smac/DIABLO, as well as several caspases (119-121).

Contrary to targets and mechanisms in which E2F1 induces the expression of genes that promote apoptosis, E2F1 can intriguingly also repress the expression of genes with prosurvival functions through both direct and indirect mechanisms. E2F1 directly represses transcription of antiapoptotic Bcl-2 family member Myeloid cell leukemia-1 (Mcl-1), which interestingly occurs in a pRb dependent manner, as deletion of the pRb-binding/transactivation domain does not abrogate its ability (122). Similarly, E2F1 directly represses the expression of human telomerase reverse transcriptase (hTERT), a gene involved in the maintenance of chromosome telomeres (123). In the death receptor mediated apoptotic pathway, TNF Receptor-Associated Factor 2 (TRAF2) inhibits apoptosis by stimulating antiapoptotic NF-kB. E2F1 can indirectly downregulate TRAF2 at the level protein though an as yet uncharacterized mechanism, providing yet another example of inhibition of survival genes mediated by E2F1 (124). Taken together, the

preceding indicates that E2F1 plays a major role in regulating apoptosis through both p53 family-dependent and –independent pathways functioning through multiple mechanisms. E2F targets and mechanisms involved in growth arrest, tumor suppression, and survival

E2F targets and mechanisms involved in growth arrest, tumor suppression, and survival

As previously discussed, under certain contexts deregulated E2F can paradoxically promote survival, induce growth arrest, or contribute to tumor suppression (38-45). In many instances these biological effects are dependent on the presence of one or more tumor suppressors such as $p19^{ARF}$, p53, p21, $p16^{INK4A}$, or Rb (42-45). The mechanism by which E2F1 regulates $p19^{ARF}$ and ATM to ultimately control p53, as well as its direct ability to transactivate p73, has been thoroughly discussed in a previous section, however in addition to these mechanisms, other tumor suppressors are also direct targets of E2F. E2F can directly induce the transcription of CKIs p21, p27, and p57, suggesting a negative-feedback mechanism limiting the activity of E2F (125-127). As exemplified by ATM, E2F can also influence the expression of multiple genes with roles in the DNA damage response and checkpoint control (3,4,8,100,128). This however leads to a rather complex web of functions, as many E2F targets involved in the DNA damage checkpoint and DNA repair also play roles in apoptosis and general DNA synthesis. In summary, under certain contexts deregulated E2F can induce that transcription of genes that inhibit cell proliferation, promote survival, or suppress tumor formation, however the contexts determining preferential transcription of these genes remains to be further explored.

The RhoBTB2 (DBC2) putative tumor suppressor gene

RhoBTB2, or *Deleted in Breast Cancer 2 (DBC2)* is a putative tumor suppressor gene located at 8p21 (129), a common spot for homozygous deletion in human malignancies arising from various tissues of origin (130-136). RhoBTB2 is the second member of a subclass within the Rho family of small GTPases proteins (RhoBTB1-3) and is highly divergent from other Rho family members. Orthologues of human *RhoBTB* genes are present in mammals, fish, flies and *D. discoideum*, yet orthologues are absent from the genomes of yeast and worms (129,137). While only a handful of studies concentrating on RhoBTB2 have been published, the following describes what is currently known.

Structure

RhoBTB2 is composed of an N-terminal RhoGTPase domain, two broad-complex bric-a-brac/poxvirus zinc finger (BTB/POZ) domains, and a conserved C-terminal domain of unknown function. The RhoGTPase domain is highly homologous to that observed in other small GTP-binding proteins, and although it contains three putative GTP-binding motifs and a GTPase motif, studies indicate that it is incapable of GTP hydrolysis (138). In contrast to other members of the Rho family, RhoBTB2 contains a tandem of BTB/POZ domains, which are evolutionarily conserved domains thought to be involved in protein-protein interactions (139). BTB/POZ domains were first identified in *Drosophila*—where such proteins act as transcriptional repressors—yet many BTB/POZ

domain-containing proteins are encoded in the human genome (139). In humans, the BTB/POZ domains of RhoBTB2 as well as other proteins have been shown to interact with the Cullin 3 (Cul3) ubiquitin ligase complex, indicating a possible mechanism of regulation or action (140-144).

Expression patterns

During mouse embryogenesis, expression of RhoBTB2 mRNA is dependent on both tissue type as well as developmental stage (145). The highest levels of RhoBTB2 expression in the developing mouse embryo are in nervous system tissues, where elevated levels of expression continue until embryonic day E16.5—when levels significantly decrease yet remain detectable throughout the remainder of development (145). The developing gut and liver also display temporal increases in RhoBTB2 expression, yet surprisingly expression in the embryonic lung and mammary gland is very weak (145). This is intriguing, as deregulation of RhoBTB2 in human malignancy is best documented in cancers of the lung and breast (146). Human multi-tissue arrays reveal RhoBTB2 expression to be weak in most tissues except neural tissues (129), while another study finds RhoBTB2 expression present in noncancerous human breast, lung, brain, and placenta samples (146). Similarly, human fetal tissues show detectable RhoBTB2 expression in the lung, heart, and brain (129). Given the variability of expression patterns between studies and the deficiencies in quantification, it is difficult to make any concrete generalizations about RhoBTB2 expression patterns in developing or mature tissues.

Deregulation in human malignancy

Alteration of RhoBTB2 in human malignancy has been described in the literature to occur by mean of deletion or loss of heterozygosity, downregulation, or point mutation (130-136,144,146). Indeed, *RhoBTB2* was first characterized in humans by virtue of its deletion in primary breast cancer samples, where it is reported to be heterozygously deleted in 3.5% of cases (146). Deletions of *RhoBTB2* have also been described in malignancies of the bladder, lung, ovary, and prostate (130-136), and ablation of RhoBTB2 expression through downregulation is reported to occur in approximately 50% of breast and lung cancers (146). In addition to deletion and downregulation, several point mutations have also been identified, with some of them effecting RhoBTB2 activity (144,146), although the biological significance of this is yet to be determined. These studies would seem to suggest that RhoBTB2 might behave as a tumor suppressor, and this idea is indeed supported by limited biological studies.

Biological functions, mechanisms, and regulation

Given the prevalence of RhoBTB2 alterations in human malignancy, one might suspect RhoBTB2 to behave biologically like a tumor suppressor, and indeed, the limited biological studies on RhoBTB2 support this hypothesis. Overexpression of RhoBTB2 in a breast cancer cell line with undetectable endogenous RhoBTB2 greatly inhibits proliferation, whereas overexpression in a cell line with endogenous RhoBTB2 has no effect on proliferation (146). Interestingly, overexpression of a BTB/POZ domain point-

mutant RhoBTB2 construct derived from a human tumor (RhoBTB2-D299N) has no effect on proliferation, suggesting a role for the BTB/POZ domain in the mechanism of RhoBTB2-mediated cell cycle inhibition (146). In addition to inhibiting proliferation under certain contexts, RhoBTB2 has been linked to the microtubule motor complex, as knockdown of RhoBTB2 in 293 cells abrogates vesicular stomatitis virus glycoprotein transport (138). Taken together, these studies suggest that under some contexts, RhoBTB2 can function as a negative regulator of proliferation, and that RhoBTB2 has a functional role in transportation along the mictrotubule motor complex. However based on gain-of-function studies, it is not possible to classify *RhoBTB2* as a tumor suppressor gene. A knockout mouse model is in order to fully examine the tumor suppressor capability of RhoBTB2.

While the mechanism by which RhoBTB2 inhibits proliferation is not clear, downregulation of cyclin D1 has been proposed. Overexpression of RhoBTB2 in a cell line deficient of endogenous RhoBTB2 expression leads to inhibition of cell cycle and downregulation of cyclin D1 protein, and overexpression of cyclin D1 upon RhoBTB2 overexpression ablates the ability of RhoBTB2 to inhibit proliferation (147). It is clear that RhoBTB2 overexpression decreases cyclin D1 protein, however the ability of enforced cyclin D1 overexpression to rescue cells from the inhibitory effect of RhoBTB2 does not demonstrate the necessity of cyclin D1 downregulation to mediate this process. It is likely that the enforced overexpression of many positive regulators of cell cycle would result in a similar effect. With this in mind, it is not clear if downregulation of cyclin D1 is a mechanism by which RhoBTB2 inhibits proliferation; studies utilizing cyclin D1 deficiencies would better address this issue. A microarray-based network

analysis of transcripts influenced by RhoBTB2 deficiency and proficiency reveal RhoBTB2 to influence pathways responsible for cell cycle, apoptosis, cytoskeleton and membrane-trafficking, however the relevance of such conclusions is not clear (148). Taken together, while it is clear that RhoBTB2 influences the expression of various genes, the mechanisms by which RhoBTB2 inhibits proliferation and influences the microtubule motor complex remain uncertain.

Only one physiological means of RhoBTB2 regulation has been reported in the literature, which involves degradation by the proteasome. RhoBTB2 binds to the Cul3 ubiquiting ligase scaffold though its first BTB/POZ domain and is also a substrate for the Cul3 ubiquitin ligase complex, which targets RhoBTB2 for degradation (144). A RhoBTB2 construct derived from a lung cancer cell line containing a point mutation (Y284D) in the first BTB/POZ domain abolishes the ability of RhoBTB2 to bind Cul3, and thereby increases its expression due to decreased degradation. The authors present an attractive model in which the tumor suppressor function of RhoBTB2 is achieved via recruiting proteins to the Cul3 ubiquitin ligase, thus targeting them for proteasomal degradation; however this model is yet to be tested.

Summary and rationale

Given the prevalence of Rb-E2F pathway deregulation in human malignancy and the detrimental biological effects associated with unrestrained E2F activity, we sought to identify novel transcriptional targets of E2F1. In this manuscript, we identify RhoBTB2 as a novel transcriptional target of E2F1. We demonstrate that overexpression of E2F1

directly activates RhoBTB2 expression, and that knockdown of E2F1 decreases the expression of RhoBTB2, thus indicating that E2F1-mediated activation of RhoBTB2 is physiologically relevant and not simply an artifact of overexpression. Furthermore, we show that RhoBTB2 is upregulated during mitosis as well as during drug-induced apoptosis, and that this activation is partially and primarily dependent on E2F1, respectively. Finally, we demonstrate that RhoBTB2 has active roles in E2F-mediated processes of cell cycle progression and apoptosis. Taken together, we describe RhoBTB2 as a novel transcriptional target of E2F1 with roles in cell cycle and apoptosis.

Experimental Procedures

Cell lines and cell culture

The H1299 cell line was a gift from Dr. Jiandong Chen (Moffitt Cancer Center, Tampa, FL) and cultured in DMEM supplemented with 2 mM L-glutamine, 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The MCF7 and MCF10A mammary fibrocystic cell lines were a gift from Dr. Richard Jove (City of Hope, Duarte, CA) and were cultured in DMEM-F12 supplemented with 2 mM L-glutamine, 10% FBS and 1% P/S. The T98G glioblastoma cell line was a gift from Dr. Joseph Nevins (Duke University, Durham, NC) and grown in DMEM supplemented with 2 mM L-glutamine, 10% FBS, and 1% P/S. The H1299-pBS/U6 and H1299-shE2F1 cell lines were constructed and cultured as previously described (125,126,149). The H1299-ER-E2F1 cell line was constructed and cultured as previously described (125,126,149,150).

Adenovirus

The Ad-GFP and Ad-E2F1-GFP adenovirus were kind gifts from Dr. Timothy Kowalik (University of Massachusetts, Worchester, MA) (20,89). The Ad-E2F1(1-283)-GFP adenovirus was constructed as previously described (42). The Ad-RhoBTB2-GFP

adenovirus was constructed using a cDNA construct of RhoBTB2 with an N-terminal 3XFlag sequence and a C-terminal *myc* tag. The entire double-tagged sequence was used for virus construction with the AdEasy™ Adenoviral Vector System (Stratagene) using the pShuttle-IRES-hrGFP-1 vector following the manufacturer's protocol. Titering was conducted using the AdEasy™ Viral Titer Kit (Stratagene).

Real-time PCR

Total cell RNA was harvested using the RNeasy Mini Kit (Qiagen) using the optional DNase treatment. Reverse Transcriptase (RT) reactions were random hexamer-primed using Applied Biosystems' (Foster City, CA) High Capacity cDNA Archive Kit. Standard curves were constructed using serial dilutions of pooled sample RNA (50, 10, 2, 0.8, 0.4, and 0.08 ng) per reverse transcriptase reaction. One 'no reverse transcriptase' control was included for the standard curve and for each sample.

TaqMan® Gene Expression Assays (Applied Biosystems) were used. The assay primer and probe sequences are proprietary. TaqMan® probe Hs01598093_g1 was used for RhoBTB2. Real-time quantitative PCR analyses were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All standards and samples were tested in triplicate wells. The no template control (H_2O), no RT controls, no amplification control (Bluescript plasmid), and No RNA control were tested in duplicate wells. PCR was carried out with the TaqMan® Universal PCR Master Mix (Applied Biosystems) using 2 µl of cDNA and 1X primers and probe in a 20-µl final reaction mixture. After a 2-min incubation at 50°C, AmpliTaq Gold was activated by a 10-min

incubation at 95°C, followed by 40 PCR cycles consisting of 15s of denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The 18s data were used for normalizing gene values: ng gene/ng 18s per well.

RhoBTB2 antibody production

Affinity-purified rabbit polyclonal antibody was generated toward a peptide corresponding to human RhoBTB2 amino acids 673-687 (KEEDHYQRARKEREK) by Pacific Immunology (Ramona, CA). Specifically, a 16-amino acid peptide (CKEEDHYQRARKEREK) was conjugated (via an artificial N-terminal cysteine residue) to Keyhole Limpet Hemocyanin and used to immunize rabbits. Serum was subjected to peptide column affinity purification prior to use in immunofluorescence. Antibody specificity was demonstrated using a previously described RhoBTB2 siRNA (148).

Plasmids, siRNA, and transfections

RhoBTB2 siRNA was custom made (Ambion) using a previously published RhoBTB2 siRNA (DBC2-γ) sequence (148). siCONTROL non-targeting siRNA (Dharmacon) was used for all negative controls. The siRNA was transfected using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocol. The pBB14 membrane GFP plasmid was a kind gift from Dr. L.W. Enquist (Princeton), constructed

as previously described (151) and transfected with Lipofectamine™ 2000 following the manufacturer's protocol.

Immunofluorescent microscopy

Cells were grown on Lab-Tek® II Chamber Slides™ (Nunc), fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X, then blocked with 2% BSA in PBS. The primary RhoBTB2 antibody was used at a 1:40 concentration, and the secondary antibody was Alexa Fluor® 555 goat anti-rabbit Ig antibody (Molecular Probes) at a concentration of 1:2000. Cover slips were mounted using ProLong® Gold antifade reagent with DAPI (Molecular Probes). Samples were viewed with a fully automated, upright Zeiss Axio- ImagerZ.1 microscope with a 40x or 63x /1.40NA oil immersion objective, and DAPI, FITC and Rhodamine filter cubes. Equal exposure times were used for each sample. Images were produced using the AxioCam MRm CCD camera and Axiovision version 4.5 software suite.

Flow cytometry

Cells were detached from culture plates via trypsin, washed twice with PBS, and then fixed in 70% ethanol. The fixed cells were washed twice with PBS and treated with RNase A and propidium iodide (PI). PI staining was used to measure for cell cycle status using a Becton-Dickinson FACScan instrument and Cell Quest software.

MTS assays

For siRNA and adenovirus based experiments, the cells were first transfected or infected as described in the results section. After 24 hours of transfection or infection the cells were trypsinized, counted, and then plated in 96-well plates. The specific drug treatments were then administered and the MTS assays were conducted using a CellTiter 96® AQ_{ueous} One Cell Proliferation Assay Kit (Promega) following the published protocol.

Results

E2F1 overexpression upregulates RhoBTB2

Using a microarray screen, we sought to identify novel targets of the E2F1 transcription factor. In this approach, we infected the H1299 cell line with adenovirus expressing either a green fluorescent protein control construct (Ad-GFP) or a GFP-fused E2F1 construct (Ad-E2F1-GFP). RNA was harvested at 24 and 48 hours and processed for microarray analysis. Among the list of genes whose transcripts were found to be highly induced by E2F1 infection was RhoBTB2.

