TRANSCRIPTIONAL REGULATION OF MYC BY THE

TUMOR SUPPRESSOR ARF

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Dissertation under the direction of Professor Stephen R. Hann

c-Myc is frequently deregulated in human cancers. While deregulated c-Myc leads to tumor growth, it also triggers apoptosis in partnership with tumor suppressors such as ARF and p53. Apoptosis induced by c-Myc is a critical fail-safe mechanism for the cell to protect against unrestrained proliferation. Despite the plethora of information on c-Myc, the molecular mechanism of how c-Myc induces both transformation and apoptosis is unclear. Oncogenic c-Myc can indirectly induce the expression of the tumor suppressor ARF, which leads to apoptosis through the stabilization of p53, but both c-Myc and ARF have apoptotic activities that are independent of p53. In cells without p53, ARF directly binds to c-Myc protein and inhibits c-Myc-induced hyperproliferation and transformation with a concomitant inhibition of canonical c-Myc target gene induction. However, ARF is an essential cofactor for p53-independent c-Myc-induced apoptosis. Here we show that ARF is necessary for c-Myc to drive transcription of a novel noncanonical target gene, *Egr1*. In contrast, c-Myc

induces another family member, *Egr2*, through a canonical mechanism that is inhibited by ARF. We further demonstrate that Egr1 is essential for p53-independent c-Myc-induced apoptosis, but not ARF-independent c-Myc-induced apoptosis. Therefore, ARF binding switches the inherent activity of c-Myc from a proliferative to apoptotic protein without p53 through a novel noncanonical transcriptional mechanism. These findings also provide evidence that cofactors can differentially regulate specific transcriptional programs of c-Myc leading to different biological outcomes.

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To my wonderful daughter, Gabrielle, my little peanut, my angel, infinitely wise,

beautiful, creative, inquisitive, caring, and joyful.

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LIST OF ABBREVIATIONS

ARF   Alternative Reading Frame protein

AR   acidic region

ATM   ataxia telangiectasia mutated protein

Asp   Aspartic acid

Ac-DEVD-pNA acetyl-Asp-Glu-Val-Asp p-nitroanilide

ATR   ataxia telangiectasia and Rad3 related

b-HLH-LZ   basic-helix-loop-helix-leucine zipper

Bax   BCL2-associated X protein

Bcl2   B-cell lymphoma 2 protein

BH3   Bcl-2 homology domain 3

Bid   BH3 interacting domain

Bak   BCL2-antagonist/killer 1

BSA   bovine serum albumin

c1,c2,c3   clone1, 2, 3

C-terminus   carboxy-terminus

CDK4   cyclin-dependent kinase 4

CDKi	cyclin-dependent kinase inhibitor
CDKN2A	cyclin-dependent kinase inhibitor 2a
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus (promoter)
CS	calf serum
CTD	carboxy-terminal domain
DKO	double knock out
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnmt3a	DNA (cytosine-5)-methyltransferase 3A
DOC	deoxycholic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
Egr	early growth response
Egr1-ER	Egr1 estrogen receptor
eIF4E	eukaryotic translation initiation factor 4E
Elf1a	eukaryotic translation initiation factor 1a

EMS	E-box Myc sequence
ER	estrogen receptor
ETOH	ethanol
FBS	fetal bovine serum
Fbw7	F-box and WD repeat domain-containing 7
fwd	forward
G1	gap_1 phase
G2	gap_2 phase
Gadd	growth arrest and DNA damage
Glu	Glutamic acid
GnRH	gonadotropin-releasing hormone
HA	hemagglutinin
HAT	histone acetyltransferase
HDAC	histone deacetylase
Hygro	hygromycin
IB	immunoblot
IgG	Immunoglobulin G
IL-3	interleukin 3

Inr	initiator element
IP	immunoprecipitation
Kbs	kilobases
KOAc	potassium acetate
Luc	luciferase
M	molarity
MBI/II/III	Myc boxI/II/III
MCS	multicloning site
MEFs	mouse embryonic fibroblasts
MgOAc	magnesium acetate
min	minute
Miz1	Myc-interacting zinc finger 1
mRNA	messanger RNA
MT-MC1	Myc Target in Myeloid Cells 1
mxi	max interacting protein
MycER	Myc estrogen receptor
Mycfl	Myc full length
MycN6KR-ER	6 lysine-to-arginine mutant in the N-terminus of MycER

N-terminus	amino-terminus
NLS	nuclear localization signal
NP40	nonyl phenoxypolyethoxylethano
NPM	nucleophosmin
NTD	amino-terminal domain
ODC	ornithine decarboxylase
OHT	4-hydroxytamoxifin
PCR	polymerase chain reaction
PDGF-β	platelet derived growth factor β receptor
PMSF	phenylmethanesulfonylfluoride
pRL-SV40	renilla luciferase Simian virus 40
pRL-TK	renilla luciferase thymidine kinase
P-TEF-b	Positive Transcription Elongation Factor b
PTEN	phosphatase and tensin homolog
Puro	puromycin
rev	reverse
RNAi	RNA interference
RNA	ribonucleic acid

RT-PCR	reverse-transcriptase polymerase chain reaction
S phase	synthesis phase
s.d.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siCtl	small interfering control
siRNA	small interfering RNA
Skp2	S-phase kinase-associated protein 2
Sp1/Sp3	specificity protein 1/3
Staph A	*Staphylococcus aureus*
Taq	*Thermus aquaticus*
Tbp	TATA-binding protein
TBS	tris-buffered saline
tert	telomerase reverse transcriptase
TFII-I	transcription factor II-I
TGFβ1	transforming growth factor beta
TRD	transcriptional regulatory domain
TRRAP	transformation/transcription domain-associated protein

WB   Western blot

WT   wild-type

Val   valine

CHAPTER I

INTRODUCTION

Biological Functions and Significance of c-Myc

The complexities of c-Myc have plagued investigators for well over the past quarter of a century. However, with the deregulation of c-Myc being one of the most prevalent alterations in many types of human cancer—Burkitt's lymphoma, myeloid and plasma cell leukemias; breast, cervical, small cell lung, and colon carcinomas; osteosarcoma; and glioblastoma (Spencer and Groudine 1991; Nesbit et al. 1999; Adhikary and Eilers 2005)—and because c-Myc has been implicated in many of the diverse biological functions leading to tumorigenesis (Pelengaris et al. 2002), it is not surprising that extensive time and effort continue to be dedicated to understanding how this small molecule functions. Through the years, researchers have discovered many diverse and sometimes contradictory, but nonetheless crucial, biological functions of c-Myc. The vital importance of c-Myc is accentuated by the embryonic lethality of *c-myc* knockout mice (Davis et al. 1993). Overexpression of c-Myc increases progression through the cell cycle by shortening G1 and G2, drives cell cycle entry in the absence of growth factors, contributes to genomic instability and chromosomal alterations, increases cell growth, prevents terminal differentiation, and causes hyperproliferation, immortalization, and transformation in cell culture

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and tumorigenesis in transgenic animals (Henriksson and Luscher 1996; Lemaitre et al. 1996; Facchini and Penn 1998; Felsher and Bishop 1999; Li and Dang 1999). In contrast, c-Myc overexpression also leads to apoptosis in cells deprived of growth factors (Askew et al. 1991; Evan et al. 1992; Bissonnette et al. 1994; Evan and Littlewood 1998). Additionally, the ability of endogenous c-Myc to drive these two conflicting processes—proliferation and apoptosis—is emphasized in *c-myc-/-* cells, which have both a slow growth phenotype due to lengthened G1 and G2 cell cycle phases and an apoptotic defect due to an unknown mechanism (Mateyak et al. 1997).

The complex and contradictory roles of c-Myc provoke many questions. How does a single protein contribute to numerous crucial and diverse biological functions? Is there a molecular mechanism to switch c-Myc from driving proliferation to initiating apoptosis? Can interaction with specific cofactors lead to differential regulation? Are there direct target genes of c-Myc that are specific to induce apoptosis? How do cancerous cells utilize c-Myc functions to survive and proliferate while bypassing its apoptotic effects? Unfortunately the answers to these important questions remain elusive. The goal of this thesis is to investigate the molecular mechanisms involved in c-Myc function, specifically focusing on the transcriptional and apoptotic regulation by the tumor suppressor ARF.

c-Myc as a transcription factor

The c-Myc protein is a transcription factor of the basic helix-loop-helix-leucine zipper (b-HLH-LZ) family that activates and represses gene expression

(Landschulz et al. 1988; Murre et al. 1989; Kato et al. 1990; Adhikary and Eilers 2005). c-Myc has a typical transcription factor modular structure with a b-HLH-LZ dimerization/DNA binding domain at the C-terminus and a transcriptional regulatory domain (TRD) at the N-terminus (Figure 1-1) (Kato et al. 1990). Dimerization with Max, a ubiquitously expressed b-HLH-LZ partner protein, and direct binding to specific DNA sequences is required for c-Myc function (Blackwood and Eisenman 1991). The E-box, CACGTG, is the 'canonical' sequence bound by Myc/Max, but many other high affinity 'non-canonical' sequences exist that Myc/Max associate with both *in vitro* and *in vivo* (Blackwell et al. 1990; Blackwell et al. 1993). Although the significance of target genes that contain these non-canonical sequences is unknown, their importance is unmistakable given the data from global binding studies that demonstrate only a minority of sites bound *in vivo* have the consensus sequence (Fernandez et al. 2003; Li et al. 2003; Adhikary and Eilers 2005).

The recruitment of multiple co-activators to E-box elements by interaction with the N-terminal domain of c-Myc is essential for target gene activation (Figure 1-1) (Cowling and Cole 2006). One such cofactor, TRRAP, is an adaptor protein that binds c-Myc at a conserved region in the N-terminus called Myc Box II (MBII) (McMahon et al. 1998). Chromatin structure of c-Myc regulated promoters is subsequently altered—acetylated—by the histone acetyltransferases (HATs), GCN5 and TIP60, bound to TRRAP (Ikura et al. 2000; Strahl and Allis 2000; Brown et al. 2001; Liu et al. 2003). Acetylated chromatin is an 'open' conformation that is accessible to general transcription factors, and this alteration

of chromatin status is a critical way that c-Myc mediates gene expression. The ATPases/helicases TIP48 and TIP49 also bind MBII and alter chromatin structure, but through a TRRAP-independent mechanism (Wood et al. 2000). Another cofactor recruited by c-Myc that affects up-regulation of target genes in a different manner is the kinase P-TEFb, which facilitates transcriptional elongation by enhancing the phosphorylation of the C-terminal domain of RNA polymerase II (Eberhardy and Farnham 2002). Furthermore, many other cofactors have been identified that are recruited to E-boxes by c-Myc that are important for different mechanisms of gene regulation (Cowling and Cole 2006). However, despite the understanding of how numerous different cofactors affect c-Myc mediated activation, it is unknown if specific cofactors can influence which of the many c-Myc target genes are regulated. Nevertheless, the discovery that TRRAP binding increases up-regulation of *cyclinD2* and *tert* but not *cad* suggests that cofactor binding can differentially regulate the induction of c-Myc target genes (Eberhardy et al. 2000; Eberhardy and Farnham 2001).

The regulation of gene expression by c-Myc is complicated by and often dependent on other transcription factors. Many other transcription factors bind and regulate the same genes and also the same sites as the Myc/Max hetero-dimer (Blackwell et al. 1990; Blackwell et al. 1993). In fact, Max can form heterodimers with several other related b-HLH proteins known as Mad, Mxi, and Mnt that bind E-box sequences and repress transcription by recruiting histone deacetylases (HDACs) through the adaptor protein SIN3 (Ayer et al. 1993; Zervos et al. 1993; Hurlin et al. 1997). Another way c-Myc is thought to activate

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Figure 1-1. Domains of c-Myc and their binding proteins. c-Myc has a typical transcription factor structure with a C-terminal domain (CTD) consisting of a basic (B) helix-loop-helix (HLH) leucine zipper (LZ) necessary for DNA binding. It also contains the nuclear localization signal (NLS). The N-terminal domain (NTD) is the transcriptional regulatory domain (TRD) that recruits cofactors to DNA to influence transcription. Conserved regions between family members and species are denoted as Myc boxes (MBI, MBII, MBIII). The function of the acidic region (AR) in the central part of the protein is unknown. The numbers under the diagram represent amino acid position. The black bars under the diagram indicate the region of interaction by the protein listed on the right. Dimerization with MAX is necessary for all of c-Myc's functions. When c-Myc interacts with Miz1 it represses transcription by preventing the transcriptional activities of Miz1. TRRAP recruits the histone acetyltransferases (HATs) GCN5 and TIP60 to the promoter. TIP48 and TIP49 are involved in chromatin remodeling. P-TEFb is a kinase that phosphorylates the c-Terminal tail of RNA polymerase II to initiate transcriptional elongation. Fbw7 is not a cofactor of c-Myc, but is an E3 ubiquitin ligase that regulates c-Myc protein stability. Skp2 is also part of an E3 ubiquitin ligase complex, but it also functions as a cofactor for c-Myc increasing the transcriptional induction of several canonical targets. The interaction with ARF switches c-Myc from driving canonical target gene up-regulation, hyperproliferation, and transformation to shutting down canonical transactivation while inducing apoptosis.
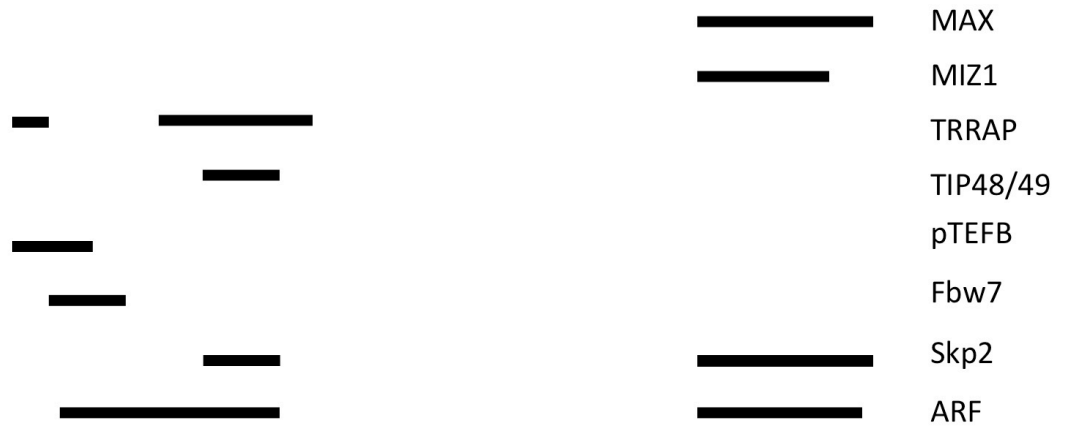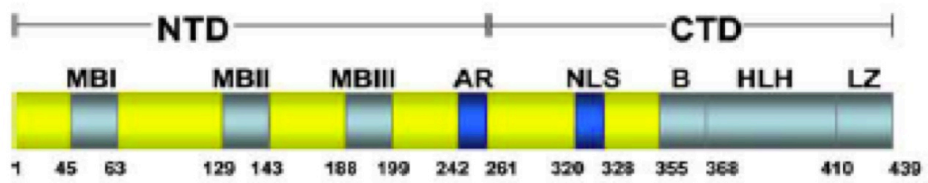
transcription is simply by de-repression, whereby Myc/Max compete with these repressive complexes that bind to E-boxes. Additionally, c-Myc can mediate repression of target genes, but this does not happen at E-boxes or any other simple consensus sequence and may not even require direct DNA binding (Kleine-Kohlbrecher et al. 2006). Instead repression by c-Myc occurs by poorly defined mechanisms that involve c-Myc binding to general transcription factors like TFII-I or other transcription factors such as Miz1, Sp1, and Sp3 at Inr elements or other regions of core promoters in target genes (Li et al. 1994; Yang et al. 2001). How c-Myc represses rather than activates these genes is unknown, but it may involve recruitment of distinct cofactors to target sites of repression. For example, instead of recruiting HATs to Miz1 bound targets of repression it was shown that c-Myc is necessary for the recruitment of the DNA methyl-transferase Dnmt3a (Brenner et al. 2005). Furthermore, though it is believed that the control of eukaryotic transcription is to a large extent combinatorial, the effects of other transcription factors binding simultaneously with c-Myc at target gene promoters have not been examined. However, a bioinformatics approach did identify a few factors, including Egr1, whose binding sites are overrepresented in c-Myc target gene promoters (Elkon et al. 2004).

c-Myc and Apoptosis

Overview of apoptosis as a fail-safe to inhibit hyperproliferation

In response to mitogens, the expression of c-Myc and other factors such as E2F are induced. They then promote cell cycle progression by up-regulation of specific genes. E2F has a logical set of target genes that elicit the onset of S phase, including cyclins, enzymes involved in maintaining nucleotide pools, and DNA polymerase itself (Patel et al. 2004). Unfortunately, the important target genes necessary for c-Myc to induce cell cycle progression have been far more difficult to elucidate due to the fact that most of the defined target genes of c-Myc are important in other biological functions, like ribosome biogenesis and metabolism. It is currently believed that the induction of *cyclinD2* and *CDK4* are important for c-Myc to drive the cell cycle (Patel et al. 2004; Adhikary and Eilers 2005). However, neither of these genes, nor any other defined target gene, can completely rescue the slow growth phenotype of Myc null cells, although ornithine decarboxylase (ODC) or MT-MC1 overexpression can partially enhance proliferation in these cells (Berns et al. 2000; Yin et al. 2002; Cohen and Prochownik 2006). Because none of c-Myc's direct targets are sufficient to induce cell cycle progression and proliferation, and because c-Myc regulates many genes involved in many functions, some have suggested that specific targets may not lead to proliferation, but rather c-Myc-induced proliferation is produced by a combined effect from a large subset of targets involved not only in the cell cycle, but also in cell growth.

A major pathway in controlling the inhibition of proliferation and the induction of apoptosis is the ARF-Mdm2-p53 pathway. ARF is an unusual tumor suppressor that is encoded by an <u>a</u>lternative <u>r</u>eading <u>f</u>rame from the evolutionarily conserved Ink4a locus, which also encodes the tumor suppressor p16 (Quelle et al. 1995). It is a highly basic protein that is typically not expressed in normal tissues, but rather is induced primarily by prolonged and sustained proliferative signals produced by active oncogenes such as c-Myc and Ras (Lowe and Sherr 2003). Because of this it is not surprising that the loss of ARF through deletion or epigenetic silencing is a common feature of many human cancers (Vonlanthen et al. 1998; Esteller et al. 2000; Sherr 2001). The prevailing view is that the major tumor suppressor functions of ARF are mediated through p53 (Sherr 2006). c-Myc overexpression/deregulation or other hyperproliferative stimuli, such as Ras, E1A, or E2F, induce ARF expression, which directly binds and inhibits Mdm2, the E3 ubiquitin ligase of p53, resulting in the stabilization of p53 (Sherr 1998). Growth arrest or apoptosis ensue due to p53 target gene up-regulation of the cyclin dependant kinase inhibitor p21, or pro-apoptotic genes such as Bax and PUMA (Yu et al. 2003). However, accumulating evidence suggests that ARF can act as a tumor suppressor, inducing apoptosis and/or inhibiting proliferation, independently of p53 by controversial and mostly unknown mechanisms. Additionally, c-Myc also causes apoptosis independently of p53, though the mechanism is unknown. However, the regulation of the Bcl-2 family members, Bim, Bax, and Bcl2, by c-Myc has proven important in this process,

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but all appear to be indirectly regulated by c-Myc (Eischen et al. 2001; Eischen et al. 2001; Egle et al. 2004; Hemann et al. 2005).

c-Myc and p53

It was surprising when c-Myc, which provides cells with survival and proliferative advantages, was shown to also induce apoptosis. Originally, it was observed that a myeloid cell line, 32D, constitutively expressing c-Myc, in response to interleukin 3 (IL-3) withdrawal, did not arrest in G1, but did rapidly initiate apoptosis (Askew et al. 1991). Soon after, others demonstrated that Rat-1 fibroblasts constitutively expressing c-Myc also did not arrest in low serum, unlike untransfected Rat-1 cells. Although the c-Myc expressing Rat-1 cells continued to proliferate, their numbers in culture did not increase because constitutive c-Myc expression was simultaneously inducing proliferation and apoptosis in the absence of mitogenic stimulation creating a nearly steady state number of cells (Evan et al. 1992). We now know that this phenomenon is not specific to these cell types or under these conditions, but instead extends to many cell types under many different conditions. In fact, many oncogenes have similar built-in fail-safe mechanisms to restrict uncontrolled proliferation—caused by their own aberrant signaling—through induction of apoptosis (Harrington et al. 1994).

Not long after the discovery of the role of c-Myc in apoptosis it was shown that activation of c-Myc stabilized p53 and induced apoptosis and cell cycle re-entry in quiescent MEFs expressing wild-type p53 (Hermeking and Eick 1994).

