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Analysis of Deleted in Breast Cancer 1 (DBC1) as a Novel Molecular Target of Resveratrol and Its Metabolites

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Analysis of Deleted in Breast Cancer 1 (DBC1) as a Novel Molecular Target of Resveratrol and Its Metabolites

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Melody Pai

2016
ABSTRACT OF THE DISSERTATION

Analysis of Deleted in Breast Cancer 1 (DBC1) as a Novel Molecular Target of Resveratrol and Its Metabolites

by

Melody Pai
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2016
Professor Jing Huang, Chair

With increasing age comes increasing susceptibility to age-related diseases, including cancer and neurodegenerative diseases. A novel approach to treating such diseases is to treat aging itself. However, not much is known about the molecular targets of the aging pathway. Utilizing the Drug Affinity Responsive Target Stability (DARTS) approach developed by our laboratory, we have been working to identify the molecular targets of the aging pathway and to analyze the anti-aging mechanisms of various small molecules that have been shown to increase life expectancy. One such small molecule, resveratrol (RSV), a polyphenol found in the skin of red grapes, has been shown to possess various health benefits, including the extension of lifespan, in several different model organisms. However, the underlying molecular mechanisms of RSV’s beneficial effects remain unclear and highly controversial. My thesis work entails...
the identification of a novel molecular target of resveratrol, deleted in breast cancer 1 (DBC1), and analysis of its mechanism of action through the protein deacetylase sirtuin 1 (SIRT1), which has been shown to be required for some of RSV’s various health benefits. I have also identified DBC1 as a target of RSV’s metabolites, the 3- and 4’-glucuronides and sulfates, and analyzed the metabolites’ mechanisms of action through SIRT1. And finally, I have discovered that resveratrol induces the phosphorylation of DBC1 and activates a distinct ATM-dependent but DBC1-independent DNA damage response from etoposide that does not result in apoptosis. Taken together, my thesis provides a novel molecular mechanism of resveratrol and its metabolites through the novel molecular target DBC1, resolving some of the controversy surrounding resveratrol’s SIRT1-dependent mode of action, and identifies a distinct ATM-dependent and DBC1-independent DNA damage response that resveratrol activates, suggesting another novel molecular mechanism of resveratrol that could contribute to its beneficial health effects.
The dissertation of Melody Pai is approved.

Catherine F. Clarke

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University of California, Los Angeles

2016
DEDICATION

To my parents for impressing on me the importance of education and for providing me with all the support I needed in pursuing my dreams.

To my sister Grace for being the best sister – keeping me sane through graduate school, always driving down to see me, and being tourists in LA with me.

To my friend Jaison for the many discussions over lunches and dinners about my research and random topics and for inspiring me with his true passion in science.

To my friend Megan for the many late nights listening to me ranting about graduate school and career decisions and for being the best roommate anyone could ask for.

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Chapter One


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Contribution: *Methods in Molecular Biology* – Melody Y. Pai and Brett Lomenick designed, performed, and analyzed experiments and wrote the manuscript. Jing Huang, Robert Schiestl, William McBride, and Joseph A. Loo provided guidance. *Cell Cycle* – Tyler J. Robinson designed, performed, and analyzed experiments and wrote the manuscript. Melody Pai designed, performed, and analyzed experiments and provided comments on the manuscript. Eldad Zacksenhaus and Jing Huang provided guidance. *Nature* – Randall M. Chin designed, performed, and analyzed experiments and wrote the manuscript. Xudong Fu, Laurent Vergnes, Melody Pai, Heejun Hwang, Brett Lomenick and Simon Diep designed, performed, and analyzed experiments and provided comments on the manuscript. Jing Huang provided ideas, helped design experiments and analyze results, and helped write the manuscript.

**Chapter Two**

Chapter two is adapted from Pai M.Y. *et al.* “Deleted in Breast Cancer 1 as a Novel Molecular Target of Resveratrol and Its Metabolites – Providing a Link Between SIRT1 and Resveratrol” (manuscript in preparation).

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Contribution: Melody Y. Pai designed, performed, and analyzed experiments and wrote the manuscript. Heejun Hwang and Laurent Vergnes performed experiments. Heping Shi and Qiaoling Wang synthesized compounds. David Sinclair graciously
provided some of the cell lines used in experiments. Jing Huang and Chuo Chen provided guidance.

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Graduate Student Researcher (June 2011 – Present)
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• Sole driver of an independent project that resolves scientific controversy surrounding the anti-aging molecule resveratrol, presenting key findings at numerous conferences and retreats
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Graduate Student Intern (June 2014 – September 2014)
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Publications


Presentations

Pai M.Y., Hwang H., Shi H., Chuo C. and Jing Huang. “RBP as a Novel Molecular Target of Resveratrol and Its Metabolites: Providing a Link between SIRT1 and Resveratrool.” Poster presentation at the UCLA Research Conference on Aging, Los Angeles, CA; July 2015.

Pai M.Y. “A Novel Molecular Target of Resveratrol and Its Metabolites: Providing a Link between SIRT1 and Resveratrool” Oral presentation at the Molecular Biology Interdepartmental Program Student Seminar, Los Angeles, CA; May 2015.

Pai M.Y., Hwang H., Shi H., Chuo C. and Jing Huang. “RBP as a Novel Molecular Target of Resveratrol and Its Metabolites: Providing a Link between SIRT1 and Resveratrool.” Poster presentation at the Molecular Biology Interdepartmental Program Annual Retreat, Los Angeles, CA; April 2015.


Pai M.Y. “Analysis of Deleted in Breast Cancer 1 (DBC1) as a Molecular Target of Resveratrol and an Important Regulator of Aging.” Oral presentation at the Molecular Biology Interdepartmental Program Student Seminar, Los Angeles, CA; April 2013.

Pai M.Y. “Analysis of Deleted in Breast Cancer 1 (DBC1) as a Molecular Target of Resveratrol and an Important Regulator of Aging.” Oral presentation at the Cell and Molecular Biology Training Grant Student Seminar, Los Angeles, CA; March 2013.
Introduction

By the year 2060, the number of individuals in the United States over the age of 65 is projected to more than double and to comprise approximately 20% of the total population\(^1\). With increasing age comes increasing susceptibility to age-related diseases, including cancer and neurodegenerative diseases\(^2\). Thus, a novel approach to treating such diseases is to treat aging itself. One of the most robust and well-studied methods to delay aging is calorie restriction (CR) (also known as dietary restriction), or the reduction of food intake without malnutrition. As early as 1935, McCay et al. showed that reducing the calorie intake of rats resulted in increased lifespan\(^3\). Continued research on CR show that this effect is conserved in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, mice, and nonhuman primates\(^4-6\). Aside from its effects on longevity, calorie restriction has also been shown to reduce the incidence of various age-related diseases\(^7-10\). Much effort has been spent to uncover the genetic mechanisms behind CR-mediated longevity. Pathways involving target of rapamycin (TOR), AMP-activated protein kinase (AMPK), and insulin-like growth factor 1 (IGF-1) receptor, to name a few, have been implicated as essential for CR-mediated longevity\(^11\).

In 2000, Lin et al. showed that the yeast Sir2 gene is required for increased yeast replicative lifespan under limiting glucose conditions, which mimics calorie restriction in yeast\(^6\). The human homolog of Sir2, SIRT1, belongs to a family of proteins called sirtuins. SIRT1 is a NAD\(^+\)-dependent protein deacetylase that deacetylates various proteins, including p53, PGC-1\(\alpha\), Ku70, and histones, thus influencing apoptosis, cell survival, metabolism, DNA repair, and gene silencing\(^12-16\). Since the discovery of SIRT1’s role in CR-mediated longevity, thousands of studies have been published on
sirtuins and aging, including some challenging data\textsuperscript{17}. Perhaps the biggest challenge to the sirtuin-CR hypothesis was published in 2011 when Burnett et al. called into question whether sirtuins are critical in mediating the effects of calorie restriction. They showed that overexpression of SIRT1 in transgenic worms does not extend lifespan, after controlling for the genetic background of the worms, contrary to earlier studies showing the opposite\textsuperscript{18}. These contradictory studies led to a re-examination of the importance of sirtuins in aging. Numerous more recent studies indicated that an overexpression or activation of SIRT1 in \textit{C. elegans}, flies, and mice does, in fact, extend lifespan\textsuperscript{19-21}, thus re-affirming the role of SIRT1 as an important mediator of longevity.

Following the discovery of SIRT1 as required for CR-mediated lifespan, screens for small molecule activators of SIRT1 were performed, and the natural product resveratrol was identified as SIRT1’s most potent activator\textsuperscript{22}. Resveratrol, a polyphenol found in the skin of red grapes and other plant sources, was proposed to act as a calorie restriction mimetic and extend the lifespan of several model organisms including \textit{S. cerevisiae}, \textit{C. elegans}, \textit{D. melanogaster}, and high-fat diet-fed mice\textsuperscript{23,24}. Several studies reported that this extension of lifespan by resveratrol along with its associated beneficial metabolic effects occur in a SIRT1-dependent manner\textsuperscript{22,25}. However, controversy surrounding the link between resveratrol and SIRT1 arose when several groups showed that the activation of SIRT1 by resveratrol required the presence of an artificial fluorophore that was conjugated to the SIRT1 substrate used in the original screen\textsuperscript{22}; without the fluorophore, SIRT1 was no longer activated, and the native substrate was no longer deacetylated\textsuperscript{26-28}. Although the link between resveratrol and SIRT1 remains to be clearly elucidated, it is likely that resveratrol activates SIRT1,
either directly or indirectly, inside the cell under certain conditions to increase longevity, but the exact mechanism(s) as to how this occurs remains unknown.

Aside from its anti-aging effects, resveratrol has also been shown to possess various health benefits, including anti-cancer and anti-diabetic effects\textsuperscript{29}, to name a few, and has been involved in over a hundred on-going or recently completed clinical trials (www.clinicaltrials.gov). Resveratrol was first indicated to possess anti-neoplastic effects in 1997 when it was shown to protect against skin cancer in a mouse model\textsuperscript{30}. Studies have suggested that resveratrol’s chemopreventive activities are dependent on the generation of DNA double-strand breaks specifically in cancer cell lines, leading to the induction of p53-dependent apoptosis through activation of the ataxia telangiectasia-mutated (ATM) kinase\textsuperscript{31-33}. The precise mechanism(s) behind resveratrol’s anti-cancer effects, however, remain largely unclear\textsuperscript{34}.

Despite resveratrol’s various documented beneficial effects \textit{in vitro} and \textit{in vivo}, to date, there is no conclusive proof that resveratrol can provide all of these benefits in humans\textsuperscript{35}. In addition, resveratrol is quickly metabolized \textit{in vivo} to the 3- and 4'-glucuronides and sulfates, leaving almost no unconjugated resveratrol in the serum\textsuperscript{36-38}. Because of resveratrol’s poor bioavailability, it has been suggested that resveratrol’s beneficial effects are, in fact, due to its metabolites\textsuperscript{39}. A recent study has suggested that resveratrol’s sulfate metabolites can generate resveratrol in the cell, and this intracellularly generated resveratrol reservoir is responsible for exerting some of resveratrol’s previously observed beneficial effects\textsuperscript{40}. However, it remains unclear how resveratrol’s metabolites behave in the cell or whether it targets the same proteins as resveratrol. Furthermore, resveratrol is a highly promiscuous small molecule and has
numerous already identified binding targets, including but not limited to the cyclooxygenases, ATP synthase, elF4A, phosphodiesterases, and tyrosyl transfer-RNA synthetase\(^{30,35,41-46}\). Therefore, it is essential to determine which previously identified and yet to be identified protein targets of resveratrol play a role in each of its various beneficial effects so that more potent and bioavailable small molecules can be discovered that target these proteins to better induce resveratrol’s beneficial effects in the cell.

In light of SIRT1’s essential role not only in aging but also various cellular processes, it has also become increasingly important to understand how SIRT1 is regulated \textit{in vivo}. Two separate groups, Zhao \textit{et al.} and Kim \textit{et al.}, identified a novel negative regulator of SIRT1 called deleted in breast cancer 1 or DBC1\(^{47,48}\). In their studies, they demonstrate that DBC1 interacts directly with SIRT1 at SIRT1’s catalytic domain to prevent its substrates from binding and thereby inhibiting its deacetylase activity. DBC1 (also known as CCAR2, KIAA1967, p30 DBC) was originally identified as being from a region that is homozygously deleted in some breast cancers\(^{49}\). However, further studies have shown that DBC1 is not deleted in all breast cancer tissues and cell lines, and the role of DBC1 in tumorigenesis and whether it is a tumor suppressor or tumor promoter remains unclear\(^{50}\). Studies have shown that DBC1 is overexpressed in some cancer types and that overexpression is correlated with poor prognosis\(^{51-53}\). Conversely, another more recent study demonstrated that DBC1 functions as a tumor suppressor by regulating the stability of the tumor suppressor p53\(^{54}\). Regardless of its unclear role in tumorigenesis, given its important role in regulating SIRT1, how the interaction between DBC1 and SIRT1 can be modulated in the cell is essential to
determine. We hypothesized that resveratrol can bind directly to DBC1 and decrease the DBC1-SIRT1 interaction, thereby indirectly activating SIRT1. To validate this, we utilized a novel small molecule target identification technique termed Drug Affinity Responsive Target Stability (DARTS)\textsuperscript{41,55-57}.

Small molecule target identification is a critical aspect of drug discovery, chemical genetics, and metabolomics\textsuperscript{41}. A variety of methods have been developed for small molecule target identification, with affinity chromatography being the most commonly used approach\textsuperscript{56,58}. However, affinity chromatography and related approaches are limited by the need to derivatize each small molecule, and many compounds cannot be modified without loss of binding specificity or affinity. These limitations have spurred the continual development of new and improved methods. Drug Affinity Responsive Target Stability (DARTS) is a paradigm-changing method developed to overcome these limitations. DARTS leverages the thermodynamic stabilization of the target protein that occurs upon small molecule binding by detecting the binding-induced increase in resistance to proteolysis\textsuperscript{41,55,56}. Unlike affinity chromatography, DARTS is also not limited by the chemistry of the small molecule of interest and does not require derivatization or immobilization of the compound. Rather, DARTS is performed by simply treating aliquots of cell lysate with the compound of interest and either vehicle control or an inactive analog, followed by limited digestion of the proteins in the cell lysate with proteases. Subsequently, the samples are separated by SDS-PAGE and stained to identify protein bands that are protected from proteolysis by the small molecule. Mass spectrometry (MS) is then used to identify the proteins present in each band. This unbiased DARTS approach has been successfully utilized to identify novel
protein targets for natural products and other bioactive small molecules\textsuperscript{41,59-61} and thus is an appropriate technique to identify novel protein targets of resveratrol.