To confirm the microarray results, we infected H1299s with either Ad-GFP, Ad-E2F1-GFP, or Ad-E2F1(1-283)-GFP, a deletion mutant of E2F1 that is lacking the transactivation domain (45). Using real-time polymerase chain reaction (PCR) to quantify RhoBTB2 mRNA expression, we found that Ad-E2F1-GFP infection does indeed induce RhoBTB2 transcript approximately 5 and 20-fold compared to that of the Ad-GFP infection at the 24- and 48-hour time points, respectively (Fig. 5A). Lack of RhoBTB2 activation by Ad-E2F1(1-283)-GFP infection confirms that upregulation of RhoBTB2 by E2F1 is dependent on E2F1's C-terminal transcription activation domain. Since all experiments conducted to this point employed the H1299 cell line, we wanted to ensure that RhoBTB2 activation by E2F1 was not cell line-dependent. To this end, we infected

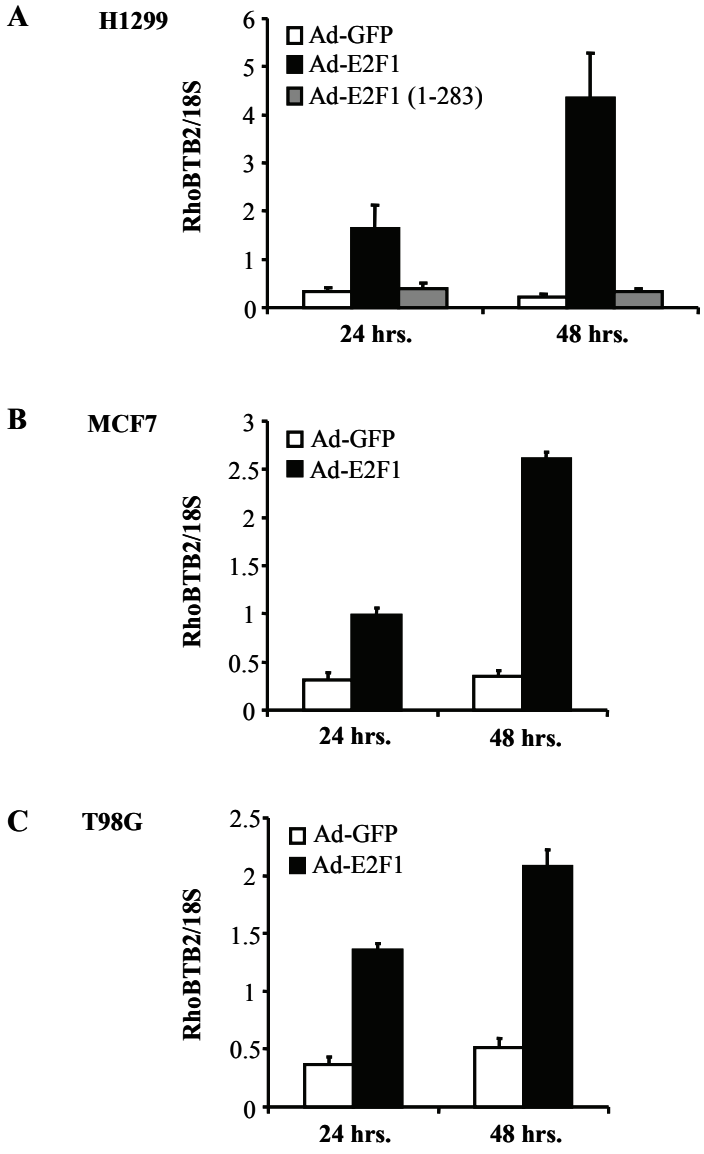


Figure 5. E2F1 overexpression upregulates RhoBTB2 mRNA. (A) H1299s were treated with either Ad-GFP, Ad-E2F1-GFP, or Ad-E2F1(1-283)-GFP adenovirus, harvested at 24 and 48 hours, with real-time PCR conducted to quantify RhoBTB2 mRNA relative to 18S. (B, C) MCF7s or T98Gs were treated with either Ad-GFP or Ad-E2F1 with subsequent real-time PCR analysis for RhoBTB2 to 18S at 24- and 48-hour time points.

the T98G and MCF7 cell lines with either Ad-GFP or Ad-E2F1 and conducted real-time PCR as in the prior experiment. We observed upregulation of RhoBTB2 similar to that which was observed in H1299s, thus confirming that RhoBTB2 upregulation by E2F1 overexpression is not cell line specific (Fig. 5B, C).

In order to conduct protein-based studies of RhoBTB2, we raised a polyclonal antibody against a 15 amino acid peptide sequence located within the C-terminus. While the antibody was not able to recognize endogenous RhoBTB2 protein in a denatured state by western blot, we were able to visualize endogenous RhoBTB2 protein via immunofluorescenct microscopy (IFM) (Fig. 6A). To confirm that the observed signal was not an artifact of non-specific binding, we transiently knocked-down RhoBTB2 expression using small inhibitory RNA (siRNA) and assayed for expression using IFM. As shown in Figure 6B, knock-down of RhoBTB2 expression diminishes the observed RhoBTB2 signal, thus confirming the specificity of the novel antibody.

Having an antibody functional for RhoBTB2 protein quantification, we sought to determine if the observed upregulation of RhoBTB2 mRNA by E2F1 overexpression resulted in a corresponding increase of RhoBTB2 at the protein level. To this end, an HA-tagged version of E2F1 (HA-E2F1), as well as a GFP-expression vector, were co-transfected into H1299s. After 24 hours the cells were stained for RhoBTB2, and GFP positive and negative cells were used to select for transfected and non-transfected cells, respectively. We found that cells positive for GFP (transfected) expressed a substantially higher level of RhoBTB2 protein as compared to adjacent GFP-negative cells (Fig. 7), thus confirming that E2F1 overexpression results in increased expression of RhoBTB2

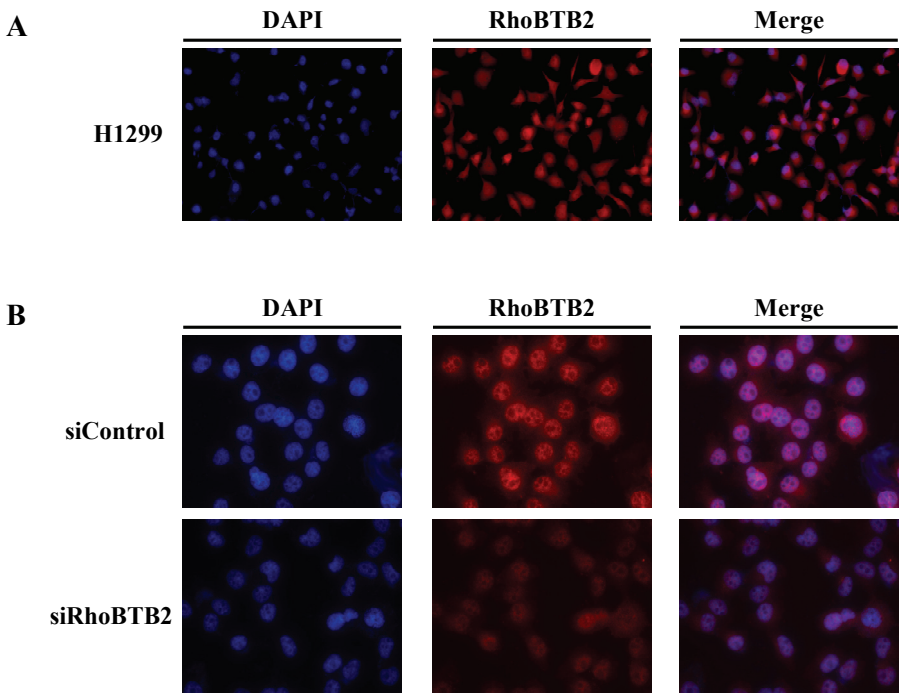


Figure 6. Novel RhoBTB2 antibody is functional in immunofluorescent microscopy and is specific for RhoBTB2. (A) Immunofluorescent microscopy (IFM) of H1299s at 40x for RhoBTB2 with a rabbit polyclonal antibody described in experimental procedures—DAPI: blue; RhoBTB2: red. (B) IFM as in 6A of H1299s transfected with either negative control siRNA (top) or siRNA to RhoBTB2 (bottom) after 48 hours.

protein. Taken together, these results demonstrate that RhoBTB2 is upregulated at both the mRNA and protein levels by E2F1 overexpression.

Upregulation of RhoBTB2 by E2F1 is direct and not dependent on artificial overexpression

We considered the possibility that RhoBTB2 might be an indirect target of E2F1; to address the issue of direct versus indirect activation, we utilized a well characterized H1299 cell line with an estrogen receptor-fused version of E2F1 stably integrated (H1299 ER-E2F1) (125,126,149,150). The result is an overexpressed version of E2F1 that is transcriptionally inactive due to estrogen receptor-mediated cytoplasmic localization. Using this system, E2F1 activity can be rapidly induced through nuclear localization by addition of the estrogen receptor ligand 4-hydroxytamoxifen (4-OHT), while simultaneously blocking new protein synthesis by means of cyclohexamide (CHX). Any transcripts found to be induced by 4-OHT in the presence of CHX can be considered direct E2F1 targets.

As shown in figure 8, RhoBTB2 mRNA expression is relatively low in the untreated H1299 ER-E2F1 cell line, as well as after 8 and 24 hours of treatment of CHX alone. As expected, upregulation of RhoBTB2 is readily observed at 8 and 24 hours after promoting E2F1 nuclear localization through treatment with 4-OHT. This activation of RhoBTB2 transcription by 4-OHT is not abrogated upon co-administration of CHX, thus confirming that RhoBTB2 is a direct transcriptional target of E2F1.

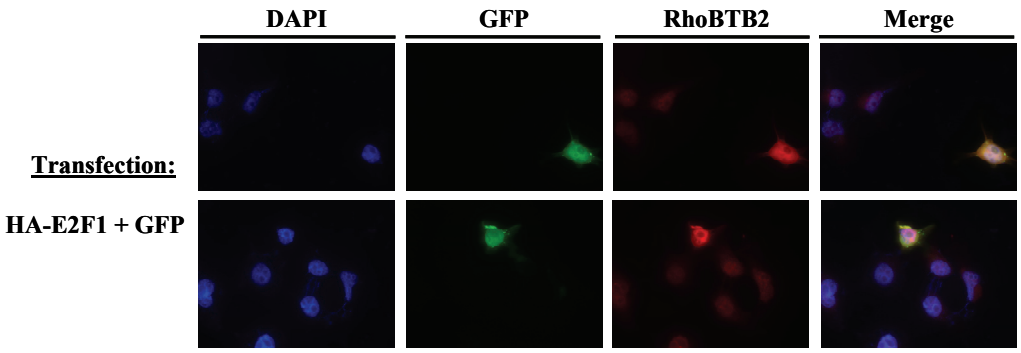


Figure 7. E2F1 overexpression upregulates RhoBTB2 protein. IFM at 63x of two different fields of H1299s 48 hours after being transiently cotransfected with pcDNA3-HA-E2F1 and pBB14, a membrane GFP plasmid—DAPI: blue; GFP (transfected cells): green; RhoBTB2: red.

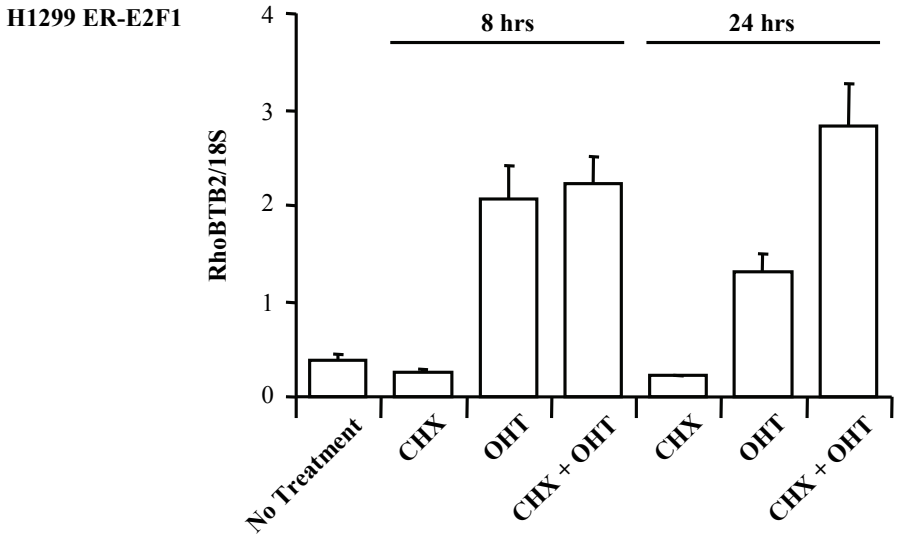


Figure 8. E2F1-mediated upregulation of RhoBTB2 is direct. The H1299-ER-E2F1 cell line was treated with either CHX, 4-OHT or both. Cells were harvested for real-time PCR analysis at 8- and 24-hour time points.

Having shown the capability of artificially overexpressed E2F1 to directly activate RhoBTB2, we next sought to determine if E2F1 plays a role in RhoBTB2 regulation under physiological conditions. To this end, we employed H1299 cell lines with a stably integrated short-hairpin inhibitory RNA corresponding to E2F1 (H1299-shE2F1) or an empty vector control (H1299-pBS/U6) (125,126,149). We observed significant knockdown of E2F1 in the H1299-shE2F1 in comparison to the H1299-pBS/U6 as previously reported (Fig. 9A) (125,126,149). We stained the cells for RhoBTB2 and compared expression levels between the two lines by means of IFM. The H1299-pBS/U6 control cell line with unaltered E2F1 expressed RhoBTB2 at levels comparable to that of the parental H1299 line (Fig. 9B). In contrast, the H1299-shE2F1 cell line displayed greatly diminished expression of RhoBTB2 when compared to that observed in the H1299-pBS/U6 cell line (Fig 9B). Given that knock-down of E2F1 diminishes RhoBTB2 expression, we conclude that E2F1 is indeed a physiological regulator of RhoBTB2.

RhoBTB2 is upregulated during mitosis, which is partially dependent on E2F1

One of the main functions of the growth promoting E2Fs is to activate the transcription of genes critical for cell cycle progression (8,9). Having identified RhoBTB2 as an E2F1 target gene, we postulated that RhoBTB2 expression may be regulated through this process. To examine RhoBTB2 expression through the cell cycle, we stained an asynchronously growing population of H1299s for RhoBTB2 and examined the population for cells in interphase as well as various stages of mitosis via

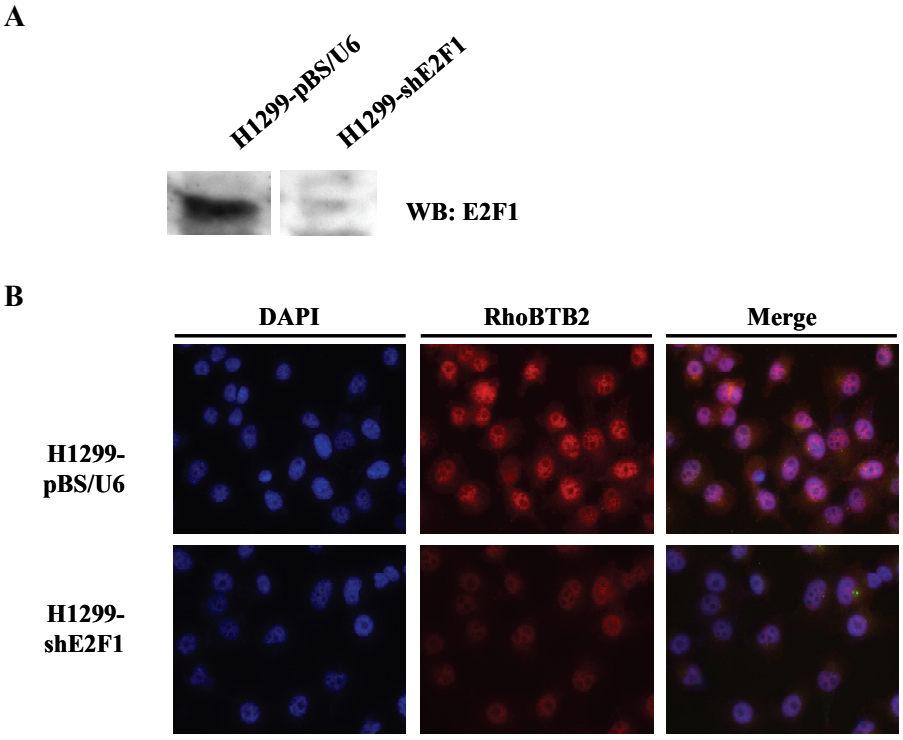


Figure 9. RhoBTB2 is a physiological target of E2F1. (A) A western blot for E2F1 in the H1299-pBS/U6 and H1299-shE2F1 cell lines demonstrating efficient knockdown of E2F1. (B) IFM at 63x using the RhoBTB2 polyclonal antibody conducted on asynchronously growing H1299-pBS/U6 and H1299-shE2F1 cell lines—DAPI: blue; RhoBTB2: red

IFM. As shown in figure 10, H1299s in interphase express a relatively low level of RhoBTB2; however, upon the initiation of prophase RhoBTB2 levels increase dramatically. RhoBTB2 expression remains highly elevated through metaphase and anaphase, and does not begin to decrease until telophase/cytokinesis.

A vast majority of cancers exhibit aberrant regulation of the RB-E2F pathway, with the end result being unrestrained E2F molecules. We considered the possibility that the observed mitotic upregulation of RhoBTB2 may be an artifact of the highly transformed H1299 phenotype. To address this issue, we conducted identical experiments in MCF10As, a non-tumorigenic mammary fibrocystic cell line. In these experiments we observed mitotic upregulation of RhoBTB2 that parallels that observed in H1299s (Fig. 10), confirming that upregulation of RhoBTB2 during mitosis is not due to the highly transformed nature of H1299s.