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Figure 1-2. c-Myc-induced apoptosis. Oncogenic c-Myc is involved in a complicated web that controls the levels of p53. c-Myc stabilizes p53 by inducing ARF, repressing Mdm2, and indirectly by inducing DNA damage. In a feedback mechanism, p53 reduces its own expression by inducing Mdm2, repressing ARF, and reducing c-Myc through the induction of miR-145. c-Myc and p53 inhibit growth and/or induce apoptosis through the regulation of common target genes. While p53 directly (solid lines) induces or represses growth inhibitory, pro-apoptotic, and anti-apoptotic targets, c-Myc appears to do so indirectly (dashed lines). The blue lines represent the direct regulation of ARF on c-Myc that results in a differential induction of c-Myc targets and p53-independent apoptosis. Exactly what targets are involved is still unknown, but the Myc/ARF interaction may explain how c-Myc indirectly regulates the pro- and anti-apoptotic factors —some of which may be the same as listed in the p53-dependent pathway— independently of p53.
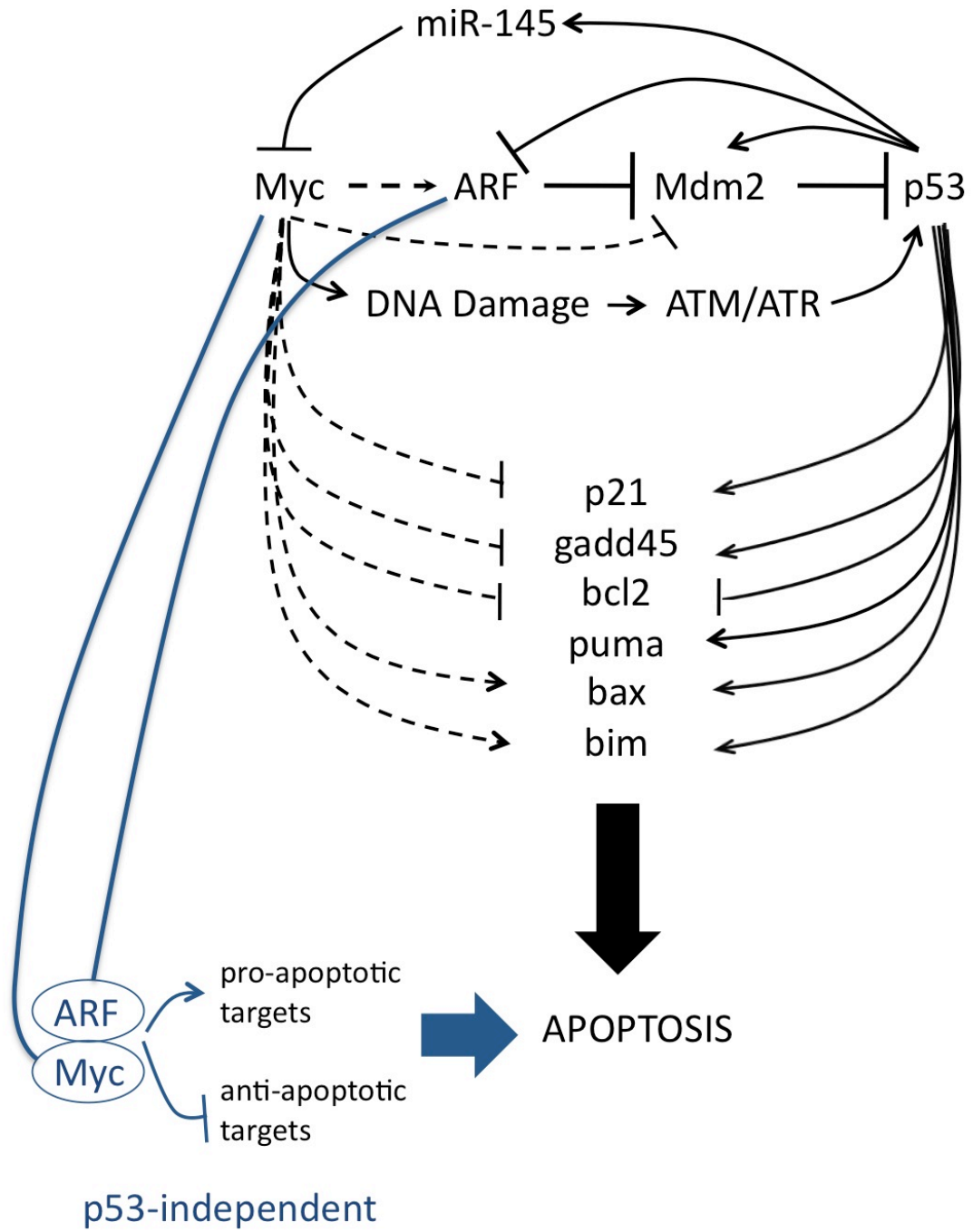
This was the birth of the connection between two of the most frequently altered proteins in human cancers (Lane 1992). The most well-established mechanism of c-Myc mediated stabilization of p53 is through the ARF/MDM2 pathway (Figure 1-2). c-Myc overexpression/deregulation, induces ARF expression through an unknown mechanism. ARF then directly binds, and inhibits Mdm2, the E3 ubiquitin ligase of p53, resulting in stabilization of p53 (Sherr 1998; Sherr 2001). There is also evidence that c-Myc binds to and represses the promoter of Mdm2, thereby c-Myc may both directly and indirectly decrease the available levels of Mdm2 leading to p53 activation (Macias et al. 2009). Additionally, through feedback mechanisms, p53 induces Mdm2 expression (Wu et al. 1993), represses ARF expression (Weber et al. 2000), and inhibits c-Myc expression through the direct induction of the tumor suppressive miR-145 (Sachdeva et al. 2009). Furthermore, overexpressed c-Myc, a common event in human cancer, causes DNA damage and genomic instability, which elicits a DNA damage response through the ataxia-telangiectasia-mutated (ATM) kinase that ultimately results in phosphorylation-dependent stabilization of p53 (Pusapati et al. 2006). Thus, c-Myc is involved in a complex and highly regulated web controlling the expression of p53 (Figure 1-2).

p53, like c-Myc, is a transcription factor that elicits its biological functions through transcriptional regulation of target genes (Zambetti et al. 1992; Zambetti and Levine 1993; White 1996). Therefore, stabilized p53 levels leads to either growth arrest or apoptosis through target gene regulation. It is proposed that oncogenic c-Myc tips the cell-fate balance toward apoptosis through regulation of

overlapping target genes (Nilsson and Cleveland 2003). p53 activates the expression of the cyclin dependent kinase inhibitor (CDKi) *p21* and the growth arrest and DNA damage gene *gadd45*, leading to cell cycle arrest (Benchimol 2001). In contrast, c-Myc represses *p21* and *gadd45*, leading to either progression through the cell cycle or apoptosis when p53 is also at high levels (Herold et al. 2002; Seoane et al. 2002; Wu et al. 2003)(Figure 1-2). However, both c-Myc and p53 can induce levels of pro-apoptotic Bcl-2 and BH3-only family members either independently or in concert, although the induction of these targets by c-Myc is controversial and appears to be indirect (Benchimol 2001; Nilsson and Cleveland 2003; Meyer et al. 2006). For example, under certain conditions both were shown to induce the expression of *Bax* and *Bak*, which are required for activation of apoptosis through permeabilization of the outer membrane of the mitochondria (Galluzzi et al. 2008) leading to cytochrome c release. Furthermore, both c-Myc and p53 transcriptionally repress the pro-survival, anti-apoptotic, *Bcl-2* gene (Eischen et al. 2001). Therefore, c-Myc inhibits p53 mediated growth arrest through repression of *p21* and *gadd* genes, while simultaneously c-Myc and p53 cooperate to induce apoptosis at the mitochondria.

p53-independent apoptosis: c-Myc and ARF

It is evident that ARF is an essential mediator between c-Myc and p53 because c-Myc induces ARF levels, which ultimately leads to p53 stabilization and apoptosis or cell cycle arrest. However, not only does ARF directly regulate

p53 levels and function, but it also directly regulates c-Myc function. We previously found that ARF directly interacts with c-Myc and inhibits c-Myc-induced hyperproliferation and transformation, while enhancing c-Myc-induced apoptosis, even in cells lacking *p53*. In fact, c-Myc fails to induce apoptosis efficiently in cells lacking both *ARF* and *p53* (Qi et al. 2004), supporting the idea that ARF is necessary for efficient p53-independent c-Myc-induced apoptosis in fibroblasts. When c-Myc is activated, nucleolar ARF translocates to the nucleoplasm and colocalizes with c-Myc (Qi et al. 2004; Gregory et al. 2005). In the nucleoplasm, we showed that c-Myc and ARF directly interact through co-IPs and bind to the same region of several c-Myc target gene promoters through ChIP assays. When ARF is highly expressed and bound to c-Myc target gene promoters with c-Myc, ARF inhibits c-Myc's ability to drive expression of well-established canonical target genes observed both in real-time RT-PCR assays following activation of the inducible c-MycER and in luciferase assays (Qi et al. 2004). However, ARF has no effect on genes commonly repressed by c-Myc. We believe that the change in target gene regulation caused by ARF led to the inability of c-Myc to cause hyperproliferation and transformation, while enhancing the ability of c-Myc to induce apoptosis. Deciphering the molecular mechanism of c-Myc-induced, ARF-dependent, p53-independent apoptosis was the goal of the studies performed for this thesis.

In order to understand how the Myc/ARF interaction induced apoptosis independently of p53, and hypothesizing that it was through a transcriptional mechanism, we performed microarrays by activating c-Myc in *p53*-/- and in

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p53/ARF double knock out (DKO) MEFs to determine if ARF inhibited the up-regulation of all genes by c-Myc. To our surprise this was not the case. In fact, our results showed that the up-regulation of certain genes like *Egr1* and *Egr4* are actually enhanced by ARF. Therefore, it appears that the ARF interaction does not completely inhibit c-Myc transcription, but rather differentially regulates the target genes induced by c-Myc. In the proceeding chapters, we address if two members of the Egr family of transcription factors, Egr1 and Egr2, are direct targets of c-Myc and if their differential regulation leads to different biological outcomes, with emphasis on whether or not Egr1 is pro-apoptotic.

Egr Transcription Factors

The Early Growth Response factors are immediate early genes like *c-myc*, *c-fos*, and *c-jun* that are rapidly induced in quiescent cells upon mitogenic stimulation even in the absence of protein synthesis (Chavrier et al. 1988; Christy and Nathans 1989). The family is a group of four zinc finger transcription factors that have highly related zinc finger DNA binding domains, but divergent transcription regulatory domains (Beckmann and Wilce 1997; Liu et al. 1998). Knockout mice have been made for all family members, and they all have distinct phenotypes, suggesting different functions (O'Donovan KJ 1999). However, there is evidence for some level of compensation between family members. For example, luteinizing hormone levels are lower in *Egr1/Egr4* double knockout mice than in Egr1 knockout mice (Tourtellotte et al. 2000). Further, Egr1 and Egr4, but not Egr2 and Egr3, respond to gonadotropin-releasing hormone

(GnRH) (Dorn et al. 1999). Interestingly, our microarray revealed that c-Myc regulates the Egr factors in this same pattern: Egr1/4 are upregulated by c-Myc in *p53*-/- MEFs with high ARF, while Egr2/3 are upregulated in *p53/ARF* DKO MEFs.

The biological functions of the Egr factors are not entirely known or understood, although numerous studies implicate a critical role for them in proliferation, apoptosis, inflammation, and differentiation. A role in proliferation for all the Egr factors is suggested because they are induced by mitogens. Additionally, it was shown that Egr2 might represent a point of convergence in the integration of different signaling pathways leading to the B cell proliferative response (Newton et al. 1996). Pre-T cells have reduced proliferation in response to pre-T cell receptor signaling in Egr3 null mice, suggesting Egr3 promotes proliferation in these cells (Xi and Kersh 2004). Egr1, the most studied family member and the only one extensively examined in MEFs, in contrast to Egr2 and Egr3, has a definitive role as a tumor suppressor by inducing apoptosis by both p53-dependent and p53-independent mechanisms (Liu et al. 1998). Studies show that the expression of Egr1 is undetectable in 72% of human breast and non-small cell lung carcinomas and is deleted in 50% of acute myeloid leukemias (Liu et al. 1998). Furthermore, *egr1-/-* mice are prone to skin cancer when challenged with the two-step carcinogenesis model (Krones-Herzig et al. 2003). Additionally, Egr1 is expressed at high levels in terminally differentiated cells, and actually Egr1 overexpression abrogates the block imparted by c-Myc on terminal M1 myeloid differentiation (Shafarenko et al.

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2005). Overexpression of Egr1 in NIH3T3 cells reversibly transformed by conditional expression of PDGF-B/v-sis leads to inhibition of proliferation, focus formation, and soft agar growth (Mercola et al. 1992). While reducing Egr1 expression through dominant-negative or RNAi approaches, inhibits radiation-induced apoptosis (Muthukkumar et al. 1995; Ahmed et al. 1996). Further, *p53* is a direct target of Egr1 as shown by ChIP and real time RT-PCR analyses (Krones-Herzig et al. 2005; Yu et al. 2007). However, even in prostate cancer cells that lack *p53*, Egr1 induction leads to apoptosis (Liu et al. 1998). Other known target genes of Egr1 are the tumor suppressors *TGFB1* and *PTEN,* as well as, the growth arrest and apoptotic genes *gadd45, Bax, Bim*, and *Fas* (Baron et al. 2006). In fact, the site of c-Myc regulation of the gadd45 promoter mapped to an Egr binding site (Amundson et al. 1998). Based on these facts and our preliminary data we hypothesized that the Egr transcription factors are direct target genes of c-Myc and that Egr1 is induced in an ARF-dependent non-canonical fashion leading to apoptosis and growth arrest through the induction of pro-apoptotic genes.

CHAPTER II

EGR1 IS A DIRECT ARF-DEPENDENT C-MYC TARGET GENE

Introduction

Deregulation or overexpression of the transcription factor c-Myc causes hyperproliferation and tumorigenesis and is a driving factor in the majority of human cancers (van Riggelen et al. 2010). Although c-Myc regulates hundreds of downstream target genes involved in many different cellular processes, it is unclear which target genes mediate specific c-Myc functions (Cohen and Prochownik 2006). Apoptosis in response to deregulated c-Myc is a major fail-safe mechanism that is essential to prevent the proliferation of tumorigenic cells (Sherr 2001). Apoptosis induced by oncogenic c-Myc occurs through both p53-dependent and independent mechanisms that are not well understood (Eischen et al. 2001; Hemann et al. 2005). A prevailing model for a p53-dependent mechanism is that the tumor suppressor ARF, which is induced by oncogenic c-Myc, causes the stabilization of the p53 protein by inhibiting its E3 ubiquitin ligase Mdm2 (Pomerantz et al. 1998; Zindy et al. 1998). Additionally, ARF, independently of p53, binds to c-Myc directly and blocks the ability of c-Myc to activate transcription of examined canonical target genes containing a CACGTG E-box Myc binding site (EMS) and also inhibits c-Myc-induced hyperproliferation and transformation (Datta et al. 2004; Qi et al. 2004). Despite this inhibition of canonical c-Myc activity, ARF has been shown to be essential for c-Myc to

induce p53-independent apoptosis in mouse embryo fibroblasts (MEFs) (Qi et al. 2004; Gregory et al. 2005). However, the mechanism of how ARF regulates c-Myc induced p53-independent apoptosis is unknown. In this chapter, we examined the transcriptional consequences of the c-Myc/ARF interaction and discovered that ARF differentially regulates the c-Myc induction of the Egr family of transcription factors. In contrast to ARF blocking the up-regulation of *Egr2* and other c-Myc canonical target genes, it is necessary for the direct transcriptional induction of *Egr1* through a novel noncanonical mechanism.

Materials and Methods

Cell culture

The *p53/ARF* double knockout (DKO) mouse embryonic fibroblasts (MEFs) were obtained from G. Zambetti (St.Jude's Children's Research Hospital, Memphis, TN). Different isolations of *p53*-/- MEFs were obtained from E. Ruley (Vanderbilt University, Nashville, TN) and C. Eischen (Vanderbilt University, Nashville, TN). The *ARF*-/- MEFs were obtained from C. Sherr (St.Jude's Children's Research Hospital, Memphis, TN). DKO, *p53*-/-, *ARF*-/-, Rat 1a embryonic fibroblasts, and psi2 ecotropic packaging cells were maintained in Dulbecco's modified Eagle medium (DMEM, high glucose; Gibco/Invitrogen) containing 10% bovine calf serum (HyClone) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The *myc*-/- (HO16) and *myc*+/+ (TGR) Rat 1a cells were obtained from J. Sedivy (Brown University, Providence, Rhode

Island) and have been described previously (Mateyak et al., 1997). TGR and H016 Rat 1a cells were maintained in 10% fetal bovine serum (FBS; Atlanta Biologicals) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The media for stable cell lines expressing c-MycER-puro or c-MycER-hygro was supplemented with 2.5 μg/ml puromycin (Calbiochem) or 50 μg/ml hygromycin B (Invitrogen) respectively. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell transfection and infection

All cells were transiently transfected with Lipofectamine 2000 (Invitrogen) or FuGENE 6 according to manufacturer's protocol with the indicated plasmids. Details for the luciferase transfections are documented in the 'Luciferase Assays' section below.

The generation of DKO MEFs, *p53-/-* MEFs, and HO16 Rat 1a cells stably expressing c-MycER or vector was described previously (Xiao et al. 1998; Qi et al. 2004). To generate stable *ARF*-/- MEFs expressing c-MycER, psi2 cells were transfected with pBabe-hygro-MycER using FuGENE 6 transfection reagent (Roche) and the virus-producing cells were selected in 400 μg/ml hygromycin B for approximately 7 days. The *ARF*-/- MEFs were then infected for 6 hours with viral supernatants, in the presence of 6 μg/ml polybrene, collected from the stable psi2 packaging cell line stably expressing c-MycER-hygro. The infected cells were selected for in 400 μg/ml hygromycin B for 2 weeks. Expression of c-MycER was verified via Western blotting.

Plasmids and expression vectors.

The expression vectors pRcCMV-Myc, pBabe-puro-MycER, pBabe-hygro-MycER, *htert*-luc, and 4x-EMS-luc, obtained from R. Eisenman (Fred Hutchison Cancer Research Center, Seattle, Washington) have been previously described (Qi et al. 2004). The 2.5kb *Egr1*-luc plasmid was constructed by removing the Egr1 promoter portion of pBLUECAT-2, obtained from B. Christy (University of Texas, San Antonio Texas), with BglII (New England Biolabs) digestion. It was then ligated (Promega) into the multiple cloning site (MCS) of linearized pGL3 (Promega). All constructs were verified by sequencing through the Vanderbilt DNA Sequencing Core.

Cell treatments

c-MycER was activated in all cells by the addition of 1-10μM 4-hydroxytamoxifen (OHT; Sigma) as indicated. To inhibit protein translation, *p53-/-* MEFs expressing c-MycER were treated with 50 μg/ml of cycloheximide (Sigma) dissolved in dimethyl sulfoxide (DMSO). Cessation of protein synthesis was confirmed by following the decay of c-Myc protein over time with Western blotting. To inhibit transcription HO16 Rat 1a cells expressing MycER were treated with 10 μg/ml Actinomycin D (Sigma) dissolved in ethanol.

The day prior to treatment, *p53-/-* MEFs expressing c-MycER were seeded at 3 x 10^6 cells per 10cm dish in antibiotic free media. The cells were treated with a final concentration of 100nM of CDKN2A (ARF) SMARTpool or Control Pool Non-Targeting #1 siRNA purchased from DHARMACON using Dharmafect Reagent 4 according to manufacturer's instructions. Approximately 16 hours later, cells were trypsinized and seeded at 5 x 10^4 cells per well in a 6 well dish. The next day MycER was activated with 5μM 4-hydroxytamoxifen (OHT; Sigma) or cells were mock treated with ethanol. The cells were harvested, RNA was isolated, and real time reverse-transcriptase polymerase chain reaction (RT-PCR) was preformed as detailed below. Knock-down of ARF was verified via Western blotting.

Antibodies

The affinity-purified rabbit polyclonal anti-Mycfl (against full-length murine c-Myc) has been previously described (Spotts et al. 1997). The c-Myc rabbit polyclonal antibody, N-262x, designed for Chromatin Immunoprecipitation (ChIP) applications was purchased from Santa Cruz Biotechnology. The affinity-purified rabbit polyclonal antibody, anti-ARF 754-5 (against murine p19 ARF) was purchased from Millipore. The rabbit polyclonal antibody, anti-CDKN2A (anti-p19 ARF), used in the ChIP assays was purchased from GeneTex. The Egr1 rabbit polyclonal antibody, anti-Egr1 C-19, was purchased from Santa Cruz Biotechnology. The mouse monoclonal anti-β-actin antibody was purchased

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from Sigma-Aldrich. Rabbit IgG used as a control for ChIP assays was purchased from Upstate.

Western blot analysis

Cell lysates were prepared in antibody buffer (20mM Tris pH7.4, 50mM NaCl, 0.5% Triton X-100, 0.5% DOC, 0.5% SDS, 1mM EDTA, 0.1M PMSF, 10mg/ml Aprotinin, 2mg/ml Leupeptin) and sonicated briefly. Total protein for each lysate was calculated using Bio-Rad D_C Protein Assay (Bio-Rad). Equal amounts of lysate (50-100 μg of total protein) were boiled for 3 minutes in Laemmli sample buffer and were resolved by 10-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight markers (Thermo Scientific and Fermentas Life Sciences) were used as standards in each gel. Proteins were transferred to nitrocellulose membranes (Protran) electrophoretically followed by blocking in 3-5% milk in Tris-buffered saline (TBS) for 30 minutes before an overnight incubation with the indicated antibodies at 4°C. The membranes were washed with TBS and then incubated with horseradish peroxidase conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies in 3-5% milk in TBS at a concentration of 1:10,000 for 45 minutes. The membranes were washed again in TBS and proteins were detected using enhanced chemiluminescence (ECL; Western Lightning) according to the manufacturer's instructions followed by development on chemiluminescence film (Amersham Hyperfilm ECL; GE).