In this dissertation, we provide a novel molecular mechanism of resveratrol and its metabolites through the novel molecular target DBC1 (discovered utilizing the DARTS method), resolving some of the controversy surrounding resveratrol's SIRT1-dependent mode of action. We also identify a distinct ATM-dependent and DBC1-independent DNA damage response that resveratrol activates, suggesting another novel molecular mechanism of resveratrol that could contribute to its beneficial health effects.
References


Chapter 1: Utilizing the Drug Affinity Responsive Target Stability (DARTS) Approach to Determine Small Molecule Protein Targets

Abstract

Drug Affinity Responsive Target Stability (DARTS) is a relatively quick and straightforward approach to identify potential protein targets for small molecules. It relies on the protection against proteolysis conferred on the target protein by interaction with a small molecule. The greatest advantage of this method is being able to use the native small molecule without having to immobilize or modify it (e.g., by incorporation of biotin, fluorescent, radioisotope, or photoaffinity labels). Here we describe examples using DARTS to assay small molecule-protein interactions, including three model drug-protein pairs – methotrexate and its protein target dihydrofolate reductase (DHFR)\(^1\), olaparib and its protein target poly(ADP-ribose) polymerase (PARP)\(^2\), and omigapil (CGP 3466B) and its protein target glyceraldehyde 3-phosphate dehydrogenase (GAPDH)\(^3\), the FDA-approved drug disulfiram used to treat chronic alcoholism\(^4\), and the lifespan-extending metabolite \(\alpha\)-ketoglutarate\(^5\).
Introduction

Small molecule target identification is a critical aspect of drug discovery, chemical genetics, and metabolomics. A variety of methods have been developed for small molecule target identification, with affinity chromatography being the most commonly used approach. However, affinity chromatography and related approaches are limited by the need to derivatize each small molecule, and many compounds cannot be modified without loss of binding specificity or affinity. On the other hand, genetic/genomic methods are limited to particular classes of compounds (e.g. those that affect fitness, transcription, localization, etc.) and, because they rely on downstream readouts, do not necessarily pinpoint the direct targets. These limitations have spurred the continual development of new and improved methods. Drug Affinity Responsive Target Stability (DARTS) is a paradigm-changing method developed to overcome these limitations. DARTS leverages the thermodynamic stabilization of the target protein that occurs upon small molecule binding by detecting the binding-induced increase in resistance to proteolysis. This is highly advantageous because it uses the native, unmodified small molecules and relies solely on the binding interaction but not downstream readouts to discover target proteins.

DARTS is a relatively simple technique that can easily be adopted by most labs. Unlike affinity chromatography, DARTS is not limited by the chemistry of the small molecule of interest and does not require derivatization or immobilization of the compound. Rather, DARTS is performed by simply treating aliquots of cell lysate with the compound of interest and either vehicle control or an inactive analog, followed by limited digestion of the proteins in the cell lysate with proteases. Subsequently, the
samples are separated by SDS-PAGE and stained to identify protein bands that are protected from proteolysis by the small molecule. Mass spectrometry (MS) is then used to identify the proteins present in each band. This unbiased DARTS approach has been successfully utilized to identify novel protein targets for natural products and other bioactive small molecules. Although this gel-based approach is the easiest to implement, more efficient gel-free proteomics approaches are also being used with DARTS to facilitate identification of the protected proteins\textsuperscript{7,9}.

While DARTS has been successfully performed in an unbiased fashion as a discovery tool to identify unknown targets of natural products and drugs (see\textsuperscript{4-6,10} for some examples), it is also powerful as a means to screen or validate binding of compounds to proteins of interest. This targeted approach has been widely used, with recombinant and/or purified proteins using gel staining, endogenous proteins in lysates using western blotting, and epitope-tagged proteins expressed in cells or in vitro and detected with epitope-specific antibodies (examples including\textsuperscript{4,6,11-15}). Moreover, the targeted approach could be used for high-throughput screening for compounds that bind a specific protein. Here we describe examples using DARTS to assay small molecule-protein interactions, including three model drug-protein pairs – methotrexate and its protein target dihydrofolate reductase (DHFR)\textsuperscript{1}, olaparib and its protein target poly(ADP-ribose) polymerase (PARP)\textsuperscript{2}, and omigapil (CGP 3466B) and its protein target glyceraldehyde 3-phosphate dehydrogenase (GAPDH)\textsuperscript{3}, the FDA-approved drug disulfiram used to treat chronic alcoholism\textsuperscript{4}, and the lifespan-extending metabolite α-ketoglutarate\textsuperscript{5}. 
Materials and Methods

Cell culture.
MDA-MB-231, BT-549, and Jurkat cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. HEK293, HEK293T, and HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown in a 5% CO₂ incubator at 37°C.

DARTS experiments with methotrexate, olaparib, and omigapil.
Jurkat and HEK293 cells were lysed with M-PER buffer (Thermo Scientific, 78501) with phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM Na₃VO₄) and protease inhibitors (Roche, 11836153001). TNC buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl₂) was added to the lysate and protein concentration was then determined using the BCA protein assay (Pierce, 23227). Cell lysates were then incubated with either vehicle (in equal volume, with final 1% DMSO) or varying concentrations of methotrexate (Sigma, M9929), olaparib (Selleck Chemicals, S1060), or omigapil (CGP 3466B) (Tocris, 2966) for 1 h on ice. Digestion was performed using varying ratios of Pronase (Roche, 10165921001), which is a cocktail of proteases, to protein at room temperature for 15 min and stopped using excess protease inhibitors with immediate transfer to ice. The resulting digests were separated by SDS-PAGE on a 4-12% Bis-Tris gradient gel (Thermo Fisher Scientific, NP0322BOX), and western blotting was performed with antibodies against dihydrofolate...
reductase (DHFR) (Santa Cruz Biotechnology, sc-377091), poly(ADP-ribose) polymerase (PARP) (Cell Signaling, 9542), eukaryotic elongation factor 1 alpha (eEF1A) (Cell Signaling, 2551), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Ambion, AM4300).

**DARTS experiments with disulfiram.**

DARTS was performed as reported previously\(^6^{,}7^{,}9\) with minor modifications. Briefly, for target identification, MDA-MB-231 cells were lysed with M-PER buffer with phosphatase inhibitors and protease inhibitors. After lysis, cold TNC buffer was added to the lysate, and protein concentration was measured via the BCA protein assay. Lysate was incubated with either vehicle (DMSO) or varying concentrations of disulfiram for 1 h at room temperature with shaking at 600 r.p.m in an Eppendorf Thermomixer. The samples were then digested with different concentrations of Pronase for 20 min at room temperature. Digestion was stopped with the addition of SDS buffer, and samples were immediately heated for 10 min at 70°C. Proteins in each sample were separated by SDS-PAGE and visualized using the ProteoSilver staining kit (Sigma, PROTSIL1). Protected bands were excised and subjected to mass spectrometry identification (Alphalyse).

For target validation, DARTS was performed as described above. Samples were separated on a 4-12% Bis-Tris gradient gel, and western blotting was performed with antibodies against IQGAP1 (Cell Signaling, 2293), MYH9 (Protein Tech Group, 11128-1-AP), β-actin (Cell Signaling, 3700), and GAPDH.
**DARTS experiments with α-ketoglutarate (α-KG).**

For unbiased target identification, Jurkat cells were lysed using M-PER buffer with the addition of protease inhibitors and phosphatase inhibitors. TNC buffer was added to the lysate, and protein concentration was then determined using the BCA protein assay. Cell lysates were then incubated with either vehicle (H₂O) or α-ketoglutarate (α-KG) (Sigma, K1128) for 1 h on ice followed by an additional 20 min at room temperature. Digestion was performed using Pronase at room temperature for 30 min and stopped using excess protease inhibitors with immediate transfer to ice. The resulting digests were separated by SDS-PAGE and visualized using SYPRO Ruby Protein Gel Stain (Thermo Scientific, S12000). The band with increased staining from the α-KG lane (corresponding to potential protein targets that are protected from proteolysis by the binding of α-KG) and the matching area of the control lane were excised, in-gel trypsin digested, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described previously. Mass spectrometry results were searched against the human Swissprot database (release 57.15) using Mascot version 2.3.0, with all the peptides meeting a significance threshold of \( P < 0.05 \).

For target verification by DARTS with western blotting, HeLa cells were lysed in M-PER buffer with the addition of protease inhibitors and phosphatase inhibitors. Chilled TNC buffer was added to the protein lysate, and protein concentration of the lysate was measured. The protein lysate was then incubated with vehicle control or varying concentrations of α-KG for 3 h at room temperature with shaking at 600 r.p.m. in an Eppendorf Thermomixer. Pronase digestions were performed for 20 min at room
temperature and stopped by adding SDS loading buffer and immediately heating at 70°C for 10 min. Samples were subjected to SDS-PAGE on 4-12% Bis-Tris gradient gel and western blotted for ATP synthase subunits ATP5B (Sigma, AV48185), ATP5O (Abcam, ab91400), and ATP5A (Abcam, ab110273). Binding between α-KG and PHD-2 (encoded by EGLN1) (Cell Signaling, 4835), for which α-KG is a co-substrate, was confirmed by DARTS. GAPDH was used as a negative control.
Results

Confirmation of Existing Small Molecule-Protein interactions Utilizing the Drug Affinity Responsive Target Stability (DARTS) Approach

Methotrexate is an antifolate drug that is used in the treatment of a variety of cancers, including breast cancer, head and neck cancer, acute lymphocytic leukemia, osteosarcoma, and primary central nervous system lymphoma\textsuperscript{16-20}. It acts by competitively binding and inhibiting dihydrofolate reductase (DHFR)\textsuperscript{1}, an enzyme that is essential for the conversion of dihydrofolate to the active tetrahydrofolate in folic acid metabolism\textsuperscript{21,22}. DARTS with methotrexate shows interaction with its known target DHFR but not eukaryotic elongation factor 1 alpha (eEF1A), which serves as a control protein (Figure 1-1A). The dissociation constant for purified recombinant DHFR is $\sim$10 nM. Its IC$_{50}$ for cell lines varies greatly, and some cells have nM IC$_{50}$ values corresponding to its binding affinity. We found that with $\sim$30 nM of methotrexate, there is the same level of protection of DHFR against proteolysis as with $\sim$100 µM of methotrexate.

Olaparib (AZD2281; KU-0059436; trade name Lynparza) is an FDA-approved drug for the treatment of ovarian cancers caused by a mutated BRCA1 or BRCA2 gene (www.cancer.org\textsuperscript{23}. It works by binding to and blocking the action of poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair\textsuperscript{2}. DARTS with olaparib confirms its interaction with its known target PARP, but not DHFR, which is instead the target for methotrexate (Figure 1-1B).
Omigapil (CGP 3466B) is a drug that was tested in clinical trials for Parkinson’s disease and amyotrophic lateral sclerosis (ALS)\textsuperscript{24-26}. It works by binding to and inhibiting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and thereby inhibiting programmed cell death (apoptosis)\textsuperscript{3,27}. DARTS with omigapil (CGP 3466B) confirms its interaction with GAPDH while eEF1A serves as a control protein (Figure 1-1C). Although omigapil was reported to show strong neuroprotective effects at 1 nM\textsuperscript{3}, it is not clear that this is mediated by GAPDH.

**Identification of IQGAP1 Scaffold Protein and Myosin Heavy Chain 9 as Binding Targets for Disulfiram**

Disulfiram (DSF) was identified to be a potent inhibitor of a panel of triple-negative breast cancer cell lines in a high-throughput screen\textsuperscript{4}. Disulfiram is currently an FDA-approved drug used to treat alcoholism based on its inhibitory effect on acetaldehyde dehydrogenases, which are encoded by 3 of the 19 different aldehyde dehydrogenase (ALDH) genes, ALDH1A1, ALDH2, and ALDH1B1/ALDH5. It has mild side effects in humans that include headache, metallic taste, and drowsiness due to increased production of tryptophol in the liver\textsuperscript{28,29}. Beyond treatment of alcoholism, disulfiram was found in non-biased drug screens to show anti-neoplastic activity against prostate cancer and glioblastoma\textsuperscript{30-32} and is the subject of ongoing clinical trials for lung and liver cancer\textsuperscript{33,34}. DSF has been implicated in multi-drug resistance, NFκB-mediated apoptosis, phosphoinositide 3-kinase signaling, and induction of p53\textsuperscript{35-38}. ALDH1A3 is commonly elevated in cancer stem cells (CSC)\textsuperscript{39}. However, this isoform is not
expressed in MDA-MB-231 cells\textsuperscript{40}, in which we showed DSF to have a potent inhibitory effect. Thus, it is unlikely that DSF exerts its effects through ALDH1A3.

To identify additional targets of disulfiram, we performed Drug Affinity Responsive Target Stability (DARTS) analysis using MDA-MB-231 and BT-549 cells\textsuperscript{6,7,9}. Cell lysates were incubated with varying concentrations of DSF (Figure 1-2A) before digestion with pronase followed by SDS-PAGE and silver staining (Figure 1-2B). DSF-protected bands were identified, extracted, and subjected to mass spectrometry analysis. Remarkably, this analysis identified 2 IQ motif-containing factors as candidate targets for DSF: IQ motif containing GTPase activating protein 1 (IQGAP1) and myosin heavy chain 9 (MYH9) (Figure 1-2C). Western blotting confirmed IQGAP1 and MYH9 as targets of disulfiram (Figure 1-3A, B). β-actin was used as a control protein to demonstrate specificity and ensure that protection of IQGAP1 and MYH9 by disulfiram was not due to interference or inhibition of the proteolysis reactions by disulfiram.