We next wanted to determine if the observed mitotic upregulation of RhoBTB2 was dependent upon E2F1. We utilized the aforementioned E2F1 proficient and knockdown cell lines H1299-pBS/U6 and H1299-shE2F1 to compare cell cycle regulation of RhoBTB2 in cells with two different levels of E2F1 expression. Asynchronously growing populations of the two cell lines were stained for RhoBTB2 and examined for cells in interphase and various stages of mitosis as previously described. As expected, mitotic upregulation of RhoBTB2 was readily observed in the H1299-pBS/U6 cell line, and was comparable to that seen in the parental H1299s (Fig. 11, top panel). During interphase, the H1299-shE2F1 cell line has lower basal expression of RhoBTB2, as previously observed. However, we noted an impaired mitotic upregulation of RhoBTB2 in the H1299-shE2F1 cell line (Fig. 11, bottom panel). While there is an

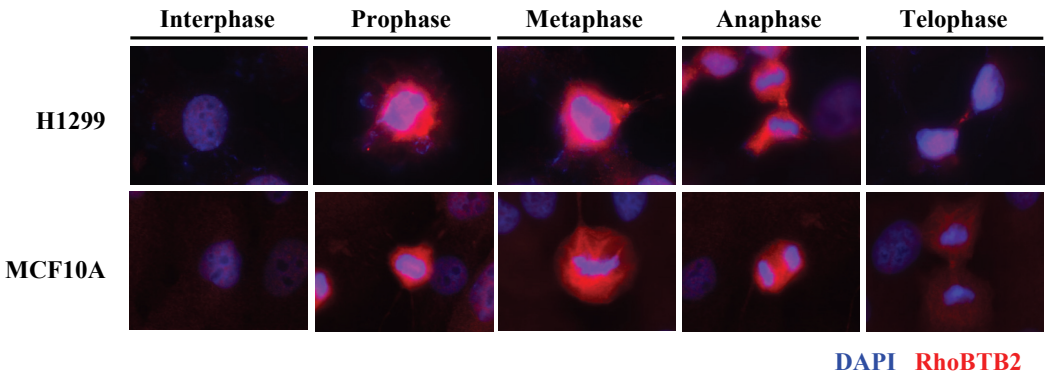


Figure 10. RhoBTB2 is upregulated during mitosis. IFM at 63x using the RhoBTB2 polyclonal antibody of representative H1299s (top) and MCF10A (bottom) cells in either interphase, prophase, metaphase, anaphase, or telophase/cytokinesis within aynchronously growing populations—DAPI: blue; RhoBTB2: red.

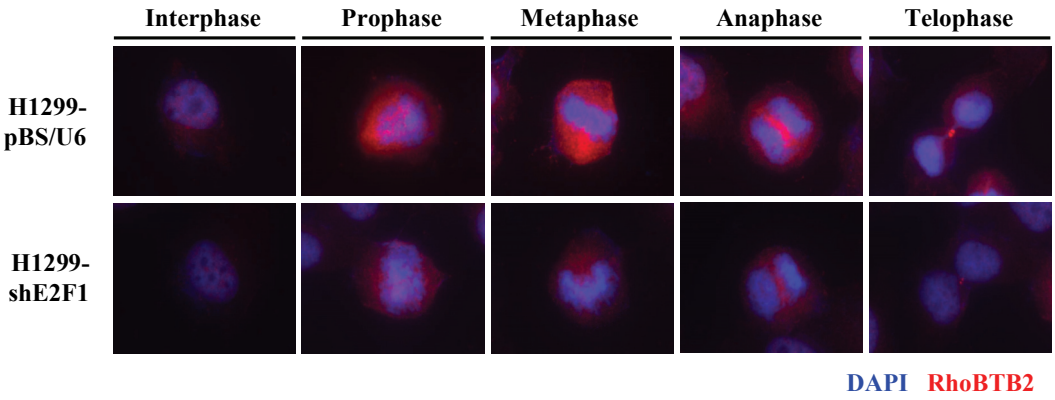


Figure 11. Mitotic upregulation of RhoBTB2 is partially dependent on E2F1. IFM at 63x using the RhoBTB2 polyclonal antibody of representative H1299-pBS/U6 (top) and H1299-shE2F1 (bottom) cells in either interphase, prophase, metaphase, anaphase, or telophase/cytokinesis within aynchronously growing populations—DAPI: blue; RhoBTB2: red.

evident upregulation of RhoBTB2 during prophase, it is significantly impaired when compared to that observed with the E2F1 proficient H1299-pBS/U6 cell line. This trend of diminished mitotic upregulation of RhoBTB2 continues to be observable throughout all of the mitotic phases examined (Fig. 11). We postulate that residual regulation ofRhoBTB2 may be mediated by additional E2F family members. Taken together, this demonstrates that RhoBTB2 is indeed upregulated during mitosis, which is partially dependent on the presence of E2F1.

Overexpression of RhoBTB2 increases the S-phase fraction and slows proliferation

Given the observation that RhoBTB2 is upregulated during M-phase of the cell cycle, we sought to determine if artificial manipulation of RhoBTB2 levels would have a functional and observable effect on cell cycle status or proliferation. To this end, we constructed an adenovirus expressing either GFP (Ad-GFP) or RhoBTB2 fused to an internal ribosome entry site (IRES) GFP construct (Ad-RhoBTB2-GFP). Asynchronously growing H1299s were then infected with equal amounts of either Ad-GFP or Ad-RhoBTB2-GFP and harvested at 48 hours for flow cytometric analysis of cell cycle status via propidium iodide (PI) staining. As shown in figure 12A, overexpression of RhoBTB2 alters the cell cycle status of H1299s by increasing the fraction of cells in S-phase. Having noted that overexpression of RhoBTB2 increased the S-phase fraction; we wanted to know how this single snap shot of cell cycle status manifested in a functional effect on cell proliferation. To test this, we infected asynchronously growing H1299s with either Ad-GFP or Ad-RhoBTB2-GFP adenovirus and conducted MTS-based

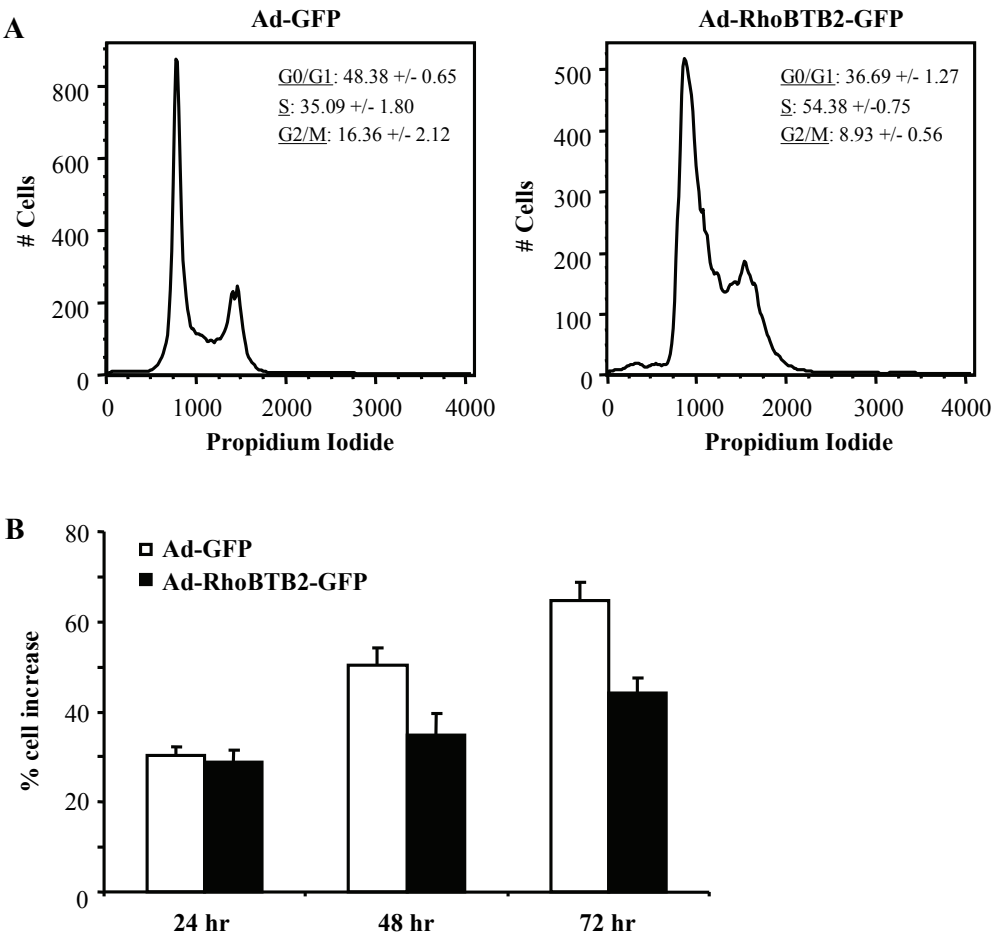


Figure 12. Overexpression of RhoBTB2 increases the S-phase fraction and slows proliferation. (A) The H1299 cell line was infected in triplicate with equal amounts of either the Ad-GFP or Ad-RhoBTB2-GFP adenovirus and harvested 48 hours post-infection for flow cytometry. Propidium Iodide was used to analyze cell cycle status. (B) H1299s were infected in triplicate with equal amounts of either the Ad-GFP or Ad-RhoBTB2-GFP adenovirus, detached at 24 post-infection, counted, and transferred to 96 well plates where an MTS assay was performed to analyze cell proliferation after 24, 48 and 72 hours.

proliferation assays. As shown in figure 12B, cells infected with RhoBTB2 adenovirus exhibited impaired cell proliferation over multiple passages as compared to those infected with the control GFP virus. Since we observed an increase in the S-phase fraction as well as slowed cell progression upon overexpression of RhoBTB2, we wanted to determine ifsiRNA-mediated knockdown of RhoBTB2 would alternatively decrease the S-phase fraction or increase the rate of proliferation. We found that depletion of RhoBTB2 did not alter the cell cycle status or the rate of proliferation, consistent with the idea of RhoBTB2 as being a negative regulator. From these observations, we conclude that the observed increase in the S-phase fraction upon overexpression of RhoBTB2 is potentially caused by a transient S-phase arrest or lengthened S-phase.

RhoBTB2 is upregulated during drug-induced apoptosis, which is primarily dependent on E2F1

E2F1 is unique among the E2F family members in that it not only has the ability to transactivate genes critical for cell cycle progression, but is also a potent inducer of apoptosis through activating the transcription of proapoptotic genes (for review, see ref. (24)). Given this fact, we investigated whether RhoBTB2 expression was effected by drug-induced apoptosis. To determine whether RhoBTB2 is regulated by apoptotic insults, we treated H1299s with either cisplatinum, flavopiridol or etoposide, chemotherapeutic agents where E2F1 is known to be a critical mediator, and conducted IFM to determine whether these cytotoxic insults had any effect on RhoBTB2 expression. As shown in figure 13A, we observed that administration of all of the chemotherapeutic

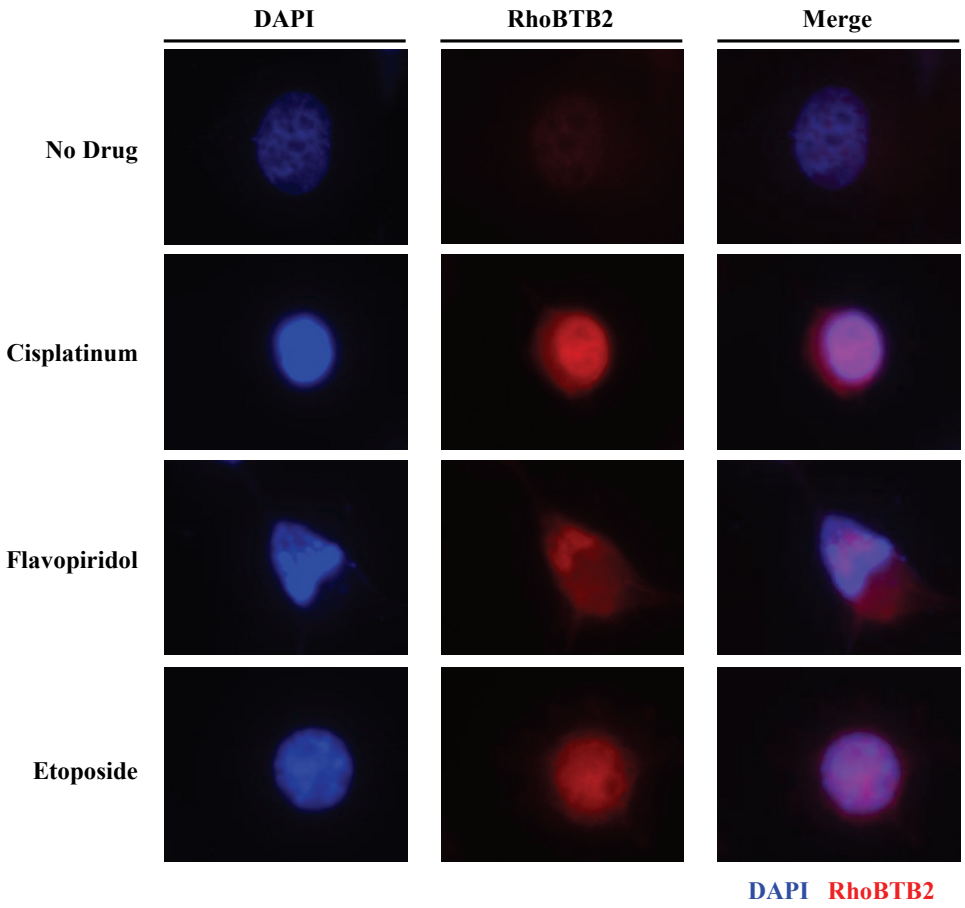


Figure 13. RhoBTB2 is upregulated during drug-induced apoptosis. IFM at 63x using the RhoBTB2 polyclonal antibody of representative cells from H1299s after 24 hours of either no treatment, 20 uM cisplatinum, 200 nM flavopiridol, or 20 uM etoposide—DAPI: blue; RhoBTB2: red.

agents tested resulted in increased RhoBTB2 protein expression, with flavopiridol exhibiting the weakest.

While we observed upregulation of RhoBTB2 during cytotoxic insult, we wanted to determine if E2F1 was responsible for this upregulation. To examine this issue, we utilized the previously described E2F1 proficient and knockdown cell lines H1299-pBS/U6 and H1299-shE2F1 and conducted IFM on cells treated with the aforementioned apoptotic stimuli. As previously observed, RhoBTB2 expression was diminished in the untreated H1299-shE2F1 cell line compared to the control H1299-pBS/U6 cell line (Fig. 14A). Upon the induction of apoptosis, the control H1299-pBS/U6 cell line behaved similar to that of the parental H1299s, with upregulation of RhoBTB2 being clearly evident after 24 hours (Fig. 14, top). In stark contrast, we observed very little upregulation of RhoBTB2 in the H1299-shE2F1 cell line (Fig. 14A, bottom). Figure 14C displays E2F1 protein levels at 24 hours post treatment, demonstrating that E2F1 upregulation does not occur in the H1299-shE2F1 cell line even in the presence of cytotoxic insult. It should be noted that in the presence of flavopiridol, we observe upregulation of E2F1 to be highest shortly after treatment (around 6 hours) and diminished by 24 hours, which explains the seemingly diminished E2F1 expression as compared to the no treatment control. Taken together, these results demonstrate that RhoBTB2 is upregulated during drug-induced apoptosis, and that this upregulation is primarily dependent on the presence of E2F1.