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MEFs and HO16 Rat 1 cells expressing c-MycER were harvested at the indicated times following the activation of c-MycER with 2-5μM OHT. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and concentration and purity was determined using a NanoDrop Spectrophotometer (ThermoScientific). Ten µgs of RNA were treated with DNA-*free* DNase (Ambion) or total RNA was treated with the RNase-free DNase set (Qiagen). Five μg of total RNA were reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed with the Bio-Rad iCycler by using iQ SYBR Green supermix (Bio-Rad). The specific primer sequences are listed below. A standard curve of 2- or 4-fold dilutions was run with each assay for each primer set. A melting curve was performed at the end of each protocol to ensure the production of only one product. Relative measurement of gene expression was calculated following manufacturer's instructions using the standard curve method. Briefly, the relative mRNA level was calculated by dividing the relative starting quantity (calculated from the standard curve) of each experimental gene by the relative starting quantity of a housekeeping gene (β-actin or TATA-binding protein). For fold induction, relative mRNA values compared with the un-activated control samples were graphed as the mean ± SD from triplicate assays.

For mRNA level analyses in HO16 and TGR cells, the cells were either harvested while growing logarithmically, or upon reaching confluence, were shifted to media containing 0.1%FBS for 48 hours before stimulation with 20%

FBS. Cells were harvested at the indicated times and the mRNA levels were determined as above. All real-time RT-PCR analyses were performed with the primers listed below and the results are reported as the mean +/- the s.d. relative to β-*actin* levels calculated as above.

Chromatin immunoprecipitation

Sixteen hours after $p53^{-/-}$ MycER and DKO MycER MEFs were plated at 6 x 10^6 cells/150mm-dish, they were treated with either 2μM OHT or ethanol for 6 hours. The cells were cross-linked for 10 minutes at room temperature by adding formaldehyde (Sigma) to a final concentration of 1% directly to the media. The cross-linking reaction was terminated through addition of glycine to a final concentration of 0.125M. The adherent cells were washed two times with phosphate buffered saline (PBS; Gibco) scraped, pooled, and pelleted with centrifugation at 1000 rpm for 10 minutes at 4°C. Nuclei were isolated by re-suspending and incubating the cells in swelling buffer (0.1M Tris pH 7.6, 10mM KOAc, 15mM MgOAc, 1% NP40, 1mM PMSF, 0.01mg/ml aprotinin, 0.01 mg/ml leupeptin) at a concentration of 1 ml per 5 x 10^7 cells for 20 minutes on ice followed by douncing the cells with 20 strokes in a 1 ml tight dounce homogenizer (Wheaton). Nuclei were collected through centrifugation at 2,500 x g for 5 minutes at 4°C and lysed in nuclei lysis buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% SDS, 1mM PMSF, 0.01mg/ml aprotinin, 0.01 mg/ml leupeptin) at a concentration of 1 ml per 1 x 10^8 cells for 10 minutes on ice. The chromatin was sheared through 8-10 pulses of sonication for 20 seconds at 25% power on

a Sonics Vibracell VC130 sonicator with 30-second rest intervals on ice between pulses. Agarose gels stained with ethidium bromide confirmed that chromatin was largely sheared to between ~100-1000 base pairs. Sheared chromatin was pre-cleared with 10 μl of blocked/washed Staphylococcus aureus (Staph A) cells —blocked in 1 mg/ml of salmon sperm DNA and 1 mg/ml of bovine serum albumin (BSA) and washed in dialysis buffer (2 mM EDTA, 50 mM Tris-Cl pH 8.0)— per 1 x 10^7 cells for 15 minutes at 4^oC. Chromatin from 1-2 x 10^7 cells was immunoprecipitated overnight with 1-2 μg of the indicated antibodies in IP dilution buffer (0.01% SDS, 1.1% Triton X 100, 1.2 mM EDTA, 16.7 mM Tris-Cl, 167 mM NaCl, 10 μl/ml PMSF, 1 μl/ml aprotinin, 1μl/ml leupeptin). The immune complexes were precipitated with blocked/washed Staph A cells (10 μl per 1 x 10^7 cells) and washed twice with dialysis buffer supplemented with 0.2% Sarkosyl and 4 times with IP wash buffer (100 mM Tris-Cl pH 9.0, 500 mM LiCl, 1% NP40, 1% deoxycholic acid (DOC) sodium salt, 1 mM PMSF) before elution in IP elution buffer (50 mM NaHCO$_3$, 1% SDS). Samples were supplemented with NaCl to a final concentration of 0.2M and incubated at 67^oC for 4 hours to reverse formaldehyde cross-links. Samples were RNAse (Clontech) and Proteinase K (Clontech) treated to remove RNA and protein respectively before DNA was purified with a PCR purification kit (Qiagen) according to manufacturer's protocol. Purified DNA was subjected to PCR amplification (Roche's Taq polymerase using Promega protocol and buffers supplemented with betaine) using specific primer sets listed below. PCR products were subjected to analysis on an ethidium bromide stained 1.5% agarose gel. For

quantitative ChIP analyses, ChIPs were performed as described above except the purified DNA was subjected to real-time PCR as described above using the primers listed below. A standard curve was used to calculate the relative starting quantity of each sample. The percent of input was calculated by dividing the relative starting quantity from each IP (Myc, ARF, IgG) by the relative starting quantity of the input and then by multiplying by 100. The results are reported as the mean +/- the s.d. from triplicate samples.

Luciferase Assays

$p53^{-/-}$ MEFs and DKO MEFs were transfected with 2 μg of Myc expression vector or empty vector, 1.9 μg of reporter plasmid, and 0.1 μg of pRL-TK (Promega) *Renilla* luciferase internal control using Lipofectamine 2000 reagent. Luciferase assays were carried out 48 hours post transfection according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System; Promega) using a single tube luminometer (Pharmingen). Results were normalized for expression of pRL-TK and are reported as the mean ± s.d. from triplicate samples. For the assays in HO16, TGR, and Rat1a cells, 3.9 μg of reporter plasmid and 0.1mg of pRL-TK or pRL-SV40 were transfected into the cells. Luciferase assays were performed, calculated, and reported as above.

Real-time RT-PCR primers

Egr1 mouse   fwd: 5' GAGGAGATGATGCTGCTGAG 3'

                 rev: 5' TGCTGCTGCTGCTATTACC 3'

Egr1 rat   fwd: 5' GAGCCCGCACCCAACAGTG 3'

                 rev: 5' TGGGGCTCAGGAAAAATGTCA 3'

Egr2 mouse   fwd: 5' CCTCCACTCACGCCACTCTC 3'

                 rev: 5' CACCACCTCCACTTGCTCCTG 3'

Egr2 rat   fwd: 5' CGCCACACCAAGATCCACC 3'

                 rev: 5' AGCCCCCAGGACCAGAGG 3'

β-actin   fwd: 5' GCTGTGCTATGTTGCTCTAG 3'

                 rev: 5' CGCTCGTTGCCAATAGTG 3'

Elf1a   fwd: 5' AAAATTGGCTACAACCCCGA 3'

                 rev: 5' CCAACCAGAAATTGGCACAA 3'

Tbp rat   fwd: 5' TGCACAGGAGCCAAGAGTGAA 3'

                 rev: 5' CACATCACAGCTCCCCACCA 3'

ChIP primers

Egr1 1   fwd: 5' CGTGCTGTTCCAGACCCTTGAAAT 3'

                rev: 5' TAGTTGTCCATGGTGGGTGAGTGA 3'

Egr1 2   fwd: 5' CCGGAACAGACCTTATTTGGGCA 3'

rev: 5' AAGTTCTGCGCGCTGGGATCTCT 3'

Egr1 3   fwd: 5' AGCCCAGGATGACGGCTGTAGAA 3'

rev: 5' TCAGGCTCCTGGAAAGCCTAGTAT 3'

Egr1 N4   fwd: 5' TTTCGATTCTGGGTGGTGCATTGG 3'

rev: 5' TGCGAGCTGGGCTAGGGAA 3'

Egr1 5   fwd: 5' CTCCCGAAATACAACCAGAGACCT 3'

rev: 5' TTGGCTACTGGTTCTTGGGACACT 3'

Egr1 N6   fwd: 5' TCCCTTTGGGTTGCTTCGGAGATA 3'

rev: 5' ACGAACCCTGCCCTGTCCTAAAGA 3'

Egr1 N7   fwd: 5' TGGAACCCTGGTTCATGTCTGGAT 3'

rev: 5' AGGGTAACACTTCCTTCACAGAGC 3'

Egr1 10   fwd: 5' GAGAAAGGAATCCATTGCCTCGTG 3'

rev: 5' TCTTCTGTGTACCCAGCACCTGTT 3'

Egr1 11   fwd: 5' ACCAGGTGCTAGATTCACCAAGTC 3'

rev: 5' GGTGAGCGTGTTTCCGAGTGAAAT 3'

Egr1 N12 fwd: 5' ACAGAGACCTTCCTGCCTGTCTTT 3'

rev: 5' CCACCAAGAAGCTTGCTGTTGCAT 3'

Egr1 N14 fwd: 5' CTGTTGTTGGTGACTTGGCTCCTT 3'

rev: 5' CCCAGGAAGAGAGATAACCTAGCA 3'

Egr1 15 fwd: 5' AAAGAGAGTCACTTCCTGAGCCCT 3'

rev: 5' GCTGGTGGCACACTTCTGTTTACT 3'

Egr1 16 fwd: 5' ACTACCATTCGATCTGCTTCAGGC 3'

rev: 5' TGGGTTTCGTCAGCACCCACAT 3'

eIF4E fwd: 5' AGAGGCCTAAATCCAACTCGGCA 3'

rev: 5' AAGGCAATACTCACCGGTTCCACA 3'

Egr2 fwd: 5' CTCCAGTCAGTTCTACCATCATCG 3'

rev: 5' TCGGGTTATGCAAATAGAGGTCCC 3'

qChIP primers

Egr1 set1 fwd: 5' CTAACCATCACAAGAACCAACAG 3'

rev: 5' ACTAATGGCAGGGTCACTTTC 3'

Egr1 set2 fwd: 5' GAGAAAGGAATCCATTGCCTCGTG 3'

rev: 5' TCTTCTGTGTACCCAGCACCTGTT 3'

Egr1 set3 fwd: 5' ACTACCATTCGATCTGCTTCAGGC 3'

rev: 5' TGGGTTTCGTCAGCACCCACAT 3'

Results

Differential induction of Egr1 and Egr2 by activated c-Myc depending on the presence of ARF

To examine whether there are c-Myc target genes that are differentially regulated due to the presence of ARF, we performed microarray analyses comparing c-MycER (Estrogen Receptor)-inducible gene expression between DKO (*p53/ARF* <u>d</u>ouble <u>k</u>nock<u>out</u>) MEFs having no ARF with *p53*$^{-/-}$ MEFs having high levels of endogenous ARF. We used genetically defined MEFs without *p53* to avoid effects caused by ARF activation of p53. Gene expression was analyzed two hours following activation of the chimaeric c-MycER protein by hydroxytamoxifen (OHT) to enrich for direct targets. Interestingly, through these microarray analyses we identified all of the Egr transcription factor family members as c-Myc-responsive genes (Table 2-1). Two of the family members (*Egr2* and *Egr3*) were up-regulated by c-Myc in DKO-MycER MEFs, but not in *p53*$^{-/-}$ MycER MEFs, suggesting that they are induced by c-Myc only in the absence of ARF, which was previously observed for the canonical c-Myc target genes *nucleolin, eIF4E, and htert* (Qi et al. 2004). Surprisingly, the other two family members (*Egr1* and *Egr4*) were up-regulated by c-Myc in *p53*$^{-/-}$ but not DKO-MycER MEFs, suggesting that their inductions are dependent on ARF expression. The differential regulation of *Egr1* and *Egr2* in *p53*$^{-/-}$ and DKO-MycER MEFs following c-Myc activation was verified by real-time RT-PCR (Fig. 2-1*A*). Further, time course analyses of *Egr1* induction by c-MycER

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Egr family member	DKO MycER (No ARF) 2 hr OHT	p53-/- MycER (High ARF) 2 hr OHT
Egr2	3.87	-
Egr3	3.73	-
Egr1	-	3.17
Egr4	-	6.87

Table 2-1. Egr factors are potential ARF-dependent targets of c-Myc. Microarray analysis of the *Egr* family members in *p53-/-* and DKO MycER MEFs. The values represent the relative expression after two hours of Myc activation in the indicated cell line compared to mock treatment in the same cells.

demonstrated that *Egr1* mRNA levels were induced by c-Myc within 1-2 hours in the $p53^{-/-}$ MycER (high levels of ARF) MEFs, but not in the DKO-MycER (no ARF) MEFs (Fig. 2-1*B*). Conversely, Egr2 mRNA levels were induced in the DKO, but not the $p53^{-/-}$ MycER MEFs with a maximal induction approximately at 2 hours (Fig 2-1*C)*. Additionally, we confirmed the induction of *Egr1* mRNA levels by c-Myc in another cell line, c-*myc*$^{-/-}$ Rat1 fibroblasts (H016) expressing c-MycER. The c-*myc*$^{-/-}$ cell line was chosen to remove the effects of endogenous c-Myc on the control of *Egr1* mRNA levels and because they have low levels of ARF expression (Qi et al. 2004). Again, real-time RT-PCR confirmed that *Egr1* levels are increased by OHT activation of c-MycER, but not by mock treatment in cells that have ARF expression (Fig 2-2), suggesting that this induction is not cell-type specific.

ARF is necessary for c-Myc to induce Egr1

Differential regulation of *Egr1* and *Egr2* by c-Myc in the presence of ARF suggests different mechanisms of induction and biological outcomes. Since ARF is essential for p53-independent c-Myc-induced apoptosis, the ARF-dependent *Egr1* putative target gene was further characterized. To determine the necessity of ARF for induction of *Egr1* by c-Myc, ARF protein expression was silenced by siRNA, as confirmed by immunoblot analysis, in $p53^{-/-}$ MycER MEFs (Fig. 2-3, lower panel). *Egr1* induction by c-Myc activation was reduced at 2 hours, the time of maximal induction, in the ARF siRNA treated cells compared to cells treated with control siRNA (Fig. 2-3, *upper panel*), suggesting that ARF is

34
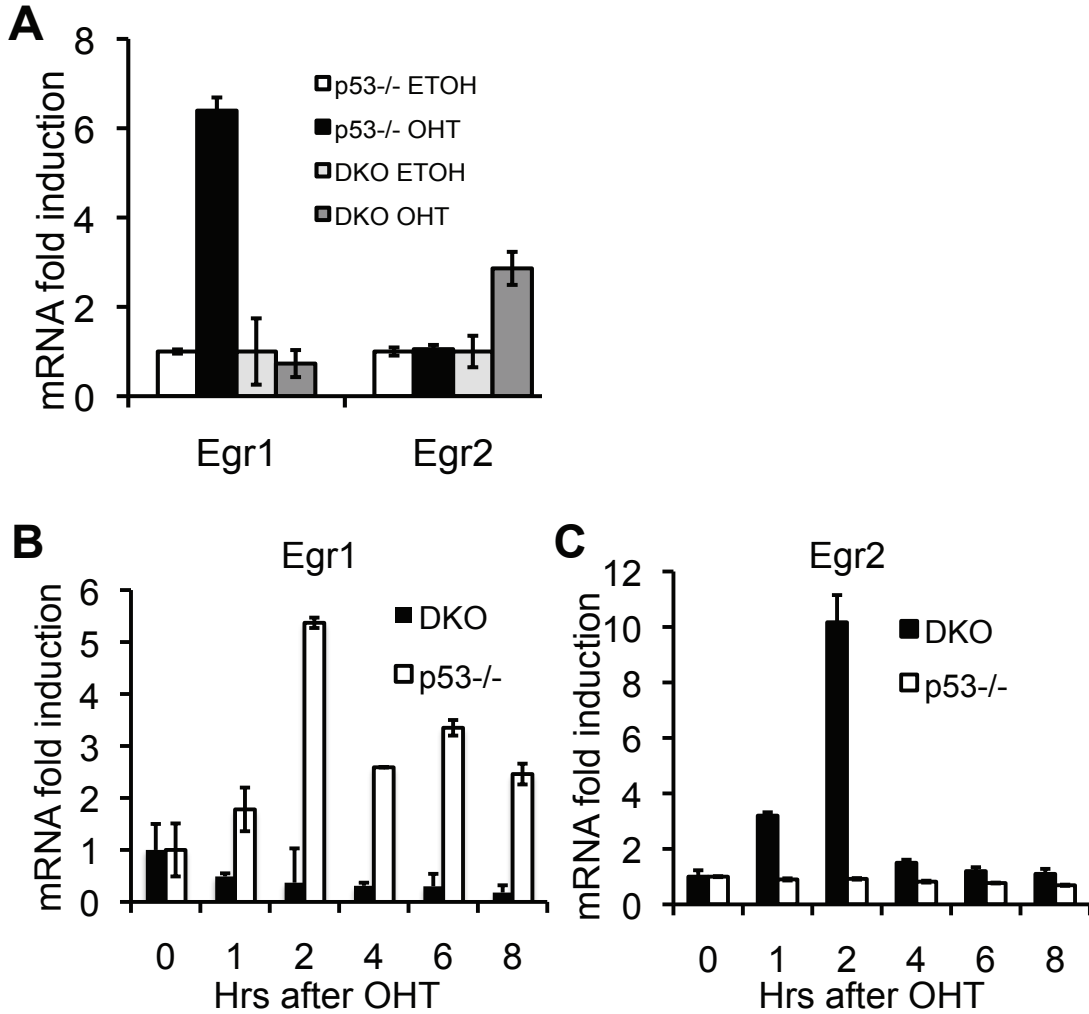
Figure 2-1. c-Myc differentially induces Egr1 and Egr2 depending on the presence of ARF. (A) Microarray verification by real-time RT-PCR of Egr1 and Egr2 mRNA levels following 2 hrs of OHT treatment in *p53−/−* and DKO MycER MEFs. (B and C) Time course analyses following MycER activation of mRNA levels of Egr1 (B) and Egr2 (C) in p53−/− and DKO MycER MEFs (± OHT) as measured by real-time RT-PCR. Results are reported as the mean of the relative mRNA levels of Egr1 or Egr2 to β-actin at each time point normalized to time 0 to give the relative fold induction ± standard deviation.
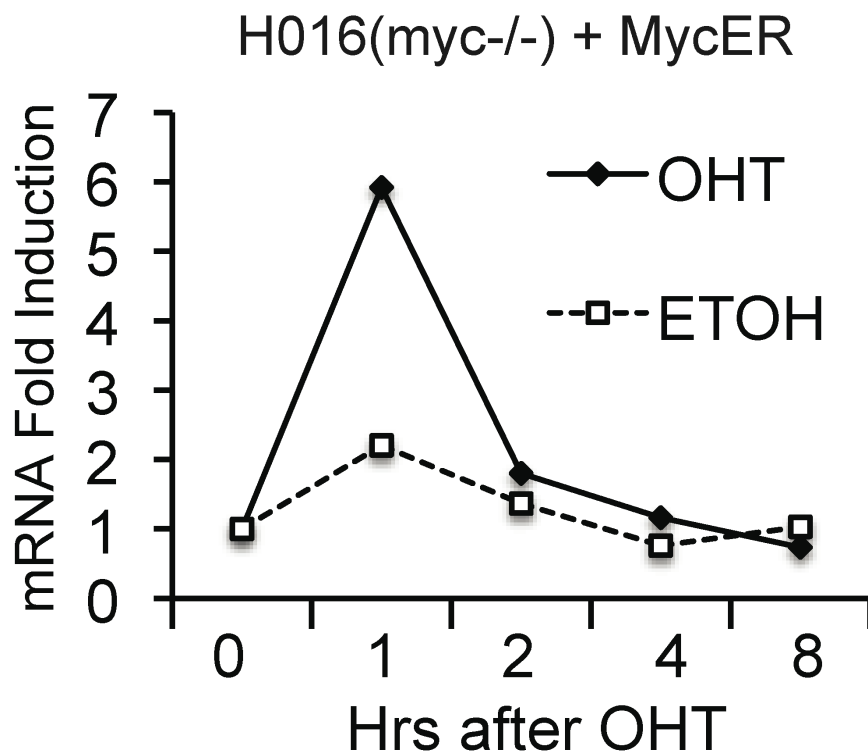
Figure 2-2. c-Myc induces *Egr1* mRNA levels in Rat1 cells. Real time RT-PCR analysis of mRNA levels of *Egr1* mRNA in *c-myc*^{-/-} (HO16) cells expressing MycER following activation or mock treatment with OHT and ethanol respectively. Results are reported as the mean of the relative mRNA levels of Egr1 to β-actin at each time point normalized to time 0 to give the relative fold induction ± standard deviation.

necessary for c-Myc driven *Egr1* induction. This reinforces the results in the genetically defined fibroblasts by providing evidence that the difference between the inductions of *Egr1* in the DKO and p53-/- MEFs by activation of c-Myc was due to the lack or presence of ARF and not a different accumulated genetic abnormality.

