

Identification of Novel Molecular Targets of Resveratrol in Colorectal Carcinogenesis

A Dissertation Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

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DEDICATION

To my father,
Willie P. Whitlock, Lt Col (Ret)

My mother,
Minnie B. Whitlock

My brother,
Willie P. Whitlock, Jr. (Preston)

My fiancé,
Korri A. Jones

And to my extended family and friends

Thank you all for your continued love and support

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ABSTRACT

Current research suggests resveratrol, a phytoalexin found predominately in grapes, may function as a chemopreventive and/or chemotherapeutic agent for various cancers, including colorectal cancer. However, the underlying mechanism(s) involved in these activities remain elusive. Thus, the objective of the studies discussed here sought to investigate the effect of resveratrol treatment on gene modulation in human colorectal cancer cells in order to identify and characterize novel molecular targets that contribute to the observed anticancer activities of resveratrol. Here, we identify activating transcription factor 3 (ATF3) and early growth response-1 (Egr-1) as novel targets of resveratrol and provide data to elucidate the mechanism(s) of regulation and how each target contributes to the anticancer effect of resveratrol in colorectal cancer cells. We demonstrate the involvement of resveratrol in ATF3 transcriptional regulation, which is facilitated by Egr-1 and Krüppel-like factor 4 interactions, and show that ATF3 contributes, at least partially, to resveratrol-induced apoptosis (**Chapter 3**). Moreover, we suggest that increased Egr-1 transcriptional activity by resveratrol requires posttranslational acetylation of Egr-1 in a SIRT1-independent manner. This acetylation by resveratrol may contribute to Egr-1-mediated expression of the pro-apoptotic protein nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) induced by the phytoalexin (**Chapter 4**). Taken together, the work presented here provide (1) novel mechanisms by which resveratrol induces ATF3 and Egr-1 expression and (2) represent additional explanations for the anti-tumorigenic/anti-carcinogenic effects of resveratrol in human colorectal cancer cells.

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LIST OF ABBREVIATIONS

AhR	aryl hydrocarbon receptor
ARE	antioxidant response element
ARNT	AhR nuclear translocator
ATF3	activating transcription factor 3
ATM	ataxia telangiectasia mutated kinase
B[a]P	benz[a]pyrene
CDK	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
COBRA	cofactor of BRCA1
COX-2	cyclooxygenase-2
CREB	cAMP response element binding protein
CSE	cigarette smoke extract
CYP	cytochrome P450
DDIT3	DNA-damage inducible transcript 3
DENA	diethyl nitrosamine
DIM	3'3-diindolymethane
DKK1	Dickkopf homologue 1
DMBA	7, 12-dimethylbenz[a]pyrene
DMSO	dimethylsulfoxide

DSS	dextran sodium sulfate
EBS	early growth response-1 binding site
Egr-1	early growth response-1
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
FANCC	Fanconi anemia, complementation group C
FoxO	forkhead box, subgroup O
GCL	glutamate cysteine ligase
HIF-1α	hypoxia inducible factor -1 α
HO-1	heme oxygenase-1
hTERT	human telomerase reverse transcriptase
IGF-1	insulin growth factor-1
IL	interleukin
Keap1	Kelch-like ECH associated protein 1
KLF4	Krüppel-like factor 4
MMP	matrix metalloproteinase
NAG-1	nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1
NF-κB	nuclear factor- κ B
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	nuclear factor E2-related factor 2
Nur77	nuclear receptor subfamily 4, group A, member 1
P(H)AH	polycyclic (poly-halogenated) aromatic hydrocarbon

PDE4B	phosphodiesterase 4B
PI3K	phosphatidylinositol-3-kinase
Plk3	polo-like kinase 3
PMA	phorbol-12-myristate-12-acetate
qRT-PCR	quantitative reverse transcription polymerase chain reaction
Resv	resveratrol
RT-PCR	reverse transcription polymerase chain reaction
SIRT1	sirtuin 1
TCDD	2, 3, 7, 8-tetrachlorodibenzo- <i>p</i> -dioxin
TNFα	tumor necrosis factor α
TPA	12- <i>o</i> -tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis inducing ligand
TSA	trichostatin A
VEGF	vascular endothelial growth factor
WT	wild-type
WNT16	wingless-type MMTV integration site family, member 16
XRE	xenobiotic response element

CHAPTER 1:
Molecular Targets of Resveratrol in Carcinogenesis

This chapter is revised based on the Baek SJ and Whitlock NC book chapter published by Springer Science+Business Media:

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My contributions to this work include: (1) gathering and reviewing the literature, (2) creating tables and figures, (3) drafting and revising the chapter, and (4) final approval of the published version. SJB revised and approved the final version for publication.

1.1. Abstract

Resveratrol, a dietary phytoalexin readily available in the diet, is reported to possess anti-tumorigenic and anti-inflammatory activity in several cancers. However, the molecular mechanisms and/or targets underlying the anti-tumorigenic activity of this compound remain largely unknown. As a potential signaling pathway modulator, resveratrol is shown to affect a multitude of signaling transduction pathways, and it is likely that this collective activity may play an important role in resveratrol-induced anti-tumorigenesis. Therefore, the elucidation of molecular targets of resveratrol is necessary to understand how this compound is beneficial in anti-tumorigenic processes. Studies to identify molecular markers have recently spawned increasing examination of possible targets of resveratrol as related to anticancer proteins. These include, but are not limited to: (1) inhibition of carcinogenic activation and induction of detoxification by phase I and phase II xenobiotic metabolizing enzymes, respectively, (2) modulation of cell survival and apoptosis, (3) suppression of pro-inflammatory signaling pathways, and (4) inhibition of angiogenesis, invasion, and

metastasis. This chapter summarizes the diverse molecular targets of resveratrol in the prevention and treatment of carcinogenesis.

1.2. Introduction

Although advances in chemoprevention and chemotherapy have been achieved, cancer remains the second leading cause of death in the United States ⁽¹⁾. As such, it has become increasingly clear that the problem of cancer cannot be solved by traditional treatment alone and an alternative approach is needed. Traditional approaches to cancer prevention have included attempts to eliminate carcinogenic agents and to detect and remove precancerous lesions; many of these efforts are focused on interrupting, reversing, or delaying the neoplastic process and remain largely ineffective and cumbersome ⁽²⁾. Yet, epidemiological and current laboratory studies suggest consumption of certain fruits and vegetables, containing phytochemicals, is associated with reduced cancer risk ⁽³⁾. It is postulated that these dietary phytochemicals can function as chemopreventive and/or adjuvant chemotherapeutic agents ⁽³⁾. However, further molecular mechanism-based studies are required to evaluate the use of dietary components in cancer chemoprevention and chemotherapy.

One such phytochemical of particular interest is resveratrol (3, 4', 5-trihydroxystilbene), a naturally occurring phytoalexin readily available in the diet and to which a plethora of health-promoting effects have been ascribed. Resveratrol, first identified as a bioactive compound in 1992, is found in several plants, particularly in the skin of red grapes ⁽⁴⁾. This compound has elicited much attention as a potential anticancer agent since its

inhibitory effect on carcinogenic processes (initiation, promotion, and progression) was first reported in 1997 ⁽⁵⁾. Subsequently, numerous studies using both *in vitro* and *in vivo* model systems have illustrated resveratrol's capacity to modulate a multitude of signaling pathways associated with cellular growth and differentiation, apoptosis, angiogenesis, invasion, and metastasis ⁽⁶⁾. Thus, resveratrol modulates multiple signaling pathways that interrupt the carcinogenic process and is also capable of extending one or more stages of this process **(Figure 1.1)**.

Despite substantial progress in understanding the molecular basis of resveratrol's anticancer activities, few clinical trials have been undertaken to confirm its use as a chemopreventive and/or adjuvant chemotherapeutic agent. Preclinical studies have demonstrated the inhibitory effects of resveratrol in different cancers **(Table 1.1)** ⁽⁷⁾ and its ability to act as an adjuvant to traditional chemotherapeutics ⁽⁸⁻¹²⁾. As an adjuvant, resveratrol potentiates the cytotoxic effects of anticancer drugs given at sub-toxic levels as well as alleviate chemotherapeutic drug-associated side effects.

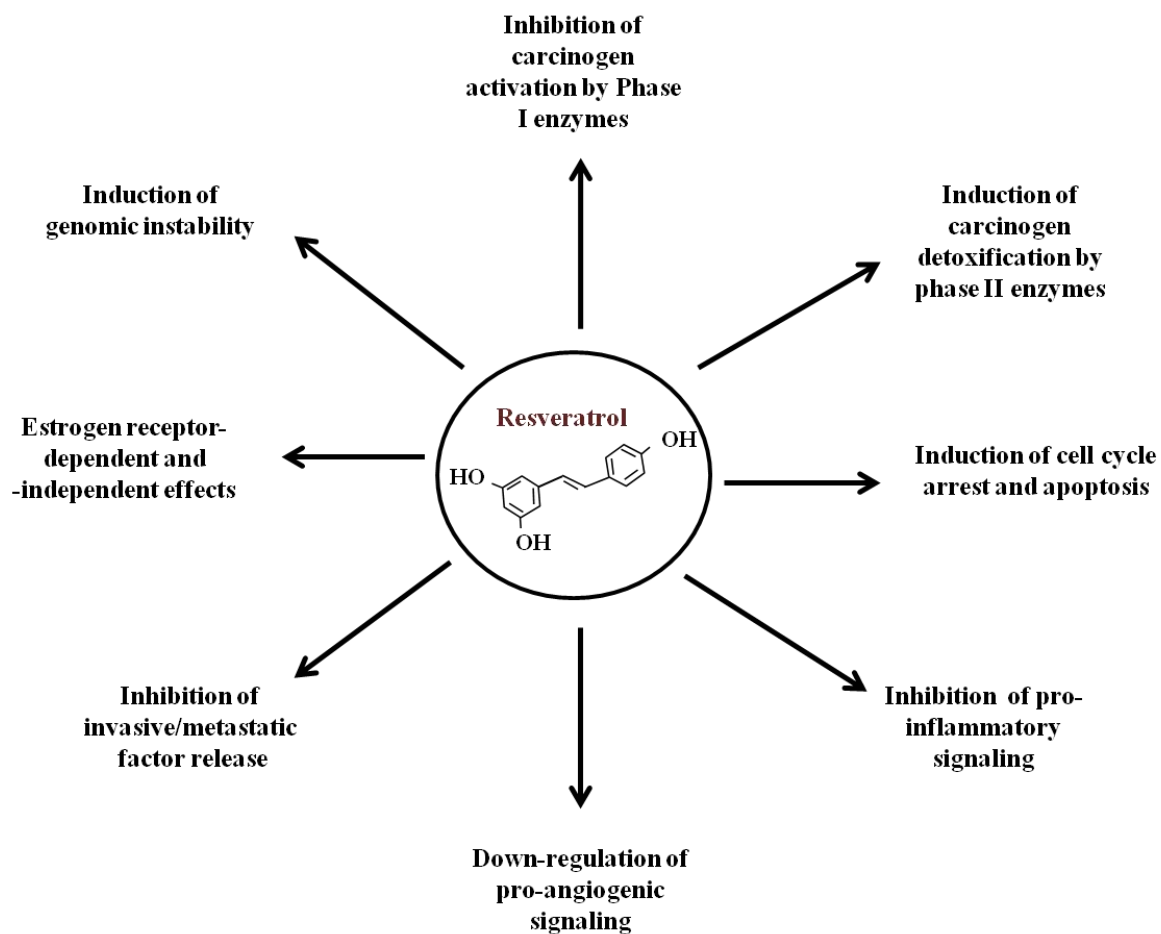


Figure 1.1. The chemopreventive and chemotherapeutic effects of resveratrol. From “Molecular targets of resveratrol in carcinogenesis” in *Evidence-based Anticancer Materia Medica* © 2011 Springer Science + Business Media. All rights reserved. Used with the kind permission of Springer Science + Business Media.

Table 1.1. Chemopreventive and chemotherapeutic effects of resveratrol: *in vivo* evidence.

<i>Experimental Model</i>	<i>Dose/Route</i>	<i>Effects</i>	<i>Mechanisms</i>	<i>References</i>
Breast cancer				
SD rats (♀), DMBA-induced	10 ppm, 127 d; diet	↑latency period, ↓incidence/multiplicity	↓NF-κB activation, ↓COX2, ↓MMP-9	(93)
Colon cancer				
C57B/6 mice, AOM + DSS-induced	300 ppm, 70 d; diet	↓incidence/multiplicity, ↓tumor volume	↓COX2, ↓TNFα, ↓iNOS	(80)
Liver cancer				
SD rats (♀), DENA + phenobarbital-induced	50-300 mg/kg/d, 20 wk; diet	↓incidence/multiplicity	↓proliferation, ↑Bax, ↓Bcl2	(63)
Pancreatic cancer				
Nude mice (♂), MIA PaCa2 cells	40 mg/kg, 4 wk; p.o.	↓tumor formation, ↓volume/growth	↓proliferation, ↓microvessel density, ↓NF-κB, ↓cyclin D1, ↓COX2, ↓MMP9, ↓survivin	(12)
Skin cancer				
B6D2F1 mice (♀), doxorubicin-resistant B16 melanoma cells	12.5 mg/kg, 30 d; s.c.	↓tumor growth, ↑survival of tumor bearing mice		(11)
SENCAR mice (♀), DMBA-induced	1-10 μM, 2/wk for 4wk; topically	↓epidermal hyperplasia		(25)
SKH-1 hairless mice (♀), UVB-induced	10 μM, 7 treatments on alternate days; topically	↓epidermal hyperplasia	↓leukocyte infiltration, ↓proliferation, ↓cdk2/6/4, ↓cyclin D1/D2, ↑p53/p21, ↓MAPK pathway	(53)
Swiss albino mice (♀), DMBA-induced	25-50 μM, 3/wk for 28 wk; topically	↓incidence/multiplicity, ↓tumor volume	↑apoptosis: ↑p53, ↑Bax, ↑cytochrome c release, ↑caspase activation, ↓Bcl2, ↓survivin, ↓PI3K/Akt pathway	(66)

From “Molecular targets of resveratrol in carcinogenesis” in *Evidence-based Anticancer Materia Medica* © 2011 Springer Science + Business Media. All rights reserved. Used with the kind permission of Springer Science + Business Media.

A Phase I clinical study is underway to determine potential adverse resveratrol-drug interaction(s) with emphasis on CYP3A4 activity; the CYP3A4 enzyme is involved in the metabolism of transplant medications/chemotherapies and is inhibited by resveratrol ⁽¹³⁾. Other clinical trials seek to determine the effects of resveratrol on Wnt signaling in normal mucosa and colon cancers (www.clinicaltrials.gov). Although shown to be well tolerated in humans, the bioavailability of resveratrol remains poor ⁽¹⁴⁾. Thus, in order to recommend use of resveratrol as a chemopreventive or adjuvant chemotherapeutic, further studies are necessary to enhance bioavailability of this phytochemical as well as to validate identified *in vitro/in vivo* targets of resveratrol. To this end, studies have begun evaluating techniques to enhance resveratrol systemic bioavailability based on combinations with compounds that inhibit the *in vivo* metabolism, nanoparticle-mediated delivery, use of naturally or synthetic derivatives, and/or use of conjugated metabolites of resveratrol ⁽¹⁵⁾. Yet, resveratrol remains a promising candidate for the prevention and treatment of cancers. This chapter summarizes identified molecular targets of resveratrol that contribute to its activities in the prevention and treatment of carcinogenesis.

1.3. Regulation of Phase I and Phase II Xenobiotic Metabolizing Enzymes

Oxidative metabolism by phase I enzymes, such as those belonging to the cytochrome P450 (CYP) family, results in the conversion of pro-carcinogens to reactive electrophilic intermediates, which are further metabolized by phase II enzymes via the conjugation of hydrophilic moieties. Resultant metabolites are detoxified and eliminated. However, inadequate detoxification by phase II enzymes potentiates genotoxicity (e.g. DNA and

protein adduct formation) of phase I products, thus initiating the carcinogenic process^(16, 17). Reports of modification of both phase I and phase II xenobiotic metabolizing enzymes by resveratrol suggest an explanation for the compound's chemopreventive effect (**Figure 1.2**). Two primary molecular mechanisms have been identified: (1) inhibition of AhR-dependent and AhR-independent activation of phase I enzymes and (2) induction of Nrf2-mediated activation of phase II enzymes.

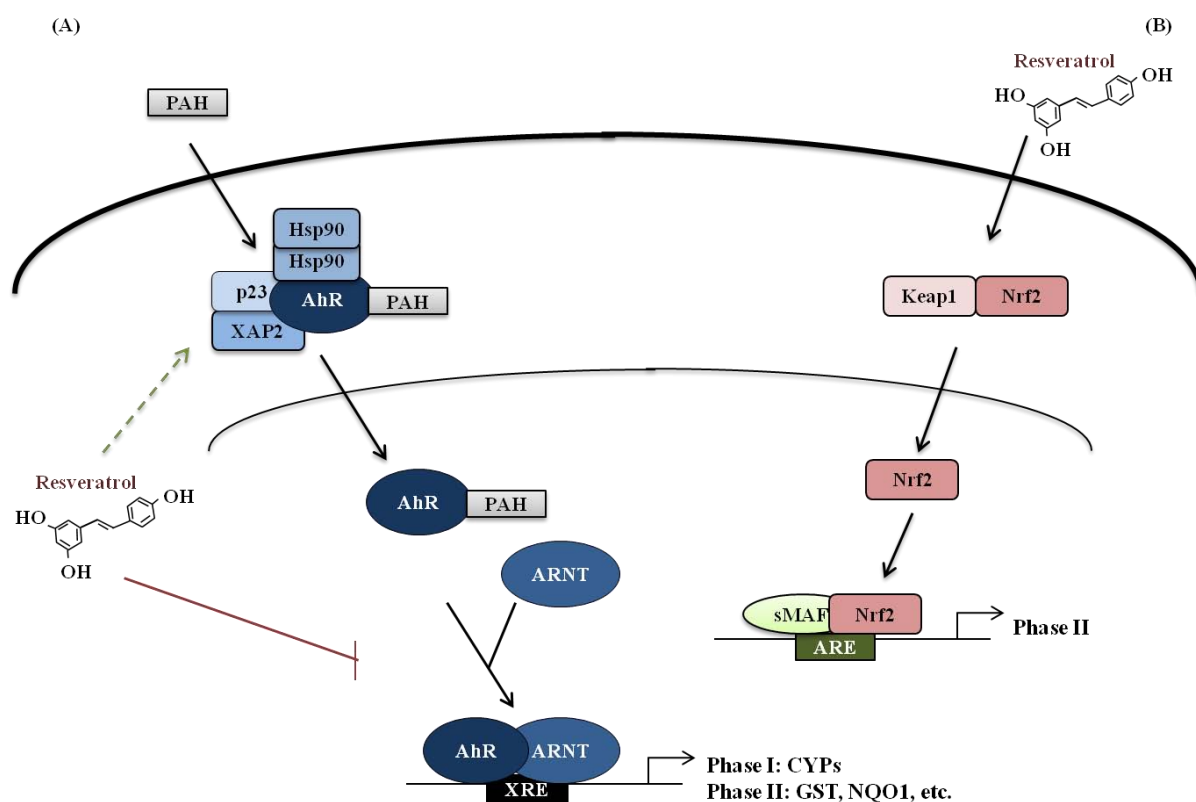


Figure 1.2. Effect of resveratrol on AhR/Nrf2 signaling pathways. See text for AhR and Nrf2 pathway description. (A) Resveratrol inhibits AhR/ARNT recruitment to XRE-driven promoters (red line) and is suggested to displace PAH binding, thus stabilizing cytosolic AhR complex (dotted line); see text for refs. (B) Resveratrol promotes Nrf2 nuclear translocation via Keap1 dissociation. This results in dimerization with small Maf (sMaf) and activation of ARE-driven promoters⁽¹⁸⁾.

1.3.1. Aryl hydrocarbon receptor (AhR)

Resveratrol is reported to alter phase I enzyme expression and activity in both an AhR-dependent and AhR-independent manner⁽¹⁹⁾. The canonical AhR-dependent signaling pathway is thought to contribute to carcinogenic initiation by phase I enzyme-activated polycyclic/poly-halogenated aromatic hydrocarbons (PAH/PHAH), and inhibition of AhR signaling by resveratrol is thought to suppress this initiative process. Under basal conditions, unbound AhR forms a multimeric complex in the cytosol. However, upon ligand binding (e.g. binding by PAH/PHAHs), AhR translocates to the nucleus (shedding the multimeric complex), forms a heterodimer with the AhR nuclear translocator (ARNT), and binds to gene promoters containing xenobiotic response elements (XREs) resulting in the transactivation of phase I enzymes⁽²⁰⁾.

Several reports demonstrate the inhibitory effects of resveratrol on AhR-mediated activation of phase I enzymes. For example, resveratrol was shown to impair TCDD (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin)-induced recruitment of AhR and ARNT to the *CYP1A1/1B1* and *CYP1A1/1A2* promoter in MCF-7 breast and HepG2 liver cancer cells, respectively, resulting in decreased expression⁽²¹⁾. Resveratrol also effectively blocked TCDD-induced, AhR-dependent transcription in both an estrogen receptor (ER)-dependent and ER-independent manner^(22, 23). Using ER-positive cancer cells, Perdew *et al.*⁽²²⁾ observed that at micromolar concentrations, resveratrol reduced the AhR/ARNT complex at the *CYP1A1* promoter, resulting in near-complete inhibition of CYP1A1 expression and metabolic activity; however, at nanomolar concentrations, resveratrol repressed AhR-mediated induction of CYP1A1 without interfering with AhR association with XREs after TCDD

exposure. On the other hand, MacPherson and Matthews ⁽²³⁾ showed resveratrol's competitive displacement of TCDD from AhR, indicating prevention of TCDD-induced CYP1A1/1B1 expression mediated by AhR/ARNT complex recruitment to the promoter in both ER-positive and ER-negative breast cancer cells. Furthermore, resveratrol was demonstrated to reverse TCDD-induced, AhR-mediated CYP1A1 and matrix metalloproteinase (MMP) 9 expressions in the gastric cancer cell line AGS ⁽²⁴⁾.

Moreover, resveratrol effects phase I enzymes in an AhR-independent manner; the phytochemical is shown to directly inhibit the expression and activity of these enzymes. Resveratrol effectively blocked CYP1A1 and/or CYP1B1 expression and activity induced by B[a]P (benz[a]pyrene), DMBA (7, 12-dimethylbenz[a]pyrene), dioxin, or TCDD in differentiating keratinocytes ^(25, 26), immortalized bronchial epithelial cells ⁽²⁷⁾, and breast cancer cells ^(19, 28, 29). Resveratrol also suppressed CYP1A1/1B1 expression *in vivo* utilizing a Balb/c animal model ^(30, 31). Furthermore, Mikstacka *et al.* ⁽³²⁾ suggest that the addition of thiol- and methoxy-groups to the resveratrol backbone increased selectivity for and inhibition of CYP1A1 and CYP1B1.

In addition to modulation of CYP1A1/CYP1B1 expression and activity, resveratrol is demonstrated to negatively regulate other CYP family members: inhibition of B[a]P-induced CYP1A2 and CYP2B expression ⁽³¹⁾ and abrogation CYP2E1 catalytic activity ⁽³³⁾ *in vivo*. CYP3A4, which is predominantly overexpressed in colon and liver cancers, has been found to be inhibited by resveratrol ⁽¹³⁾. Thus, resveratrol plays a suppressive role in AhR-dependent and AhR-independent (e.g. effecting CYPs directly) activation of phase I enzymes, contributing to the anticancer activity of this dietary phytoalexin.

1.3.2. Nuclear factor E2-related factor 2 (Nrf2)

Induction of Nrf2 signaling by resveratrol is thought to confer protection against phase I enzyme-activated carcinogens and associated carcinogenicity via the transactivation of antioxidant and phase II detoxifying enzymes⁽³⁴⁾. Under basal conditions, Kelch-like ECH-associated protein 1 (Keap1) sequesters Nrf2 in the cytoplasm, targeting the transcription factor for proteasomal degradation. However, when induced by electrophiles, reactive oxygen species, or dietary phytochemicals, Nrf2 dissociates from Keap1 and translocates to the nucleus where it dimerizes with small Maf proteins and activates antioxidant response element (ARE)-driven promoters^(18, 34).