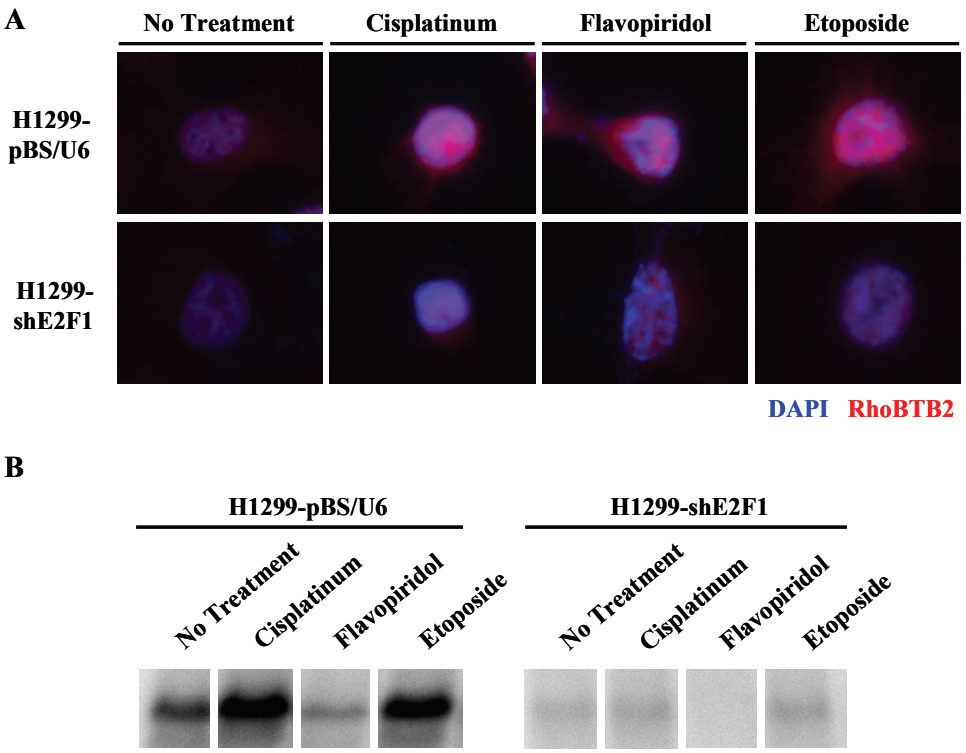


Figure 14. Upregulation of RhoBTB2 during drug-induced apoptosis is primarily dependent on E2F1. (A) IFM at 63x using the RhoBTB2 polyclonal antibody of representative H1299-pBS/U6 (top) or H1299-shE2F1 (bottom) cells after 24 hours of either no treatment, 20 uM cisplatinum, 200 nM flavopiridol, or 20 uM etoposide—DAPI: blue; RhoBTB2: red.

Knockdown of RhoBTB2 expression by siRNA impairs the induction of drug-induced apoptosis

Previous experiments demonstrated that RhoBTB2 is upregulated during drug-induced apoptosis in an E2F1-dependent manner; we therefore wanted to explore whether disruption of RhoBTB2 activity would have a functional effect on drug-induced apoptosis. To address this issue, we transiently depleted RhoBTB2 in H1299s via siRNA-mediated knockdown of RhoBTB2, induced apoptosis using the drug treatments previously employed, and conducted MTS assays to measure cell viability over the span of three days. While loss of viability occurred in both the siControl and siRhoBTB2 transfected cell lines upon cytotoxic drug treatment, this loss of viability was abrogated in cells lacking RhoBTB2 (Fig. 15). We observed similar results in all drug treatments used, implying that RhoBTB2 may play a more ubiquitous role in apoptosis. Since we observed abrogated induction of apoptosis in upon siRNA-mediated knockdown of RhoBTB2, we wanted to determine if overexpression of RhoBTB2 would alternatively hasten the induction of apoptosis. We found that adenovirus-mediated overexpression of RhoBTB2 did not hasten the induction of apoptosis. We interpret these data as for the first time demonstrating that RhoBTB2 plays a direct and important role in the implementation of apoptosis.

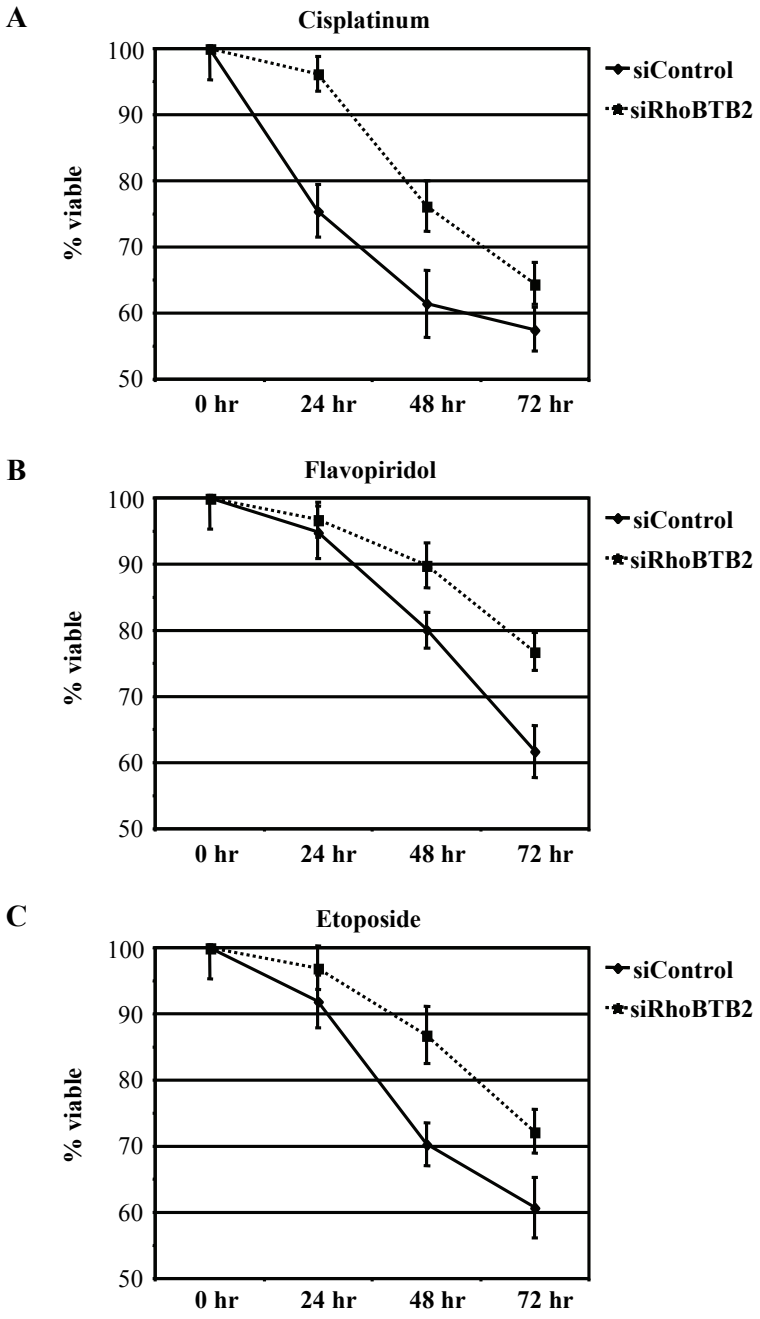


Figure 15. Knockdown of RhoBTB2 via siRNA impairs the induction of drug-induced apoptosis. H1299s were transiently transfected with either a negative control siRNA, or siRNA against RhoBTB2, detached at 24 post-transfection, counted, and transferred to 96 well plates where an MTS assay was performed to analyze cell viability after 24, 48 and 72 hours of treatment with either 20 uM cisplatinum (A), 200 nM flavopiridol (B), or 20 uM etoposide (C).

Discussion

E2F is perhaps best known for its ability to promote the transcription of genes involved in the G1/S-phase transition; however an increasing amount of evidence implicates a role for E2F in the regulation of genes with mitotic functions. Overexpression of E2F1 or E2F2 induces a subset of genes with mitotic functions, and E2F1 can be found at the promoters of genes with mitotic functions (101-105). Furthermore, targets of E2F1 and E2F2 tend to be physiologically regulated temporally at two distinct cell cycle stages: G1/S and G2, implicating a role for E2F-mediated transcription long after E2F is thought to be inactive (101).

While a number of mitotic E2F targets have been identified, few have been characterized. In this work, we demonstrate that RhoBTB2 is a direct target of E2F1 that is physiologically upregulated during mitosis. We further show that mitotic upregulation of RhoBTB2 is partially dependent of E2F1, as knockdown of E2F1 expression via shRNA abrogates mitotic upregulation of RhoBTB2. It is possible that the remaining mitotic upregulation of RhoBTB2 in the absence of E2F1 is dependent on E2F2 or E2F3a; however we have not pursued this hypothesis.

In addition to being a mitotic target of E2F1, we also find that RhoBTB2 is an apoptotic target of E2F1 as well. RhoBTB2 is upregulated upon treatment with chemotherapeutic drugs, which is primarily independent on E2F1 as knockdown of E2F1

with shRNA abrogates this effect as well. We see a greater dependence on E2F1 for apoptosis-induced upregulation as opposed to mitotic upregulation, and this may be due to an inability of E2F2 or E2F3a to compensate, as E2F1 is the primary inducer of apoptosis among the activating E2Fs.

In order to further explore the significance of E2F-mediated regulation of RhoBTB2, we examined a functional role for RhoBTB2 in either of these processes. Overexpression of RhoBTB2 increases the fraction of cells in S-phase and significantly impairs cell proliferation, which we interpret as possibly being a transient S-phase block, as we only see a partial block in cell proliferation. In the case of apoptosis, we find that depletion of RhoBTB2 by siRNA slows the induction of drug-induced apoptosis. While deciphering mechanisms by which RhoBTB2 acts in cell cycle inhibition and the induction of apoptosis was beyond the scope of this study, published reports on RhoBTB2 have led to some intriguing hypotheses.

In agreement with our observations, RhoBTB2 was shown to inhibit cell proliferation in a breast cancer cell line deficient for RhoBTB2 (146). Futher studies asserted that RhoBTB2-mediated downregulation of cyclin D1 was obligatory for this effect (147). Another study utilizing pathway-based analysis of gene expression patterns found RhoBTB2 to effect the expression of genes associated with cell cycle, apoptosis, cytoskeleton and membrane-trafficking pathways (148). But perhaps the most intriguing study of found that RhoBTB2 direct bound and was a substrate of the Cul3 ubiquitin ligase (144). The authors proposed a hypothesis in which RhoBTB2 served as a scaffold that recruited proteins to the Cul3 complex to be targeted for degradation. This seems

quite rational, as other BTB/POZ domain-containing proteins have similar functions (140-144).

Given the previously mentioned studies coupled with our own observations, we believe that the functional significance of E2F1-mediated upregulation of RhoBTB2 could be directly related to the ability of RhoBTB2 to recruit proteins to the Cul3 complex to be targeted for degradation. We propose a model in which the physiological role of RhoBTB2 in mitosis and apoptosis is to recruit proteins to the Cul3 complex to be targeted for degradation, and that the cell cycle inhibition observed during overexpression may be a non-physiological response from RhoBTB2 targeting proteins to Cul3 in phases of the cell cycle where RhoBTB2 would not normally be present (Fig. 16). While cyclin D1 would seem like an attractive candidate to mediate this effect, one would not expect to see an arrest occurring in S-phase or G2/M upon loss of cyclin D1. Additionally, cyclin D2 or D3 might be expected to compensate. While the mechanisms behind the biological functions of RhoBTB2 are yet to be determined, it is clear that RhoBTB2 is indeed a physiologically relevant direct target of E2F1.

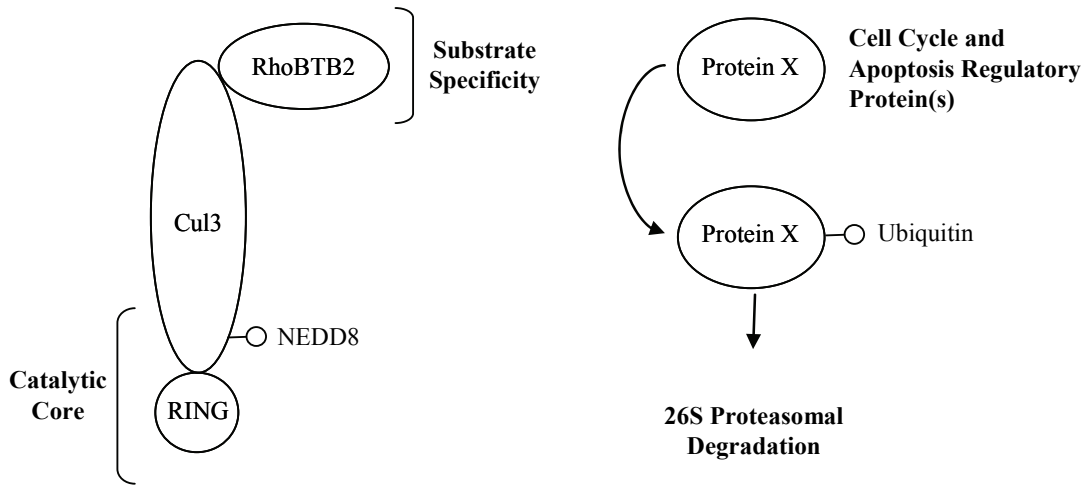


Figure 16. A proposed mechanistic model for RhoBTB2 activity. In this model, we propose that RhoBTB2 exerts its cell cycle and apoptotic biological effects by facilitating uibiquitination and subsequent degradation of cell cycle and apoptosis regulatory proteins. We propose that RhoBTB2 acts as a substrate-specific adaptor for the Cul3 ubiquitin ligase.

PART II

IDENTIFICATION AND CHARACTERIZATION OF TWO NOVEL MCL-1 PROMOTER POLYMORPHISMS

Abstract

A publication from Moshynska et al. identified two novel sequence variants of the MCL-1 promoter within lymphocytes from chronic lymphocytic leukemia patients (CLL), but not within noncancerous tissue from the same individuals or in lymphocytes from 18 healthy control subjects (1). This result suggested that the variants—insertions of 6 or 18 nucleotides at position –188 relative to the transcription start site—were CLL-related somatic oncogenic mutations. Moshynska et al. also determined that the 6- and 18-nucleotide insertions were associated elevated Mcl-1 expression, and proposed that the variant promoters could be used as a prognostic marker. We independently identified and cloned the three observed sequence variants from cancer cell lines hereby referred to as the Mcl-1 +0, +6 or +18 promoters. In contrast to Moshynska et al., we find the variant promoters to be identically present in both cancerous and adjacent noncancerous clinical lung samples, suggesting that the variants are germ-line encoded. We also find the three variant promoters prevalent in genomic DNA derived from healthy control samples and present at frequencies similar to that observed in cancerous cell lines. Furthermore, activity analysis of the three variant promoters reveals the Mcl-1 +6 and +18 promoters to be less active than the Mcl-1 +0 promoter, both during normal cellular homeostasis and under conditions that actively induce Mcl-1 transcription. Given our results, we

conclude that the Mcl-1 +6 and +18 promoters are likely benign polymorphisms and do no represent a reliable prognostic marker.

Introduction

Mcl-1 and the Bcl-2 family of proteins

Mcl-1 is an antiapoptotic member of the Bcl-2 family of proteins. The Bcl-2 family is a group of proteins involved in the intrinsic stress-mediated apoptotic pathway whose primary role is to regulate the release of cytochrome c and other apoptotic factors from the mitochondria and possibly the endoplasmic reticulum (ER) (152,153). Upon release from the mitochondria, cytochrome c forms a complex with Apaf-1 which cleaves and activates effector caspases, thus initiating apoptosis (154,155). The Bcl-2 family is divided into three subfamilies that play distinct roles in both promoting and inhibiting the integrity of the mitochondrial membrane and ultimately—the release of cytochrome c and the initiation of apoptotsis. These subfamilies consist of the proapoptotic BH3-only subfamily, the antiapoptotic Bcl-2 subfamily, and the proapoptotic Bax subfamily. The roles of each family member in the intrinsic apoptotic pathway are illustrated in figure 17.

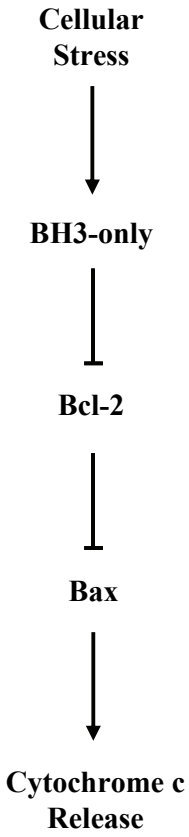


Figure 17. The Bcl-2 family and the intrinsic stress-induced apoptotic pathway. Cellular stress activates proapoptotic BH3-only Bcl-2 subfamily members through various mechanisms. BH3-only subfamily members block the ability of antiapoptotic Bcl-2 subfamily members to restrain the activity of proapoptotic Bax subfamily members. This leads to oligomerization of Bax subfamily members in the mitochondrial membrane, which promotes the release of cytochrome c and other apoptotic factors, thus initiating apoptosis.

The BH3-only subfamily

The BH3-only subfamily consists of multiple family members that are the first responders to cellular stress within the intrinsic apoptotic pathway (Fig. 13). Depending on the nature of the cellular stress, individual or multiple BH3-only proteins may become activated. Activation can occur through transcriptional regulation, post-translational modification, or both, and is largely family member-dependent. These subfamily members are proapoptotic, and promote the release of apoptotic factors from the mitochondria. While there is significant disagreement as to the exact mechanism by which Bcl-2 family members interact to disrupt mitochondrial membrane integrity, it can be generally stated that the primary role of the BH3-only proteins is to antagonize the inhibitory action of antiapoptotic Bcl-2 family members through direct binding. Indeed, the BH3-only family is named such due to the presence of a single BH3 domain which, depending on the subfamily member, binds to the receptor domain of one or more antiapoptotic Bcl-2 subfamily proteins (152,153).

