2024

Temporal And Epigenetic Mechanisms Underlying Dietary Phytochemicals In Breast Cancer Prevention

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TEMPORAL AND EPIGENETIC MECHANISMS UNDERLYING DIETARY PHYTOCHEMICALS IN BREAST CANCER PREVENTION

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2024

TEMPORAL AND EPIGENETIC MECHANISMS UNDERLYING DIETARY PHYTOCHEMICALS IN BREAST CANCER PREVENTION

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BIOLOGY

ABSTRACT

Breast cancer (BC) is a widespread malignancy that, despite advancements in treatments and improvements in survival, had an estimated 297,790 new cases and 43,170 mortalities in 2023. With around 1 in every 8 women in the US expected to be diagnosed with BC at some point in their lives, the economic, societal, and healthcare burdens associated with this disease cannot be understated. Because of this, treatments and preventions that are affordable, efficacious, and easily administered are in high demand. Preventive therapies have shown great promise in reducing disease burden with relatively few serious side effects. BC prevention through the consumption of epigenetically active plant-based compounds known as dietary phytochemicals (DP) has become a large area of research due to their potency, relative low cost, and little to no side effects. However, questions remain as to the best timeline of administration and mechanisms of action for many phytochemicals. Our studies aimed to address these questions for the phytochemicals sulforaphane (SFN) and withaferin a (WFA), respectively. Our results indicate that SFN-containing broccoli sprout extract administered during the peripubertal period may be important for later-life BC prevention and that decreases in promoter methylation of the tumor suppressor gene *p21* and resulting *p53*-independent increases in its gene expression may be vital for the anticancer function of WFA. These results indicate that puberty may be a critical period for the effective administration of phytochemicals and an important time to target for the prevention of breast cancer. These

results also suggest that WFA may have value in the prevention of highly prevalent and pathogenic *p53*-mutant BC.

Keywords: Breast Cancer Prevention, DNA Methylation, Sulforaphane, Withaferin A, Transgenic Mice, Epigenetic Editing

DEDICATION

I dedicate this work to my friends, family, and all those who supported me throughout my time as a graduate student. My parents, Julie and Cliff Brane as well as my brother Grayson have shown unwavering support as I pursued this degree. I also dedicate this work to my late grandmother, Alice Lorene Brane, who was always excited about and encouraging towards my studies. Finally, I dedicate this work to my constant companion Kiko, who has remained by my side throughout my studies.

ACKNOWLEDGEMENTS

I want to thank my mentor and dissertation committee chair Dr. Trygve Tollefsbol, who has provided constant support, advice, and opportunities in both my research and professional development. Through his guidance and creative allowance, I was able to develop my projects and techniques. His support has been unconditional throughout my time at UAB, and my appreciation for this cannot be understated. I also want to thank my previous mentors, including Dr. Leslie Goertzen and Dr. Jeremy Day. A special thanks goes to Dr. Faraz Sultan, who assisted in troubleshooting my CRISPR project. I thank Dr. Steven Watts for allowing me to become a part of the UAB biology department. The department has been a great environment for research and scholarship.

I also express my gratitude towards my full committee, including Dr. Melissa Harris, Dr. Liou Sun, Dr. Edwin Aroke, and Dr. Doug Hurst. Their insight and expertise have been invaluable to both the development of my projects and my development as a scientist. I appreciate the commitment of their valuable time towards this work, and it would not have been possible without their contributions.

I thank all the members of the Cancer Prevention and Control Training Program T32 that I have been a part of since 2020. Dr. Lyse Norian has been a champion for all the students in the program, and she has been a major help during my time at graduate school. I also want to thank the program directors Dr. Ritu Aneja and Dr. Ellen Smith for

their contributions to this T32. This program has done so much for me and is a major contributor to my successes.

Finally, I'd like to thank the Tollefsbol Laboratory members, both past and present for their help and comradery throughout the years. The assistance and advice of Dr. Huixin Wu was invaluable to the mouse work I did, and I thank Mijanur Rahman for his help with my CRISPR work. I also thank Sebanti Ganguly, Manvi Sharma, Itika Arora, Brittany Witt, and Dr. Shizhao Li for their help and constructive feedback throughout the years.

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PERIPUBERTAL NUTRITIONAL PREVENTION OF CANCER-ASSOCIATED GENE EXPRESSION AND PHENOTYPES

P21 PROMOTER METHYLATION IS VITAL FOR THE ANTICANCER ACTIVITY OF WITHAFERIN A

LIST OF ABBREVIATIONS

ALT	Alternative means of telomere lengthening
A/PI	Asian/Pacific Islander women
BC	Breast cancer
BSp	Broccoli Sprout extract
CIRP	Cold inducible RNA-binding protein
CP	Critical period
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR-associated
DCIS	Ductal Carcinoma in situ
DEG	Differentially expressed genes
DMG	Differentially methylated genes
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
DNMT2	DNA methyltransferase 2
DNMT3a	DNA methyltransferase 3a
DP	Dietary phytochemical
DSB	Double strand breaks
EGFP	Enhanced green fluorescent protein
ER(-)	Estrogen receptor-negative
FDR	False discovery rate

GO	Gene ontology
HDAC	Histone deacetylase
HER2	HER2-enriched
Her2/neu	FVB/N-Tg(MMTVneu)202Mu
hTERT	Human telomerase reverse transcriptase
NAF1	Nuclear assembly factor 1
PD	Parkinson’s disease
RRBS	Reduced representation bisulfite sequencing
SFN	Sulforaphane
SV40	C3(1)-SV40 Tag (FVB-Tg(C3-1-TAg)cJeg/JegJ)
TERC	RNA component of telomerase
TERT	Telomerase reverse transcriptase
Tet	Ten eleven translocation
TET1	Ten-eleven translocation enzyme 1
TNBC	Triple negative breast cancer
VO	Vaginal Opening
WFA	Withaferin A

GENERAL INTRODUCTION

Breast cancer (BC) is a widespread disease that affects the lives of millions of women each year. While the incidence of some forms of cancer has dropped in recent years, the number of new BC cases has continued to rise [1]. The American Cancer Society estimates that there were around 297,790 new BC cases in 2023, accounting for around 30% of all new cancer diagnoses in women. Additionally, BC is behind only lung cancer in terms of lethality and results in over 43,000 deaths per year. In the United States, both BC incidence and mortality vary greatly with ethnic background [2]. Both Non-Hispanic White and Non-Hispanic Black women are around 25% more likely to develop BC than Asian/Pacific Islander (A/PI) women. This has been partially explained through differences in diet, as A/PI women are more likely to consume diets high in fruit, vegetables, and soy, all of which have been associated with decreased breast cancer risk and mortality [3]. The efficacy of these plant-based compounds, known as dietary phytochemicals (DPs), combined with their relative low cost and ease of application, makes them an attractive target for studies of BC prevention. Mechanistically, consumption of DPs has been linked with changes to the epigenome through the modulation of epigenetic modifiers such as histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) [4]. However, questions remain as to the importance of timing and specific genetic/epigenetic mechanisms of action behind DPs.

To address these questions, we designed experiments utilizing the DPs sulforaphane (SFN) and withaferin a (WFA) to investigate temporal and mechanistic aspects of dietary

Chemoprevention, respectively. For the former experiments, our ideas were based on the growing body of scientific literature that seems to indicate there are windows of time, known as critical periods (CPs), wherein external effectors may have a greater influence on later life BC development [5-7]. These CPs are thought to arise from phases of rapid cellular growth and development that leave the cell vulnerable to genetic and epigenetic damage [8]. For breast tissue, CPs include the perinatal, peripubertal, and peripregnancy periods, and tissues incur the largest amount of growth and differentiation during these times [9]. Past studies have linked environmental exposures such as the pesticide DDT and the miscarriage prevention drug diethylstilbestrol during CPs to increased BC risk later in life [8,10,11]. In particular, the peripubertal period has drawn interest in its connections to BC risk and prevention, as it is also associated with large fluctuations in hormone levels and early, prolonged puberty has been positively associated with BC risk [12,13]. Observations on environmental exposures during puberty in the context of BC have also extended to diet and nutrition, with high fat diets during the peripubertal period being associated with increased BC risk [14]. While there are few studies investigating the protective effects of diet during puberty, there is some evidence that diets high in fruits, vegetables, and fiber may have some protective effect against BC [15]. However, there is currently little work directly linking peripubertal consumption of specific dietary factors to BC risk, and both the clinical and preclinical literature on how DPs interact with CPs remains sparse.

SFN, contained within cruciferous vegetables, is a strong candidate as a chemopreventive compound, and both clinical and mouse model studies indicate that it may be an important DP for BC prevention [16,17]. To glean a better understanding of

the importance of diet, CPs, and BC prevention, we designed an experiment where we administered the DP SFN contained within broccoli sprout extracts (BSp) during the peripubertal period of two transgenic mouse models for BC: the C3(1)-SV40 Tag (FVB-Tg(C3-1-TAg)cJeg/JegJ) (SV40) and FVB/N-Tg(MMTVneu)202Mu (Her2/neu) models. We measured the effects of this BSp on tumor size, incidence, and latency. We also measured the long-term effects of this treatment on gene and protein expression through a combination of RT-qPCR, western blotting, and whole genome RNA sequencing. To measure effects on DNA methylation profiles, we also performed whole genome reduced representation bisulfite sequencing (RRBS).

To expand on our understanding of the importance of DPs' effects on the epigenome, we focused our second set of experiments on WFA and its effects on the promoter DNA methylation states of key cancer-associated genes. WFA, found in Ashwagandha root, has long been used in traditional medicines for its cardioprotective and anti-inflammatory properties, but recently it has received increased scientific attention due to its anti-cancer modulation of pathways associated with proliferation, apoptosis, and metastasis [18,19]. Many of these effects are thought to arise from WFAs inhibitory effect on the class of epigenetic writers known as DNMTs, but previous studies in our laboratory and others have indicated that WFA has differential effects on gene expression and promoter methylation states [20-22]. Specifically, the tumor suppressors *p21* and *p53* as well as the oncogene *CCND1* have been highlighted for potential importance in the function of WFA [20, 23-25]. There is little known about which of these genes' specific promoter methylation states, if any, contribute to the anticancer properties of WFA. To address this, we designed an experiment utilizing CRISPR-dCas technology tied to methylation

modifiers in both the absence and presence of WFA in two BC cell lines: the ERα (+) MCF7 cell line and ERα (-) MDA-MB-231 cell line. Within these cells, we measured the effects of modulating the promoter methylation states of *p21*, *p53,* and *CCND1* on gene expression and BC cell viability using a CRISPR-dCas construct tied to a ten-eleven translocation enzyme 1 (Tet1) for DNA demethylation and a DNA methyltransferase 3a (DNMT3a) for DNA methylation. We also measured the effects of WFA administration alone on promoter methylation, gene expression and BC cell viability. Finally, we used our CRISPR constructs to reverse WFA-associated methylation changes to *p21*, *p53*, and *CCND1* and measured how this influenced the expression of these genes as well as how this modulated the anti-cancer effects of WFA.

Overall, the scope of this work was to gain a greater understanding of the efficacy of DP administration on both temporal and mechanistic levels. Knowledge on the importance and effectiveness of chemoprevention during a critical period such as puberty sets the groundwork for future clinical research which may help inform nutritional prevention strategies utilizing the DP SFN in the wider population, especially for individuals who have contributing risk factors or a genetic predisposition to BC. Additionally, a gene-specific understanding of epigenetic mechanisms behind the DP WFA allows for increased precision when selecting a BC prevention regimen and, if combined with precision medicine, may lead to greater efficacy and fewer side effects in a clinical setting.

TARGETING TELOMERES AND TELOMERASE: STUDIES IN AGING AND DISEASE UTILIZING CRISPR/CAS9 TECHNOLOGY

by

ANDREW BRANE AND TRYGVE O. TOLLEFSBOL

Cells

Format adapted for dissertation

ABSTRACT

Telomeres and telomerase provide a unique and important avenue of study in improving both life expectancy and quality of life due to their close association with aging and disease. While major advances in our understanding of these two biological mediators have characterized the last two decades, previous studies have been limited by the inability to affect change in real time within living cells. The last three years, however, have witnessed a huge step forward to overcome this limitation. The advent of the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system has led to a wide array of targeted genetic studies that are already being employed to modify telomeres and telomerase, as well as the genes that affect them. In this review, we analyze studies utilizing the technology to target and modify telomeres, telomerase, and their closely associated genes. We also discuss how these studies can provide insight into the biology and mechanisms that underlie aging, cancer, and other diseases.

Keywords: telomeres, telomerase, CRISPR, CRISPR/Cas9, Cas9, dCas9, cancer, aging

INTRODUCTION

1.1 Telomeres and Telomerase

Since their discovery, telomeres have been at the forefront of research in both aging and disease. There were observations implicating structures on chromosome ends assisting in stability as far back as the 1930's, but it was not until Blackburn and Gall's pioneering paper that the modern idea of a telomere emerged [1,2]. Their initial study showed that telomeres consist of linked, repeating nucleotide hexamers. Further study revealed a variety of possible sequences among clades of organisms [3]. Within these clades, however, function is highly conserved, with telomeres being transferred from distantly related species that are able to maintain biological activity [4].

This activity is important for mitigating damage to genes during chromosomal replication. DNA polymerase is unable to replicate the ends of chromosomes, due to the nature of DNA replication. Telomeres act as buffer zones, which prevent the gradual degradation of genes. Over the lifetime of a cell, telomeres become shorter, and cells will become senescent once a critical length is reached [5]. Because it is necessary to restore telomeres to continue division and reproduction, the discovery of telomeres posed a mechanistic question as to how they are built and maintained. The answer to this question followed shortly after, when Greider and Blackburn discovered and isolated the protein that they called terminal transferase [6]. This protein is today known as telomerase.

As a ribonucleoprotein, telomerase is composed of both RNA and proteins, and it consists of two molecules each of telomerase reverse transcriptase, telomere RNA, and dyskerin

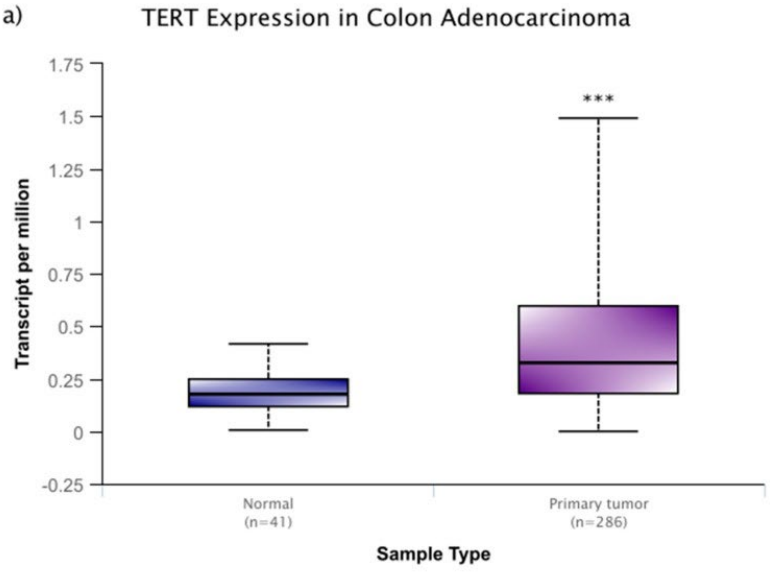
[7,8,9]. While each subunit is necessary for proper biological function, the catalytic portion that is known as telomerase reverse transcriptase (TERT and hTERT in humans) is normally the limiting factor for telomerase activity and telomere elongation [10]. It is for this reason that a majority of research regarding telomere biology focuses on TERT.

Because of their intimate association with cell replication and senescence, telomeres and, by extension, telomerase, have been implicated in disease and aging since the 1980's [5]. Mice that were bred to be deficient in telomerase showed a marked decrease in health and, after several generations, lose the ability to breed completely [11]. Studies have shown that telomere length decreases in older organisms and that this effect is not simple correlation [12,13]. Within tissues, the ablation of telomeres results in spatially specific, age-associated damage [13,14]. On a cellular level, the loss of telomere function is linked with decreased ability for cellular division, and TERT overexpression is linked with an increase in cellular proliferation [15]. The effect is especially pronounced in stem cells, where telomerase is normally upregulated to a high degree [16,17]. When telomerase activity is disrupted in these cells, they lose replicative capacity and lose their pluripotency. In addition, this disruption leads to an increase in cellular oxidative stress [18].

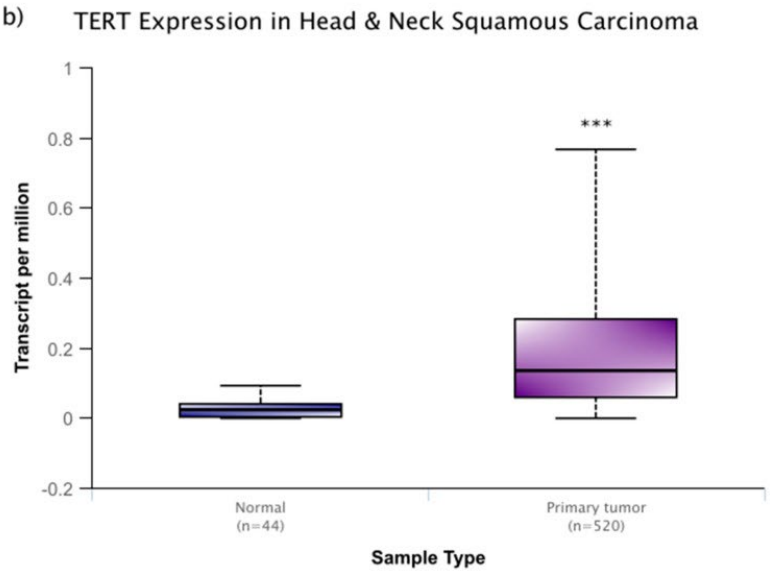
However, these changes in activity are not solely linked with aging. As is the case with many cellular processes, disruptions to the normal function of telomeres and telomerase are associated with human disease. One of the most closely associated of these diseases is, perhaps, cancer. In around 90% of cancers, the expression of telomerase is increased,

while similar, yet benign, tumors do not display this increase in telomerase [19]. In a sample that was derived from cancer patient data within the National Cancer Institute Genomic Data Commons, this increase is seen across several cancer types (Figure 1a–c) and it remains high through progressive breast cancer stages (Figure 1c) [20]. In addition, more severe, metastatic stages of cancer experience higher expression levels of telomerase, and disrupting telomerase activity may have some efficacy in preventing metastasis [10,21]. It is thought that these high levels of expression prevent cellular senescence and they allow for aggressive, rapidly dividing cancer lines.

a)


TERT Expression in Colon Adenocarcinoma
1.75
1.5
1.25
1
0.75
0.5
0.25
0
-0.25
***
Transcript per million
Normal
(n=41)
Primary tumor
(n=286)
Sample Type

b)


TERT Expression in Head & Neck Squamous Carcinoma
1
0.8
0.6
0.4
0.2
0
-0.2
***
Transcript per million
Normal
(n=44)
Primary tumor
(n=520)
Sample Type

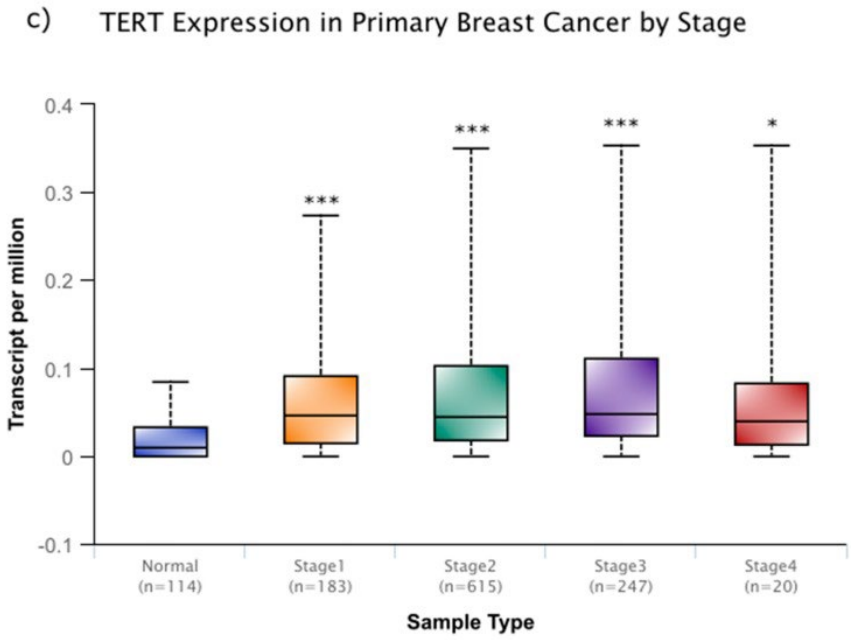


Figure 1. Telomerase reverse transcriptase (TERT) expression of colon adenocarcinoma (a), and head and neck squamous carcinoma (b), and primary breast cancer (c) within the publicly available National Cancer Institute Genomic Data Commons. In (c), TERT expression data is expanded based on stage of breast cancer sampled. While there is no significant difference among cancer stages, each stage of cancer displays significantly higher expression than normal tissue. Data were accessed through the University of Alabama at Birmingham UALCAN cancer transcriptome database (http://ualcan.path.uab.edu) [20]. These box and whisper plots encompass all transcriptome data recorded, with the upper and lower bars giving the total range of data recorded. The bolded middle line represents the mean of the data, and the second and third quartiles are contained within the box. Significance in relation to normal tissue is denoted by asterisks, with * representing a $p < 0.05$ and *** representing a $p < 0.001$. These types of cancer (a–c) were chosen for their relatively high sample sizes, but these trends carry across many other cancer types.

1.2 The CRISPR-Cas System

While major strides in the understanding of the function and dysfunction of telomeres and telomerase have been apparent, a crucial hurdle in this study has been the inability to affect and observe changes within living systems. However, recently a solution to this issue has emerged. The clustered regularly interspaced short palindromic repeats-CRISPR-associated (CRISPR-Cas) system was first described as an adaptive bacterial immune system in the early 2000's and it functions to attack foreign bacterial and viral DNA in prokaryotes [22]. This discovery, named for its genomic clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein, drew interest from an evolutionary standpoint at the time. It was not until eight years later that an application for this system would be discovered and would draw wider acclaim. Between 2013 and 2014, the laboratories of Drs. Feng Zhang and George Church described methods of editing DNA in vitro utilizing the CRISPR-Cas system [23,24]. It was not long after that many other investigators began to adopt and modify the system.

This system is comprised of two major parts, which can be roughly broken down into a guiding and an affecting portion. Responsible for specificity, the guiding portion consists of a single stranded RNA molecule, which is called single guide RNA (sgRNA) [25]. This RNA component targets a genomic region by complementing a specific DNA sequence and is associated with the affecting portion by way of a fused portion of scaffold RNA. This affecting component is comprised of one of several Cas proteins, the most common being Cas9. In its native state, this protein has double-stranded endonuclease activity [24,26]. After producing a cut in the DNA, donor DNA with a

desired sequence can be added to the target site [27]. Combining this endonuclease activity with the aforementioned RNA guide allows for highly specific, tightly regulated editing of genetic information in vitro and in vivo.

The CRISPR-Cas system is not, however, limited to inducing double-stranded breaks. The Cas protein can be modified to retain its targeting ability while losing its endonuclease activity [25]. This catalytically inactive Cas (dCas) can be used as is, or further modified with a number of different functional groups. Molecules (visualized in Figure 2) can be attached to dCas. These molecules can then be brought into proximity or attached to specified regions of the genome.