Bishayee *et al.*⁽³⁵⁾ demonstrated that attenuation of DENA (diethyl nitrosamine)-induced liver carcinogenesis by resveratrol was mediated by increased Nrf2 expression. Resveratrol induction of NAD(P)H quinone oxidoreductase (NQO1) expression in TCDD-treated MCF10F immortalized breast cells involved Nrf2, leading to suppression of DNA adduct formation⁽²⁸⁾. NQO1 was also increased by resveratrol after 4-hydroxyestradiol and estradiol-3-4-quinone treatment⁽³⁶⁾. Induction of Nrf2 signaling by resveratrol resulted in increased expression of NQO1, heme-oxygenase-1 (HO-1), and glutamate cysteine ligase (GCL) catalytic subunit in cigarette smoke extract (CSE)-treated bronchial epithelial cells⁽³⁷⁾. Kode *et al.*⁽³⁸⁾ observed restored glutathione levels in CSE-treated A549 lung alveolar epithelial cancer cells by resveratrol; this effect was mediated via Nrf2-induced GCL expression and activity through the inhibition of CSE-modified Nrf2 posttranslation. Resveratrol protected primary hepatocytes exposed to oxidative stress via increased Nrf2-mediated NQO1, catalase, superoxide dismutase, glutathione reductase, glutathione

peroxidase, and glutathione-S-transferase expression⁽³⁹⁾. Furthermore, resveratrol increased NQO1 expression and activity in K562 leukemia cell line, which was associated with resveratrol-induced Nrf2/Keap1 complex disruption, Nrf2 nuclear translocation, and subsequent binding to ARE within the NQO1 promoter⁽⁴⁰⁾. These results indicate that Nrf2 is a key protein that controls resveratrol-induced anti-tumorigenesis in several cancers. In contrast, Kawai *et al.*⁽⁴¹⁾ observed Nrf2 cytoplasmic accumulation and inhibition of Nrf2-dependent transcription by resveratrol, presumably mediated through induced sirtuin 1 (SIRT1) deacetylase activity, in both K562 leukemic and HepG2 hepatocellular carcinoma cell lines. Thus, resveratrol could affect not only translocation but also accumulation of Nrf2, potentially contributing to these context-dependent observations.

1.4. Modulation of Cell Cycle Progression and Apoptosis

The deregulation of cell cycle machinery and evasion of apoptosis, which contribute to aberrant cell proliferation and survival, are considered a fundamental hallmark of cancer development^(42, 43). Therefore, identification of agents that target this abnormal signaling offers an important strategy for both chemoprevention and chemotherapy. Resveratrol is one such compound; several studies have demonstrated that the anti-proliferative effect of resveratrol on cancer cells is attributable to inhibition of multiple pro-survival signaling pathways^(44, 45).

1.4.1. Cell cycle proteins

Eukaryotic cell replication involves a series of processes collectively known as the cell cycle ⁽⁴⁶⁾. The cell cycle is divided into two major events: (1) interphase (divided into four phases: G0, G1, S, and G2), in which the cell grows and accumulates nutrients, and (2) mitosis (M), where the cells divide into two daughter cells. Proper functioning of the cell cycle is important; the presence of numerous internal checkpoints allows the cell to detect genetic damage and stalls cell cycle progression until these aberrations are repaired or apoptosis is triggered. Failure of DNA quality control by checkpoints (and loss of regulatory balance) facilitates uncontrolled cell proliferation and the development of cancer ⁽⁴⁶⁾.

Cyclins and cyclin-dependent kinases (CDKs) are key regulatory molecules governing the ordered processes of the cell cycle. Activation of CDKs by cyclins results in the phosphorylation of target proteins, leading to the activation or inactivation of these targets, and facilitates progression into the next phase of the cell cycle. Of the many cyclins, cyclin D1 is an important cell cycle regulator involved in the G1/S phase transition and in the regulation of proliferation and differentiation. Cyclin D1, produced in response to growth factors, is often increased in various cancers due to deregulated degradation ⁽⁴³⁾.

Resveratrol is reported to induce G1/S or G2/M phase cell cycle arrest in many cancer cell lines, attributable to the modulation of cyclin-associated CDK proteins ⁽⁴⁴⁾. Experiments in cell culture revealed that down-regulation of cyclin D1 and/or CDK4/6 by resveratrol resulted in the inhibition of cell proliferation, growth arrest, and subsequent induction of apoptosis. Such an effect was observed in studies of the bladder ⁽⁴⁷⁾, breast ⁽⁴⁸⁾, colon ⁽⁴⁹⁾, liver ⁽⁵⁰⁾, multiple myeloma ⁽⁸⁾, pancreas ⁽¹²⁾, prostate ^(51, 52), and skin cancer cells ^(11, 53).

These results were also observed *in vivo* utilizing an UVB-induced model of skin tumorigenesis ⁽⁵⁴⁾. Resveratrol treatment of colorectal cancer cells also inhibited expression of cyclin D1 and its complex formation with CDK4 ⁽⁵⁵⁾; it is likely that resveratrol-induced cyclin D1 suppression may result from β -catenin inactivation in colorectal cancer cells and should be considered a molecular target in these cancers ⁽⁴⁹⁾. Moreover, treatment with resveratrol potentiated the cytotoxic effects of the chemotherapeutic drugs doxorubicin and gemcitabine in chemo-resistant B16 melanoma and pancreatic cancer cells, respectively, *in vitro* and inhibited tumor growth *in vivo* ^(11, 12).

The CDK inhibitor p21 interacts with cyclin-CDK complexes to negatively regulate their expression and is responsible for the G1 phase arrest phenotype ⁽⁴³⁾. In contrast to cyclin D1, resveratrol-mediated cell cycle arrest is associated with p53-mediated p21 expression. Activation of p21 or p27 by resveratrol was observed in bladder ⁽⁴⁷⁾, breast ⁽⁴⁸⁾, liver ⁽⁵⁶⁾, lung ⁽⁵⁷⁾, prostate ⁽⁵¹⁾, and skin cancer cells ^(53, 54). Cell cycle arrest was associated with apoptosis. Interestingly, Castello and Tessitore ⁽⁵⁸⁾ demonstrated resveratrol inhibition of U937 leukemic monocyte lymphoma cell proliferation was associated with increased cyclin A and cyclin E expression, concomitant p21 and ribonucleotide reductase inhibition, and the absence of apoptosis.

1.4.2. Apoptosis

Apoptosis, or programmed cell death, involves a series of biochemical events that lead to a variety of morphological changes such as blebbing, cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal

DNA fragmentation⁽⁵⁹⁾. Apoptosis removes damaged and unwanted cells and maintains tissue integrity and function⁽⁵⁹⁾.

p53 tumor suppressor: The tumor suppressor protein p53 is a critical transcription factor involved in the regulation of cell proliferation and apoptosis; as such, it is a key mediator in the prevention of carcinogenesis⁽⁶⁰⁾. It has been shown that resveratrol can cause apoptosis through p53-dependent and p53-independent pathways⁽⁴⁴⁾. Several groups, including our laboratory, have implicated the activation of p53-dependent pathway(s) in the observed anti-proliferative effects of resveratrol. For example, Heiss *et al.*⁽⁶¹⁾ demonstrated that chronic administration of resveratrol at a sub-apoptotic dose resulted in senescent-like growth arrest in different carcinoma cell lines. This effect was due to increased reactive oxygen species generation, ataxia telangiectasia mutated kinase (ATM) and p53 activation (via p38MAPK-mediated p53 phosphorylation at serine 15), induction of p21, and subsequent induction of senescence. Utilizing both *in vitro* and *in vivo* models of tumorigenesis and carcinogenesis, resveratrol is also shown to induce apoptosis via activation of both intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) pathways⁽⁴⁴⁾. Resveratrol altered the Bax:Bcl2 protein ratio in the A431 epidermoid cancer cell line, leading to mitochondrial membrane depolarization and subsequent induction of caspase-dependent apoptosis, presumably mediated through p53 activation⁽⁶²⁾. Similar results were observed in breast⁽⁶³⁾ and in the DENA-initiated, phenobarbital-promoted *in vivo* model of liver carcinogenesis⁽⁶⁴⁾. Resveratrol increased p53-mediated expression of pro-apoptotic proteins (e.g. Bax, Bak, Bim, PUMA, Noxa, etc.) and the release of mitochondrial proteins (e.g. cytochrome *c*, Smac/DIABLO, etc.) to the cytosol, thus triggering suppression of

inhibitors of apoptosis proteins (Bcl2, Bcl-XL, survivin, XIAP, etc.) and caspase activation in various cancer cell lines^(9, 10, 65-67). In addition, resveratrol induced the redistribution of death receptors to membrane lipid rafts in colorectal and multiple myeloma/T cell leukemia cancer cells, thus sensitizing these cells to death receptor-mediated apoptosis^(68, 69). Furthermore, resveratrol sensitized TNF-related apoptosis-inducing ligand (TRAIL)-dependent cell death in androgen-sensitive and androgen-insensitive prostate cancer cells^(9, 10).

How resveratrol increases p53 expression is not clear; it is believed that resveratrol treatment causes DNA damage, which facilitates p53 activation. Tyagi *et al.*⁽⁷⁰⁾ observed resveratrol-induced cell cycle arrest was mediated through increased Cdc2 tyrosine 15 phosphorylation via the activation of the DNA damage pathway (i.e. ATM/ATR-Chk1/2-Cdc25 pathway). Resveratrol treatment also suppressed the metastasis-associated protein-1/nucleosome remodeling deacetylation complex, allowing for increased p53 acetylation and subsequent activation of pro-apoptotic genes⁽⁷¹⁾.

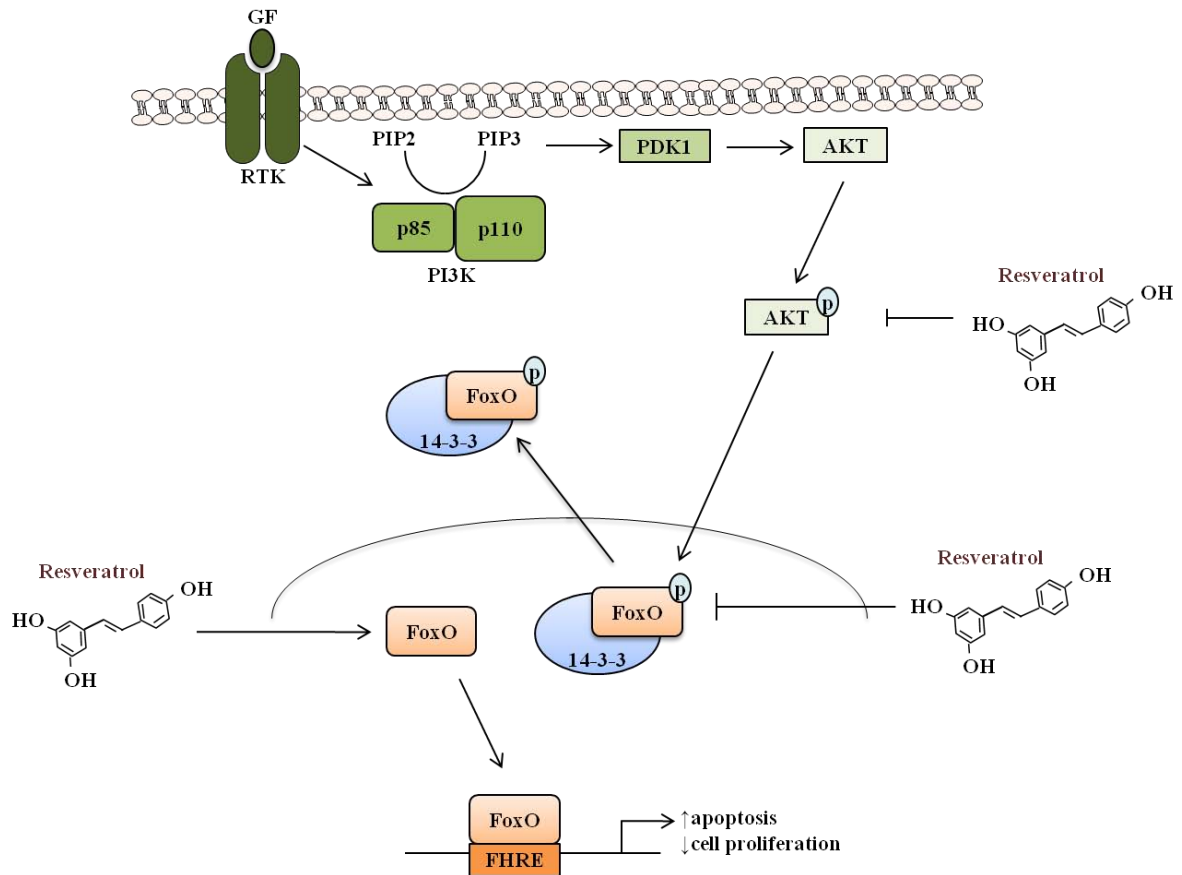
Forkhead box, subgroup O (FoxO) transcription factor: Proteins known as FoxO, divergent members of the Fox/winged-helix transcription factor superfamily, are recognized tumor suppressors that play a critical role in cell fate decisions⁽⁷²⁾. Composed of four members (FoxO1, 3, 4, and 6), FoxO transcription factors coordinate the expression of genes involved in diverse cellular processes, including cell cycle progression, apoptosis, and oxidative stress response. The function of FoxO proteins is regulated by posttranslational modification(s), specifically phosphorylation and acetylation, which dictates their subcellular localization and transcriptional activity⁽⁷³⁾.

Abrogation of FoxO function occurs in numerous cancers due in large part to the constitutive activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, a key regulator of FoxO activity. PI3K/Akt-mediated phosphorylation results in the inactivation of FoxO transcription factors. FoxO phosphorylation by PI3K/Akt facilitates their interactions with 14-3-3 chaperone proteins and nuclear export; cytoplasmic sequestration inhibits FoxO-dependent transcription⁽⁷²⁾. FoxOs are also regulated by de/acetylation in response to oxidative stress. Several studies identify FoxOs as targets of the NAD-dependent class III histone/protein deacetylase SIRT1. Under conditions of oxidative stress, SIRT1 forms a complex with and deacetylates FoxO transcription factors, resulting in the preferential activation of cell cycle arrest/stress resistance-related genes, thereby promoting cell survival⁽⁷⁴⁻⁷⁶⁾. Furthermore, silencing of SIRT1 resulted in FoxO4-mediated apoptosis in epithelial-derived cancer cells⁽⁷⁷⁾. Thus, targeting these inhibitory pathways may prove advantageous in the prevention/treatment of cancers.

The activation of FoxO transcription factors is implicated in the observed anticancer activities of resveratrol. Using prostate cancer cells, Chen *et al.*⁽⁷⁸⁾ demonstrated resveratrol's ability to inhibit the phosphorylation of PI3K/Akt (i.e. PI3K/Akt inactivation) resulted in decreased FoxO phosphorylation. Resveratrol increased nuclear translocation, DNA binding affinity, and transcriptional activity of FoxOs. Furthermore, the anti-proliferative/pro-apoptotic effect (Bim/TRAIL/DR4/DR5/p27^{Kip} induction and cyclin D1 inhibition) of resveratrol on prostate cancer cells is FoxO-dependent. Similar results were also observed *in vivo*⁽⁷⁹⁾. A schematic representation of resveratrol-mediated FoxO regulation is shown in **Figure 1.3**.

As described, SIRT1 induced FoxO deacetylation results in the preferential activation of cell survival-related genes. Being that resveratrol is considered a SIRT1 agonist, although currently controversial, one could speculate that the activation of a FoxO-mediated pro-survival mechanism may be induced by resveratrol-increased SIRT1 deacetylase activity. Indeed, this is demonstrated in cardiomyocytes; resveratrol, presumably through SIRT1, increased FoxO transcriptional activation of stress resistance-related genes and subsequent phosphorylation by Akt⁽⁸⁰⁾. However, currently, the resveratrol/SIRT1/FoxO signaling axis has not been studied in cancer cells thus allowing one to infer such an effect is context dependent.

Figure 1.3. Effects of resveratrol on the FoxO signaling pathway. See text for FoxO pathway description. Resveratrol is demonstrated to inhibit Akt activation and subsequent FoxO phosphorylation and nuclear export. In contrast, resveratrol-induced FoxO expression facilitates transactivation of forkhead response element (FHRE)-driven promoters (see text for refs.) GF, growth factor; RTK; receptor tyrosine kinase.



1.5. Suppression of the Inflammatory Signaling Pathway

Chronic inflammation contributes to the development of many diseases, including cancer⁽⁸¹⁾. For example, chronic colitis is associated with an increased risk of developing colorectal cancer, and the susceptibility to cancer increases when the tissue is chronically inflamed⁽⁸²⁾. Many tumors contain non-cancer cells, including immune and vascular cells that are important in inflammation. The critical molecular interactions between neoplastic cells and inflammatory cells remain unknown, but both pro- and anti-inflammatory mediators are likely involved⁽⁸¹⁾. Thus, it is clear the link between inflammation and cancer is strong and that the interplay between the immune system and the development and remission of

cancer is critical. These inflammatory processes contribute to the development and progression of carcinogenesis, including tumor growth, angiogenesis, invasion, and metastasis ⁽⁸¹⁾.

1.5.1. Nuclear factor- κ B (NF- κ B)

A key mediator of inflammation-induced cellular transformation is the transcription factor NF- κ B. Cytoplasmic sequestration by I κ B α prevents NF- κ B-dependent transactivation. Upon activation, IKK β phosphorylates I κ B α resulting in its degradation, and facilitates NF- κ B nuclear translocation and subsequent activation of transcription ⁽⁸³⁾.

It has been postulated that the anticancer effects of resveratrol are attributable to the inactivation of NF- κ B-dependent signaling. Resveratrol inhibited IKK β -mediated I κ B α phosphorylation, resulting in increased I κ B α expression, NF- κ B cytoplasmic retention, and subsequent NF- κ B inactivation ^(84, 85). Resveratrol also blocked interleukin-1 β (IL-1 β)-, tumor necrosis factor α (TNF α)-, and HO-1-induced NF- κ B activation ⁽⁸⁶⁻⁸⁸⁾. Resveratrol treatment of multiple myeloma cells resulted in cell cycle arrest and suppression of NF- κ B-dependent signaling related to proliferation (cyclin D1), survival (Bcl-2, Bcl-XL, XIAP, c-IAP, and survivin), angiogenesis (vascular endothelial growth factor, VEGF), and metastasis (MMP9 and IL-6) ^(8, 89). Similar results were observed *in vivo* using a DENA-initiated liver carcinogenesis model ⁽⁹⁰⁾ and Mia PaCa-2 orthotopic model of pancreatic cancer ⁽¹²⁾. Furthermore, resveratrol prevented PMA (phorbol 12-myristate 12-acetate)-induced HT1080 fibrosarcoma cell adhesion to endothelial cells via modulation of intercellular adhesion molecule-1 expression and NF- κ B activity ⁽⁹¹⁾. Taken together, it is clear that resveratrol not

only curbs expression of NF- κ B, but also impedes the phosphorylation of I κ B α thereby keeping the constitutive NF- κ B subunit in an inactive state, resulting in suppression of the inflammatory and pro-tumorigenic changes associated with this pathway (**Figure 1.4**).

1.5.2. Cyclooxygenase-2 (COX-2)

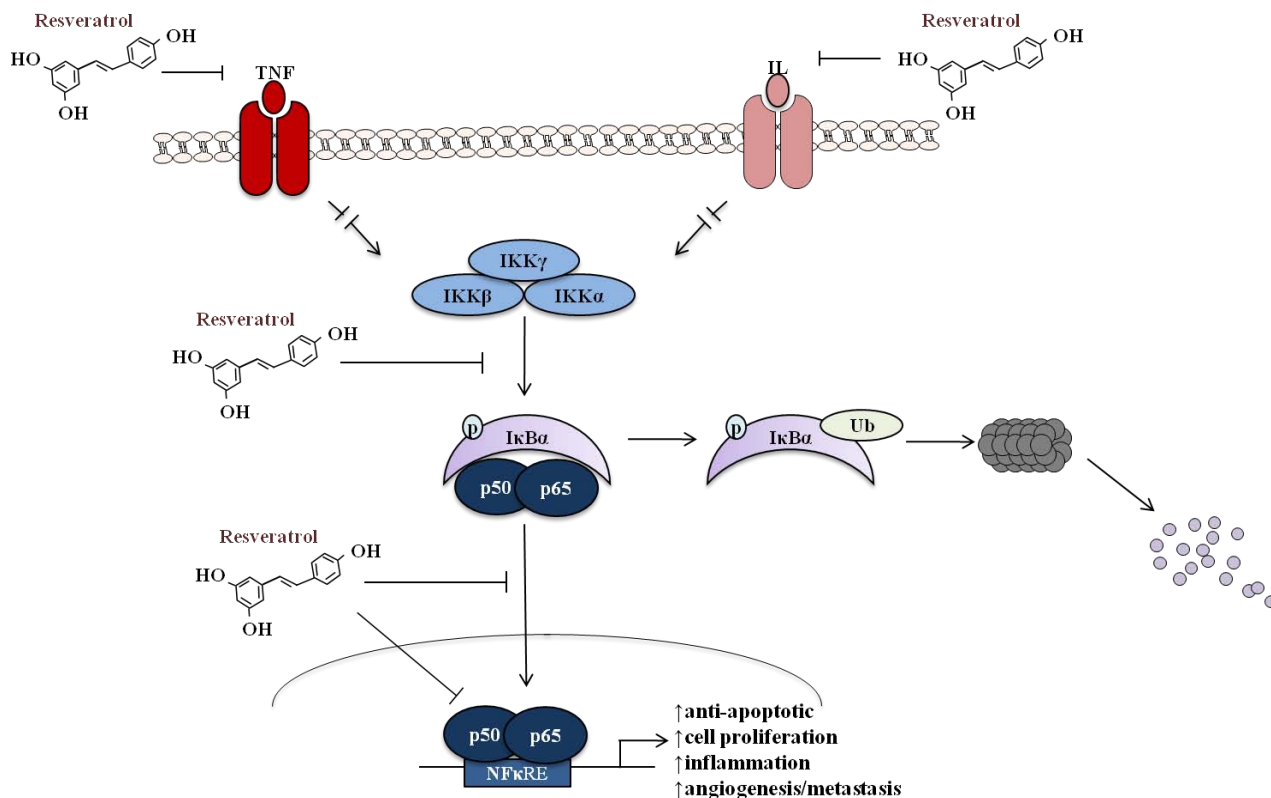
Overexpression of COX-2 is suggested to contribute to tumor development via modulation of the multiple stages of tumorigenesis: cell survival signaling pathways, angiogenesis, invasion, and metastasis⁽⁹²⁾. Resveratrol inhibited the expression and activity of COX-2 through direct interaction with the enzyme's catalytic domain⁽⁹³⁾. Zykova *et al.*⁽⁹⁴⁾ demonstrated inhibition of COX-2 activity (prostaglandin E2 synthesis), inhibition of colony formation, and suppression of anchorage-independent growth in HT29 colon cancer cells by this compound; COX-2 inhibition was absolutely required for resveratrol-mediated activity. Resveratrol also reduced COX-2 expression in DENA-initiated liver⁽⁹⁰⁾, DMBA-initiated mammary⁽⁹⁵⁾, and TPA (12-*o*-tetradecanoylphorbol-13-acetate)-initiated skin⁽⁹⁶⁾ carcinogenesis. Furthermore, using a DSS (dextran sodium sulfate)-induced mouse model of colitis, resveratrol treatment was shown to reduce COX-2 expression^(97, 98); similar results were observed in DSS-induced, azoxymethane-promoted model of colitis-driven colon cancer⁽⁸²⁾.

1.5.3. Pro-inflammatory cytokines: Tumor necrosis factor α (TNF α) and interleukins (ILs)

Pro-inflammatory cytokines, such as TNF α and ILs, are important mediators of both inflammation-related cancer and cancer-related inflammation. Expression of these cytokines

is associated with carcinogenesis mediated via the activation of the NF- κ B signaling pathway^(81, 99). Resveratrol reversed colitis-associated expression of TNF α , IL-6, and IL-1 β induced by DSS^(82, 97, 98). Resveratrol suppressed IL-1 β -stimulated cell proliferation, reduced IL-1 β -production, and IL-1 β -induced NF- κ B activation in acute myeloid leukemia cells⁽⁸⁶⁾. Using streptozotocin-nicotinamide-induced diabetic rats, Palsamy and Subramanian⁽¹⁰⁰⁾ reported that resveratrol ameliorates induction of pro-inflammatory cytokines (e.g. TNF α , IL-1 β , IL-6) in pancreatic β -cells. Leiro *et al.*⁽¹⁰¹⁾ proposed the anti-inflammatory activity ascribed to resveratrol is related, in part, to the inhibition of TNF α and IL-1 β mRNA processing, which resulted in reduced migration of head and kidney leukocytes in response to inflammatory stimuli.