The Bcl-2 subfamily

The second subfamily within the Bcl-2 family is the Bcl-2 subfamily, and their primary role is to promote cell survival. Like the BH3-only subfamily, the Bcl-2 subfamily consists of multiple members; however the most notable and likely most relevant members are Bcl-2, Mcl-1 and Bcl-x_L. Bcl-2 subfamily proteins act downstream of BH3-only proteins and, in the absence of activated BH3-only proteins, maintain

mitochondrial membrane integrity at least in part by antagonizing the activity of Bax subfamily members. Structurally, Bcl-2 subfamily members contain a transmembrane domain (TM), and BH1-4 domains. The TM domain is thought to function as an anchor for integration into the membranes of the mitochondria and endoplasmic reticulum, and BH1, BH2 and BH3 domains collectively form a receptor domain specific for the BH3 motif. In the absence of cellular stress, Bcl-2 subfamily proteins are thought to restrain the proapoptotic activity of Bax subfamily proteins at least in part through direct binding of the Bcl-2 receptor domain to the BH3 domain of Bax subfamily members. However in the presence of cellular stress and activated BH3-only proteins, the BH3 domain of BH3-only proteins is thought to directly bind to the receptor domain of Bcl-2 subfamily members and prevent Bcl-2 subfamily members from restraining the activity of Bax or Bak (152,153).

The Bax subfamily

The final subfamily within the Bcl-2 family is the proapoptotic Bax subfamily, which consists of only two members: Bax and Bak. Bax and Bak are thought to be functionally redundant, as inactivation of either family member alone has little effect on apoptotsis, while inactivation of both significantly inhibits apoptosis (156). Structurally, Bax and Bak contain a TM domain and BH1-3 domains. Cellular stress induces Bax and Bak to make a conformational change and form homo-oligomers within the mitochondrial membrane. Oligomerization of Bax and Bak in the mitochondrial membrane disrupts membrane integrity and promotes the release of apoptotic factors. The

mechanism by which oligomerized Bax and Bak compromises mitochondrial membrane integrity has not been fully elucidated, although several models have been proposed (152,153).

Mcl-1 is an inhibitor of apoptosis

As illustrated in the previously described model, the physiological role of Mcl-1 and other antiapoptotic subfamily members is to promote survival through the maintenance of mitochondrial membrane integrity (Fig. 13). This function is well documented in both cell culture-based and *in vivo* experiments utilizing both gain-of-function and loss-of-function techniques. While the experiments that lend support for this model of activity are best described for Bcl-2, studies focusing on Mcl-1 will be the topic of discussion in the following paragraphs.

In cell culture-based assays, Mcl-1 overexpression inhibits the induction of apoptosis in multiple models, and is exemplified by the ability of overexpressed Mcl-1 to inhibit apoptosis induced by staurosporin or transient c-Myc overexpression in Chinese hamster ovary cells (157,158). Additionally, in murine myeloid progenitor cells, overexpression of Mcl-1 delays apoptosis induced by cytotoxic agents or growth factor withdrawal (159). In agreement with *in vitro* studies, transgenic expression of Mcl-1 in hematolymphoid tissues results in increased viability in various cells of lymphoid and myeloid origin—occuring at both mature and immature stages of development (160,161). Transgenic expression of Mcl-1 also promotes the development of certain hyperplasias and malignancies, which may be the result of an inhibition of apoptosis (160,162).

Studies dealing with the effects of deregulated Mcl-1 expression on aberrant tissue proliferation are addressed in more detail in the section regarding Mcl-1 and oncogenic transformation.

Similar to overexpression studies, depletion of Mcl-1 by means of antisense RNA or siRNA in cell culture assays can either promote spontaneous apoptosis or sensitize to apoptosis. This effect is well documented and holds true in multiple *in vitro* model systems. Indeed, several studies demonstrate the feasibility of utilizing siRNA against Mcl-1 as a therapeutic intervention in malignancy (163-166). Since disruption of both *Mcl-1* alleles in mice results in peri-implantation embryonic lethality (167), various targeted disruptions have been developed to examine the role of disrupted Mcl-1 expression *in vivo*. Targeted deletion of *Mcl-1* in the T or B cell lineages results in a significant reduction of B and T lymphocytes, and when deleted in the same lineage during lymphocyte development, increased apoptosis and developmental arrest is observed (168). Furthermore, deletion of *Mcl-1* in mature lymphocytes leads to a loss of viability (168). Induced deletion of *Mcl-1* in mature mice leads to depletion of the bone marrow due to decreased cell survival (169), and targeted deletion to macrophages and neutrophils results in decreased neutrophil survival as manifested by an increased rate of apoptosis in granulocytic compartments (170). Collectively, these experiments describe the crucial role that Mcl-1 plays in regulating apoptosis both *in vitro* and *in vivo*.

Mcl-1 and oncogenic transformation

One of the defining features of malignant transformation is an ability to evade apoptotic signals. Given Mcl-1's potent ability to promote survival, it is not surprising that Mcl-1 can contribute to oncogenic transformation under certain contexts. Much of the support for this stems from experiments utilizing mice transgenic for Mcl-1. Long-term expression of transgenic Mcl-1 targeted to hematolyphoid tissues results in the development of B-cell lymphoma (160), and explantation and culture of myeloid cells derived from Mcl-1 transgenic mice in the presence of interleukin (IL)-3 can induce immortalization (161). Additionally, transgenic expression of murine Mcl-1 leads to islet cell hyperplasia (162). It is unlikely that Mcl-1 action alone is sufficient for oncogenic transformation and likely cooperates with one or many oncogenic mutations to promote characteristics of a malignant phenotype. It is clear however, that under the proper conditions, enforced Mcl-1 expression can contribute to the development of an oncogenic phenotype.

Mechanisms regulating Mcl-1 expression

Mcl-1 expression is regulated at multiple levels including regulation of transcription, modification of transcript, and post-translation modification (Fig. 14). The Mcl-1 protein has a very short half-life (171,172), and as such many of the primary means of regulation are dependent on transcriptional mechanisms. Physiological mechanisms governing Mcl-1 transcription are highly dependent on cell type and

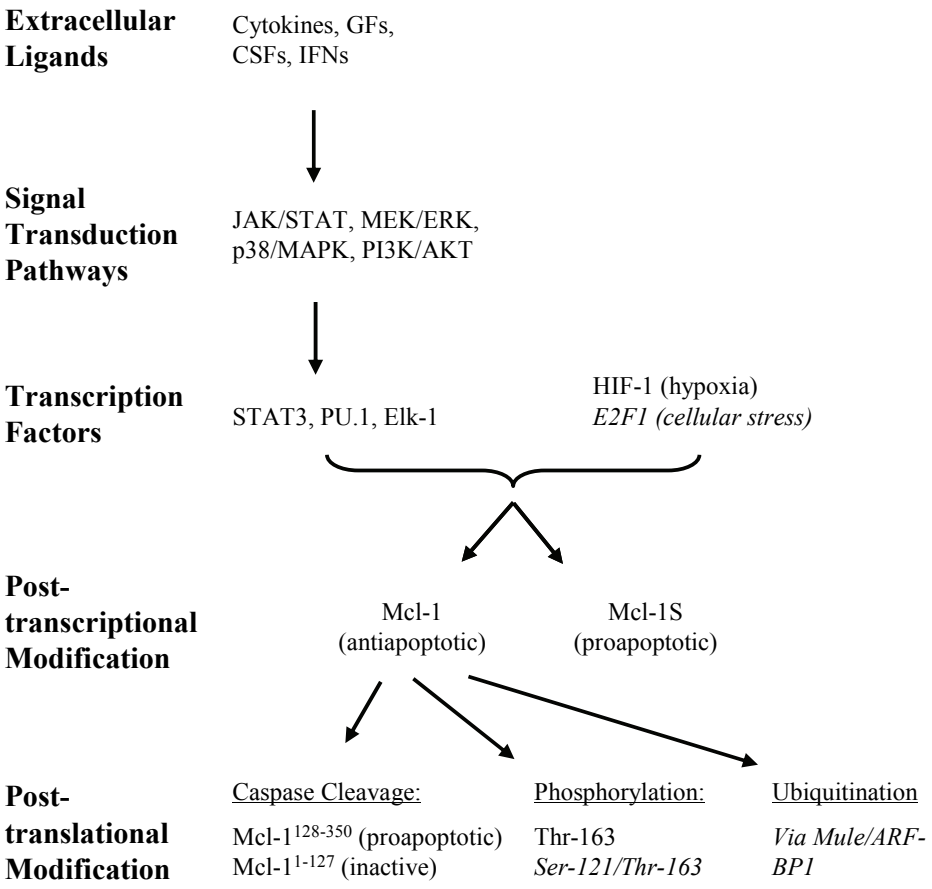


Figure 18. Mechanisms regulating Mcl-1. Mcl-1 is regulated in a context-dependent manner by transcriptional, post-transcriptional, and post-translation mechanisms. Extracellular ligands signal through multiple transduction pathways to positively influence Mcl-1 transcription in part through STAT3, PU.1, and Elk-1. Hypoxia also directly upregulates Mcl-1 transcription through HIF-1, whereas stress-induced upregulation of E2F1 directly represses Mcl-1 transcription (*italics: negative regulation*). Mcl-1 transcript may also be alternatively spliced to create a shorter form of Mcl-1 termed Mcl-1S, which is proapoptotic in nature. Finally, Mcl-1 may be regulated post-translationally through caspase cleavage, phosphorylation, or ubiquitination.

developmental state, and since much of the research on Mcl-1 has concentrated on hematopoietic tissues, many of the mechanisms regulating Mcl-1 transcription have been identified in that context. Transcription of Mcl-1 can be positively influenced by various extracellular stimuli including cytokines, growth factors, colony stimulating factors, and interferons (173,174). Depending on the stimuli, these signals are mediated by one or more transduction pathways including the JAK/STAT, MEK/ERK, p38/MAPK and PI3K/AKT signal transduction pathways (173,174).

Transcription factors residing at the ends of signal transduction pathways that positively regulate Mcl-1 transcription work primarily through three response elements within the MCL-1 promoter. Induction of Mcl-1 transcription through the PI3K/AKT pathway is mediated through the cAMP response element-binding (CREB) transcription factor, which directly binds a cAMP response element (CRE)-2 upstream of the transcription start site (175). Activation via the JAK/STAT pathway is the result of STAT binding to a serum-inducible element (SIE) (176-178), and activation through p38/MAPK-mediated pathways results in Ets family member PU.1 also binding to the SIE element (179). Additionally, activation through MEK/ERK also functions through the SIE element as mediated by Ets member Elk-1 (180). In addition to transcriptional control mediated by signal transduction pathways, Mcl-1 is also induced in hypoxic conditions through during binding of HIF-1 to the promoter (181). As previously eluded to, many of the previously described mechanisms regulating Mcl-1 transcription have been identified in hematopoietic tissue development, and it is not clear what role these ligands and signal transduction pathways play in regulating Mcl-1 transciption in other tissues.

While mechanisms positively regulating Mcl-1 transcription in the context of development and maturation of hematopoietic tissues are well described, less is known about how Mcl-1 is transcriptionally regulated in other tissues and other contexts—specifically how it is repressed during the induction of apoptosis. Given the lability of the Mcl-1 protein, it reasonable to hypothesize that transcriptional downregulation of Mcl-1 is not due to direct repression, but rather from a lack of positive signaling. Previous studies from our lab, however, have identified the E2F1 transcription factor as directly binding to and repressing the MCL-1 promoter (122). Interestingly, this is independent of pRb family member binding, although the exact mechanism has not yet been determined. As described in the following section, Mcl-1 is thought to play a critical role in promoting the survival of malignant cells, and as such elucidating mechanisms regulating Mcl-1 transcription in this context is of great importance.

In addition to transcriptional regulation, Mcl-1 is also subject to post-transcriptional and post-translational modification. In some contexts, Mcl-1 undergoes alternative splicing to produce a shorter from of Mcl-1 (termed Mcl-1S) (182,183). This modification results in the loss of the TM domain as well as BH1-2 domains, giving way to an alternate Mcl-1 protein with a structure similar to that of the BH3-only subfamily members (182,183). Indeed, Mcl-1S does not bind to proapoptotic Bcl-2 family members but instead binds to antiapoptotic members, and its overexpression is sufficient to induce apoptosis (182,183). Mechanisms of post-translational modification include cleavage, phosphorylation, and ubquitination. During apoptosis, Mcl-1 is subject to caspase-mediated cleavage at conserved aspartic acid residues (184,185), with one the resultant cleavage products being proapoptotic in nature in overexpression assays (184). In the

case of phosphorylation, Mcl-1 is phosphorylated through multiple mechanisms at two specific serine residues that can either positively or negatively influence Mcl-1 protein levels. Oxidative stress inactivates Mcl-1 via JNK-mediated phosphoryalation at serine-121 and threonine Thr-163 (186), and Mcl-1 is also phosphorylated at Thr-163 via TPA by Erk-dependent mechanisms—leading to increased protein stability (187,188). There are purported to be other phosphorylation sites in Mcl-1, but they have yet to be characterized (188). Finally, Mcl-1 is subject to ubiquitination by Mule/ARF-BP1, which negatively regulates Mcl-1 protein by targeting it for proteasomal degradation (189). Taken together, Mcl-1 is subject to both positive and negative regulation at multiple levels through multiple mechanisms.

Mcl-1 and human malignancy

While in itself not a direct oncogene, as described in a previous section Mcl-1 may behave as an oncogene when present in combination with other oncogenic mutations due to its potent ability to promote survival. This is partially exemplified by studies demonstrating correlations between Mcl-1 expression and disease outcomes. In chronic lymphocytic leukemia, a higher level of Mcl-1 expression correlates with failure to achieve complete remission, and in breast cancer Mcl-1 expression associates with poor prognosis (190,191). Additionally, Mcl-1 expression is associated with disease progression in melanoma, and is also a predictor of survival in gastric carcinoma (192,193). Experiments utilizing siRNA-mediated knockdown of Mcl-1 in cancerous cells has also pointed at the integral role Mcl-1 may play promoting malignant cell

viability. Indeed, antisense depletion of Mcl-1 in malignant cell can lead to decreased viability and the induction apoptosis—suggesting that interfering with Mcl-1 expression may prove to be a rational therapy for human malignancies (163-166).

Summary and rationale

Mcl-1 is an antiapoptotic Bcl-2 family member, and as such may act as a potent oncogene due to its ability to promote cell survival. Indeed, antisense depletion of Mcl-1 can induce loss of viability and apoptosis in malignant cells—exemplifying the necessity of Mcl-1 expression to their survival (163-166). Since Mcl-1 is a labile protein, much of its regulation is thought to be dependent on transcriptional mechanisms. A publication from Moshynska et al. identified two novel sequence variants of the MCL-1 promoter within lymphocytes from chronic lymphocytic leukemia patients, but not within noncancerous tissue from the same individuals or in lymphocytes from 18 healthy control subjects (1). This result suggested that the variants—insertions of 6 or 18 nucleotides at position –188 relative to the transcription start site (194)—were CLL-related somatic oncogenic mutations. Moshynska et al. also determined that the 6- and 18-nucleotide insertions were associated with elevated Mcl-1 expression, and proposed that the variant promoters could be used as a prognostic marker. We independently identified and cloned the three observed sequence variants from cancer cell lines hereby referred to as the MCL-1 +0, +6 or +18 promoters. In contrast to Moshynska et al., we find the variant promoters to be identically present in both cancerous and adjacent noncancerous clinical lung samples, suggesting that the variants are germ-line encoded. We also find the three

variant promoters prevalent in genomic DNA derived from healthy control samples and present at frequencies similar to that observed in cancerous cell lines. Furthermore, activity analysis of the three variant promoters reveals the MCL-1 +6 and +18 promoters to be less active than the MCL-1 +0 promoter, both during normal cellular homeostasis and under conditions that actively induce MCL-1 transcription. Given our results, we conclude that the MCL-1 +6 and +18 promoters are likely benign polymorphisms and do no represent a reliable prognostic marker for CLL.

Experimental Procedures

Promoter identification and screening

Genomic DNA was extracted from cell lines as described (Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001, p. 8.46-8.53). Mcl-1 promoter sequence from cell lines representative of the three aberrances, namely H1299, K562, and T98G, was amplified from genomic DNA using a primer pair that spanned bases –223 to –246 (5'-AGG CCC GAG GTG CTC ATG GAA AGA-3') and +72 to +93 (5'-TTG AGG CCA AAC ATT GCC AGT CA-3') of what is referred to as the Mcl-1 +0 promoter. The resulting products were cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen) and the products sequenced by the Moffitt Molecular Biology Core. For larger scale screening purposes, a primer pair that spanned bases –110 to –129 (5'-AGC TTC CGG AGG GTT GCG CA-3') and –162 to –182 (5'-GGC ACT CAG AGC CTC CGA AGA-3') were used to amplify the Mcl-1 promoter with the resulting products resolved on a 6% polyacrylamide gel and visualized after exposure to ethidium bromide.