<u>*Egr1 is a direct target gene of c-Myc*</u>

The relatively rapid induction of *Egr1* mRNA in *p53$^{-/-}$* MycER MEFs by activated c-MycER suggests that it is a direct target. To test if c-Myc can induce *Egr1* mRNA levels in the absence of protein synthesis, *p53$^{-/-}$* MycER MEFs were treated with cycloheximide to inhibit translation before c-MycER activation. Cycloheximide treatment alone caused *Elf1a,* a gene not regulated by c-Myc (Knoepfler et al. 2006), and *Egr1* mRNA levels to stabilize (data not shown), as previously observed with many transcripts (Lemaire et al. 1988). However, activation of c-MycER in the presence of cycloheximide increased *Egr1* levels by ~4 fold in two hours over cycloheximide treatment alone, suggesting that c-Myc can induce *Egr1* without de novo protein synthesis (Fig. 2-4, *upper panel*), even with relatively modest levels of c-MycER remaining (Fig. 2-4, *lower panel*). In contrast, activated c-MycER did not enhance levels of *Elf1a*, a gene known not to be regulated by c-Myc, in the presence or absence of cycloheximide (Fig. 2-4). This suggests that *Egr1* is a direct transcriptional target of c-Myc. However, c-Myc may also control the levels of *Egr1* by other mechanisms, such as inhibition of mRNA degradation. To determine whether c-Myc influenced *Egr1* mRNA
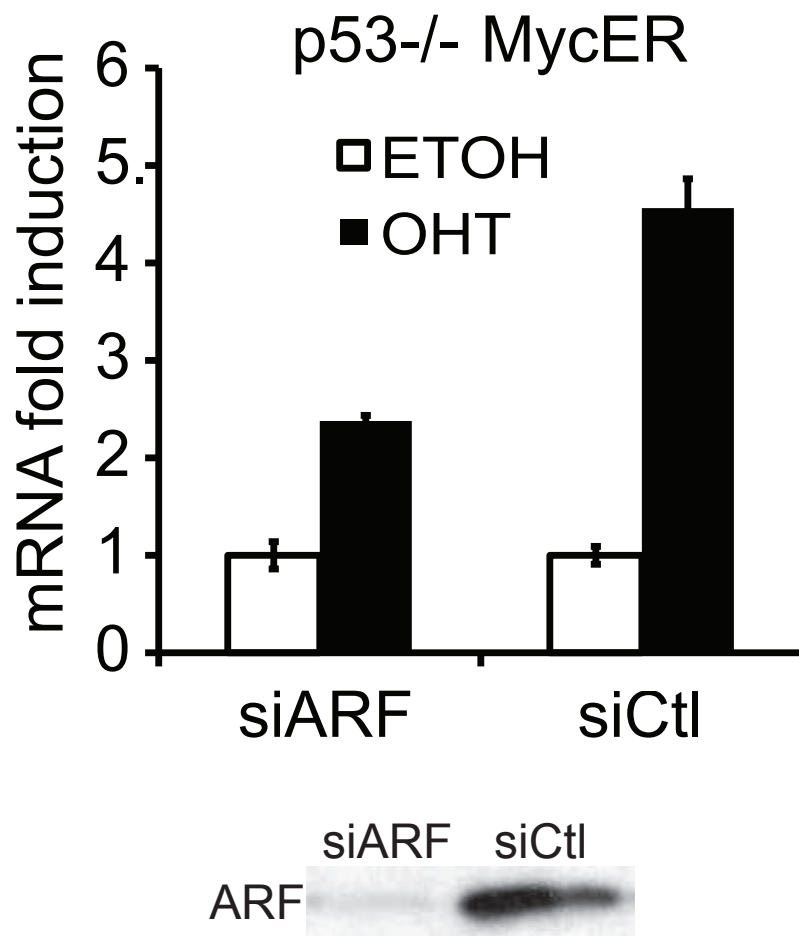
Figure 2-3. ARF is necessary for the c-Myc induction of *Egr1*. (*top panel*) Real-time RT-PCR analysis of *Egr1* mRNA levels following c-Myc activation with OHT for two hours in *p53$^{-/-}$* MycER MEFs treated first for 48 hours with either siARF or control SMART pool. Results are reported as the mean of the relative mRNA levels of Egr1 to β-actin at each time point normalized to time 0 to give the relative fold induction ± standard deviation. *(lower panel)* Immunoblot showing ARF protein levels following siRNA treatment.
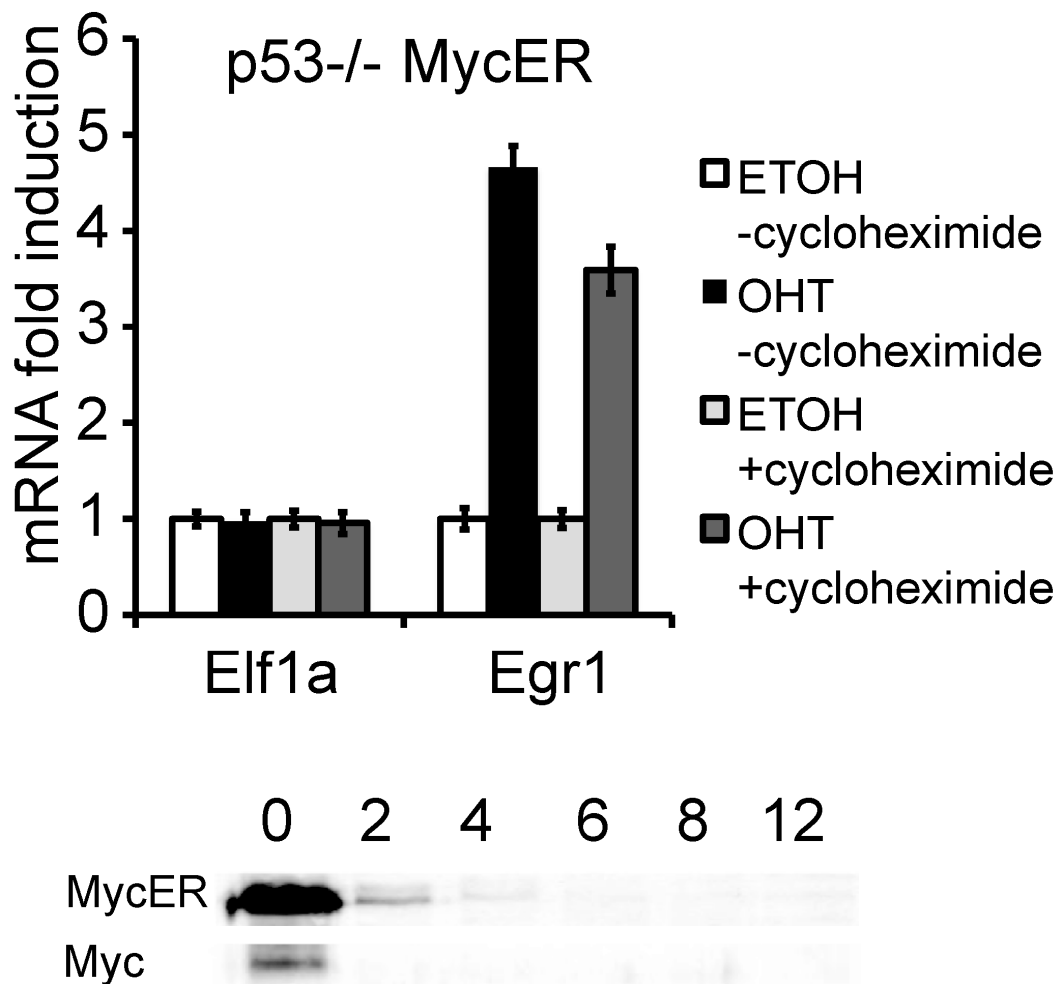
Figure 2-4. c-Myc induces *Egr1* mRNA in the absence of protein synthesis. Real-time RT-PCR analysis of *Egr1* or *Elf1a* in *p53$^{-/-}$*MycER MEFs (+/-OHT 2hrs) treated first with cycloheximide—or mock treated with DMSO—for 30 minutes to inhibit protein synthesis. Results are graphed as the mean of the relative mRNA fold induction over mock treated (ETOH) controls +/- s.d. Immunoblot shows c-Myc and c-MycER protein following cycloheximide addition.

degradation, real-time RT-PCR analysis was performed following transcriptional inhibition with actinomycin D treatment in *c-myc-/-* (H016) cells expressing c-MycER. The decay of *Egr1* mRNA levels was the same with c-MycER activation or mock treatment (Fig. 2-5).   Therefore, activation of c-MycER primarily increases transcription of *Egr1* in cells with ARF expression, rather than enhancing *Egr1* mRNA stability.

Egr1 protein induction by c-Myc

In order to demonstrate that the regulation of *Egr1* mRNA levels by activated c-Myc correlates with protein levels, we examined the effects of c-Myc activation on Egr1 protein expression through Western Blotting (WB). Immunoblot analyses revealed that activated c-MycER induced the expression of Egr1 protein in *p53*$^{-/-}$ MycER MEFs within 3-4 hours, with maximal induction by 8 hours (Fig. 2-6A), but failed to induce Egr1 levels in *ARF*$^{-/-}$ MycER MEFs (Fig. 2-6B).   Egr1 levels were also not induced by OHT treatment alone in *p53*$^{-/-}$ MEFs with vector (Fig. 2-6D), nor by activated c-MycER in DKO MEFs (Fig. 2-6C), indicating that Egr1 protein levels are only increased by activated c-Myc in the presence of ARF.
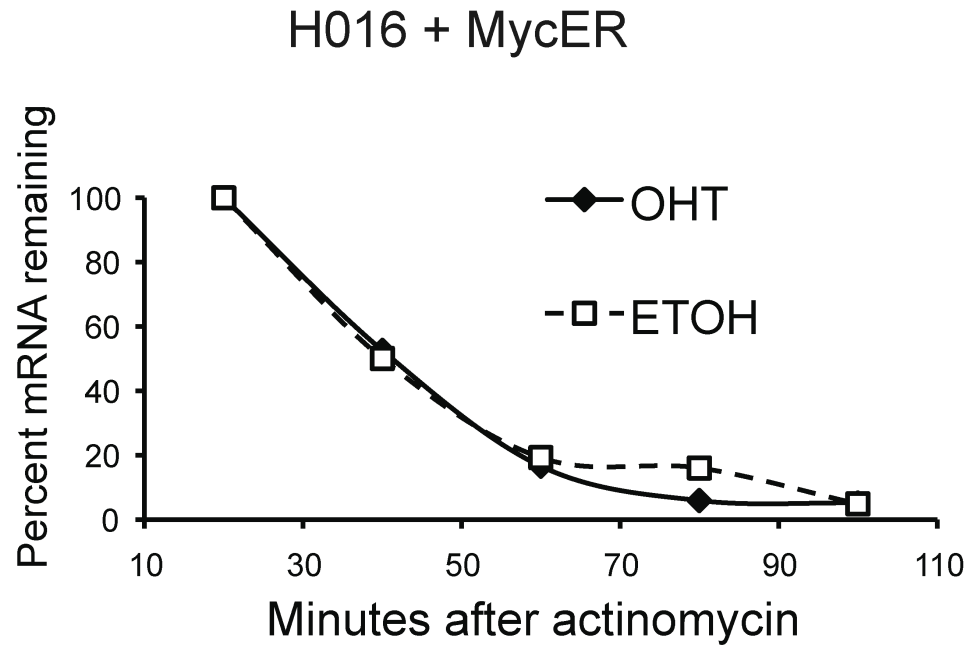
Figure 2-5. c-Myc does not influence the stability of *Egr1* mRNA. Real time RT-PCR analysis of the decay of *Egr1* mRNA levels in HO16 cells expressing c-MycER (+/-OHT) at the indicated times following 15 minutes of actinomycin D treatment to prevent transcription. Results are reported as the mean of the relative mRNA levels of Egr1 to β-actin at each time point divided by the original amount at time 0 and multiplied by 100 to give the percent mRNA remaining.
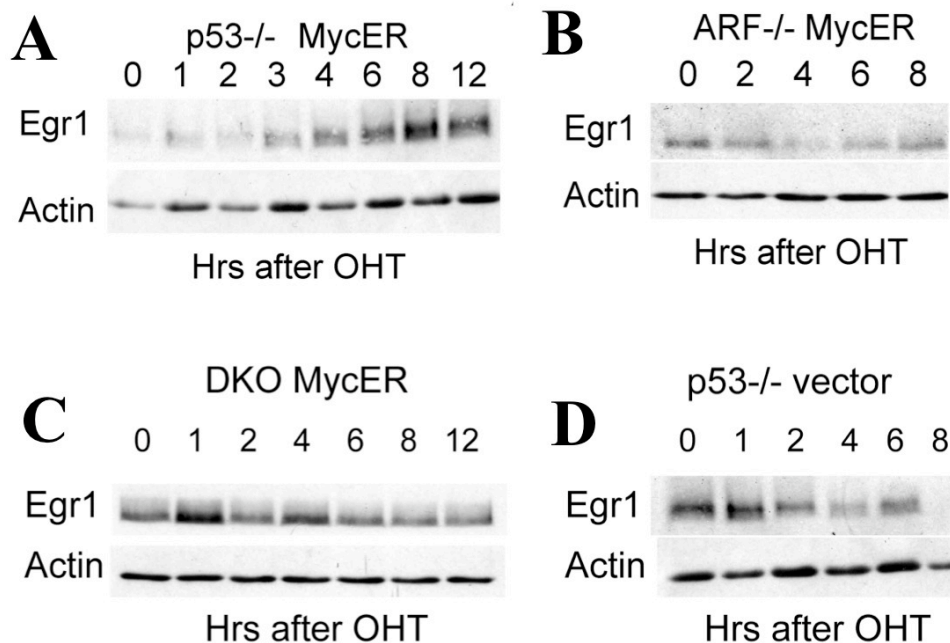
Figure 2-6. Activation of c-Myc induces Egr1 protein levels only in the presence of ARF. (*A-C*) Western blot (WB) analyses of Egr1 protein levels following OHT activation in *p53$^{-/-}$* MycER MEFs (*A*), *ARF$^{-/-}$* MycER MEFs (*B*), and DKO MycER MEFs. (*D*) WB analyses of Egr1 protein levels after the indicated times following OHT treatment in *p53$^{-/-}$* MEFs with vector instead of MycER to demonstrate that Egr1 protein is not induced by OHT treatment alone. A WB analysis of β-actin expression demonstrates equal loading for each cell type and time point.

Endogenous c-Myc regulates Egr1 levels.

Thus far in this chapter, we have shown that the activation of an exogenously expressed c-MycER directly induces the mRNA and protein levels of Egr1 only in cells with ARF. To determine whether endogenous c-Myc also regulates _Egr1_ expression, logarithmically growing _c-myc_[-/-] (HO16) and parental wild-type (TGR) Rat1 cells were harvested and the relative expression of _Egr1_ and the negative control _Elf1a_ was determined with real-time RT-PCR. _Egr1_ was expressed substantially higher in the cells with c-Myc, unlike _Elf1a_, which was expressed equally in the two cell lines (Fig. 2-7_A_), suggesting that _Egr1_ expression is controlled by endogenous c-Myc.

Since the _Egr_ genes are also known to be immediate early genes like c-Myc that are induced by serum (Christy and Nathans 1989), we examined the influence of endogenous c-Myc expression on the serum induction of _Egr1_. The TGR and HO16 cells were made quiescent by serum deprivation and then the expression of _Egr1_ was followed after serum stimulation. _Egr1_ levels were substantially increased and sustained to a greater extent in the cells with c-Myc compared to cells without c-Myc (Fig. 2-7_B_). These results suggest that _Egr1_ gene expression is controlled by endogenous c-Myc and that c-Myc is necessary for the full serum induction of _Egr1_.
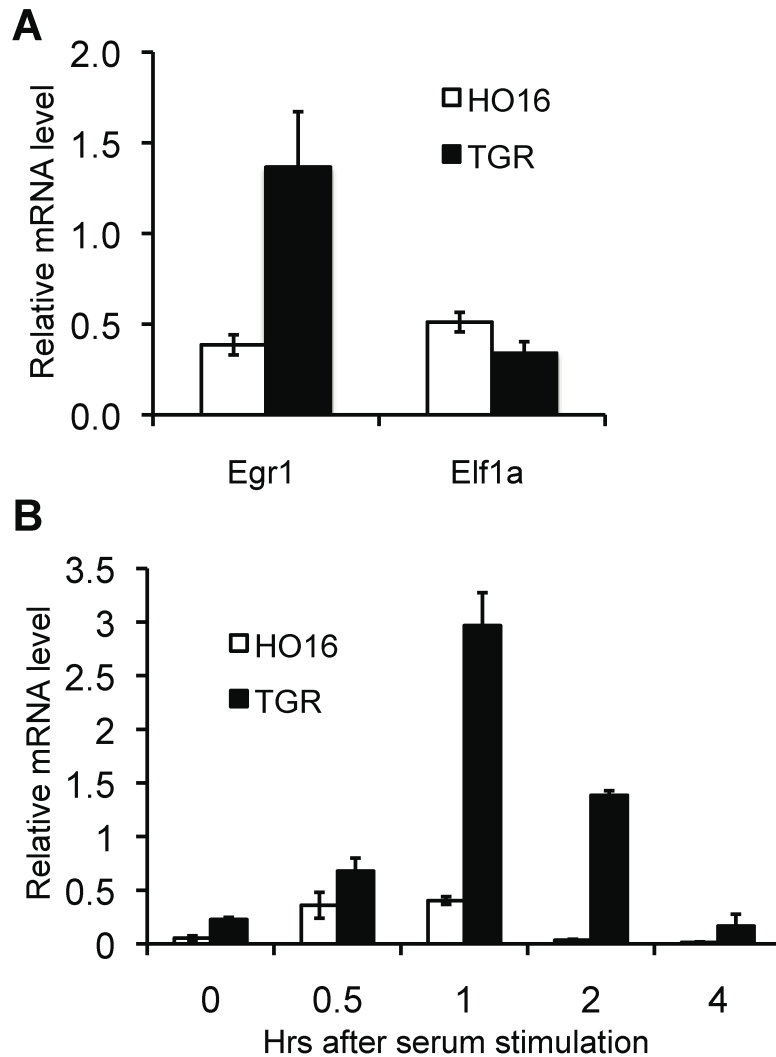
Figure 2-7. Endogenous c-Myc is necessary for the full expression and induction of _Egr1_. (_A_) Real-time RT-PCR analysis of _Egr1_ mRNA levels in logarithmically growing _c-myc^{-/-}_ (H016) and parental (TGR) rat1 cells. (_B_) Real-time RT-PCR analysis of _Egr1_ mRNA levels following serum stimulation of serum-starved H016 and TGR cells. Results are graphed as the mean of the relative mRNA levels of _Egr1_ to _TATA binding protein_ (_Tbp_) +/- s.d.

c-Myc and ARF are recruited to the Egr1 promoter

If _Egr1_ is a direct target as suggested by the preceding data, then c-Myc must be recruited to the _Egr1_ promoter. However, since the proximal promoter of _Egr1_—at least 15 kilobases upstream and downstream of the transcriptional start site—does not contain any canonical E-box Myc sites (CACGTG), we used a scanning chromatin immunoprecipitation (ChIP) approach to identify the c-Myc binding region. ChIP was performed using _p53_$^{-/-}$ MycER MEFs with partially overlapping primers spanning 5 kilobases (kbs) upstream of the start site. We found that activated c-Myc was recruited to one region of the _Egr1_ promoter (-904 to -1319) (Fig. 2-8). As a positive control, primers for the c-Myc canonical target gene _eIF4E_ promoter were used (Fig. 2-8). Since _Egr1_ induction by c-MycER is dependent on the presence of ARF, we determined whether ARF is also recruited to the _Egr1_ promoter. ChIP analysis revealed that ARF was recruited to the same region as c-Myc upon OHT activation, but not to other regions of the promoter (Fig. 2-9_A_, right panel). However, ARF was not detected at the _Egr1_ promoter without OHT activation (Fig. 2-9_A,_ left panel), suggesting that ARF is only recruited to the _Egr1_ promoter upon c-Myc activation, as previously observed with canonical target genes (Qi et al. 2004). This observation agrees with the finding that ARF is mostly nucleolar in _p53_$^{-/-}$ MEFs until c-Myc activation causes ARF to be translocated to the nucleoplasm (Qi et al. 2004; Gregory et al. 2005). Conversely, to determine whether c-Myc can be recruited to the _Egr1_ promoter without ARF we used DKO-MycER MEFs. ChIP

45
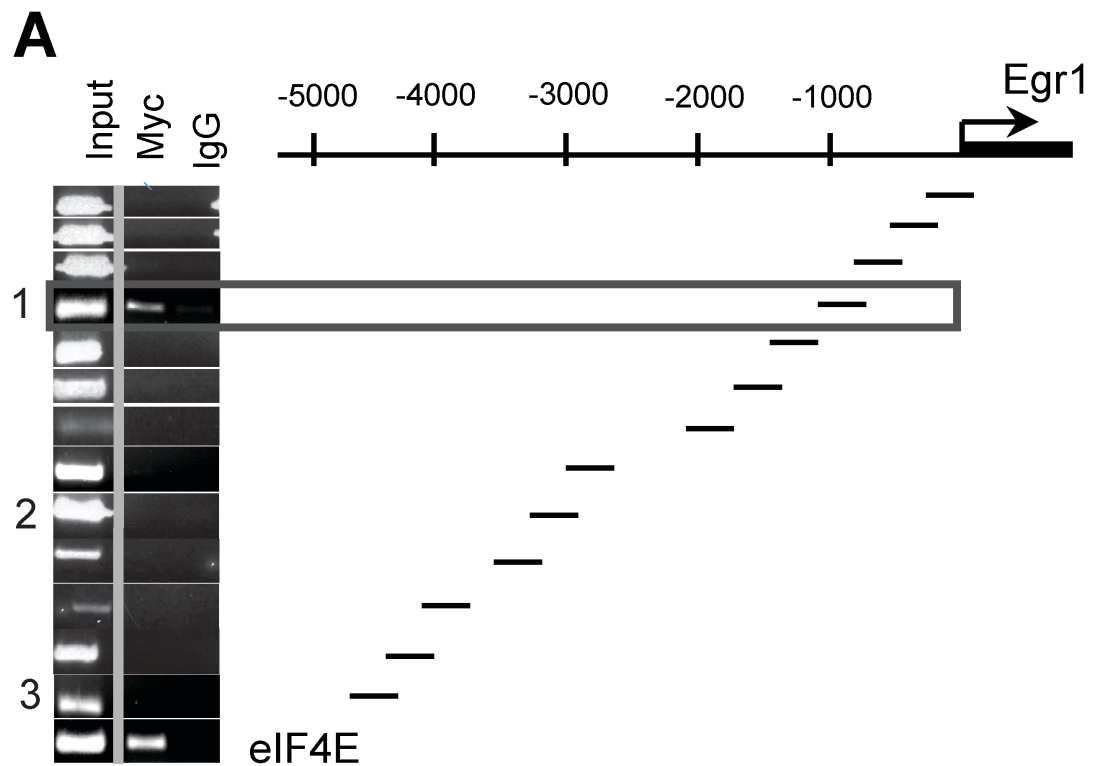
Figure 2-8. c-Myc is recruited to the *Egr1* promoter at a noncanonical binding site. Chromatin prepared from *p53^-/-^* MycER MEFs (+OHT) was subjected to IP using anti-Myc or IgG followed by PCR using the indicated panel of partially overlapping primers spanning 5kb of the *Egr1* promoter. The box indicates a region (-904 to -1319) to which c-Myc is recruited. *eIF4*E primers spanning an established EMS binding sequence were used as a positive control. The numbers on the left indicate primer pairs amplifying a specific region of the promoter referred to in Figure 2-9.

analysis revealed that activated c-MycER was recruited to the *Egr1* promoter without ARF (Fig. 2-9*B*, right panel), suggesting that although ARF cannot associate with the Egr1 promoter without Myc that Myc can associate without ARF.