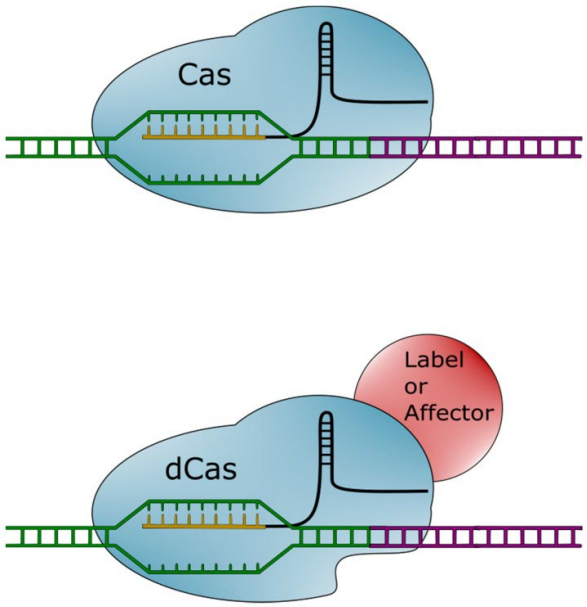


Figure 2. Diagram of clustered regularly interspaced short palindromic repeats-associated (Cas) system variants. The Cas (upper) system targets a specific genomic region (green) with its single guide RNA (sgRNA) (gold and black). The Cas protein will then make a cut in the adjacent DNA region (purple). The catalytically inactive Cas (dCas) (lower) system targets a genomic region with an identical system. However, the dCas protein lacks endonuclease activity. Various molecules (red) can be fused to the dCas protein. These include labels and affectors. Labels bring a fluorescent signal in close proximity to target DNA, while affectors can modify characteristics, such as the epigenetic state of DNA.

2. Telomeres – Imaging

One emerging use of this CRISPR-dCas system involves targeting telomeres for imaging. This system has a number of advantages over other systems, most of which stem from the dynamic and sustained nature of the CRISPR system. In one of the first imaging experiments involving CRISPR, Chen et al. were able to label telomeres in HEK293T, UMUC3, and HeLa cell lines with enhanced green fluorescent protein (EGFP) [28]. Within these cells, telomere movements were observed with a labeling efficiency and intensity akin to the well-established DNA FISH protocol. Further improvements that were made on this system may, in fact, result in greater labeling efficiency and specificity [29]. By replacing the EGFP with the brighter mClover fluorescent tag, labeled telomeres became even easier to detect and only produced negligible off-target effects [30]. Imaging telomeres is not in itself a new idea, but the unprecedented precision and efficiency of the CRISPR-Cas system provides a novel way to quickly and efficiently track telomeres.

The true advantage of CRISPR imaging is, however, its ability to be directly applied to living systems. While there are other methods of fluorescently labeling genomic elements, these are toxic to the cell and can result in irreparable DNA damage [31]. This limitation has disallowed the uninterrupted recording of telomeres and other genomic elements in vitro. Shao et al. were among the first to establish that labeling with the CRISPR-dCas had minimal cytotoxicity and was suitable for continuous viewing [32]. Their system was used to track telomeres and centromeres over a five-minute period and measured the relative movements of each during interphase.

Building on these ideas, Dreissig et al. were able to track telomere movements in leaf cells of Nicotiana benthamiana using dCas that was labeled with both EGFP and mRuby2 fluorescent tags [33]. Within the nucleus, they observed telomere movements of up to 2 μm during interphase. In addition, combining this technique with fluorescently labeled proteins allowed for the visualization of live protein-telomere interactions. By labeling both telomeres and end-binding protein TRB1, they found that these leaf cells appeared to contain chromosomes with both blunt and overhanging ends, a phenomenon that is not observed in mammals or fungi. While this study was limited to this single, specific protein interaction, future work could lead to an understanding of how telomeres interact with any relevant portions of the proteome.

More recently, this technique has been extended to transgenic mouse models [34]. By expressing dCas-GFP throughout a mouse, the guides for telomeres could be inserted into specific tissues for labeling. The group used this technology, combined with CRISPR-interference of the TRF1 gene, to observe the aggregation and fusion of telomeres in real time. This technology has the potential to be extended to other genes, allowing for the study of real-time changes in telomere dynamics after genetic manipulations.

3. Telomeres – Editing

As discussed earlier, disruptions and damage to telomeres can lead to a wide array of cellular dysfunction. The CRISPR-Cas system's ability to cut and insert genes allows for the real-time, in vivo study of telomere damage. Using this system to induce double strand breaks (DSBs) in telomeres resulted in the activation of a telomeric repair system

that was regulated by the Rad51 gene [35]. This study differs from previous conflicting and ambiguous results, which are likely due to a lack of precision in non-CRISPR induction of DSBs. Previous findings were clouded by the initiation of senescence and apoptotic pathways in DSB-induced cells, and this study provides an example of how the Cas system can be utilized to remove the noise from results.

Taking these ideas a step further, Kim et al. were able to completely remove telomeres in bone marrow neuroblasts and measure the effects on cellular function and senescence [36]. Telomere removal led to a cascade of cellular changes, chiefly a loss of mitochondrial function and an aggregation of Parkinson's disease (PD) associated proteins. This change lowered cellular viability and it has the potential to model both aging and PD in cells. Because this method only removes telomeres, it allows for the study of how this specific process contributes to cellular aging [13,14,36]. This process is important in establishing causality and removing ambiguity that could be associated with other cellular aging models.

CRISPR-Cas can also be employed to create more minor changes to telomeres. While the changes can be as small as a single nucleotide, they can have a major impact on a cell's biology. After inducing a mutation to a subtelomeric CTCF binding site, known as TERRA, the cells exhibited a loss of sister telomeres and reduced capacity for replication [37]. These issues were exacerbated by the induction of replication stress and it led to a higher rate of apoptosis. This study implicates CTCF and TERRA sites as being vital for

successful telomere replication and maintenance and elucidates their importance for the overall maintenance and stability of chromosomes.

While current research involving CRISPR-Cas ablation of telomeres has been limited, the ability to use the technology in any cell type allows for the study of a variety of different diseases. By utilizing these methods in different tissues, the effect of aging can be measured across a broad array of conditions. Further study has the potential to answer both biological and mechanistic questions regarding telomere loss and the disease states that it causes.

4. Telomerase – Imaging

While imaging the genomic region containing TERT with dCas is possible, it is not the nucleotide sequence itself that is primarily associated with biological function. Because of this, targeting and modifying the protein telomerase appears to be the most effective way to image and study its dynamics. By introducing a fluorescent marker at the TERT locus, Schmidt, Zaug, and Cech were able to distinguish three stages of telomerase movement [38]. The stages can be characterized as a rapid diffusion stage, a frequent, transient telomere-associating stage, and a rarer, long-term association stage that results in a majority of telomere elongation. In addition, telomerase appears to bind with the ssDNA overhangs and add multiple hexamer repeats in tandem [39]. Taking these results together provides a novel model for telomere formation; wherein, telomeres are elongated in short controlled periods following longer periods of transient association. Labeling and the subsequent imaging of telomerase using the CRISPR-Cas system allows for an unprecedented ability to study the spatiotemporal dynamics of telomerase movements

and recruitment. Understanding these dynamics is vital in the study of diseases, such as cancer, which utilize the protein to facilitate rapid, aggressive division.

5. Telomerase – Editing

One of the most common and valuable tools in biotechnology is modulating gene expression by knocking out or knocking in a gene. When doing so, comparing differences to wild type organisms allows for the parsing of genetic function. By targeting the promoter of hTERT, CRISPR-Cas can be used to both ablate and enhance TERT expression [40]. Mutations that led to silencing resulted in normally immortal cell lines senescing and eventually dying, while those that increased expression saw TERT levels that were akin to those found in tumor cell lines. As an unintended consequence of adding a protein Halo tag to the N-terminus of TERT, Chiba et al. found that they could modulate expression between these two extremes [41]. Additionally, they found a reduction of telomere lengths within these cell lines, implicating steric hindrance as a factor in telomere lengthening.

A different study by Xi et al. explored similar ideas, focusing on urothelial cancer cells [42]. These cells contain a single DNA substitution mutation in their hTERT promoter, which had previously been associated with high expression levels. The group used the CRISPR-Cas system to revert this mutation and observed a restoration in the baseline hTERT levels.

In a later study, the mechanism behind this phenomenon was explored. In this experiment, promoter mutations were induced by the CRISPR-Cas system [43]. These

mutants saw an increase in chromatin interactions upstream of the gene, as well as a recruitment of the transcription factor GABPA. This transcription factor directly recruits DNA polymerase II and it provides a possible mechanism for the activation of TERT caused by promoter mutations across multiple cancer types.

While targeting telomerase directly has important clinical implications in cancer treatment, there are still 10–15% of cancer cases that exhibit telomere lengthening without a corresponding increase in telomerase activity [19]. These remaining cancers are still able to replicate rapidly, so they must be lengthening their telomeres and preventing senescence by some other means. This alternative means of telomere lengthening (ALT) is the proposed mechanism for this prevention and it must be studied in order to achieve a full understanding of cancer proliferation [44]. One way to increase understanding of these ALT pathways is to generate a continuously dividing cell line that lacks telomerase activities. By utilizing CRISPR to knockdown both TERT and a cell death pathway that is known as ATRX/DAXX, cells that exhibited ALT pathways were formed. This ALT pathway can also be achieved through CRISPR mediated knockout of the RNA component of telomerase (TERC) [45]. The ALT pathway arose in only a tiny fraction of cells and led to telomere generation with large overhangs on the lagging strands. While ALT telomere elongation appears to be rare and it is still not fully understood, unraveling the mechanism and biology of the process is important for studying cancer that does not employ telomerase-associated telomere lengthening.

Due to their prevalence in the disease, understanding the significance of TERT mutations is vital in the understanding of cancer cell growth. CRISPR-Cas provides a powerful tool for affecting these mutations in live cells and it allows for the modeling of rapidly dividing cell lines. However, this modeling is not limited to cancer research. It has great potential to model aging as well as diseases that are characterized by cellular aging. The technique has already been used to reprogram hTERT in fibroblast cells, creating a novel model for Werner's syndrome [46]. With the ever-increasing understanding of disease mechanisms, it is even more important to be able to create accurate cellular models for these diseases. These models serve as a platform to test new drugs and therapies. It is therefore important that they have accurate, specific genetic states to ensure that treatments will translate from the laboratory into patients.

6. Genes that Affect Telomeres and Telomerase

As with any gene or protein within a biological system, telomeres and telomerase are affected by a suite of different genes. The CRISPR-Cas system allows for the identification and subsequent modification of these genes. Modifications to these genes often induce changes that are associated with cancer and aging, but some disease models can be induced independently of the two. One such gene is nuclear assembly factor 1 (NAF1) [47]. Within the cellular and mouse models CRISPR induced mutations of the NAF1 gene result in a loss of around half of cellular TERT activity. This mutation and the resulting expression loss form a profile that matches that of pulmonary fibrosis-emphysema. It is likely that this disease progresses by disrupting telomere homeostasis, a process that many aging and cancer-associated genes also influence.

In one of the earlier experiments to utilize the technology, CRISPR-Cas was used to confirm that the cold inducible RNA-binding protein (CIRP) functions in telomere maintenance at all temperatures and it modulates TERT expression at low temperatures [48]. With this gene knocked out, the overall telomere length was shorter than the controls. These results indicate that CIRP is necessary for mediating telomerase activity during hypothermia as well as under normal cellular conditions.

Likewise, Notch1, a gene that is normally involved in development, was also found to be necessary for proper telomerase function [49]. Without it, telomeres shortened and expressed phenotypes that are typical of aging cells. This result also exemplifies Notch1's role in cancer, due to evidence that telomeric shortening is important for early tumorigenesis [12,49]. The loss of telomeres leads to chromosomal instability, which is conducive to the development of cancer phenotypes. This change may also explain Notch1's pleiotropic association with tumor suppression and oncogenesis, as the shortening of telomeres is also associated with decreased replicative capacity [50].

Gu et al. saw similar results with a different gene and mechanism; the group found that, by disrupting CTC1, a part of the telomere-regulating complex, telomeres would undergo rapid elongation, followed by an acute breakdown [51]. Similar to Notch1, this breakdown leads to chromosomal instability. Another gene that was implicated as important for genomic stability was POLD3 [52]. Without it, cells lost telomeres, and these losses were likely due to the induction of DSBs. Cells that were deficient in POLD3 were unable to replicate efficiently and tended to have micronuclei. Ablating genes, such

as these with CRISPR/Cas, allows for the systematic study of the mechanisms of telomere and telomerase loss.

In some cancer types, telomere fusions result in massive rearrangements of genes on chromosomes as well as on localized hypermutation [53]. By using the CRISPR knockdown array, the group found evidence that these genomic events are caused by recombination after activity from the cytoplasmic nuclease TREX1. As both rearrangements and hypermutation can lead to complications in recognizing and treating cancer cells, understanding how they are formed as well as what genetic factors contribute to them further the understanding of the disease itself. The identification of TREX1 provides an important screening target for clinicians when considering treatment and genetic counseling.

7. Epigenetics – Editing

A critically important, yet understudied, avenue of telomerase biology is epigenetics. As epigenetic modifications can affect expression to a large degree, genes that modulate this expression are important targets for study. While there are no CRISPR-based studies involving the direct methylation or demethylation of the hTERT gene, there is some interest in the genes that enact these epigenetic changes. Cells that were deprived of the DNA methyltransferase 2 (DNMT2), which catalyzes the addition of methyl groups to tRNA, suffered both a decrease in telomere lengths and telomerase activity [54,55]. Interestingly, the loss of DNMT2 resulted in the compensatory upregulation of other DNA methyltransferases, including Dnmt1, Dnmt3a, and Dnmt3b. These methyltransferases primarily methylate DNA, which led to global DNA

hypermethylation. In turn, this hypermethylation induced cellular senescence apoptotic pathways. These results suggest that DNMT2 could serve as an important target for cancer and other telomere-associated disease.

Conversely, Cas-mediated knockout of the ten eleven translocation (Tet) proteins, which facilitate DNA demethylation, resulted in an elongation of telomeres [56]. Although the loss of a demethylator would suggest a higher level of methylation and results that are in line with those of the DNMT2 study, the lack of compensatory global methylation likely prevented telomere shortening and cell senescence [55,56]. This result directly implicates Tet in the maintenance of normal telomere lengths and underscores its importance as a target for cancer and aging therapies. Understanding how changes in methylation state are induced at the TERT locus is vital, as they may provide insight as to why the expression levels differ in disease states with no obvious mutation.

8. Conclusions

Aging is a pervasive and complicated process that results from the body's limited capacity to regenerate itself. Cancer is an almost as pervasive and equally complicated disease that hijacks these regenerative capabilities to proliferate unchecked. Due to their complex nature, parsing out the mechanisms of each appears to be a daunting task. However, the inception of CRISPR-Cas technology has provided a powerful tool that can be used to fashion rapid and specific genomic changes in living organisms. This system, as overviewed in Figure 3, has already made major contributions to the understanding of telomeres and telomerase in the context of aging and disease and it will undoubtedly continue to do so as the technology develops.

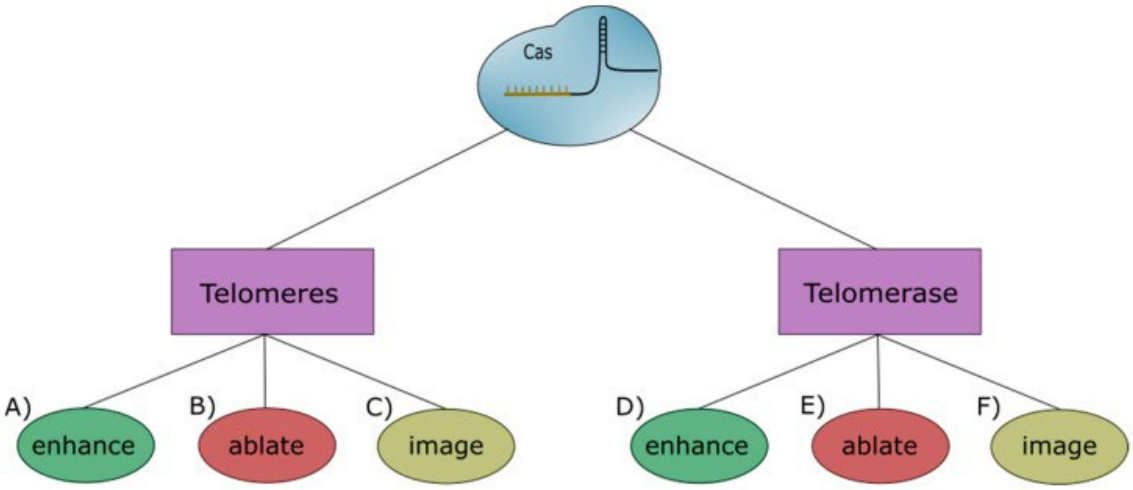


Figure 3. Overview of the uses for clustered regularly interspaced short palindromic repeats (CRISPR) in regards to telomeres and telomerase. Studies measuring an increase in telomere length (A) achieve this by activating telomere repair systems or increasing gene expression of proteins that build directly onto telomeres. As a whole, these studies observe normal to enhanced replicative capacity. Conversely, CRISPR-mediated ablation of telomeres occurs through direct removal and damage (B). These studies witness an upregulation of repair mechanisms as well as decreases in both cellular health and viability. Studies involved in imaging telomeres largely utilize fluorescently labeled dCas (C) and observe minimal cytotoxicity and high efficiency. These properties allow for real-time, in vivo study of telomere movements and interactions. Enhancing telomerase activity through interactions with other proteins and epigenetic changes (D) leads to increases in telomere length that can result in chromosomal instability. These changes can increase replicative capacity and led to cell phenotypes similar to cancer cell lines. The ablation of the telomerase gene occurs through direct action of the Cas protein or by activating genes that inhibit its transcription (E). Overall, these changes lead to a decrease in replicative capacity and/or an upregulation in alternate telomere lengthening mechanisms. Imaging telomerase involves directly introducing a fluorescent tag onto the TERT protein (F). This allows for the study of telomerase activity as well as the dynamics of telomere formation.

Acknowledgements

We would like to acknowledge the members of the Tollefsbol lab for their advice and support.

Author Contributions

A.C.B. and T.O.T. conceived of this review article and participated in all drafts of the manuscript. A.C.B. wrote the first draft of the manuscript with guidance from T.O.T.

Final editing and approval of the final draft were performed by T.O.T. All authors read and approved the final draft.

Funding

This work was supported in part by grants from the National Cancer Institute (R01 CA178441 and R01 CA204346).

Conflicts of Interest

The authors declare no conflicts of interest.

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PERIPUBERTAL NUTRITIONAL PREVENTION OF CANCER-ASSOCIATED GENE EXPRESSION AND PHENOTYPES

by

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Cancers (Basel)

Format adapted for dissertation

ABSTRACT

Simple Summary

Certain life stages, known as critical periods, during growth and development are thought to be important for later-life breast cancer initiation and progression. Nutritional factors, especially those found in plant-based diets, are believed to be key to the impact of these critical periods on cancer. However, there is currently little known with respect to how nutrition during critical periods can affect breast cancer. In this study we evaluated nutritional intervention during the critical period of puberty and whether it could have a significant effect on tumor phenotype, as well as underlying gene expression, protein expression and DNA methylation patterns. We found that sulforaphane-containing broccoli sprout extracts administered during the peripubertal period in mice were able to reduce tumor size and incidence while delaying latency. We also found gross changes to gene expression patterns, including many cancer-associated genes, as well as potentially important changes to methylation profiles in our treatment groups.

Abstract

Breast cancer (BC) is a nearly ubiquitous malignancy that effects the lives of millions worldwide. Recently, nutritional prevention of BC has received increased attention due to its efficacy and ease of application. Chief among chemopreventive compounds are plant-based substances known as dietary phytochemicals. Sulforaphane (SFN), an epigenetically active phytochemical found in cruciferous vegetables, has shown promise in BC prevention. In addition, observational studies suggest that the life stage of phytochemical consumption may influence its anticancer properties. These life stages, called critical periods (CPs), are associated with rapid development and increased

susceptibility to cellular damage. Puberty, a CP in which female breast tissue undergoes proliferation and differentiation, is of particular interest for later-life BC development. However, little is known about the importance of nutritional chemoprevention to CPs. We sought to address this by utilizing two estrogen receptor-negative [ER(-)] transgenic mouse models fed SFN-containing broccoli sprout extract during the critical period of puberty. We found that this treatment resulted in a significant decrease in tumor incidence and weight, as well as an increase in tumor latency. Further, we found significant alterations in the long-term expression of cancer-associated genes, including p21, p53, and BRCA2. Additionally, our transcriptomic analyses identified expressional changes in many cancer-associated genes, and bisulfite sequencing revealed that the antiproliferation-associated gene Erich4 was both hypomethylated and overexpressed in our experimental group. Our study indicates that dietary interventions during the CP of puberty may be important for later-life ER(-) BC prevention and highlights potential important genetic and epigenetic targets for treatment and study of the more deadly variants of BC.

Keywords: breast cancer, puberty, critical periods, cancer prevention, nutrition, gene expression, DNA methylation

INTRODUCTION

Breast cancer (BC) is a widespread malignancy and major source of financial, social, and medical hardship in the United States. BC is expected to account for ~15% of all cancer cases in women, and trails behind only lung cancer in terms of female cancer mortality [1,2]. Despite this, BC generally has favorable survival outcomes when compared to other cancer types. While many increases in survival are attributed to advances in early detection and treatments, the large remaining disparity between survival outlook and mortality is due to differences in disease severity among molecular subtypes of BC [3]. These subtypes are commonly categorized by the receptors present on the cell surface and include Luminal A, Luminal B, HER2-enriched, and triple negative (TNBC). As many of the most efficacious treatments are targeted to these receptors, the receptor-poor HER2 and receptor-negative TNBC are the most deadly and difficult to treat [3,4].

Interest in a cure for BC remains high, with over 500 clinical trials sponsored by the NIH today [5]. However, true cures for the deadliest forms of BC remain elusive, and successful treatment of any case of BC can result in a wide array of deleterious side effects, including loss of bone density, neuropathy, and cognitive decline [6,7,8]. Further, treatment can also result in financial hardships due to both the cost of treatment and loss of the ability to work post-therapy [9]. For these reasons, BC control through prevention is an appealing target for study that has the potential to minimize human suffering that arises from the disease.

Historically, cancer prevention has primarily been accomplished through avoidance of risk factors associated with specific cancer types, such as tobacco use for lung and oral cancers or alcohol consumption for stomach, breast, and prostate cancers [10,11]. However, cancer prevention can also be achieved though chemoprotective compounds administered before the onset of the disease. The two most common drugs used for BC prevention are Tamoxifen and Raloxifene, and both have been shown to reduce BC risk by around 40% [12]. However, these drugs can also lead to severe side effects, including induction of menopausal symptoms, blood clots, and, in the case of Tamoxifen, increased risk of uterine cancer. As a result, these drugs are often only prescribed to high-risk individuals for the purpose of cancer prevention.