**Identification of ATP Synthase, and not TOR, as a Binding Target for the Lifespan-Extending Metabolite α-Ketoglutarate**

The tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (α-KG) was discovered to delay aging and extend the lifespan of adult *Caenorhabditis elegans* by ~50\textsuperscript{5}. To investigate the molecular mechanism(s) of longevity by α-KG, we took advantage of an unbiased biochemical approach, DARTS\textsuperscript{6,7,9}. As we proposed that key target(s) of α-KG are likely to be conserved and ubiquitously expressed, we used a human cell line (Jurkat) that is easy to culture as the protein source for DARTS (Figure 1-4A). Mass spectrometry identified ATP5B, the β subunit of the catalytic core of the
ATP synthase, among the most abundant and enriched proteins present in the α-KG-treated samples; the homologous α subunit ATP5A was also enriched but to a lesser extent (Figure 1-4B). The interaction between α-KG and ATP5B was verified using additional cell lines (Figure 1-5A). ATP5B was validated to be a legitimate target of α-KG in vivo, as α-KG cannot further extend the lifespan of C. elegans lacking atp-2, the ortholog of ATP5B.

Furthermore, we showed that despite the additional requirement of target of rapamycin (TOR) for the longevity benefit of α-KG, α-KG does not directly interact with TOR. Susceptibility of the mammalian TOR (mTOR) protein to Pronase digestion is unchanged in the presence of α-KG, whereas, as expected, Pronase resistance in the presence of α-KG is increased for ATP5B, which we identified as a new binding target of α-KG (Figure 1-5B, C). We showed that despite not interacting directly with TOR, α-KG decreases TOR pathway activity by acting upstream of TOR through the inhibition of ATP synthase.
Discussion

As proof-of-concept for the Drug Affinity Responsive Target Stability (DARTS) method’s ability to identify protein targets for small molecules, we performed DARTS experiments with three drugs with well-known protein targets – methotrexate, olaparib, and omigapil. We show here that DARTS successfully confirms the binding of these drugs with their protein targets as well as the specificity of these drugs for their protein targets – methotrexate with dihydrofolate reductase, olaparib with poly(ADP-ribose) polymerase, and omigapil with glyceraldehyde 3-phosphate dehydrogenase. Therefore, we confidently utilize the DARTS approach to identify novel protein targets for the FDA-approved drug disulfiram used to treat chronic alcoholism and the lifespan-extending metabolite α-ketoglutarate.

Identification of IQGAP1 Scaffold Protein and Myosin Heavy Chain 9 as Binding Targets for Disulfiram

Using the DARTS method, we identified and confirmed IQ motif containing GTPase activating protein 1 (IQGAP1) and myosin heavy-chain 9 (MYH9) as direct binding targets of disulfiram (DSF), a potent inhibitor of triple-negative breast cancer (TNBC) cell lines. IQGAP1 is a ubiquitously expressed scaffold protein involved in regulation of the actin cytoskeleton, transcription, cellular adhesion, and the cell cycle\textsuperscript{41-45}. Recent studies suggest a role for IQGAP1 in several human cancers, such as thyroid, colorectal, gastric, and breast cancer, where it acts to modulate oncogenic pathways\textsuperscript{46-52}. IQGAP1 interacts with at least 90 proteins and behaves as a control hub for mTOR, ERK-MAPK, and Wnt/β-catenin pathways\textsuperscript{41,52-54}. The IQGAP1 family
includes IQGAP1-3, of which IQGAP2 has also been implicated in cancer\textsuperscript{52,55,56}. However, neither homolog is expressed ubiquitously. IQGAP2 is expressed at highest levels in the liver, kidney, and platelets\textsuperscript{57,58}, while IQGAP3 is predominately expressed in brain tissue, where it promotes neurite growth\textsuperscript{59}.

MYH9 is a subunit of myosin IIA that plays a role in cell motility, maintenance of cell shape, and cytokinesis\textsuperscript{60-62}. Furthermore, myosin heavy chain 9 has been implicated in cancer metastasis\textsuperscript{63-65} and regulation of EGFR-cytoskeletal interaction\textsuperscript{66,67}. Binding of disulfiram with these two IQ motif-containing proteins is consistent with pathway analysis of MDA-MB-231 cells treated with DSF, which identified divergent signaling pathways affected by DSF treatment\textsuperscript{4}. In addition, knockdown of each protein resulted in ~25-30\% inhibition of TNBC cell growth\textsuperscript{4}. Our results suggest that IQGAP1 and MYH9 should be further investigated as potential therapeutic targets for the treatment of triple-negative breast cancers.

**Identification of ATP Synthase, and not TOR, as a Binding Target for the Lifespan-Extending Metabolite α-Ketoglutarate**

Metabolism and aging are intimately linked. Compared with \textit{ad libitum} feeding, dietary restriction consistently extends lifespan and delays age-related diseases in evolutionarily diverse organisms\textsuperscript{68,69}. Similar conditions of nutrient limitation and genetic or pharmacological perturbations of nutrient or energy metabolism also have longevity benefits\textsuperscript{70,71}. Recently, several metabolites have been identified that modulate aging\textsuperscript{72,73}; however, the molecular mechanisms underlying this are largely undefined. We have discovered α-ketoglutarate (α-KG), a tricarboxylic acid cycle intermediate,
extends the lifespan of adult *Caenorhabditis elegans*. This longevity effect is mediated by inhibition of ATP synthase, which we identified as a direct binding target of α-KG utilizing DARTS, and TOR, a major effector of dietary restriction but not a direct binding target of α-KG. Identification of new protein targets of α-KG illustrates that regulatory networks acted upon by metabolites are probably more complex than appreciated at present, and that DARTS is a useful method for discovering new protein targets and regulatory functions of metabolites. Our findings demonstrate a novel mechanism for extending lifespan that is mediated by the regulation of cellular energy metabolism by a key metabolite. Such moderation of ATP synthesis by metabolite(s) has probably evolved to ensure energy efficiency by the organism in response to nutrient availability. We suggest that this system may be exploited to confer a dietary-restriction-like state that favors maintenance over growth, and thereby delays aging and prevents age-related diseases. Taken together, our analyses uncover new molecular links between a common metabolite, a universal cellular energy generator, and dietary restriction in the regulation of organismal lifespan, thus suggesting new strategies and therapeutic targets for the prevention and treatment of aging and age-related diseases.

In summary, we have shown here that the Drug Affinity Responsive Target Stability (DARTS) approach is a relatively simple and quick method that can be used to not only confirm small molecule-protein interactions (methotrexate-DHFR; olaparib-PARP; omigapil-GAPDH) but also, more importantly, to identify new protein targets for existing drugs (disulfiram) and endogenous metabolites (α-ketoglutarate), providing potential therapeutic targets for various diseases. In the next chapter, we utilize the DARTS method to identify a novel protein target for the natural product resveratrol.
Figure 1-1: DARTS confirms well-known small molecule-protein interactions. **(A)** Jurkat lysates were incubated with varying concentrations of methotrexate (Mtx) or vehicle (in equal volume, with final 1% DMSO), followed by digestion with 1:900 Pronase:protein ratios for 15 min. **(B)** Performed as in (A) using varying concentrations of olaparib or vehicle (in equal volume, with final 1% DMSO). **(C)** HEK293 cell lysates were incubated with 100 µM omigapil (CGP) or 1% DMSO, followed by digestion with 1:1600, 1:800, 1:400, and 1:200 Pronase:protein ratios for 15 min.
Figure 1-2: DARTS identifies IQGAP1 and MYH9 as protein targets for disulfiram. (A) Structure of disulfiram. (B) Unbiased DARTS was performed on MDA-MB-231 cell lysates incubated with either vehicle (DMSO) or varying concentrations of disulfiram and visualized via silver staining. Protected bands (arrows) were excised along with corresponding control bands and were sent for mass spectrometry analysis. (C) Mass spectrometry analysis identifies IQGAP1 and MYH9 in disulfiram-treated bands.
Figure 1-3: DARTS-western blotting confirms IQGAP1 and MYH9 as targets of disulfiram. (A) DARTS-western blot analysis was performed in MDA-MB-231 cells to validate IQGAP1 and MYH9 as binding targets of disulfiram. β-actin was used as a negative control. IQGAP1 and MYH9 over β-actin ratios, calculated from ImageJ analysis\(^\text{74}\), are shown. Arrow indicates band used to calculate enrichment ratio over control. (B) DARTS-western blot analysis was performed in BT-549 cells. GAPDH was used as a negative control.
Figure 1-4: DARTS identifies ATP5B as an α-ketoglutarate-binding protein. (A) Unbiased DARTS was performed in Jurkat cells with varying concentrations of α-ketoglutarate (α-KG). The red arrowhead represents the protected band that was excised (along with the proper control band) for mass spectrometry analysis. (B) Mass spectrometry results showing enriched proteins in the α-KG-treated DARTS sample. Only showing those proteins with at least 15 spectra in α-ketoglutarate sample and enriched at least 1.5 fold.
Figure 1-5: DARTS-western blotting confirms ATP5B, and not mTOR, as binding targets of α-ketoglutarate (α-KG). (A) DARTS-western blot analysis using HeLa cells confirms α-KG binding specifically to ATP5B. GAPDH was used as a negative control. (B) DARTS-western blot analysis using HEK293 cells confirms that α-KG does not bind to TOR directly. ImageJ was used to quantify the mammalian TOR/GAPDH and ATP5B/GAPDH ratios. (C) DARTS-western blot analysis using HeLa cells confirms that α-KG does not bind to TOR directly. ImageJ was used to quantify the mammalian TOR/GAPDH and ATP5B/GAPDH ratios.
References


Chapter 2: Deleted in Breast Cancer 1 as a Novel Molecular Target of Resveratrol and its Metabolites – Providing a Link between SIRT1 and Resveratrol

Abstract

Resveratrol, a plant polyphenol, has been shown to possess various health benefits, including chemoprevention and lifespan extension, in model organisms\textsuperscript{1,2}. However, the underlying molecular mechanisms of resveratrol’s beneficial effects remain unclear. Whereas many studies postulate that resveratrol acts as a calorie restriction (CR) mimetic and directly activates sirtuin 1 (SIRT1) to extend lifespan\textsuperscript{3,4}, other studies suggest that SIRT1 is not a direct target of resveratrol and is not essential for CR- or RSV-mediated lifespan extension\textsuperscript{5-7}. Here we identify a novel target of resveratrol, deleted in breast cancer 1 (DBC1), which potentially reconciles the controversy regarding the mechanism of SIRT1 activation by resveratrol. DBC1 binds directly to SIRT1 and acts as a negative regulator of SIRT1\textsuperscript{8,9}. We show that resveratrol binds directly to DBC1 and decreases DBC1-SIRT1 interaction. Consequently, SIRT1 is released and promotes the deacetylation of p53, which is a downstream effector of SIRT1. In addition, we demonstrate that resveratrol sulfates and glucuronides, the major metabolites of resveratrol\textsuperscript{10-12}, also bind to DBC1 and promote SIRT1 activity. Taken together, we provide evidence that resveratrol indirectly activates SIRT1 by binding to and inhibiting DBC1, providing a novel molecular mechanism of its SIRT1-dependent beneficial effects.
Introduction

Aging and its associated diseases have significant health and economic impacts. With increasing age comes increasing susceptibility to cancer and neurodegenerative diseases\textsuperscript{13}. One of the most robust and well-studied methods to delay aging is calorie restriction (CR). As early as 1935, McCay \textit{et al.} showed that reducing the calorie intake of rats resulted in increased lifespan\textsuperscript{14}. Continued research on CR show that this effect is conserved in yeast, \textit{C. elegans}, mice, and primates\textsuperscript{15-17}. Much effort has been spent to uncover the genetic mechanisms behind CR-mediated longevity. Pathways involving target of rapamycin (TOR) and insulin-like growth factor 1 (IGF-1) receptor, to name a few, have been implicated as essential for CR-mediated longevity\textsuperscript{18}.

In 2000, Lin \textit{et al.} showed that the yeast \textit{Sir2} gene is required for increased yeast replicative lifespan under limiting glucose conditions, which mimics calorie restriction in yeast\textsuperscript{17}. The human homolog of Sir2, SIRT1, belongs to a family of proteins called sirtuins. SIRT1 is a NAD\textsuperscript{+}-dependent protein deacetylase that deacetylates various proteins, including p53, PGC-1\textalpha{}, Ku70, and histones, thus influencing apoptosis, cell survival, metabolism, DNA repair, and gene silencing\textsuperscript{19-23}. Following the discovery of SIRT1 as a gene required for CR-mediated lifespan, screens for small molecule activators of SIRT1 were performed, and the natural product resveratrol was identified as SIRT1’s most potent activator\textsuperscript{3}.

Resveratrol, a polyphenol found in the skin of red grapes and other plant sources, was proposed to act as a calorie restriction mimetic and extend the lifespan of several model organisms including yeast, worms, flies, and high-fat diet-fed mice\textsuperscript{1,24}. Several studies reported that this extension of lifespan by resveratrol along with its
associated beneficial metabolic effects occur in a SIRT1-dependent manner. However, controversy surrounding the link between resveratrol and SIRT1 arose when several groups showed that the activation of SIRT1 by resveratrol required the presence of an artificial fluorophore that was conjugated to the SIRT1 substrate used in the original screen; without the fluorophore, SIRT1 was no longer activated, and the native substrate was no longer deacetylated. Furthermore, Burnett et al. called into question whether sirtuins are critical in mediating the effects of calorie restriction, showing that overexpression of SIRT1 in transgenic worms does not extend lifespan, after controlling for the genetic background of the worms, contrary to earlier studies showing the opposite.

These contradictory studies led to a re-examination of the importance of sirtuins in aging. Numerous more recent studies indicated that an overexpression or activation of SIRT1 in worms, flies, and mice extends lifespan, thus re-affirming the role of SIRT1 as an important mediator of longevity. Although the link between resveratrol and SIRT1 remains to be elucidated, it is likely that resveratrol activates SIRT1, possibly indirectly, inside the cell under certain conditions to increase longevity. Since resveratrol has many identified binding targets, including COX-2, ATP synthase, eIF4A, phosphodiesterases, and tyrosyl transfer-RNA synthetase, its activation of SIRT1 may be mediated through these or other resveratrol-binding proteins.