To quantitatively determine the relative amounts of c-Myc and ARF recruited to the *Egr1* promoter under different conditions, we used real-time PCR to analyze the ChIP assays. We verified that c-Myc and ARF were both recruited to the *Egr1* promoter after MycER activation, but ARF was not detected without activation of c-Myc in $p53^{-/-}$ MycER MEFs (Fig. 2-9*C*). Without ARF, activated c-MycER was still recruited to the *Egr1* promoter in DKO-MycER MEFs (Fig. 2-9*D*), but at lower levels compared to $p53^{-/-}$ MycER MEFs (Fig. 2-9*C*), suggesting that ARF enhances the recruitment of c-Myc to the *Egr1* promoter. Without OHT activation we detected low levels of c-Myc at the *Egr1* promoter in *p53$^{-/-}$* MycER MEFs (Fig. 2-9*C*), but not in DKO-MycER MEFs (Fig. 2-9*D*), suggesting that c-MycER is partially active without OHT in the $p53^{-/-}$ MycER MEFs. Taken together, the results suggest that the interaction of activated c-Myc with ARF enhances the recruitment of c-Myc to the *Egr1* promoter, but that ARF alone cannot be detected.

In contrast to *Egr1*, *Egr2* is induced by c-MycER in DKO-MycER MEFs and the *Egr2* promoter contains three putative canonical CACGTG sites. ChIP analysis using DKO-MycER MEFs revealed that activated c-Myc was recruited to the canonical CACGTG in the *Egr2* promoter located at -2400 (Fig. 2-10*A*), but not to the other two CACGTG sequences. As a positive control, primers for the

47

Figure 2-9. **ARF is recruited with c-Myc to the *Egr1* promoter**. (*A*) ChIP performed as in Fig. 2-8 except MycER was either activated with OHT treatment (*right panel)* or mock treated with ethanol *(left panel)* for 6 hours and chromatin was subjected to IP with anti-Myc, anti-ARF, or IgG. (*B*) Chromatin prepared from DKO MycER MEFs (+/-OHT) was subjected to IP using anti-Myc, IgG, or no antibody followed by PCR using the indicated primers that span the binding site in the *Egr1* promoter. (*C*) ChIPs as in (*A)* in p53$^{-/-}$ MycER MEFs +/- OHT except purified DNA was subjected to real-time PCR. Results are reported as the mean of the percent of input +/- s.d. The numbers below the x-axis indicate the region of the *Egr1* promoter being amplified as demonstrated in Fig. 2-8 and *(A). (D)* Quantitative ChIPs as in *(C)* but in DKO MycER MEFs +/- OHT.

A

ETOH   p53-/- MycER   OHT

B

ETOH   DKO MycER   OHT

C
p53-/- MycER

D
DKO MycER
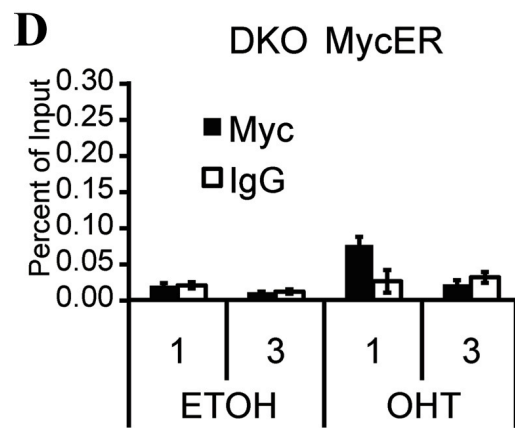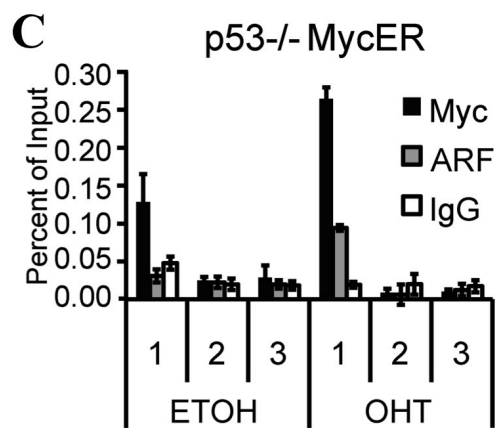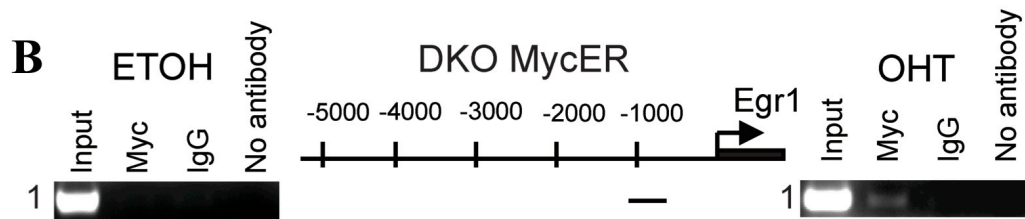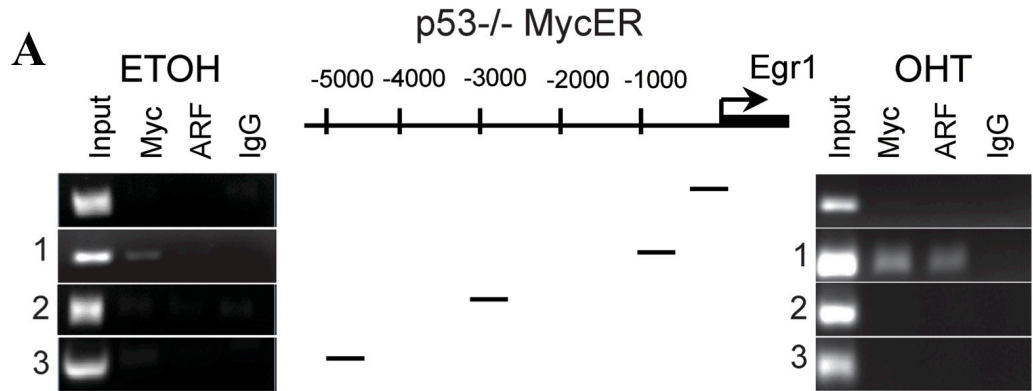
established c-Myc target *eIF4E* promoter were used (Fig. 2-10*B*). This suggests that like Egr1, Egr2 is also a direct target of c-Myc.

<u>*c-Myc and ARF regulate the Egr1 promoter*</u>

　　　　To further examine the regulation of the *Egr1* promoter by c-Myc and ARF we performed luciferase assays with a 2.5kb fragment of the *Egr1* promoter containing the putative c-Myc binding site identified above. The *Egr1* promoter was induced in *p53$^{-/-}$* MEFs overexpressing c-Myc (Fig. 2-11*A*), demonstrating that c-Myc activates the *Egr1* promoter. In contrast, the *Egr1* promoter was slightly inhibited in DKO MEFs expressing c-Myc (Fig 2-11*B*), confirming the dependence on ARF for the c-Myc activation of the *Egr1* promoter. As previously reported (Qi et al. 2004), the *htert* promoter and the 4XEMS promoter were both induced by c-Myc in DKO MEFs (Fig. 2-11*B*), but the *htert* promoter induction was blocked in *p53$^{-/-}$* MEFs with high ARF expression (Fig. 2-11*A*). In addition, the *Egr1* promoter was induced by c-Myc in Rat1a cells (Fig. 2-11*C*), confirming that this regulation is not cell-type specific. To determine whether the *Egr1* promoter is regulated by endogenous c-Myc, the activity of the *Egr1* luciferase promoter was compared between TGR (*c-myc* WT) and HO16 (*c-myc$^{-/-}$*) Rat1 cells. The *Egr1* promoter was more active in TGR cells than in the HO16 cells, similar to the levels observed with the artificial canonical 4XEMS promoter (Fig. 2-11*D*). In comparison to the endogenous *Egr1* expression induced by c-MycER (Fig. 2-11B) the activity of the *Egr1* promoter induced by c-Myc in transient luciferase assays is relatively modest, suggesting that the chromatin environment
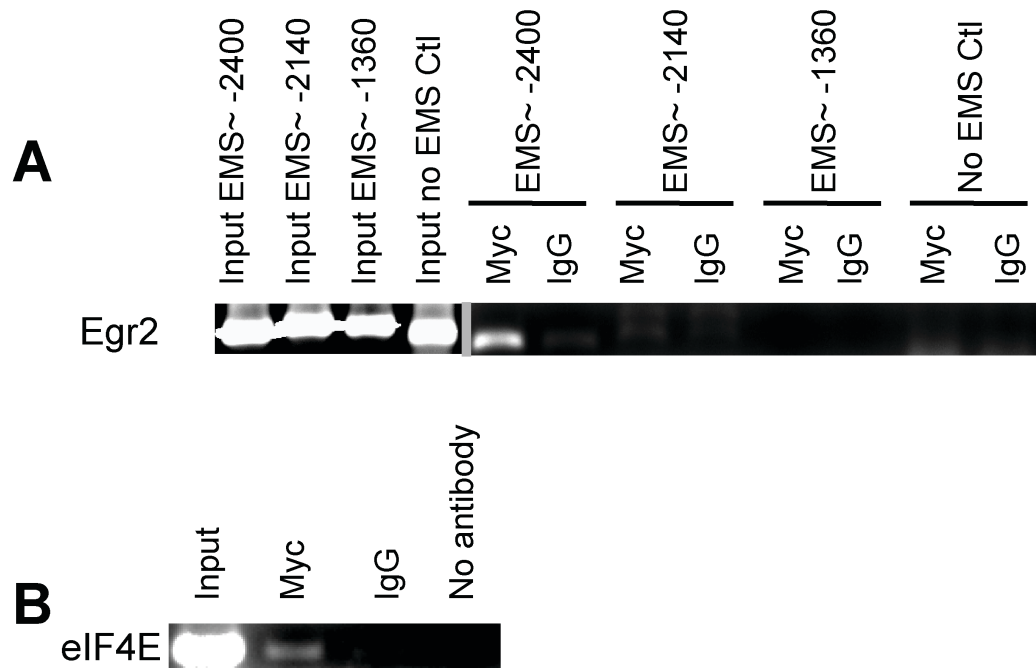
Figure 2-10. c-Myc is recruited to the *Egr2* promoter at a canonical binding site. *(A-B)* Chromatin prepared from DKO MycER MEFs (+OHT) was subjected to ChIP using anti-Myc or IgG followed by PCR amplification using primers spanning the EMS of *(A) eif4E* and the *(B)* 3 EMS sites in the *Egr2* promoter gel.

Figure 2-11. c-Myc activates the *Egr1* promoter in the presence of ARF. (*A-B*) Reporter constructs for *Egr1* (2.5kb upstream), *htert*, and 4XEMS promoters were transiently transfected into *p53$^{-/-}$* MEFs (*A*) and DKO MEFs (*B*) with exogenous c-Myc or vector control and a thymidine kinase renilla luciferase (pRL-TK) transfection control. The mean of the relative luciferase reporter activity to pRL-TK is reported +/- s.d. (*C)* The *Egr1* reporter construct and pRL-TK were transfected into Rat1A cells that constitutively express c-Myc or vector. Relative luciferase activity was determined as above and then normalized to the vector control. *(D)* The *Egr1* reporter construct or 4xEMS were transfected into *c-myc-/-* (H016) and WT (TGR) cells and relative luciferase activity was determined and results were normalized to the activity in *c-myc-/-* cells.
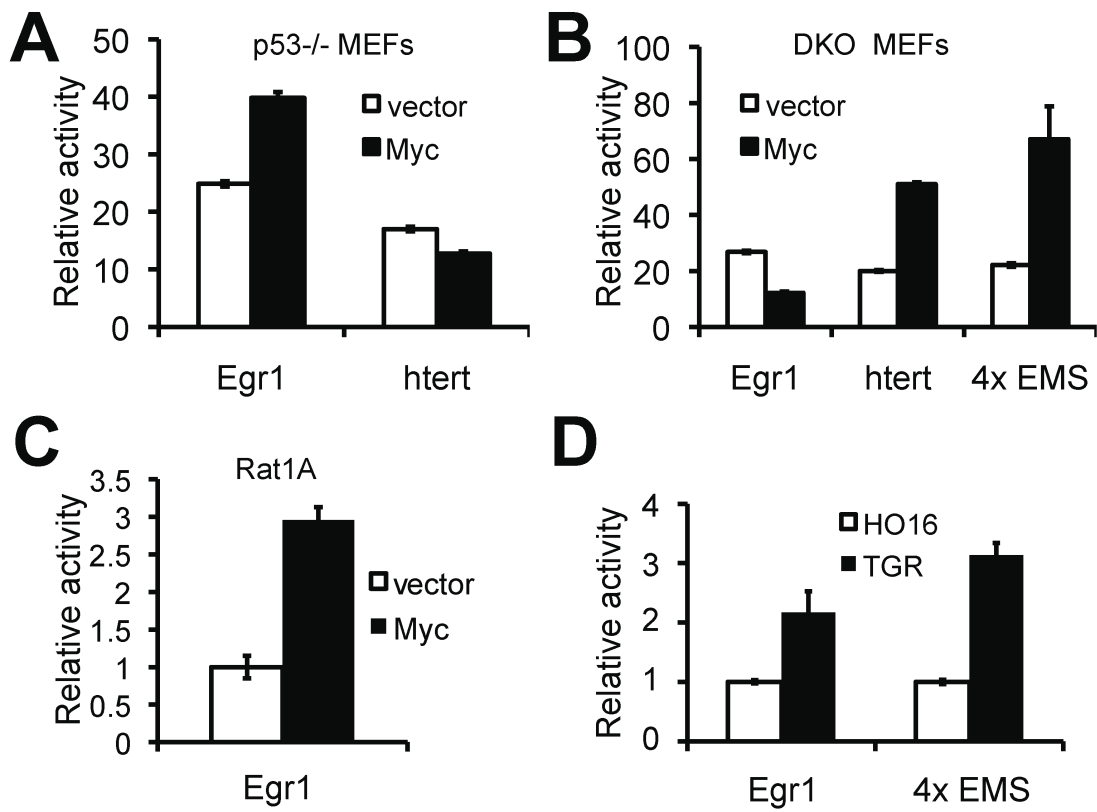
and/or the different assay conditions influence the regulation of the *Egr1* promoter by c-Myc. Taken together these results suggest that both exogenous and endogenous c-Myc induce the *Egr1* promoter by a novel ARF-dependent noncanonical transcriptional mechanism, unlike other c-Myc target gene promoters containing the canonical CACGTG binding site that are inhibited by ARF.

Loss of N-terminal ubiquitination mimics ARF control of c-Myc-Induced Egr1 expression.

In collaboration with Dr. Q. Zhang and Dr. M. Gregory, we observed that the interaction with ARF reduced N-terminal ubiquitination of c-Myc (Zhang and Gregory, unpublished observations). Because there is evidence from several labs that ubiquitin plays a role in transcription beyond its function in proteolysis (Kim et al. 2003; von der Lehr et al. 2003) and because our data demonstrate that ARF binding simultaneously influences c-Myc ubiquitination and transcriptional regulation, we hypothesized that ARF may affect c-Myc transcriptional functions by preventing N-terminal ubiquitination. To determine whether inhibiting ubiquitination of the N-terminal transcriptional regulatory domain (TRD) affects c-Myc mediated transcription, we generated a full-length expression vector of c-MycER that has all six lysines (51, 52, 127, 144, 149 and 158) in the TRD changed to arginines (Figure 2-12), which we termed c-MycN6KR-ER.

We first examined the effects of our mutant on c-Myc ubiquitination, and the c-MycN6KR-ER is less ubiquitinated compared with wild-type c-Myc. Next, we tested the effects of inhibiting N-terminal ubiquitination on c-Myc transcription in MEFs without ARF. In DKO MEFs wild-type c-MycER induced the well-characterized canonical c-Myc target genes *nucleolin*, *eIF4E* and *Rcl*, in the typical fashion, whereas c-MycN6KR-ER, which lacks the 6 lysines in the N-terminal TRD, was unable to induce their expression (Figure 2-13A-C) much like wild-type c-Myc cannot induce canonical targets when interacting with ARF. In marked contrast, c-MycN6KR-ER was able to induce *Egr1* expression, even without ARF, to significantly higher levels than wild-type c-MycER (Figure 2-13D). Taken together, these results suggest that the loss of c-Myc N-terminal ubiquitination mimics the effects of the ARF interaction on c-Myc-driven transcription. When either c-Myc binds to ARF, or in the absence of ARF, when N-terminal ubiquitination of c-Myc is prevented through mutation, then c-Myc no longer induces canonical target genes, but instead induces *Egr1*. Therefore, this suggests that a possible mechanism for ARF control on the transcriptional activities of c-Myc is through preventing the ubiquitination of the TRD of c-Myc.

Discussion

Our results suggest that there is a novel ARF-dependent mechanism of c-Myc transcriptional regulation, whereby ARF binds with c-Myc at promoters and selectively and differentially induces c-Myc target genes. Previously, we
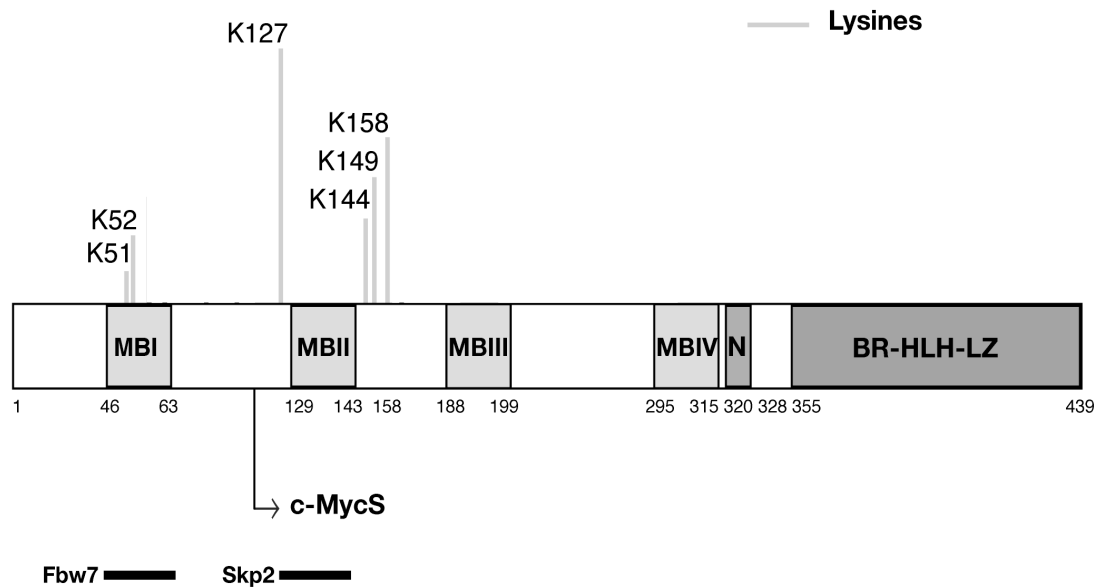
Figure 2-12. Diagrammatic representation showing c-Myc protein structure and lysines mutated in MycN6KR. Sites of N-terminal lysine residues mutated in MycN6KR are shown. The sites of interactions of known N-terminal E3 ubiquitin ligases Fbw7 and Skp2 are indicated below the diagram. Numbers under diagram indicate relative amino acid position of specific domains. Notable sequence motifs are denoted on the diagram. The relative position of an alternative translation initiation site, c-MycS, is shown on the bottom of the diagram.