Because of this, preventive interventions with wider scopes of use are in high demand. In particular, nutritional prevention of BC is a growing field that has potential for widespread use that is relatively inexpensive and efficacious with few to no side effects [13]. A group of chemopreventive compounds of high interest are those found within a plant-based diet and are collectively termed dietary phytochemicals. Sulforaphane (SFN), a dietary phytochemical found within cruciferous vegetables, has been shown to be efficacious in the prevention of BC within multiple mouse models [14]. These results are backed by clinical studies that inventoried the nutritional habits of BC patients and women in the wider population [15].

A major question that remains regarding nutritional prevention of BC concerns the timing of nutritional interventions. Specifically, it has been suggested that there are certain

windows of susceptibility in which breast tissue is both more vulnerable to damage and responsive to preventive measures [16,17,18]. For breast tissue, growth and development primarily occurs during the prenatal period, puberty, and time of first pregnancy [19,20]. Environmental exposures during these windows, termed critical periods (CPs) in the context of cancer development, include prenatal exposure to the miscarriage prevention drug diethylstilbestrol, as well as peripubertal/peripregnancy exposure to the pesticide DDT, and have been linked to increased risk for BC development [21,22,23]. The CP of puberty has drawn particular interest due to its close association with rapid breast development, as well as fluctuations in hormone levels, which are important for the development of many types of BC [24].

Initial studies most commonly linked early onset of puberty to increased BC risk, reasoning that an increased duration of hormonal exposure resulted in increased BC risk [18,24,25]. It has also been reported that specific foods consumed during puberty may have an effect on later-life BC development [26]. Specifically, there are some indications that diets high in fats during puberty may increase BC risk later in life [27]. Few studies, however, have explored how peripubertal diet can decrease risk for BC, and fewer still have evaluated this in a basic science setting.

In this study, we utilized two transgenic mouse models for estrogen receptor-negative BC to elucidate the effects of SFN-containing broccoli sprout extract (BSp) administered during the critical period of puberty. Our aim was to determine whether or not BSp given during only the peripubertal period could have a long-term effect on BC tumor

morphology, gene expression, and DNA methylation. We hypothesized that peripubertal BSp treatment would result in a decrease in tumor size and number and an increased latency period, as well as having long-term effects on both gene expression and global methylation patterns when compared to a standard diet during this time. To test this, we fed our mouse models SFN-containing BSp over a 5-week peripubertal period and measured tumor characteristics throughout life, as well as molecular changes that occurred at experimental termination. An overview of our experimental design is outlined in Figure 1 below.

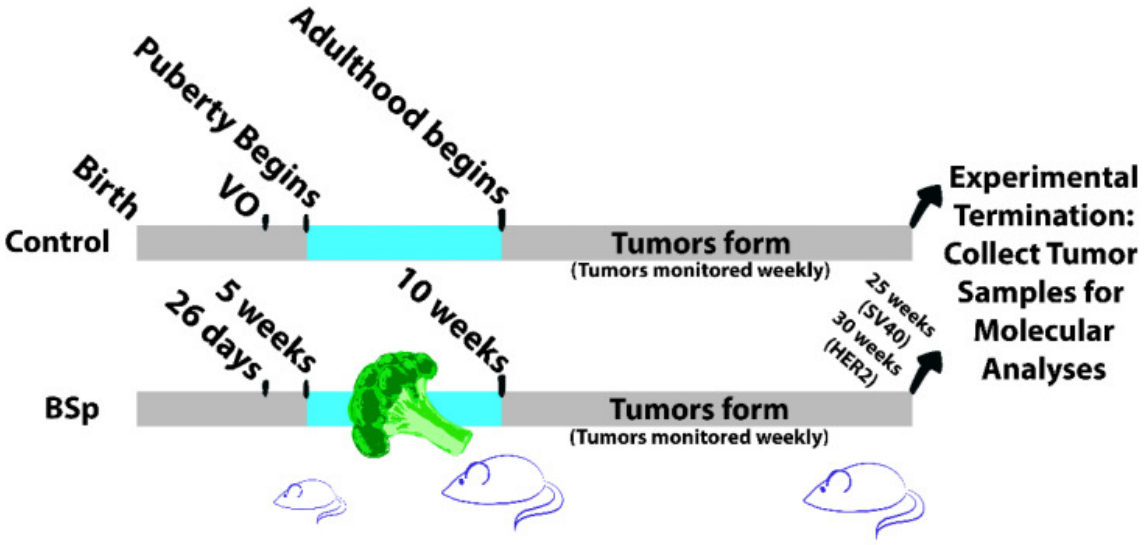

Figure 1. Overview of experimental design. Experiments were conducted in both SV40 and HER2/neu transgenic mouse lines. Puberty in mice begins about 10 days following vaginal opening (VO). Mice in the experimental (BSp) group received chow infused with SFN-containing BSp for a total of 5 weeks, beginning on the first day of the 5th week and ending on the last day of the 9th week. Mice were monitored for tumor formation beginning at 10 weeks of age. Experiments were terminated and tumor samples were collected for downstream analysis when tumor size reached 1 cm3. For all groups n = 24.

MATERIALS AND METHODS

Animal Housing and Husbandry

Animal Housing and Experimental Design

All mice were housed in the UAB Campbell Hall animal facility. Mice were bred at around 8–10 weeks of age and weaned at 21 days old, with genotyping performed at the time of weaning. Mice were fed and given water ad libitum. At five weeks of age, experimental group mice were given the BSp diet until 10 weeks of age. During this time, control mice were continuously fed with NIH-31 variety mouse chow. For both experimental and control groups, n = 24. This treatment window lasted a total of 5 weeks. Beginning at 10 weeks, both groups were fed NIH-31 variety. Beginning after weaning, puberty was monitored daily and confirmed in all females through observation of vaginal opening (VO). VO is a readily observable characteristic that occurs 7–10 days prior to the first ovulation in mice [28,29]. While mice typically reach sexual maturity between 6–8 weeks of age, additional behavioral and developmental changes continue to occur until around 10 weeks of age [30]. Throughout their lives, tumor size and incidence were measured in each individual on a weekly basis. For in vivo experiments n = 24.

Transgenic Mouse Lines

The C3(1)-SV40 Tag (FVB-Tg(C3-1-TAg)cJeg/JegJ) (SV40) mouse line typically develops tumors resembling Ductal Carcinoma in situ (DCIS) within the mammary epithelium at approximately 15 weeks of age, with 100% of female mice developing tumors by around 6 months of age [31]. The FVB/N-Tg(MMTVneu)202Mu (Her2/neu)

Her2/neu mouse line develops ER(-) mammary tumors beginning at 20 weeks of age with a median age of 30 weeks [32]. Both were available from the Jackson Laboratories as breeder pairs from 4 weeks of age.

Animal Diet

Chow infused with 26% broccoli sprouts (BSp) is commercially available from TestDiet and was produced by infusing control chow with BSp. The BSp was obtained through Natural Sprout Company (Springfield, MO) and was infused into pellets by TestDiet (Branchburg, NJ). This amount was equivalent to the consumption of 266 g (~4 cups) of BSp per day in humans, and this amount has already been shown to be both realistic and efficacious [33]. Total SFN content for BSp food at the concentration used (26%) is between 5.13 and 6.60 μM per gram of BSp [34]. Full information on the contents of experimental food is available in Supplementary Data S1. Control chow is the AIN-93G variety, and both BSp and control chow are administered ad libitum, with no detectable difference in the total volume of chow consumed. Diets were confirmed to have no effect on oncogenic driver expression.

Tissue Collection

Upon experiment termination, mice were sacrificed using CO2 according to ARP protocols. Breast tumor samples for experimental protocols were collected subdermally, flash frozen, and stored at −80 °C. Tumor weight was recorded at the time of termination. For potential future experiments, additional blood samples were collected through an

intracardiac puncture along with normal breast tissue that was collected subdermally. Organ tissues were collected from the thoracic cavity. All samples were stored at −80 °C.

Nucleic Acid Extraction and Analysis

DNA and RNA Extraction

All nucleic acid extractions were performed on frozen breast tumor samples from control and BSp-fed groups. Total RNA for qPCR was extracted utilizing a Qiagen RNeasy kit per the manufacturer's instructions. Total RNA for sequencing was extracted using TRIzol reagent based on the manufacturer's protocols. Genomic DNA was extracted using the Qiagen DNEasy kit according to the manufacturer's instructions. All nucleic acids were assessed for purity and concentration using a Nanodrop spectrophotometer.

qPCR

cDNA was synthesized per the manufacturer's instructions from 250 ng of RNA using iScript Reverse Transcription Supermix for RT–qPCR (BIORAD). Using the cDNA generated from this protocol, primers obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA), and SsoAdvanced Universal SYBR® Green Supermix (BIORAD, Hercules, CA, USA), quantitative real-time PCR was performed. These reactions were performed in triplicate using the CFX Connect Real-Time PCR Detection System (BIORAD). Thermal cycling began at 94 °C and was followed by 35 cycles of PCR (94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s). GAPDH served as an endogenous control, and a vehicle control was used for calibration. Relative changes in gene expression were

calculated through the 2-ΔΔCQ method, where ΔΔCQ = [ΔCQ(treatment group) − ΔCQ(control group)] and ΔCQ = [CQ(gene of interest)-CQ(GAPDH)] [35]. Relative expression levels of these genes were compared between treatment and control groups. For all qPCR experiments, n = 10. A full list of primers can be found in Supplementary Table S1.

Western Blotting

Total protein from around 50 mg of flash-frozen mammary tumors was extracted with T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, St. Louis, MO, USA), according to the manufacturer's protocol. Protein concentrations were ascertained utilizing a Bradford Assay, and denatured samples were subjected to electrophoresis on 4–15% NuPAGE Tris-HCl precast gels (Invitrogen, Waltham, MA, USA). Proteins were transferred onto nitrocellulose membranes and subsequently probed with antibodies to p21, p53, and BRCA2. Actβ was used as the loading control for each membrane. Antibody details can be found in Supplementary Table S2. Protein bands were visualized using Clarity Max™ Western ECL Blotting Substrates (Bio-Rad, Hercules, CA, USA) on a ChemiDoc™ XRS + System (Bio-Rad). Protein expression was quantified using ImageJ. For Western Blot experiments n = 6.

RNA Sequencing

RNA sequencing was performed in a manner similar to previous work in our laboratory on tumor samples taken from control and BSp-fed groups [36]. RNA-seq was performed on extracted RNA by the UAB Heflin Genomics Core utilizing an Illumina NextSeq500

(Illumina, San Diego, CA, USA). Samples were assessed for quality using FastQC (v0.11.4) and aligned to the mouse reference genome GRCm38/mm10 using the default parameter settings of Kallisto. Further BAM file processing was performed with Kallisto, and transcription-level abundance estimates were generated for each sample file [37]. Following this, these estimates were input into the tximport package in R, allowing for gene-level expressional analysis [36,38]. Identification of differentially expressed genes (DEGs) was conducted with the Limma package in R, wherein the significant threshold for DEGs was set to $|\log2(\text{fold-change})| > 2$ and false discovery rate (FDR) ≤ 0.01. To identify enriched pathways, we utilized the web-based gene ontology analysis tool WebGestalt with our enriched gene list [39]. For RNAseq analyses, n = 7.

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS was performed similarly to previous work in our laboratory on breast tumor samples taken from both control and BSp-fed groups, and pair-end libraries were generated and sequenced by the UAB Heflin genomics core using an Illumina NextSeq500 [36]. Samples were assessed for quality using FastQC and trimmed using trim_galore based on the NuGEN Ovation RRBS system. These reads were aligned to the aforementioned mouse genome using Bismark alignment with default parameter settings. Utilizing the bismark_methylation extractor, CpG site call files were generated.

Analysis of differentially methylated regions and genes (DMRs and DMGs) was conducted with the methylKit package in R (v 3.6.1) utilizing the call files generated from the Bismark_methylation extractor [36,40]. DMRs and DMGs were identified based

on a false discovery rate of ≤0.05, and methylation profiles between the control and BSp-treated group were generated through hierarchical clustering with the hclust package in R.

To build on our understanding of the association between methylation and gene expression, identified DMRs were analyzed for correlation with DEGs. DMR-DEG pairs that were significantly correlated ($p < 0.05$) were identified. For RRBS, $n = 7$.

Statistical Analyses

For all experiments, the statistical significance of expression differences, as well as tumor latency and size between experimental and control samples were determined using a Student's T-test performed in Microsoft Excel. For tumor incidence, additional tests for significance were performed using a Chi-Squared test in SPSS statistical software (IBM) [34]. For all tests, a cutoff of $p < 0.05$ was considered statistically significant, with $p < 0.05$ being indicated by *, $p < 0.01$ being indicated by **, and $p < 0.001$ being indicated by ***. A minimum sample size of 11 was calculated using the 2-Sample, 1-Sided online power calculator found at powerandsamplesize.com [41]. For this calculation, a power of 0.8 and a significance of $p = 0.05$ were used.

RESULTS

BSp Administration during the Peripubertal Period Resulted in a Decrease in Mammary Tumor Formation in Both SV40 and HER2/neu Mice

As depicted in Figure 2, overall tumor incidence for both SV40 and HER2/neu mice was significantly reduced for mice treated with BSp-infused chow. In SV40 mice, tumor formation began around 16 to 17 weeks in controls, with a more noticeable separation between control and treatments groups occurring after 21 weeks. In HER2/neu mice, tumor formation began around 21–22 weeks in controls, with a gap in incidence forming around 26 weeks. However, as HER2/neu mice reached 100% incidence, this separation closed. For HER2/neu mice, peripubertal BSp treatment also resulted in a significant decrease in tumor weight of approximately 0.9 g (Figure 3a) and a significant increase in tumor latency (Figure 3b). In SV40 mice, tumor latency followed a similar trend, but was not at significant levels (Supplementary Figure S1) and tumor weight was insignificant. In addition, BSp treatment had no significant effect on overall body weight (Supplementary Figure S2) or the timing of VO (Supplementary Figure S3). For both breeds of mice, the approximate tumor size was larger in control mice throughout life (Supplementary Figures S4 and S5).

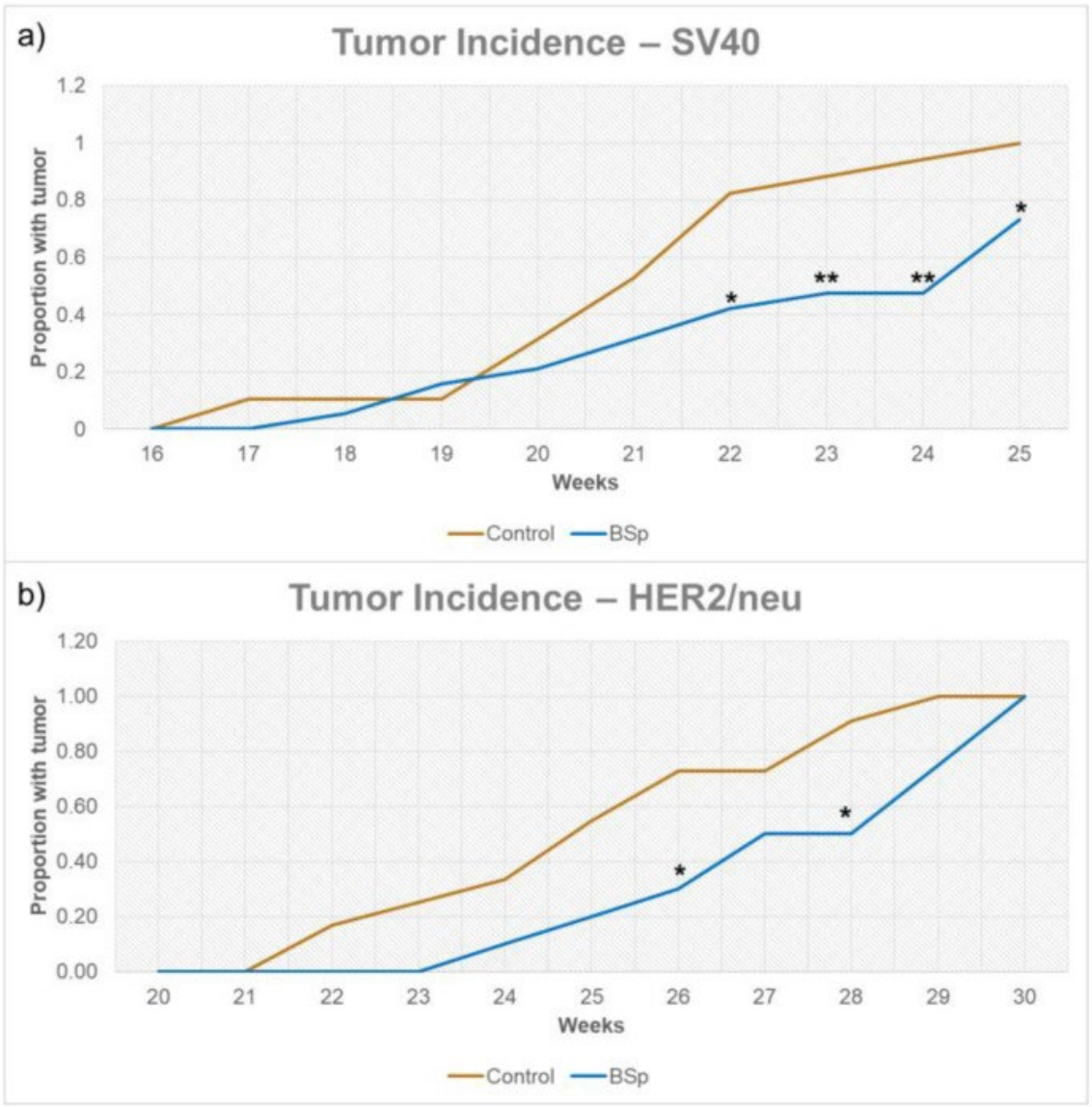

Figure 2. Tumor incidence for SV40 (a) and HER2/neu (b) mice. Tumor formation began around 16 weeks for SV40 mice and 21 weeks for HER2/neu mice. For both controls and experimental mice in both SV40 and HER2 experiments n = 24 mice. * = $p < 0.05$ and ** = $p < 0.01$.

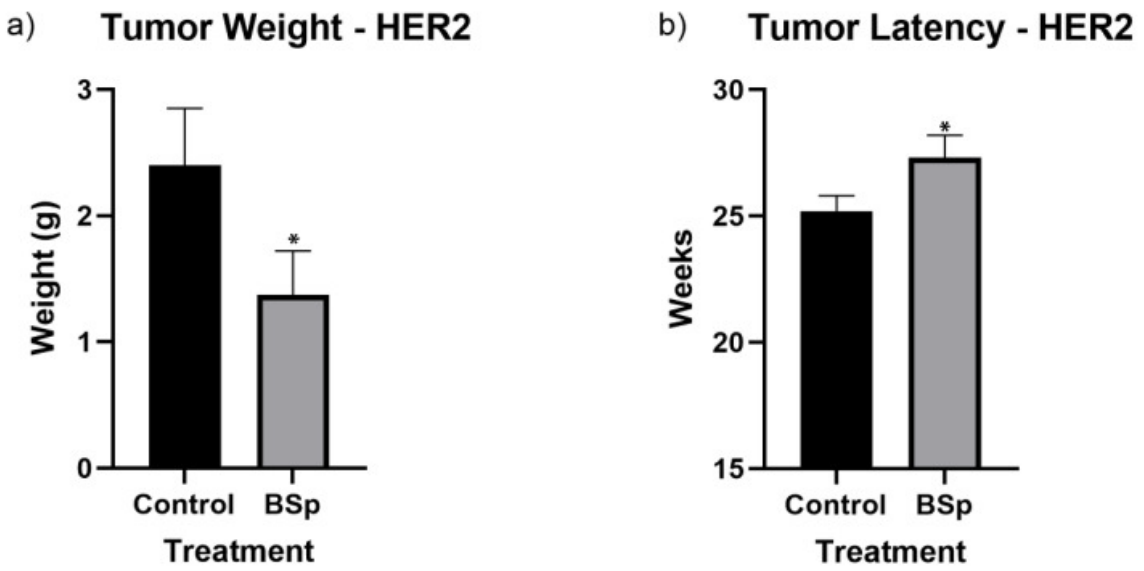

Figure 3. Tumor weight (a) and latency (b) of HER2/neu mice. BSp treatment significantly decreased mean tumor weight by 0.9 g and significantly increased mean tumor latency. For each group n = 24 mice. * = $p < 0.05$.

BSp Administration during the Peripubertal Period Resulted in an Increase in Gene Expression of Key Cancer-Associated Genes in HER2/neu Mice

In order to ascertain how molecular mechanisms may be affecting our observed changes in tumor incidence, we performed RT-qPCR and Western blot analyses on key cancer-associated genes (Figure 4). Because the HER2/neu mice had a more robust response to BSp treatment, further molecular analyses were conducted on HER2/neu tumor samples. We found that in HER2/neu tumor samples, p21 (a), p53 (b), and BRCA2 (c), gene expressions were significantly upregulated. We also found significant increases in the protein expression of both p53 (d and g), p21 (e and h), and BRCA2 (f and i). We also evaluated expressional changes in BRCA1 and tert, but there was no significant difference in expression levels.

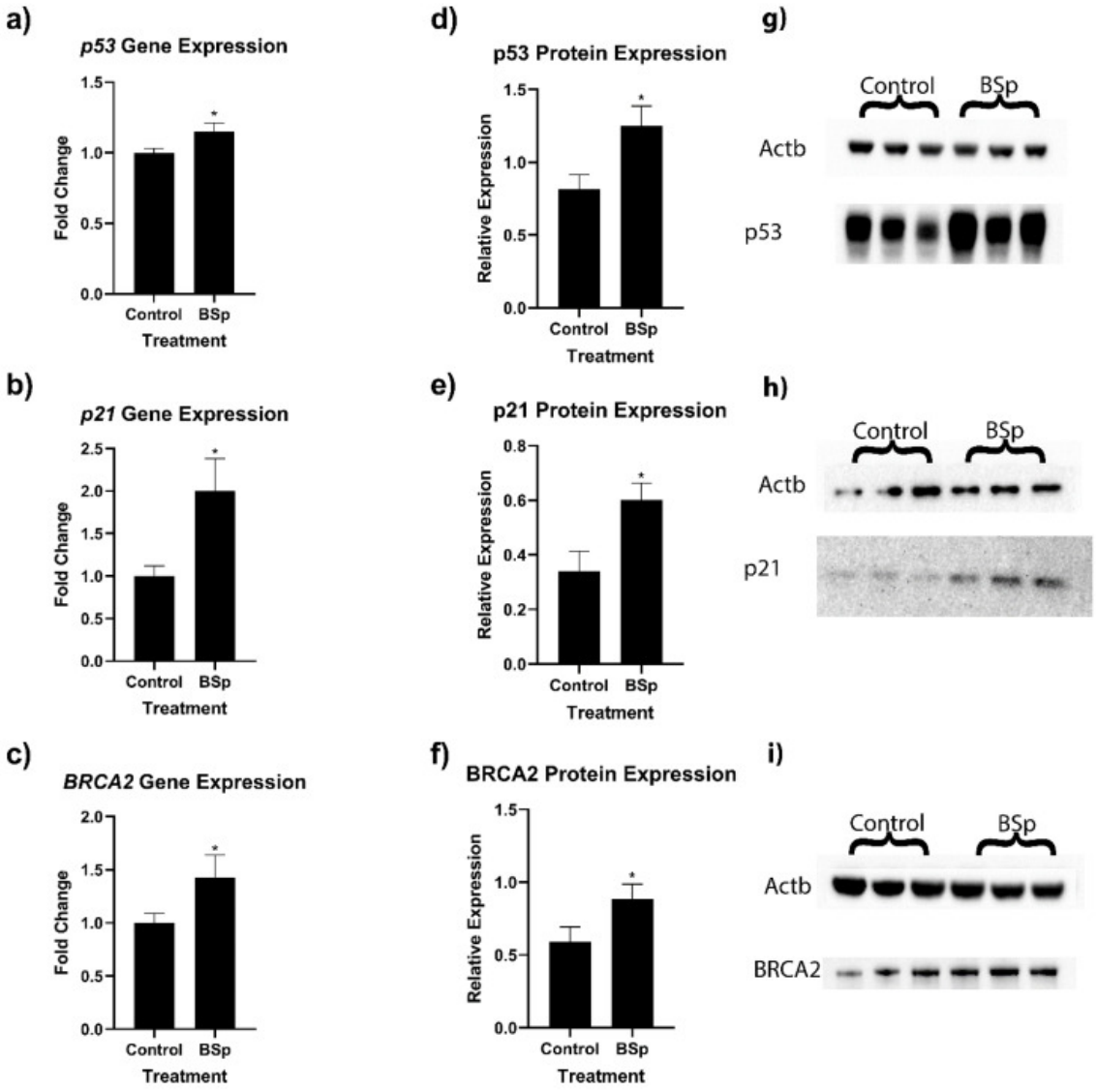

Figure 4. Expression of key tumor suppressor genes in HER2/neu mouse breast tumors. We observed significant increases in relative gene expression in p53 (a), p21 (b), and BRCA2 (c). We also observed significant increases in protein expression for p53 (d,g), p21 (e,h), and BRCA2 (f,i). For RT-qPCR, n = 10, and for Western blots, n = 6. * = $p < 0.05$. The uncropped blots are shown in File S1.