Figure 1.4. Effects of resveratrol on the NF- κ B signaling pathway. See text for NF- κ B pathway description. Resveratrol is demonstrated it inhibit NF- κ B signaling at all steps (see text for refs).



1.6. Inhibition of Angiogenesis, Invasion, and Metastasis

Angiogenesis, the formation of new blood vessels, is critical to tumor development and growth, invasion, and metastasis⁽¹⁰²⁾. Neovascularization facilitates cell dissociation from the primary tumor site, entry into circulation, and establishment of the tumor(s) at a secondary site (a process termed metastasis)⁽¹⁰³⁻¹⁰⁵⁾. Disruption of the angiogenic and metastatic signaling cascades is another mechanism by which resveratrol exerts its chemotherapeutic effect.

1.6.1. Hypoxia inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF)

Adaptation to hypoxia by tumor cells is mediated by HIF-1 α , which regulates the expression of proteins, such as VEGF, associated with angiogenic and invasive signaling cascades ⁽¹⁰⁶⁾. Treatment with resveratrol reduced expression of HIF-1 α and VEGF in ovarian cancer cells; resveratrol-mediated activity was attributed to inactivation of PI3K/Akt and protein translational regulators, enhancement of HIF-1 α proteasomal degradation, and subsequent suppression of VEGF ⁽¹⁰⁷⁾. These results were also observed in tongue squamous cell carcinoma ⁽¹⁰⁸⁾ and lysophosphatidic acid-stimulated ovarian cancer cells ⁽¹⁰⁹⁾. VEGF expression was also reduced in multiple myeloma ⁽⁸⁹⁾ and endometrial cancer cells ⁽¹¹⁰⁾. Additionally, the anti-migratory/angiogenic effects of resveratrol observed in human umbilical vein epithelial cells was FoxO-dependent and predicated on PI3K/Akt pathway inhibition; this observation was associated with decreased VEGF expression ⁽¹¹¹⁾.

1.6.2. Matrix metalloproteinase 2 and 9 (MMP2 and MMP9)

Cell invasion and metastasis are facilitated via the proteolytic degradation of basement membrane and connective tissue; this degradation of extracellular matrices creates a channel by which tumors escape into circulation. MMPs are essential factors in this process ^(81, 103). Numerous reports illustrate the anti-invasive and anti-metastatic properties of resveratrol. Treatment with resveratrol inhibited heregulin- β 1-mediated MMP9 expression in breast cancer cells through the inactivation of ERK1/2 signaling resulting in suppression of invasion ⁽¹¹²⁾. TNF α -mediated MMP9 expression and invasion was also reduced in resveratrol-treated hepatocellular carcinoma cells; this suppression was associated with

down-regulation of NF- κ B signaling⁽⁸⁷⁾. Inactivation of NF- κ B signaling in lung adenocarcinoma cells also contributed to the anti-invasive effect of resveratrol and led to inhibition of HO-1-induced MMP9 expression, migration, and invasion⁽⁸⁸⁾. Resveratrol blocked insulin growth factor-1 (IGF-1)-mediated cell migration *in vitro* through inactivation of the PI3K/Akt signaling pathway and down-regulation of MMP2 induced by IGF-1⁽¹¹³⁾. Moreover, resveratrol blunted TNF α -induced monocyte adhesion and migration through modulation of adhesion molecules and MMP2 enzymatic activity⁽¹¹⁴⁾.

1.7. Other Molecular Targets

1.7.1. Topoisomerase II

Topoisomerases are ubiquitously expressed enzymes that regulate the topological state of DNA; these enzymes remove knots and tangles from the genome through the winding and unwinding of DNA, generating transient double-stranded breaks in the DNA double helix to control cell division and proliferation. In malignant tumor cells, this enzyme is overexpressed when compared to normal, quiescent cells; thus, topoisomerase II has been suggested as a potential chemopreventive target⁽¹¹⁵⁾. Resveratrol is demonstrated to inhibit topoisomerase activity. Treatment of glioblastoma cells with the compound interfered with decatenation (i.e. unwinding of DNA) by topoisomerase; these authors suggest resveratrol acted as a “topoisomerase poison” due to the prolongation of the topoisomerase-DNA complex⁽¹¹⁶⁾.

1.7.2. Human telomerase reverse transcriptase (hTERT)

Addition of hexameric repetitive sequences (referred to as telomeres) to chromosomal ends by telomerase ensures and maintains genomic stability. With each cell division, telomeres progressively shorten until a critical length is reached and the cell undergoes senescence or apoptosis. In most cancers, telomerase is highly activated resulting in tumor cell immortalization due to the maintenance of telomeres. This immortalization is associated with increased expression of the telomerase catalytic subunit, hTERT⁽¹¹⁷⁾. Therefore, targeting telomerase and its catalytic subunit hTERT provides an additional mechanism by which anticancer agents exert chemopreventive/chemotherapeutic effects. Resveratrol has been shown to suppress telomerase activity in breast⁽¹¹⁸⁾ and colon⁽¹¹⁹⁾ cancer cells associated with decreased nuclear levels of hTERT. Furthermore, resveratrol treatment of osteosarcoma and lung cancer cells induced telomeric instability, phosphorylation of histone H2AX and p53, and activation of DNA damage signaling⁽¹²⁰⁾.

1.8. Identification of Novel Molecular Targets

To identify novel molecular targets that mediate the anti-cancer activities of resveratrol, our laboratory performed microarray analysis using HCT-116 human colorectal cancer cells treated with resveratrol (50 μ M) for 24 h (**Table 1.2**). Among the 519 genes altered by resveratrol, three genes, *ATF3*, *NAG-1*, and *DDIT3* were of particular interest due to their suggested involvement as negative regulators of tumorigenesis and carcinogenesis.

Table 1.2. Alteration of gene expression profiles of HCT-116 colorectal cancer cells by resveratrol (50 μ M). Select up- and down-regulated genes from microarray analysis.

<i>Gene Symbol</i>	<i>Gene Name</i>	<i>GenBank No.</i>	<i>Up/Down</i>
<i>DDIT3</i>	DNA-damage-inducible transcript 3	NM_004083	Up
<i>ATF3</i>	Activating transcription factor 3	NM_004024	Up
<i>COBRA1</i>	Cofactor of BRCA1	NM_015456	Up
<i>OR8B8</i>	Olfactory receptor, family 8, subfamily B, member 8	NM_012378	Up
<i>AADACL1</i>	Arylacetamide deacetylase-like 1	NM_020792	Up
<i>PLK3</i>	Polo-like kinase 3 (<i>Drosophila</i>)	NM_004073	Up
<i>PCK2</i>	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	NM_004563	Up
<i>NAG-1</i>	NSAID-activated gene-1	NM_004864	Up
<i>WNT16</i>	Wingless-type MMTV integration site family, member 16	NM_016087	Down
<i>C4orf18</i>	Chromosome 4 open reading frame 18	NM_016613	Down
<i>FANCC</i>	Fanconi anemia, complementation group C	NM_000136	Down
<i>PDE4B</i>	Phosphodiesterase 4B	NM_001037	Down
<i>IGFBP7</i>	Insulin-like growth factor binding protein 7	NM_001553	Down

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1.8.1. Activating transcription factor 3 (*ATF3*)

ATF3 is a member of the ATF/cAMP response element binding protein (CREB) family of bZIP transcription factors and is characterized as a stress inducible/adaptive response gene⁽¹²¹⁾. Much controversy exists as to the role of *ATF3* in tumorigenesis due in part to the observation of both positive and negative effects. Recently, a dichotomous role was reported for *ATF3* in cancer development; the authors concluded its role as a tumor suppressor or oncogene is dependent on cell context and the extent of malignancy⁽¹²²⁾. However, several lines of evidence suggest that *ATF3* may function as a tumor suppressor, which will be discussed further in **Chapter 3** in addition to our recent findings.

1.8.2. Nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1)

NAG-1, a divergent member of the transforming growth factor β superfamily of cytokines, is suggested to play a role in a number of cellular processes, including inflammation and cell survival. NAG-1 was first identified by Baek et al.⁽¹²³⁾ through PCR-based subtractive hybridization from an NSAID-induced library in human colorectal cancer cells. NAG-1 was shown to be highly expressed in mature intestinal epithelial cells and significantly reduced in human colorectal carcinomas and neoplastic intestinal polyps of *Min* mice⁽¹²⁴⁾. NAG-1 overexpression reduced cell viability of breast cancer cells by up to 80%⁽¹²⁵⁾ and treatment of prostate cancer cells with purified NAG-1 induced apoptosis⁽¹²⁶⁾. In addition, NAG-1 transgenic mice are resistant to the development of intestinal tumors following treatment with azoxymethane or by introduction of a mutant *APC* gene⁽¹²⁷⁾. Furthermore, subsequent studies have identified NAG-1 induction by a number of dietary and naturally occurring compounds demonstrated to possess anticancer activity. These include genistein⁽¹²⁸⁾, epicatechin gallate⁽¹²⁹⁾, indole-3-carbinol⁽¹³⁰⁾, conjugated linoleic acid⁽¹³¹⁾, berberine^(132, 133), 6-gingerol⁽¹³⁴⁾, platycodon D⁽¹³⁵⁾, capsaicin⁽¹³⁶⁾, *Astragalus saponins*⁽¹³⁷⁾, and the isoflavonoid formononetin⁽¹³⁸⁾. Increased NAG-1 expression contributed to the inhibition of cell proliferation and induction of apoptosis by these compounds. Additionally, we and others have shown NAG-1 up-regulation by resveratrol resulted in increased p53-mediated cell death in colorectal, lung, and osteosarcoma cancer cells⁽¹³⁹⁾ and inhibition of cell proliferation in pancreatic cancer cells⁽¹⁴⁰⁾.

1.8.3. DNA-damage-inducible transcript 3 (DDIT3)

DDIT3 is a critical mediator in the induction of cell cycle arrest and/or apoptosis in response to ER stress. Transcriptional activation by ER stress results in downstream regulation of genes involved in the modulation of cell survival. For example, DDIT3 expression inhibits Bcl2 and leads to Bax translocation to the mitochondria resulting in activation of the intrinsic pathway of apoptosis⁽¹⁴¹⁾. In several studies, resveratrol is demonstrated to increase DDIT3 expression in colon^(142, 143) and leukemia⁽¹⁴⁴⁾ cancer cells. Increased DDIT3 expression contributed to induction of cell cycle arrest and/or induction of apoptosis by resveratrol.

1.8.4. Other potential targets

Although gene alteration by resveratrol has yet to be validated, each of the aforementioned genes (refer to **Table 1.2**) is suggested to act as either a negative or positive regulator of tumorigenesis. COBRA1, an integral component of negative elongation factor complex, is demonstrated to inhibit estrogen-dependent transcription and growth of breast cancer cells *in vitro*. Furthermore, loss of COBRA1 is associated with breast cancer progression and metastasis⁽¹⁴⁵⁾. Plk3, whose expression is rapidly increased in response to DNA damage and cellular stress, is believed to behave as a tumor suppressor gene and serve as a “functional link” in p53-induced cell cycle arrest and apoptosis⁽¹⁴⁶⁾. Thus, induction of COBRA1 or Plk3 may play a role in resveratrol-mediated inhibition of proliferation or induction of apoptosis in colorectal and other cancers. On the other hand, down-regulation of WNT16, FANCC, or PDE4B by resveratrol may lead to enhanced inhibition of cell growth

or sensitization to chemotherapy. For example, overexpression of WNT16 is associated with oncogene-induced senescence via regulation of p53 activity⁽¹⁴⁷⁾ and enhanced cell proliferation and clonogenicity of basal cell carcinomas⁽¹⁴⁸⁾. Disruption of FANCC, which results in deactivation of the Fanconi anemia pathway, sensitizes cancer cells to DNA damage and growth inhibition induced by DNA interstrand-cross-linking agents thereby abrogating chemo-resistance⁽¹⁴⁹⁻¹⁵¹⁾. Moreover, increased PDE4B expression is associated with inactivation of cAMP and abrogation of growth inhibitory effects of cAMP^(152, 153).

1.9. Summary

As discussed in previous sections, resveratrol is demonstrated to modulate the expression and/or activity of proteins involved in critical pathways of carcinogenesis. These include carcinogen activation and detoxification, inflammation, angiogenesis, invasion and metastasis. Although numerous molecular targets have been identified, the underlying mechanisms involved in the antitumor/anticancer activities of resveratrol are not completely understood. Thus, the objective of the studies discussed herein sought to investigate the effect of resveratrol treatment on gene modulation in human colorectal cancer cells in order to identify and characterize a novel molecular target(s) that may mediate the anticancer activities of resveratrol.

CHAPTER 2:
Materials and Experimental Methods

2.1. Cell Lines and Reagents

All human cancer cell lines were purchased from American Type Culture Collection (Manassas, VA) unless otherwise stated; authentication occurred via short tandem repeat profiling, monitoring of cell morphology, and karyotyping. SqCC/Y1 head and neck squamous cell carcinoma cell line was generously provided by Dr. Dong M. Shin (Emory University, Atlanta, GA) and characterized previously⁽¹⁵⁴⁾. HCT-116 and HT-29 colorectal carcinoma cells were maintained in McCoy's 5A culture medium; SW480 colorectal carcinoma cells were maintained in RPMI-1640 culture medium. Caco-2 colorectal carcinoma cells and SqCC/Y1 were grown in DMEM culture medium. A549 lung epithelial carcinoma cells, LoVo colorectal carcinoma cells, and PC3 prostate adenocarcinoma cells were cultured in Ham's F12 medium. All media were supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). Resveratrol was purchased from Alexis Biochemicals (San Diego, CA), 3'-diindolymethane (DIM) from Sigma Aldrich (St. Louis, MO), and trichostatin A (TSA) and cycloheximide (CHX) from Fisher Scientific (Pittsburgh, PA). All chemicals were dissolved in either dimethylsulfoxide (DMSO) or ethanol. ATF3 (sc-188), Egr-1 (sc-110), KLF4 (GKLF4; sc-20691), and β -actin (sc-47778 HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acetylated Lysine (#9441), phosphor-SIRT1 (Ser27; #2327), and phosphor-SIRT1 (Ser47; #2314) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-SIRT1 (clone 10E4; #04-1557) antibody was purchased from Millipore (Billerica, MA).

2.2. Construction of Plasmids

For deletion analysis of the *ATF3* promoter, *pATF3 -1850/+34*⁽¹⁵⁵⁾ was serially deleted using the Erase-a-Base System (Promega, Madison, WI) according to manufacturer's protocol. The *pATF3 -514 del Egr-1* reporter construct was previously described⁽¹⁵⁶⁾. Putative binding sites of KLF4 within the -514 bp region of the *ATF3* promoter were deleted using Stratagene's QuikChange II Site Directed Mutagenesis Kit (La Jolla, CA) with KLF4-A and KLF4-B deletion primers (see **Table 2.1**). *pATF3 -514 del Egr-1 KLF4* reporter construct was generated using *pATF3 -514 del Egr-1* and KLF4-A deletion primers as described. Deletions were confirmed by DNA sequencing. The ATF3 (pCG-ATF3) and SIRT1 (pBABE/T1) expression vectors were kindly provided by Drs. Tsonwin Hai (Ohio State University, Columbus, OH) and Thomas E. Eling (NIEHS, Research Triangle Park, NC), respectively. KLF4 (pcDNA3.1/His/V5/KLF4) expression vector was generated using human KLF4 specific primers (see **Table 2.1**). Egr-1 wild type (pcDNA3.1/NEO/Egr-1; WT) expression vector was described previously⁽¹⁵⁷⁾; Egr-1 del AD, del ZD2, and KDKK→aDaa mutation constructs were generated using specific primers (see **Table 2.1**). Sp1 (pCMV-Sp1) and p53 (pcDNA3.1/myc/His/p53) expression vectors were previously described^(139, 158). The *pNAG-133/+41* and pEBS1⁴luc reporter constructs were described by Baek *et al.*^(157, 158).

2.3. Microarray Analysis

HCT-116 colorectal cancer cells were treated with DMSO or resveratrol (50 or 100 µM) for 24 h. Total RNA was amplified using Agilent Low RNA Input Fluorescent Linear

Amplification Kit (Santa Clara, CA) protocol. Starting with 500 ng of total RNA, Cy3- or Cy5-labeled cRNAs was produced according to the manufacturer's protocol. For each two-color comparison, 750 ng of each Cy3- and Cy5-labeled cRNAs was mixed and fragmented using Agilent *in situ* Hybridization Kit protocol. Hybridizations were performed for 16 h in a rotating hybridization oven using Agilent 60-mer oligo microarray processing protocol. Data was obtained using Agilent Feature Extraction Software (v7.5) using defaults for all parameters. Intensity plots were rated for each ratio experiment, and genes were considered "signature genes" if the *p* value was less than 0.001. Functional annotation of genes was performed according to the Gene Ontology Consortium (<http://www.geneontology.org/index.shtml>) by Gene Spring (v7.3).

2.4. Reverse Transcription and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR and qRT-PCR)

Total RNA was isolated from HCT-116 cells treated with DMSO or resveratrol (50 μ M) using 5 Prime Perfect RNA Cell/Tissue Kit (Gaithersburg, MD) and 1 μ g of RNA was reverse transcribed using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Rockford, IL). One μ L of synthesized DNA was added to 24 μ L of PCR reaction mixture containing human specific gene primers (see **Table 2.2**). Thermo cycling occurred as follows: initial denaturation at 94°C for 3 min followed by 30 (*ATF3*, *DKK1*, *Egr-1*, *HO-1*, *KLF4*, *NAG-1*, *Nur77* and *PDE4B*) and 25 (*GAPDH*) cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were electrophoresed on 1.4% agarose gel and photographed under ultraviolet light. The intensity of each band was

quantified using Scion Image software (Scion Corp., Frederick, MD) and normalized to *GAPDH*, an internal control. Quantitative reverse transcription PCR (qRT-PCR) was performed according to Fast SYBR Green Master Mix protocol (Applied Biosystems, Carlsbad, CA) and analyzed using MyIQ Single Color Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

Table 2.1. Primers used for the construction of plasmids.

<i>Construct</i>	<i>Forward Primer (5' to 3')</i>	<i>Reverse Primer (5' to 3')</i>
del KLF4-A	ccccctctcttcggcccgccctggccccc	cggggccgaaagagaggggggcactggatg
del KLF4-B	ggccctcctcctcctccgctcggttcgg	cggaggaaggaggaggggccaaggcggggc
pM/Egr-1	ttgaattcgccgcgccaaggccgag	ttaagcttgcaaatccaattgtcc
del AD	catcacctatggcctagtgcacatgaccaaccc ac	tcactaggccataggtgatggggggcagtcgagt g
del ZD2	gccctcccagttcgctgcgacatctgtggaag aa	cgcaggcgaactggaagggtcttgccctgtgtg g
KDKK→aDaa (Egr-1 aDaa)	cggcaggcggacgcggcagcagacaaaagt gtgtggcctcttcg	gtctgctgccgcgtccgctgccgcaagtggatct tggtatgcc
KLF4 and pVP16/KLF4	cgaattctatggctgtcagcgacgcg	cccaagcttttaaaatgcctcttcattgtgaaggc

Table 2.2. Primers used for RT-PCR and/or qRT-PCR.

<i>Gene</i>	<i>Forward Primer (5' to 3')</i>	<i>Reverse Primer (5' to 3')</i>
ATF3	gtttgaggatttgctaacctgac	agctgcaatctatttcttctcgt
DKK-1	catcagactgtgcctcagga	ggcaagacagaccttctcca
Egr-1	ctgcgacatctgtggaagaa	tgtcctgggagaaaagggtg
HO-1	atgacaccaaggaccagagc	gtgtaaggaccatcggaga
KLF4	cgaattctatggctgtcagcgacgcg	cccaagcttttaaaatgcctcttcattgtgaaggc
NAG-1	acgctacgaggacctgctaa	agattctgccagcagttggt
Nur77	cacagcttgctgtcgatgt	tcttgcaatgatgggtgga
PDE4B	ataccgatcgattcaggtc	agtgggtggtagggactttg
GAPDH	tcaacggatttggtcgtatt	ctgtggtcatgagtcctcc

2.5. RNA Stability

HCT-116 cells were grown to 60% to 80% confluence and pretreated with DMSO or resveratrol (50 μ M) for 24 h in serum-free media. After pretreatment, actinomycin D was added directly to the media at a dose of 5 μ M to terminate transcription as indicated. Total RNA was isolated and reverse transcribed. Reverse transcription PCR (RT-PCR) for *ATF3* and *GAPDH* occurred as previously described.

2.6. Western Blot Analysis

Cells were grown to 60% to 80% confluence in 60 mm plates. For transient transfections, plasmid mixtures containing 2.5 μ g of Egr-1 WT or aDaa expression vector were transfected for 5 h in serum-free media as described. Cells were serum starved overnight and treated with resveratrol and/or trichostatin A (TSA) as indicated in serum-free media. Protein lysates were isolated in ice-cold RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease (1 mM PMSF, 1 μ g/mL aprotinin, and 1 μ g/mL leupeptin) and phosphatase (0.1 nM Na_3VO_4 and 25 mM NaF) inhibitors. Protein concentrations were measured using BCA protein assay (Thermo Fisher Scientific) with bovine serum albumin as the standard. Total protein was subjected to Western blot analysis as described ⁽¹⁵⁶⁾. Briefly, proteins (30 μ g or 50 μ g) were separated by SDS-PAGE (8-12%) and transferred to nitrocellulose membrane (Pall Life Sciences, Pensacola, FL). Membranes were blocked in 5% skim milk in TBS/0.05% Tween-20 (TBS-T) for 1 h and incubated with specified antibodies at 4°C overnight. After three washes with TBS-T, blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed three times.

Protein signal was detected using Enhanced ChemiLuminescence System (Thermo Fisher Scientific).

2.7. *de novo* Protein Synthesis

HCT-116 cells were grown to 60% to 80% confluence in 60 mm plates and pretreated with CHX (10 mg/mL) for 30 min in serum-free media. After pretreatment, DMSO or resveratrol (50 μ M) was added directly to the media and incubated for 24 h. Total RNA was isolated and reverse transcribed. Quantitative RT-PCR for *ATF3* and *GAPDH* occurred as described.

2.8. Transfection using Luciferase Reporter System

Transient transfections were performed using LipofectAMINE (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. HCT-116 cells were seeded in 12-well plates at a concentration of 2.0×10^5 cells per well. The next day, plasmid mixtures containing *ATF3* promoter (0.5 μ g) and *pRL-null* vector (0.05 μ g) were co-transfected for 5 h in serum-free media. For co-transfection experiments, 0.25 μ g of the *ATF3* promoter and 0.25 μ g of expression vector were co-transfected with 0.05 μ g *pRL-null* vector. For competition assays, plasmid mixtures were prepared as indicated in **Figure A.9**. To measure Egr-1 and NAG-1 activity, plasmid mixtures containing *pEBS1⁴luc* (0.375 μ g) and Egr-1 or SIRT1 expression vector (0.375 μ g or 0.25 μ g) were co-transfected with 0.0075 μ g *pRL-null* vector, as indicated in **Figures B.1** and **B.4**, using PolyJet DNA *in vitro* Transfection Reagent (SignaGen Laboratories, Rockville, MD). After transfection, cells were treated with

resveratrol (50 μ M) in serum-free media for 24 h. Cells were harvested in 1X passive lysis buffer, and luciferase activity was measured and normalized to *pRL-null* luciferase activity using DualGlo Luciferase Assay Kit (Promega).

2.9. RNA Interference

Egr-1 sense and antisense oligonucleotides were previously described⁽¹⁵⁶⁾. *ATF3* and *KLF4* siRNA were purchased from Santa Cruz Biotechnology; control siRNA was purchased from Ambion (Austin, TX). HCT-116 cells were transfected with either 100 nM (*ATF3* and *KLF4*) or 200 nM (*Egr-1*) of each construct using TransIT-TKO transfection reagent (Mirus, Madison, WI). After transfection for 24 h, cells were serum starved overnight and treated as indicated in **Figure A.5**. Total protein was subjected to Western blot analysis as described.