Figure 2-13. MycN6KR induces Egr1 but not canonical c-Myc target genes in cells without ARF. Serum starved DKO MEFs expressing MycER or MycN6KR-ER were induced with OHT. RNA was isolated at the indicated times and was reverse transcribed using the RT-PCR system (Promega). Quantitative real-time PCR was performed using the iCycler and SYBR green dye (Bio-Rad) with primers for (A) *eif4E*, (B) *nucleolin*, (C) *rcl*, and (D) *egr1*. Relative measurement of gene expression was calculated following manufacturer's instructions using the standard curve method. Relative mRNA levels compared to β-actin mRNA were graphed as the mean ±s.d. from triplicate assays.

A *eif4e*

B *nucleolin*

C *RCL*

D *Egr1*

Hours after OHT

58
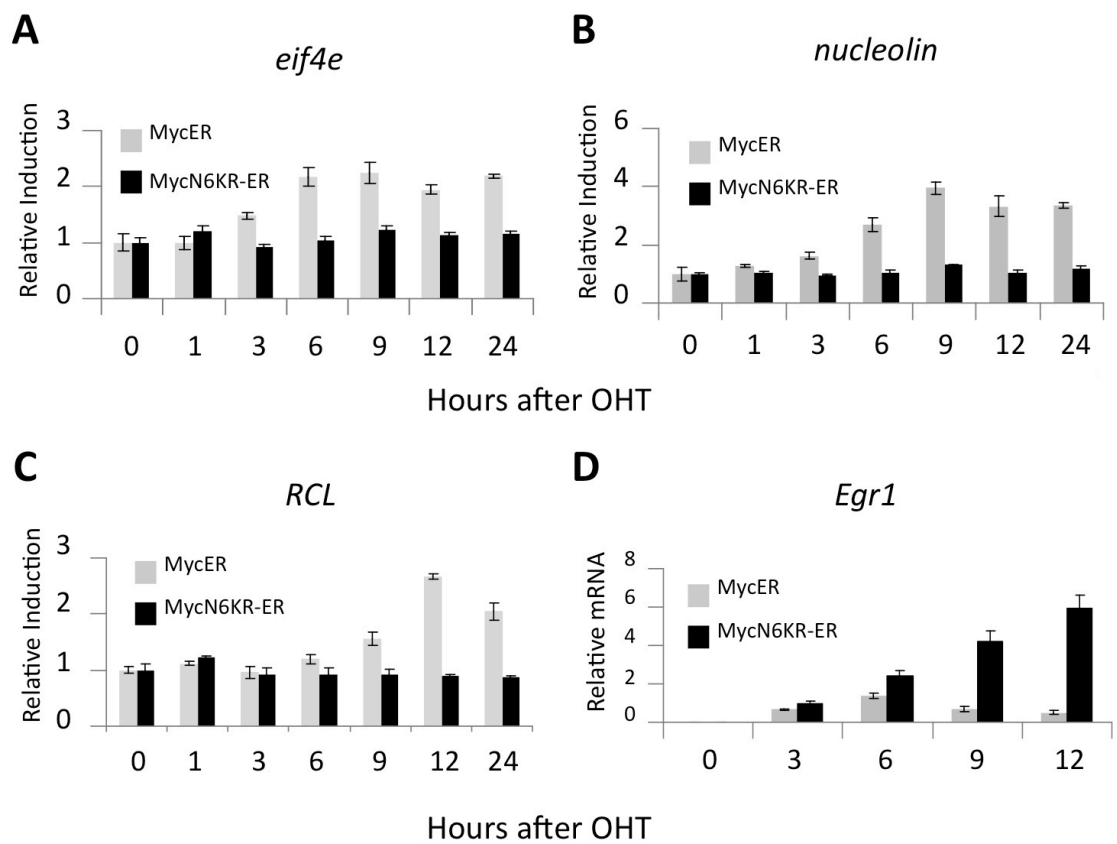
demonstrated that ARF inhibits well-established target genes with canonical Myc binding sites (Qi et al. 2004), as exemplified by *Egr2* shown in this chapter. In contrast, we have now established that ARF is necessary for c-Myc to directly induce transcription of a novel target gene, *Egr1*. This is the first c-Myc target gene that has been shown to be dependent on ARF. Although the regulation of any c-Myc target gene may be cell-type specific, Egr1 was also recently found to be induced by c-Myc in a microarray using B cells (Fan et al. 2010).

Since c-Myc is recruited to the *Egr1* promoter without ARF, albeit at lesser amounts than with ARF, we propose that ARF may not only enhance recruitment of c-Myc to the *Egr1* promoter, but also influences the transcriptional activity of c-Myc target genes post-DNA binding. Substantial control of c-Myc target genes after DNA binding is supported by the previous observation that c-Myc was directly recruited to approximately 3,000 genes in human B cells, but only 406 were induced by activated c-Myc (Zeller et al. 2006). Influencing the transcriptional up-regulation of a c-Myc target gene promoter by interaction with ARF may involve a number of mechanisms, including modulating RNA polymerase elongation, chromatin modification/remodeling, and/or derepression. Unfortunately, how ARF influences c-Myc transcription remains unknown, however in this chapter we demonstrate that ARF reduces N-terminal ubiquitination of c-Myc. We further show that this change in post-translational modification results in the same differential induction of targets genes—Egr1 is induced, while canonical targets are not—observed after the interaction of c-Myc

with ARF. Whether or not the differential induction of targets is important for the

biological effects exerted by the Myc/ARF interaction will be the focus of Chapter

III. Specifically, we will address whether or not the ARF-dependent induction of

Egr1 is necessary and sufficient for c-Myc-induced p53-independent apoptosis.

CHAPTER III

EGR1 IS NECESSARY FOR C-MYC-INDUCED P53-INDEPENDENT

APOPTOSIS

Introduction

Hyperproliferation and transformation as a result of oncogenic c-Myc signaling is limited by the simultaneous sensitization of cells to apoptosis by that same c-Myc signaling (Nilsson and Cleveland 2003). As discussed in the previous chapters, c-Myc is known to induce apoptosis by increasing p53 levels through the induction of the tumor suppressor ARF (Sherr 1998). Through a p53-independent mechanism, ARF also directly inhibits the ability of Myc to induce hyperproliferation and transformation, while synergizing with Myc to induce apoptosis (Qi et al. 2004). In the preceding chapter, we demonstrated that the interaction with ARF switched the induction of c-Myc targets. In the presence of ARF, *Egr2* and canonical target genes were inhibited, while ARF expression was necessary for c-Myc to induce *Egr1*. In this chapter, we show that the differential induction of target genes caused by the ARF interaction is necessary for c-Myc to induce apoptosis. Specifically we demonstrate that the ARF-dependent direct c-Myc target gene Egr1 is necessary and sufficient for p53-independent c-Myc-induced apoptosis.

Materials and Methods

Cell culture

In addition to the cell lines described in the preceding chapter, we obtained *Egr1-/-* and wild-type (WT) MEFs from D. Mercola (University of California, Irvine, California) and E. Adamson (Burnham Institute, La Jolla, California). They were maintained in 10% fetal bovine serum (FBS; Atlanta Biologicals) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The media for stable cell lines expressing c-MycER-puro or c-MycER-hygro was supplemented with 2.5 μg/ml puromycin (Calbiochem) and 50 μg/ml hygromycin B (Invitrogen) respectively. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Retroviral infection

To generate stable cell lines of *Egr1-/-* and wild-type MEFs expressing c-MycER or vector, psi2 cells seeded at 6 x 10^5 cells/60mm dish were transfected with 2μg of pBabe-puro-MycER using 6μl of FuGENE 6 transfection reagent (Roche) and the virus-producing cells were selected in 400 μg/ml hygromycin B for approximately 7 days. The *Egr1-/-* and WT MEFs were then infected for 6 hours with viral supernatants, in the presence of 6 μg/ml polybrene, collected from the psi2 packaging cell line stably expressing c-MycER-puro. The infected cells were selected for in 400 μg/ml hygromycin B for 3 weeks. Expression of c-

MycER was verified via Western blotting. *p53-/-* MEFs expressing Egr1ER were also generated using the above protocol.

Plasmids and expression vectors.

In addition to the constructs described in the preceding chapter, pCB6-HA-Egr1 was obtained from J. Svaren (Waisman Center, Madison, Wisconsin). pBabepuro-Egr1ER was constructed by first removing the silent restriction sites in pCB6-HA-Egr1 with mutagenesis PCR (Stratagene). Next, using PCR the HA tag and the stop codon were removed and an optimal start codon consensus sequence was added, as were BamHI restriction sites to both the N and C-termini. pBabepuro-MycER was digested with BamHI to remove c-Myc. The amplified product was also digested with BamHI and ligated into pBabe-puro-ER. The construct was verified by sequencing.

Cell treatments

c-MycER was activated in all cells by the addition of 1-2μM OHT as indicated. Egr1ER was activated in cells by the addition of 1-2μM OHT. As a positive control to induce apoptosis, *p53-/-* MEFs expressing MycER were treated with 1μM of staurosporine (Upstate) dissolved in dimethyl sulfoxide (DMSO).

The day prior to treatment *p53$^{-/-}$, ARF$^{-/-}$, DKO,* and wild-type MEFs expressing c-MycER were seeded at 4.5 x 10^6 cells per 10cm dish in antibiotic free media. The cells were treated with a final concentration of 100nM of Egr1 SMARTpool or Control Pool Non-Targeting #1 siRNA purchased from DHARMACON using Dharmafect Reagent 4 according to manufacturer's instructions. Approximately 16 hours later, cells were trypsinized and seeded as described in the apoptosis assay section below. In order to knock-down expression of Egr1 and ARF simultaneously, *p53-/-* MEFs expressing c-MycER were seeded at 3 x 10^6 cells per 10cm dish in antibiotic free media. The following day cells were treated with a final concentration of 50nM of Egr1 SMARTpool and 50nM of CDKNDA SMARTpool using Dharmafect Reagent 4 according to manufacturer's protocol. For the treatment of cells with individual siRNAs, *p53$^{-/-}$* MEFs expressing MycER, seeded at 2x10^6 cells per 10cm dish, were treated with a final concentration of 100nM of each individual siRNA used to make the Egr1 SMARTpool, as well as, Control Pool Non-Targeting #2 purchased from DHARMACON using Dharmafect Reagent 4 according to manufacturer's instructions. The effectiveness of the siRNA treatments to reduce protein expression was determined by Western Blotting.

Antibodies

All antibodes used were described in Chapter II.

<u>*Western blot analysis*</u>

Western blotting was performed as in Chapter II.

<u>*Apoptosis assays*</u>

Two days after plating *Egr1*$^{-/-}$ MycER and WT MycER MEFs at 1×10^5 cells/well in 6 well dishes, the cells were shifted into media containing 0.5% FBS with or without 2μM OHT (added daily). The numbers of floating (apoptotic) and attached (living) cells were determined in triplicate at the indicated times with a hemacytometer. Results are reported as a ratio of dead to living cells over time. To confirm the viability of the cells being counted, one part cell suspension was mixed with one part 0.4% Trypan blue (Sigma-Aldrich) before counting.

For cell death assays performed on RNAi treated cells, *p53*$^{-/-}$ MycER, *ARF*$^{-/-}$ MycER, DKO-MycER, and WT-MycER MEFs were treated with siRNA as described in the RNA interference section above. After 16-24hrs the cells were seeded 2×10^5 cells/well in 6 well dishes in media containing 2% CS with or without 2μM OHT (added daily). The numbers of living and dead cells were determined after three days of OHT treatment as described above in triplicate in at least three different experiments with two different polyclonal cell lines and reported as the number of dead cells/number of total cells multiplied by 100 (percent dead).

For the cell viability assays in p53-/- MEFs expressing Egr1ER or vector, the cells were seeded at 2×10^5 cells/well in 6 well dishes in media containing

10% calf serum. The next day the media was replaced with low serum media containing 2% calf serum with 2μM OHT or ethanol. OHT or ethanol was added daily for three days before the number of living and dead cells were determined and reported as above.

Apoptosis was confirmed with an activated caspase-3 colorimetric assay (Sigma-Aldrich) according to manufacture protocol. Briefly, *p53*-/- MEFs expressing c-MycER were treated with siEgr1 or siControl as described above. The next day the cells were seeded at 1.2 x 10^6 cells per 10cm dish in media containing 2% calf serum with 2μM OHT (added daily) or ethanol as a control. After the third day of c-MycER activation, the cells were washed, lysed, and treated according to protocol. The amount of p-Nitroaniline released due to the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3 in the lysate was determine by reading the absorbance values at 405 nm after a 19 hr incubation at 37^oC. The average caspase 3 activity, determined according to manufacturer instructions, was reported +/- the standard deviation from three independent samples.

Results

Egr1-/- MEFs are deficient in c-Myc-induced apoptosis

Since c-Myc induces both apoptosis and Egr1 expression in an ARF-dependent manner, and because Egr1 is necessary for apoptosis in several cell

types (Virolle et al. 2001; Yu et al. 2007), we examined whether Egr1 might be involved in *p53*-independent c-Myc-mediated apoptosis. We obtained *Egr1*$^{-/-}$ MEFs and parental wild-type (WT) MEFs and generated lines that express comparable levels of c-MycER (Fig. 3-1*, lower panel*) through retroviral infection. Activated c-MycER induced apoptosis in the WT MEFs, as others have shown, but failed to induce apoptosis in the *Egr1*$^{-/-}$ MEFs, suggesting that Egr1 expression is necessary for c-Myc-induced apoptosis (Fig. 3-1*, upper panel*).

Reduced Egr1 and/or ARF expression inhibits c-Myc induced p53-independent apoptosis.

To confirm the necessity of Egr1 for c-Myc induced apoptosis using another approach, we treated *p53*$^{-/-}$ MycER MEFs with Egr1 siRNA or control siRNA. Egr1 protein expression was effectively inhibited by Egr1 siRNA for up to 5 days—longer than the duration of an apoptosis assay—with or without OHT treatment (Fig. 3-2). Inhibiting Egr1 expression significantly reduced c-Myc-induced apoptosis in *p53*$^{-/-}$ MycER MEFs using a pool of Egr1 siRNA oligomers (Fig. 3-3). In addition, to confirm the necessity of ARF for p53-independent c-Myc-induced apoptosis, we inhibited ARF protein expression using ARF siRNA as performed in Chapter II. The reduction of c-Myc-induced apoptosis by inhibition of ARF expression was comparable to the reduction observed with inhibition of Egr1 expression (Fig. 3-3). Additionally, the combined inhibition of both ARF and Egr1 expression did not further reduce c-Myc-induced apoptosis (Fig. 3-3), suggesting that ARF and Egr1 function in the same pathway to induce
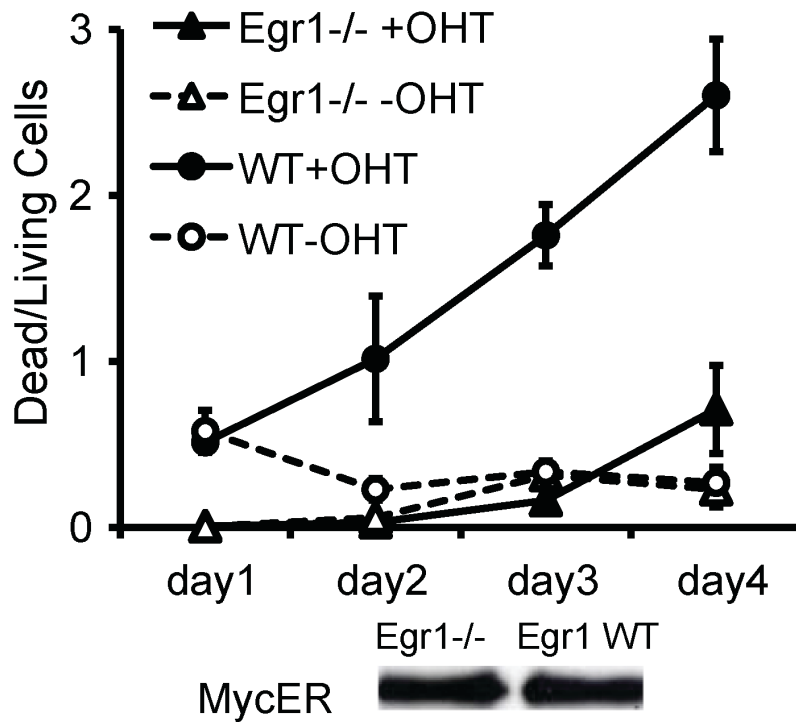
Figure 3-1. Activated c-Myc cannot induce cell death in Egr1-/- MEFs. *Egr1*^{-/-} and parental wild-type MEFs expressing equal levels of c-MycER (*lower panel*) were assayed for cell viability (+/-OHT) in low serum (0.5% calf serum) by counting living and dead cells with a hemacytometer. Viability was confirmed using trypan blue exclusion. The results are reported as the mean of the ratio of dead to living cells from three independent assays +/- standard deviation.
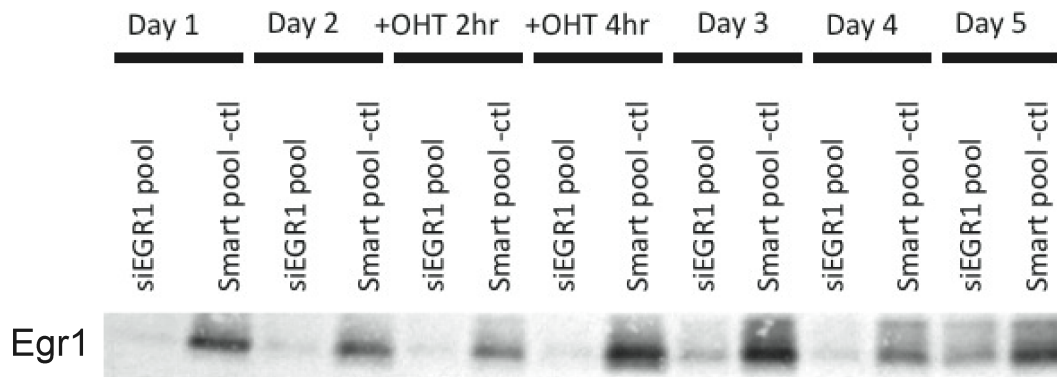
Figure 3-2. siRNA treatment suppresses Egr1 protein for 5 days and following MycER activation. Western Blot analysis of Egr1 protein at the indicated times in *p53*$^{-/-}$ MycER MEFs (+OHT on day 2) following treatment with either a siEgr1 SMARTpool or a negative control SMARTpool #1 as described in Materials and Methods.

apoptosis. Our results demonstrating that c-Myc induces Egr1 only in the presence of ARF, also suggests that ARF and Egr1 are functioning in the same pathway. Reduced apoptosis due to loss of Egr1 expression was confirmed by a decrease in caspase-3 activation (Fig. 3-4) as determined by a colorimetric assay that measures the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by activated caspase 3 isolated from cell lysates of treated cells.

In order to verify that the reduction of Egr1 through siRNA reduces apoptosis in another way, the different Egr1 siRNA oligomers from the pool were used to individually knockdown Egr1 expression. Three of the four oligos significantly reduced Egr1 expression as confirmed by Western Blotting (Fig. 3-5, *lower panel*). The two most efficient oligos (termed C1 and C3) were used to treat $p53^{-/-}$ MycER MEFs independently before assaying for apoptosis in low serum with or without activation of MycER with OHT. As was the case with the SMARTpool, inhibiting Egr1 expression with either of the individual oligos significantly reduced c-Myc-induced apoptosis in $p53^{-/-}$ MycER MEFs as compared to control treated cells (Fig. 3-5). Confirmation of this result with two different individual siRNA oligos and a smartpool decreases the likelihood that the reduction of apoptosis is due to off-target effects.