Cell lines

Breast cancer cell lines screened consists of: MDA-MB-231, MDA-MB-361, MDA-MB-361, MDA-MB-435s, MDA-MB-453, MDA-MB-468, MCF7, SK-BR-3 and T47D. Lung cancer lines screened consist of: H322, H358, H324, H661, H522, H146, H209, H417, H82 and H211.

Paired clinical lung samples

All patient and control donors provided informed consent as approved by the Institutional Review Board. Lung tumor and corresponding normal lung tissue specimens were collected from patients undergoing routine thoracotomy for surgical resection of their malignancy. Resected specimens were briefly inspected by a surgical pathologist and then snap-frozen in liquid nitrogen. Frozen sections were microscopically viewed to assess the proportion of tumor cells, normal cells, and necrotic cells in tumor specimens to ensure absence of malignant cells in normal specimens. None of the patients had received radiation or chemotherapy prior to sample collection.

Healthy control samples

Peripheral blood mononuclear cells were collected from healthy normal volunteers. None of the volunteers had a known malignancy or illness. All were

Caucasian except for one Hispanic and one Asian volunteer. The ages ranged from 19 to 62, and 34 of the volunteers were female and 25 were male.

Luciferase assays

The above described pCR2.1-TOPO Mcl-1 +0, +6, and +18 constructs employed for the initial screening were shuttled into the pGL3 (Invitrogen) luciferase vectors, and the sequences were verified via sequencing by the Moffitt Molecular Biology Core. Luciferase assays were conducted using the Dual-Luciferase Reporter Assay System (Promega) following the published protocol. NIH/3T3 and K562 cells were grown to ~70% confluency in 60 mm2 plates, and K562s were transfected at a density of 1.5 × 106 cells per 60 mm2 plate. Lipofectamine and PLUS reagent (Invitrogen) were used for transfections following the published protocol. All transfections were conducted in triplicate. DNA concentrations per transfection were as follows: 1 μg pGL3/derivative, 0.1 μg pRLTK internal control plasmid, and 0.9 μg carrier DNA. Cells were washed once with phosphate-buffered saline (PBS) and given new media at 4 hours after transfection. At 24 hours, cells were either harvested for analysis or induced to differentiate with 100 n*M* phorbol 12-myristae 13-acetate (PMA). PMA treated cells were collected for analysis at 12 hours post treatment. For analysis of activity, luciferase activity was normalized to the internal renilla activity.

Results

Identification of two novel MCL-1 promoter variants

An article entitled "Prognostic Significance of a Short Sequence Insertion in the MCL-1 Promoter in Chronic Lymphocytic Leukemia" identified two novel sequence variants of the Mcl-1 promoter within lymphocytes from chronic lymphocytic leukemia patients, but not within noncancerous tissue from the same individual or in lymphocytes from 18 healthy control subjects (1). This result suggested that the variants—insertions of 6 and 18 nucleotides at position –188 relative to the transcription start site as mapped by akgul et al. (194)—were CLL-related somatic oncogenic mutations. Moshynska et al. also determined that the 6- and 18-nucleotide insertions were associated with higher Mcl-1 mRNA and protein, and may therefore hold prognostic significance.

In the course of analyzing the E2F1-mediated transcriptional repression of Mcl-1, we independently identified and cloned the three observed sequence variants from three human cancer cell lines, H1299$^{(MCL\text{-}1\ +0/+0)}$ lung cancer cells, K562$^{(MCL\text{-}1\ +6/+6)}$ erythroleukemia cells, and T98G$^{(MCL\text{-}1\ +0/+18)}$ glioblastoma cells, representing the MCL-1 +0, +6, and +18 alleles, respectively. We next used polymerase chain reaction, followed by resolution of the PCR products on acrylamide gels, to determine MCL-1 promoter status in a large number of cell lines and solid tumors. The MCL-1 +6 and MCL-1 +18

promoters occurred with a relatively high frequency in genomic DNA derived from both breast and lung cancer cell lines, although the common MCL-1 +0 allele was the most prevalent (Table 1).

The MCL-1 +6 and MCL-1 +18 promoter variants are not the result of somatic mutation

We next wanted to determine if the variant promoters were somatic in origin. To address this issue, we analyzed the MCL-1 promoter status of genomic DNA derived from 15 sets of paired lung cancer and adjacent normal lung tissue from patients undergoing routine thoracotomy for surgical resection of their malignancy. All samples were provided in deidentified fashion, and all patients provided informed consent as approved by the Institutional Review Board. In all 15 samples, the MCL-1 promoter profile was identical in cancerous and normal tissue (Fig. 18). Given this observation, we conclude that the variant promoters are not somatic in origin and are germ-line encoded.

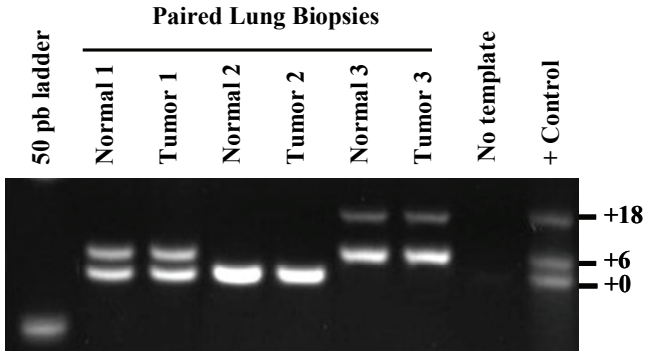
The MCLl-1 +6 and MCL-1 +18 promoters are common polymorphisms

While we established that the variant promoters were not the result of somatic mutation, we considered the possibility that the MCL-1 promoter variants may predispose to malignancy. To this end, we screened genomic DNA derived from 59 healthy individuals, all of whom provided informed consent, for the presence of the variant Mcl-1 promoters. Nearly half of the total alleles had one or both insertions, and the insertions occurred at frequencies similar to that observed in cancer cell lines (Fig. 19). Thus, it

Table 1. The allelic frequencies of the MCL-1 +0, MCL-1 +6, and MCL-1 +18 promoters in breast and lung cancer cell lines.

Allele	Breast Lines (N=16)	Lung Lines (N=20)
MCL-1 +0	9 (56%)	10 (50%)
MCL-1 +6	3 (19%)	3 (15%)
MCL-1 +18	4 (25%)	7 (35%)

A

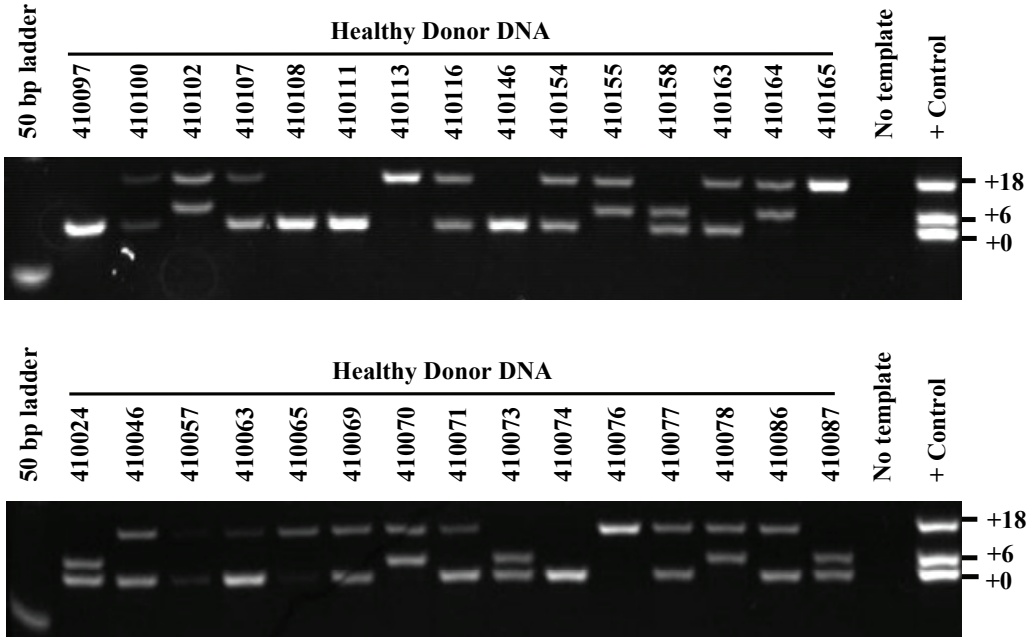


B

Allele	Normal Lung (N=36)	Lung Tumor (N=36)
MCL-1 +0	25 (70%)	25 (70%)
MCL-1 +6	5 (14%)	5 (14%)
MCL-1 +18	6 (16%)	6 (16%)

Figure 19. The variant MCL-1 promoters are not the result of somatic mutation. (A) Representative samples of the MCL-1 promoter status in genomic DNA from paired lung tumor biopsy and adjacent normal tissue derived from patients undergoing routine thoracotomy as determined by resolving PCR products from the MCL-1 promoter on a polyacrylamide gel with subsequent visualization. (B) The resultant allelic frequencies of all paired samples examined.

A



B

Allele	Normal (N=118)
MCL-1 +0	55 (47%)
MCL-1 +6	24 (20%)
MCL-1 +18	14 (33%)

Figure 20. The variant MCL-1 promoters are prevalent in genomic DNA derived from healthy controls. (A) Representative samples of the MCL-1 promoter status in genomic DNA from healthy donor peripheral blood mononuclear cells as determined by resolving PCR products from the MCL-1 promoter on a polyacrylamide gel with subsequent visualization. (B) The resultant allelic frequencies of all healthy control samples examined.

appears likely that the MCL-1 +6 and +18 promoter variants are likely common benign polymorphisms.

The MCL-1 +6 and MCL-1 +18 promoters are less active than the common MCL-1 +0 promoter

Mcl-1 belongs to the Bcl-1 family of proteins and may be a potent oncogene due to its ability to block apoptosis. Although we found the MCL-1 +6 and +18 polymorphisms to be quite common, we considered it possible that they could contribute to oncogenesis by rendering the promoter more active, thereby increasing the expression of MCL-1. To explore this possibility, we cloned the MCL-1 +0, +6 and +18 promoters into a pGL3 luciferase vector, transfected the constructs into multiple cell lines, and determined promoter activity. Surprisingly the variant promoters displayed decreased activity—both during normal cellular homeostasis and under conditions that actively induce Mcl-1 transcription (i.e., treatment with phorbol 12-myristate 13-acetate (PMA)), with the +18 promoter displaying approximately half the activity of the MCL-1 +0 promoter (Fig. 20A, B and C). Taken together, we conclude that the MCL-1 +6 and +18 variant promoters likely represent benign polymorphisms that probably do not represent a reliable prognostic marker.

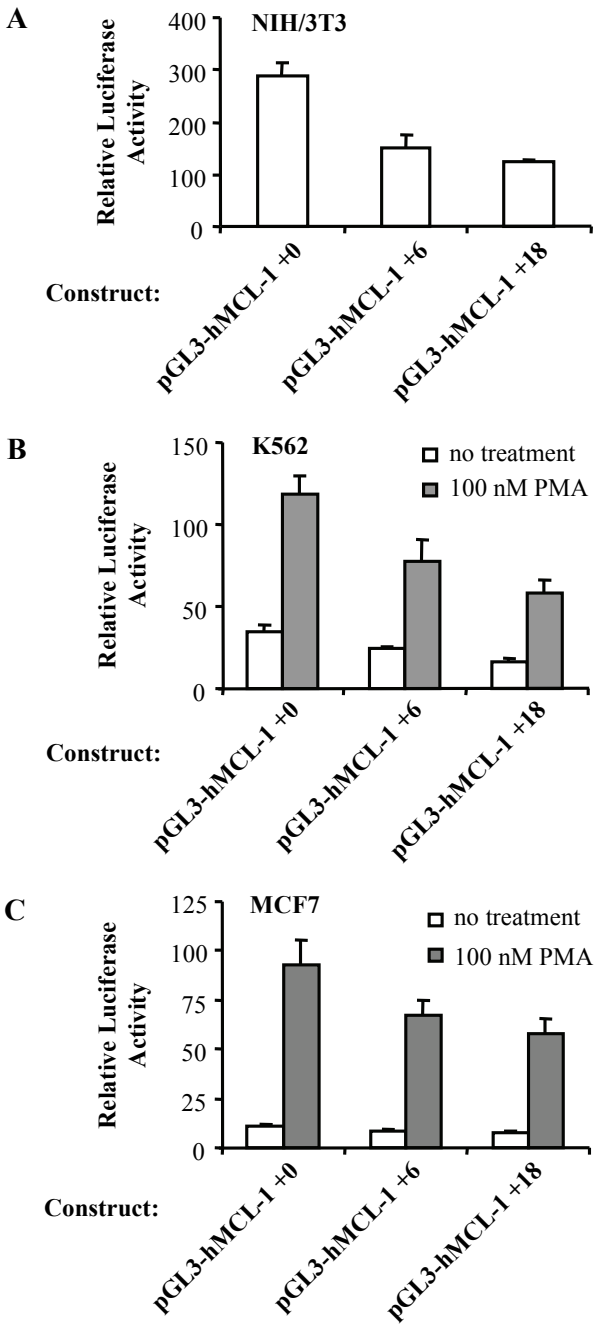


Figure 21. The MCL-1 +6 and MCL-1 +18 promoters are less active than the MCL-1 +0 promoter. (A) Luciferase constructs representing the three variant MCL-1 promoters and a renilla control construct were cotransfected into NIH/3T3s with the MCL-1 promoter constructs assayed for activity relative to the renilla internal control 24 hours post transfection. (B and C) Transfections conducted as in figure 20A, except MCF7 and K562 cells were treated in parallel plus or minus PMA to induce Mcl-1 transcription. Cells were harvested after 12 hours of PMA treatment.

Discussion

While there are significant disagreements between our data and that published by Moshynska et al. (1), there is agreement as to the existence, location, and composition of the MCL-1 +6 and +18 variant promoters. Beyond that however, there is little common ground. Moshynska et al. claim that to have specifically found the MCL-1 +6 and +18 promoters only in genomic DNA derived from lymphocytes from CLL patients and not within cancerous tissue derived from the same individuals or in lymphocytes from healthy control subjects, suggesting that the variants are CLL-related oncogenic mutations. In contrast, we find the variant promoters identically present in paired samples of cancerous and adjacent noncancerous lung, and also find the promoters prevalent in genomic DNA derived from healthy volunteers (195). Similar studies investigating the variant MCL-1 promoters are in agreement with our results. Vargas et al. report the variant promoters present in 24 healthy control samples, and Iglesias-Serret et al. find the variant promoters present in lymphocytes from CLL patients as well as lymphocytes from 10 control subjects and mouth epithelial cells from 10 additional healthy control subjects (196,197). Experiments by Dicker et al. find MCL-1 promoter status identical between that observed in lymphocytes from CLL patients and in genomic DNA derived from buccal swabs, and further find the MCL-1 +6 and +18 promoters common DNA samples from health control individuals (198). Coenen et al. have also reported similar results

(199). Given the bulk of evidence in support of our data, we confidently conclude that the variant MCL-1 promoters are not the result of a CLL-related oncogenic mutation and instead represent common benign polymorphisms.

A second assertion from the Moshynska publication was that the presence of the MCL-1 +6 and +18 promoters correlated with increased expression of MCL-1 mRNA and protein (1). To investigate the effect of the MCL-1 +6 and +18 variants on promoter activity, we cloned the MCL-1 +0, +6, and +18 promoters into luciferase vectors and assayed them for activity in multiple cell lines and found the MCL-1 +6 and +18 promoters to be less active than the common MCL-1 +0 promoter, both during normal cellular homeostasis and under conditions that actively induce Mcl-1 transcription (195). Unfortunately, most studies investigating the variant MCL-1 promoters did not conduct expression assays, however one study did compare MCL-1 promoter status to Mcl-1 expression in CLL patients via microarray, but observed no correlation (198). It is possible that the reported positive correlation by Moshynska et al. is real, however our promoter activity assay would argue otherwise. Therefore, we assert that under our experimental conditions, the variant MCL-1 +6 and +18 promoters are less active than the common MCL-1 +0 promoter.

Another major finding from the Moshynska et al. publication is that the MCL-1 +6 and +18 variant promoters positively correlate with risk of dying and decreased disease-free survival in CLL patients (1). Since our studies did not examine an association with CLL, we do not definitively disagree with this statement. However, the finding by ourselves and others that the MCL-1 +6 and +8 promoters are actually common polymorphisms with no discernable correlation to malignancy highlights a

fundamental flaw in either the screening technique, data reporting, or both employed by Moshynska et al. This in itself is sufficient to be skeptical of any analyses and conclusions inferred from their data, yet other studies conducting similar sets of experiments have thoroughly demonstrated that the MCL-1 +6 or +18 promoters do not correlate with disease outcomes in either CLL or acute lymphoblastic leukemia (ALL) (198-201). Thus, whether an error in the screening technique or an error in reporting, it is clear that the MCL-1 +6 and +18 promoters hold no prognostic significance to CLL.