2.10. Electrophoretic Mobility Shift Assay (EMSA)

HCT-116 cells were grown to 80% confluence. After overnight serum starvation, cells were treated with resveratrol (50 μ M) for 24 h. Cells were then washed with PBS and nuclear extracts were prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to protocol. Double-stranded oligonucleotides corresponding to (F, 5'-GTGAGCGAGGGCGGGG-3') and KLF4 (F, 5'-TCCACCCCTTCCACCCCT-3') binding sites were synthesized and end-labeled with biotin (Operon, Huntsville, AL). Mutant oligonucleotides of *Egr-1* (F, 5'-GTGAGCGAAAACGGGG-3') and KLF4 (F, 5'-TCCAAAACTTCCAAAACT-3') were also synthesized. To ensure specific binding of *Egr-1* and KLF4, recombinant proteins were generated using TNT Quick Coupled

Transcription/Translation System (Promega). EMSA was done using the LightShift Chemiluminescent EMSA kit according to the manufacturer's protocol (Thermo Fisher Scientific). Briefly, biotin-labeled oligonucleotide (10 nM) was incubated with nuclear extract (5 µg) or recombinant protein and 1X binding buffer (Promega) at room temperature for 20 min. For competition assay, proteins were pre-incubated with unlabeled oligonucleotide (10- or 100-fold) for 10 min.

2.11. Chromatin Immunoprecipitation

HCT-116 cells were grown to 80% confluence and treated with resveratrol (50 µM). After 24 h, cells were fixed with 1% formaldehyde at 37°C for 10 min. The fixed cells were scraped into conical tubes, pelleted, and lysed in lysis buffer containing protease and phosphatase inhibitors. DNA was sheared into fragments by sonication 4 times at 10 s at 50% constant maximal power. The sonicated cell supernatant was diluted 10-fold in immunoprecipitation buffer and 1% of diluted cell supernatant was kept as a control (input). The chromatin was pre-cleared with protein A/G agarose, 20 µg heat-denatured salmon sperm DNA, 50 µg bovine serum albumin, and 10 µg antibodies against Egr-1, KLF4, or IgG overnight at 4°C. The immunocomplexes were washed five times with wash buffer and eluted with elution buffer. Protein-DNA cross-links were reversed with 27 µL of 5 M NaCl and 1 µL of 10 mg/mL RNase A at 65°C for 4 h and incubated with 2 µL of 20 mg/mL proteinase K at 45°C for 1 h. The DNA was purified by phenol extraction and ethanol precipitation. The region between -298 and -114 bp of the human *ATF3* promoter was amplified by qRT-PCR and RT-PCR using F, 5'-CGGCTCCGGTCCTGATATGG-3' and R,

5'-AGAACCGGCCGAACGGAGCG-3' as described. For *NAG-1*, the region between -133 and +41 bp of the promoter was amplified using F, 5'-CCAGAAATGTGCCCTAGCTT-3' and R, 5'-GACCGGGACTGACCAGATG-3'. The 184 bp (ATF3) or 202 bp (*NAG-1*) product was resolved on 2% agarose gel and visualized under ultraviolet light.

2.12. Mammalian 2-Hybrid Assay

The pM/Egr-1 and pVP16/KLF4 vectors for mammalian-2-hybrid were generated. PCR fragments were amplified from pcDNA3.1/NEO/Egr-1 and pcDNA3.1/His/V5/KLF4 using Egr-1 and KLF4-specific primers, respectively (see **Table 2.1**). After PCR, fragments were cloned into pCR2.1/TOPO vector (Invitrogen), digested with *EcoRI* and *HindIII* restriction enzymes, and cloned into pM or pVP16 vectors. Deletion clones of pM/Egr-1 were generated as described using del AD and del ZD2 primers (**Table 2.1**). Mammalian-2-hybrid assay was performed according to Matchmaker Mammalian Assay Kit 2 protocol (Clontech, Mountain View, CA). Transfection with LipofectAMINE occurred as described using plasmid mixtures containing 0.2 µg of pM/Egr-1 or deletion construct, 0.2 µg of pVP16/KLF4, 0.2 µg of pG5Luc, and 0.06 µg *pRL-null* vector.

2.13. Immunoprecipitation

For immunoprecipitation of Egr-1 (WT and deletions, generated as described) and KLF4, each expression vector was transfected into HCT-116 cells as indicated and grown to confluence (see **Figure A.11**). For acetylation experiments, Egr-1 WT or aDaa mutation expression vector was transfected into HCT-116 cells. Cells were then serum starved

overnight and treated with resveratrol (50 μ M) for 2 h. After washing with ice-cold PBS, cells were harvested in RIPA buffer containing inhibitors and incubated on ice for 30 min. Cell suspensions were centrifuged for 10 min, supernatant collected, and protein concentrations were measured. Approximately 600 μ g of protein and 5 μ g of antibody (IgG, Egr-1 or KLF4) were incubated overnight at 4°C with shaking. The following day, lysate-antibody mix was added to 50 μ L protein A/G resin beads (Santa Cruz Biotechnology), which was washed and re-suspended in RIPA buffer, and incubated overnight at 4°C with shaking. After overnight incubation, samples were centrifuged for 30 s and the supernatant removed. Protein A/G resin beads were washed 5 times with 500 μ L ice-cold PBS, with complete removal of PBS after each wash to prevent background. After the last wash, 60 μ L of 2X loading buffer was added to the resin beads, vortexed vigorously, and boiled at 100°C for 5 min to denature and separate the protein from the beads. Samples were then centrifuged, supernatant collected, and Western blot analysis was performed using 10 μ L of sample as described.

2.14. Caspase 3/7 Enzymatic Activity

Apoptosis was measured using Apo-ONE Homogenous Caspase-Glo-3/7 Assay kit (Promega) according to manufacturer's protocol. Cells were harvested in RIPA buffer containing protease and phosphatase inhibitors. The same volume of Caspase-Glo-3/7 reagent was added to cell lysates (30 μ g protein) in 96-well plates and incubated at room temperature in the dark for 1 h. Luminescence was measured using FLX800 microplate reader (BioTek, Winooski, VT).

2.15. Statistical Analyses

SAS for Windows (v9.2; SAS Institute, Inc., Cary, NC) statistical analysis software was used. For multiple group comparisons, analysis of variance with Tukey's multiple comparison test was used to compare mean values. The Student's *t* test was used to analyze differences between samples. Results were considered statistically significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Chapter 3:

**Role of Egr-1, KLF4, and ATF3 in Resveratrol-Mediated
Anticancer Activity**

This chapter is revised based on the article published by Whitlock *et al.*:

Whitlock NC, Bahn JH, Lee SH, Eling TE, and Baek SJ (2011). Resveratrol-induced apoptosis is mediated by early growth response-1, Krüppel-like factor 4, and activating transcription factor 3. *Cancer Prevention Research* **4**(1): 116-127.

My contributions to this work include: (1) experimental design, (2) acquisition, analysis, and interpretation of data, (3) gathering and reviewing the literature, (4) creating figures/tables, and (5) drafting and revising the article. JBH generated the KLF4 and pVP16/KLF4 constructs. SHL performed *ATF3* promoter assay with Egr-1 overexpression. All authors reviewed and approved the final version for publication.

3.1. Abstract

Resveratrol, a dietary phytoalexin readily available in the diet, is reported to possess anti-tumorigenic properties in several cancers, including colorectal cancer. However, the underlying mechanism(s) involved is not completely understood. In the present study, we investigated the effect of resveratrol treatment on gene modulation in human colorectal cancer cells and identified activating transcription factor 3 (ATF3) as the most highly induced gene after treatment. We confirmed that resveratrol up-regulates ATF3 expression, both at the mRNA and protein level, and showed resveratrol involvement in ATF3 transcriptional regulation. Analysis of the *ATF3* promoter revealed the importance of early growth response-1 (Egr-1; located at -245 to -236) and Krüppel-like factor 4 (KLF4; located at -178 to -174) putative binding sites in resveratrol-mediated ATF3 transactivation. Specificity of these sites to the Egr-1 and KLF4 protein was confirmed by electrophoretic mobility shift and chromatin immunoprecipitation assays. Resveratrol increased Egr-1 and KLF4 expression, which preceded ATF3 expression, and further suggests Egr-1 and KLF4

involvement in resveratrol-mediated activity. We provide evidence for Egr-1 and KLF4 interaction in the presence of resveratrol, which may facilitate ATF3 transcriptional regulation by this compound. Furthermore, we demonstrate that induction of apoptosis by resveratrol is mediated, in part, by increased ATF3 expression. Taken together, these results provide a novel mechanism by which resveratrol induces ATF3 expression and represent an additional explanation of how resveratrol exerts its anti-tumorigenic effects in human colorectal cancer cells.

3.2. Introduction

Current research suggests that various dietary phytochemicals function as chemopreventive and/or adjuvant chemotherapeutic agents, adding to the paradigm that a diet high in fruit and vegetable content confers protection against chronic disease⁽³⁾. One such phytochemical is resveratrol (3, 4', 5-trihydroxystilbene), a naturally occurring phytoalexin readily available in the diet and to which a plethora of health-promoting effects have been ascribed⁽⁴⁾. Resveratrol has elicited much attention as a potential anticancer agent since the inhibitory effect of this compound on carcinogenic processes was first reported in 1997⁽⁵⁾. Subsequently, numerous studies have illustrated the anti-proliferative effect of resveratrol on cancer cells, which is believed attributable to induction of cell cycle arrest in the G1/S or G2/M phase and induction of apoptosis and related proteins^(44, 45). More importantly, treatment with resveratrol inhibited tumorigenesis *in vivo*^(7, 82, 159). However, the underlying mechanism(s) involved in the anti-tumorigenic/anti-carcinogenic activities of resveratrol remain poorly defined due to its capacity to modulate a multitude of signaling pathways.

Activating transcription factor 3 (ATF3), a member of the ATF/CREB family of bZIP transcription factors, is characterized as a stress-inducible or adaptive response gene⁽¹²¹⁾. Much controversy exists as to the physiological role of ATF3 in tumorigenesis, and ATF3 is demonstrated to a positive and/or negative modulator of tumor progression. Recently, a dichotomous role was reported for ATF3 in cancer development; the authors concluded its role as a tumor suppressor or oncogene is largely dependent on cellular context and extent of malignancy⁽¹²²⁾. However, several lines of evidence suggest that ATF3 may function as a tumor suppressor gene in colorectal carcinogenesis. Firstly, ATF3 expression is markedly reduced in cancer tissues, including colon, when compared to normal adjacent tissue^(160, 161). Secondly, ATF3 overexpression is demonstrated to elicit a number of cellular responses. These include (1) the induction of cell cycle arrest with concomitant inhibition of proliferation⁽¹⁶²⁾, (2) induction of apoptosis *in vitro* and *in vivo*^(155, 163-165), inhibition of invasion and associated genes⁽¹⁶⁶⁻¹⁶⁸⁾, and retardation of tumor formation *in vivo*^(163, 168). Finally, ATF3 is reported to mediate or enhance induction of apoptosis by compounds demonstrated to possess antitumor and anticancer properties^(130, 155, 169-171). Thus, it is believed that ATF3 plays an anti-tumorigenic and anti-carcinogenic role in colorectal cancer.

In the study described here, we examined the effect of resveratrol treatment on gene modulation in HCT-116 human colorectal cancer cells. We identified ATF3 as the most highly induced gene after treatment and sought to investigate the transcriptional mechanism and biological consequence of ATF3 expression in response to resveratrol. Here, we report that early growth response-1 (Egr-1) and Krüppel-like factor 4 (KLF4) mediates *ATF3* transactivation by resveratrol. We show Egr-1 and KLF4 interaction in the presence of

resveratrol, which may facilitate ATF3 transcriptional regulation by this compound. Furthermore, we demonstrate induction of apoptosis by resveratrol is mediated, at least in part, by ATF3.

3.3. Results

3.3.1. Effect of Resveratrol on Gene Expression in HCT-116 Cancer Cells

The mechanism(s) underlying the anti-tumorigenic properties of resveratrol in colorectal cancer remain mostly unclear. To investigate how resveratrol alters gene expression in colorectal cancer cells, microarray analysis was performed using HCT-116 cells treated with resveratrol (100 μ M). Among the genes up-regulated by the treatment, ATF3 was identified as most highly induced (**Table 3.1**). We confirmed resveratrol-induced *ATF3* transcript using reverse transcription PCR (**Figure A.1**). The alteration of mRNA transcripts of five additional genes (*HO-1*, *DKK1*, *NAG-1*, *Nur77*, and *PDE4B*) by resveratrol was also confirmed, each of which are suggested to play a role in tumorigenesis ⁽¹⁷²⁻¹⁷⁶⁾.

Table 3.1. Alteration of gene expression profiles of HCT-116 colorectal cancer cells by resveratrol (100 μ M). HCT-116 human colorectal cancer cells were treated with resveratrol (100 μ M) for 24 h and microarray analysis was performed. The top 13 up- and down-regulated genes are listed.

<i>Gene Symbol</i>	<i>Gene Name</i>	<i>Accession No.</i>	<i>Fold Change</i>
ATF3	Activating transcription factor 3	I_929428	20.37
MTHFD2	Methylenetetrahydrofolate (NAD-dependent) 2, Methenyltetrahydrofolate cyclohydrolase	I_939586	13.57
HMOX1	Heme oxygenase 1 (HO-1)	I_961390	13.13
PLAB	Macrophage inhibitory cytokine 1 (NAG-1)	I_966585	12.59
ASNS	Asparagine synthase	I_930098	10.56
S100A2	Homo sapiens S100 calcium binding protein A2, mRNA	NM_005978.2	9.88
C20orf97	Protein with high similarity to neuronal cell death Inducible Putative Kinase	I_961961	8.93
SULF2	Member of the sulfatase family	I_962934	8.23
CNK	Cytokine inducible kinase	I_964814	7.80
FUS	RNA-binding protein FUS	I_965527	7.78
DKK1	Dickkopf homolog 1	I_932501	7.47
HERPUD1	Homocysteine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain member 1	I_959712	7.16
NR4A1	Nuclear receptor subfamily 4 group A member 1 (Nur77)	I_932430	7.13
NR2F1	Homo sapiens nuclear receptor subfamily 2, group F, member 1 (NR2F1), mRNA	NM_005654.2	-3.46
HNRPA2B1	Heterogenous nuclear ribonucleoprotein A2/B1	I_930777	-3.47
HMGA2	High-mobility group (nonhistone chromosomal) protein isoform I-C	I_963472	-3.48
HAIK2	Member of the intermediate filament family, has moderate similarity to keratin 19 (human KRT19)	I_958778	-3.65
PRKACB	cAMP dependent protein kinase catalytic subunit beta	I_931783	-3.69
WNT16	Wingless-type MMTV integration site family member 16	I_929920	-3.77
PDE4B	Phosphodiesterase 4B	I_929286	-3.79
SCG2	Secretogranin II (chromogranin C)	I_928496	-3.81
GPR110	Member of the secretin family of G protein-coupled receptors	I_957588	-3.98
VSNL1	Visinin-like Protein 1	I_940805	-4.16
DKFZP434P1735	Protein containing 10 armadillo/beta-catenin repeats, weak similarity to <i>S. cerevisiae</i> Vac8p	I_931567	-4.26
CPO	Coproporphyrinogen oxidase	I_956835	-4.54
KITLG	Stem cell factor, ligand of KIT tyrosine kinase receptor	I_944208	-5.04

3.3.2. Resveratrol Increases ATF3 Expression in Cancer Cells

We next determined whether resveratrol increases ATF3 expression at the protein level. As shown in **Figure A.2A** and **A.2B**, resveratrol increased ATF3 in a concentration- and time-dependent manner in HCT-116 cells. Furthermore, increased ATF3 expression was observed in both colorectal (**Figure A.2C**) and non-colorectal (**Figure A.2D**) cancer cells after treatment; however, we did not observe ATF3 induction in LoVo colorectal and SqCC/Y1 head and neck cancer cells. Absence of ATF3 induction by resveratrol in these cells is most likely due to high endogenous expression of ATF3 (LoVo) or the lack of proper signaling pathway(s) needed for ATF3 increase by resveratrol (SqCC/Y1). Because resveratrol increased ATF3 expression at the mRNA and protein level, we sought to characterize ATF3 as a molecular target of this compound at the transcription level.

3.3.3. Egr-1 and KLF4 are involved in Resveratrol-Induced ATF3 Expression

To identify the transcriptional binding site(s) responsible for resveratrol-mediated ATF3 expression, HCT-116 cells were transfected with different promoter constructs spanning the -1850 to +34 bp promoter region (**Figure A.3A**). Resveratrol treatment resulted in a significant increase in ATF3 promoter activity for all reporter constructs; however, the greatest induction was observed within the -514 and +34 bp region, suggesting that a major response element(s) may be present. This region was further analyzed using three independent transcription factor search engines (Genomatix-MatInspector, TFSearch, and Transcription Element Search System) and five possible factors were commonly identified: p53, Sp1, Egr-1, KLF4, and ATF/CREB (**Figure A.3B**).

To investigate the role of identified *cis*-acting elements in resveratrol-mediated transcriptional regulation of ATF3, co-transfection experiments were performed utilizing expression vectors of the aforementioned factors and *ATF3* promoter constructs as indicated (**Figure A.4**). Expression of each vector, with the exception of ATF3, resulted in increased *pATF3* -514/+34 luciferase activity after resveratrol treatment compared to pcDNA3.1 empty vector control (**Figure A.4A**). Interestingly, cells over-expressing KLF4 and Egr-1 showed the greatest increase in resveratrol-induced *ATF3* promoter activity, resulting in a 4.1- and 4.3-fold induction, respectively. A similar pattern was observed for the -132 to +34 bp region (**Figure A.4B**); however, co-transfection of these expression vectors with *pATF3* -84/+34 reporter construct had no effect on promoter activity after treatment (**Figure A.4C**). Of note, suppression of *ATF3* promoter was observed in cells with ATF3 overexpression, consistent with its role as a negative self-regulator⁽¹⁷⁷⁾. These results identify putative resveratrol response elements located within the -514 and -84 bp region of the *ATF3* promoter and suggest that both Egr-1 and KLF4 may play a role in ATF3 regulation by resveratrol.

To clarify the importance of Egr-1 and KLF4 in resveratrol-mediated activation of ATF3, internal deletion clones lacking the Egr-1 and KLF4 binding sites were generated and transfected into HCT-116 cells. Deletion of Egr-1 or KLF4-A binding site markedly reduced *ATF3* promoter activity in response to resveratrol treatment when compared to wild type (**Figure A.5A**). Deletion of the KLF4-B binding site did not change resveratrol-induced transactivation. Furthermore, because deletion of binding sites corresponding to either Egr-1 or KLF4-A resulted in a significant decrease in resveratrol-induced promoter activity, a

deletion clone lacking both these sites was generated. Resveratrol-induced activity was dramatically reduced in cells transfected with the double deletion promoter construct compared to wild type (**Figure A.5A**). Moreover, suppression of either endogenous Egr-1 or KLF4 decreased ATF3 expression in the presence of resveratrol (**Figure A.5B**), confirming that both Egr-1 and KLF4 contribute to resveratrol-induced ATF3 expression.

3.3.4. *Egr-1 and KLF4 bind to the ATF3 Promoter*

To determine whether Egr-1 and KLF4 bind to the *ATF3* promoter, EMSA was performed using biotin-labeled oligonucleotides containing 1-2 copies of the corresponding promoter binding site. First, we examined the effect of resveratrol on promoter binding by these factors using nuclear extracts prepared as described in **Chapter 2**. Pre-incubation of resveratrol-treated nuclear extracts with Egr-1 and KLF4 oligonucleotides resulted in DNA/protein complex formation that was competed out with addition of 10X and 100X molar excess of unlabeled wild type oligonucleotides (**Figure A.6, left, lanes 3-4**), whereas pre-incubation with unlabeled mutant oligonucleotides had no effect on DNA/protein complex formation (**Figure A.6, left, lanes 5-6**). This suggests that both Egr-1 and KLF4 are able to bind to the *ATF3* promoter. Secondly, we verified the specificity of the binding sites for Egr-1 and KLF4 using *in vitro* translated proteins mixed with their respective oligonucleotides. As shown in **Figure A.6**, both Egr-1 and KLF4 bind to their specific binding sites as evidenced by the formation of shift bands that were competed out by 10X and 100X molar excesses of unlabeled oligonucleotides (**Figure A.6, right, lanes 10-11**).

Addition of mutant oligonucleotides did not affect binding of Egr-1 or KLF4 to the *ATF3* promoter (**Figure A.6**, left, lanes 12-13).

Finally, a chromatin immunoprecipitation (ChIP) assay was performed to further confirm that Egr-1 and KLF4 bind to the *ATF3* promoter after resveratrol treatment. As shown in **Figure A.7**, immunoprecipitation of the chromatin/protein complex with Egr-1 and KLF4 resulted in the enrichment of these proteins at the *ATF3* promoter (*left*) and the visualization of a 184 bp band of the amplified promoter (*right*). In conjunction with results described above, these data suggest that both Egr-1 and KLF4 bind to the *ATF3* promoter and activate transcription in the presence of resveratrol. However, Egr-1 may contribute more to resveratrol-induced *ATF3* expression, since resveratrol enhances Egr-1 binding capacity to the *ATF3* promoter.

3.3.5. *de novo* Synthesis is required to Increase *ATF3* Expression

We then examined the requirement of *de novo* protein synthesis for resveratrol-mediated *ATF3* expression because Egr-1 and KLF4 appear to be required for induction of *ATF3*. HCT-116 cells were treated with CHX and resveratrol (50 μ M). In the presence of CHX, resveratrol was unable to increase *ATF3* expression, suggesting that *ATF3* increase by resveratrol is dependent on *de novo* protein synthesis (**Figure A.8A**). We next determined whether resveratrol could alter expression of Egr-1 and KLF4. Resveratrol treatment increased *Egr-1* and *KLF4* mRNA transcript (**Figure A.8B**) and protein expression (**Figure A.8C**). It should be noted that induction of both Egr-1 and KLF4, which occurred at approximately 1 h of treatment and continued until 3 h, preceded that of *ATF3*, which began

after 6 h. Together, these results are compatible with the notion that observed increase in ATF3 expression by resveratrol requires synthesis of Egr-1 and KLF4.

3.3.6. *Egr-1 and KLF4 Interaction Facilitates Resveratrol-Mediated ATF3 Induction*

Because both Egr-1 and KLF4 are involved in resveratrol-mediated activity, we examined whether these factors coordinate or compete in ATF3 expression. HCT-116 cells were co-transfected with *pATF3 -514/+34* and expression vectors and treated as described. As observed previously, both Egr-1 and KLF4 increased *ATF3* promoter activity in the presence of resveratrol (**Figure A.9**). Moreover, co-expression of these factors resulted in enhanced transactivation of ATF3 at both the basal level and after resveratrol treatment.

Previous studies have shown interaction between two Zn finger transcription factors⁽¹⁷⁸⁾ and that this interaction facilitates transcriptional activation⁽¹⁷⁹⁾. To investigate the potential for Egr-1 and KLF4 interaction, mammalian-2-hybrid assay was performed. Briefly, Egr-1 and KLF4 were cloned into the pM vector containing a DNA binding domain and pVP16 vector containing an activation domain, respectively. These constructs were then co-transfected with the luciferase reporter construct pG5Luc into HCT-116 cells and treated with resveratrol. If there is interaction, luciferase activity should be detected resulting from the formation of a complex between the two vectors and subsequent binding to the DNA binding domain of the pG5Luc vector; this results in pG5Luc promoter activation.

As shown in **Figure A.10A**, Egr-1 and KLF4 bind to each other at the basal level and this interaction is enhanced after resveratrol treatment (7.2-fold vs. 52-fold increase). Increased Egr-1 and KLF4 interaction was also observed after DIM treatment. DIM

increases ATF3 expression⁽¹³⁰⁾. To exclude the potential for direct binding of pVP16/KLF4 to the DNA binding domain of the pM empty vector, the vectors were co-transfected and mammalian-2-hybrid assay was performed; no interaction was detected, allowing one to infer the interaction of Egr-1 and KLF4 is genuine (data not shown).

We next sought to tentatively identify the region that may facilitate this interaction and generated pM/Egr-1 deletion constructs lacking (1) 133-159 aa of the activation domain and (2) 370-395 aa of the Zn finger domain (**Figure A.10B, top**). Both the partial deletion of the activation domain and deletion of the second Zn finger domain of Egr-1 resulted in decreased interaction with KLF4 (**Figure A.10B, bottom**). We confirmed mammalian-2-hybrid assay by Egr-1 and KLF4 immunoprecipitation experiments (**Figure A.11**). Egr-1 and KLF4 are co-immunoprecipitated with either antibody, whereas Egr-1 deletion clones had diminished binding capacity to KLF4. Together, these data suggest that Egr-1 and KLF4 physically interact, which is increased by resveratrol, and that the Egr-1 activation or Zn finger domain may mediate this interaction.