Egr1 is necessary specifically for c-Myc-induced p53-independent apoptosis

To compare the effects of Egr1 inhibition on c-Myc-induced apoptosis in MEFs with different genetic backgrounds, we treated $p53^{-/-}$, DKO, $ARF^{-/-}$, and
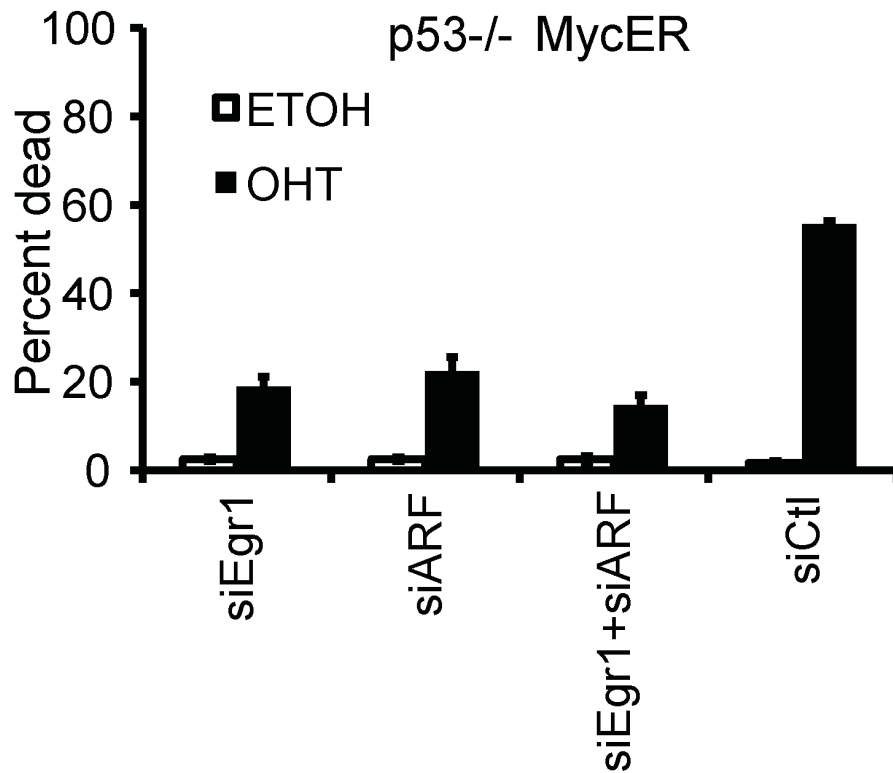
Figure 3-3. Egr1 and ARF are necessary for c-Myc induced p53-independent cell death. *p53$^{-/-}$* MycER MEFs (+/-OHT) treated with siEgr1, siARF, siEgr1 and siARF, or control SMARTpool #1 for 24 hrs were assayed for apoptosis in low serum three days following OHT activation by counting living and dead cells with a hemacytometer. The results are reported as the average number of dead cells multiplied by 100 divided by the number of total cells +/- standard deviation.
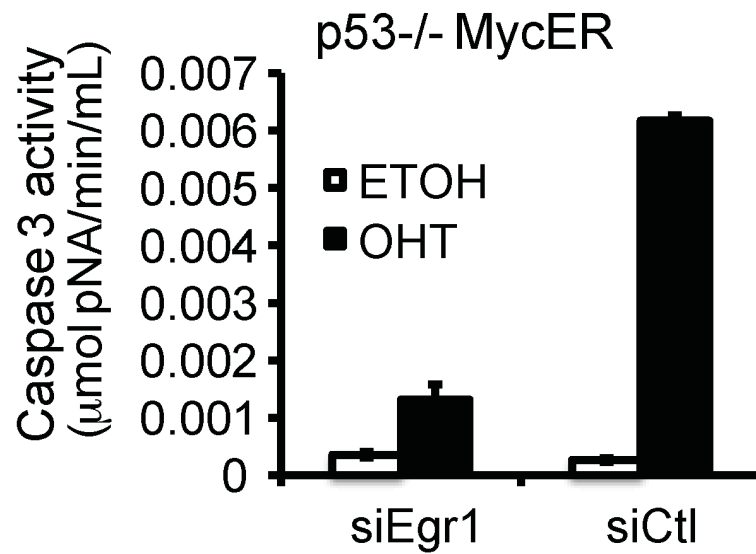
Figure 3-4. Egr1 is necessary for c-Myc induced p53-independent apoptosis. Caspase-3 activity was determined in siEgr1 or siCtl treated p53$^{-/-}$ MycER MEFs +/- OHT for three days as described in the Materials and Methods. Results are reported as the average μmol pNA hydrolyzed per min per ml of cell lysate +/- standard deviation.

WT-MycER MEFs with Egr1 siRNA. As previously shown, activation of c-MycER did not induce apoptosis in MEFs lacking *p53* and *ARF* (Qi et al. 2004), and inhibition of Egr1 expression had no effect on the cells (Fig. 3-6). However, activation of c-MycER in both *ARF*$^{-/-}$ and in wild type MycER MEFs did cause apoptosis (Fig. 3-6). This confirms that c-Myc can induce apoptosis independently of ARF in cells with p53, which has been previously shown (Eischen et al. 1999). Importantly, inhibition of Egr1 by siRNA had no effect on p53-dependent, ARF-independent c-Myc-induced apoptosis observed in *ARF*$^{-/-}$ MEFs (Fig. 3-6) or apoptosis caused by staurosporine treatment (Fig. 3-7), suggesting that reducing Egr1 levels does not cause a general defect in apoptosis, but rather Egr1 is specifically necessary for c-Myc-induced, p53-independent apoptosis.

Egr1 is sufficient for p53-independent apoptosis

Finally, to determine whether Egr1 is capable of inducing apoptosis without activated c-Myc or p53, we generated *p53*$^{-/-}$ MEFs expressing an Egr1-ER fusion protein (Fig. 3-8, *lower panel*). Upon four days of activation with OHT treatment, Egr1-ER efficiently induced apoptosis in low serum unlike OHT treatment in cells with vector alone (Fig. 3-8, *upper panel*). Overall, these results suggest that Egr1 is necessary and sufficient for mediating p53-independent and ARF-dependent c-Myc-induced apoptosis.
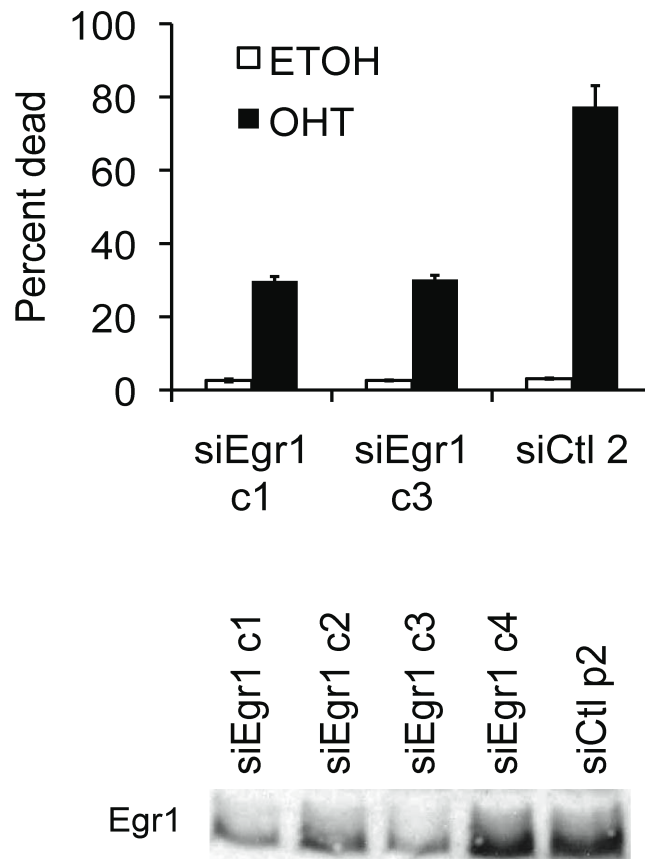
Figure 3-5. Treatment with single siRNA oligos targeting Egr1 is sufficient to reduce c-Myc-induced p53-independent apoptosis. The four siRNAs targeting *Egr1* in the SMARTpool were individually (designated as siEgr1 c1, c2, etc.) used to knockdown Egr1 expression. (lower panel) The Western Blot analysis demonstrates that c1 and c3 most effectively reduced Egr1 levels compared to non-targeting control pool #2 in *p53$^{-/-}$* MycER MEFs. *(top panel) p53$^{-/-}$* MycER MEFs (+/- OHT) treated with siEgr1 c1, siEgr1 c2, or control SMARTpool #2 for 24 hrs were assayed for apoptosis in low serum 3 days following OHT activation as described in the Materials and Methods. The average percent dead, as calculated in Fig. 3-3, is reported +/- standard deviation.
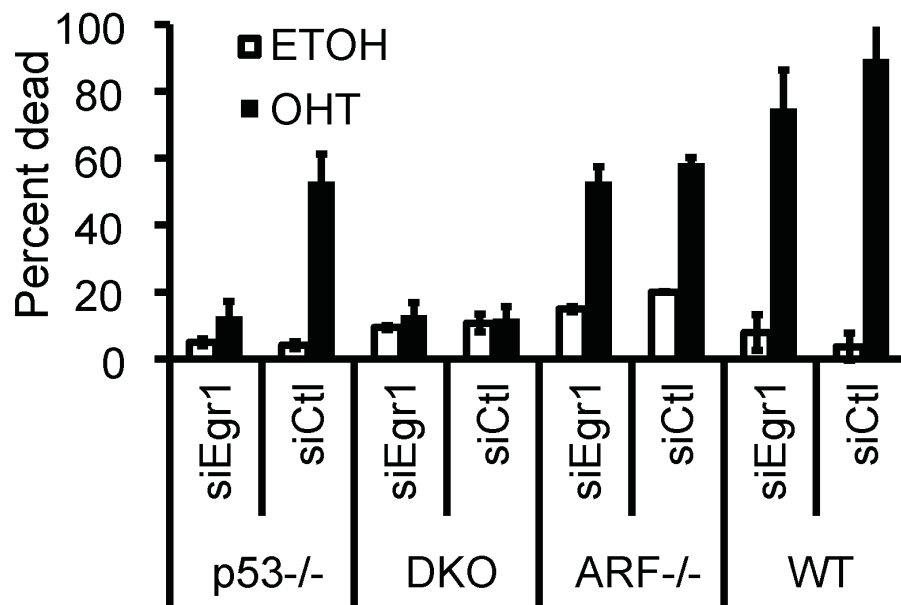
Figure 3-6. **Egr1 is necessary for c-Myc-induced p53-independent apoptosis, but not p53-dependent apoptosis.** p53$^{-/-}$ MycER, DKO-MycER, ARF$^{-/-}$ MycER, and WT-MycER MEFs treated with either siEgr1 or siCtl were assayed for apoptosis and the results are calculated and reported as in Fig 3-3.

Egr1 is necessary for apoptosis induced by c-MycN6KR-ER

If the ARF interaction is switching c-Myc from driving canonical target gene activation to inducing Egr1 by inhibiting N-terminal ubiquitination, as suggested in the previous chapter, and if the induction of apoptosis is a result of the differential induction of target genes, as suggested in this chapter, then the activation of the N-terminal ubiquitination mutant, c-MycN6KR-ER, in DKO MEFs without ARF expression, should limit hyperproliferation and induce apoptosis as if ARF is present and interacting with c-Myc. To test this idea, we examined the ability of c-MycN6KR-ER to induce hyperproliferation, transformation, and apoptosis compared to wild type c-MycER. We first examined the ability of wild-type c-Myc to induce hyperproliferation in DKO MEFs lacking both *ARF* and *p53*. Activated wild-type c-MycER induces hyperproliferation by approximately 2-fold (Zhang and Gregory, unpublished observations). In contrast, activation of c-MycN6KR-ER resulted in a two-fold decrease in proliferation (Zhang and Gregory, unpublished observations). Additionally, the ability of c-MycN6KR-ER to induce anchorage-independent growth in DKO MEFs is significantly reduced compared to wild-type c-MycER (Zhang and Gregory, unpublished observations). The inhibition of c-Myc-induced proliferation and transformation through inhibition of N-terminal ubiquitination is similar to the published effects of ARF on c-Myc (Qi et al. 2004). To observe the effects of the loss of c-Myc N-terminal ubiquitination on apoptosis in a standard apoptotic assay, we placed DKO MEFs expressing either c-MycER or c-MycN6KR-ER in low serum and activated the ER-fused Myc proteins with OHT. As we found previously, activated wild-type c-MycER did not

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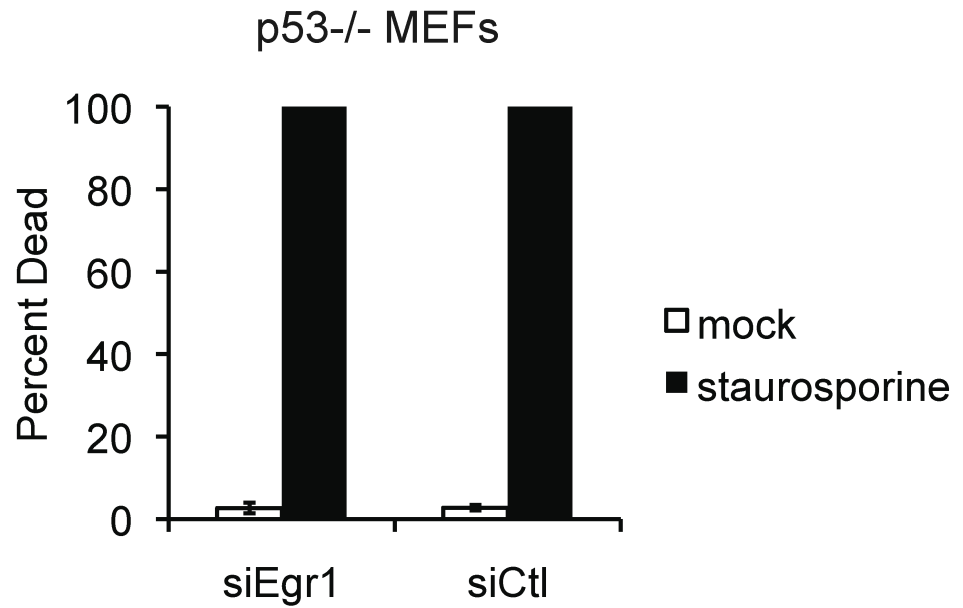
Figure 3-7. **Inhibition of Egr1 expression does not inhibit staurosporine-induced apoptosis.** *p53*$^{-/-}$ MycER MEFs treated with siEgr1 or control SMARTpool for 24 hrs were treated with or without staurosporine. Apoptosis was assayed 24 hours later by counting living and dead cells with a hemacytometer. Results were calculated and are reported as in Fig. 3-3.
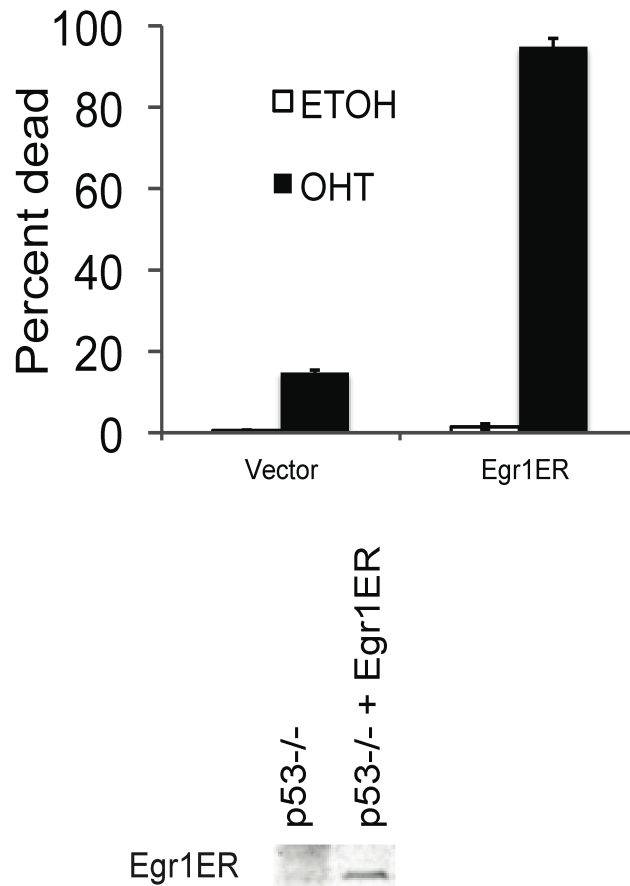
Figure 3-8. Activation of Egr1ER induces apoptosis in *p53^-/-* MEFs. (upper panel) *p53^-/-* Egr1ER and *p53^-/-* pBabe vector MEFs were assayed for apoptosis. Results are reported as the average percent dead +/- s.d. following four days of treatment (+/- OHT) in 1% serum media. (lower panel) Western Blot shows Egr1ER expression following stable retroviral infection in *p53^-/-* MEFs.

induce apoptosis in cells lacking ARF (Figure 3-9, *upper panel*) (Qi et al. 2004).

In contrast, c-MycN6KR-ER efficiently induced rapid cell death (Figure 3-9, *upper panel*). Apoptosis was confirmed by Western blot analysis of active caspase 3 (data not shown). Furthermore, we demonstrated that inhibition of Egr1 expression by siRNA also significantly inhibited the ability of c-MycN6KR to induce apoptosis in DKO MEFs (Figure 3-9, *lower panel*), just as it prevented apoptosis by the activation of wild-type MycER in *p53*-/- MEFs. These results suggest that the N-terminal ubiquitination mutant of c-Myc is inducing p53-independent apoptosis through Egr1, just like wild-type c-Myc does in the presence of ARF.

Discussion

The apoptotic potential of c-Myc acts as a built-in fail-safe mechanism that limits hyperproliferation and transformation caused by deregulated expression of itself. One prevailing view is that c-Myc induces apoptosis through the indirect induction of ARF that ultimately leads to stabilization of p53 (Sherr 1998). However, c-Myc can also induce apoptosis though less-defined p53-independent mechanisms (Meyer et al. 2006). In one such mechanism, ARF directly interacts with c-Myc leading to a reduction of hyperproliferation and transformation and the induction of apoptosis (Qi et al. 2004). In this chapter, we explored how the c-Myc/ARF interaction leads to p53-independent apoptosis. Specifically, we
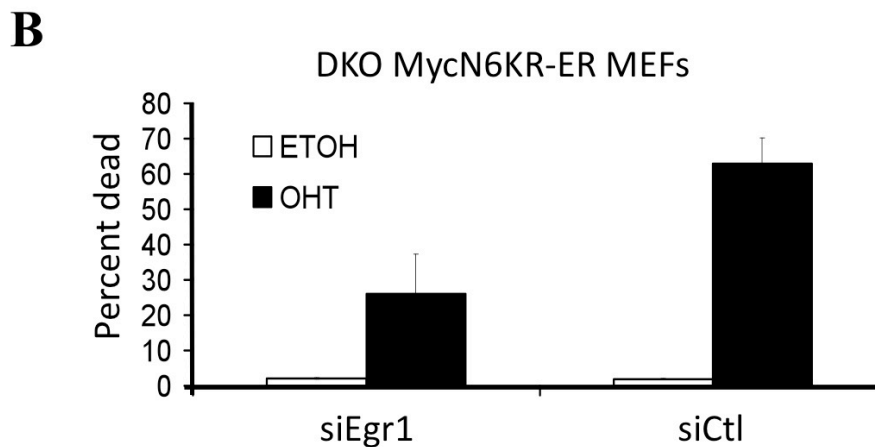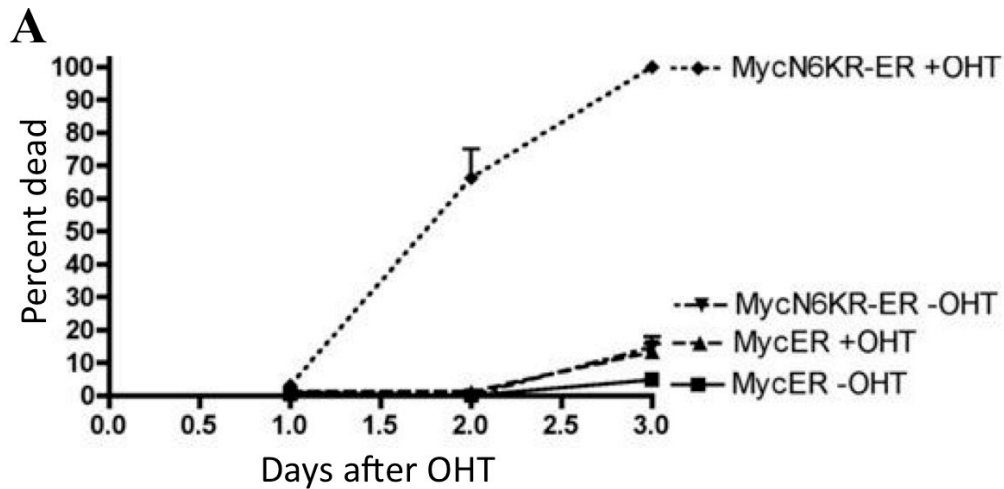
Figure 3-9. c-MycN6KR induces apoptosis in p53/ARF double knockout MEFs. A) One day after seeding at 1×10^5 cells/35mm dish in media containing 10% BCS, MycER and MycN6KR-ER DKO MEFs were shifted into media containing 1% BCS and the cells were treated with 2 μM OHT as indicated and re-fed with media containing 2 μM OHT daily. B) MycN6KR-ER DKO MEFs were treated with either siGENOME Smart Pool targeting Egr1 or Smart Pool control. The next day the cells were split into triplicate plates in DMEM containing 2%CS with either OHT or ethanol. The average percent dead +/- standard deviation was determined and calculated as described in the Materials and Methods.

demonstrated that the ARF-dependent noncanonical induction of *Egr1* is essential for c-Myc to induce apoptosis independently of p53. Thus, ARF binding essentially switches the inherent activity of c-Myc to an apoptotic protein through transcriptional regulation. Several previously identified canonical c-Myc target genes can induce apoptosis, including ODC (Packham and Cleveland 1994) and MT-MC1 (Yin et al. 2002); however, it has not been shown conclusively that reduced expression of either of these or any other canonical direct target gene disrupts c-Myc-induced apoptosis independently of p53. Therefore, we demonstrate the first ARF-dependent c-Myc target gene that is necessary for p53-independent apoptosis.