BSp Administration during the Peripubertal Period in HER2/neu Mice Resulted in Gross Changes to Gene Expression Profiles, with Effects on Many Key Cancer-Associated Genes and Pathways

To achieve a more wholistic view of gene expression changes, as well as to identify potentially important candidate genes, we performed RNA-seq analyses. We found significant expressional changes in 174 genes in the BSp-treated group when compared to the control. Overall, there were 92 genes downregulated and 82 genes upregulated in the BSp-treated group, and the top 20 downregulated and upregulated genes ranked by gene-expression fold change are displayed in Table 1 and Table 2, respectively. Several candidate genes were selected for PCR verification, including candidate oncogenes Chrdl2, Pcsk1, and Slc51b from the downregulated gene list and candidate tumor suppressors Lman1I, Clec4e, and Parp6 from the upregulated gene list (Supplementary Figure S6). A full list of genes along with their expressional changes is available in Supplementary Data S2.

Table 1. Top 20 downregulated genes in BSp-treated breast tumor samples vs. control tumor samples. For both groups n = 7 mice and significance cutoff was an adjusted p value < 0.05.

Gene ID	Name	Log FC	Avg. Exp.	adj. *p* Value
Pcdhb1	Protocadherin Beta 1	−3.39609	−4.2328285	0.0008754
Vmn2r29	Vomeronasal 2, receptor 29	−2.91192	−4.2173375	0.04718332
Gm21962	Predicted gene, 21962	−2.86629	−3.8732011	0.02353836
Gm28778	Predicted gene 28778	−2.681	−4.5447846	0.0008754
Mroh8	Maestro Heat Like Repeat Family Member 8	−2.55521	−4.6539474	0.00107869
Olfr798	Olfactory receptor 798	−2.33029	−4.2038604	0.00553995
Prss39	Protease, serine 39	−2.30801	−4.0433775	0.04061123
Olfr837	Olfactory receptor 837	−2.27784	−4.3915511	0.00413192
Olfr592	Olfactory receptor 592	−2.21951	−4.1896308	0.00413192
Olfr1181	Olfactory receptor 1181	−2.21231	−4.669899	0.04061123
Neurod6	Neuronal Differentiation 6	−2.12643	−4.5476479	0.00802338
Gm2897	Predicted gene 2897	−2.11836	−4.4376761	0.04726518
Slc51b	Solute Carrier Family 51 Subunit Beta	−2.11418	−4.6957968	0.02815029
Vmn1r210	Vomeronasal 1 receptor 210	−2.11002	−4.4579116	0.02274392
Olfr1228	Olfactory receptor 1228	−2.06907	−4.8911034	0.00490394

Gene ID	Name	Log FC	Avg. Exp.	adj. *p* Value
Pcsk1	Proprotein convertase subtilisin/kexin type 1	−1.95999	−2.733677	0.00720816
Srarp	Steroid Receptor Associated And Regulated Protein	−1.9012	−4.7484372	0.04726518
Hoxc10	Homeobox C10	−1.89364	−4.2726565	0.01709571
Chrdl2	Chordin Like 2	−1.799	−4.4780566	0.04187643
Slc6a21	Solute carrier family 6 member 21	−1.77935	−5.1522347	0.02831963

Table 2. Top 20 upregulated genes in BSp-treated breast tumor samples vs. control tumor samples. For both groups n = 7 mice and significance cutoff was an adjusted p Value < 0.05.

Gene ID	Name	Log FC	Avg. Exp.	adj. *p* value
Gm2237	Predicted gene 2237	3.158839	−4.602845	0.0008754
Lman1l	Lectin, Mannose Binding 1 Like	2.951392	−4.1578837	0.00107869
Clec4e	C-Type Lectin Domain Family 4 Member E	2.886333	−3.9958048	0.01925175
Kif19b	Kinesin Family Member 19	2.583289	−4.5418484	0.0008754
Armc12	Armadillo Repeat Containing 12	2.483762	−4.2946274	0.00113373
Tmem200c	Transmembrane Protein 200C	2.018997	−4.6153797	0.02619552
Olfr361	Olfactory receptor 361	1.983424	−4.2906929	0.04288585
Sncb	Synuclein Beta	1.923413	−4.9915667	0.01746493
Tmem59l	Transmembrane Protein 59 Like	1.858611	−4.7316851	0.02557444
Gm11168	Predicted gene 11168	1.802597	−4.6737187	0.03114816
Erich4	Glutamate Rich 4	1.791582	−4.6379376	0.04726518
Treml1	Triggering Receptor Expressed On Myeloid Cells Like 1	1.710026	−4.8032762	0.01326325
Olfr922	Olfactory receptor 922	1.645667	−4.8070893	0.01625371
Mmp13	Matrix metallopeptidase 13	1.119203	1.333663	0.0463051
Prmt6	Protein Arginine Methyltransferase 6	1.092539	3.0074275	0.00815202
Tspan4	Tetraspanin 4	0.904403	5.6742821	0.04244361
Sphk1	Sphingosine Kinase 1	0.898623	4.7451435	0.01787144
Parp6	Poly(ADP-Ribose) Polymerase Family Member 6	0.806976	2.9441413	0.0113267
Adam8	ADAM metallopeptidase domain 8	0.779123	3.0242021	0.04726518
Coq10b	Coenzyme Q10B	0.622019	4.0602847	0.02931014

To understand the biological processes that were affected by gene expression changes, we utilized our RNA sequencing results for gene ontology analysis. For both downregulated and upregulated genes, we identified significantly affected pathways (adj $p < 0.05$) in the categories of biological processes, molecular function, and cellular components. A summary of the five largest groupings, as well as those with known effects on cancer biology are outlined in Figure 5. These groupings are not mutually exclusive, and some genes were excluded due to a lack of information on their known biological function. A full list of genes within their respective GO groupings can be found in Supplementary Data S3 and S4 for downregulated and upregulated genes, respectively. In addition, full figures generated by the Webgestalt program are available as Supplementary Figures S7 and S8.

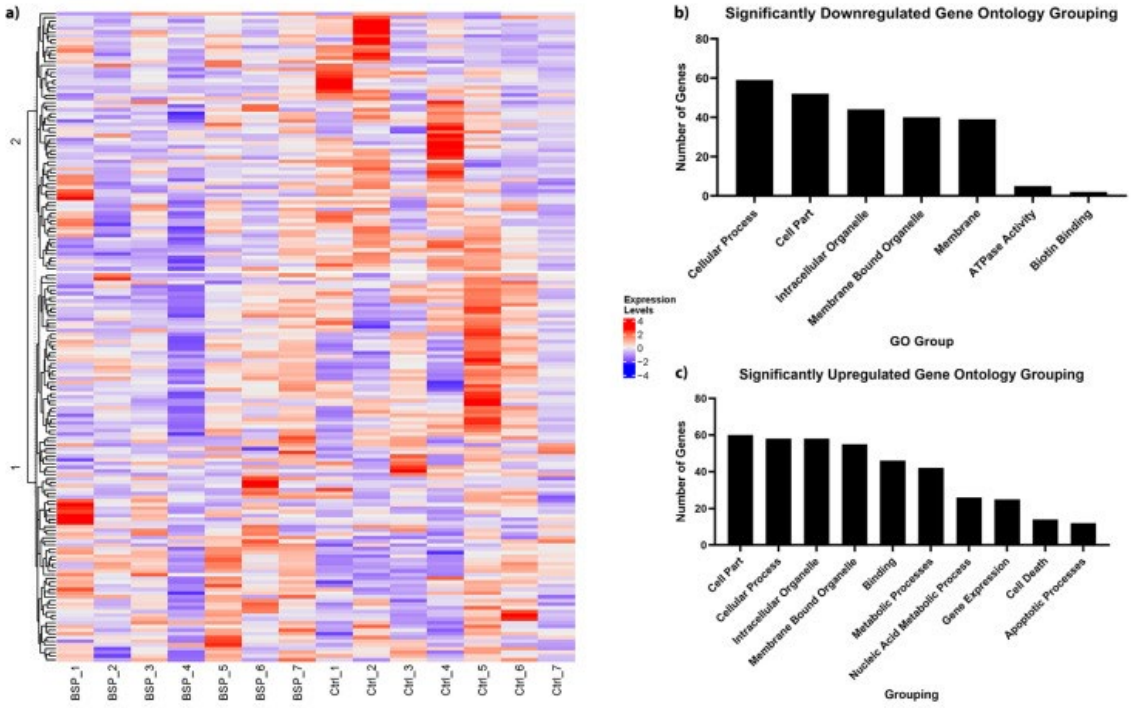

Figure 5. RNA sequencing heatmap (a), downregulated gene ontology (b), and upregulated gene ontology (c) analyses for breast tumor samples of peripubertal BSp-treated HER2/neu mice. For RNA-sequencing (a), n = 7 and genes were deemed significant if their adjusted p-value was $p < 0.05$. RNA sequencing results informed GO analyses in (b,c). For GO analyses, a cutoff of adjusted $p < 0.05$ was used to determine significant enrichment. The histograms shown here represent the GO groupings with the top 5 highest gene counts, as well as other groupings with physiological relevance to cancer biology.

BSp Administration during the Peripubertal Period in HER2/neu Mice Had a Lasting Effect on Genome-Wide Methylation, and was Associated with Increases in Expression to the Anti-Proliferation Linked Erich4 Gene

To build on our understanding of how epigenetic effects may be playing a role in expressional and phenotypic changes, we followed our RNA sequencing work with whole genome RRBS analysis. Overall, we found CpG methylation changes in 243 genes, with increases in methylation levels for 113 genes and decreases in methylation for 130 genes

in the BSp group relative to control (Figure 6a). To ascertain how these methylation changes may affect expression, we integrated our RNA sequencing and RRBS analyses. We found that out of the 243 differentially methylated genes and 174 differentially expressed genes, the gene Erich4 was differentially methylated and expressed (Figure 6b). In the BSp-treated group, Erich4 was both hypomethylated and overexpressed, and these high expression levels were verified with RT-qPCR (Figure 6c). A full list of differentially methylated genes is available in Supplementary Data S5.

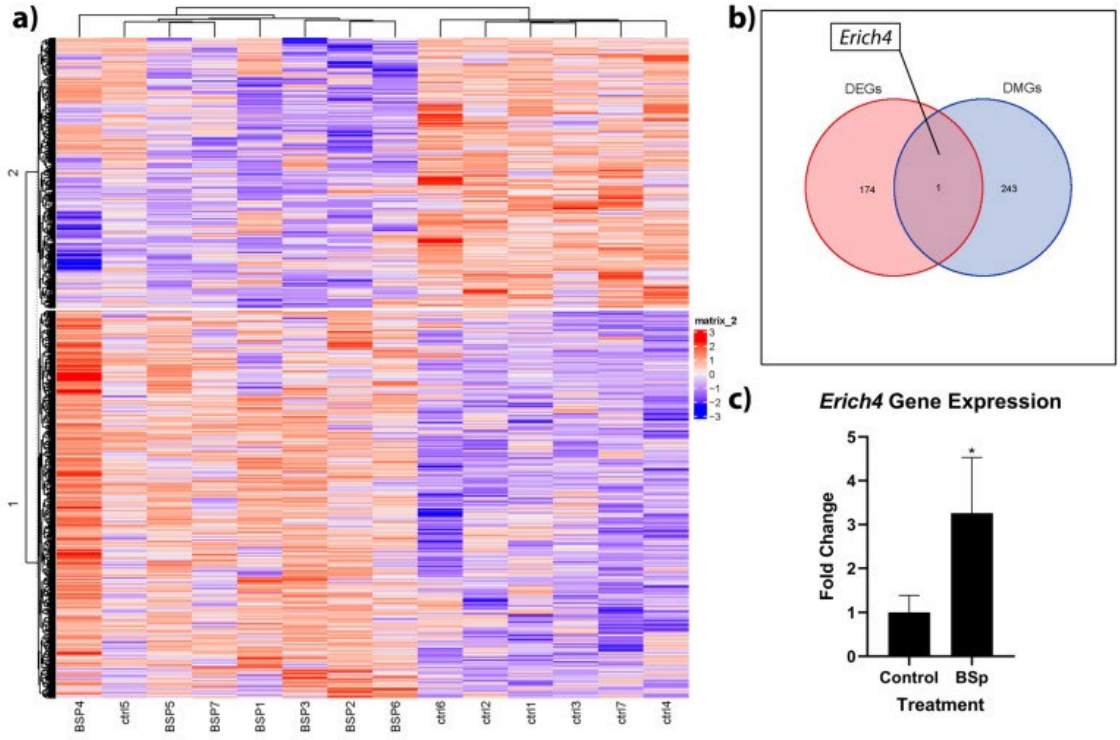

Figure 6. Heatmap of differentially methylated genes within breast tumor samples of BSp-treated mice vs. controls (a), as well as subsequent Venn diagram (b) of overlap between differentially expressed (red) and differentially methylated (blue) genes. Erich4 was hypomethylated and overexpressed, and these results were verified with RT-qPCR (c). For RRBS and RNAseq, n = 7. For RT-qPCR, n = 10. * = $p < 0.05$.

DISCUSSION

Despite continued advances in BC detection and therapies, BC mortality remains a leading cause of death for women worldwide. Preventive interventions have the potential to greatly reduce disease burden, thereby saving lives while simultaneously minimizing the economic strain associated with conventional therapies. In particular, nutrition-based prevention is relatively inexpensive, easy to implement, and has nearly no detectable negative side effects. Based on clinical observations of BC patients, there is evidence that nutritional prevention may be important at key life stages such as puberty [26]. Because the peripubertal period is a time in which nutritional compliance is feasible through school and parental supervision, understanding the relevance of this time period for later-life BC prevention may be vital for cancer control planning. Our work is among the first to study the effects of a known chemopreventive administered during puberty in a basic science setting. Our results indicate that SFN-containing BSp administered during puberty is sufficient to reduce tumor burden, which includes a significant decrease in size, a decrease in incidence, and an increase in latency, as well as having a profound effect on long-term gene expression.

Earlier work from our lab indicates that BSp treatment has no significant effect on tumor incidence or latency when it begins to be administered at adulthood (defined in that study as beyond 8 weeks of age) and continues until termination [42]. This contrasts the effects witnessed in both SV40 and HER2/neu mice when BSp treatment is administered during the peripubertal period (beginning at 5 weeks of age and ending at 10 weeks of age). When compared to previous work in which BSp was administered throughout life, our data, as expected, showed more modest effects on tumor size, latency, and incidence [42].

However, our treatment window lasted only 5 weeks (vs. 29 weeks in the prior lifelong study), and our significant results indicate that BSp intervention during this period alone can result in significant decreases to both tumor burden and incidence. While the idea that pubertal diet can have an effect on later life BC development has been documented in clinical cohorts, a majority of these studies only examined high-fat diets as a mechanism of increased risk [26,27,43,44]. In addition to previous work from our laboratory, clinical observations have indicated that diets rich in cruciferous vegetables and, by extension, SFN have a chemoprotective effect, resulting in decreased chances of developing or dying from BC [34,42,45]. However, this study is among the first to show that intervention during critical periods such as puberty may be important for the prevention or delay of later-life BC development.

Along with observing these phenotypic changes, we also generated a unique expression profile for our peripubertal BSp treatment. To determine the potential for long-term gene expressional changes, we performed RT-qPCR on the BC-associated genes p21, p53, BRCA1, BRCA2, and tert. Our results indicated significant increases in expression for the tumor suppressor genes p21, p53, and BRCA2 in HER2/neu mice. Protein validation found that there were significantly higher levels of p53, p21, and BRCA2 proteins at the time of experimental termination, indicating that these expressional changes were robust. Overall, these genes are closely tied to BC, and overexpression of each of these genes has importance for curbing BC development. Specifically, p21 is not typically mutationally deactivated in the course of BC development, so upregulation of this gene may have preventive and therapeutic potential [46]. p21 has also been shown to reduce BC burden

and can be upregulated by the BC drug Valtrate [46,47,48]. p53 is among the mostly widely studied tumor suppressor genes and its mutational loss is vital to approximately 35% of BC patients and 80% of TNBC cases [49]. Because of this, upregulation of a mutated p53 is unlikely to achieve therapeutic success. However, from a prevention standpoint, upregulation of WT-p53 in a precancerous cell could result in apoptotic destruction of the cell before full cancer cell transformation could occur. BRCA2 is well known for its impact on BC, with its heritable mutation to either BRCA1 or BRCA2 being responsible for up to 10% of all BC cases in Western countries [50,51]. Much of the current body of research on BRCA2 is associated with its loss, but lower expression levels are associated with decreased ability to repair DNA double-strand breaks, as well as an increased risk of developing BC [50,52]. In the context of our study, these three upregulated tumor suppressors have well documented anticancer effects, and it is likely that their increase in expression is responsible in part for the more favorable tumor characteristics we observed.

To build on our understanding of how peripubertal BSp treatment could affect gene expression patterns, we performed RNA sequencing. We identified 174 DEGs in the BSp-treated group, with a total of 82 upregulated and 92 downregulated genes. Within these groups, there are several genes that may be responsible for the phenotypic effects we observed, and several are known or are candidate tumor suppressors and oncogenes.

Within our top 20 downregulated genes, our treatment had a significant long-term effect on the oncogenes Chrdl2, Pcsk1, and Slc51b. Chrdl2 is an oncogene that has been

associated with poor prognosis in colorectal cancer cells where it is known to be an inhibitor of apoptosis [53]. In BC, its overexpression is associated with increased capacity for bone metastases, and decreased expression results in decreased proliferative capacity of osteosarcoma cells [54]. Taken with our results, this suggests that BSp treatment during the peripubertal period could result in less severe BC outcomes, as well as fewer metastases. Pcsk1 is over-expressed in breast and colorectal cancers, with poor prognoses being associated with this expression [55,56]. The current consensus is that Pcsk1 is important for tumorigenesis, so downregulation of this gene may be important for the anticancer effects that we observed [55]. Slc51b is indicative of poor prognoses in hepatic cancer and its overexpression results in increased proliferation and invasion [57]. Downregulation of this gene with our BSp treatment may explain the differences we found in tumor size in our HER2/neu mouse lines.

Conversely, within the top 20 upregulated genes, our treatment had significant long-term effects on the tumor suppressors Lman1I, Clec4e, and Parp6. Lman1l is necessary for proper excretion of the angiogenesis and tumor growth inhibitor A1AT, and loss of Lman1l has been associated with both colorectal and prostate cancers [58,59]. While there is little current research on the importance of Lman1l in BC, its high expression combined with its known molecular function may explain the lower tumor weight we observed in BSp-treated HER2/neu mice. Clec4e is an important regulator of the immune response, and higher levels of its expression are correlated with increased immune cell infiltration in hepatocellular carcinoma [60]. As infiltration of CD4+ and γδ T cells is associated with better overall and disease-free survival in BC patients, our overexpression

findings may explain the increase in latency in HER2/neu mice, as well as the decrease in incidence we observed in both mouse strains [61]. Parp6 is a member of the PARP family, which are typically known as oncogenes [62]. However, Parp6 has been shown to be a negative regulator of cell proliferation function via downregulation of Survivin in colorectal cancer and high PARP6 expression has been correlated with better tumor cell differentiation [62,63]. If these effects extend to our BC model, this may explain a portion of the reduction in tumor size and incidence witnessed in our peripubertal treatment group.

For a more wholistic understanding of the pathways and cellular functions associated with DEGs we identified, we utilized WebGestalt, a web-based gene ontology toolkit [39]. For the 21 significantly downregulated gene pathways and 65 significantly upregulated pathways, there were several standout groups with respect to cancer biology. Within the downregulated genes we discovered that both ATPase activity pathways (five genes) and biotin binding pathways (two genes) were significantly enriched. High expression of vacuolar ATPases have been implicated in cancer cell survival, development of drug resistance, and metastasis [64]. Interestingly, Wrn, Abcd4, Ythdc2, and Dync1h1 within this group are noted to have oncogenic function in colon, breast, gastric, and colorectal cancers, respectively [65,66,67,68]. Downregulation of these genes and this pathway may explain the differences we found between the control and peripubertal BSp-treatment groups. Biotin can act as an alternate energy source to sustain tumor cell proliferation and biotin buildup, and increased transporter expression often occurs in cancer cells, including BC cells [69,70]. Because of this, biotin-bound

molecules can also more easily enter cancer cells. In our study, we observed downregulation of biotin-binding genes HLCS and ACACA. HLCS is a gene important for biotin transport and is predictive of lymph node metastases and poor prognosis in BC [69]. In glioblastoma xenograft models its overexpression is predictive of poor prognosis and depletion disrupts tumorigenicity [71]. ACACA is relatively more poorly understood, but downregulation in mouse models has been found to suppress prostate cancer progression and lower tumor volume [72]. Our treatment's downregulation of these genes correlated with our anticancer in vivo results and were congruent with these studies, so it is possible that changes in expression of these genes are responsible, in part, for our observations.

Within our upregulated gene enrichment set, there was a large number of metabolic processes affected (42 genes) with a particular focus on gene sets that affect gene expression (25 genes) and nucleic acid metabolism (26 genes). Upregulation of these gene sets may give us some indication as to how peripubertal BSp treatment alone could have such a profound long-term effect on overall gene expression patterns. Possibly more significant, however, is our treatment's effect on genes within the cell death grouping (14 genes) and its subset of apoptotic processes (12 genes). Several genes within this group, including BLCAP, CCAR1, and TRIM39, have expressions that are linked directly to apoptosis or cell cycle arrest [73,74,75]. Upregulation of these and other apoptosis-associated genes may explain the more favorable tumor phenotype we observed in the peripubertal BSp-treated group.