3.3.7. Knockdown of ATF3 Suppresses Resveratrol-Induced Apoptosis

We have identified ATF3 as a molecular target of resveratrol; however, the biological consequence(s) of ATF3 induction by resveratrol in colorectal tumorigenesis is unknown. As described in the introduction, pharmaceuticals and phytochemicals' increase of ATF3 mediated or enhanced apoptosis by these compounds^(130, 155, 169-171). To determine if this is true for resveratrol, we measured caspase-3/7 enzyme activity for resveratrol-treated HCT-116 and HT29 cancer cells. As depicted in **Figure A.12A**, resveratrol treatment increased

apoptosis in a dose-dependent manner. Furthermore, ATF3 overexpression in these cells also increased apoptosis, which depend on caspase activation (**Figure A.12B**). Together, these data suggest that both resveratrol and ATF3 expression induce apoptosis in our model system. Next, to investigate whether resveratrol-induced apoptosis is mediated by ATF3, ATF3 expression was knocked down by siRNA followed by resveratrol treatment for 24 h. As depicted in **Figure A.12C**, knockdown of ATF3 significantly abrogated caspase-3/7 enzymatic activity by resveratrol. This suggests that ATF3 plays a role in resveratrol-induced apoptosis.

3.3.8. Egr-1 and KLF4 Transactivate ATF3 Independent of Resveratrol

Results herein suggest Egr-1 and KLF4 involvement in resveratrol-mediated ATF3 activation. To determine if these transcription factors are able to affect *ATF3* transcription individually, promoter activity was measured after co-transfection with Egr-1 or KLF4 expression vectors. As shown in **Figure A.13**, both Egr-1 and KLF4 are able to increase *ATF3* promoter activity. Of note, the minimum region required for Egr-1 or KLF4-mediated induction may be independent of that shown to be required for resveratrol (see **Figure A.4**). For Egr-1, the -1850 bp of the *ATF3* promoter showed the highest luciferase activity and for KLF4, this region lies within the -1420 bp of the *ATF3* promoter. This result suggests that binding sites of Egr-1 and KLF4 within the -514 bp of the *ATF3* promoter play an important role in resveratrol-induced ATF3 expression; however, Egr-1 and KLF4 also increase *ATF3* promoter activity in the absence of resveratrol through different promoter regions.

3.3.9. Posttranscriptional Regulation of ATF3 by Resveratrol as an Alternate Regulatory Pathway

Posttranscriptional control of eukaryotic gene expression provides an alternative regulatory process(s) to control protein abundance ⁽¹⁸⁰⁾. To determine if such processes account for ATF3 regulation by resveratrol, *ATF3* mRNA stability was measured as described in **Chapter 2**. Briefly, HCT-116 cells were pre-treated with resveratrol (50 μ M) for 24 h followed by actinomycin D, to terminate transcription, for the indicated times. As depicted in figure **A.14**, resveratrol stabilized *ATF3* mRNA and increased the half-life from approximately 7 h to 14 h, suggesting that posttranscriptional control of ATF3 by resveratrol may account for the observed increase in ATF3 protein level.

3.4. Discussion

Resveratrol, a dietary phytoalexin readily available in the diet, has garnered much attention as a potential chemopreventive and/or chemotherapeutic agent. Numerous studies, utilizing *in vitro* and *in vivo* model systems, have illustrated resveratrol's capacity to inhibit the stages of carcinogenesis (initiation, promotion, and progression) and modulate a multitude of signaling pathways associated with cellular growth and division, apoptosis, angiogenesis, and metastasis ⁽⁶⁾. However, the underlying mechanism(s) involved in the anti-tumorigenic/anti-carcinogenic activities of resveratrol, especially in colorectal cancer, are complex and remain poorly defined. The present study sought to investigate the effect of resveratrol on gene modulation in HCT-116 human colorectal cancer cells in order to identify a novel target that may mediate the anticancer activities of this compound and determine the

mechanism involved in its regulation. Our data identified ATF3 as the most highly expressed gene after resveratrol treatment (**Table 3.1**) and showed ATF3 was indeed up-regulated at both the mRNA (**Figure A.1**) and protein (**Figure A.2**) level by the compound. We also demonstrate increased stability of the *ATF3* transcript by resveratrol, which may contribute to the observed ATF3 expression in colorectal cancer cells highly induced by resveratrol (**Figure A.14**).

Recently, a dichotomous role in cancer development was reported for ATF3; the authors of that study demonstrated both a tumor suppressive (early-stage tumorigenesis) and oncogenic (late-stage tumorigenesis) role in breast cancer cell lines derived from similar genetic backgrounds with varying degrees of malignancy⁽¹²²⁾. Similarly, a duality of function was demonstrated for ATF3 in cancer studies of the prostate^(164, 181) and of the colon^(168, 182). Yet, as described in the introduction, several lines of evidence suggest ATF3 behaves as a negative regulator of tumorigenesis. For example, ATF3 expression is induced by several compounds demonstrated to possess anti-tumorigenic properties^(130, 155, 169-171). The results presented here add to the list of compounds that induce ATF3 with mechanistic data.

To elucidate the molecular mechanism by which resveratrol induced ATF3 expression, the promoter region spanning -1850 to +34 bp was assessed by luciferase assay in response to resveratrol. We found that resveratrol transactivated the *ATF3* promoter and identified putative response elements located within the -514 to -84 bp (**Figure A.3**). Co-transfection experiments suggested the involvement of the transcription factors Egr-1 and KLF4 (**Figure A.4**). Egr-1 and KLF4 participation in resveratrol-mediated activation of

ATF3 was confirmed by deletion (**Figure A.5A**) and knockdown (**Figure A.5B**) experiments. Interestingly, deletion of both Egr-1 and KLF4-A binding sites from the *ATF3* promoter dramatically reduced transactivation by resveratrol, indicating that the combination of these two factors is important for *ATF3* transcriptional regulation by resveratrol. Yet, we cannot exclude the involvement of other cofactors, such as p53 and Sp1 (**Figure A.4**), in the compound's-mediated activity. *ATF3* regulation by p53 is documented in the literature⁽¹⁸³⁾ and p53 is increased by resveratrol⁽¹³⁹⁾, which suggests a potential role for p53 in resveratrol-mediated *ATF3* activation. Furthermore, Sp1 was recently demonstrated to regulate *ATF3* expression in colon cancers cells⁽¹⁸⁴⁾; however, we did not observe Sp1 induction by resveratrol (data not shown). Here, we focused on Egr-1 and KLF4 because both increased resveratrol-mediated *ATF3* activation and were modulated by the compound. In addition, as shown in **Figure A.13**, both Egr-1 and KLF4 activate *ATF3* promoter activity in the absence of resveratrol, indicating the pleiotropic role of both Egr-1 and KLF4 in *ATF3* transcriptional regulation. Because resveratrol can increase Egr-1 and KLF4 expression and transcription activity, one could speculate that observed anticancer properties of resveratrol may be facilitated through either an Egr-1-mediated or KLF4-mediated mechanism.

Egr-1 and KLF4 belong to a family of immediate early response genes whose expression is transiently induced in response to various environmental stimuli^(185, 186). Each encode a transcription factor containing three carboxyl C2H2 type zinc finger motifs that coordinate the expression of genes associated with cell proliferation, differentiation, and apoptosis⁽¹⁸⁶⁻¹⁸⁸⁾. In addition, Egr-1 and KLF4 are suggested to act as master regulatory proteins involved in cell fate decisions^(189, 190). Nonetheless, as with *ATF3*, much

controversy exists as to the role of Egr-1 and KLF4 in cancer development and their biological function appears largely context dependent. Several studies have demonstrated that Egr-1 and KLF4 expression facilitate tumor progression *in vivo*⁽¹⁹¹⁻¹⁹⁴⁾; however, there is ample evidence supporting a tumor suppressive role for both transcription factors⁽¹⁹⁵⁻²⁰²⁾.

Our data indicates that Egr-1 and KLF4 are able to bind to the *ATF3* promoter (**Figure A.6 and A.7**) and that their biosynthesis is necessary for resveratrol-mediated activity (**Figure A.8**). Moreover, these transcription factors cooperate in *ATF3* transactivation (**Figure A.9**). From this, two inferences can be made: (1) synergism between Egr-1 and KLF4 promote *ATF3* activation, which is enhanced after resveratrol treatment and (2) interaction between the two facilitate transcriptional regulation by *ATF3* by the compound. Indeed, the results ascertained by mammalian 2-hybrid assay and immunoprecipitation experiments corroborate Egr-1 and KLF4 interaction (**Figure A.10 and A.11**, respectively). Furthermore, resveratrol and DIM increased Egr-1 and KLF4 interaction. These results imply that a similar mechanism may contribute to induction of *ATF3* by these and possibly other phytochemicals that increase *ATF3* expression.

Current research suggests the importance of C2H2 type zinc finger domains in protein-protein interaction^(203, 204). Consistently, we found the zinc finger domain of Egr-1 as a possible region responsible for interaction with KLF4. Our data also identified the activation domain of Egr-1 as responsible for mediating the interaction with KLF4 (**Figure A.10 and A.11**). To our knowledge, this is the first report identifying the involvement of the activation domain in protein-protein interaction of two zinc finger transcription factors. Thus, the activation domain contributes not only to the initiation of transcription but also

contribute to protein-protein interactions with other transcription factors; interaction via the activation domain or the zinc finger domain leads to enhanced compound-induced transcription.

Results identify ATF3 as a molecular target of resveratrol whose regulation is mediated by Egr-1 and KLF4 interaction. However, the biological consequence(s) of ATF3 induction by the compound is unknown in colorectal tumorigenesis. To answer this question, we examined induction of apoptosis by resveratrol and ATF3 in colorectal cancer cells because, as described, ATF3 mediates or enhances apoptosis by compounds with known antitumor activities. As depicted in **Figure A.12**, resveratrol and ATF3 overexpression increased caspase 3/7 enzymatic activity in HCT-116 and HT29 cancer cells. Furthermore, knockdown of ATF3 expression resulted in reduced apoptosis by resveratrol and suggests that ATF3 plays a role in resveratrol-induced apoptosis. However, further study is required to determine whether ATF3 contributes to resveratrol anticancer effects *in vivo*.

In conclusion, we found the stress-inducible and/or adaptive response gene ATF3 as the most highly induced gene after resveratrol treatment in human colorectal cancer cells. Here, we report for the first time the involvement of resveratrol in the transcriptional regulation of ATF3 and that the C2H2 type zinc finger transcription factors Egr-1 and KLF4 mediate this regulation. We demonstrate that Egr-1 and KLF4 interact with each other in the presence of resveratrol, which may facilitate ATF3 transcriptional regulation by this compound. Furthermore, increased ATF3 expression by resveratrol facilitates induction of apoptosis by the compound (**Figure. 3.1**).

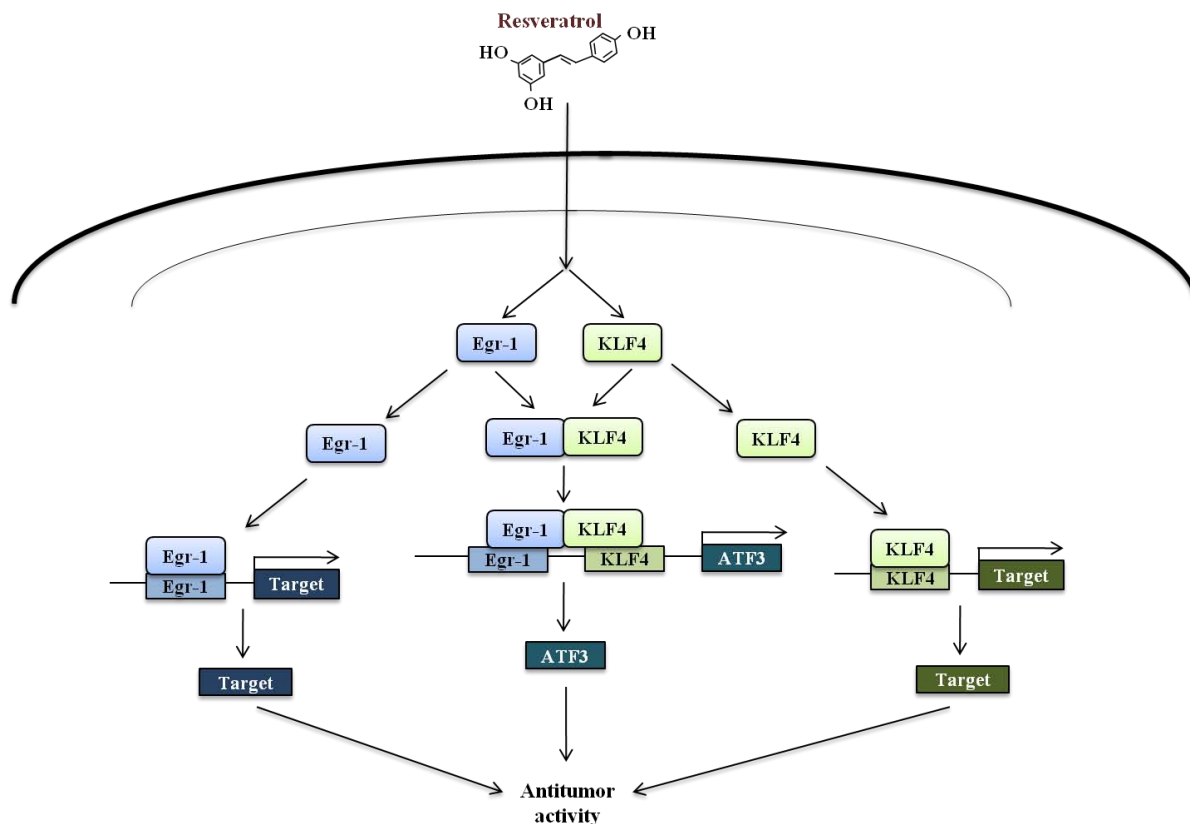


Figure 3.1. Schematic diagram of ATF3-mediated resveratrol action in colorectal cancer cells. Egr-1 and KLF4 are involved in the induction of ATF3 by resveratrol. Resveratrol increases the expression of both Egr-1 and KLF4; this in turn results in the interaction of Egr-1 with KLF4, potentially facilitated by the activation or zinc finger domain of Egr-1, leading to promoter binding and transactivation of ATF3. Increased ATF3 expression results in increase of apoptosis in colorectal cancer cells. Alternatively, both Egr-1 and KLF4 activate *ATF3* promoter in the absence of resveratrol.

Chapter 4:

**Role of Post-translational Modification of Egr-1 in
Resveratrol-Mediated Anticancer Activity**

4.1. Abstract

Resveratrol, a dietary phytochemical readily available in the diet, has elicited much attention as a potential chemopreventive and/or chemotherapeutic agent in several cancers, including colorectal cancer. However, the underlying mechanism(s) involved is not completely understood. Recently, we identified the transcription factor early growth response-1 (Egr-1) as a molecular target of resveratrol in colorectal cancer cells that is involved in resveratrol-mediated anticancer effects. In the present study, we sought to investigate the role of the sirtuin 1 (SIRT1) deacetylase, which is suggested to account for the observed anticancer activities of resveratrol, in increased Egr-1 transcriptional activity by the phytochemical. Here, we report a SIRT1-independent mechanism for resveratrol-induced Egr-1 activity due to decreased SIRT1 phosphorylation, resulting in suppression of SIRT1 deacetylase activity. We show that acetylation contributes to Egr-1-induced nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) expression through increased Egr-1 binding to the *NAG-1* promoter and enhanced transactivation. Thus, acetylation of Egr-1 plays an important role in resveratrol-mediated transactivation of NAG-1. Taken together, these results provide a novel mechanism by which resveratrol induces Egr-1 transcriptional activity that is independent of SIRT1 expression.

4.2. Introduction

Resveratrol (3, 4', 5-trihydroxystilbene), a naturally occurring dietary phytoalexin to which a plethora of health-promoting benefits have been ascribed ⁽⁴⁾, has elicited much attention as a potential cancer chemopreventive and/or chemotherapeutic agent since the

inhibitory effect of the compound on carcinogenesis was first reported by Jang *et al.* ⁽⁵⁾. Subsequently, numerous studies have illustrated the anti-proliferative effect of resveratrol on cancer cells, which is attributable to induction of cell cycle arrest and/or apoptosis and related proteins ^(45, 69, 205). Treatment with resveratrol has also been shown to result in induction of autophagocytosis and senescence in ovarian and colorectal cancer cells, respectively ^(61, 206). Furthermore, resveratrol treatment resulted in the inhibition of angiogenic, invasive, and metastatic factor release ⁽⁶⁾ as well as inhibition of tumorigenesis *in vivo* ^(67, 159, 207). However, the underlying mechanism(s) involved in the anticancer activities of resveratrol remain poorly defined.

The seven member mammalian sirtuin family of histone deacetylases have gained considerable attention as novel targets for the treatment of chronic diseases, such as cancer ⁽²⁰⁸⁾. Sirtuin 1 (SIRT1), a NAD-dependent type III histone and non-histone deacetylase, is reported to play a critical role in various physiological and pathological conditions, including carcinogenesis ⁽²⁰⁸⁾. Yet, as to whether the inhibition or activation of SIRT1 contributes to the development of cancer is debatable due to its involvement in the inhibition of key tumor suppressor and proto-oncogene proteins ⁽²⁰⁹⁾. In colorectal carcinogenesis, SIRT1 expression is demonstrated to inhibit tumor formation and proliferation *in vitro* and *in vivo* ^(210, 211). Moreover, resveratrol is identified as a potent activator of SIRT1 ⁽²¹²⁾ and activation of SIRT1 by resveratrol reduces tumorigenesis *in vivo* ⁽²¹³⁾. Furthermore, Boily *et al.* ⁽²¹⁴⁾ suggest that activation of the deacetylase by the phytoalexin is required, at least partially, for the observed anticancer activities of resveratrol.

Previously, we identified the transcription factor early growth response-1 (Egr-1) as a molecular target of resveratrol in colorectal cancer cells and demonstrate Egr-1's role in resveratrol-mediated activating transcription factor 3 (ATF3) activation and subsequent anticancer effects ⁽²¹⁵⁾. In the study described here, we sought to investigate the role of SIRT1 in increased Egr-1 transcriptional activity by the phytoalexin. Here, we demonstrate a SIRT1-independent mechanism for resveratrol-induced Egr-1 activity and suggest the importance of acetylation, at least partially, in this response.

4.3. Results

4.3.1. Increased Egr-1 Activity by Resveratrol is SIRT1 Independent

Recently, Boily *et al.* ⁽²¹⁴⁾ suggested that the anticancer effects of resveratrol are partially dependent on the presence of SIRT1 protein. Thus, to investigate the role of SIRT1 in resveratrol-induced Egr-1 transcriptional activity, we first examined the effect of resveratrol on SIRT1 phosphorylation status in HCT-116 human colorectal cancer cells. Current research suggests SIRT1 phosphorylation, particularly at serine residues 27 and 47, is associated with increased deacetylase activity ⁽²¹⁶⁻²¹⁸⁾. As demonstrated in **Figure B.1A** and **B.1B**, resveratrol treatment did not alter total SIRT1 protein expression; however, we observed a dramatic decrease in SIRT1 phosphorylation at serine 27 but not serine 47, which occurred in a concentration- and time-dependent manner (**Figure B.1A** and **B.1B**, respectively).

Next, we determined if SIRT1 could affect resveratrol-induced Egr-1 transcriptional activity. To accomplish this, we performed co-transfection experiments using the pEBS1⁴luc reporter construct, which contains four copies of the Egr-1 binding site (EBS) and is used as

an indirect measure of transcriptional activity. HCT-116 cancer cells were co-transfected as indicated in **Figure B.2A** and treated with resveratrol (50 μ M) for 24 h. As shown in **Figure B.2A**, resveratrol increased the luciferase activity of pEBS1⁴luc-transfected cells in the presence of endogenous and exogenous Egr-1 levels (pBABE). Moreover, SIRT1 overexpression had no effect on resveratrol-induced pEBS14luc activity. A similar effect was observed with *pEgr-1 -1260/+35* reporter construct (**Figure B.2B**). Taken together, these results suggest that (1) resveratrol decreases SIRT1 phosphorylation, presumably resulting in suppression of SIRT1 deacetylase activity, and (2) resveratrol increases Egr-1 transcriptional activity independent of SIRT1 activation.

4.3.2. *Egr-1 aDaa Mutation Diminishes Resveratrol-Induced Egr-1 Activity*

Acetylation of Egr-1 is reported to result in the preferential activation of Egr-1 pro-survival target genes in prostate cancer ⁽²¹⁹⁾; Egr-1 is suggested to behave as an oncogene in these cancers. However, in our model system, Egr-1 facilitates the anticancer activities of resveratrol ⁽²¹⁵⁾; therefore, we hypothesize that acetylation of Egr-1, through inhibition of SIRT1 deacetylase activity (**Figure B.1**), may be required for observed resveratrol-induced expression mediated by the transcription factor. To test this hypothesis, we first generated an acetylation deficient Egr-1 construct as described in **Chapter 2** based upon the identified putative acetylation site (KDKK) within Egr-1's weak transactivation domain (see **Figure B.3** for amino acid sequence ⁽²¹⁹⁾).

We next determined whether resveratrol increased Egr-1 acetylation and whether it occurred at the KDKK site. HCT-116 cells were transiently transfected with either control or

Egr-1 (WT and acetylation deficient, aDaa) constructs. Culture of transfected cells with resveratrol (50 μ M) for 2 h resulted in increased acetylation of both endogenous and exogenous Egr-1 as demonstrated using an anti-acetylated lysine antibody (**Figure B.4A**). However, Egr-1 acetylation by resveratrol was not observed in the acetylation deficient Egr-1 aDaa construct (**Figure B.4A**). Furthermore, resveratrol-induced Egr-1 transcriptional activity was diminished in the presence of the acetylation deficient construct (**Figure B.4B**). These results suggest that resveratrol acetylates Egr-1 and that this posttranslational modification, at least partially, is important in resveratrol-induced Egr-1 transcriptional activity.

4.3.3. Acetylation Contributes to Activation of the Egr-1 Target Gene, NAG-1

As previously described, Egr-1 acetylation status is associated with preferential transactivation of either survival-related (acetylated) or apoptosis-related (unacetylated) Egr-1 gene targets in prostate cancers⁽²¹⁹⁾; yet, in colorectal cancer, acetylation of Egr-1 may facilitate anti-proliferative and/or pro-apoptotic gene activation. We therefore studied three known Egr-1 target genes to determine whether acetylated Egr-1 produced a different result compared to unacetylated Egr-1. An expression study was performed in resveratrol-treated HCT-116 cancer cells by measuring protein (**Figure B.5A**) and mRNA (**Figure B.5B**) levels of activating transcription factor 3 (ATF3), NSAID-activated gene-1 (NAG-1), and p53 after transfection with Egr-1 WT or Egr-1 aDaa. **Figure B.5A** shows that resveratrol strongly induced the expression of the Egr-1 target genes examined (*lanes 2, 6, and 10*). Similar results were observed in the presence of Egr-1 WT overexpression (*lanes 5 and 6*).

However, Egr-1 aDaa was only less active in inducing NAG-1 expression when compared to WT in vehicle- and resveratrol-treated HCT-116 cancer cells (**Figure B.5A**, lanes 5 and 6 vs. lanes 9 and 10). Decreased Egr-1 aDaa induced NAG-1 mRNA expression was also observed in control- and resveratrol-treated HCT-116 cancer cells (**Figure B.5B**).

Acetylation of non-histone proteins is suggested to alter the DNA binding affinity of transcription factors for their target gene⁽²²⁰⁾. Because resveratrol treatment increases Egr-1 acetylation and subsequent expression of the Egr-1 target gene NAG-1, we sought to determine if this increased expression was due to increased Egr-1 binding affinity to the *NAG-1* promoter resulting in its transcriptional activation by Egr-1. We performed a chromatin immunoprecipitation (ChIP) assay to examine whether unacetylated Egr-1 (Egr-1 aDaa) had less affinity for the *NAG-1* promoter as compared to acetylated Egr-1 (Egr-1 WT). As shown in **Figure B.6A**, immunoprecipitation of chromatin/protein complex with Egr-1 resulted in the visualization of a 202-bp band of the amplified *NAG-1* promoter. Interestingly, in Egr-1 aDaa-transfected HCT-116 cancer cells had less promoter binding as compared to Egr-1 WT-transfected cells for both vehicle- and resveratrol-treated cells. The observed decrease in Egr-1 aDaa binding affinity for the *NAG-1* promoter also resulted in reduced Egr-1 induced transcriptional activation (**Figure B.6B**). Taken together, these results suggest that Egr-1 acetylation, at least partially, contributes to Egr-1-induced NAG-1 expression and to resveratrol-induced Egr-1-mediated NAG-1 expression in our model system.