It is unclear why there are so many discrepancies between the work of ourselves and others and that presented by Moshynska et al. As previously mentioned, the gross amounts of errors are likely the result of a flawed screening technique, flawed data reporting, or both. In our experiments, we utilized a PCR-based screening technique that allowed for a clear distinction of both MCL-1 promoter alleles of a sample by virtue of differences in migration within the different sized PCR products. However, the technique employed by Moshynska et al. consisted of direct sequencing of PCR products, which in theory should be a reliable technique. And, given the complete absence of the MCL-1 +6 and MCL-1 +18 promoters in every sample they examined except CLL cells, it is unlikely that this scenario could occur completely by chance. Taken together, we conclude that the MCL-1 +6 and +18 promoters are common benign polymorphisms that likely hold no prognostic value for CLL, that the MCL-1 +6 and +18 promoters are less active than the MCL-1 +0 promoter, and that the discrepancies found within the Moshynska publication are likely the result of an error in data reporting.

LIST OF REFERENCES

1. Moshynska, O., Sankaran, K., Pahwa, P., and Saxena, A. (2004) *J Natl Cancer Inst* **96**(9), 673-682
2. Bracken, A. P., Ciro, M., Cocito, A., and Helin, K. (2004) *Trends Biochem Sci* **29**(8), 409-417
3. DeGregori, J., and Johnson, D. G. (2006) *Curr Mol Med* **6**(7), 739-748
4. Johnson, D. G., and Degregori, J. (2006) *Curr Mol Med* **6**(7), 731-738
5. Cam, H., and Dynlacht, B. D. (2003) *Cancer Cell* **3**(4), 311-316
6. Harbour, J. W., and Dean, D. C. (2000) *Genes Dev* **14**(19), 2393-2409
7. Nevins, J. R. (2001) *Hum Mol Genet* **10**(7), 699-703
8. DeGregori, J. (2002) *Biochim Biophys Acta* **1602**(2), 131-150
9. Dimova, D. K., and Dyson, N. J. (2005) *Oncogene* **24**(17), 2810-2826
10. Dyson, N. (1998) *Genes Dev* **12**(15), 2245-2262
11. Trimarchi, J. M., and Lees, J. A. (2002) *Nat Rev Mol Cell Biol* **3**(1), 11-20
12. Wang, S., Ghosh, R. N., and Chellappan, S. P. (1998) *Mol Cell Biol* **18**(12), 7487-7498
13. Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993) *Genes Dev* **7**(3), 331-342
14. Meyerson, M., and Harlow, E. (1994) *Mol Cell Biol* **14**(3), 2077-2086
15. Johnson, D. G., Ohtani, K., and Nevins, J. R. (1994) *Genes Dev* **8**(13), 1514-1525

16. Hsiao, K. M., McMahon, S. L., and Farnham, P. J. (1994) *Genes Dev* **8**(13), 1526-1537

17. Neuman, E., Flemington, E. K., Sellers, W. R., and Kaelin, W. G., Jr. (1994) *Mol Cell Biol* **14**(10), 6607-6615

18. Ohtani, K., DeGregori, J., and Nevins, J. R. (1995) *Proc Natl Acad Sci U S A* **92**(26), 12146-12150

19. Dynlacht, B. D., Flores, O., Lees, J. A., and Harlow, E. (1994) *Genes Dev* **8**(15), 1772-1786

20. DeGregori, J., Kowalik, T., and Nevins, J. R. (1995) *Molecular and cellular biology* **15**(8), 4215-4224

21. Zhang, L., and Wang, C. (2006) *Oncogene* **25**(18), 2615-2627

22. Xu, M., Sheppard, K. A., Peng, C. Y., Yee, A. S., and Piwnica-Worms, H. (1994) *Mol Cell Biol* **14**(12), 8420-8431

23. Marti, A., Wirbelauer, C., Scheffner, M., and Krek, W. (1999) *Nat Cell Biol* **1**(1), 14-19

24. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. (1986) *Nature* **323**(6089), 643-646

25. Knudson, A. G., Jr. (1971) *Proc Natl Acad Sci U S A* **68**(4), 820-823

26. Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature* **366**(6456), 704-707

27. Veltman, J. A., Fridlyand, J., Pejavar, S., Olshen, A. B., Korkola, J. E., DeVries, S., Carroll, P., Kuo, W. L., Pinkel, D., Albertson, D., Cordon-Cardo, C., Jain, A. N., and Waldman, F. M. (2003) *Cancer Res* **63**(11), 2872-2880

28. Orlic, M., Spencer, C. E., Wang, L., and Gallie, B. L. (2006) *Genes Chromosomes Cancer* **45**(1), 72-82

29. Oeggerli, M., Tomovska, S., Schraml, P., Calvano-Forte, D., Schafroth, S., Simon, R., Gasser, T., Mihatsch, M. J., and Sauter, G. (2004) *Oncogene* **23**(33), 5616-5623

30. Foster, C. S., Falconer, A., Dodson, A. R., Norman, A. R., Dennis, N., Fletcher, A., Southgate, C., Dowe, A., Dearnaley, D., Jhavar, S., Eeles, R., Feber, A., and Cooper, C. S. (2004) *Oncogene* **23**(35), 5871-5879

31. Grasemann, C., Gratias, S., Stephan, H., Schuler, A., Schramm, A., Klein-Hitpass, L., Rieder, H., Schneider, S., Kappes, F., Eggert, A., and Lohmann, D. R. (2005) *Oncogene* **24**(42), 6441-6449

32. Saito, M., Helin, K., Valentine, M. B., Griffith, B. B., Willman, C. L., Harlow, E., and Look, A. T. (1995) *Genomics* **25**(1), 130-138

33. Fujita, Y., Sakakura, C., Shimomura, K., Nakanishi, M., Yasuoka, R., Aragane, H., Hagiwara, A., Abe, T., Inazawa, J., and Yamagishi, H. (2003) *Hepatogastroenterology* **50**(54), 1857-1863

34. Brookman-Amissah, N., Duchesnes, C., Williamson, M. P., Wang, Q., Ahmed, A., Feneley, M. R., Mackay, A., Freeman, A., Fenwick, K., Iravani, M., Weber, B., Ashworth, A., and Masters, J. R. (2005) *Prostate cancer and prostatic diseases* **8**(4), 335-343

35. Watanabe, T., Imoto, I., Katahira, T., Hirasawa, A., Ishiwata, I., Emi, M., Takayama, M., Sato, A., and Inazawa, J. (2002) *Jpn J Cancer Res* **93**(10), 1114-1122

36. Postma, C., Hermsen, M. A., Coffa, J., Baak, J. P., Mueller, J. D., Mueller, E., Bethke, B., Schouten, J. P., Stolte, M., and Meijer, G. A. (2005) *J Pathol* **205**(4), 514-521

37. Suzuki, T., Yasui, W., Yokozaki, H., Naka, K., Ishikawa, T., and Tahara, E. (1999) *Int J Cancer* **81**(4), 535-538

38. Lee, T. A., and Farnham, P. J. (2000) *Oncogene* **19**(18), 2257-2268

39. Melillo, R. M., Helin, K., Lowy, D. R., and Schiller, J. T. (1994) *Mol Cell Biol* **14**(12), 8241-8249

40. Pierce, A. M., Schneider-Broussard, R., Gimenez-Conti, I. B., Russell, J. L., Conti, C. J., and Johnson, D. G. (1999) *Mol Cell Biol* **19**(9), 6408-6414

41. Russell, J. L., Weaks, R. L., Berton, T. R., and Johnson, D. G. (2006) *Oncogene* **25**(6), 867-876

42. Frame, F. M., Rogoff, H. A., Pickering, M. T., Cress, W. D., and Kowalik, T. F. (2006) *Oncogene* **25**(23), 3258-3266

43. Dimri, G. P., Itahana, K., Acosta, M., and Campisi, J. (2000) *Mol Cell Biol* **20**(1), 273-285

44. Lomazzi, M., Moroni, M. C., Jensen, M. R., Frittoli, E., and Helin, K. (2002) *Nat Genet* **31**(2), 190-194

45. Lazzerini Denchi, E., Attwooll, C., Pasini, D., and Helin, K. (2005) *Mol Cell Biol* **25**(7), 2660-2672

46. Yee, A. S., Reichel, R., Kovesdi, I., and Nevins, J. R. (1987) *Embo J* **6**(7), 2061-2068

47. Ivey-Hoyle, M., Conroy, R., Huber, H. E., Goodhart, P. J., Oliff, A., and Heimbrook, D. C. (1993) *Mol Cell Biol* **13**(12), 7802-7812

48. Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993) *Mol Cell Biol* **13**(12), 7813-7825

49. Leone, G., Nuckolls, F., Ishida, S., Adams, M., Sears, R., Jakoi, L., Miron, A., and Nevins, J. R. (2000) *Mol Cell Biol* **20**(10), 3626-3632

50. He, Y., Armanious, M. K., Thomas, M. J., and Cress, W. D. (2000) *Oncogene* **19**(30), 3422-3433

51. Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z. X., Xu, G., Wydner, K. L., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994) *Genes Dev* **8**(22), 2665-2679

52. Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M., and Bernards, R. (1994) *Genes Dev* **8**(22), 2680-2690

53. Buck, V., Allen, K. E., Sorensen, T., Bybee, A., Hijmans, E. M., Voorhoeve, P. M., Bernards, R., and La Thangue, N. B. (1995) *Oncogene* **11**(1), 31-38

54. Itoh, A., Levinson, S. F., Morita, T., Kourembanas, S., Brody, J. S., and Mitsialis, S. A. (1995) *Cell Mol Biol Res* **41**(3), 147-154

55. Trimarchi, J. M., Fairchild, B., Verona, R., Moberg, K., Andon, N., and Lees, J. A. (1998) *Proc Natl Acad Sci U S A* **95**(6), 2850-2855

56. Cartwright, P., Muller, H., Wagener, C., Holm, K., and Helin, K. (1998) *Oncogene* **17**(5), 611-623

57. Gaubatz, S., Wood, J. G., and Livingston, D. M. (1998) *Proc Natl Acad Sci U S A* **95**(16), 9190-9195

58. de Bruin, A., Maiti, B., Jakoi, L., Timmers, C., Buerki, R., and Leone, G. (2003) *J Biol Chem* **278**(43), 42041-42049

59. Di Stefano, L., Jensen, M. R., and Helin, K. (2003) *Embo J* **22**(23), 6289-6298

60. Logan, N., Delavaine, L., Graham, A., Reilly, C., Wilson, J., Brummelkamp, T. R., Hijmans, E. M., Bernards, R., and La Thangue, N. B. (2004) *Oncogene* **23**(30), 5138-5150

61. Maiti, B., Li, J., de Bruin, A., Gordon, F., Timmers, C., Opavsky, R., Patil, K., Tuttle, J., Cleghorn, W., and Leone, G. (2005) *J Biol Chem* **280**(18), 18211-18220

62. Christensen, J., Cloos, P., Toftegaard, U., Klinkenberg, D., Bracken, A. P., Trinh, E., Heeran, M., Di Stefano, L., and Helin, K. (2005) *Nucleic Acids Res* **33**(17), 5458-5470

63. Sears, R., Ohtani, K., and Nevins, J. R. (1997) *Mol Cell Biol* **17**(9), 5227-5235

64. Trimarchi, J. M., Fairchild, B., Wen, J., and Lees, J. A. (2001) *Proc Natl Acad Sci U S A* **98**(4), 1519-1524

65. Logan, N., Graham, A., Zhao, X., Fisher, R., Maiti, B., Leone, G., and La Thangue, N. B. (2005) *Oncogene* **24**(31), 5000-5004

66. Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993) *Nature* **365**(6444), 349-352

67. Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994) *Proc Natl Acad Sci U S A* **91**(23), 10918-10922

68. DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997) *Proc Natl Acad Sci U S A* **94**(14), 7245-7250

69. Lukas, J., Petersen, B. O., Holm, K., Bartek, J., and Helin, K. (1996) *Mol Cell Biol* **16**(3), 1047-1057

70. DeGregori, J., Leone, G., Ohtani, K., Miron, A., and Nevins, J. R. (1995) *Genes Dev* **9**(23), 2873-2887

71. Schwarz, J. K., Bassing, C. H., Kovesdi, I., Datto, M. B., Blazing, M., George, S., Wang, X. F., and Nevins, J. R. (1995) *Proc Natl Acad Sci U S A* **92**(2), 483-487

72. Mann, D. J., and Jones, N. C. (1996) *Curr Biol* **6**(4), 474-483

73. Johnson, D. G., Cress, W. D., Jakoi, L., and Nevins, J. R. (1994) *Proc Natl Acad Sci U S A* **91**(26), 12823-12827

74. Shan, B., and Lee, W. H. (1994) *Mol Cell Biol* **14**(12), 8166-8173

75. Singh, P., Wong, S. H., and Hong, W. (1994) *Embo J* **13**(14), 3329-3338

76. Xu, G., Livingston, D. M., and Krek, W. (1995) *Proc Natl Acad Sci U S A* **92**(5), 1357-1361

77. Chen, Q., Liang, D., Yang, T., Leone, G., and Overbeek, P. A. (2004) *Dev Neurosci* **26**(5-6), 435-445

78. Chen, Q., Hung, F. C., Fromm, L., and Overbeek, P. A. (2000) *Invest Ophthalmol Vis Sci* **41**(13), 4223-4231

79. Guy, C. T., Zhou, W., Kaufman, S., and Robinson, M. O. (1996) *Mol Cell Biol* **16**(2), 685-693

80. Pierce, A. M., Fisher, S. M., Conti, C. J., and Johnson, D. G. (1998) *Oncogene* **16**(10), 1267-1276

81. Paulson, Q. X., McArthur, M. J., and Johnson, D. G. (2006) *Cell Cycle* **5**(2), 184-190

82. Scheijen, B., Bronk, M., van der Meer, T., De Jong, D., and Bernards, R. (2004) *J Biol Chem* **279**(11), 10476-10483

83. Conner, E. A., Lemmer, E. R., Omori, M., Wirth, P. J., Factor, V. M., and Thorgeirsson, S. S. (2000) *Oncogene* **19**(44), 5054-5062

84. Agger, K., Santoni-Rugiu, E., Holmberg, C., Karlstrom, O., and Helin, K. (2005) *Oncogene* **24**(5), 780-789

85. Pierce, A. M., Gimenez-Conti, I. B., Schneider-Broussard, R., Martinez, L. A., Conti, C. J., and Johnson, D. G. (1998) *Proc Natl Acad Sci U S A* **95**(15), 8858-8863

86. Vigo, E., Muller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M. C., and Helin, K. (1999) *Mol Cell Biol* **19**(9), 6379-6395

87. Kowalik, T. F., DeGregori, J., Leone, G., Jakoi, L., and Nevins, J. R. (1998) *Cell Growth Differ* **9**(2), 113-118

88. Wu, X., and Levine, A. J. (1994) *Proc Natl Acad Sci U S A* **91**(9), 3602-3606

89. Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995) *Journal of virology* **69**(4), 2491-2500

90. Holmberg, C., Helin, K., Sehested, M., and Karlstrom, O. (1998) *Oncogene* **17**(2), 143-155

91. Lazzerini Denchi, E., and Helin, K. (2005) *EMBO Rep* **6**(7), 661-668

92. Berton, T. R., Mitchell, D. L., Guo, R., and Johnson, D. G. (2005) *Oncogene* **24**(15), 2449-2460

93. Wikonkal, N. M., Remenyik, E., Knezevic, D., Zhang, W., Liu, M., Zhao, H., Berton, T. R., Johnson, D. G., and Brash, D. E. (2003) *Nat Cell Biol* **5**(7), 655-660

94. Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. (1996) *Cell* **85**(4), 537-548

95. Cloud, J. E., Rogers, C., Reza, T. L., Ziebold, U., Stone, J. R., Picard, M. H., Caron, A. M., Bronson, R. T., and Lees, J. A. (2002) *Mol Cell Biol* **22**(8), 2663-2672

96. Zhu, J. W., Field, S. J., Gore, L., Thompson, M., Yang, H., Fujiwara, Y., Cardiff, R. D., Greenberg, M., Orkin, S. H., and DeGregori, J. (2001) *Mol Cell Biol* **21**(24), 8547-8564

97. Murga, M., Fernandez-Capetillo, O., Field, S. J., Moreno, B., Borlado, L. R., Fujiwara, Y., Balomenos, D., Vicario, A., Carrera, A. C., Orkin, S. H., Greenberg, M. E., and Zubiaga, A. M. (2001) *Immunity* **15**(6), 959-970

98. DeRyckere, D., and DeGregori, J. (2005) *J Immunol* **175**(2), 647-655

99. Li, F. X., Zhu, J. W., Hogan, C. J., and DeGregori, J. (2003) *Mol Cell Biol* **23**(10), 3607-3622

100. Stevaux, O., and Dyson, N. J. (2002) *Curr Opin Cell Biol* **14**(6), 684-691

101. Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M., and Nevins, J. R. (2001) *Mol Cell Biol* **21**(14), 4684-4699

102. Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A., and Dynlacht, B. D. (2002) *Genes Dev* **16**(2), 245-256