Finally, we sought to determine if peripubertal BSp treatment could have long-term effects on the methylome and what part, if any, these methylation changes had on our DEG profiles. Past work in our lab has implicated SFN within BSp as having a significant effect on DNMTs and global methylation patterns, both in vitro and in vivo [36,76]. We measured a total of 243 differentially methylated genes (DMGs), with hypermethylation in 113 genes and hypomethylation in 130 genes. Our results indicate that peripubertal BSp treatment does have an effect on methylation patterns. While there is in vitro evidence that SFN contained within BSp acts as a DNMT inhibitor; our results, as well as those of our previous work, indicate that in vivo effects of SFN are more complex, having both hypo- and hyper-methylating effects on the epigenome [36,76,77].

With 174 DEGs and 243 differentially methylated genes, we identified a key gene, Erich4, that was differentially methylated and expressed. Additionally, known as glutamate rich 4, Erich4 is relatively poorly understood, but its low expression has been associated with renal cell carcinoma and its mutational loss has been recorded in basal cell carcinoma [78,79]. Interestingly, one of its few known interactions is with PPP2R5A, a gene implicated in negative control of cell growth [80]. If this interaction is important in stemming cancer cell proliferation, it is possible that the hypomethylation and overexpression of Erich4 with BSp treatment contributed to the anticancer effects that we found in vivo.

This study provides an important first step in understanding how dietary phytochemicals, such as SFN-containing BSp, can provide long-term protective effects when administered

during a CP. We observed significant reductions to tumor severity with peripubertal treatment, but future work remains on the impact of other CPs and their relative importance to overall BC prevention. We also observed changes in the expression of many genes, including known and potential cancer-associated genes, and further studies will be required to determine which of these genes are more important to tumor progression and morphology. Finally, our results indicate that our peripubertal treatment can have a significant impact on global DNA methylation patterns. Further study will be necessary in understanding the mechanistic basis of the expressional changes we observed.

CONCLUSIONS

Overall, we found long-term reductions in the cancer phenotype, as well as changes to gene expression and methylation profiles due to dietary intervention administered during puberty alone. While this is in accordance with previous work indicating that adolescent diet could have an effect on BC development, our study is among the first to indicate that a peripubertal intervention with dietary phytochemicals can have an effect on later-life BC development [26]. We also developed gene expression and methylation profiles for peripubertal BSp exposure. Within this profile we identified tumor suppressor genes, including p21, p53, and BRCA2, that were upregulated in HER2/neu BSp-treated mice. In addition to these genes, we identified a suite of upregulated and downregulated genes, many of which have known or potential tumor suppressor or oncogenic functions. Although we did not find a large number of both differentially methylated and expressed genes, global bisulfite sequencing did reveal a potential tumor suppressor, Erich4, that appears to be under epigenetic control. Taken together, our results indicate that nutritional prevention of later-life BC utilizing critical periods, such as puberty alone, is feasible. These results indicate that the administration of nutritional interventions for BC prevention may be key during critical periods such as puberty, and further clinical and laboratory studies of how substances, such as dietary phytochemicals, administered during these periods affect BC development and have the potential to inform decisions involving BC prevention and control.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15030674/s1. Data S1: Diet Composition;

Data S2: Full DEG list; Data S3: GO Downregulated; Data S4: GO Upregulated; Data S5: Full DMR list; Table S1: Primer sequences for RT-Qpcr; Table S2: Antibodies for Western blots; Figure S1: Average tumor latency observed in SV40 mice; Figure S2: Mouse body weight for SV40 (a) and HER2/neu (b) mice; Figure S3: Average day of vaginal opening (VO) for SV40 (a) and HER2/neu (b) mice; Figure S4. Approximate tumor size for HER2/neu mice in both control (a) and BSp (b) fed mice; Figure S5. Approximate tumor size for SV40 mice in both control (a) and BSp (b) fed mice; Figure S6: PCR verification of tumor suppressors Lman1I (a), Clec4e (b), Parp6 €, as well as oncogenes Chrdl2 (d), Pcsk1 €, and Slc51b (f); Figure S7: Gene ontology analysis for downregulated genes identified by RNA sequencing; Figure S8: Gene ontology analysis for upregulated genes identified by RNA sequencing.

Funding Statement

This work was supported by grants from the National Institutes of Health (NCI R01 CA178441 and NCI R01 CA204346 to TT), as well as in part by grant number T32 CA47888, Cancer Prevention and Control Training Program grant, funded by the National Cancer Institute, National Institutes of Health.

Author Contributions

Conceptualization, A.B. and T.O.T.; methodology, A.B., I.A. and T.O.T.; software, I.A.; validation, A.B. and T.O.T.; formal analysis, A.B.; investigation, A.B.; resources, T.O.T.; data curation, A.B. and I.A.; writing—original draft preparation, A.B.; writing—review and editing, T.O.T.; visualization, T.O.T.; supervision, T.O.T.; project administration, T.O.T.; funding acquisition, T.O.T. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Animal procedures in this study were approved by UAB IACUC (Animal Project Numbers: 20653, 10088).

Informed Consent Statement

Not Applicable.

Data Availability Statement

Data are contained within the article or Supplementary Materials.

Conflicts of Interest

The authors declare no conflict of interest.

Footnotes

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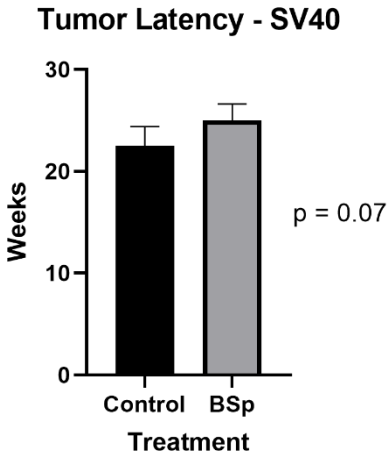
SUPPLEMENTAL MATERIALS

Supplementary Table 1. Primer sequences for RT-qPCR.

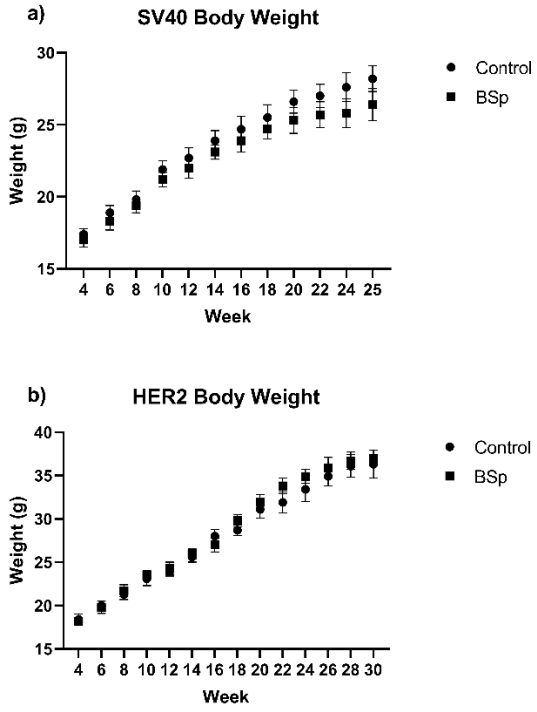
GAPDH	GTGGAGTCATACTGGAACATGTAG AATGGTGAAGGTCGGTGTG
P53	TGAAAATGTCTCCTGGCTCAG CTAGCATTCAGGCCCTCATC
P21	GAAGAGACAACGGCACACT CAGATCCACAGCGATATCCAG
BRCA2	TGTGTCATCCCTCTCCAGTATC GATGCCTAAACCCAGAAAGAGT
Erich4	CCCCATCTCCAGTCCCA TCAGGCCCTTATGTCTTCAG
Lman1I	ATCAAAGTAGATCCCGATGCC GAGATGCAGATGAGAGTGACTG
Clec4e	TGAGAGCTGCGATATGTTACG ATCCCACCACACAGAGAGA
Parp6	GCATACTTCATAATCTGGACAAGG GTAGAGGTGTTTGGCTATCCC
Chrdl2	TCTCAGTTGTCCTGTCTTTGC AGTGTGTCCTGTGTAGCTGTA
Pcsk1	TCACAGTTATCTCCCTGACGA CCCAGAAGGCATTTGAATATGG
Slc51b	GCTGCTTCTTTCGATTTCTGTT GCTTTGGTATTTTCGTGCAGA

Supplementary Table 2. Antibodies for Western blots.

P53	Anti-p53 antibody (ab131442)
P21	Recombinant Anti-p21 antibody [EPR18021] (ab188224)
BRCA2	Anti-BRCA2 antibody (ab216972)
ActB	β-Actin (13E5) Rabbit mAb #4970



Supplementary Figure 1. Average tumor latency observed in SV40 mice. BSp-treated mice developed tumors approximately 2 weeks later than controls in this mouse model. p = 0.07 and n = 24.



Supplementary Figure 2. Mouse body weight for SV40 (a) and HER2/neu (b) mice. Treatment did not have a significant effect on total body weight in either mouse strain. n = 24 for both strains.

P21 PROMOTER METHYLATION IS VITAL FOR THE ANTICANCER ACTIVITY OF WITHAFERIN A

by

ANDREW BRANE, MADELINE SUTKO, AND TRYGVE O. TOLLEFSBOL

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ABSTRACT

Breast Cancer (BC) is a widespread malignancy that affects the lives of millions of women each year, and its resulting financial and healthcare hardships cannot be understated. These issues in combination with side effects and obstacles associated with the current standard of care generate considerable interest in new potential targets for treatment as well as means for BC prevention. One potential preventive compound is Withaferin A (WFA), a traditional medicinal compound found in winter cherries. WFA has shown promise as an anticancer agent and is thought to act primarily through its effects on the epigenome, in particular the methylome. However, the relative importance of specific genes' methylation states to WFA function remains unclear. To address this, we utilized human BC cell lines in combination with CRISPR-dCas fused to DNA methylation modifiers (i.e, epigenetic editors) to elucidate the importance of specific genes' promoter methylation states to WFA function and cancer cell viability. We found that targeted demethylation of promoters of the tumor suppressors *p21* and *p53* resulted in increased gene expression, while targeted methylation of the promoter of the oncogene *CCND1* resulted in decreased gene expression. These changes were also associated with decreases in cell viability. When given in combination with WFA in both *p53* mutant and wild type cells, we discovered that targeted methylation of the *p21* promoter was able to modulate the anticancer effects of WFA, while targeted methylation or demethylation of the promoters of *p53* and *CCND1*, respectively, had no significant effect on viability decreases from WFA treatment. Taken together, these results indicate that *p21*, *p53*, and *CCND1* may be important targets for epigenetic editing therapies and that WFA may have

utility in the prevention of BC through its effect on *p21* promoter methylation independent of *p53* function.

Keywords: DNA methylation, Breast Cancer, Phytochemicals, Prevention, Epigenetics, CRISPR-dCas, Withaferin A

INTRODUCTION

Despite recent advances that have significantly improved both quality of life and outcomes for breast cancer (BC) patients, the disease remains a global burden for women's health. Unlike many other cancer types, BC case numbers continue to rise on a yearly basis [1]. Nearly 300,000 BC cases and over 43,000 mortalities were expected in 2023, and disease severity and prognosis vary greatly according to the molecular subtype of the cancer [1-3]. While luminal A and B subtypes are often seen as more treatable, manageable diseases, the HER2-enriched (HER2) and triple-negative (TNBC) subtypes have less favorable outcomes. TNBC has the poorest response to the current standard of care and has a mortality rate of around 22% [3,4]. Because of both the continuing rise in BC cases and the difficulty associated with treating HER2 and TNBC subtypes, interest in BC prevention is at an all-time high.

BC incidence rates, particularly TNBC, are strongly associated with racial backgrounds, with Non-Hispanic White and Non-Hispanic Black being around 25% more likely to develop BC than Asian/Pacific Islander women (A/PI) [3,5]. These differences can be partially explained through differences in diet, where A/PI often consume diets rich in fruits, vegetables, and soy. These foods contain substances known as dietary phytochemicals: bioactive compounds with known anticancer effects [6]. Mechanistically, these dietary phytochemicals are thought to function through both increasing cellular antioxidant levels and modulation of cells' epigenetic profiles [6,7].

Withaferin A (WFA) is one such dietary phytochemical and is found within winter cherries, also known as Ashwagandha root [8]. While WFA has been used since ancient times as a means of preventing stress and increasing longevity, scientific research in the

last half century has established its anti-inflammatory, cardioprotective, and anticancer properties [8-10]. WFA exhibits anticancer effects on a number of cellular pathways relevant to BC cell biology, including proliferation, apoptosis, and metastasis [8]. Although the specific mechanisms behind these changes are poorly understood, much of WFA's function is thought to derive from its inhibition of the family of methylation writers known as DNA methyltransferases (DNMTs) [11-14].

Past studies, including those of our laboratory, have indicated that WFA has differential effects on specific genes' expression levels and maintains its anticancer effects in *p53* mutant cell lines [13, 15]. Many of these changes are thought to arise from WFA's inhibitory effect on many classes of DNMTs, but WFA has differential effects on DNA methylation states that vary by genomic site [13,16,17]. While a great number of genes within these and other studies have been correlated with WFA's effect on cancer initiation and progression, genes associated with apoptosis and cell cycle control appear to be of particular importance. Specifically, the tumor suppressors *p21* and *p53* along with the oncogene *CCND1* have been implicated in WFA function in multiple cancer cell lines [13, 18-20]. The anticancer effects of WFA also extend to in vivo experiments, with WFA treatment reducing tumor size in mouse models of prostate, ovarian, and breast cancers [21-23]. However, questions remain as to how specific genes' methylation states affect cancer cell viability as well as how they contribute to the anticancer functions of WFA.

In this study, we utilized CRISPR-dCas technology to parse the effects of modulating cancer-associated promoter methylation on the viability of two breast cancer cell lines. We also modulated the methylation states of these genes in combination with

WFA treatment. Our aim was to determine which genes' promoter methylation states influence the viability of BC cells and to ascertain which of these genes' methylation states were important to the anticancer function of WFA. We hypothesized that demethylating the promoter and increasing the expression of the tumor suppressors *p21* and *p53* and methylating the promoter and decreasing the expression of the oncogene *CCND1* would result in significant decreases in BC cell viability. In addition, we hypothesized that ablating the methylation/expression changes associated with WFA treatment on one or more of these genes would restore cancer cell viability loss associated with WFA treatment. To test this, we transfected BC cell lines with CRISPR-dCas9 constructs fused with epigenetic modifiers alongside guides to the promoters of the tumor suppressor *p21*, the tumor suppressor *p53*, and the oncogene *CCND1*. To understand the impact of gene-specific methylation state on overall cancer cell viability, we transfected these constructs alone. Additionally, we administered WFA alongside these constructs to determine the importance of these genes' methylation states to the anticancer function of WFA. Background and experimental design for this study can be found in Figure 1.

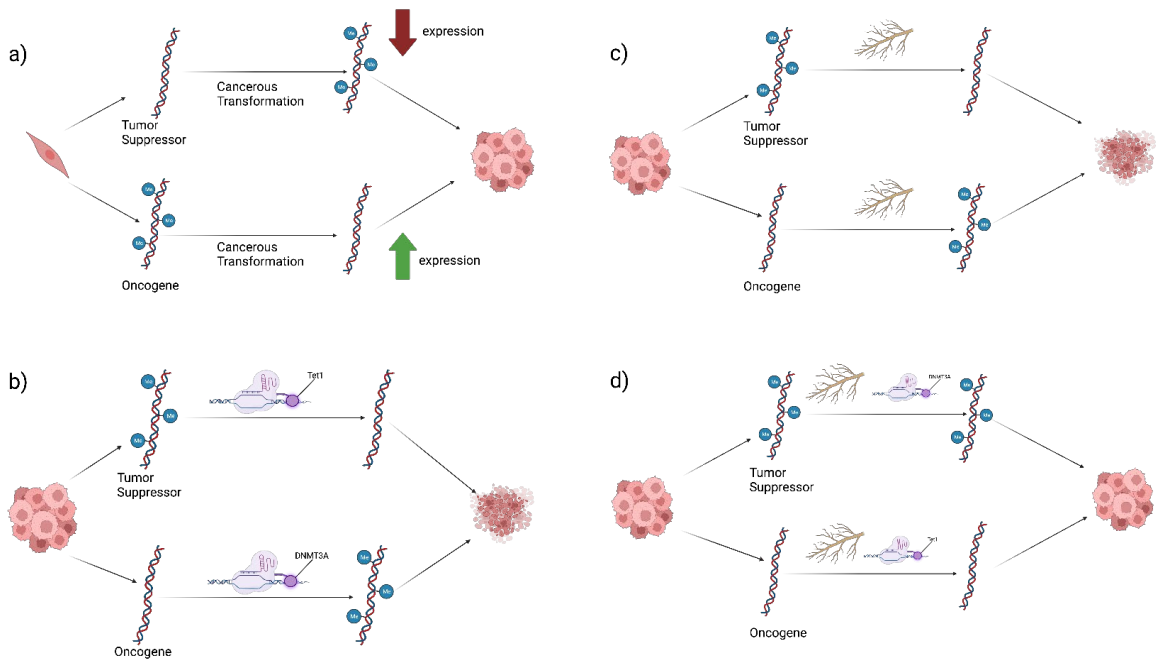

Figure 1. Justification (a) and experimental design (b-d) for this study. During cancerous transformation, tumor suppressors such as *p21* and *p53* can become methylated and oncogenes such as *CCND1* can become demethylated, leading to changes in expression levels (a). In initial experiments (b) the tumor suppressors *p21* and *p53* were targeted with Tet1 for promoter demethylation and the oncogene *CCND1* was targeted with DNMT3A for methylation. In subsequent experiments, WFA was applied to cells (c), and changes to promoter methylation of these tumor suppressors and oncogenes were measured. These methods were combined (d), with CRISPR constructs acting antagonistically towards WFA methylation changes. All experiments were conducted in MCF7 and MDA-MB-231 breast cancer cells. Figure created with BioRender.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The ERα (+) MCF7 cell line and ERα (-) MDA-MB-231 breast cancer cell lines were utilized in this study. In addition, the MCF10A human mammary epithelial cells served as a control for selecting the effective concentration of withaferin A (WFA) in subsequent experiments. MCF7 and MDA-MB-231 cells were grown in DMEM (Corning Inc., Corning, NY) media containing 10% FBS and 100 units/mL penicillin streptomycin. MCF10A cells were grown in 50/50 DMEM F12 media containing 5% donor horse serum, 100 µL of 20 ng/mL EGF, 50 µL of 100 ng/mL cholera endotoxin, 100 µL of 0.05 µg/mL hydrocortisone, 0.292 g of 2 mmol/L L-glutamine, and 5 mL of 100 units/mL penicillin streptomycin. All cells were subcultured upon reaching ~90% confluence and maintained in a 5% CO_2 incubator at 37 ºC.

Withaferin A and Cell Treatment

WFA was sourced from LKT Laboratories (Minneapolis, MN) and has a molecular weight of 470.606 g/mol. Stock concentrations were frozen at -20 ºC in DMSO (Sigma Aldrich, St Louis, MO) at a concentration of 100 mmol/mL.

Cells were seeded and allowed 48 h to adhere to plates and enable CRISPR treatment. Following this, cells were treated over a three-day period with either WFA or DMSO as a vehicle control at indicated concentrations.

Isolation and Growth of CRISPR Constructs

CRISPR constructs and guides are contained within circular, bacterial plasmids with mammalian promoters 5’ of the genes necessary for CRISPR expression. These plasmids were delivered as E. coli bacterial stabs and contain ampicillin resistance genes rendering

them suitable for selection and growth in ampicillin-treated Terrific Broth (Fisher Scientific, Mapton, NH) medium and agar plates. Bacterial stabs are spread on Amp-TB plates and incubated for 18 h at 30 ºC. Single colonies are chosen from these plates and used to inoculate 100 mL of amp-TB broth. This broth is incubated for 18 h at 30 ºC in a shaker incubator. Plasmids are extracted using a Quiagen MIDIprep kit and stored in a concentrated form at -20 ºC. Purified CRISPR constructs contain a deactivated Cas protein tied to one of two molecules and were obtained from Addgene (Watertown, MA). In experiments designed for targeted demethylation of promoters we utilized pINDUCER dCas9-TET1CD , which was a gift from Danwei Huangfu (Addgene plasmid # 101921 ; http://n2t.net/addgene:101921 ; RRID:Addgene_101921 ; RRID:Addgene_129025) [24]. In experiments designed for targeted methylation of promoters we employed pdCas9-DNMT3A-EGFP, a gift from Vlatka Zoldoš (Addgene plasmid # 71666 ; http://n2t.net/addgene:71666 ; RRID:Addgene_71666) [25]. All CRISPR experiments also utilize the pDECKO_mCherry plasmid (pDECKO), which was a gift from Roderic Guigo & Rory Johnson (Addgene plasmid # 78535 ; http://n2t.net/addgene:78535 ; RRID:Addgene_78535) [26].

Guide Selection and Cloning

Genes of interest were screened for differences in promoter methylation utilizing the UAB UALCAN database, which contains promoter methylation data for both breast tumor and normal breast tissues [27]. Potential genes were selected for CRISPR experiments based on significantly different promoter methylation levels in tumor tissues compared to normal breast tissue. Guide sequences were designed using the University of California Santa Cruz genome browser CRISPR guide design tool on the GRCh38/hg38

human genome assembly [28]. At least four potential guides approximately 50 bp upstream of each targeted promoter CpG island were selected for further screening with RT-qPCR. To increase the probability of quickly finding efficient and specific guides, only guides with MIT Guide specificity scores of >70 and a Doensch et al. 2016 score of greater than 55 were selected [28-30]. Guide oligonucleotides were created by Integrated DNA Technologies, Inc. (Coralville, IA) and cloned into the pDECKO plasmid using the protocol described by Vojita et al. [26]. Within this plasmid, guide sequences were cloned into the scaffold adjacent to the U6 promoter using the BsmBI restriction enzyme (Fisher). Experimental guide sequences can be found in Supplementary Table 1. Following this, guide plasmids were transformed into NEBExpress® Competent E. coli (High Efficiency) (New England Biolabs, Ipswitch, MA) according to manufacturer's protocol. Cells were grown and maintained identically to those containing construct plasmids.

Nucleic Acid Extraction

Nucleic acids were isolated from cell pellets derived from cell treatments performed in 24 well plates. Extractions were performed on fresh cell pellets or frozen pellets stored in DNA/RNA shield reagent (Zymo, Irvine, CA) after experimental conclusion. DNA and RNA were extracted concurrently using a Zymo Research Corporation Quick DNA/RNA Miniprep Plus Kit according to manufacturer's instructions.

RT-qPCR

cDNA was synthesized per the manufacturer's instructions from 250 ng of RNA using iScript Reverse Transcription Supermix for RT–qPCR (BIORAD). Using the cDNA generated from this protocol, primers obtained from Integrated DNA Technologies, Inc.

and SsoAdvanced Universal SYBR® Green Supermix (BIORAD, Hercules, CA, USA), quantitative real-time PCR was performed. These reactions were performed in triplicate using the CFX Connect Real-Time PCR Detection System (BIORAD). Thermal cycling began at 94 °C and was followed by 35 cycles of PCR (94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s). GAPDH served as an endogenous control, and a vehicle control was used for calibration. Relative changes in gene expression were calculated through the 2-ΔΔCQ method, where ΔΔCQ = [ΔCQ(treatment group) − ΔCQ(control group)] and ΔCQ = [CQ(gene of interest)-CQ(GAPDH)] [30]. Relative expression levels of these genes were compared between treatment and control groups. A full list of primers can be found in Supplementary Table 2.