4.4. Discussion

The dietary phytochemical resveratrol has elicited much attention as a putative anticancer agent; current research demonstrates resveratrol-mediated inhibition of carcinogenesis and modulation of cancer-associated signal transduction pathways ⁽⁶⁾. Yet, despite the identification of molecular targets, the underlying mechanisms that mediate resveratrol's anticancer activities remain elusive, especially in colorectal cancer. In this present study, we sought to investigate the role of SIRT1 in resveratrol-induced Egr-1 transcriptional activity. Our data revealed a SIRT1-independent mechanism for resveratrol-induced Egr-1 activity and suggest the importance of acetylation, at least partially, in this response.

Recently, it has been suggested that SIRT1 contributes to the anticancer activity(s) of resveratrol; the authors of that study demonstrate reduced resveratrol-mediated tumor inhibition (incidence, load, and volume) in SIRT1-null mice as compared to normal using a two-stage model of skin carcinogenesis ⁽²¹⁴⁾. Similarly, Wang *et al.* ⁽²¹³⁾ showed that activation of SIRT1 by resveratrol could partially inhibit tumor formation attributable to delayed tumor onset. Moreover, the tumors that developed in resveratrol-treated mice did not contain SIRT1, thus explaining why resveratrol did not inhibit tumor formation in this group ⁽²¹³⁾. Based on these studies, one could infer that the inhibitory effect of resveratrol on carcinogenesis is predicated on SIRT1 activity. Yet, as we demonstrate here, enhanced Egr-1 transcriptional activity by resveratrol occurs in a SIRT1-independent manner (**Figure B.1. and B.2**). This contradicts the notion of a SIRT1-dependent component to the anticancer effects of resveratrol. Recent evidence; however, suggests that resveratrol is not a direct

activator of SIRT1⁽²²¹⁾ and resveratrol inhibition of tumor growth is SIRT1-independent⁽²²²⁾. The results presented here are in agreement with these latest findings.

Acetylation of non-histone proteins at lysine residues is suggested to modulate protein function (e.g. signal transduction, DNA binding, protein stability, and localization)⁽²²⁰⁾. Furthermore, as in the case of the tumor suppressor p53, acetylation of transcription factors at lysine residues by the p300/CBP acetyltransferase may increase DNA binding affinity followed by subsequent transcriptional activation of their target genes⁽²²³⁾.

Therefore, inhibition of SIRT1 deacetylase activity by resveratrol may contribute to Egr-1 acetylation and increased Egr-1 transcriptional activity. Indeed, our results suggest that resveratrol-induced Egr-1 activity is associated with the transcription factor's acetylation (**Figure B.4**). However, the acetyltransferase responsible for Egr-1 acetylation remains to be identified, although p300/CBP involvement is likely⁽²¹⁹⁾.

Egr-1 belongs to a family of immediate early response genes whose expression is transiently induced in response to various environmental stimuli⁽¹⁸⁵⁾ and is suggested to act as a master regulatory protein involved in cell fate decisions⁽¹⁸⁹⁾. Several reports indicate Egr-1 involvement in the activation of the pro-apoptotic proteins ATF3^(155, 156, 215, 224), NAG-1⁽²²⁵⁻²²⁹⁾, and p53^(228, 230, 231). Additionally, Yu *et al.*⁽²¹⁹⁾ reported that acetylation of Egr-1 at the KDKK motif, within its weak transactivation domain, dictated its role in cell fate decisions in prostate carcinogenesis. Thus, to determine if Egr-1 acetylation status is associated with Egr-1 mediated cell fate decisions in our HCT-116 colorectal cancer cell model, we examined the expression of these three Egr-1 targets. As shown in **Figure B.5**, we observed less NAG-1 expression for acetylation deficient Egr-1 aDaa construct-

transfected HCT-116 colorectal cancer cells as compared to Egr-1 WT-transfected cells. Decreased NAG-1 expression by the acetylation deficient Egr-1 occurred, presumably, through decreased *NAG-1* promoter binding followed by diminished NAG-1 transactivation (**Figure B.6**). However, expression of Egr-1 aDaa had no appreciable effect on ATF3 or p53 expression; this observation may be due to the involvement of other transcription factors in resveratrol-mediated ATF3 or p53 activation.

In conclusion, we demonstrate here a SIRT1-independent mechanism for increased Egr-1 transcriptional activity by resveratrol, associated with the inhibition of SIRT1 deacetylase activity (**Figure 4.1**). The study also demonstrates the importance of acetylation in resveratrol-induced Egr-1 activity. Acetylation facilitates Egr-1-induced NAG-1 expression through increased Egr-1 binding to the *NAG-1* promoter followed by enhanced transactivation in the presence of resveratrol. Thus, acetylation of Egr-1 plays an important role in resveratrol-mediated transactivation of NAG-1.

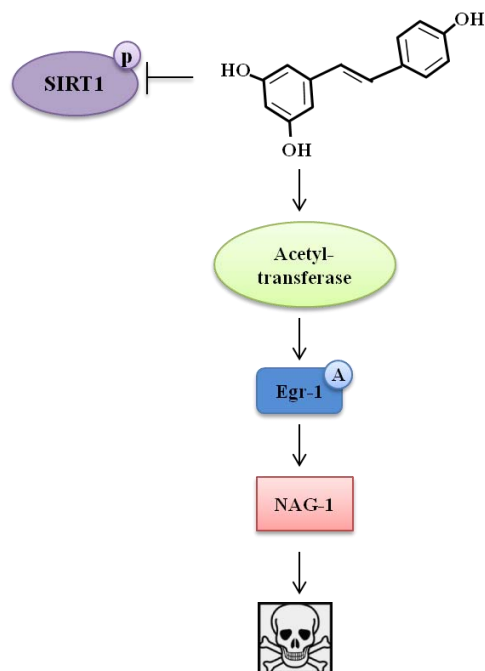


Figure 4.1. Schematic diagram of the role of resveratrol-induced Egr-1 acetylation in colon cancer cells. In colorectal cancer, resveratrol increases Egr-1 acetylation through a yet to be identified acetyltransferase, resulting in increased NAG-1 expression and subsequent induction of anticancer effects such as apoptosis.

Chapter 5:

Conclusions and Future Perspectives

The continued identification of dietary phytochemicals and their related derivatives with chemopreventive and/or chemotherapeutic activities offers an alternative and complementary approach to the prevention and treatment of cancers. Studies investigating the use of these compounds for cancer prevention or as adjuvants to traditional therapies have revealed several potential benefits: (1) suppression of tumorigenesis and carcinogenesis *in vitro* and *in vivo*, (2) sensitization of cancer cells to drug-induced growth inhibition, and (3) minimization of adverse effects associated with conventional therapies. Most importantly, dietary phytochemicals are demonstrated to target multiple signal transduction pathways involved in cancer development, an important advantage due to the inherent heterogeneity of cancers.

Resveratrol (3, 4', 5-trihydroxystilbene), a naturally-occurring phytoalexin, has been identified in extracts from more than 70 plant species, including grapes (predominant source), berries, jackfruit, and peanuts ⁽²³²⁾. Since Jang *et al.* ⁽⁵⁾ first identified the inhibitory effect of resveratrol on carcinogenesis, numerous studies have demonstrated the capacity of resveratrol to modulate a multitude of signaling pathways associated with cancer development (see **Chapter 1**). As a result, we postulate that resveratrol may function as a potential signaling pathway modulator and it is likely that this collective activity, rather than just a single effect, plays an important role in the anticancer properties of resveratrol.

However, despite the identification of numerous molecular targets, the underlying mechanisms involved in the antitumor/anticancer activities of resveratrol, especially in colorectal cancer, are complex and remain poorly defined. Thus, we sought to investigate the effect of resveratrol on gene modulation in human colorectal cancer cells in order to identify

and characterize novel molecular targets that may contribute to observed antitumor activities of resveratrol. The data presented in **Chapter 3** and in **Chapter 4** suggest that activating transcription factor 3 (ATF3) and early growth response-1 (Egr-1), respectively, are novel molecular targets of resveratrol in colorectal carcinogenesis.

ATF3, a stress-inducible or adaptive response gene, is suggested to function as a tumor suppressor gene in colorectal carcinogenesis due to its inhibitory effects on tumor cell proliferation, tumor formation, and invasion (see **Chapter 3** for references). Here, we identify ATF3 as a target of resveratrol that contributes to apoptosis induced by the dietary phytoalexin *in vitro*. Furthermore, resveratrol-increased ATF3 expression is mediated through Egr-1 and Krüppel-like factor 4 (KLF4) interactions, which occurs via the activation of zinc-finger domain of Egr-1. These findings show, for the first time, the involvement of resveratrol in the transcriptional regulation of ATF3, mediated by Egr-1 and KLF4, and that increased ATF3 expression facilitates induction of apoptosis by resveratrol.

Egr-1, characterized as an immediate early response transcription factor, is also suggested to behave as a tumor suppressor in colorectal cancer (refer to **Chapter 3** and **Chapter 4**). The data presented in **Chapter 4** suggest that increased Egr-1 transcriptional activity by resveratrol is dependent on the resveratrol-induced posttranslational acetylation of Egr-1, which is SIRT1-independent *in vitro*. Thus, resveratrol not only affects Egr-1 at the level of transcription (**Chapter 3**), but affects Egr-1 at the level of translation as well. Egr-1 acetylation by resveratrol contributes, at least partially, to Egr-1 induced expression of the pro-apoptotic protein NAG-1. Again, these findings represent a novel mechanism by which resveratrol induces Egr-1 transcriptional activity that is independent of SIRT1 expression.

Yet, in order to identify ATF3 or Egr-1 (and even KLF4) as genuine targets of resveratrol, we must validate the importance of these proteins in the anticancer activity of resveratrol *in vivo*. This can be readily achieved using an azoxymethane-induced model of colorectal carcinogenesis with ATF3-null⁽²³³⁾ and Egr-1-null⁽²³⁴⁾ mice. With this model we can examine the involvement of ATF3 and Egr-1 in resveratrol-mediated chemoprevention (administration of resveratrol prior to azoxymethane injection) and chemotherapy (azoxymethane injection prior to resveratrol administration). It would also be interesting to examine the role of ATF3 and Egr-1 using *APC*^{min/+} mice, a genetic model that results in adenoma formation in the small intestine.

Therefore, in addition to the classical approaches to chemotherapy, the role of resveratrol as an adjuvant therapy to standard cancer treatments should be evaluated. As discussed, resveratrol is demonstrated to potentiate the cytotoxic effects of known chemotherapeutic drugs and could alleviate associated adverse effects. Most patients would readily accept the addition of a low toxicity agent such as resveratrol as adjuvants to chemotherapy. Additionally, the molecular targets described in **Chapter 1** should be evaluated in an adjuvant setting to determine its contributions to the anticancer benefits of resveratrol; validation of such targets could identify novel therapeutic targets for the treatment of cancer and justify the use of resveratrol as an anticancer drug. Moreover, the identification of the novel molecular targets ATF3 (**Chapter 3**) and Egr-1 (**Chapter 4**), and subsequent validation as a target *in vivo*, provide an additional explanation of how resveratrol exerts its anti-tumorigenic and anti-carcinogenic effects in colorectal cancer.

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APPENDICES

APPENDIX A:
Figures for Chapter 3

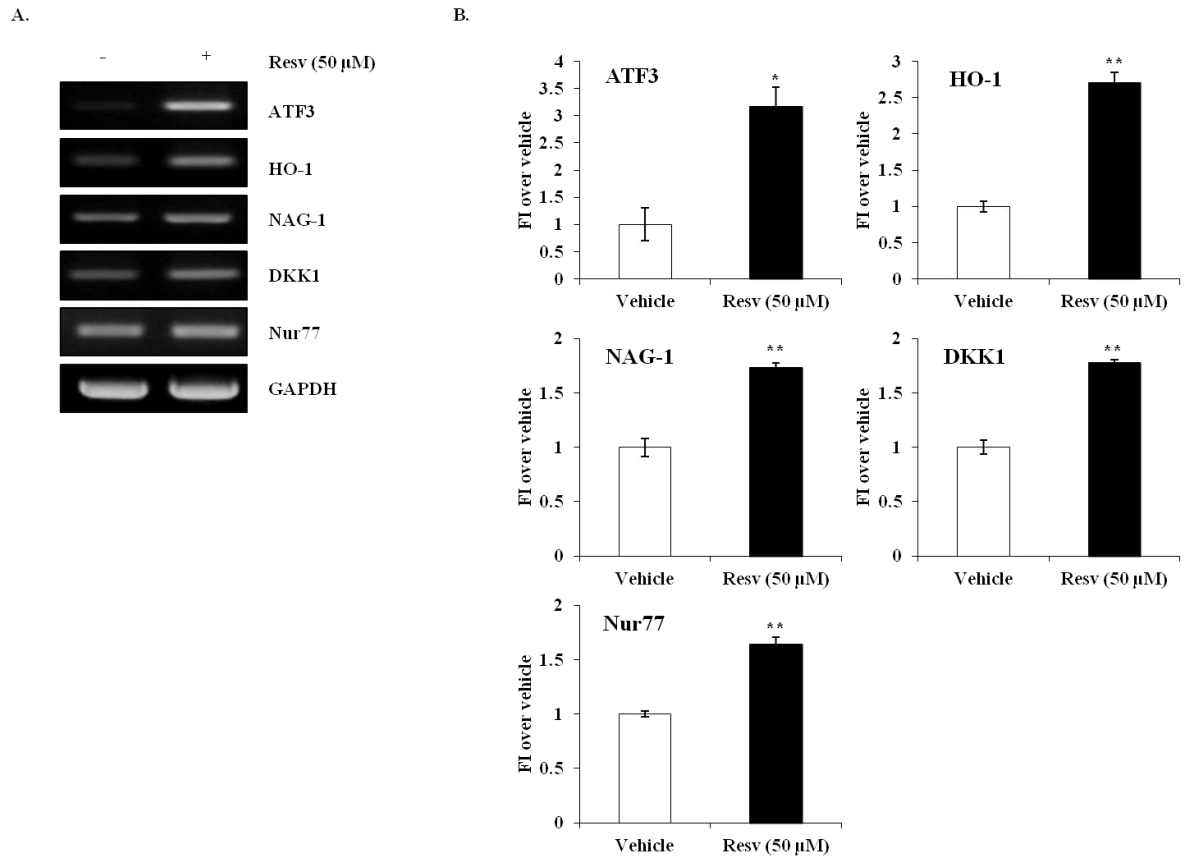


Figure A.1. Effect of resveratrol on gene expression in HCT-116 cells. (A) HCT-116 cells were treated with vehicle or resveratrol (Resv) in serum-free media for 24 h. RT-PCR was performed using specified human primers as described in **Chapter 2**. *GAPDH* served as a loading control. Representative gel pictures of three independent experiments are shown. (B) Gel densitometry results from three experiments for the respective genes in (A). Values are expressed as fold induction relative to vehicle-treated cells adjusted to *GAPDH*. Statistical significance is shown as $p < 0.05$ * and $p < 0.01$ **.

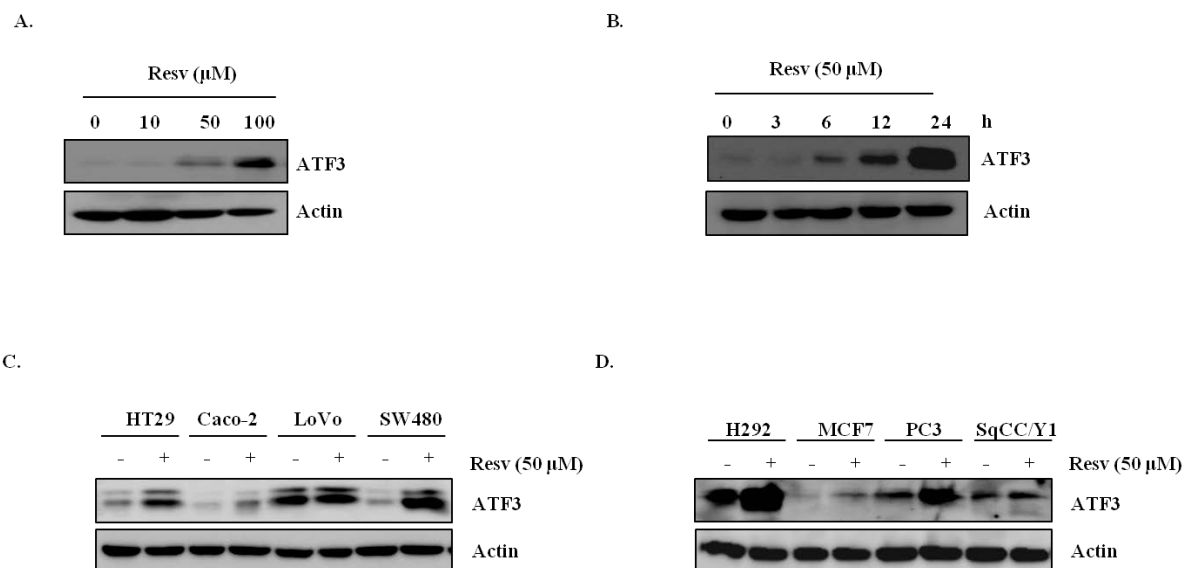


Figure A.2. Resveratrol increased ATF3 expression in cancer cells. Cancer cells were seeded and grown to 60% to 80% confluence, serum starved overnight, and treated with Resv as indicated in serum-free media. Protein lysates were harvested and subjected to Western blot analysis using ATF3 and Actin antibodies. (A) HCT-116 cells were treated with 0, 10, 50, and 100 μ M Resv for 24 h. (B) HCT-116 cells were incubated with Resv (50 μ M) for the indicated times. (C) Colorectal (HT29, Caco-2, LoVo, and SW480) and (D) non-colorectal (NCI-H292 lung, MCF7 breast, PC3 prostate, and SqCC/Y1 head and neck) cancer cells were treated with vehicle or Resv (50 μ M) for 24 h.

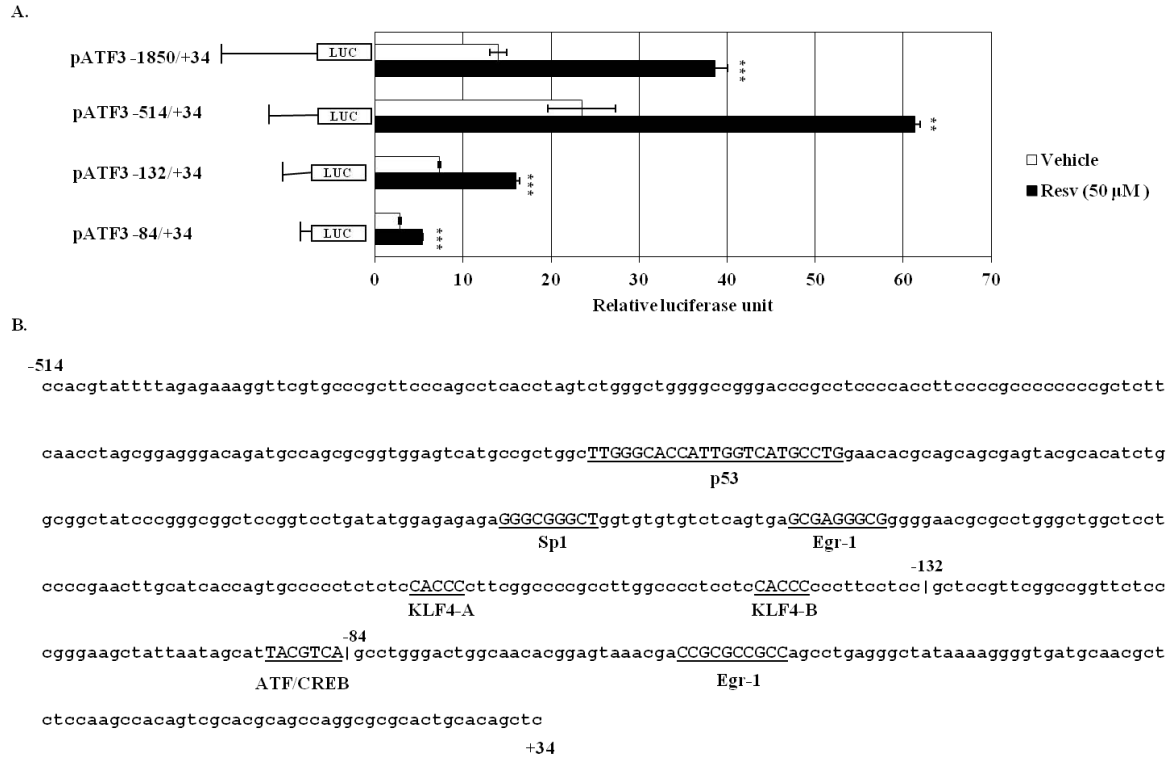


Figure A.3. Resveratrol response element located within the -514 to +34 bp region of the *ATF3* promoter. (A) Each indicated construct of the *ATF3* promoter (0.5 μg) and *pRL-null* (0.05 μg) were transiently transfected into HCT-116 cells and treated with vehicle or Resv (50 μM) for 24 h. The promoter activity was measured as a ratio of firefly luciferase signal/renilla luciferase signal. The x-axis shows relative luciferase unit of each construct. The results are the mean ± SD of 3 replicates. $p < 0.05^*$; $p < 0.01^{**}$; and $p < 0.001^{***}$, on the basis of Student's *t* test. (B) Nucleotide sequence of the -514 to +34 bp region of the *ATF3* promoter. Predicted binding sites of identified transcription factors are capitalized and underlined with name located underneath.

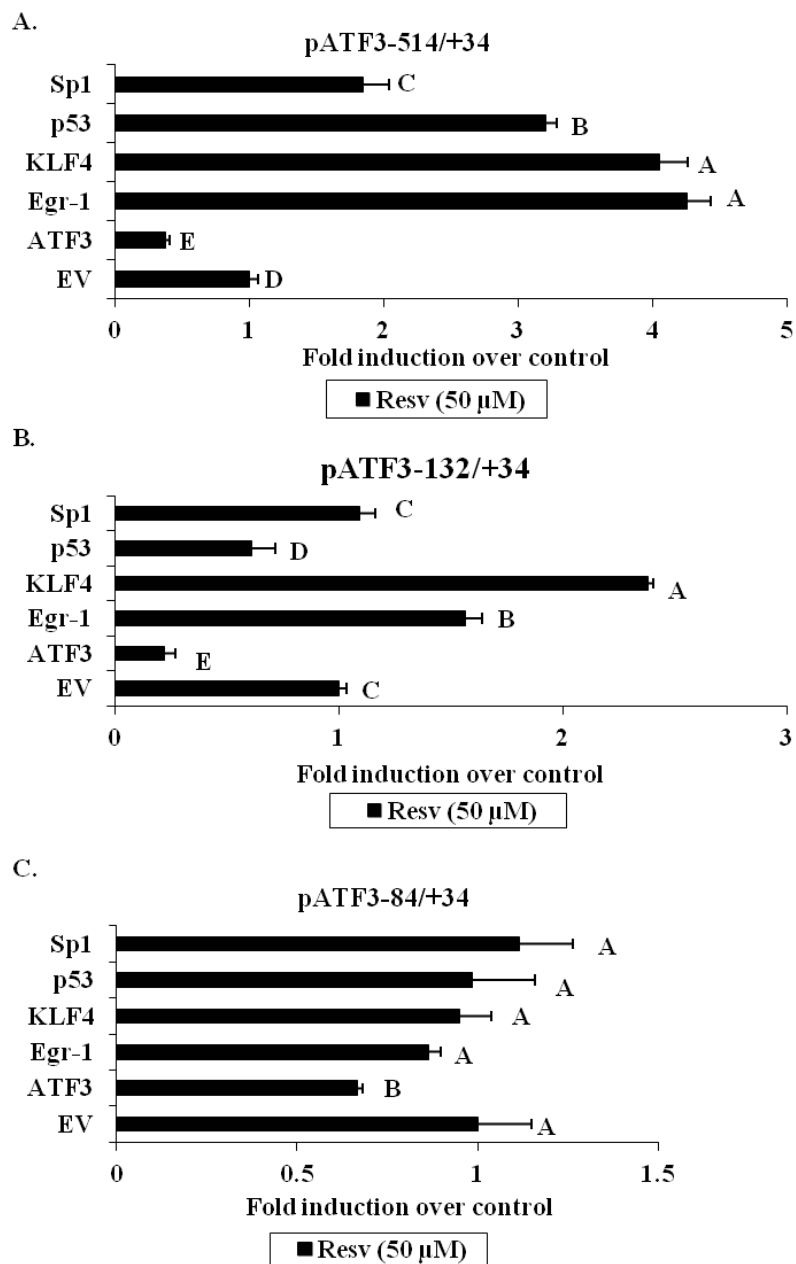
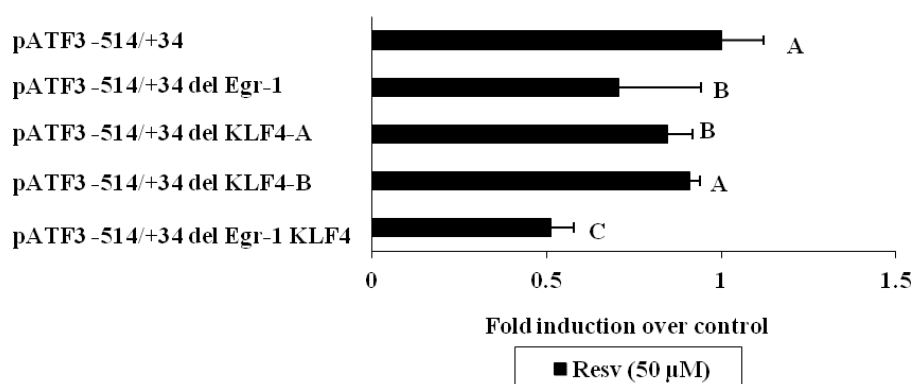


Figure A.4. Egr-1 and KLF4 contribute to resveratrol-induced ATF3 expression. Empty vector (pcDNA3.1/NEO, EV) or the indicated expression vector (0.25 μ g each) was co-transfected with *pATF3-514/+34* (A), *pATF3-132/+34* (B), or *pATF3-84/+34* (C) and *pRL-null* vector (0.05 μ g) into HCT-116 cells. Cells were then treated with vehicle or Resv (50 μ M) in serum-free media for 24 h. The x-axis shows fold induction relative to EV control. Data analyzed using Tukey's multiple comparison tests. Means with same letters indicate no significance ($p < 0.05$).