In light of the new evidence for SIRT1’s essential role in aging, it has become increasingly important to understand how SIRT1 is regulated in vivo. One of the negative regulators of SIRT1, deleted in breast cancer 1 (DBC1; also known as KIAA1967, p30 DBC, CCAR2), was initially identified from a region of chromosome...
8p21 that is homozygously deleted in some human breast cancer cell lines\textsuperscript{34}. However, DBC1’s link to tumorigenesis is still unclear\textsuperscript{35}. DBC1 was shown to interact directly with SIRT1 and inhibit its deacetylase activity both \textit{in vitro} and \textit{in vivo}\textsuperscript{8,9}. We hypothesized that resveratrol may indirectly activate SIRT1 by binding to DBC1 and preventing DBC1 from inhibiting SIRT1, providing a possible molecular mechanism underlying the health benefits of resveratrol. Utilizing the drug target identification approach DARTS\textsuperscript{29}, we show that DBC1 is a novel target of resveratrol. Resveratrol binds directly to DBC1 at its N-terminus and disrupts the DBC1-SIRT1 complex. The decreased interaction of SIRT1 from its negative regulator DBC1 by resveratrol promotes the deacetylation of the SIRT1 substrate p53. In addition, we demonstrate that resveratrol sulfates and glucuronides, some of the major metabolites of resveratrol that are more physiologically relevant, behave similarly to resveratrol and bind DBC1 to indirectly activate SIRT1. Therefore, DBC1 may be a legitimate target of resveratrol \textit{in vivo}. 
Materials and Methods

Synthesis of resveratrol metabolites.
The 3- and 4'-glucuronide and sulfate metabolites of resveratrol were synthesized as described previously \(^{36,37}\).

Cell culture.
HEK293, HEK293T and mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown in a 5% CO\(_2\) incubator at 37°C.

DARTS experiments with resveratrol.
For target verification by DARTS-Western blotting, HEK293, HeLa, and mouse embryonic fibroblast (MEF) cells were lysed in M-PER buffer (Thermo Scientific, 78501) supplemented with protease inhibitors (Roche, 11836153001) and phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM Na\(_3\)VO\(_4\)). After 10 min incubation on ice with the lysis buffer, samples were centrifuged to clear cellular debris. Supernatant was collected and measured for protein concentration by the BCA Protein Assay kit (Pierce, 23227). The protein lysate was then incubated with vehicle control (DMSO) or varying concentrations of resveratrol (Calbiochem, 554325) for 30 min or 1 hr at room temperature with shaking at 600 r.p.m in an Eppendorf Thermomixer. Pronase (Roche, 10165921001) digestions were performed for 20 min at room temperature and stopped by adding protease inhibitor.
and/or SDS loading buffer and heating at 70°C for 10 min. Samples were subjected to SDS-PAGE on 4-12% Bis-Tris gradient gel (Thermo Fisher Scientific, NP0322BOX) and Western blotted for DBC1 (Bethyl Laboratories, A300-432A), SIRT1 (Cell Signaling, 2496), FLAG (Sigma, A8592), and GAPDH (Ambion, AM4300).

**Creation of DBC1 truncation and domain mutants.**

Full length DBC1 and the DBC1 truncation and domain mutants were cloned out of a DBC1 cDNA clone (Origene, SC324243) into the pcDNA3.1(-) mammalian expression vector (Thermo Fisher Scientific, V795-20). Primers used are provided (Table 2-1).

**DARTS experiments with DBC1 truncation and domain mutants.**

HEK293T cells were transfected with the DBC1 truncation and domain mutants using Xfect transfection reagent (Clontech, 631317). After 24 hours, cells were lysed with supplemented M-PER buffer and DARTS was performed as described above.

**Co-immunoprecipitation assay.**

HEK293 cells and HEK293T cells transfected with FLAG-tagged wild-type DBC1 were incubated with either vehicle (DMSO) or 100 µM resveratrol for 2 hours before harvested. Cells were lysed with non-denaturing NETN lysis buffer as described previously⁹, composed of 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% Igepal CA-630, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, and protease inhibitors (Roche, 11836153001), for 30 min at 4°C on a rotator. Samples were then centrifuged to clear cellular debris, and supernatant was collected and measured for protein concentration.
by the BCA Protein Assay kit (Pierce, 23227). 1 mg of protein from HEK293 sample lysates was incubated with 40 µL of recombinant protein G-sepharose (Thermo Fisher Scientific, 101241) bead slurry and either 1 µg of rabbit IgG antibody (Santa Cruz Biotechnology, sc-2027) as control or DBC1 antibody (Bethyl Laboratories, A300-432A) for 1 hr at 4°C on a rotator. Alternatively, 1 mg of protein from HEK293T sample lysates were incubated with 30 µL of anti-FLAG beads (Sigma, F2426) for 1 hr at 4°C on a rotator. Samples were then washed with NETN buffer two times before elution with 2X SDS loading buffer.

**Acetylation assay.**

HEK293T cells were transfected with empty vector or FLAG-tagged full length DBC1 for 24 hours. Cells were then treated with DMSO or 100 µM resveratrol for 2 hours. After drug incubation, cells were treated with 10 µM MG132 (Sigma, C2211) for 20 minutes and with or without 20 µM etoposide (VP-16) (Sigma, E1383) for one hour before lysing with NETN buffer. Lysates were incubated with recombinant protein G-sepharose beads (Thermo Fisher Scientific, 101241) and 1 µg p53 antibody (Santa Cruz Biotechnology, sc-98) at 4°C overnight on a rotator. Samples were then washed with NETN buffer two times before elution with 2X SDS loading buffer. Membranes were blotted with antibody against acetylated p53 (K382) (Abcam, 75754).
Results

Deleted in Breast Cancer 1 Is a Novel Target of Resveratrol

The Drug Affinity Responsive Target Stability (DARTS) approach relies on the theory that small molecule binding to its protein target(s) results in increased thermodynamic stability of the protein target(s) and thereby confers protection of the target(s) against degradation by proteases\textsuperscript{29,38,39}. Using lysates from human embryonic kidney 293 (HEK293) cells, we observed that with increasing concentrations of resveratrol (Figure 2-1A), there is increased protection of DBC1 against protease degradation (Figure 2-1B). We have confirmed that the increased protection of DBC1 against protease digestion is seen across multiple cell lines, including HeLa cells and mouse embryonic fibroblast cells (Figure 2-2). In contrast, resveratrol does not protect SIRT1 against protease digestion (Figure 2-1B, 2-1C). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a control protein to demonstrate specificity and ensure that protection of DBC1 by resveratrol is not due to interference or inhibition of the proteolysis reactions by resveratrol. A complete dose curve with resveratrol reveals that resveratrol binds to DBC1 and confers protection against proteolysis at concentrations in the micromolar range (Figure 2-1C). In addition, the resveratrol analog, pterostilbene, does not protect DBC1 against protease digestion, suggesting that the interaction between DBC1 and resveratrol is specific (Figure 2-1D). Together, these results show that DBC1 is a novel and specific binding target of resveratrol.
Resveratrol Interacts with DBC1 at Its N-Terminus

DBC1 has several distinct domains, including an S1-like RNA binding domain (RBD), a nuclear localization signal (NLS), a leucine zipper domain (LZ), a Nudix domain (ND), an EF hand (EF), and a coiled-coil domain (CC) (Figure 2-3A). We observed that DARTS performed with cell lysates transfected with N-terminal FLAG-tagged full-length DBC1 resulted in three digested fragments (Figure 2-3B, arrows). In the presence of resveratrol, these fragments also show increased resistance to proteolysis. Protection of these lower molecular weight fragments and detection via FLAG antibody suggest that resveratrol binds at the N-terminus of DBC1, which interacts with SIRT18. The LZ domain has been shown to be required for binding to several epigenetic modifiers, including the deacetylase SIRT18. We thus constructed N- and C-terminal truncation mutants of DBC1 (Figure 2-3A) and performed DARTS with cell lysates transfected with the mutants to map the resveratrol-binding region on DBC1. Through these experiments, we confirm that resveratrol interacts with the N-terminus of DBC1 (Figure 2-3B). The ΔC mutant, along with WT DBC1, is protected against proteolysis in the presence of resveratrol, but the ΔN mutant is not. In addition, the N-terminal domain, but not the core or the C-terminal domains of DBC1, is sufficient for resveratrol to confer protection against protease digestion (Figure 2-3C, 2-3D). Combined, these results suggest that the N-terminus of DBC1 containing the RBD, NLS, and LZ domains, and not the core region or the C-terminus, is required for resveratrol to bind and confer protection against proteolysis.
Resveratrol Decreases DBC1-SIRT1 Interaction and Affects Downstream SIRT1 Activity in a DBC1-Dependent Manner

Since the N-terminal region of DBC1, more specifically the leucine zipper domain of DBC1, is necessary for its interaction with SIRT1, we tested whether resveratrol binding to the N-terminus of DBC1 affects the DBC1-SIRT1 interaction. Indeed, in the presence of resveratrol, less SIRT1 is co-immunoprecipitated with DBC1 (Figure 2-4A), suggesting that resveratrol decreases DBC1-SIRT1 interaction. DBC1 interacts with SIRT1 on SIRT1’s catalytic domain and when bound, prevents SIRT1 from deacetylating its substrates, including the tumor suppressor protein p53. SIRT1 regulates p53 to inhibit apoptosis and promote cell survival under genotoxic stress.

In Kim et al., the authors observed that DBC1 overexpression resulted in hyperacetylation of p53 in response to genotoxic stress induced by etoposide treatment, suggesting that overexpression of DBC1 inhibits SIRT1 activity. Given our observation that resveratrol decreases DBC1-SIRT1 interaction, we examined resveratrol's effects on downstream SIRT1 activity. We observed that resveratrol treatment abolishes the effect of DBC1 overexpression on the acetylation levels of p53 at Lysine 382 (Figure 2-4B). Consistent with the hypothesis that resveratrol binds to DBC1 and prevents its binding to SIRT1, p53 is no longer hyperacetylated in the presence of resveratrol under DBC1 overexpression conditions. These experiments suggest a possible novel mechanism that links resveratrol to its reported biological effects on SIRT1 through DBC1, and thus potentially reconciles the controversy surrounding the mechanism of resveratrol in SIRT1 activation (Figure 2-4C).
Resveratrol’s Metabolites Bind to DBC1 and Affect Downstream SIRT1 Activity

Resveratrol is quickly metabolized \textit{in vivo} to the 3- and 4’-glucuronides and sulfates, leaving almost no unconjugated resveratrol in the serum\textsuperscript{10-12}. It is therefore possible that the \textit{in vivo} effects observed with resveratrol treatment are, in part, due to its metabolites. To address this hypothesis, we synthesized the resveratrol metabolites (Figure 2-5A) as described previously\textsuperscript{36,37} and tested their binding to DBC1 using DARTS. Similarly to resveratrol, both the 3- and 4’-sulfates and the 3- and 4’-glucuronides, albeit to a much lower extent, protected DBC1 from protease digestion (Figure 2-5B). To test whether the binding of resveratrol’s metabolites to DBC1 also has a functional effect on SIRT1, we analyzed the acetylation levels of p53 under DBC1 overexpression conditions. Similar to resveratrol treatment, treatment of cells with resveratrol’s 3- and 4’-sulfate metabolites ameliorates the effect of DBC1 overexpression on the acetylation levels of p53, albeit to a lower extent than resveratrol (Figure 2-5C). Taken together, our evidence suggests that the more physiologically relevant metabolites of resveratrol behave similarly to resveratrol itself and thus DBC1 can be considered a legitimate target of resveratrol \textit{in vivo}.
Discussion

Our data supports the hypothesis that deleted in breast cancer 1 (DBC1), the negative regulator of sirtuin 1 (SIRT1), is a direct binding target of resveratrol. Resveratrol binds to the N-terminal region of DBC1, which is also the region required for SIRT1 binding to DBC1. Resveratrol binding to DBC1 decreases DBC1-SIRT1 interaction. This decrease in interaction abolishes the effect of DBC1 overexpression on p53 acetylation levels and affects downstream SIRT1 activity in a DBC1-dependent manner, suggesting a novel mechanism of resveratrol’s activation of SIRT1. In addition, the 3- and 4’-sulfate and glucuronide metabolites of resveratrol behave similarly to resveratrol, binding to DBC1 and affecting downstream SIRT1 activity in a DBC1-dependent manner. This finding potentially reconciles the controversy surrounding the mechanism of SIRT1 activation by resveratrol.

Currently, there is no direct evidence that resveratrol binds to SIRT1 in vivo. Although we did not observe protection of SIRT1 from proteolysis by resveratrol in our DARTS experiments, we have shown here that resveratrol can indirectly activate SIRT1 by decreasing the DBC1-SIRT1 interaction, confirming resveratrol’s role as a SIRT1 activator. In addition, we are not excluding the contribution of other resveratrol targets to its downstream effects on SIRT1. Resveratrol is a highly promiscuous small molecule and has numerous known protein targets, including, but not limited to, the cyclooxygenases (COXs), the mitochondrial F1F0-ATPase (Complex V), phosphodiesterases (PDEs), and the more recently discovered tyrosyl transfer-RNA synthetase2,30-33,40. Even though we have shown here that resveratrol binds to DBC1 to indirectly activate SIRT1, activation of SIRT1 may also require contribution from or can
be achieved by any of resveratrol’s other target proteins. For instance, Price et al. showed that resveratrol improves mitochondrial function and increases mitochondrial biogenesis in a SIRT1-dependent manner\textsuperscript{41}. We have found that resveratrol’s beneficial effects on mitochondrial function are not dependent on DBC1 (Figure 2-6).