Bisulfite Conversion and Sequencing

Bisulfite conversion was performed on genomic DNA samples using a Zymo Research Corporation EZ DNA Methylation-Gold™ Kit (Zymo) according to manufacturer's instructions. Bisulfite treated DNA samples were then subjected to PCR amplification of promoter CpG islands. Potential bisulfite primers were selected utilizing the MethPrimer tool with inputs of gene-of-interest promoter CpG islands 250 bp up and downstream of the promoter [32]. Confirmation of PCR products was performed by gel electrophoresis with 0.5 µL of PCR product on a 2% agarose gel. A full list of bisulfite primers can be found in Supplementary Table 3. For each sample, 5 µL of PCR product was purified using Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup (Fisher) according to manufacturer's instructions. Samples were subjected to Sanger sequencing at the UAB Genomics core, utilizing the forward bisulfite primers found in ST3.

MTT Analysis

Cell viability assays were performed in 96-well plates (Corning) seeded with 5x103 cells following WFA and/or CRISPR treatments. Viability was measured by the uptake of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT). MTT was added to the media of cells in 96-well plates where it was converted to a purple insoluble formazan by mitochondrial enzymes. Following a 4-hour incubation, media was removed and formazan crystals were dissolved in DMSO. Wells were then read at 595 nm using a microplate reader (Epoch model, Biotek, Winooski, VT, USA).

Statistical Analysis

For all experiments involving only two groups, statistical significance between experimental and control samples were determined using a Students T-test performed in Microsoft Excel. For all experiments with more than two groups, one-way independent ANOVA, followed by Tukey's post-hoc test was performed in SPSS statistical software (IBM) [33]. For all tests, a cutoff of $p<0.05$ was be considered statistically significant, with $p<0.05$ being indicated by *, $p<0.01$ being indicated by **, and $p<0.001$ being indicated by ***. Sample sizes for our studies were determined using an online power calculator found at powerandsamplesize.com [34]. Graphs were created using GraphPad Prism (version 9.5.0) or BioRender (BioRender.com).

p21, *p53*, and *CCND1* are differentially methylated in breast cancer patients versus normal breast tissue

In order to glean a better understanding of whether our targeted genes of interest may have translational value, we acquired promoter methylation data from the UALCAN database of patient breast tumor samples [27]. As depicted in Figure 2, UALCAN database samples indicated that patients with breast cancer carcinomas had significantly higher methylation levels of *p21* (Fig 2a) and *p53* (Fig 2b) but lower methylation levels of *CCND1* (Fig 2c) when compared to normal breast tissue samples.

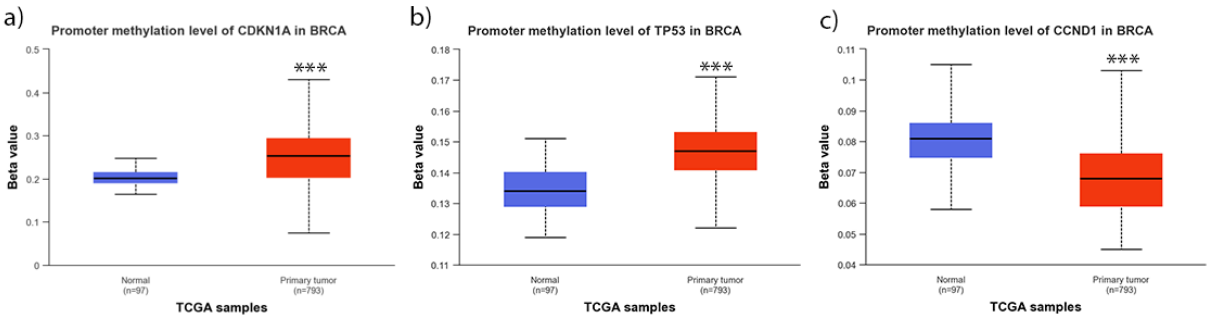

Figure 2. Average promoter methylation levels in breast tumor tissue compared to controls for *p21* (CDKN1A) (a), *p53* (TP53) (b), and *CCND1* (c). For normal tissue n=93 and for primary tumor tissue n=793. Data were acquired from the UAB UALCAN database [27]. *** indicates that $p < 0.001$.

Targeted demethylation of the *p21* promoter increased *p21* expression and decreased cancer cell viability

To parse the effects of *p21* promoter methylation on BC cells, we targeted the methylation eraser Tet1 to the promoter region of *p21*. Transfection of dCas9-Tet1 constructs alongside guide constructs resulted in significant increases in the gene expression of *p21* in both MCF7 and MB-MDA-231 breast cancer cell lines (Fig 3a).

This change in expression was accompanied by significant decreases in BC cell viability, depicted in Figure 3b. Representative images of these control and experimental cells are depicted in Supplementary Figure 1.

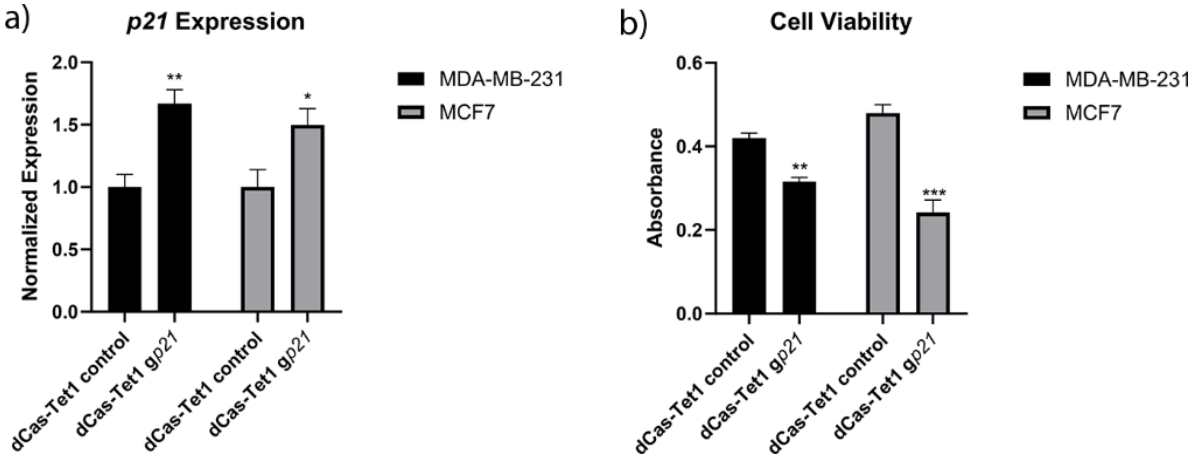

Figure 3. Expressional changes (a) and viability changes (b) resulting after transfection of a CRISPR-dCa9-Tet1 construct into BC cell lines. CRISPR constructs were transfected alongside empty guide vectors as controls or guides with a promoter for *p21* cloned into their sgRNA scaffold. For these experiments, n=6. * indicates that $p < 0.05$, ** indicates that $p < 0.01$, and *** indicates that $p < 0.001$.

Targeted demethylation of the p53 promoter increased p53 expression and decreased cancer cell viability

To achieve this same understanding of *p53*, we targeted Tet1 to the promoter region of *p53*. Transfection of dCas9-Tet1 constructs alongside guide constructs resulted in significant increases in the gene expression of *p53* in both MCF7 and MB-MDA-231 breast cancer cell lines (Fig 4a). However, this change in expression was only accompanied by significant reductions in cell viability within MCF7 cells (Fig 4b). Representative images of these control and experimental cells are depicted in Supplementary Figure 2.

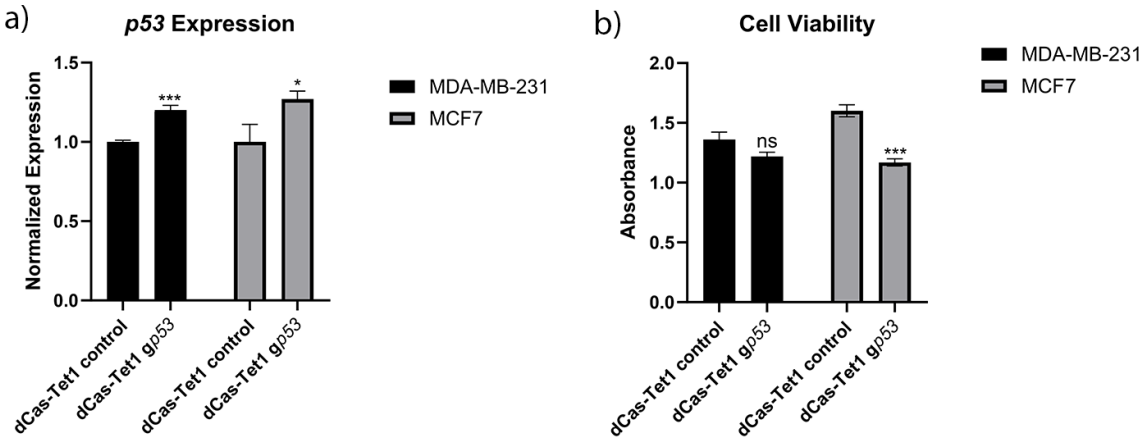

Figure 4. Expressional changes (a) and viability changes (b) resulting after transfection of a CRISPR-dCa9-Tet1 construct into BC cell lines. CRISPR constructs were transfected alongside empty guide vectors as controls or guides with a promoter for *p53* cloned into their sgRNA scaffold. For these experiments, n=6. * indicates that $p < 0.05$ and *** indicates that $p < 0.001$.

Targeted methylation of the CCND1 promoter decreased CCND1 expression and decreased cancer cell viability

To build on our understanding of *CCND1*, we targeted DNMT3A to the promoter region of *CCND1*. Transfection of dCas9-DNMT3A constructs alongside guide constructs resulted in significant decreases in the gene expression of *CCND1* in both MCF7 and MB-MDA-231 breast cancer cell lines (Fig 5a). This change in expression was accompanied by significant decreases in BC cell viability (Fig. 5b). Representative images of these control and experimental cells are depicted in Supplementary Figure 3.

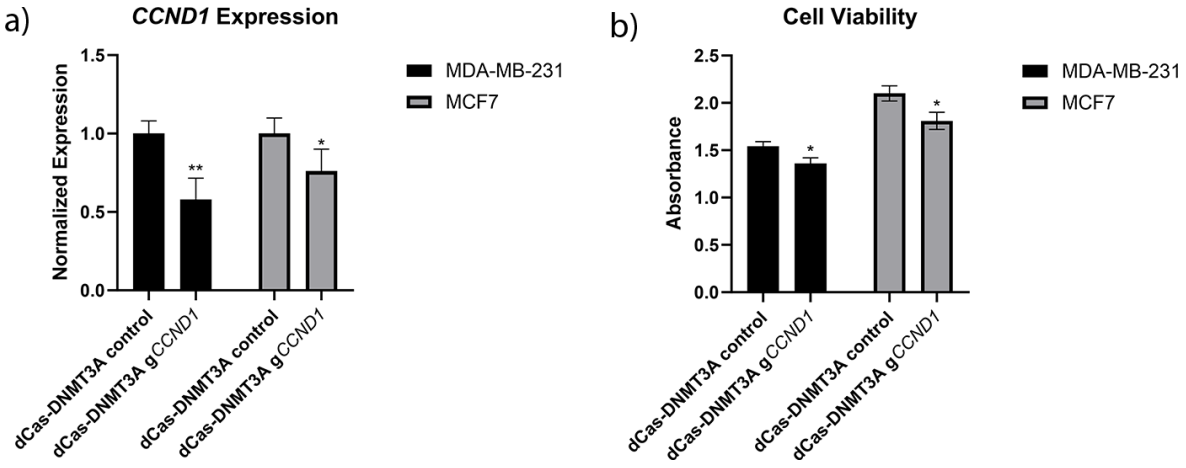

Figure 5. Expressional changes (a) and viability changes (b) resulting after transfection of a CRISPR-dCa9-Tet1 construct into BC cell lines. CRISPR constructs were transfected alongside empty guide vectors as controls or guides with a promoter for *CCND1* cloned into their sgRNA scaffold. For these experiments, n=6. * indicates that $p < 0.05$, and ** indicates that $p < 0.01$.

Targeted methylation of the p21 promoter in combination with WFA ablates p21 expression changes and resulted in a loss of WFA anticancer function

Before performing experiments alongside WFA, we established the optimal concentration of WFA for our experiments to be 0.5 μM utilizing a dose response assay with MCF10A cells as a noncancer control. We found that 0.5 μM was the highest concentration that would significantly reduce the viability of our cancer cell lines without impacting the viability of our control cell line (Supplementary Figure 4). For the remainder of our experiments, we used this concentration of WFA within our treatment groups. To determine the importance of methylation and gene expression changes to WFA anticancer function, we transfected a guide for the promoter of *p21* alongside dCas9-DNMT3A. We found that these constructs ablated increases in p21 gene expression associated with WFA treatment (Fig 6 a-d) in both MCF7 and MB-MDA-231 cells. Constructs also modulated the anticancer effects of WFA, resulting in a loss of significant viability decreases

associated with WFA administration (Fig 6 e & f). Representative images of these cells are depicted in Supplementary Figure 5. Alongside this, we performed bisulfite sequencing on the promoter of *p21*. We found that WFA treatment resulted in significantly fewer methylated CpGs at the promoter of *p21*, and this significant decrease was lost when WFA was administered alongside CRISPR-dCas-DNMT3A and a guide for *p21* (Figure 7a).

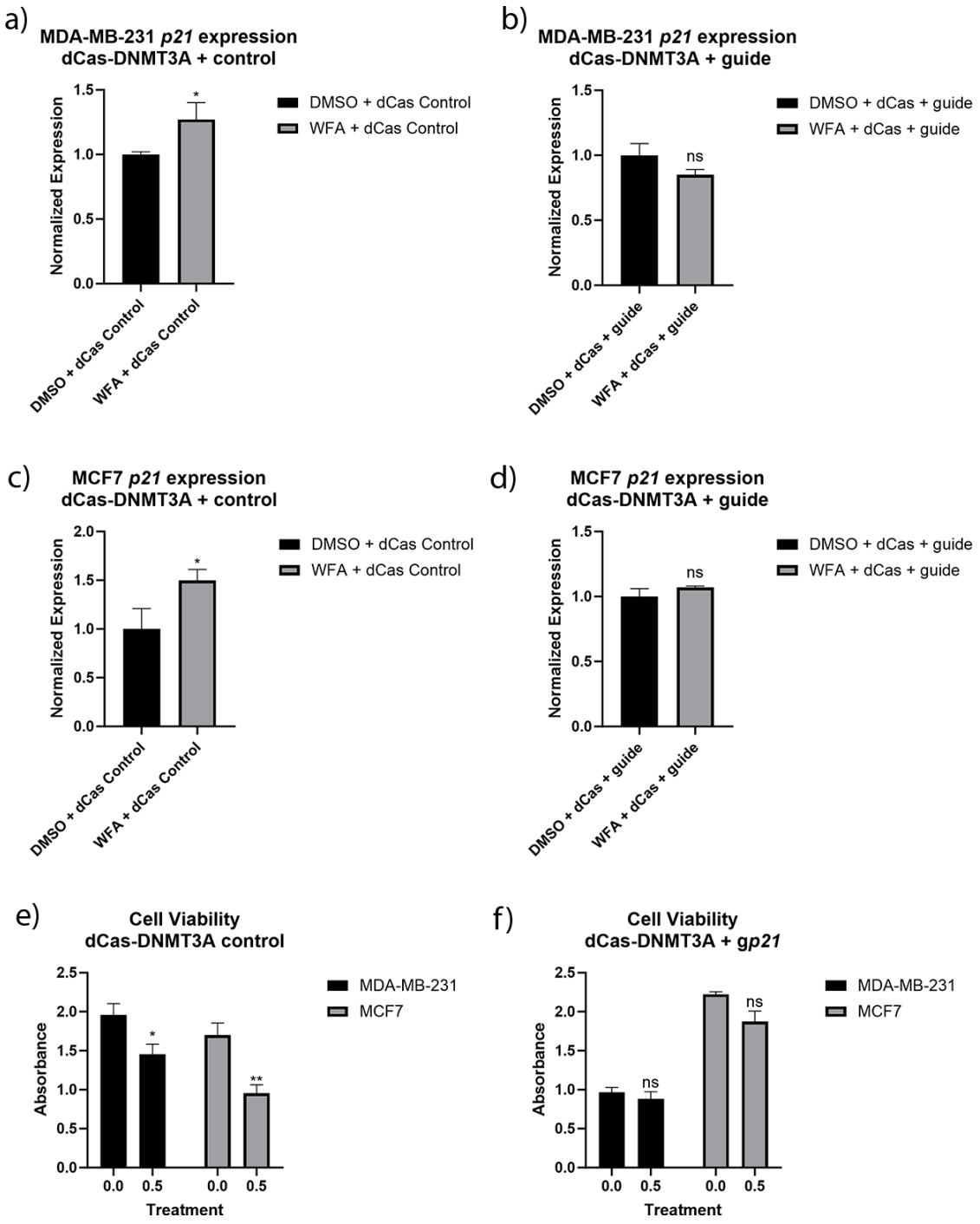

Figure 6. Expressional changes of *p21* after WFA treatment with and without a guide for p21 in both MDA-MB-231 (a & b) and MCF7 (c & d) cells. Cell viability was also performed in control (e) and g*p21* (f) cells. All experiments had an n = 6. * indicates that $p < 0.05$.

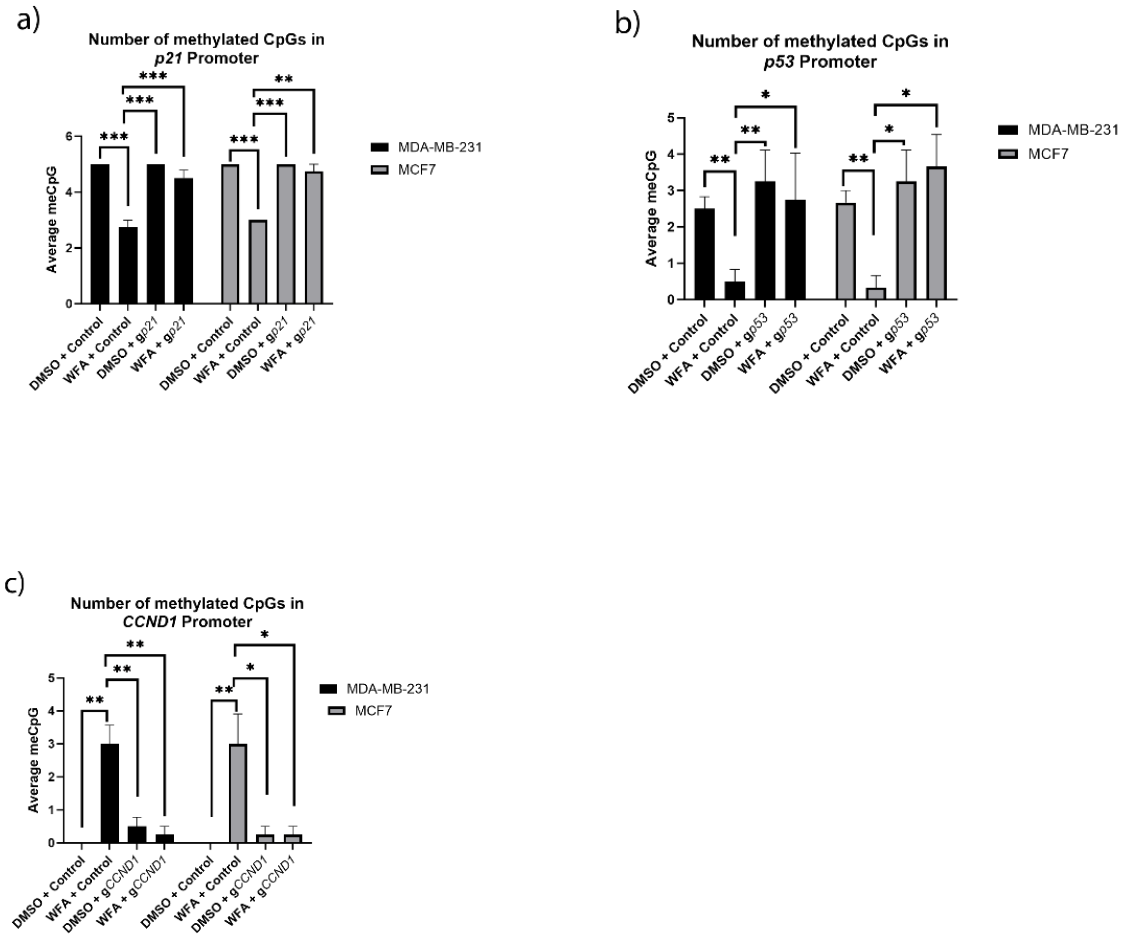

Figure 7. Promoter methylation status of cell lines in experiments involving *p21* (a), *p53* (b), and *CCND1* (c). For *p21* our bisulfite primers covered 5 CpGs, for *p53* our primers covered 5 CpGs, and for *CCND1* our primers covered 7 CpGs. For all experiments, n = 6. * indicates that $p < 0.05$, ** indicates that $p < 0.01$, and *** indicates that $p < 0.001$.

Targeted methylation of the p53 promoter in combination with WFA ablates p53 expression changes with no significant loss of WFA anticancer function

We next extended our study of WFA function to the p53 gene by transfecting a guide for the promoter of *p53* alongside dCas9-DNMT3A. This resulted in a loss of increases in *p53* expression associated with WFA treatment in both cell lines (Fig 8 a-d). However, WFA-treated cells still experienced a significant loss of overall cell viability, indicating that modulation of *p53* alone was not sufficient to inhibit WFA's effects (Fig 8 e & f).

Representative images of these cells are depicted in Supplementary Figure 6. Alongside this, we performed bisulfite sequencing on the promoter of *p53*. We found that WFA treatment resulted in significantly fewer methylated CpGs at the promoter of *p53*, and this significant decrease was lost when WFA was administered alongside CRISPR-dCas-DNMT3A and a guide for *p53* (Figure 7b).