A.



B.

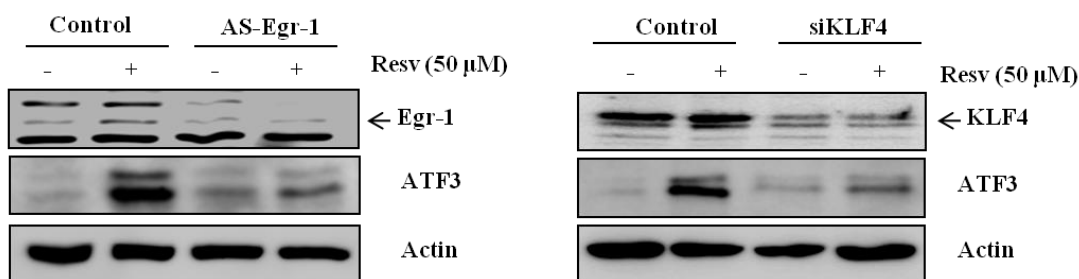


Figure A.5. Both *Egr*-1 and *KLF4* are involved in resveratrol induction of ATF3. (A) *pATF3*-514/+34 or its internal deletion clones (0.5 μg) were transfected into HCT-116 cells followed by treatment with vehicle or Resv (50 μM) in serum-free media for 24 h. Values were normalized to vehicle treatment. The x-axis represents fold induction relative to the *pATF3*-514/+34 control. Data analyzed using Tukey's multiple comparison test; mean values with the same letters indicate no significance ($p < 0.05$). (B) HCT-116 cells were transfected with *Egr*-1 sense/antisense oligonucleotides or control/*KLF4* siRNA using TransIT-TKO transfection reagent. Cells were serum starved overnight and treated with vehicle or Resv (50 μM) for 6 h (*KLF4*) or 24 h (*Egr*-1). Protein lysates were harvested and subjected to Western blot analysis for *Egr*-1 or *KLF4*, ATF3, and Actin.

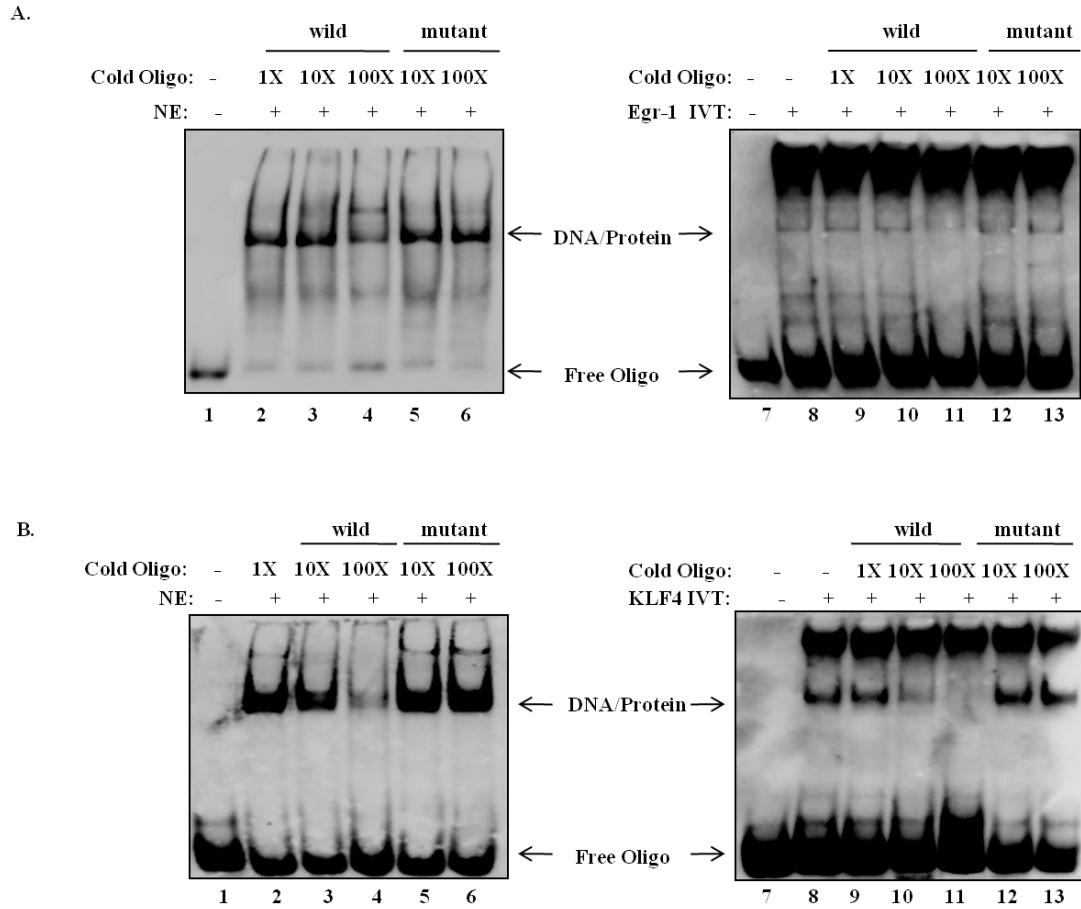


Figure A.6. Egr-1 and KLF4 bind to the ATF3 promoter, EMSA. Gel shift assays were performed using nuclear extracts (NE) from Resv (50 μ M)-treated HCT-116 cells for 24 h or *in vitro* translated (IVT) proteins as described in **Chapter 2**. Competitions were done in the presence of 10X and 100X excess of unlabeled oligonucleotides (lanes 3-4 and 10-11). Specificity of the DNA/protein complex was confirmed by the absence of competition with an excess of unlabeled mutated oligonucleotide (lanes 5-6 and 12-13). A, *Egr-1* and B, *KLF4*. Arrows, DNA/protein complexes.

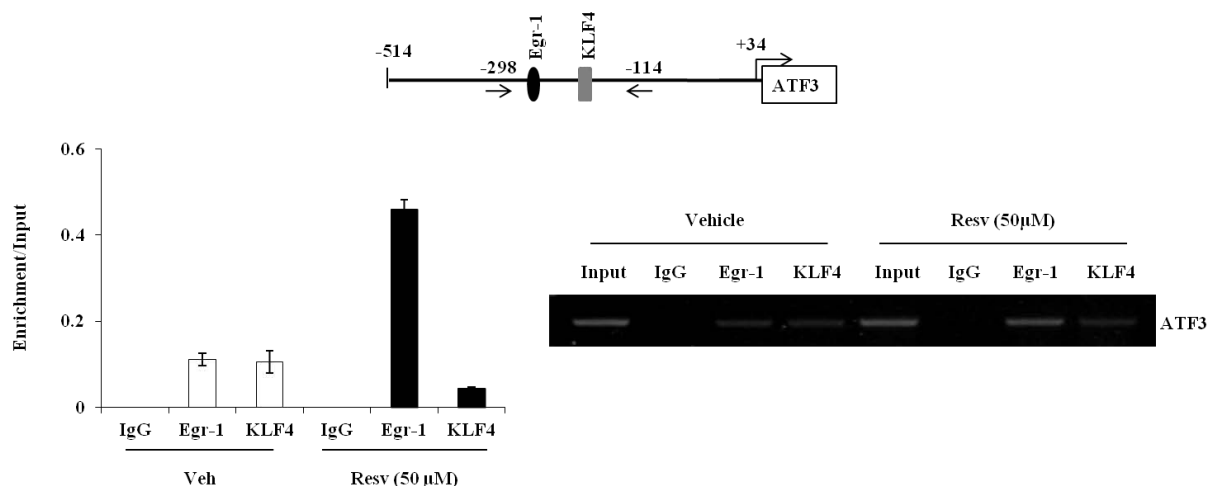


Figure A.7. Egr-1 and KLF4 bind to the ATF3 promoter, ChIP. HCT-116 cells were treated with vehicle or Resv (50 μ M) for 24 h. The chromatin/protein complexes were cross-linked by formaldehyde treatment, and chromatin pellets were extracted and sonicated. The associated *Egr-1* and *KLF4* DNA was isolated as described in **Chapter 2**. The sequence of the human *ATF3* promoter (-298/-114) was amplified by PCR primer pairs (arrows). The input represents PCR products obtained from 1% aliquots of chromatin pellets before immunoprecipitation. *Left*, ChIP qRT-PCR. The x-axis shows enrichment relative to input. The results are the mean $\pm$ SD of three independent experiments. *Right*, RT-PCR; representative gel picture of three experiments is shown.

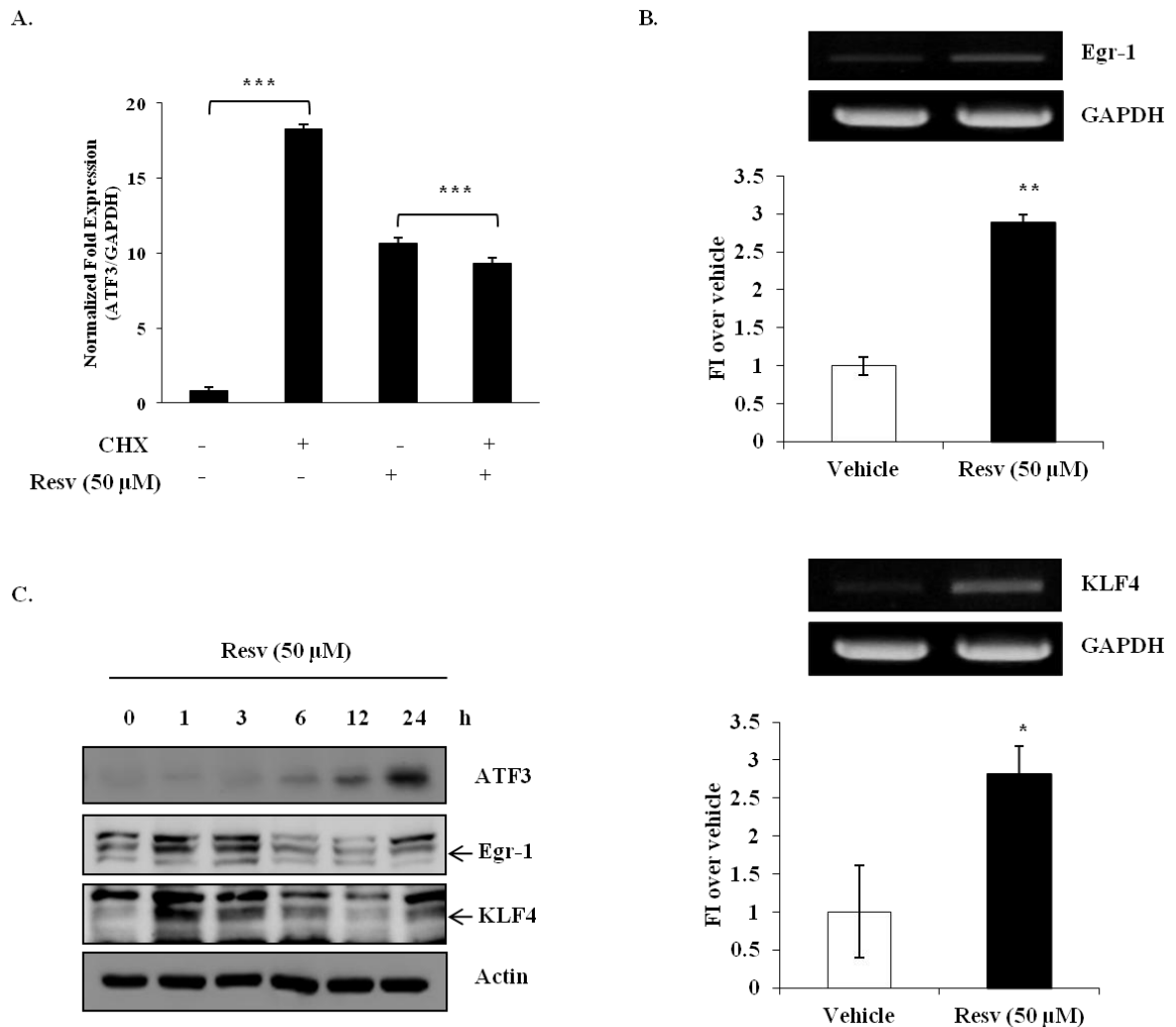


Figure A.8. *de novo* protein synthesis of Egr-1 and KLF4 is necessary for resveratrol-mediated ATF3 activation. (A) HCT-116 cells were pretreated with cycloheximide (CHX, 10 mg/mL) for 30 min in serum-free media followed by treatment with Resv (50 μ M) for 24 h. Values are normalized fold induction relative to GAPDH expression. The data are representative of three independent experiments. (B) HCT-116 cells were treated with vehicle or Resv (50 μ M) in serum-free media for 24 h. RT-PCR was performed using *Egr-1*, *KLF4*, and *GAPDH* human primers; the latter served as a loading control. Representative gel pictures (*top*) and gel densitometry (*bottom*) of three independent experiments are shown. Values are expressed as fold induction relative to vehicle-treated cells adjusted to GAPDH. $p < 0.05^*$ and $p < 0.01^{**}$, based on Student's *t* test. (C) HCT-116 cells were serum starved overnight and treated with Resv (50 μ M) for the indicated times. Protein lysates were harvested and subjected to Western blot analysis using ATF3, Egr-1, KLF4, and Actin antibodies.

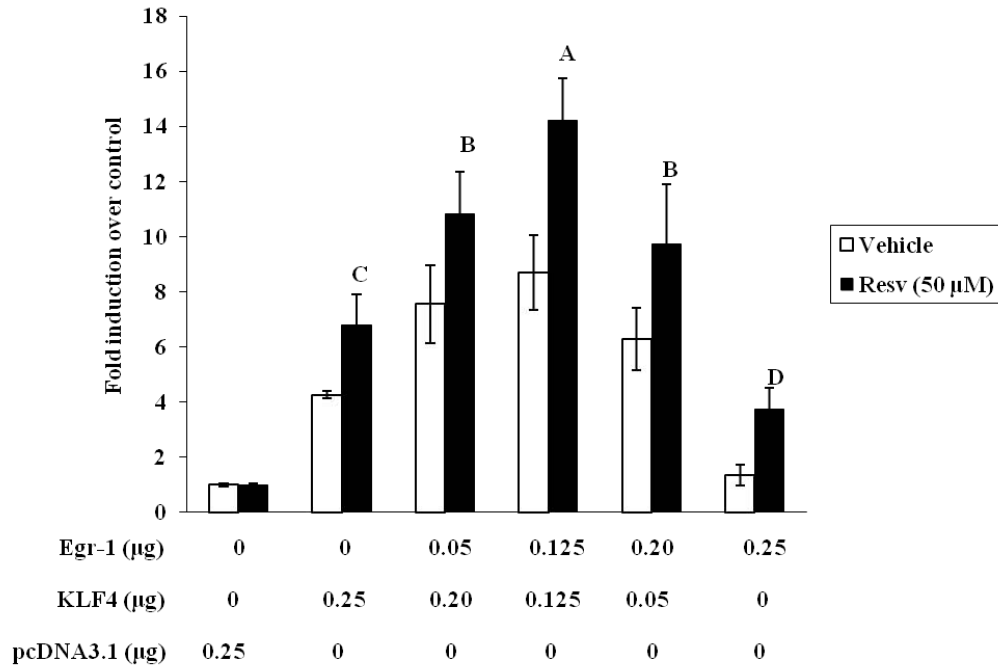


Figure A.9. Egr-1 and KLF4 coordinate the expression of resveratrol-induced ATF3 expression. The *pATF3-514/+34* reporter construct (0.25 μg) was co-transfected with pcDNA3.1 (empty), Egr-1, and KLF4 expression vectors as indicated in the presence of *pRL-null* vector (0.05 μg). Cells were treated with vehicle or Resv (50 μM) in serum-free media for 24 h. The y-axis shows fold induction relative to pcDNA3.1 control. The results are the mean ± SD of three replicates. Data analyzed using Tukey's multiple comparison test; mean values with the same letters indicate no significance ($p < 0.05$).

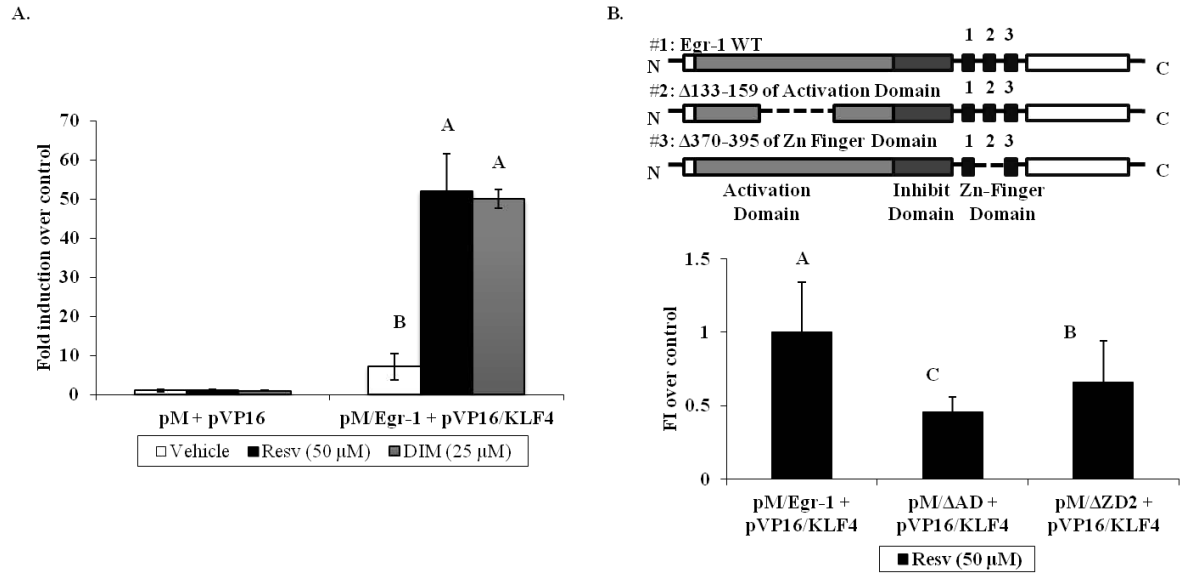


Figure A.10. Increased Egr-1 and KLF4 interact after resveratrol treatment. Mammalian 2-hybrid assay was performed as described in **Chapter 2**. (A) HCT-116 cells were co-transfected with pM and pVP16 vectors or pM/Egr-1 and pVP16/KLF4 vectors (0.2 μ g each) in the presence of pG5luc vector (0.2 μ g) and *pRL-null* vector (0.06 μ g). Cells were treated with vehicle, Resv (50 μ M), or DIM (25 μ M) for 24 h in serum-free media. Promoter activity was measured as a ratio of firefly luciferase/renilla luciferase signal, and the results are expressed as the mean $\pm$ SD of three independent experiments. The y-axis shows fold induction over control vectors. Data analyzed using Tukey's multiple comparison test; mean values with the same letters indicate no significance ($p < 0.05$). (B) *Top*, schematic diagram of Egr-1 protein structure and locations used to generate internal deletions. *Bottom*, HCT-116 cells were transfected with pM/Egr-1 internal deletion constructs, pVP16/KLF4 vector, and pG5luc vector. Cells were treated with vehicle or Resv (50 μ M) in serum-free media for 24 h. Values are normalized to vehicle treatment. The y-axis represents fold induction relative to pM/Egr-1 and pVP16/KLF4 control. Data analyzed using Tukey's multiple comparison test; mean values with the same letters indicate no significance ($p < 0.05$).

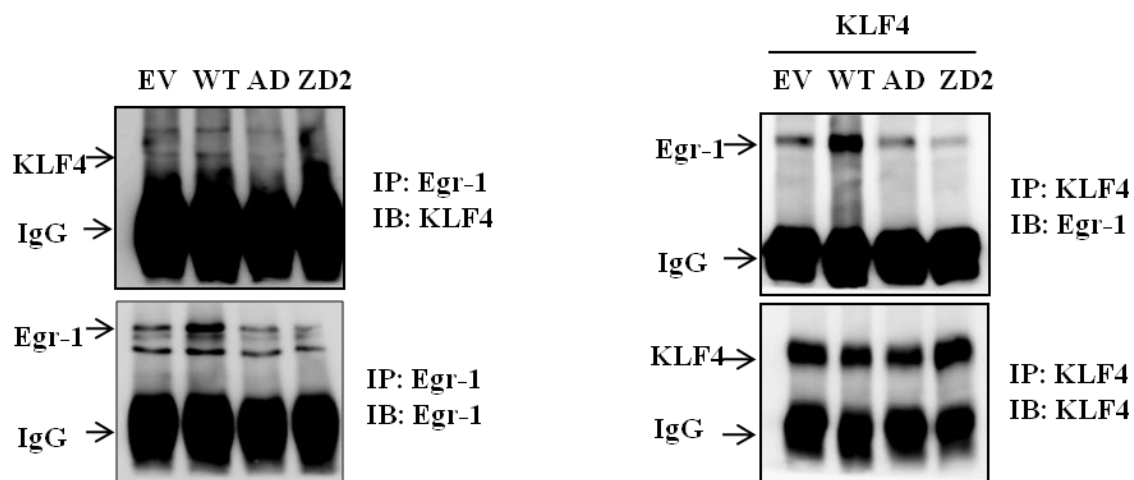


Figure A.11. Confirmation of Egr-1 and KLF4 interaction in the presence of resveratrol. HCT-116 cells were transfected with empty (EV), Egr-1 (WT or deletion), or co-transfected with KLF4 expression vectors as indicated and grown to 80% confluence. Cells were serum starved overnight, treated with Resv (50 μ M) for 2 h, and harvested as described in **Chapter 2**. Cell extracts were immunoprecipitated and immunoblotted with antibody against Egr-1 or KLF4.