Although our data points to an exciting novel mechanism of resveratrol’s indirect activation of SIRT1, we do not attempt to explain the mechanism behind all of resveratrol’s beneficial effects. Resveratrol may also activate SIRT1 by binding to, for example, active regulator of SIRT1 (AROS), which positively regulates SIRT1\textsuperscript{42}, and hypermethylated in cancer 1 (HIC1), which negatively regulates SIRT1\textsuperscript{43}. In addition, DBC1 is known to interact with and regulate other proteins \textit{in vivo}, including epigenetic modifiers, such as the deacetylase HDAC3 and the methyltransferase SUV39H1, as well as nuclear receptors, such as estrogen receptor (ER), androgen receptor (AR), retinoic acid receptor (RAR), and Rev-erb\textsubscript{a}\textsuperscript{44-51}. It is possible that resveratrol prevents DBC1 from binding to these other substrates, further contributing to the health benefits observed with resveratrol treatment.

Due to resveratrol’s rapid metabolism and thus poor bioavailability, it is likely that its metabolites are responsible for the biological effects observed\textsuperscript{11,52}. Based on our data, resveratrol’s metabolites behave similarly to resveratrol, interacting with DBC1 to indirectly activate SIRT1, albeit to a lesser extent. Interestingly, the 3- and 4’-sulfates appear to bind to DBC1 with greater affinity and indirectly activate SIRT1 much better than the 3- and 4’-glucuronides. Additional functional roles of resveratrol sulfates and glucuronides await further investigation.
Finally, it remains to be determined whether DBC1 is required for resveratrol's beneficial effects on lifespan or any of its other numerous health benefits in vivo. DBC1 is conserved in mice; however, there is no ortholog of DBC1 in S. cerevisiae, the organism in which resveratrol was first discovered to extend lifespan. In C. elegans, the closest ortholog of DBC1 is lateral signaling target 3 or lst-3, with only 17% sequence similarity to human DBC1 (Figure 2-7A). Preliminary lifespan analyses show that knockdown of lst-3 in C. elegans increases mean lifespan by approximately 30% (Figure 2-7B). This corresponds with our hypothesis that resveratrol's beneficial effects on lifespan could be due to its interaction with DBC1 and dissociation of the DBC1-SIRT1 complex to indirectly increase SIRT1 activity. However, further experiments need to be performed to determine whether lst-3 is indeed a negative regulator of sir-2.1 in C. elegans as well as whether lifespan extension with resveratrol treatment is attenuated in lst-3 knockdown worms.

Despite all that remains unknown, we have shown here a novel connection between resveratrol and SIRT1. Resveratrol indirectly activates SIRT1 by directly interacting with its endogenous inhibitor DBC1 to prevent the inhibition of SIRT1. With the discovery of this novel mechanism, it will be important to perform small molecule screens to find more specific inhibitors of DBC1 that may be more efficacious than resveratrol in treating cancer or increasing healthspan and lifespan.
Figures

Figure 2-1. DARTS-Western blotting identifies DBC1 as a novel target of resveratrol. (A) Structure of resveratrol. (B) DARTS was performed with lysates from HEK293 cells. Lysates were incubated with DMSO, 100 µM, or 500 µM resveratrol (RSV) for 1 hr at room temperature before adding protease and analyzing via Western blotting. Increasing concentrations of resveratrol result in increased protection of DBC1 against proteolysis. FL – full length. (C) DARTS was performed with lysates from HEK293T cells transfected with N-terminal FLAG-tagged wild-type DBC1. Lysates were incubated with DMSO or resveratrol at concentrations ranging from 10 nM to 200 µM for 30 min at room temperature with shaking before adding protease and analyzing via Western blotting. (D) DARTS was performed with lysates from HEK293 cells. Lysates were incubated with DMSO or varying concentrations of resveratrol or pterostilbene for 1 hr at room temperature before adding protease and analyzing via Western blotting.
Figure 2-2. Resveratrol protects DBC1 from protease digestion in HeLa and mouse embryonic fibroblast (MEF) cells. (A) DARTS was performed with lysates from HeLa cells as described in Figure 2-1B. Lysates were incubated with DMSO, 100 µM, or 500 µM resveratrol for 1 hr at room temperature before adding protease and analyzing via Western blotting. Increasing concentrations of resveratrol result in increased protection of DBC1 against proteolysis. (B) DARTS was performed with lysates from wild-type MEF and SIRT1 knockout MEF cells also as described in Figure 2-1B. Resveratrol protects DBC1 against proteolysis in both WT and SIRT1 knockout MEFs. Both cell lines were a gift from the David Sinclair laboratory.
Figure 2-3. DARTS with wild type DBC1 and DBC1 truncation and domain mutants identify the N-terminus of DBC1 as the resveratrol-binding region. (A) Map of DBC1 and DBC1 truncation mutants. (B) DARTS was performed with lysates from HEK293T cells transfected (Tf) with DBC1 truncation mutants. Lysates were incubated with DMSO or 200 µM resveratrol for 30 min at room temperature before adding protease and analyzing via Western blotting. The WT and ΔC mutants are protected in the presence of resveratrol, but the ΔN mutant is not. Arrows indicate digested fragments of DBC1 (that have intact N-termini) that are protected from proteolysis with resveratrol. (C) Map of DBC1 domain mutants. (D) DARTS was performed as in (B) with lysates from HEK293T cells transfected (Tf) with DBC1 domain mutants. The N-terminal domain, and not the core or the C-terminal domains, is sufficient to bind resveratrol and confer protection against proteolysis. FLAG/GAPDH ratios were calculated using ImageJ.
Figure 2-4. Functional analysis of resveratrol’s interaction with DBC1. (A) On the left, HEK293 cells were treated with 100 µM resveratrol for 2 hours and co-immunoprecipitation was performed using anti-DBC1 antibody. On the right, HEK293T cells transfected with FLAG-tagged wild-type DBC1 were treated with 100 µM resveratrol for 2 hours and co-immunoprecipitation was performed using anti-FLAG beads. Resveratrol decreases DBC1-SIRT1 interaction as less SIRT1 co-immunoprecipitates with DBC1 in the presence of resveratrol. (B) HEK293T cells were transfected with control vector or wild-type DBC1 and treated with DMSO or 100 µM resveratrol for 2 hours. After resveratrol treatment, cells were treated with 10 µM MG132 for 20 min to stabilize p53 levels, followed by 20 µM etoposide (VP-16) for 1 hour to induce genotoxic stress. Co-immunoprecipitations were performed with p53 antibody and acetylation levels of p53 (K382) were assessed. Resveratrol abolishes the effect of DBC1 overexpression on p53 acetylation levels. (C) Schema of resveratrol’s mechanism of action. In the absence of resveratrol, DBC1 binds to and inhibits SIRT1, preventing SIRT1 from deacetylating its substrates. In the presence of resveratrol, resveratrol binds to DBC1 directly and decreases DBC1-SIRT1 interaction. This results in increased SIRT1 activity and thus increased deacetylation of SIRT1’s substrates.
Figure 2-5. Target identification and functional analysis of resveratrol’s metabolites. (A) Structures of synthesized 3- and 4’-glucuronide and sulfate metabolites of resveratrol. The metabolites were synthesized as described previously.36,37 (B) DARTS was performed with lysates from HEK293T cells transfected with full-length wild-type DBC1. Lysates were incubated with DMSO or 200 µM resveratrol and the metabolites for 30 min at room temperature with shaking before adding protease and analyzing via Western blotting. The 3- and 4’-sulfate metabolites, along with resveratrol, protect DBC1 against protease digestion, whereas the 3- and 4’-glucuronide metabolites do to a much lower extent. 1) DMSO, 2) 200 µM RS, 3) 200 µM R-3-G, 4) 200 µM R-3-S, 5) 200 µM R-4-G, 6) 200 µM R-4-S. (C) HEK293T cells were transfected with control vector or wild-type DBC1 and treated with DMSO or 100 µM resveratrol and its metabolites for 2 hours. After resveratrol treatment, cells were treated with 10 µM MG132 for 20 min to stabilize p53 levels, followed by 20 µM etoposide (VP-16) for 1 hour to cause genotoxic stress. Co-immunoprecipitations were performed with p53 antibody and acetylation levels of p53 (K382) were assessed. Resveratrol and its metabolites mitigate the effects of DBC1 overexpression on p53 acetylation levels.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer Sequence 5’→3’</th>
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| Wild-type DBC1 | F: ATGCGAATTCGCCATGGACTACAAGGATGACGATGAC  
                     AAGTCCCAGTTTTAAGCGCCAGCGG  
                     R: ATGCGGTACCTCAGTTGCTAGGTGCGCGGCTCCTC  |
| ΔN DBC1        | F: ATGCGAATTCGCCATGGACTACAAGGATGACGATGAC  
                     AAGTCAGCCCTCCCCCTGAGCCAG  
                     R: same as wild-type reverse primer |
| ΔC DBC1        | F: same as wild-type forward primer  
                     R: ATGCGGTACCTCATAAGGGGAGCAGCAGAGGG  |
| N-terminus     | F: same as wild-type forward primer  
                     R: ATGCGGTACCTCATAAGGGGAGCAGCAGAGGG  |
| Core           | F: same as ΔN forward primer  
                     R: same as ΔC reverse primer |
| C-terminus     | F: ATGCGAATTCGCCATGGACTACAAGGATGACGATGAC  
                     AAGGACTGTCTGCTTTTCTTTTGTGCC  
                     R: same as wild-type reverse primer |

**Table 2-1.** Primers used to create DBC1 truncation and domain mutants.
Resveratrol’s beneficial effects on mitochondrial function are DBC1-independent. (A) HEK293 cells were transfected with control or DBC1 siRNA for 48 hours before treatment with 20 µM resveratrol for 24 hrs. Genomic DNA was extracted, and qPCR was performed as described previously\textsuperscript{53}. (B) Wild-type MEF cells and DBC1 knockout MEF cells were treated with 25 µM resveratrol for 24 hrs. Genomic DNA was extracted and qPCR was performed using D-loop primers to measure mitochondrial DNA and telomerase reverse transcriptase (TERT) primers to measure nuclear DNA. D-loop expression levels were normalized to TERT expression levels. (C) HEK293T cells were transfected with control or DBC1 siRNA for 72 hours before treatment with increasing concentrations of resveratrol (12.5 µM to 100 µM) for 2 hrs followed by analysis of ATP content using Promega’s CellTiter-Glo. (D) Wild-type MEF cells and DBC1 knockout MEF cells were treated with increasing concentrations of resveratrol (12.5 µM to 100 µM) for 2 hrs followed by analysis of ATP content using Promega’s CellTiter-Glo.
Figure 2-7. Knockdown of DBC1 homolog in *C. elegans* extends lifespan. (A) Sequence alignment between human DBC1 and *C. elegans* *lst-3* reveals approximately 17% sequence similarity. (B) RNAi knockdown of *lst-3* results in a ~30.3% increase in mean lifespan compared to *gfp* RNAi knockdown worms.
References


Chapter 3: Resveratrol Induces an ATM-Dependent DNA Damage Response Distinct from Etoposide in a DBC1-Independent Manner

Abstract

Deleted in Breast Cancer 1 (DBC1) is a negative regulator of sirtuin 1 (SIRT1) that interacts directly with and inhibits its deacetylase activity\(^1,2\). Previously, it has been shown that the commonly used genotoxic agent etoposide (VP-16) induces phosphorylation of DBC1 upon genotoxic stress to enhance DBC1-SIRT1 binding, promoting p53-dependent apoptosis\(^3\). Resveratrol, a plant polyphenol, has also been shown to induce p53-dependent apoptosis, but its role in this DBC1-SIRT1-p53 axis is unclear\(^4\). Here we demonstrate that resveratrol induces the phosphorylation of H2A.X (\(\gamma\)H2A.X), resulting in the phosphorylation of DBC1 by the ATM and ATR kinases, but to a lower extent than the commonly used genotoxic agent etoposide (VP-16). However, phosphorylation of DBC1 by resveratrol does not appear to enhance DBC1-SIRT1 binding or have functional significance in downstream DNA damage response. Interestingly, resveratrol activates a distinct ATM-dependent DNA damage response that does not result in activation of apoptosis, whereas etoposide, titrated down to induce the same levels of phosphorylated DBC1 and \(\gamma\)H2A.X accumulation as resveratrol, activates an ATM-dependent DNA damage response that does result in activation of apoptosis. Furthermore, resveratrol's physiologically relevant metabolites do not induce \(\gamma\)H2A.X accumulation, phosphorylation of DBC1, or activation of the DNA damage response. Taken together, we provide evidence that resveratrol induces an ATM-dependent DNA damage response distinct from that induced by etoposide in a DBC1-independent manner.
Introduction

SIRT1 is a NAD⁺-dependent protein deacetylase that deacetylates various proteins, including p53, PGC-1α, Ku70, and histones (to name a few), thus influencing apoptosis, cell survival, metabolism, DNA repair, and gene silencing⁵⁻⁹. Given its important role in these cellular processes, it is important to understand how SIRT1 is regulated in the cell. Two separate groups, Zhao et al. and Kim et al., identified a novel negative regulator of SIRT1 called deleted in breast cancer 1 or DBC1¹⁻². In their studies, they demonstrate that DBC1 interacts directly with SIRT1 at its catalytic domain to prevent its substrates from binding and thereby inhibiting its deacetylase activity.

DBC1 (also known as CCAR2, KIAA1967, p30 DBC) was originally identified as being from a region that is homozygously deleted in some breast cancers¹⁰. However, further studies have shown that DBC1 is not deleted in all breast cancer tissues and cell lines, and the role of DBC1 in tumorigenesis and whether it is a tumor suppressor or tumor promoter remains unclear¹¹. Studies have shown that DBC1 is overexpressed in some cancer types and that overexpression is correlated with poor prognosis¹²⁻¹⁴. Conversely, another more recent study demonstrates that DBC1 functions as a tumor suppressor by regulating the stability of the tumor suppressor p53¹⁵. Regardless of its unclear role in tumorigenesis, given its important role in regulating SIRT1, how the interaction between DBC1 and SIRT1 can be modulated in the cell is essential to determine.