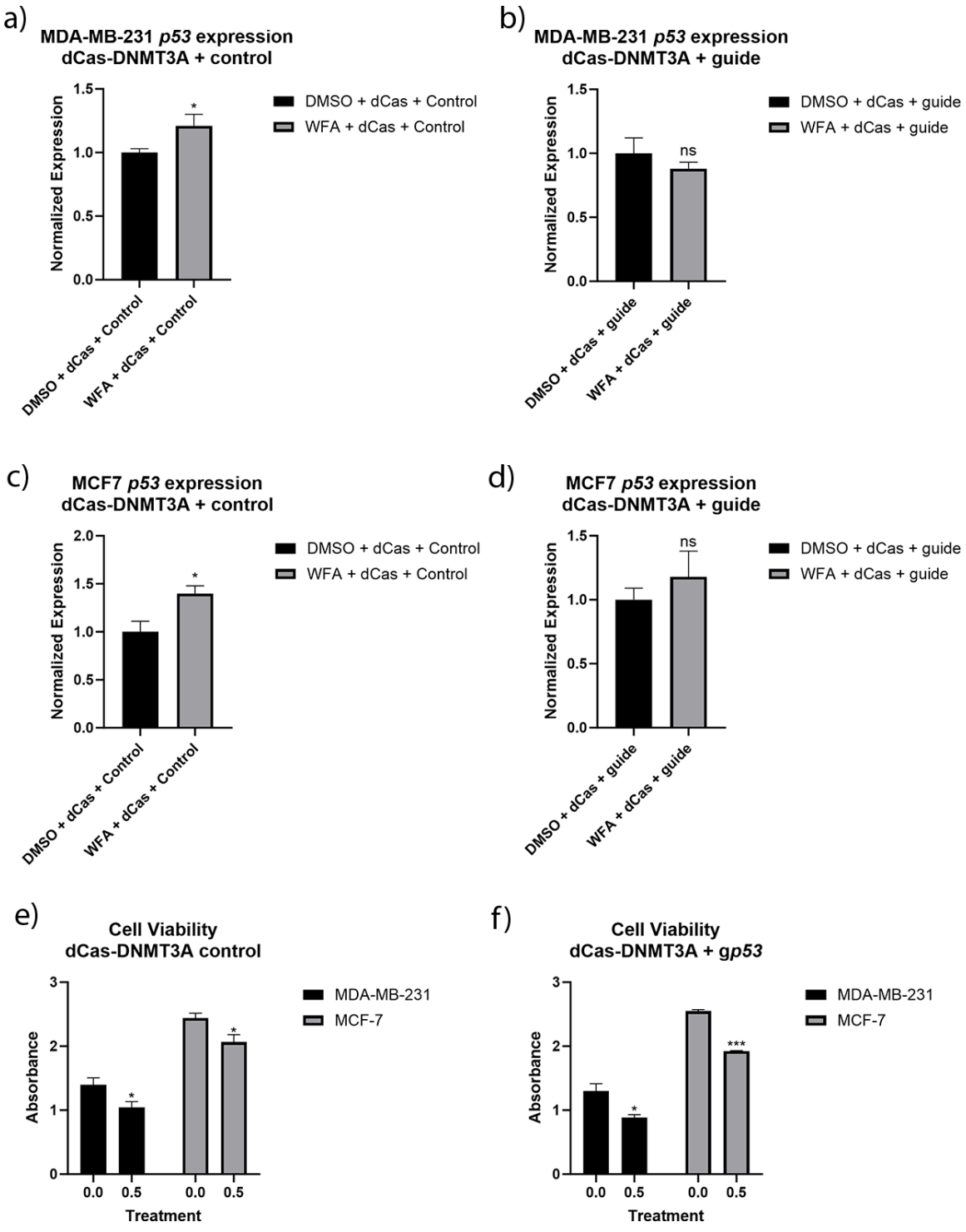

Figure 8. Expressional changes of p53 after WFA treatment with and without a guide for *p53* in both MDA-MB-231 (a & b) and MCF7 (c & d) cells. Cell viability was also performed in control (e) and g*p53* (f) cells. All experiments had an n = 6. * indicates that $p < 0.05$ and *** indicates that $p < 0.001$.

Targeted demethylation of the CCND1 promoter in combination with WFA ablates CCND1 expression changes with no significant loss of WFA anticancer function

Following this, we sought to determine the influence of *CCND1* promoter methylation on WFA function by transfecting a guide for the promoter of *CCND1* alongside dCas9-Tet1. In both MB-MDA-231 and MCF7 cells, transfecting constructs restored decreases of *CCND1* expression associated with WFA treatment (Fig 9 a-d). Despite this, WFA maintained its significant decreases in viability in both cancer cell lines (Figure 9 e&f). Representative images of these cells are depicted in Supplementary Figure 7. Alongside this, we performed bisulfite sequencing on the promoter of *CCND1*. We found that WFA treatment resulted in significantly more methylated CpGs at the promoter of *CCND1*, and this significant increase was lost when WFA was administered alongside CRISPR-dCas-Tet1 and a guide for *CCND1* (Figure 7c).

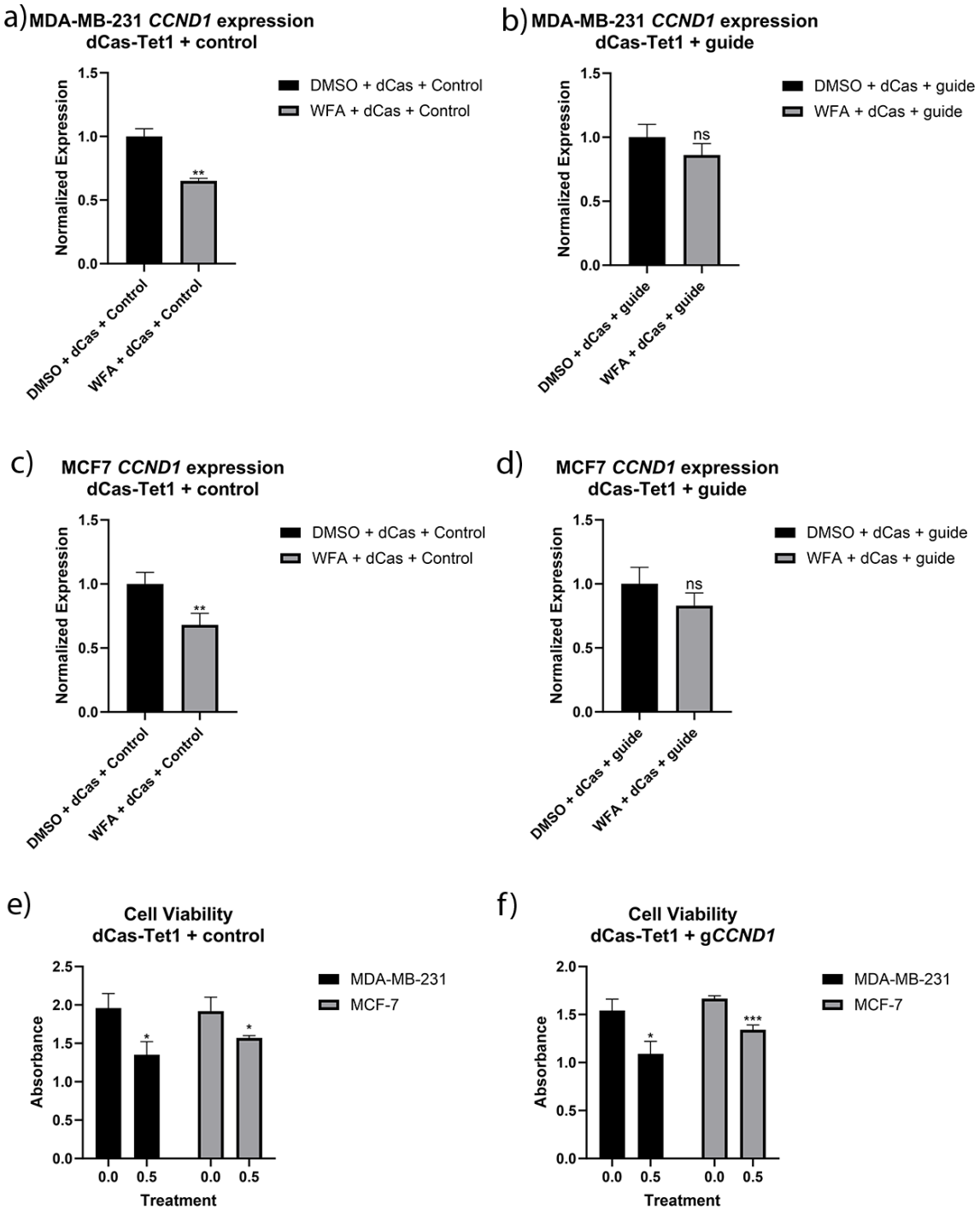

Figure 9. Expressional changes of *CCND1* after WFA treatment with and without a guide for *CCND1* in both MDA-MB-231 (a & b) and MCF7 (c & d) cells. Cell viability was also performed in control (e) and g*CCND1* (f) cells.. All experiments had an n = 6. * indicates that $p < 0.05$, ** indicates that $p < 0.01$, and *** indicates that $p < 0.001$.

DISCUSSION

Although continued advances in treatments have undoubtedly improved the lives of patients worldwide, BC continues to be a leading cause of mortality in women and its burden on our healthcare system cannot be understated. As our understanding of BC has progressed, it has become apparent that progression can vary greatly among individual cases, and the most successful treatments are likely to be those that take unique tumor profiles into account [35]. Because of this, interest in therapeutic targets is high, with hopes that targeted anticancer modifications can be matched to genetic and epigenetic aberrations within patients [35-37]. To achieve this, we require an understanding of how gene-specific effects can influence cancer viability. Epigenetic effects are an attractive target due to their specificity to cancer type and stage as well as their potential for reversal in real time [38,39]. Drugs targeting the epigenetics of DNA methylation patterns, including 5′-azacytidine and 5-aza-2′-deoxycytidine, have resulted in therapeutic success and have been utilized to treat TNBC [40,41]. These drugs, however, are non-specific in nature and can result in adverse events such as nausea, vomiting, diarrhea, and fatigue [41,42]. Increasing the specificity of epigenetics-based therapies may alleviate many of the side effects they are associated with, and, in combination with precision profiling of patient cancers, could result in more effective therapies. This idea extends to cancer prevention, wherein therapies tailored to individuals are likely to see greater success. Epigenome-affecting phytochemical treatments such as WFA provide an attractive avenue for cancer prevention due to their low cost and few side effects [43,44]. However, the gene specific effects of WFA on the methylome are poorly understood, limiting their utility for cancer prevention. Our study is among the first to modify the

methylation state of specific gene promoters while measuring their effects on cancer cell viability as well as measure the relative importance of gene-specific promoter methylation to the anticancer function of a phytochemical. Our results indicate that the genes *p21*, *p53*, and *CCND1* can be expressionally controlled through promoter methylation modifications, and these changes in expression are associated with decreases in BC cell viability. Our results also indicate that, despite affecting the expression and methylation of each of these genes, WFA anticancer function appears to be linked to changes in expression and DNA methylation associated with the p21 promoter.

P21, *p53*, and *CCND1* as molecular targets for epigenetic therapies

P21 loss has long been a focus of cancer research, due largely to its connections to cell cycle progression and apoptosis [45]. In BC, *p21* is not typically mutated, and therapies that upregulate *p21* expression have shown promise in treatment of the disease [46-48]. Additionally, epigenetic modifications, particularly changes to DNA methylation patterns, are important for the expression of *p21* and often dysregulated in cancers [49]. These ideas are supported by our findings derived from the UALCAN database, wherein patients suffering from breast carcinomas had significantly higher *p21* promoter methylation (Figure 2a) [27]. Our experimental results are also in accordance with these ideas, with both MDA-MB-231 and MCF7 cells having lower viability following increases in induced *p21* expression (Figure 3 a&b). These results highlight that targeted demethylation of the *p21* promoter may have therapeutic utility in treating TNBC.

To, perhaps, an even greater extent than *p21*, *p53* loss is also heavily associated with cancer initiation and progression. Often referred to as “the guardian of the genome”, *p53*

expression is involved in cell cycle, apoptosis, and genomic stability [50]. Because of difficulties in targeting *p53* and the high rate of *p53* mutations in cancer, therapies aimed specifically at the gene remain both attractive and elusive [50,51]. While its expression is not associated as heavily with its promoter methylation status as *p21*, promoter hypermethylation has been linked to decreases in expression as well as poor prognoses in various cancer types [52,53]. Similarly to *p21*, patient samples within the UALCAN database indicate that BC patients had significantly higher levels of *p53* promoter methylation (Figure 2b) [27]. We found that targeted upregulation of *p53* resulted in increases in expression for both MDA-MB-231 and MCF7 cells (Figure 4a). However, significant decreases in cancer cell viability were only observed in MCF7 cells (Figure 4b). As MDA-MB-231 cells possess a mutated form of *p53*, these results indicate that only upregulation of WT-*p53* resulted in decreases in cancer cell viability [54]. As with *p21*, targeted demethylation of the *p53* promoter may be useful from a therapeutic perspective, but it is important to note that p53 mutation occurs in over 50% of cancers, so any potential treatment should be tailored to an individual [55].

Conversely to *p21* and *p53*, the oncogene *CCND1* is not as widely associated with general carcinomas, with alterations in only around 4% of all cancer cases [56]. However, in breast cancers, it is overexpressed in around 50% of all cancers, with overexpression of *CCND1* associated with poor outcomes [56,57]. This matches the data derived from the UALCAN database, with breast carcinomas being associated with hypomethylation of its promoter relative to normal tissues (Figure 2c) [27]. We found that targeting DNMT3A to the promoter of *CCND1* resulted in significant decreases in its expression (Figure 5a) alongside significant decreases in viability (Figure 5b). These results support the

importance of *CCND1* in BC pathogenesis and indicate that targeted methylation of *CCND1* may lower disease burden.

Genetic targets and their relationship to WFA function

Studies evaluating the efficacy of WFA in cancer prevention have implicated the *p53/p21/CCND1* pathway to be of primary importance for its function. However, there is still no consensus on which of these genes, if any, is primarily responsible for WFA's anticancer function. Some studies have indicated that the restoration of *p53* expression and subsequent apoptosis induction and cell cycle arrest is WFA's primary mechanism of action [58-60]. However, WFA maintains anticancer function in cell lines possessing mutant *p53*, and it has been reported that WFA can upregulate *p21* independently from *p53* [15,18,61]. Previous in vitro work within our laboratory has also indicated that *p21* may be important for the anticancer activity of WFA independent of *p53* function [13]. This same study also indicated that WFA treatment decreased expression of *CCND1*, a cell-cycle oncogene associated with increased cell proliferation in cancers [13,62]. *CCND1* expression is typically negatively associated with *p21* levels, and WFA lowering its expression may be of particular importance in BC [56,57,63]. In this study, our experimental results with WFA were in accordance with our previous work, wherein WFA treatment raised the expression of *p21* (Figure 6 a & c), raised the expression of *p53* (Figure 8 a & c), and lowered the expression of *CCND1* (Figure 9 a & c) [13]. WFA treatment also resulted in lower levels of cancer cell viability at a concentration of 0.5 μM, and this concentration had no significant effect on MCF10A control cells (Supplementary Figure 1). In mouse models, treatments as low as 4 mg/kg have resulted

in blood concentrations of 2 μM [64]. Additionally, WFA, has been approved for treatments at far higher dosage than this level, with maximum doses of 325 mg/kg/day and a recommended starting dose of 65 mg/kg/day [65]. These studies indicate that our WFA dosage may be both feasible and efficacious in a clinical cancer prevention setting.

To ascertain which of these genes' methylation/expressional states was important to the anticancer function of WFA, we combined WFA treatments with CRISPR constructs that were antagonistic to WFA's effect on these genes. For *p21*, *p53*, and *CCND1*, we were able to ablate changes in gene expression (Figures 6,8, and 9) and DNA methylation (Figure 7) associated with WFA treatment, returning the expression level of these genes to levels similar to DMSO control treatments. For cells treated with a guide for *p21*, this was accompanied by a restoration of cancer cell viability (Figure 6f). Interestingly, this restoration of cell viability did not extend to cells treated with guides for *p53* (Figure 8f) or *CCND1* (Figure 9f), despite having a significant impact on their gene expression. Taken together, these results suggest that demethylation of the *p21* promoter and its resulting increase in *p21* gene expression are vital for the anticancer function of WFA.

While our understanding of risk factors contributing to cancer incidence continues to improve, the rate of new cancer cases remains stagnant for men and is gradually rising for women [66]. Because of this, interest in cancer preventive interventions remains high. For these treatments to be successful, they must be safe, efficacious, and easy to administer. Because dietary phytochemicals are generally present in safe levels within relatively accessible food products, there has been a wealth of research on their effectiveness in cancer prevention. Preventive effects have been reported in a wide array

of edible plants, including cruciferous vegetables, grapes, and green tea [67-69]. Ashwagandha-derived WFA, while less commonly found in Western diets and supplements, appears to have therapeutic potential through its effects on DNA methylation [8, 17, 70]. However, questions remain as to what types of cancer and what genetic/epigenetic profiles WFA administration may benefit.

Through this study, we sought answers to these questions by parsing the gene-specific epigenetic mechanisms behind WFA function. Our study is among the first to elucidate these mechanisms behind a dietary phytochemical, and our work indicates that WFA may be suitable for the prevention of BC with non-mutant *p21*. Additionally, these ideas highlight the therapeutic potential of WFA in *p53*-mutant BC prevention. As over 50% of BC cases have mutationally deactivated *p53*, WFA may provide an effective means of prevention in this subset of cancer cases [55]. Additionally, these results suggest that WFA may have utility in the prevention of cancers associated with inherited mutations of *p53*, such as Li Fraumeni syndrome [71].

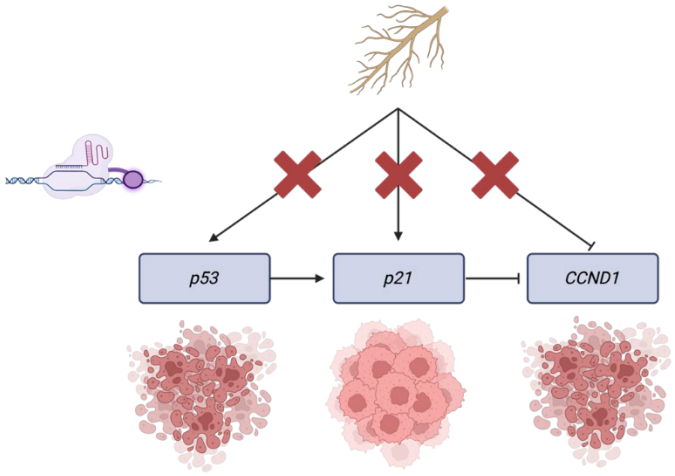

Figure 10. Simple summary of overall findings for this manuscript. We found that by systematically blocking methylation changes associated with WFA treatment, only *p21* methylation changes were required to maintain WFA's significant anticancer function. Figure created with BioRender.

Limitations

While the scope of this study was to establish and verify potential methylation targets for WFA, this work could be expanded upon utilizing CRISPR constructs delivered to in vivo mouse models with a lentiviral vector. For future studies, it will be important to establish that WFA's reliance on *p21* is maintained in a true tumor microenvironment. Our study was also only limited to the genes *p53*, *p21*, and *CCND1*. While these genes were selected due to their close ties to BC biology and disparate methylation state in cancerous tissues, there are undoubtedly other genes with methylation states associated in some part to the function of WFA. Despite this, our study is among the first to link a specific gene's methylation state to the anticancer function of a phytochemical. In addition, our study suggests that WFA may be a useful cancer preventative agent in the highly prevalent *p53*-mutant BC.

Acknowledgements

This research was funded by grants from the National Institutes of Health (NCI R01 CA178441 and NCI R01 CA204346) as well as in part by grant number T32 CA47888, Cancer Prevention and Control Training Program grant, funded by the National Cancer Institute, National Institutes of Health. Figures 1 and 10 were created using BioRender (Biorender.com).

Author Contributions

Research design: A.B. and T.O.T.; Conducting Experiments: A.B. and M.S.; Data Analysis: A.B. and M.S.; First Draft: A.B.; Editing and subsequent drafts: A.B. and T.O.T All authors read and approved the final manuscript.

Declaration of Interests

The authors declare no competing interests.

INCLUSION AND DIVERSITY

Both the University of Alabama at Birmingham and the Tollefsbol Laboratory support inclusive, diverse, and equitable conduct of research.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. List of final guide sequences used for this experiment. Guides were synthesized by IDT.

Guide Name	Sequence
Guide *p21*	GCGCGGGTCCCGCCTCCTTG
Guide *p53*	AATATTAATGAGGAAGACCT
Guide *CCND1*	TGGCATCGGGGTACGCGCGG

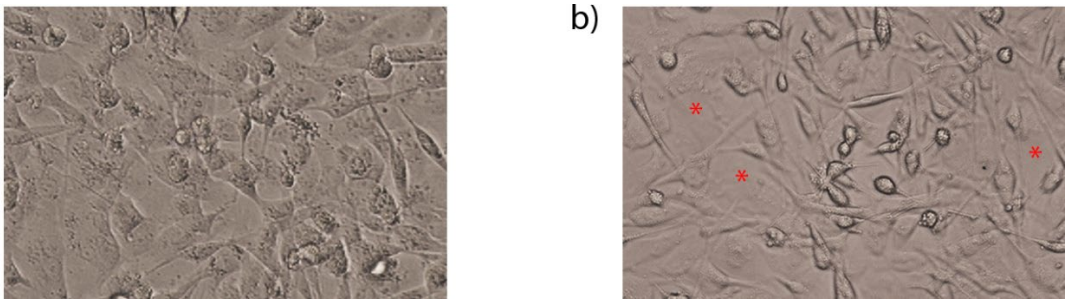
Supplementary Table 2. List of qPCR primer sequences used for this experiment. Primers were synthesized by IDT.

Name	Sequence
P21 Forward qPCR	TGGAGACTCTCAGGGTCGAAA
P21 Reverse qPCR	GGCGTTTGGAGTGGTAGAAATC
P53 Forward qPCR	GAGGTTGGCTCTGACTGTACC
P53 Reverse qPCR	TCCGTCCCAGTAGATTACCAC
CCND1 Forward qPCR	AGCGGTCCAGGTAGTTCA
CCND1 Reverse qPCR	GTGTCCTACTTCAAATGTGTGC
GAPDH Forward qPCR	GGCAAATTCAACGGCACAGT
GAPDH Reverse qPCR	AGATGGTGATGGGCTTCCC

Supplementary Table 3. List of Bisulfite primers used for amplification and sequencing. Primers were synthesized by IDT.