103. Weinmann, A. S., Yan, P. S., Oberley, M. J., Huang, T. H., and Farnham, P. J. (2002) *Genes Dev* **16**(2), 235-244

104. Zhu, W., Giangrande, P. H., and Nevins, J. R. (2004) *Embo J* **23**(23), 4615-4626

105. Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., Lowe, S. W., and Cordon-Cardo, C. (2004) *Nature* **430**(7001), 797-802

106. Hiebert, S. W., Packham, G., Strom, D. K., Haffner, R., Oren, M., Zambetti, G., and Cleveland, J. L. (1995) *Mol Cell Biol* **15**(12), 6864-6874

107. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998) *Nature* **395**(6698), 124-125

108. Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F., and Sherr, C. J. (1998) *Proc Natl Acad Sci U S A* **95**(14), 8292-8297

109. Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) *Cell* **92**(6), 725-734

110. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387**(6630), 296-299

111. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387**(6630), 299-303

112. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) *Science* **281**(5383), 1677-1679

113. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) *Science* **281**(5383), 1674-1677

114. Irwin, M., Marin, M. C., Phillips, A. C., Seelan, R. S., Smith, D. I., Liu, W., Flores, E. R., Tsai, K. Y., Jacks, T., Vousden, K. H., and Kaelin, W. G., Jr. (2000) *Nature* **407**(6804), 645-648

115. Stiewe, T., and Putzer, B. M. (2000) *Nat Genet* **26**(4), 464-469

116. Hershko, T., Chaussepied, M., Oren, M., and Ginsberg, D. (2005) *Cell Death Differ* **12**(4), 377-383

117. Real, P. J., Sanz, C., Gutierrez, O., Pipaon, C., Zubiaga, A. M., and Fernandez-Luna, J. L. (2006) *FEBS Lett* **580**(25), 5905-5909

118. Rodriguez, J. M., Glozak, M. A., Ma, Y., and Cress, W. D. (2006) *J Biol Chem* **281**(32), 22729-22735

119. Moroni, M. C., Hickman, E. S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Muller, H., and Helin, K. (2001) *Nat Cell Biol* **3**(6), 552-558

120. Xie, W., Jiang, P., Miao, L., Zhao, Y., Zhimin, Z., Qing, L., Zhu, W. G., and Wu, M. (2006) *Nucleic Acids Res* **34**(7), 2046-2055

121. Nahle, Z., Polakoff, J., Davuluri, R. V., McCurrach, M. E., Jacobson, M. D., Narita, M., Zhang, M. Q., Lazebnik, Y., Bar-Sagi, D., and Lowe, S. W. (2002) *Nat Cell Biol* **4**(11), 859-864

122. Croxton, R., Ma, Y., Song, L., Haura, E. B., and Cress, W. D. (2002) *Oncogene* **21**(9), 1359-1369

123. Crowe, D. L., Nguyen, D. C., Tsang, K. J., and Kyo, S. (2001) *Nucleic Acids Res* **29**(13), 2789-2794

124. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995) *Science* **269**(5229), 1424-1427

125. Ma, Y., and Cress, W. D. (2007) *Oncogene* **26**(24), 3532-3540

126. Wang, C., Hou, X., Mohapatra, S., Ma, Y., Cress, W. D., Pledger, W. J., and Chen, J. (2005) *The Journal of biological chemistry* **280**(13), 12339-12343

127. Radhakrishnan, S. K., Feliciano, C. S., Najmabadi, F., Haegebarth, A., Kandel, E. S., Tyner, A. L., and Gartel, A. L. (2004) *Oncogene* **23**(23), 4173-4176

128. Stevens, C., and La Thangue, N. B. (2004) *DNA Repair (Amst)* **3**(8-9), 1071-1079

129. Ramos, S., Khademi, F., Somesh, B. P., and Rivero, F. (2002) *Gene* **298**(2), 147-157

130. Lundgren, R., Mandahl, N., Heim, S., Limon, J., Henrikson, H., and Mitelman, F. (1992) *Genes Chromosomes Cancer* **4**(1), 16-24

131. Emi, M., Fujiwara, Y., Nakajima, T., Tsuchiya, E., Tsuda, H., Hirohashi, S., Maeda, Y., Tsuruta, K., Miyaki, M., and Nakamura, Y. (1992) *Cancer Res* **52**(19), 5368-5372

132. Bova, G. S., Carter, B. S., Bussemakers, M. J., Emi, M., Fujiwara, Y., Kyprianou, N., Jacobs, S. C., Robinson, J. C., Epstein, J. I., Walsh, P. C., and et al. (1993) *Cancer Res* **53**(17), 3869-3873

133. Fujiwara, Y., Emi, M., Ohata, H., Kato, Y., Nakajima, T., Mori, T., and Nakamura, Y. (1993) *Cancer Res* **53**(5), 1172-1174

134. Sunwoo, J. B., Holt, M. S., Radford, D. M., Deeker, C., and Scholnick, S. B. (1996) *Genes Chromosomes Cancer* **16**(3), 164-169

135. Brown, M. R., Chuaqui, R., Vocke, C. D., Berchuck, A., Middleton, L. P., Emmert-Buck, M. R., and Kohn, E. C. (1999) *Gynecol Oncol* **74**(1), 98-102

136. Wistuba, II, Behrens, C., Virmani, A. K., Milchgrub, S., Syed, S., Lam, S., Mackay, B., Minna, J. D., and Gazdar, A. F. (1999) *Cancer Res* **59**(8), 1973-1979

137. Rivero, F., Dislich, H., Glockner, G., and Noegel, A. A. (2001) *Nucleic Acids Res* **29**(5), 1068-1079

138. Chang, F. K., Sato, N., Kobayashi-Simorowski, N., Yoshihara, T., Meth, J. L., and Hamaguchi, M. (2006) *J Mol Biol* **364**(3), 302-308

139. Collins, T., Stone, J. R., and Williams, A. J. (2001) *Mol Cell Biol* **21**(11), 3609-3615

140. Furukawa, M., He, Y. J., Borchers, C., and Xiong, Y. (2003) *Nat Cell Biol* **5**(11), 1001-1007

141. Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D. A. (2003) *Mol Cell* **12**(3), 783-790

142. Pintard, L., Willis, J. H., Willems, A., Johnson, J. L., Srayko, M., Kurz, T., Glaser, S., Mains, P. E., Tyers, M., Bowerman, B., and Peter, M. (2003) *Nature* **425**(6955), 311-316

143. Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T. H., Vidal, M., Elledge, S. J., and Harper, J. W. (2003) *Nature* **425**(6955), 316-321

144. Wilkins, A., Ping, Q., and Carpenter, C. L. (2004) *Genes Dev* **18**(8), 856-861

145. St-Pierre, B., Jiang, Z., Egan, S. E., and Zacksenhaus, E. (2004) *Gene Expr Patterns* **5**(2), 245-251

146. Hamaguchi, M., Meth, J. L., von Klitzing, C., Wei, W., Esposito, D., Rodgers, L., Walsh, T., Welcsh, P., King, M. C., and Wigler, M. H. (2002) *Proc Natl Acad Sci U S A* **99**(21), 13647-13652

147. Yoshihara, T., Collado, D., and Hamaguchi, M. (2007) *Biochem Biophys Res Commun* **358**(4), 1076-1079

148. Siripurapu, V., Meth, J., Kobayashi, N., and Hamaguchi, M. (2005) *J Mol Biol* **346**(1), 83-89

149. Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress, W. D., and Chen, J. (2006) *Nature cell biology* **8**(9), 1025-1031

150. Ma, Y., Cress, W. D., and Haura, E. B. (2003) *Molecular cancer therapeutics* **2**(1), 73-81

151. Kalejta, R. F., Shenk, T., and Beavis, A. J. (1997) *Cytometry* **29**(4), 286-291

152. Cory, S., Huang, D. C., and Adams, J. M. (2003) *Oncogene* **22**(53), 8590-8607

153. Adams, J. M., and Cory, S. (2007) *Oncogene* **26**(9), 1324-1337

154. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Cell* **90**(3), 405-413

155. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91**(4), 479-489

156. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) *Science* **292**(5517), 727-730

157. Reynolds, J. E., Li, J., Craig, R. W., and Eastman, A. (1996) *Exp Cell Res* **225**(2), 430-436

158. Reynolds, J. E., Yang, T., Qian, L., Jenkinson, J. D., Zhou, P., Eastman, A., and Craig, R. W. (1994) *Cancer Res* **54**(24), 6348-6352

159. Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. (1997) *Blood* **89**(2), 630-643

160. Zhou, P., Levy, N. B., Xie, H., Qian, L., Lee, C. Y., Gascoyne, R. D., and Craig, R. W. (2001) *Blood* **97**(12), 3902-3909

161. Zhou, P., Qian, L., Bieszczad, C. K., Noelle, R., Binder, M., Levy, N. B., and Craig, R. W. (1998) *Blood* **92**(9), 3226-3239

162. Matsushita, K., Okita, H., Suzuki, A., Shimoda, K., Fukuma, M., Yamada, T., Urano, F., Honda, T., Sano, M., Iwanaga, S., Ogawa, S., Hata, J., and Umezawa, A. (2003) *Mol Cell Endocrinol* **203**(1-2), 105-116

163. Moulding, D. A., Giles, R. V., Spiller, D. G., White, M. R., Tidd, D. M., and Edwards, S. W. (2000) *Blood* **96**(5), 1756-1763

164. Zhang, B., Gojo, I., and Fenton, R. G. (2002) *Blood* **99**(6), 1885-1893

165. Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., and Amiot, M. (2002) *Blood* **100**(1), 194-199

166. Leuenroth, S. J., Grutkoski, P. S., Ayala, A., and Simms, H. H. (2000) *J Leukoc Biol* **68**(1), 158-166

167. Rinkenberger, J. L., Horning, S., Klocke, B., Roth, K., and Korsmeyer, S. J. (2000) *Genes Dev* **14**(1), 23-27

168. Opferman, J. T., Letai, A., Beard, C., Sorcinelli, M. D., Ong, C. C., and Korsmeyer, S. J. (2003) *Nature* **426**(6967), 671-676

169. Opferman, J. T., Iwasaki, H., Ong, C. C., Suh, H., Mizuno, S., Akashi, K., and Korsmeyer, S. J. (2005) *Science* **307**(5712), 1101-1104

170. Dzhagalov, I., St John, A., and He, Y. W. (2007) *Blood* **109**(4), 1620-1626

171. Chao, J. R., Wang, J. M., Lee, S. F., Peng, H. W., Lin, Y. H., Chou, C. H., Li, J. C., Huang, H. M., Chou, C. K., Kuo, M. L., Yen, J. J., and Yang-Yen, H. F. (1998) *Mol Cell Biol* **18**(8), 4883-4898

172. Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., and Wang, X. (2003) *Genes Dev* **17**(12), 1475-1486

173. Craig, R. W. (2002) *Leukemia* **16**(4), 444-454

174. Le Gouill, S., Podar, K., Harousseau, J. L., and Anderson, K. C. (2004) *Cell Cycle* **3**(10), 1259-1262

175. Wang, J. M., Chao, J. R., Chen, W., Kuo, M. L., Yen, J. J., and Yang-Yen, H. F. (1999) *Mol Cell Biol* **19**(9), 6195-6206

176. Puthier, D., Bataille, R., and Amiot, M. (1999) *Eur J Immunol* **29**(12), 3945-3950

177. Epling-Burnette, P. K., Liu, J. H., Catlett-Falcone, R., Turkson, J., Oshiro, M., Kothapalli, R., Li, Y., Wang, J. M., Yang-Yen, H. F., Karras, J., Jove, R., and Loughran, T. P., Jr. (2001) *J Clin Invest* **107**(3), 351-362

178. Epling-Burnette, P. K., Zhong, B., Bai, F., Jiang, K., Bailey, R. D., Garcia, R., Jove, R., Djeu, J. Y., Loughran, T. P., Jr., and Wei, S. (2001) *J Immunol* **166**(12), 7486-7495

179. Wang, J. M., Lai, M. Z., and Yang-Yen, H. F. (2003) *Mol Cell Biol* **23**(6), 1896-1909

180. Townsend, K. J., Zhou, P., Qian, L., Bieszczad, C. K., Lowrey, C. H., Yen, A., and Craig, R. W. (1999) *J Biol Chem* **274**(3), 1801-1813

181. Piret, J. P., Minet, E., Cosse, J. P., Ninane, N., Debacq, C., Raes, M., and Michiels, C. (2005) *J Biol Chem* **280**(10), 9336-9344

182. Bae, J., Leo, C. P., Hsu, S. Y., and Hsueh, A. J. (2000) *J Biol Chem* **275**(33), 25255-25261

183. Bingle, C. D., Craig, R. W., Swales, B. M., Singleton, V., Zhou, P., and Whyte, M. K. (2000) *J Biol Chem* **275**(29), 22136-22146

184. Michels, J., O'Neill, J. W., Dallman, C. L., Mouzakiti, A., Habens, F., Brimmell, M., Zhang, K. Y., Craig, R. W., Marcusson, E. G., Johnson, P. W., and Packham, G. (2004) *Oncogene* **23**(28), 4818-4827

185. Clohessy, J. G., Zhuang, J., and Brady, H. J. (2004) *Br J Haematol* **125**(5), 655-665

186. Inoshita, S., Takeda, K., Hatai, T., Terada, Y., Sano, M., Hata, J., Umezawa, A., and Ichijo, H. (2002) *J Biol Chem* **277**(46), 43730-43734

187. Domina, A. M., Smith, J. H., and Craig, R. W. (2000) *J Biol Chem* **275**(28), 21688-21694

188. Domina, A. M., Vrana, J. A., Gregory, M. A., Hann, S. R., and Craig, R. W. (2004) *Oncogene* **23**(31), 5301-5315

189. Zhong, Q., Gao, W., Du, F., and Wang, X. (2005) *Cell* **121**(7), 1085-1095

190. Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M., Rai, K., Hines, J., and Reed, J. C. (1998) *Blood* **91**(9), 3379-3389

191. Ding, Q., He, X., Xia, W., Hsu, J. M., Chen, C. T., Li, L. Y., Lee, D. F., Yang, J. Y., Xie, X., Liu, J. C., and Hung, M. C. (2007) *Cancer Res* **67**(10), 4564-4571

192. Zhuang, L., Lee, C. S., Scolyer, R. A., McCarthy, S. W., Zhang, X. D., Thompson, J. F., and Hersey, P. (2007) *Mod Pathol* **20**(4), 416-426

193. Maeta, Y., Tsujitani, S., Matsumoto, S., Yamaguchi, K., Tatebe, S., Kondo, A., Ikeguchi, M., and Kaibara, N. (2004) *Gastric Cancer* **7**(2), 78-84

194. Akgul, C., Turner, P. C., White, M. R., and Edwards, S. W. (2000) *Cell Mol Life Sci* **57**(4), 684-691

195. Freeman, S. N., Bepler, G., Haura, E., Sutphen, R., and Cress, W. D. (2005) *J Natl Cancer Inst* **97**(14), 1088-1089; author reply 1093-1085

196. Vargas, R. L., Felgar, R. E., and Rothberg, P. G. (2005) *J Natl Cancer Inst* **97**(14), 1089-1090; author reply 1093-1085

197. Iglesias-Serret, D., Coll-Mulet, L., Santidrian, A. F., Navarro-Sabate, A., Domingo, A., Pons, G., and Gil, J. (2005) *J Natl Cancer Inst* **97**(14), 1090-1091; author reply 1093-1095

198. Dicker, F., Rauhut, S., Kohlmann, A., Kern, W., Schoch, C., Haferlach, T., and Schnittger, S. (2005) *J Natl Cancer Inst* **97**(14), 1092-1093; author reply 1093-1095

199. Coenen, S., Pickering, B., Potter, K. N., Johnson, P. W., Stevenson, F. K., and Packham, G. (2005) *Haematologica* **90**(9), 1285-1286

200. Tobin, G., Skogsberg, A., Thunberg, U., Laurell, A., Aleskog, A., Merup, M., Sundstrom, C., Roos, G., Nilsson, K., and Rosenquist, R. (2005) *Leukemia* **19**(5), 871-873

201. Nenning, U. C., Eckert, C., Wellmann, S., Barth, A., Henze, G., and Seeger, K. (2005) *J Natl Cancer Inst* **97**(14), 1091-1092; author reply 1093-1095

ABOUT THE AUTHOR

Scott N. Freeman received his B.S. degree *Cum Laude* with a major in Biology and a minor in Chemistry from Central Michigan University in Mount Pleasant, Michigan in 2002. Scott N. Freeman continued his education by accepting a position in the Cancer Biology Ph.D. program at the University of South Florida where he conducted his graduate research under the supervision of W. Douglas Cress, Ph.D. at the H. Lee Moffitt Cancer Center and Research Institute. Scott N. Freeman has one first author publication in *The Journal of the National Cancer Institute*, a second author publication in *Cancer Biology and Therapy*, and at the time of this publication, has a first author publication pending for *The Journal of Biological Chemistry*.