In a recent study, we showed that the compound resveratrol modulates the DBC1-SIRT1 interaction. Resveratrol, a polyphenol found in the skin of red grapes and other plant sources, has been shown to possess various health benefits, but the
mechanism(s) of resveratrol's beneficial effects on health still remains, in large part, unclear\textsuperscript{16}. We have identified a novel target of resveratrol, deleted in breast cancer 1 (DBC1) (Pai \textit{et al.}, manuscript in preparation; see Chapter 2). Briefly, we observed that resveratrol interacts directly with DBC1 to promote dissociation of the DBC1-SIRT1 complex, thereby indirectly activating downstream SIRT1 deacetylase activity, suggesting a novel mechanism for resveratrol's SIRT1-dependent beneficial effects.

Various posttranslational modifications of DBC1 have also been shown to modulate the DBC1-SIRT1 interaction. Zannini \textit{et al.} showed that the DNA damaging agent etoposide increases the DBC1-SIRT1 interaction through phosphorylation of DBC1. DBC1 phosphorylation by the ATM and ATR kinases, in response to DNA damage induced by etoposide, increases DBC1's interaction with SIRT1 thus inhibiting downstream SIRT1 activity\textsuperscript{3}. Zheng \textit{et al.} showed that etoposide increases the DBC1-SIRT1 interaction through decreased acetylation of DBC1. Decreased DBC1 acetylation by human MOF, in response to etoposide, enhances DBC1's interaction with SIRT1, resulting in inhibition of SIRT1 activity\textsuperscript{17}. And finally, Park \textit{et al.} showed that etoposide increases the DBC1-SIRT1 interaction through increased sumoylation of DBC1. Sumoylation of DBC1 by Small Ubiquitin-like Modifier 2/3 (SUMO 2/3) causes an increase in the DBC1-SIRT1 interaction, resulting in the release of p53 from SIRT1 for promotion of p53-mediated apoptosis\textsuperscript{18}.

Similarly to etoposide, resveratrol has also been shown to induce DNA damage by poisoning topoisomerase II, resulting in activation of p53-dependent apoptosis in cancer cells\textsuperscript{19-21}. Given its DNA damaging and apoptosis-inducing activity, we wanted to investigate resveratrol's role in the ATM/ATR-DBC1-SIRT1-p53 axis. Here we show that
resveratrol treatment of cells induces the phosphorylation of DBC1 in an ATM- and ATR-dependent manner, albeit to a much lower extent than etoposide. Surprisingly, this phosphorylation of DBC1 by resveratrol does not have functional significance in downstream DNA damage response in non-cancer cells. Interestingly though, we observed that resveratrol treatment activates an ATM-dependent DNA damage response (DDR) distinct from that activated by etoposide in a DBC1-independent manner. In addition, resveratrol's metabolites do not induce the phosphorylation of DBC1 or activation of the DNA damage response.
Materials and Methods

Synthesis of resveratrol metabolites.
The 3- and 4’-glucuronide and sulfate metabolites of resveratrol were synthesized as described previously\textsuperscript{22,23}.

Drug treatments.
Cells were treated with 100 µM resveratrol (Sigma Aldrich, R5010) dissolved in DMSO for 2 hours, 20 µM etoposide (Sigma Aldrich, E1383) dissolved in DMSO for 2 hours, 10 µM KU-55933 (Tocris, 3544) dissolved in DMSO for 1 hour prior to resveratrol or etoposide treatment as described previously\textsuperscript{24}, 50 µM Pterostilbene (Sigma Aldrich, P1499) dissolved in DMSO for 2 hours, and 100 µM resveratrol 3- and 4'-glucuronides and sulfates dissolved in DMSO for 2 hours, unless indicated otherwise.

Cell culture.
HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, 11995-065) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, 16000-044) and 1% penicillin-streptomycin (Thermo Fisher Scientific, 15140-122) and grown at 37°C in a 5% CO\textsubscript{2} incubator.

Creation of FLAG-tagged wild-type DBC1 and truncation mutants.
FLAG-tagged full length DBC1 and DBC1 truncation mutants were cloned out of a DBC1 cDNA clone (Origene, SC324243) into the pcDNA3.1(-) mammalian expression
vector (Thermo Fisher Scientific, V795-20) as described previously (Pai et al., manuscript in preparation; see Chapter 2).

**Immunoprecipitation assay.**

HEK293T cells were transfected with FLAG-tagged wild-type DBC1 or DBC1 truncation mutants using Xfect transfection reagent (Clontech, 631317). Non-transfected or transfected cells were incubated with vehicle (DMSO), etoposide (VP-16), or resveratrol before harvesting. Cells were lysed with non-denaturing NETN lysis buffer, composed of 20 mM Tris- HCl pH 8.0, 100 mM NaCl, 0.5% Igepal CA-630, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, and protease inhibitors (Roche, 11836153001), as described previously\(^1\) for 30 min at 4°C on a rotator. Samples were then centrifuged to clear cellular debris, and supernatant was collected and measured for protein concentration by the BCA protein assay (Pierce, 23227). Approximately 1 mg of protein from sample lysates was incubated with 35 µL of recombinant protein G-sepharose bead slurry (Thermo Fisher Scientific, 101241) and either 1 µg of rabbit IgG antibody (Santa Cruz Biotechnology, sc-2027) as control or DBC1 antibody (Cell Signaling, 5693) for 1 hr at 4°C on a rotator. Alternatively, protein lysates were incubated with 25 µL anti-FLAG beads (Sigma, F2426) for 1 hr at 4°C on a rotator. Samples were then washed with NETN buffer two times before elution with 2X SDS loading buffer and analyzed via Western blotting.
**Western blotting.**

All protein samples were subjected to SDS-PAGE on 4-12% Bis-Tris gradient gels (Thermo Fisher Scientific, NP0321BOX, NP0322BOX). The following antibodies were used for Western blot analysis: phospho-ATM (Cell Signaling, 13050), ATM (Cell Signaling, 2873), phospho-ATR (Genetex, GTX128145), ATR (Cell Signaling, 13934), DBC1 (Cell Signaling, 5693), SIRT1 (Cell Signaling, 2496), phospho-CHK1 (Cell Signaling, 2348), phospho-CHK2 (Cell Signaling, 2197), CHK1 (Cell Signaling, 2360), CHK2 (Cell Signaling, 3440), phospho-p53 (Cell Signaling, 9284), p53 (Santa Cruz Biotechnology, sc-98), GAPDH (Ambion, AM4300), p21 (Cell Signaling, 2947), phospho-DBC1 (Cell Signaling, 4880), cleaved PARP (Cell Signaling, 9546), and FLAG (Sigma, A8592).

**Immunofluorescence staining.**

HEK293T cells were plated on glass coverslips in a 6-well plate at a concentration of 1.5 x 10^5 cells per well and treated with DMSO or 20 µM and 5 µM etoposide, 100 µM resveratrol, 100 µM of resveratrol's metabolites, or 50 µM pterostilbene for 2 hrs. After incubation, cells were washed with PBS, fixed with ice-cold methanol for 15 min at room temperature, and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. Cells were then washed with PBS three times for 5 min each before blocking with 1% BSA in PBS-T (PBS with 0.1% Tween-20) for at least 30 min at room temperature. Cells were then incubated with primary antibody γ-H2A.X (Cell Signaling, 9718) at 1:400 dilution in 1% BSA in PBS-T overnight at 4°C. Following overnight incubation, cells were washed 3 times with 1% BSA in PBS-T before incubation with
secondary antibody anti-rabbit IgG Cy2 at 1:1000 dilution in 1% BSA in PBS-T for at least one hour at room temperature. Cells were then washed again, incubated with Topro-3 nuclear stain at 1:1000 dilution in 1% BSA in PBS-T for 15 min at room temperature, and mounted on slides using Vectashield mounting medium (Vector Laboratories, H-1000). Samples were imaged using a Zeiss confocal microscope (LSM 700 Imager M2 using Zen 2009) and analyzed for fluorescence intensity using ImageJ software.

**Knockdown of ATM, ATR, and DBC1.**

Cells were transfected with 25 nM non-targeting siRNA (Dharmacon, D-001810-10-05), SMARTpool ATM siRNA (Dharmacon, L-003201), SMARTpool ATR siRNA (Dharmacon, L-003202), or SMARTpool DBC1 siRNA (Dharmacon, L-010427) using DharmaFECT 1 transfection reagent (Dharmacon, T-2001-01). Cells were treated with indicated drug treatments 72 hours after siRNA treatment and harvested for further analysis.

**Statistical analyses.**

Experiments were performed at least two times with identical or similar results. Data shown represent biological replicates. All statistical analyses were performed using Prism software, and all experimental p-values were obtained using student’s t-test with Welch’s correction.
Results

Resveratrol Induces γH2A.X Accumulation, Resulting in the Phosphorylation of DBC1

High dose resveratrol treatment (100 µM) of cells induces phosphorylation of both endogenous and exogenously expressed DBC1 at Threonine 454, albeit to a much lower extent than 20 µM etoposide (Figure 3-1A, B). A dose curve of resveratrol suggests that resveratrol’s ability to induce phosphorylation of DBC1 peaks at around 100 µM concentration, as a higher dose (250 µM) does not induce higher levels of phosphorylation of DBC1 (Figure 3-2A, B). Furthermore, we previously reported that resveratrol binds to the N-terminus of DBC1 (Pai et al., manuscript in preparation; see Chapter 2). However, we observed that the direct interaction between DBC1 and resveratrol is not necessary for resveratrol to induce DBC1 phosphorylation. The N-terminal mutant of DBC1, which we have shown previously to be unable to directly interact with resveratrol, is still phosphorylated upon resveratrol treatment (Figure 3-1C). This suggests that resveratrol’s indirect activation of SIRT1 through direct binding to DBC1 is independent of resveratrol’s ability to induce the phosphorylation of DBC1.

Since the phosphorylation of DBC1 has been shown to occur in response to DNA damage\(^3\), we examined whether resveratrol was inducing DNA damage in cells. Previous reports have shown that resveratrol is a topoisomerase II inhibitor, similar to etoposide, and induces DNA double-strand breaks\(^{19-21}\). We observed that resveratrol induces an accumulation of histone H2A.X phosphorylation (γH2A.X), a common indicator of DNA double-strand breaks (DSBs)\(^{25}\), upon resveratrol treatment (Figure 3-
3A, B). The levels of γH2A.X accumulation induced by etoposide and resveratrol treatment also correspond to the levels of DBC1 phosphorylation, with resveratrol inducing less γH2A.X accumulation than etoposide. Taken together, these data suggest that resveratrol induces DNA damage in the cell, to a lower extent than etoposide, which results in the phosphorylation of DBC1.

**Resveratrol Induces DBC1 Phosphorylation in an ATM- and ATR-dependent Manner**

Zannini et al. showed that the phosphorylation of DBC1 that occurs in response to DNA damage caused by etoposide is dependent on the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3 related (ATR) kinases, the master regulators of the cellular response to genotoxic stress. Therefore, we examined whether the phosphorylation of DBC1 upon resveratrol treatment is also a result of ATM and ATR kinase activity. In cells knocked down for ATM and ATR, phosphorylation levels of DBC1 are reduced, but not completely abolished, upon etoposide and resveratrol treatment (Figure 3-4A, C). Similarly, in cells treated with the ATM-specific inhibitor KU-55933, phosphorylation levels of DBC1 are reduced, but not completely abolished upon etoposide and resveratrol treatment (Figure 3-4B). This suggests that similarly to etoposide, the phosphorylation of DBC1 that occurs in response to DNA damage caused by resveratrol is, in part, dependent on the ATM and ATR kinases.

Despite resveratrol’s widely known anti-oxidant properties, it has also been shown to possess pro-oxidant properties. In addition, activation of ATM has been shown to occur by oxidative stress in the absence of DNA double-strand breaks. In
fact, Lee et al. showed that under oxidizing conditions and without the presence of DNA damage, resveratrol could directly activate ATM\textsuperscript{31}. Therefore, we tested whether the phosphorylation of DBC1 upon resveratrol treatment was also partially a result of oxidative stress-induced ATM activation. We observed that under our specific treatment conditions, 100 µM of resveratrol for 2 hours, activation of ATM and subsequent phosphorylation of DBC1 occurs independently of oxidative stress. Treatment of cells with the anti-oxidant N-acetyl cysteine (NAC) does not reduce the phosphorylation levels of DBC1 upon resveratrol treatment (Figure 3-5A). And, under our treatment conditions, resveratrol actually results in slightly reduced reactive oxygen species (ROS) levels (Figure 3-5B). Taken together, these data suggest that resveratrol’s induction of DBC1 phosphorylation is dependent specifically on the activation of the ATM and ATR kinases upon DNA damage and not upon activation of ATM upon oxidative stress. It remains possible that there are additional factors responsible for resveratrol’s induction of DBC1 phosphorylation, given resveratrol’s many identified targets in the cell\textsuperscript{32-38}.

**Phosphorylation of DBC1 in Response to Resveratrol Does Not Increase Binding to SIRT1 or Inhibit SIRT1 Activity**

Upon identifying DBC1 as a novel ATM/ATR substrate, Zannini et al. showed that ATM- and ATR-dependent phosphorylation of DBC1 upon genotoxic stress enhances DBC1-SIRT1 binding. This increase in binding results in inhibition of downstream SIRT1 deacetylase activity, as seen by increased p53 acetylation levels and subsequent p53-mediated apoptosis, thus providing a novel link between the ATM/ATR kinases and the
DBC1-SIRT1-p53 apoptotic axis. Therefore, we wanted to examine whether resveratrol's induction of DBC1 phosphorylation by the ATM/ATR kinases is also involved in this pathway. However, with our treatment conditions, we do not observe a difference in DBC1-SIRT1 binding upon either etoposide or resveratrol treatment (Figure 3-1A, B). In addition, the phosphorylation levels of DBC1 do not correlate with downstream acetylation levels of p53 as expected, as both etoposide and resveratrol treatments result in similar levels of acetylated p53 despite significantly different levels of phosphorylated DBC1 (Figure 3-1A, B; 3-6A). This suggests that under our treatment conditions, phosphorylation of DBC1 does not have functional significance in the downstream SIRT1-p53 axis. Therefore, we wanted to determine what functional significance, if any, DBC1 phosphorylation has in the DNA damage response.