As summarized in Fig. 3-10, our model is that under normal physiological low ARF conditions, c-Myc induces canonical target genes, such as *cyclin D2, cdk4, nucleolin, eIF4E, and Egr2*, which stimulate cell cycle progression and cell growth. Under normal conditions p53 levels are also low due to Mdm2-mediated degradation of p53 protein. Upon oncogenic activation, c-Myc causes both an increase in ARF expression and a relocalization of ARF from the nucleolus to the nucleoplasm, independently of p53 (Qi et al. 2004). In a direct feedback mechanism, ARF binds with c-Myc to inhibit canonical c-Myc target gene induction and proliferation, while inducing noncanonical expression of *Egr1* and Egr1-mediated apoptosis. In cells with wild-type p53, elevated ARF also inhibits Mdm2 activity, leading to p53 protein stabilization and p53-induced apoptosis. In cells that lack functioning ARF, but have a wild-type p53, c-Myc induces p53-

Figure 3-10. Model of c-Myc-ARF-Egr1 pathway. c-Myc induces canonical target genes under normal physiological conditions when ARF is low, leading to proliferation (*top panel*). p53 levels are also low under normal conditions due to Mdm2-mediated degradation of p53 protein. Upon oncogenic activation of c-Myc, ARF directly interacts with c-Myc and inhibits canonical target gene expression necessary for proliferation. ARF also switches c-Myc to an apoptotic protein that induces Egr1 through a noncanonical mechanism, which then mediates c-Myc-induced apoptosis independently of p53 (*lower panel*). In p53-dependent apoptosis, elevated ARF induced by oncogenic c-Myc also binds to and inhibits Mdm2 activity, leading to p53 stabilization and apoptosis. c-Myc-induced apoptosis can also occur independently of ARF through alternate p53-dependent mechanisms.
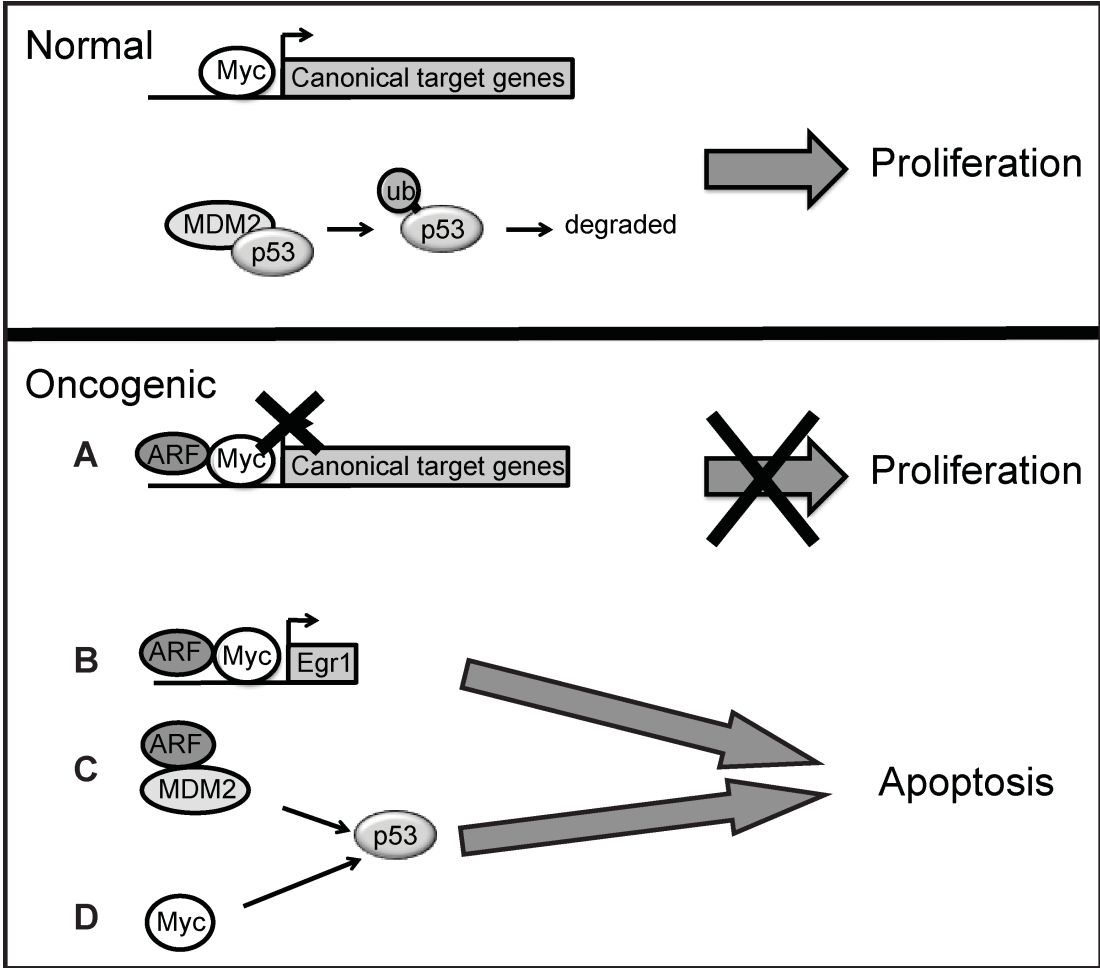
dependent apoptosis through less-defined mechanisms (Zindy et al. 1998;

Pusapati et al. 2006).

Finally, in this chapter, we showed that the inhibition of c-Myc N-terminal ubiquitination can mimic or substitute for the necessity of ARF in c-Myc-mediated apoptosis and that the ubiquitination status of c-Myc controls the ability of c-Myc to induce apoptosis in fibroblasts. Therefore, both the interaction of ARF with c-Myc and the loss of N-terminal domain ubiquitination, switch c-Myc from a proliferative to an apoptotic protein and induces a novel apoptotic pathway through Egr1 expression.

CHAPTER IV

CONCLUSIONS

The Myc/ARF/Egr1 pathway as a fail-safe mechanism

The c-myc proto-oncogene encodes an enigmatic transcription factor that paradoxically has essential roles in the regulation of both proliferation and apoptosis. Additionally, deregulated c-*myc* expression combined with a loss of tumor suppressors, manifested in many types of human cancers, causes hyperproliferation, transformation, and tumorigenesis. Despite the extensive insight gained through meticulous examination of Myc over the past 30 years, questions remain about the molecular mechanisms governing the diverse and critical roles of c-Myc. How does a single protein govern both survival and death? Is there a signal that switches the function of c-Myc from driving proliferation to initiating apoptosis? How do cancerous cells overcome this signal? Distinct threshold levels of c-Myc, which are regulated by various signaling pathways or deregulated in cancer, are correlated with different biological outcomes, such as transformation or apoptosis (Murphy et al. 2008). But it is unclear if elevated c-Myc levels lead to merely an amplification of the same target genes induced by lower levels of c-Myc, or if an increase in c-Myc expression leads to the binding and activation of additional targets. Considering that cell context also influences the biological outcomes initiated by elevated c-Myc, it is possible that additional cellular factors directly or indirectly modulate specific c-Myc target genes. In this dissertation, it was shown

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that a cofactor of c-Myc, the ARF tumor suppressor, switches the inherent function of c-Myc from a proliferative protein to an apoptotic protein through the transcriptional regulation of Egr1 (Boone et al. 2011). Hopefully, through further understanding of this and other natural mechanisms that protect cells from uncontrolled c-Myc expression, specific therapies can be developed to prevent or treat c-Myc-driven human cancers.

Apoptosis induced by oncogenic c-Myc occurs with or without p53 (Meyer et al. 2006). In the ARF-MDM2-p53 pathway elevated or deregulated c-Myc levels indirectly induce ARF, which then stabilizes p53 through direct binding and inhibition of MDM2, the E3 ubiquitin ligase of p53 (Sherr 1998). The increased levels of p53 then induce apoptosis through transactivation of direct pro-apoptotic target genes, such as Bax and PUMA (Zambetti et al. 1992). c-Myc can also indirectly cause p53-dependent apoptosis without ARF by inducing a DNA damage response leading to activation of the ATM/ATR kinases and phosphorylation-mediated stabilization of p53 (Pusapati et al. 2006). However, how c-Myc induces apoptosis independently of p53 has not been described. We now demonstrate that ARF is necessary for the induction of a novel c-Myc target gene, Egr1, which is essential for c-Myc-driven p53-independent apoptosis (Boone et al. 2011). Although, this Myc-ARF-Egr1 pathway is critical for the ability of c-Myc to induce apoptosis independently of p53, it is not necessary for p53-dependent c-Myc-induced apoptosis, suggesting that it is a distinct and alternative fail-safe mechanism.

ARF: an unusual cofactor

In addition to ARF, there are many other cofactors that are necessary for or influence c-Myc transcriptional activity and biological functions. Max, TRRAP, Mediator, and PTEFb are examples of cofactors that are essential for basic transcriptional activities such as DNA binding, recruiting general machinery for chromatin modification and remodeling, and activation of RNA polymerase II (Cowling and Cole 2006). Other c-Myc cofactors, such as the ubiquitin E3 ligases Skp2 (Kim et al. 2003; von der Lehr et al. 2003) and HectH9 (Adhikary et al. 2005), stimulate canonical transcription and cell cycle progression. Another cofactor, nucleophosmin (NPM), also enhances canonical transcription and significantly stimulates c-Myc-induced hyperproliferation and transformation (Li et al. 2008). In contrast, interaction of c-Myc with cofactor Bin1 inhibits c-Myc transactivation and induces caspase-independent apoptosis through an unknown mechanism (Sakamuro et al. 1996). Other proteins also interact with c-Myc to influence apoptosis through different mechanisms. For example, c-Myc appears to be a cofactor of the transcription factor Miz1, since Miz1 recruits c-Myc to Miz1 target genes to inhibit transcription, leading to an induction of apoptosis and an inhibition of cell adhesion, perhaps through the repression of anti-apoptotic genes (Adhikary et al. 2005; Herkert et al.). However, we now show that ARF is unlike any of these known cofactors in that it *differentially* regulates which c-Myc target genes are expressed. Through this differential regulation of target genes, the interaction of ARF switches the inherent function of c-Myc from driving proliferation to initiating apoptosis.

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ARF has a dual role in regulating c-Myc transcriptional activity. ARF interacts directly with c-Myc and inhibits target genes, such as *cdk4, tert, eIF4E*, and *Egr2*, which possess canonical Myc E-box binding sites (CACGTG) (Qi et al. 2004). Interestingly, ARF is recruited to these canonical target genes with c-Myc to inhibit transcription, while also being recruited with c-Myc to the noncanonical target gene *Egr1* to activate transcription (Boone et al. 2011). While c-Myc can be recruited to the *Egr1* promoter without ARF, ARF significantly increases recruitment of c-Myc to the promoter and is necessary for transcriptional induction of Egr1 by c-Myc, which is necessary and sufficient to induce p53-independent apoptosis (Boone et al. 2011). In agreement with our results, Egr1 overexpression is sufficient to induce or enhance apoptosis in several different cell types, including MEFs (Das et al. 2001; Yu et al. 2007). A pro-apoptotic, tumor suppressive role for Egr1, is also suggested by the findings that its expression is low or lost in many human tumors (Joslin et al. 2007; Liu et al. 2007). Furthermore, although we have not established a role for Egr2 in c-Myc mediated biology, opposing roles of Egr1 and Egr2 have been found during adipocyte differentiation (Boyle et al. 2009) and T cell function (Collins et al. 2008), supporting our model that Egr1 and Egr2 have different roles in mediating c-Myc function.

Transcriptional regulation by ARF: a p53-independent function

In addition to transcriptional regulation of c-Myc, ARF regulates gene expression by directly interacting with and altering the location and/or the transactivaton/transrepression ability of multiple other transcription factors such

as E2F, Foxm1β, BCL6, p63, and interestingly, Egr1 (Datta et al. 2003; Calabro et al. 2004; Kalinichenko et al. 2004; Datta et al. 2005; Suzuki et al. 2005; Yu et al. 2009). One study demonstrated that ARF directly inhibited E2F mediated transcription by directly binding and preventing the dimerization of E2F with DP1 (Datta et al. 2003; Datta et al. 2005), suggesting that one possible mechanism of ARF regulation of transcription factors is through the competition with cofactors. Alternatively, the ARF interaction with Egr1 controls Egr1 transcriptional functions through sumoylation mediated by binding the SUMO-conjugating enzyme Ubc9 (Yu et al. 2009). In fact, ARF-mediated sumoylation of Egr1 is necessary for Egr1 to induce the tumor suppressor PTEN, suggesting that another possible mechanism of ARF regulation of transcription factors is through alterations of post-translational modifications (Yu et al. 2009). These facts combined with our observations that the interaction of ARF with c-Myc reduces ubiquitination, and that the c-MycN6KR mutant mimics the Myc/ARF interaction in that it induces Egr1 and apoptosis independently of p53, suggests that the ARF interaction is differentially affecting c-Myc transcriptional and biological activities through a change in c-Myc post-translational modifications. Because both sumoylation and ubiquitination occur on lysine residues, it is possible that the change in ubiquitination levels could be due to ARF-mediated sumoylation of the N-terminal lysines, thus leading to a blockage of ubiquitination. In contrast, it could also be that the ARF interaction is preventing the binding of an E3 ubiquitin ligase of c-Myc, thus inhibiting ubiquitination. Or it could be a combination of both mechanisms. Regardless, our results solidify that a p53-independent function of

ARF is transcriptional regulation. The first indication of p53-independent tumor suppressor functions of ARF came from knockout mice. Mice that are null for *ARF*, *p53*, and *mdm2* develop a wider variety of tumors and at a frequency greater than those observed in mice lacking *p53/mdm2* or *p53* alone (Weber et al. 2000). Further, reintroduction of ARF into *ARF/mdm2/p53* triple null MEFs caused growth arrest of these cells and caused inhibition of proliferation and apoptosis in *p53*-deleted SAOS-2 osteosarcoma cells (Korgaonkar et al. 2002; Yarbrough et al. 2002). However, despite years of research since those findings, describing the p53-independent functions of ARF has been largely controversial.

The cofactor switch model

Few direct c-Myc target genes like Egr1 have been identified that mediate a specific c-Myc function, giving rise to the idea that it takes multiple target genes to mediate any c-Myc function. Interestingly, Egr1 is a transcription factor, like c-Myc, that regulates the expression of a large number of target genes, some of which have been shown to be indirect targets of c-Myc (Krones-Herzig et al. 2003; Krones-Herzig et al. 2005; Rogulski et al. 2005; Kubosaki et al. 2009). In addition, the Egr1 binding site is the most commonly occurring cis-regulatory element found near c-Myc binding sites throughout the genome (Elkon et al. 2004), suggesting that Egr1 and c-Myc may cooperatively or antagonistically regulate certain genes. Furthermore, MT-MC1, another c-Myc target gene that can substitute for c-Myc in sensitizing cells to apoptosis, is a DNA binding protein that alters the expression of 47 different genes, all of which have both c-Myc and

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Egr1 binding sites in their promoters (Rogulski et al. 2005). In fact, there is experimental evidence that c-Myc and Egr1 bind simultaneously to 7 different promoters (Elkon et al. 2004). Therefore, we speculate the importance of Egr1 as a target gene, is due to sequential and/or simultaneous, cooperative and/or antagonistic, regulation of other targets—in other words, c-Myc initiates a wave of transcription through Egr1. This could be a possible explanation for the large number of generally non-overlapping potential c-Myc target genes that have been identified by numerous studies using different cell types and conditions. Perhaps c-Myc, in different cell contexts depending on the interaction of specific cofactors like ARF, directly induces a variety of transcription factors, in this case Egr1, which in turn can sequentially or cooperatively regulate a large number of genes. In this general "Cofactor Switch" model depicted in Figure 4-1, a cofactor like ARF could control a large number of genes for specific c-Myc functions (Figure 4-1).

It is understood, however, that this simple model becomes very complex when you consider that there are many cofactors of c-Myc that are simultaneously expressed and competing for binding. It is unknown how the relative abundance of these different cofactors and/or their differential binding affinities controls c-Myc function. Additionally, under certain conditions, there may be a heterogeneous mixture of c-Myc complexes that directly compete against one another. For example, under low ARF conditions, when there is a stoichiometric excess of c-Myc, there could be both c-Myc that is bound to ARF and c-Myc that is not. In this scenario, some molecules of c-Myc could be
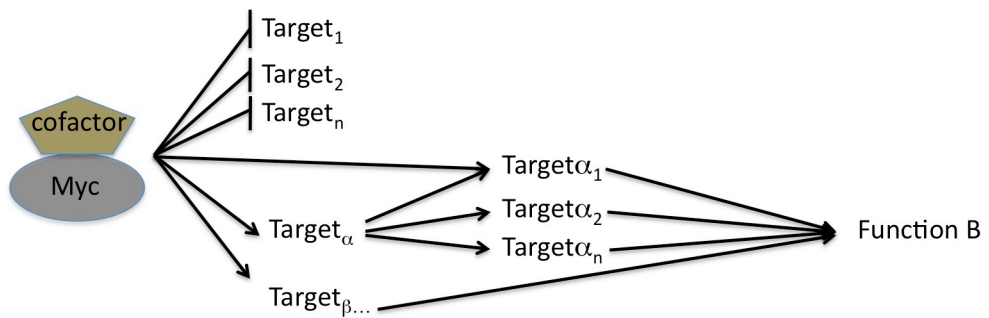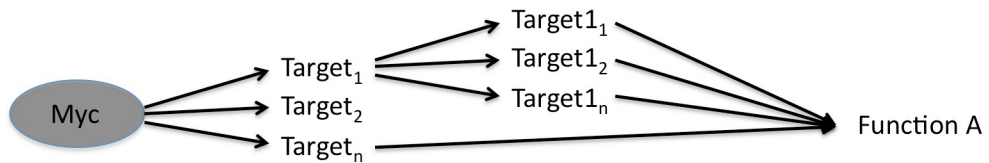
Figure 4-1. Cofactor Switch Model. In addition to cofactors being able to inhibit or enhance the c-Myc transcriptional response, ARF, and we propose that other cofactors, can also differentially switch the response, whereby shutting off targets induced before binding, such as $Target_1$, $Target_2$..., while being necessary to induce new targets, such as $Target\alpha$, $Target\beta$... Furthermore, if the new targets are transcription factors or other proteins important for gene regulation, like $Target\alpha$ then this could elicit a wave of transcription —induction of new targets such as $Target\alpha_1$, $Target\alpha_2$...— that ultimately leads to specific biological functions.

inducing the pro-apoptotic target Egr1, while others could be inducing proliferative targets like cyclinD2. How the cell would resolve this conflict is unknown. Another of many layers of complexity comes from the fact that not a single c-Myc target gene has been found that is only regulated by c-Myc. So, when considering the ultimate transcriptional regulation of any target and the consequential biological outcome(s), we must take into consideration that other signaling pathways indirectly influence the ability of c-Myc to regulate its targets.

Future Directions

Though we have now established the Myc/ARF/Egr1 apoptotic pathway, many questions remain. How does ARF differentially regulate c-Myc targets? Does it compete with other cofactors? Does it recruit new cofactors to c-Myc or the DNA of target genes? Does it affect important posttranslational modifications of c-Myc? Though the data that ARF inhibits ubiquitination and that the MycN6KR mutant mimics ARF's effects on c-Myc's transcription and biological functions does suggest that ARF is influencing Myc transcription through the alteration of ubiquitination, we still do not know by what mechanism. We are currently investigating if ARF is competing for Myc binding with known ubiquitin ligases. Additionally, it is difficult to envision a clear mechanism for how the Myc/ARF complex binds and inhibits certain targets, but is necessary for the induction of another target, Egr1. It could be that the regulation is promoter specific. Because of this, it will be important to identify the exact noncanonical site through which c-Myc and ARF regulate the Egr1 promoter, and to determine

if the regulation of this noncanonical site is crucial for other c-Myc targets. Once promoter bashing is complete and the site or sites are identified, it would be interesting to combine a siRNA screen with luciferase assays to determine if other factors are important for the c-Myc/ARF induction of Egr1. Furthermore, we still do not understand the mechanism of how induction of Egr1 stimulates apoptosis. Others have shown that Egr1 has many downstream targets that are important apoptotic genes, like PTEN and Bax (Virolle et al. 2001; Krones-Herzig et al. 2003; Baron et al. 2006; Yu et al. 2007). However, it will be essential to identify what is downstream of Egr1 that induces c-Myc-driven p53-independent apoptosis. While a 'gene at a time' approach could identify an important downstream target, a combination of global gene expression and a large-scale apoptotic screen after treatment with a siRNA library, would be ideal to fully understand the pathway. Finally, it would be interesting to understand how c-Myc and Egr1 regulate genes together. As stated above, the two transcription factors share many targets, and it has been demonstrated through ChIP analyses that for at least some targets, they bind to the same promoter at the same time (Elkon et al. 2004). However, it is not known if they regulate genes sequentially and/or simultaneously. Nor is it known if they act cooperatively and/or antagonistically on target gene promoters. Understanding how Myc functions and interacts with other transcription factors on promoters, as well as, waves of transcription elicited by c-Myc could be key to comprehending the broad reach of c-Myc's biological functions.

The Myc-ARF-Egr1 pathway may play an important role in restraining c-Myc-induced tumorigenesis. Our finding that Egr1 is essential for p53-independent c-Myc-induced apoptosis and the observations that Egr1 expression is low or lost in many human tumors suggest that the Myc-ARF-Egr1 pathway plays a key role in c-Myc-induced apoptosis and indicates that this pathway is disabled in tumors even with an intact ARF gene. Successful chemotherapy strategies depend on induction of cell death in the targeted tumor. Further understanding the mechanism controlling this pathway could be critical for future cancer therapeutic approaches by unleashing the inherent apoptotic function of c-Myc.

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