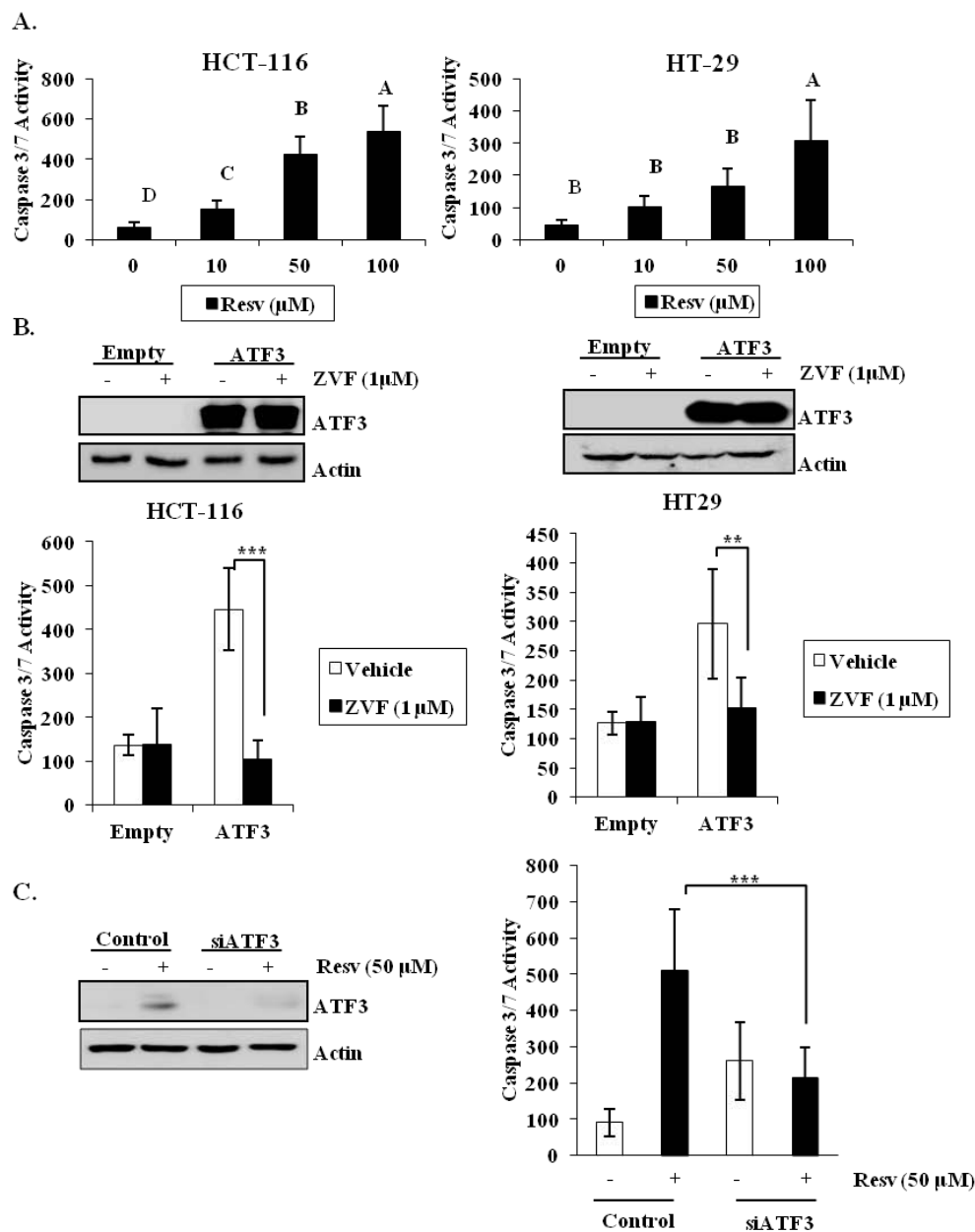
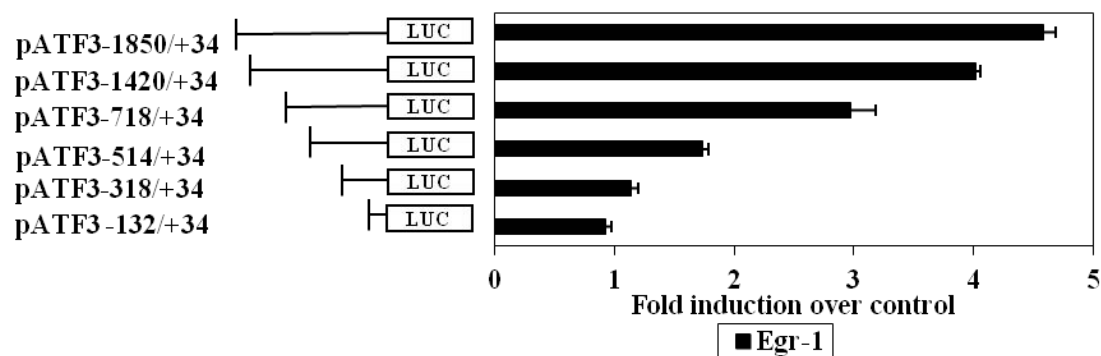


Figure A.12. Relevance of ATF3 in resveratrol-induced apoptosis. Caspase 3/7 enzymatic activity was measured as described in **Chapter 2**. (A) HCT-116 and HT29 cells were treated with 0, 10, 50, and 100 μM Resv for 24 h. Data analyzed using Tukey's multiple comparison test; mean values with the same letter indicate no significance ($p < 0.05$). (B) HCT-116 and HT29 cells were transfected with empty or ATF3 expression vector and grown to 60% to 80% confluence. Cells were serum starved overnight and treated with the pan caspase inhibitor z-vad-fmk (ZVF, 1 μM) for 3 h. ATF3 overexpression was validated (*top*). (C) HCT-116 cells were transfected with control/ATF3 siRNA using TransIT-TKO transfection reagent and treated with Resv (50 μM) for 24 h. ATF3 knockdown was validated (*left*). $p < 0.01^{**}$ and $p < 0.001^{***}$, on the basis of Student's t test.

A.



B.

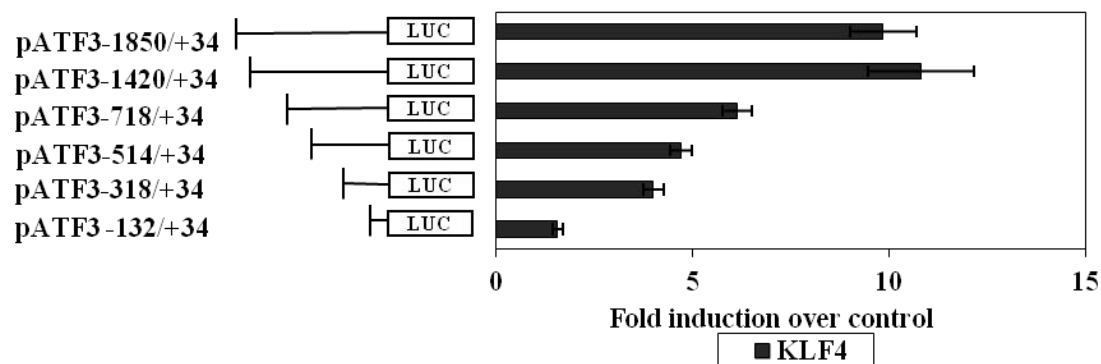
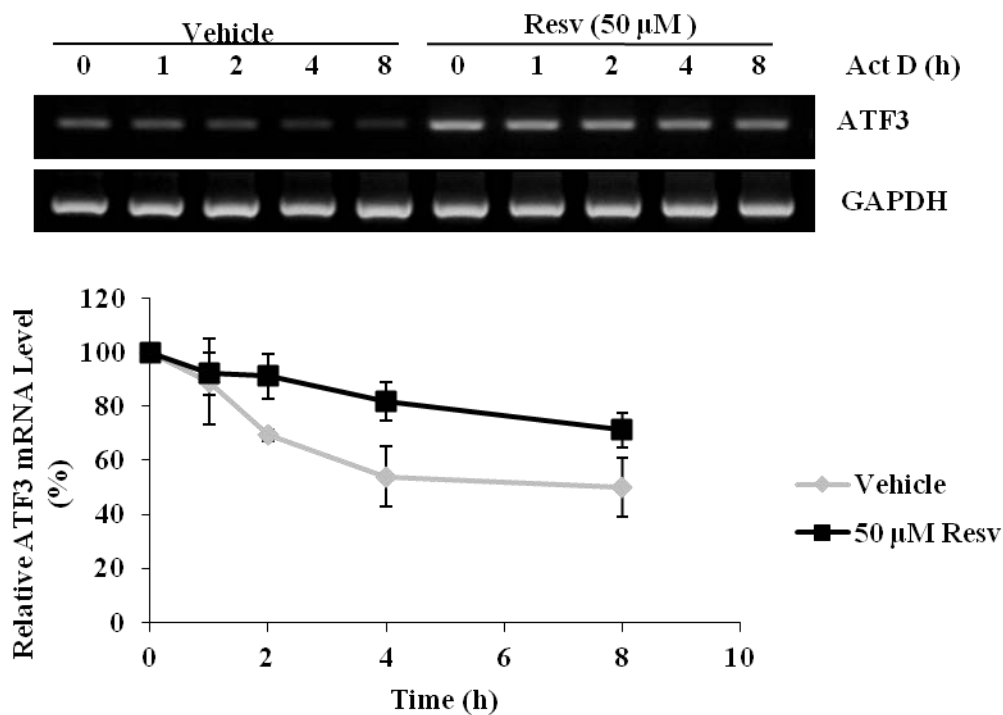


Figure A.13. Egr-1 and KLF4 activate ATF3 independent of resveratrol. Empty vector or the indicated expression vector (0.25 μ g each) was co-transfected with the *ATF3* promoter construct in the presence of *pRL-null* vector (0.05 μ g) into HCT-116 cells. Promoter activity was measured as a ratio of firefly luciferase signal/renilla luciferase signal. The x-axis shows fold induction relative to pcDNA3.1 control. The results are the means $\pm$ SD of three replicates. A, *Egr-1* and B, *KLF4*.



A.14. Posttranscriptional regulation of ATF3 as an alternate regulatory pathway. HCT-116 cells were incubated with vehicle or Resv (50 μ M) for 24 h and subsequently treated with antinomycin D (Act D, 5 μ M). At the indicated times, total RNA was isolated, and RT-PCR was performed using *ATF3* and *GAPDH* human specific primers, the latter served as a loading control. Representative gel pictures (*top*) and gel densitometry (*bottom*) of three independent experiments are shown. Values are expressed as percentage of relative *ATF3* transcript present at Time 0 of Act D treatment.

APPENDIX B:
Figures for Chapter 4

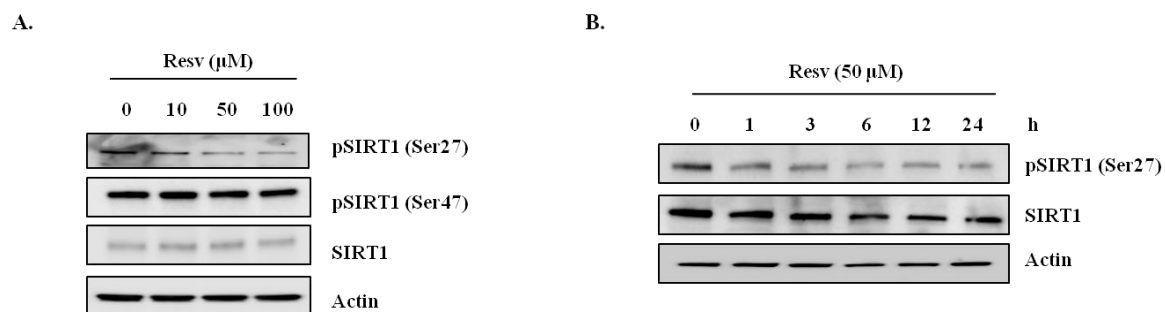
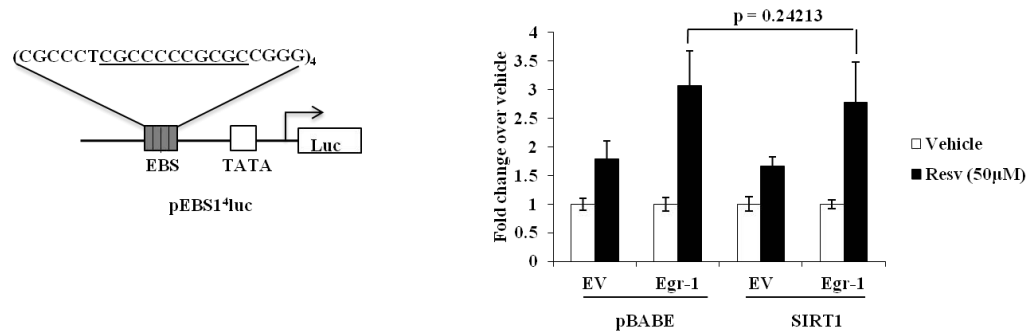


Figure B.1. Resveratrol decreases serine 27 SIRT1 phosphorylation. HCT-116 cells were seeded and grown to 60% to 80% confluence, serum starved overnight, and treated with Resv as indicated in serum-free media. Protein lysates were harvested and subjected to Western blot analysis using pSIRT1 (Ser 27), pSIRT1 (Ser 47), SIRT1, and Actin antibodies.

A.



B.

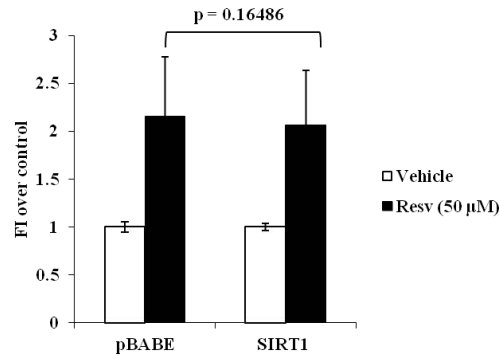


Figure B.2. Increased Egr-1 activity by resveratrol is SIRT1-independent. (A) HCT-116 cells were co-transfected with EV and *pEBS1⁴luc* vectors (0.25 μg each) or Egr-1 and *pEBS1⁴luc* vectors (0.25 μg each) in the presence of SIRT1 expression vector (0.25 μg) and *pRL-null* vector (0.0075 μg). Cells were treated with vehicle or Resv (50 μM) for 24 h in serum-free media. (B) pBABE or SIRT1 (0.375 μg) was co-transfected with *pEgr-1 -1260/+35* (0.375 μg) or *pRL-null* vector (0.0075 μg) into HCT-116 cells and treated with vehicle or Resv (50 μM) in serum-free media for 24 h. Promoter activity for (A) and (B) was measured as a ratio of firefly luciferase signal/renilla luciferase signal, and the results are expressed as the mean ± SD of 3 replicates. The y-axis shows fold induction over vehicle control.

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1  MAAKAEMQL MSPLQISDPF GSFPHSPTMD NYPKLEEMML LSNQAPQFLG AAGAPEGSGS
61  NSSSSSSGGG GGGGGGSNSS SSSSTFNPQA DTGEQPYEHL TAESFPDISL NNEKVLVETS
121 YPSQTTRLPP ITYTGRFSLE PAPNSGNTLW PEPLFSLVSG LVSMTNPPAS SSSAPSPAAS
181 SASASQSPPL SCAVPSNDSS PIYSAAPTFP TPNTDIFPEP QSQAFFGSAG TALQYPPPAY
241 PAAKGGFQVP MIPDYLFPQQ QGDLGLGTPD QKPFQGLESR TQQPSLTPLS TIKAFATQSG
301 SQDLKALNTS YQSOLIKPSR MRKYPNRPSK TPPHERPYAC PVESCDRRFS RSDDELTRHIR
361 IHTGQKPFQC RICMRNFSRS DHLTTHIRTH TGEKPFACDI CGRKFARSDE RKRHTKIHLR
421 QKDKKADKSV VASSATSSLS SYPSPVATSY PSPVTTSYPS PATTSYPSPV PTSFSSPGSS
481 TYPSPVHSGF PSPSVATTYS SVPPAFPAQV SSFPSSAVTN SFSASTGLSD MTATFSPRTI
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Figure B.3. Egr-1 amino acid sequence and predicted acetylation site.

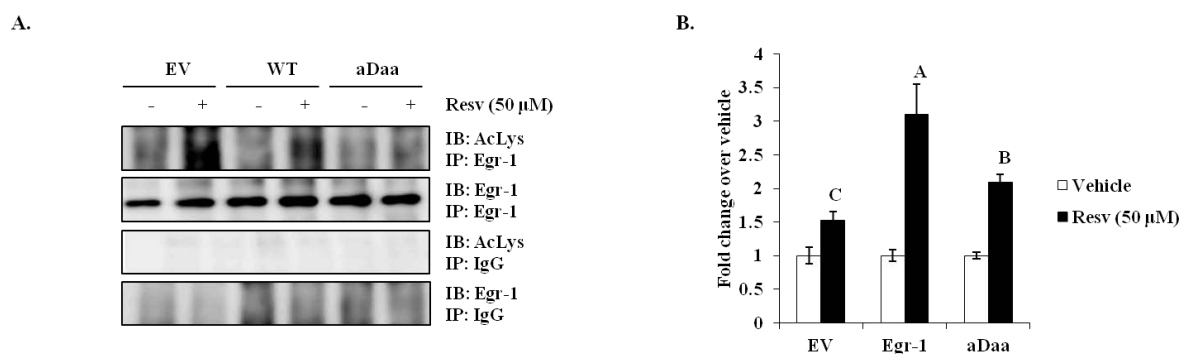
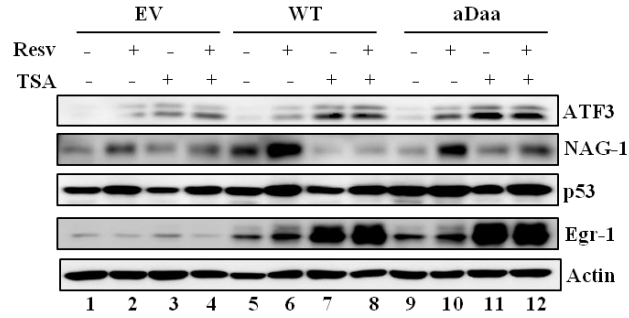


Figure B.4. Egr-1 aDaa mutation diminishes resveratrol-induced Egr-1 activity. (A) HCT-116 cells were transfected with EV, Egr-1 WT, or Egr-1 aDaa vectors and grown to 80% confluence. Cells were serum starved overnight, treated with Resv (50 μ M) for 2 h, and harvested as described in **Chapter 2**. The cell extracts were immunoprecipitated with Egr-1 and immunoblotted with antibody against Egr-1 and acetylated lysine. (B) EV, Egr-1, or Egr-1 aDaa expression vector (0.375 μ g each) was co-transfected with *pEBS1⁴luc* (0.375 μ g) and *pRL-null* vector (0.0075 μ g) into HCT-116 cells. Cells were then treated with vehicle or Resv (50 μ M) in serum-free media for 24 h. The promoter activity was measured as a ratio of firefly luciferase signal/renilla luciferase signal. The y-axis shows fold change relative to vehicle control. The results are the mean $\pm$ SD of 3 replicates. Data analyzed using Tukey's multiple comparison test; mean values with same letters indicate no significance ($P < 0.05$).

A.



B.

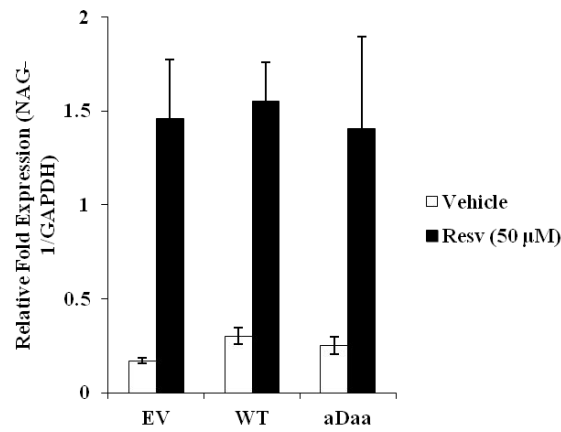
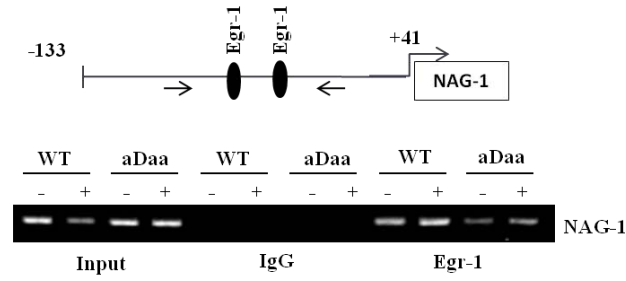


Figure B.5. Acetylation contributes to activation of the Egr-1 target gene, NAG-1. HCT-116 cells were transfected with EV, Egr-1 WT, or Egr-1 aDaa and grown to 80% confluence. (A) Cells were serum starved overnight and pre-treated with Resv (50 μ M) for 2 h followed by TSA (1 μ M) for 24 h as indicated. Protein lysates were harvested and subjected to Western blot analysis using ATF3, NAG-1, p53, Egr-1, and Actin antibodies. (B) qRT-PCR was performed as described in **Chapter 2** for NAG-1. Values are normalized to fold induction relative to GAPDH expression. The data are representative of 3 independent experiments.

A.



B.

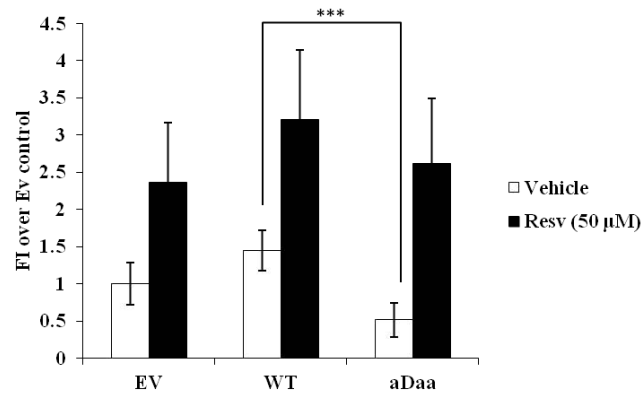


Figure B.6. Acetylation contributes to NAG-1 promoter binding and transactivation by Egr-1. HCT-116 cells were transfected with EV, Egr-1 WT, or Egr-1 aDaa vectors and grown to 80% confluence. (A) The sequence of the human NAG-1 promoter (-133/+31) was amplified by PCR primer pairs. The input represents PCR products obtained from 1% aliquots of chromatin pellets before immunoprecipitation. Representative gel picture of three experiments is shown. (B) Each vector (0.375 μg) was co-transfected with *pNAG-1 -133/+41* (0.375 μg) or *pRL-null* vector (0.0075 μg) into HCT-116 cells and treated with vehicle or Resv (50 μM) in serum-free media for 24 h. Promoter activity for was measured as a ratio of firefly luciferase signal/renilla luciferase signal, and the results are expressed as the mean ± SD of 3 replicates. The y-axis shows fold induction over vehicle control. $p < 0.001^{***}$, on the basis of Student's *t* test.

VITA

Nichelle Chantil Whitlock was born in Richmond, VA, to Willie and Minnie Whitlock; she has a younger brother named Willie Preston Jr., but everyone calls him Preston. As a child, because of her father's military service (GO ARMY BEAT NAVY!), Nichelle had the opportunity to live in West Germany (Ansbach and Wurzburg) where she spent preschool through first grades. After her father's tour of duty, the family moved to Springfield, VA, for two years and ultimately settled in Stafford, VA. She went to North Stafford High School/the Commonwealth Governor's School, a high school program for gifted and highly motivated students, and participated in FIRST (For Inspiration and Recognition of Science and Technology) robotics. After graduation, she attended the University of Tennessee, Knoxville (UT, GO BIG ORANGE!) and was introduced to the field of cancer prevention research in the Laboratory of Environmental Carcinogenesis led by Dr. Seung J. Baek. Nichelle obtained a Bachelor's of Science degree in Biological Sciences-Microbiology in 2006 and accepted a position as a graduate research assistant in the Comparative and Experimental Medicine (CEM) Program at the UT with Dr. Baek as her Ph.D. advisor. As a doctoral candidate, Nichelle sought to identify novel targets of resveratrol (a dietary phytochemical) in colorectal carcinogenesis. During the course of her studies, Nichelle received three travel awards to attend the Experimental Biology Meetings to present her research findings (CEM graduate program travel award, MARC/ASPET graduate student travel award, and APS/NIDDK minority travel fellowship award). She received the CEM Teaching Fellowship in 2010 and 2011. She has two first author publication in *Cancer Prevention Research* (2011) and *The Veterinary Journal* (2008; co-first author) and several

co-author publications in *Oncogene* (2010) and *Molecular Cancer Therapeutics* (2008); she also recently contributed a book chapter to *Evidence-Based Anticancer Materia Medica* (2011). Nichelle graduated with a Ph.D. in Comparative and Experimental Medicine in December 2011 and will continue to pursue a career in the interdisciplinary field of cancer prevention research.