**Resveratrol Activates the DNA Damage Response in an ATM- and ATR-Dependent Manner but in a Manner Distinct from Etoposide**

Because we observed that resveratrol treatment induces DNA damage similarly to etoposide, albeit to lower levels, we wanted to determine whether resveratrol also activates the DNA damage response similarly to etoposide and whether it is dependent on the phosphorylation of DBC1. Upon DNA damage, the ATM and ATR kinases are activated, resulting in a phosphorylation cascade in which transducer/mediator and effector proteins are phosphorylated and activated in order to determine the appropriate response by the cell, either cell cycle arrest and DNA repair or induction of apoptosis. Among these transducer/mediator proteins are the CHK2 and CHK1 kinases that are phosphorylated by ATM and ATR, respectively. One of the main effector proteins
is p53, which can be phosphorylated and subsequently stabilized by various kinases, including CHK1 and CHK2\textsuperscript{43}. p53 is one of the key effector proteins that is responsible for determining the fate of the cell upon genotoxic stress, transcriptionally activating either a cell cycle arrest and DNA repair response or an apoptosis response\textsuperscript{43,44}. The combination of detection of DNA damage, signaling the presence of DNA damage – through activation of downstream transducer/mediator proteins (CHK1 and CHK2), and promoting either DNA repair or apoptosis – through activation of downstream effector proteins (p53) – is known as the DNA damage response or DDR.

Resveratrol treatment activates the DNA damage response, as evidenced by phosphorylation of the CHK2 (Thr68) and CHK1 (Ser345) kinases and p53 (Ser15) (Figure 3-6A). The activation of the DNA damage response, similarly to the induction of DBC1 phosphorylation, is dependent on the ATM and ATR kinases as loss of ATM results in decreased phosphorylation of the CHK2 kinase and loss of ATR results in decreased phosphorylation of the CHK1 kinase upon resveratrol treatment. Interestingly, we also observed a decrease in p21 levels in response to resveratrol treatment that occurs independently of the ATM and ATR kinases. Importantly, despite the phosphorylation of DBC1 that is induced upon DNA damage by etoposide and resveratrol, the DNA damage response does not appear to be dependent on DBC1, as both loss and overexpression of DBC1 does not affect the phosphorylation levels of the ATM and ATR kinases, the CHK2 and CHK1 kinases, or p53 (Figure 3-6B, C, D). This correlates with our previous observation that, with our treatment conditions, phosphorylation of DBC1 does not have functional significance in the ATM- and ATR-dependent DNA damage response.
In addition, we observed that the levels of phosphorylation of these transducer and effector proteins vary between etoposide and resveratrol treatment and do not necessarily correlate with the levels of DNA damage or DBC1 phosphorylation. Therefore, we performed a time course analysis of etoposide and resveratrol treatment to more closely examine the kinetics of the DNA damage response. In the time course with resveratrol, we observed that phosphorylation of ATM (Ser1981) increases gradually, and phosphorylation of CHK2 also increases gradually and peaks around 2 hours before decreasing (Figure 3-7A, B). This curve of gradual increase of phosphorylation of CHK2 seems to occur with a concomitant decrease in p21 expression levels. In contrast, in the time course with etoposide, we observed that phosphorylation of ATM and CHK2 occurs immediately (15-30 minutes after treatment) and is reduced within a few hours of treatment (Figure 3-7C, D). Also, levels of p21 do not decrease but instead increase dramatically around 12 hours of treatment. In both the resveratrol and etoposide time courses, phosphorylation of ATR (Thr1989) increases gradually, and phosphorylation of CHK1 increases gradually and peaks around 4 hours before decreasing (Figure 3-7A-D). This data shows that the ATM-dependent DNA damage response kinetics upon etoposide and resveratrol treatment are clearly distinct. Furthermore, sustained etoposide treatment for 24 hours results in eventual induction of apoptosis, as evidenced by the cleavage of PARP-1 (Figure 3-7E). Interestingly, sustained resveratrol treatment for 24 hours does not result in the cleavage of PARP-1. Taken together, this suggests that resveratrol treatment induces a distinct ATM-dependent response from that of etoposide that does not activate
apoptosis, whereas etoposide treatment induces a distinct ATM-dependent response that does activate apoptosis.

Despite the DBC1-independent activation of the DNA damage response by resveratrol and etoposide, we posited that perhaps the phosphorylation levels of DBC1 could somehow be used to predict the molecular signature of downstream DNA damage response – that higher levels of phosphorylated DBC1 and DNA damage is correlated with activation of an ATM-dependent DDR that results in apoptosis and lower levels of phosphorylated DBC1 and DNA damage is correlated with activation of an ATM-dependent DDR that does not result in apoptosis. Thus, we performed a dose curve of etoposide to determine a concentration that induced similar levels of DNA damage as resveratrol, as assessed by phosphorylation levels of DBC1 and γH2A.X accumulation. From our dose curve analysis, 5 µM of etoposide induces phosphorylation of DBC1 and γH2A.X accumulation to similar levels of resveratrol-induced DBC1 phosphorylation and γH2A.X accumulation (Figure 3-8A, B). We then performed a time course analysis using the lower dose of etoposide (5 µM) to observe if the lower amount of DNA damage induced can result in an ATM-dependent DNA damage response more closely resembling that of resveratrol’s. Surprisingly, we observed that treatment of cells with the lower dose of etoposide still does not result in an ATM-dependent DNA damage response similar to that of resveratrol’s and in fact results in a response that more closely resembles the higher dose of etoposide’s response – with immediate phosphorylation of CHK2 and ATM 15-30 minutes following treatment and reduction within a few hours (Figure 3-8C, D). In addition, sustained etoposide treatment for 24 hours with the low dose of etoposide also results in eventual induction of apoptosis, as
evidenced by the cleavage of PARP-1, but to a lower extent than with the high dose of etoposide (Figure 3-8E). Taken together, these data suggest that despite inducing the same amount of DNA damage, these two DNA damaging agents, etoposide and resveratrol, activate distinct ATM-dependent and DBC1-independent DNA damage responses.

**Resveratrol’s Metabolites Do Not Induce DNA Damage, Phosphorylation of DBC1, or the DNA Damage Response**

Resveratrol is quickly metabolized *in vivo* to the 3- and 4'-glucuronides and sulfates\(^ {45-47}\). Because of resveratrol’s poor bioavailability, it has been suggested that resveratrol’s beneficial effects are, in fact, due to its metabolites\(^ {48}\). In our previous report, we showed that resveratrol’s 3- and 4'-sulfate metabolites behaved similarly to resveratrol, directly binding to DBC1 to indirectly activate downstream SIRT1 activity (Pai *et al.*, manuscript in preparation; see Chapter 2). Therefore, we examined whether resveratrol’s metabolites also induced DNA damage, resulting in phosphorylation of DBC1 and activation of the DNA damage response. Interestingly, we observed that 2 hour treatment of cells with 100 µM of resveratrol’s metabolites does not induce DNA damage, as assessed by accumulation of γH2A.X phosphorylation, or the phosphorylation of DBC1 (Figure 3-9A, B, C). In addition, treatment of cells with the metabolites does not activate the ATM and ATR kinases, the CHK1 and CHK2 kinases, or p53 (Figure 3-9D). Taken together, this suggests that resveratrol has the ability to induce DNA damage, but once it is metabolized in the body, its metabolites do not induce DNA damage or activate the DNA damage response.
Discussion

Our studies demonstrate that resveratrol induces DNA damage, as indicated by γH2A.X accumulation, resulting in the phosphorylation of DBC1 and downstream activation of the DNA damage response. However, the phosphorylation of DBC1 does not seem to have functional significance in the DDR in our context. Interestingly, we observed that resveratrol activates a distinct ATM-dependent DNA damage response that does not result in apoptosis, whereas etoposide, titrated down to induce the same levels of phosphorylated DBC1 and γH2A.X accumulation as resveratrol, activates an ATM-dependent DNA damage response that does result in apoptosis. Furthermore, resveratrol’s physiologically relevant metabolites do not induce DNA damage, phosphorylation of DBC1, or the DNA damage response.

Previous studies have reported that resveratrol does not induce DNA damage and instead activates ATM directly under oxidizing conditions. Furthermore, studies have shown that γH2A.X foci formation can occur in the absence of DNA damage, and thus γH2A.X accumulation is not always an indicator of DNA damage. Although we do not provide direct evidence that resveratrol treatment of cells results in double-strand breaks and only examine γH2A.X accumulation as a marker for DNA damage, we do provide evidence that resveratrol is not activating ATM directly under oxidizing conditions, as pre-treatment of cells with the antioxidant N-acetyl cysteine does not abolish resveratrol’s effects on DBC1 phosphorylation (Figure 3-5). In addition, other studies have reported that resveratrol, at concentrations similar to the one used in our experiments, inhibit topoisomerase II activity and induce double-strand breaks and micronuclei formation in non-cancer cells. Thus, it is likely that the ATM- and ATR-
dependent phosphorylation of DBC1 that is induced by resveratrol is occurring in response to DNA damage.

In contrast to a previous report, under our treatment conditions, we do not see a functional significance of phosphorylated DBC1 in the DNA damage response. It is well understood that resveratrol induces different effects in the cell depending on the cell type, the dose used, and the length of treatment\textsuperscript{51}. Zannini et al. observed phosphorylated DBC1-dependent effects in DNA damage response and p53-dependent apoptosis in U2OS cells, an osteosarcoma cell line\textsuperscript{3}. The DBC1 phosphorylation and DNA damage response experiments presented here were performed in HEK293T cells, and thus phosphorylated DBC1 could have different functional effects in cancerous versus non-cancerous cells. Although HEK293T cells are not cancer-derived cells, it will be important to perform these experiments in normal untransformed cells to determine if resveratrol has similar effects in a more normal background and to determine if its phosphorylated DBC1-related effects are truly dependent on a cancerous background. It will also be important to determine the functional significance, if any, of resveratrol's induction of phosphorylation of DBC1 and activation of the DNA damage response in non-cancerous cells. In addition, other posttranslational modifications of DBC1 have also been shown to modulate the DBC1-SIRT1 interaction, including acetylation and sumoylation\textsuperscript{17,18}. It will be interesting to see whether resveratrol has any effects on the acetylation and sumoylation levels of DBC1 and, if so, whether these posttranslational modifications have functional significance in downstream DNA damage response.

Despite resveratrol's DBC1-independent effects on DNA damage response, we observed that resveratrol treatment activates an ATM-dependent DDR that does not
result in apoptosis, which is distinct from the ATM-dependent DDR activated upon etoposide treatment that does result in apoptosis. We hypothesize that, in the context of non-cancerous cell lines, resveratrol has a more protective role, activating the DNA damage response and DNA repair pathways to prime and protect cells from further and more extensive DNA insult. Similarly to the theory of mitohormesis, which suggests that bursts of reactive oxygen species (ROS) leads to increased oxidative stress resistance and overall survival rates\textsuperscript{52,53}, resveratrol could be inducing a small “burst” of DNA damage, activating the DNA damage response and DNA repair pathways to allow cells to be better adapted to the many different types of endogenous and exogenous DNA insults that occur in cells every day. In fact, the notion of small amounts of DNA damage contributing to a “survival response” that promotes longevity is not a new one\textsuperscript{54}.

In addition, in agreement with this hypothesis, we observed that resveratrol’s metabolites do not induce DNA damage or activate the DDR. It is therefore possible that once resveratrol enters the cell, it causes activation of the DNA damage response, but because resveratrol is quickly metabolized and these metabolites do not further activate the DDR, the DNA damaging agent is no longer present (providing that “burst” of DNA damage), but the DDR has been slightly activated, allowing the cells to respond more quickly to further DNA insult. Further experiments need to be performed to validate this hypothesis, such as pre-treating cells with resveratrol for an extended amount of time and then treating with etoposide to see if they respond differently from non-pre-treated cells. If valid, this could provide a novel mechanism of resveratrol’s beneficial effects.

And finally, due to resveratrol’s poor bioavailability, the structurally similar natural compound pterostilbene, found primarily in blueberries, with approximately four
times greater bioavailability compared to resveratrol, has been investigated for its potential therapeutic benefits\textsuperscript{55}. We performed a dose curve of pterostilbene to determine a concentration that induced similar levels of DNA damage, if any, as resveratrol, as indicated by phosphorylation levels of DBC1 and γH2A.X accumulation. From our dose curve analysis, 50 µM of pterostilbene induces phosphorylation of DBC1 to similar levels of resveratrol-induced DBC1 phosphorylation and γH2A.X accumulation (Figure 3-10A, B). We then performed a time course analysis using this concentration of pterostilbene to observe the DNA damage response. We observed that treatment of cells with pterostilbene results in a DNA damage response similar to that of resveratrol’s (Figure 3-10C, D). In addition, sustained pterostilbene treatment for 24 hours also does not result in induction of apoptosis, as evidenced by the lack of cleavage of PARP-1 (Figure 3-10E). These data suggest that, similarly to resveratrol, pterostilbene can induce phosphorylation of DBC1 in response to DNA damage and induce an ATM-dependent DDR similar to resveratrol’s and distinct from etoposide’s. It remains to be seen whether pterostilbene is acting through the theory of "DNA damage hormesis", but a recent report has shown that pterostilbene protects cells from chromosomal damage induced by ionizing radiation\textsuperscript{56}, further suggesting that resveratrol and its analogs could be providing beneficial effects through this pathway.