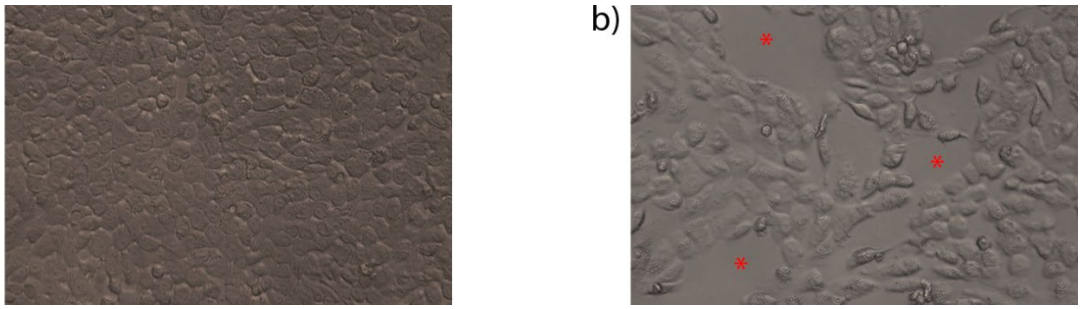
Name	Sequence
P21 Forward Bisulfite	GTTAGTTGAGGTGTGAGTAGTT
P21 Reverse Bisulfite	CTCTCTCACCTCCTCTAAATAC
P53 Forward Bisulfite	AGGATTTATTAAGTTTAGTTAGGAGTTT
P53 Reverse Bisulfite	ATTTTAAACTTCTCAAAAATCTAAAACC
CCND1 Forward Bisulfite	AAGTTGTAAAGTTTTGGAGTTTTTAG
CCND1 Reverse Bisulfite	AACTAATATTCCATAACTAAAACTCTTC

a) b)



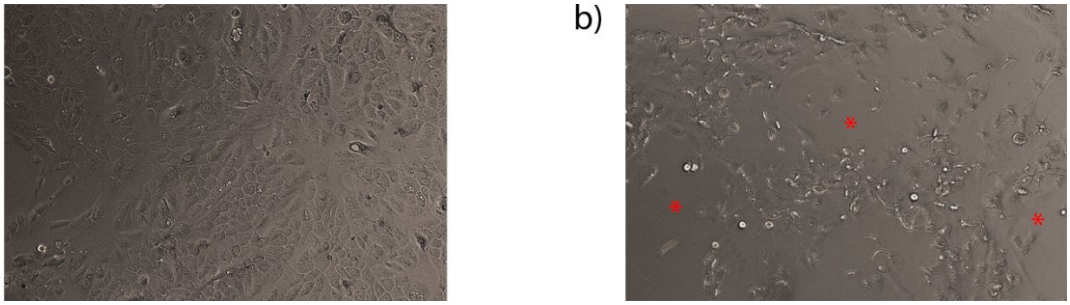
Supplementary Figure 1. Representative images of dCas-Tet1 control (a) and dCas-DNMT3A + gp21 (b) cells. Areas of lower cell density are highlighted here with red asterisks. Cells pictured are MCF7 cells and images have had color saturation reduced for increased clarity.

a) b)

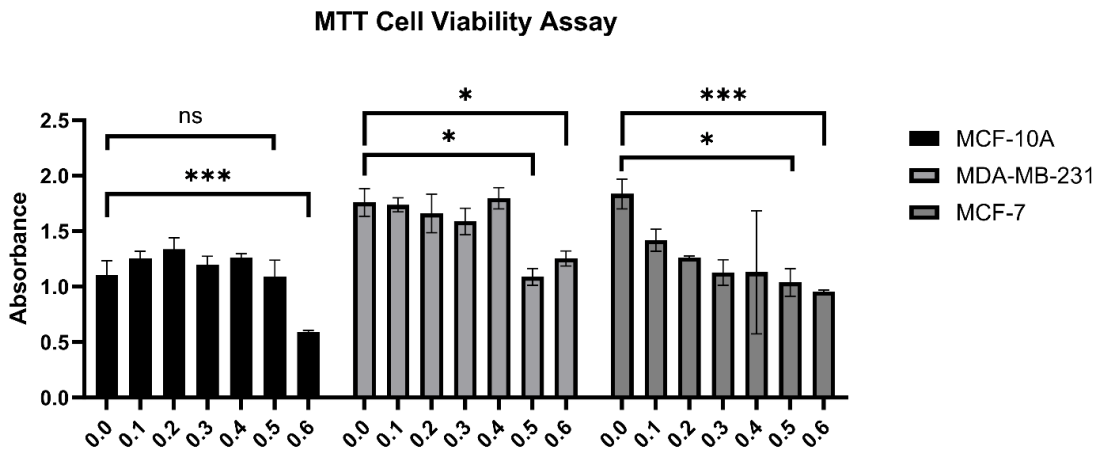


Supplementary Figure 2. Representative images of dCas-Tet1 control (a) and dCas-DNMT3A + gp53 (b) cells. Areas of lower cell density are highlighted here with red asterisks. Cells pictured are MCF7 cells and images have had color saturation reduced for increased clarity.

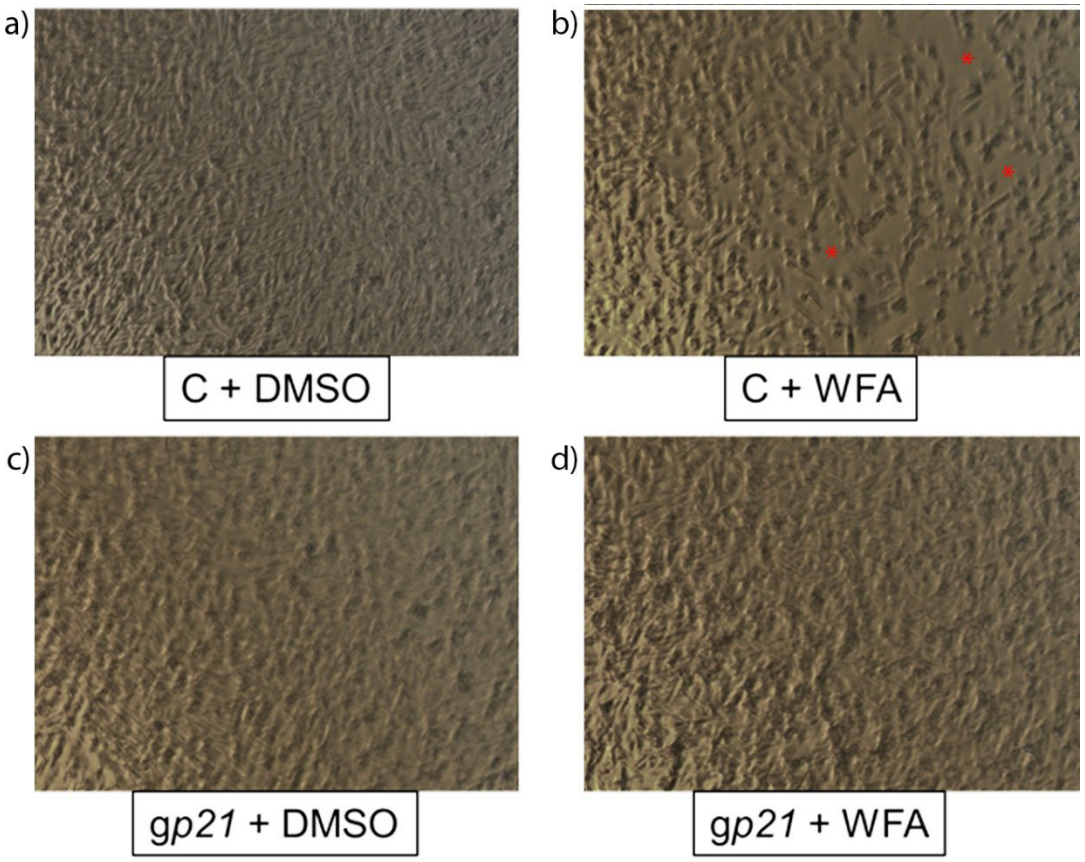
a) b)



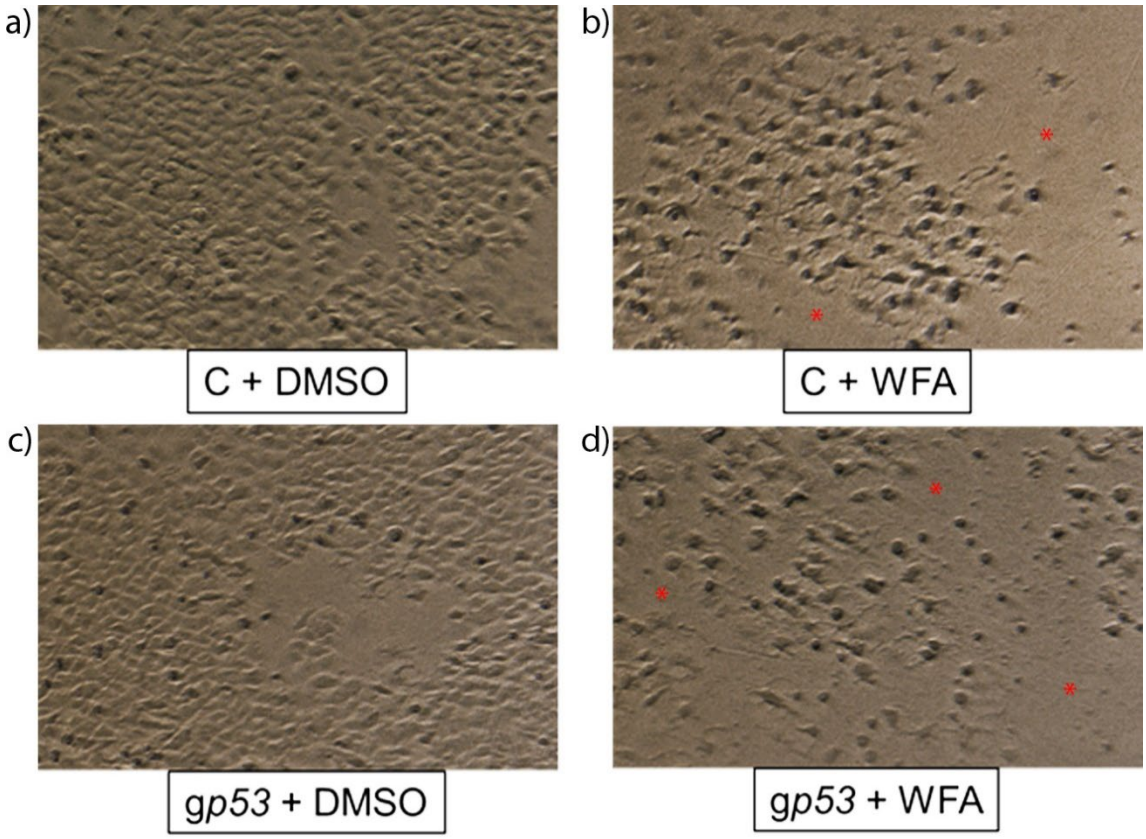
Supplementary Figure 3. Representative images of dCas-DNMT3A control (a) and dCas-DNMT3A + gCCND1 (b) cells. Areas of lower cell density are highlighted here with red asterisks. Cells pictured are MCF7 cells and images have had color saturation reduced for increased clarity.



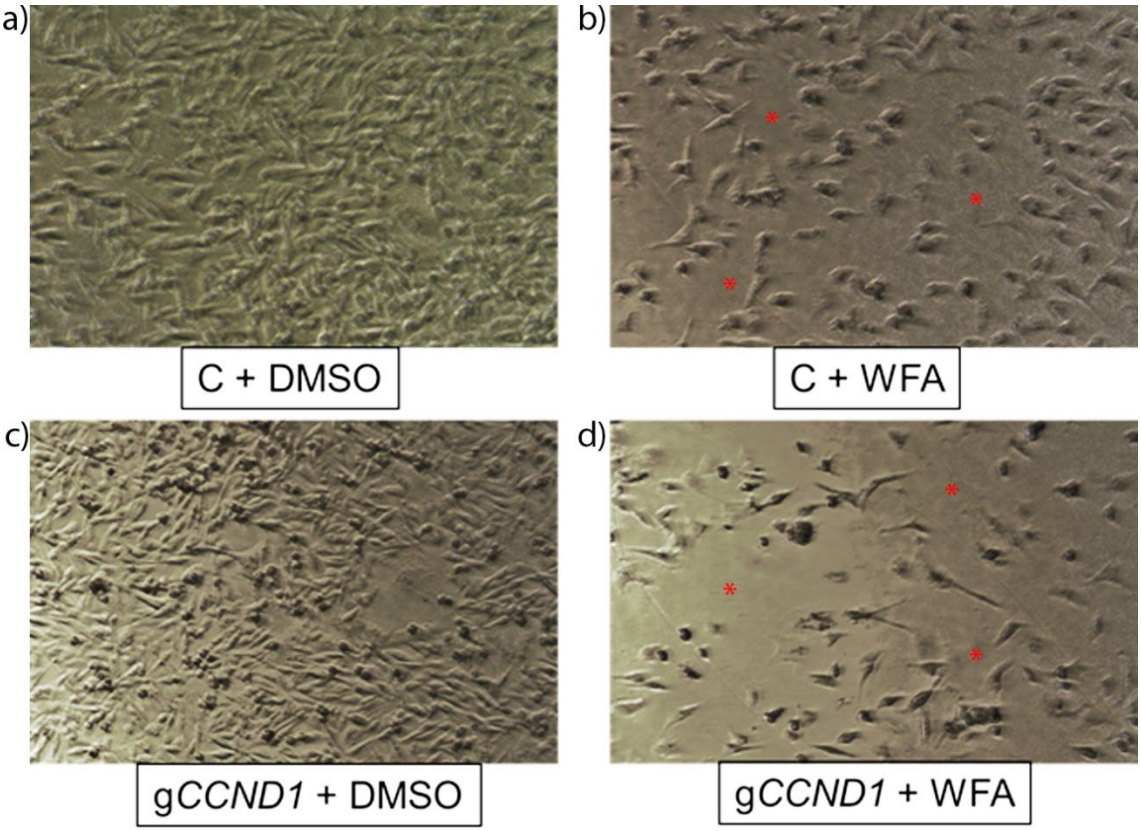
Supplementary Figure 4. MTT assay using ascending concentrations of WFA. MCF10A cells served as controls and did not have significant reductions in viability until 0.6 µM. For each group n = 6.



Supplementary Figure 5. Representative images of dCas-DNMT3A + control guides alongside DMSO (a), dCas-DNMT3A + control guides alongside WFA (b), dCas-DNMT3A + gp21 alongside DMSO (c), and dCas-DNMT3A + gp21 alongside WFA (d). Areas of lower cell density are highlighted here with red asterisks. Cells pictured are MCF7 cells and images have had color saturation reduced for increased clarity.



Supplementary Figure 6. Representative images of dCas-DNMT3A + control guides alongside DMSO (a), dCas-DNMT3A + control guides alongside WFA (b), dCas-DNMT3A + gp53 alongside DMSO (c), and dCas-DNMT3A + gp53 alongside WFA (d). Areas of lower cell density are highlighted here with red asterisks. Cells pictured are MCF7 cells and images have had color saturation reduced for increased clarity.



Supplementary Figure 7. Representative images of dCas-Tet1 + control guides alongside DMSO (a), dCas-Tet1 + control guides alongside WFA (b), dCas-Tet1 + gCCND1 alongside DMSO (c), and dCas-Tet1 + gCCND1 alongside WFA (d). Areas of lower cell density are highlighted here with red asterisks. Cells pictured are MCF7 cells and images have had color saturation reduced for increased clarity.

GENERAL DISCUSSION

While advancements in detection and treatment over the last century have led to radical decreases in BC mortality, similar advances for incidence rates remain elusive [1, 26]. Additionally, hormone receptor-poor subtypes of breast cancer, including HER2+ and triple-negative breast cancer (TNBC), have shown more modest reductions in mortality and remain significant public health concerns [26]. Additionally, side effects from current standard of care can vary greatly in severity and duration, and include acute side effects like fatigue, nausea, and pain as well as long-term side effects like lymphedema, cognitive decline, and neuropathy [27,28]. Because of this, new and more effective measures for BC prevention are in high demand.

One avenue of prevention where its importance is becoming increasingly apparent is that of nutritional chemoprevention. Nutritionally derived DPs such as SFN and WFA have shown promise in *in vitro* and *in vivo* preclinical models of cancer prevention, and these experiments are backed by clinical observations of diet and its influence on BC incidence [16,17,22, 29]. However, there is less research involving DPs as a dietary intervention over long time scales in premalignant models, and many questions remain on the importance of these time scales as well as the genetic and epigenetic mechanisms behind these DPs.

To address the first of these questions, we utilized the dietary phytochemical SFN contained within BSp in two transgenic mouse models. SFN is an isothiocyanate found in

cruciferous vegetables that, through past work in our laboratory, has been shown to have inhibitory effects on DNMTs and HDACs [30]. Work in our lab has also shown that SFN administration during adulthood before manifestation of tumors was insufficient for achieving a significant reduction in cancer-associated phenotypes [31]. Because there is a growing body of evidence indicating that environmental exposures during CPs such as puberty are important for later-life cancer risk and puberty is a period of high levels of hormone exposure often associated with BC, we designed our first set of experiments around the CP of puberty from a chemoprotective standpoint [10-13]. We administered SFN-containing BSp to an experimental group in SV40 and HER2/neu mouse models of BC during the 5-week period surrounding puberty confirmed visually with the vaginal opening (VO) method. Following this, mice were fed a control diet that extended well into adulthood for both strains of mice. We found that this treatment resulted in significant decreases in tumor incidence in both models, and for HER2/neu mice, tumor latency was significantly increased, and tumor weight was significantly decreased. These results indicate that a peripubertal DP treatment is sufficient to enact significant phenotypic changes in both tumor presence and morphology.

To parse potential mechanisms behind these changes we performed RT-qPCR and western blots on key cancer associated genes. We found that our treatment induced significant long-term increases in the gene and protein expression of the tumor suppressors *p53*, *p21*, and *BRCA2*. These genes, associated largely with apoptosis, cell cycle, and DNA repair, respectively, are important molecular targets for various anticancer therapies [32-34]. The upregulation of these tumor suppressor genes through peripubertal SFN administration provides an interesting and potentially therapeutically

relevant avenue for BC prevention. To expand on our molecular understanding of this peripubertal treatment, we followed these experiments up with whole genome RNA sequencing. From this we identified 82 upregulated genes and 92 downregulated genes. After analyzing these data, we found a number of cancer-associated genes that were differentially expressed. These included the upregulation of *Lman1l*, *Clec4e*, and *Parp6*, all of which have some varying form of identified tumor suppressor function. Additionally, our downregulated gene set included the genes *Chrdl2*, *Pcsk1*, and *Slc51b*, all of which have some identified oncogenic or pro-cancer function. It is possible that changes in these genes expression states are in some part responsible for the phenotypic changes we observed. Further ontological gene pathway analyses performed on our RNA sequencing data revealed a number of pathways that were significantly modulated in our SFN-containing BSp peripubertal treatment group.

Within the overexpressed gene set, the significantly upregulated pathways of cell death (14 genes total) and apoptosis (12 genes total) may have particular importance to anticancer activity in our BSp-treated group. Due to the close relationship between aberrant cell death regulation and cancer, it is likely that increased expression of these pathways was in some part responsible for the reduction of cancer cell phenotypes we observed. While these are newly described, preclinical findings, it is possible that these changes extend to peripubertal consumption of SFN in humans. As apoptosis is a major mechanism of cancer prevention, upregulation of these pathways using safe, non-toxic dietary measures may have clinical value [35,36].

Within the underexpressed gene set, there were two significantly downregulated pathways with connections to cancer cell biology: ATPase activity (5 genes) and biotin

binding pathways (2 genes). Both are related to increased energy and metabolic demands associated with cancer cells, and both are often significantly upregulated in rapidly dividing, aggressive forms of cancer [37-39]. Downregulation of these pathways may be responsible for the significant reduction in tumor size witnessed in our peripubertal treatment group, and, if these long-term changes extend to human peripubertal SFN consumption, they could lead to fewer and less aggressive cases of BC.

Finally, we utilized global RRBS to ascertain whether a peripubertal SFN-containing BSp treatment could have long-term effects on the methylome. We found that our treatment resulted in significant changes to promoter methylation in 243 total genes, with hypermethylation of 113 genes and hypomethylation of 130 genes. Although SFN is most often regarded as an HDAC inhibitor, these results are in accordance with previous work from our lab and indicate that SFN treatment can have significant effects on the methylome [30,40]. Building on this, we combined our RRBS data with our RNA sequencing data to ascertain which genes, if any, were both differentially methylated and expressed. We found one gene, *Erich4*, that was both hypomethylated and over expressed. Although relatively little is known about this gene, its expression is positively associated with the expression of the antiproliferation-associated gene *PPP2R5A* [41]. If the interaction between these two genes is important, it is possible that our treatment's upregulation of *Erich4* has a significant effect on our observed reduction in tumor severity, and *Erich4* may be an important target for future study.

While this work helped to elucidate the importance of CPs such as puberty in DP-associated cancer prevention, we also sought to explore the molecular epigenetic mechanisms behind DP function, specifically how changes to gene-specific methylation

states can affect DP function. To accomplish this, we utilized the DP WFA, a steroidal lactone and DNMT inhibitor isolated from ashwagandha root, in conjunction with CRISPR-dCas technology that allows for gene-specific manipulation of promoter methylation state [29,42]. Using the MDA-MB-231 and MCF7 cell lines, we focused on three genes tied closely with breast cancer cell biology. *P21* is an important tumor suppressor gene with ties to cell cycle progression and apoptosis [43]. Although it is not typically mutated in BC cases, its expression and promoter methylation are often dysregulated, and restoration of these factors to typical biological levels could have utility from both a prevention and therapeutic standpoint [32,43]. We found that targeting the demethylator Tet1 to the promoter of *p21* was able to significantly increase its gene expression and decrease cell viability in both MCF7 and MDA-MB-231 cells. *P53* is a tumor suppressor that is vital for the regulation of cell homeostasis and prevention of cancerous cellular progression [44]. The mutation rate for *p53* is high in BC cases, and in cases where the gene is not mutated the promoter is often hypermethylated [44,45]. We found that targeting the demethylator Tet1 to the promoter of *p53* led to a significant increase in its gene expression in both MCF7 and MDA-MB-231 cells. However, we only observed significant decreases in cell viability for the non-mutant *p53* MCF7 cell line. *CCND1* is an oncogene that, while not typically dysregulated in wider cancer types, is overexpressed in over 50% of cancer cases and associated with poor outcomes [46,47]. We found that targeting the methylator DNMT3A to the promoter of *CCND1* was able to significantly decrease its gene expression and decrease cell viability in both MCF7 and MDA-MB-231 cells. Taken together, these results indicate that each of these genes

respond to methylation-induced regulation in our cell models and they may serve as important potential targets for methylation-based therapies or prevention of BC.

Previous studies implicated these three genes in the function of WFA, and treatment of our cell lines with 0.5 μM WFA resulted in significant decreases in cancer cell viability but not MCF10A breast control cells [20, 48-50]. We confirmed that WFA treatment significantly decreased the promoter methylation state and increased the expression of *p21* and *p53*. WFA treatment also significantly increased the promoter methylation state and decreased the expression of *CCND1*. To determine which of these genes' methylation states were important to the anticancer function of WFA, we combined this treatment with a guided epigenetic modifier that would counter our WFA-induced changes to methylation state. We found that by guiding DNMT3A methylator to the promoter of *p21* in the presence of WFA, we were able to ablate the methylation and expressional changes associated with WFA treatment. In addition, targeted methylation of the *p21* promoter restored the WFA-associated losses in cell viability. Similarly to *p21*, we found that targeted methylation of the *p53* promoter in conjunction with WFA resulted in a loss of WFA induced promoter demethylation and its associated gene expression increases. Despite this, cells treated with WFA and these constructs maintained the WFA-associated cell viability losses, indicating that epigenetic manipulation of this gene was not sufficient to remove WFA's anticancer function. Targeted demethylation of the *CCND1* promoter alongside WFA treatment led to restoration of *CCND1* expression and promoter methylation to baseline levels. As with *p53*, these changes did not restore BC viability within our BC cell lines. Taken together, these results indicate that decreases in promoter methylation and subsequent increases in gene expression of *p21* are vital for the function

of WFA, as ablation of WFA-induced changes to *p21* was sufficient for the reversal of associated viability losses. While methylation and expressional changes associated with modulating *p53* and *CCND1* can have significant impacts on BC cell viability, removal of WFA induced changes in these genes alone does not appear sufficient to remove the anticancer function of WFA. As *p21* mutations in BC are rare, WFA induced increases in expression could have important therapeutic and chemopreventive potential [32]. Additionally, WFA's continued function in the presence of downregulated and methylated *p53* implicates its utility in the prevention of *p53* mutant BC. As over 50% of BC cases have a mutant form of *p53*, our study underlines the wide potential for this DP [51]. These results also indicate that WFA may have utility for BC prevention in individuals with inherited *p53* mutations such as Li Fraumeni syndrome [52].

Overall, our studies provide important insights into the temporal and epigenetic mechanisms behind the DPs SFN and WFA, respectively. We are among the first to both implicate the CP of puberty as a target for chemoprevention in a basic science setting as well as elucidate the importance of gene specific methylation states to the function of a DP. Our work lays important groundwork for establishing and understanding the temporal role that DPs may play in BC prevention and identification of key genes and pathways with which these DPs interact. In addition, our *in vitro* experiments reveal the potential for modulating specific methylation states on cancer cell viability and provide evidence for the utility of WFA for the prevention of *p21* wild-type cancers.

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