Despite all that remains unknown, we have shown here that resveratrol activates a distinct ATM-dependent DNA damage response from etoposide that does not result in apoptosis in non-cancerous cells. Importantly, we suggest a novel mechanism of action for resveratrol that could contribute to its beneficial effects \textit{in vivo} and warrants further investigation.
**Figures**

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**Figure 3-1.** Resveratrol induces the phosphorylation of DBC1. (A) HEK293T cells were treated with 20 µM etoposide (VP-16) or 100 µM resveratrol (RSV) for 2 hours before lysis for immunoprecipitation with DBC1 antibody and subsequent analysis via Western blotting. (B) HEK293T cells transfected with wild-type and T454A mutant DBC1 were treated as described in (A) before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (C) Map of wild-type DBC1 and DBC1 truncation mutants. HEK293T cells transfected with wild-type and truncations mutants of DBC1 were treated as described in (A) before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting.
Figure 3-2. Resveratrol dose curve analysis. (A) HEK293T cells transfected with wild-type DBC1 were treated with increasing concentrations of resveratrol (10, 25, 50, 100, and 250 μM) for 2 hours before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (B) Quantification of p-DBC1/DBC1 levels from (A).
Figure 3-3. Resveratrol induces γH2A.X accumulation. (A) HEK293T cells were treated with 20 µM VP-16 or 100 µM RSV for 2 hours before staining with γH2A.X antibody and Topro-3 and imaging with a confocal microscope. (B) Quantification of relative fluorescence intensity of (A) performed using ImageJ and Prism software, p < 0.0001.
Figure 3-4. Resveratrol induces phosphorylation of DBC1 in an ATM- and ATR-dependent manner. (A) HEK293T cells were transfected with control and ATM siRNA for 48 hours before transfection with wild-type DBC1 for 24 hours. Cells were then treated with 20 µM VP-16 or 100 µM RSV for 2 hours before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (B) HEK293T cells were transfected with wild-type DBC1 and treated with 10 µM KU-559933 for 1 hour, followed by treatment with VP-16 and RSV as in (A) before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (C) HEK293T cells were transfected with control and ATR siRNA for 48 hours before transfection with wild-type DBC1 for 24 hours. Cells were then treated as in (A) before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting.
Figure 3-5. Resveratrol does not induce phosphorylation of DBC1 in a ROS-dependent manner. (A) HEK293T cells transfected with wild-type DBC1 were treated with 10 mM N-acetyl cysteine (NAC) for 40 min, followed by treatment with 20 µM VP-16 or 100 µM RSV for 2 hours before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (B) HEK293T cells were stained with 2’,7’-dichlorofluorescin diacetate (DCFDA) (Sigma, D6883) followed by treatment with 20 µM VP-16 and 100 µM RSV for 2 hours before analysis of fluorescence levels on a microplate reader.
Figure 3-6. Resveratrol's effects on the DNA damage response are ATM- and ATR-dependent but not DBC1-dependent. (A) HEK293T cells were transfected with control, ATM, and ATR siRNA for 72 hours before drug treatment. Cells were then treated with 20 µM VP-16 or 100 µM RSV for 2 hours before lysis for Western blot analysis. (B) HEK293T cells were transfected with control and wild-type DBC1 plasmid vectors for 24 hours before drug treatment. Cells were then treated as in (A) before lysis for Western blot analysis. (C) HEK293T cells were transfected with control and DBC1 siRNA for 72 hours before drug treatment. Cells were then treated as in (A) before lysis for Western blot analysis. (D) Quantification of (C).
Figure 3-7. Time course analysis of resveratrol and etoposide on the DNA damage response. (A) HEK293T cells were treated with 100 µM RSV for 15 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, and 24 hrs before lysis for Western blot analysis. (B) Graphical representation and quantification of (A). (C) HEK293T cells were treated with 20 µM VP-16 for varying times described in (A) before lysis for Western blot analysis. (D) Graphical representation and quantification of (C). (E) HEK293T cells were treated with 20 µM VP-16 and 100 µM RSV for 24 hrs before lysis for Western blot analysis to observe cleaved PARP-1 levels.
Figure 3-8. Low dose etoposide does not induce the same DNA damage response as resveratrol. (A) HEK293T cells transfected with wild-type DBC1 were treated with 100 µM RSV and varying concentrations of VP-16 (1 µM, 5 µM, 10 µM, and 20 µM) for 2 hours before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (B) HEK293T cells were treated with 20 µM VP-16, 5 µM VP-16 or 100 µM RSV for 2 hours before staining with γH2A.X antibody and Topro-3 and imaging with a confocal microscope. Quantification of relative fluorescence intensity performed using ImageJ and Prism software, p < 0.0001. (C) HEK293T cells were treated with 5 µM VP-16 for 15 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, and 24 hrs before lysis for Western blot analysis. (D) Graphical representation and quantification of (C). (E) HEK293T cells were treated with 20 µM VP-16, 5 µM VP-16, and 100 µM RSV for 24 hrs before lysis for Western blot analysis to observe cleaved PARP-1 levels.
Figure 3-9. Resveratrol's metabolites do not induce γH2A.X accumulation, phosphorylation of DBC1, or the DNA damage response. (A) HEK293T cells were treated with 100 µM of RSV and its 3- and 4'-glucuronide and sulfate metabolites for 2 hours before staining with γH2A.X antibody and Topro-3 and imaging with a confocal microscope. (B) Quantification of relative fluorescence intensity of (A) performed using ImageJ and Prism software, p < 0.0001. (C) HEK239T cells transfected with wild-type DBC1 were treated as described in (A) before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (D) HEK293T cells were treated as described in (A) before lysis for Western blot analysis.
Figure 3-10. Pterostilbene induces a similar DNA damage response as resveratrol. (A) HEK293T cells transfected with wild-type DBC1 were treated with 100 µM RSV, 50 µM pterostilbene, and 100 µM pterostilbene for 2 hours before lysis for FLAG immunoprecipitation and subsequent analysis via Western blotting. (B) HEK293T cells were treated with 100 µM RSV or 50 µM pterostilbene for 2 hours before staining with γH2AX antibody and Topro-3 and imaging with a confocal microscope. Quantification of relative fluorescence intensity performed using ImageJ and Prism software, p < 0.0001. (C) HEK293T cells were treated with 50 µM pterostilbene for 15 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, and 24 hrs before lysis for Western blot analysis. (D) Graphical representation and quantification of (C). (E) HEK293T cells were treated with 20 µM VP-16, 5 µM VP-16, 100 µM RSV, and 50 µM pterostilbene for 24 hrs before lysis for Western blot analysis to observe cleaved PARP-1 levels.
References


Conclusions and Future Directions

In this dissertation, we show that the Drug Affinity Responsive Target Stability (DARTS) approach can be used to identify novel protein targets for small molecules, including the FDA-approved drug disulfiram that was shown to be a potent inhibitor of triple-negative breast cancer cell lines\(^1\) and the lifespan-extending endogenous metabolite α-ketoglutarate\(^2\). In particular, we utilize DARTS to identify deleted in breast cancer 1 (DBC1) as a novel protein target for the anti-aging compound resveratrol and examine resveratrol’s DBC1-dependent effects in the cell. We also identify a distinct ATM-dependent and DBC1-independent DNA damage response that is activated upon resveratrol treatment. Taken together, our data provides evidence of a novel protein target and mechanism(s) of action for resveratrol that should be further investigated for their roles in the aging pathway.

In Chapter 1, we demonstrate the utility of the DARTS approach. We performed DARTS experiments using three drugs with well-known protein targets and successfully confirmed the binding of these drugs with their protein targets – methotrexate with dihydrofolate reductase\(^3\), olaparib with poly(ADP-ribose) polymerase\(^4\), and omigapil with glyceraldehyde 3-phosphate dehydrogenase\(^5\). Therefore, we confidently utilized the DARTS approach to identify novel protein targets for the FDA-approved drug disulfiram used to treat chronic alcoholism and the lifespan-extending metabolite α-ketoglutarate.

Using the DARTS method, we identified and confirmed IQ motif containing GTPase activating protein 1 (IQGAP1) and myosin heavy-chain 9 (MYH9) as direct binding targets of disulfiram (DSF), a potent inhibitor of triple-negative breast cancer (TNBC) cell lines\(^1\). Binding of disulfiram with these two IQ motif-containing proteins is
consistent with pathway analysis of MDA-MB-231 cells treated with DSF, which identified divergent signaling pathways affected by DSF treatment. In addition, knockdown of each protein resulted in ~25-30% inhibition of TNBC cell growth. In contrast, disulfiram treatment alone resulted in ~75% inhibition of TNBC cell growth, suggesting that additional factors, possibly other IQ-motif containing proteins, likely interact with and contribute to the global effect of DSF. Nonetheless, our results suggest that IQGAP1 and MYH9 should be further investigated as potential therapeutic targets for the treatment of triple-negative breast cancers.

Utilizing the DARTS method, we also identified and confirmed ATP5B, the β subunit of the catalytic core of the ATP synthase, as a direct binding target for the lifespan-extending metabolite α-ketoglutarate. This tricarboxylic acid cycle intermediate extends the lifespan of *Caenorhabditis elegans* by inhibition of ATP synthase and TOR, a major effector of dietary restriction but not a direct binding target of α-KG (as determined via DARTS). Longevity molecules that delay aging and extend lifespan have long been a dream of humanity. Endogenous metabolites such as α-ketoglutarate that can alter *Caenorhabditis elegans* lifespan suggest that an internal mechanism may exist that is accessible to intervention; whether this can translate into manipulating the aging process in humans remains to be seen.

In Chapter 2, we again utilize the DARTS method to identify deleted in breast cancer 1 (DBC1), the negative regulator of sirtuin 1 (SIRT1) as a direct binding target of resveratrol. We show that resveratrol binds to the N-terminal region of DBC1, decreasing the DBC1-SIRT1 interaction. This decrease in interaction abolishes the effect of DBC1 overexpression on p53 acetylation levels and affects downstream SIRT1
activity in a DBC1-dependent manner, suggesting a novel mechanism of resveratrol's activation of SIRT1. In addition, the 3- and 4'-glucuronide and sulfate metabolites of resveratrol behave similarly to resveratrol, binding to DBC1 and affecting downstream SIRT1 activity in a DBC1-dependent manner.

Although our data points to an exciting novel mechanism of resveratrol's indirect activation of SIRT1, we are not excluding the contribution of other resveratrol targets to its downstream effects on SIRT1. Activation of SIRT1 may also require contribution from or can be achieved by any of resveratrol's other target proteins, including, but not limited to, the cyclooxygenases (COXs), the mitochondrial F1F0-ATPase (Complex V), phosphodiesterases (PDEs), and the more recently discovered tyrosyl transfer-RNA synthetase. We also do not attempt to explain the mechanism behind all of resveratrol's beneficial effects. Resveratrol may also activate SIRT1 by binding to, for example, active regulator of SIRT1 (AROS), which positively regulates SIRT1, or hypermethylated in cancer 1 (HIC1), which negatively regulates SIRT1. In addition, DBC1 is known to interact with and regulate other proteins in vivo, including epigenetic modifiers, such as the deacetylase HDAC3 and the methyltransferase SUV39H1, as well as nuclear receptors, such as estrogen receptor (ER), androgen receptor (AR), retinoic acid receptor (RAR), and Rev-erbα. It is possible that resveratrol prevents DBC1 from binding to these other substrates, further contributing to its health benefits.

In addition, it remains to be determined whether DBC1 is required for resveratrol's beneficial effects on lifespan or any of its other numerous health benefits in vivo. DBC1 is conserved in mice; however, the closest ortholog of DBC1 in C. elegans is lateral signaling target 3 or lst-3, with only 17% sequence similarity to human DBC1.
Further experiments need to be performed to determine whether *lst*-3 is indeed a negative regulator of *sir*-2.1 in *C. elegans* and whether lifespan extension with resveratrol treatment is attenuated in *lst*-3 knockdown worms as well as in more complex organisms.

And finally, in Chapter 3, we demonstrate that resveratrol induces DNA damage, as indicated by γH2A.X accumulation, resulting in the phosphorylation of DBC1 and downstream activation of the DNA damage response. However, the phosphorylation of DBC1 does not seem to have functional significance in the DDR in our context. Interestingly, we observed that resveratrol activates a distinct ATM-dependent DNA damage response that does not result in apoptosis, whereas etoposide, titrated down to induce the same levels of phosphorylated DBC1 and γH2A.X accumulation as resveratrol, activates an ATM-dependent DNA damage response that does result in apoptosis. Furthermore, resveratrol’s physiologically relevant metabolites do not induce DNA damage, phosphorylation of DBC1, or the DNA damage response.

In contrast to a previous report, under our treatment conditions, we do not see a functional significance of phosphorylated DBC1 in the DNA damage response. It is well understood that resveratrol induces different effects in the cell depending on the cell type, the dose used, and the length of treatment\(^2^3\). Zannini *et al.* observed phosphorylated DBC1-dependent effects in DNA damage response and p53-dependent apoptosis in U2OS cells, an osteosarcoma cell line\(^2^4\). The DBC1 phosphorylation and DNA damage response experiments presented here were performed in HEK293T cells, and thus we propose that phosphorylated DBC1 could have different functional effects in cancerous versus non-cancerous cells. Although HEK293T cells are not cancer-
derived cells, it will be important to perform these experiments in normal untransformed cells to determine if resveratrol has similar effects in a more normal background and to determine if its phosphorylated DBC1-related effects are truly dependent on a cancerous background. It will also be important to determine the functional significance, if any, of resveratrol's induction of phosphorylation of DBC1 and activation of the DNA damage response in non-cancerous cells.

We hypothesize that resveratrol's activation of an ATM-dependent DNA damage response distinct from etoposide's may contribute to its health benefits. Similarly to the theory of mitohormesis, which suggests that bursts of reactive oxygen species (ROS) leads to increased oxidative stress resistance and overall survival rates\textsuperscript{25,26}, resveratrol could be inducing a small “burst” of DNA damage, activating the DNA damage response and DNA repair pathways to allow cells to be better adapted to the many different types of endogenous and exogenous DNA insults that occur in cells every day. Further experiments need to be performed to validate this hypothesis, such as pre-treating cells with resveratrol for an extended amount of time and then treating with etoposide to see if they respond differently from non-pre-treated cells. One of the hallmarks of aging is the accumulation of DNA damage\textsuperscript{27}, and if our hypothesis is valid, this could provide a novel mechanism of resveratrol's anti-aging effects.

Despite all that remains unknown, we have identified a potentially novel target of the aging pathway. With the discovery of DBC1 as a novel protein target of resveratrol, it will be important to perform small molecule screens to find more specific inhibitors of DBC1 that may be more efficacious than resveratrol in treating cancer or increasing healthspan and lifespan. Furthermore, with the discovery of resveratrol's activation of an
ATM-dependent DNA damage response distinct from etoposide, we suggest a novel mechanism of action for resveratrol that could contribute to its beneficial effects \textit{in vivo} and warrants further investigation.
References


