Broccoli sprouts delay prostate cancer formation and decrease prostate cancer severity with a concurrent decrease in HDAC3 protein expression in TRAMP mice.

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Abbreviations: HDACs, histone deacetylases; H3K9ac, acetyl-histone H3 lysine 9; H3K9me3, trimethyl-histone H3 lysine 9; H3K18ac, acetyl-histone H3 lysine 18; H&E, hematoxylin and eosin; mPIN, mouse prostatic intraepithelial neoplasia; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; qPCR, quantitative real-time PCR; RBBP8, retinoblastoma binding protein 8, endonuclease; SERPINB5, serpin family B member 5; SFN, sulforaphane; SFN-CG, sulforaphane-cysteinylglycine; SFN-Cys, sulforaphane-cysteine; SFN-GSH,
sulforaphane-glutathione; SFN-NAC, sulforaphane-N-acetylcysteine; STAT3, signal transducer and activator of transcription 3; TRAMP, transgenic adenocarcinoma of the mouse prostate

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Abstract

Background: Cruciferous vegetables have been associated with the chemoprevention of cancer. Epigenetic regulators have been identified as important targets for prostate cancer chemoprevention. Treatment of human prostate cancer cells with sulforaphane (SFN), a chemical from broccoli and broccoli sprouts, inhibits epigenetic regulators such as histone deacetylase (HDAC) enzymes, but it is not known whether consumption of a diet high in broccoli sprouts impacts epigenetic mechanisms in an *in vivo* model of prostate cancer. Objective: In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, we tested the hypothesis that a broccoli sprout diet suppresses prostate cancer, inhibits HDAC expression, alters histone modifications, and changes the expression of genes regulated by HDACs. Methods: TRAMP mice were fed a 15% broccoli sprout, or control AIN93G diet; tissue samples were collected at 12 and 28 weeks of age. Results: Mice fed broccoli sprouts had detectable levels of SFN metabolites in liver, kidney, colon, and prostate tissues. Broccoli sprouts reduced prostate cancer incidence, and progression to invasive cancer by 11- and 2.4-fold at 12 and 28 weeks of age, respectively. There was a significant decline in HDAC3 protein expression in the epithelial cells of prostate ventral and anterior lobes at 12 weeks. Broccoli sprout consumption also decreased histone H3 lysine 9 tri-methylation in the ventral lobe (12 week), and decreased histone H3 lysine 18 acetylation in all prostate lobes (28 weeks). A decline in p16 mRNA levels, a gene regulated by HDAC3, was associated with broccoli sprouts consumption, but no significant changes were noted at the protein level. Conclusions: Broccoli sprout intake caused a decline in prostate cancer occurrence and HDAC3 protein expression in the prostate, extending prior work.
that implicated loss of HDAC3/corepressor interactions as a key preventive mechanism by SFN *in vivo*.

**Keywords:** broccoli, chemoprevention, histone deacetylase (HDAC), histone H3 lysine 18 acetylation (H3K18ac), epigenetics, prostate cancer, TRAMP mouse model, sulforaphane
Introduction

Prostate cancer is the second most frequently diagnosed cancer among men globally, and is a leading cause of cancer-related deaths in the United States (1, 2). The disease is typically slow growing, and although abnormalities in the prostate epithelium can be observed in males in their twenties or thirties, prostate cancer generally does not become of clinical concern until later in life (3-5). The long latency period of prostate cancer suggests that therapeutic strategies that slow disease progression could be beneficial by delaying full disease onset and possibly decreasing invasive surgical procedures like prostatectomy. Increasing the latency period of prostate cancer could also be beneficial by increasing the period of time during which a therapeutic intervention could occur. Characterization of the molecular mechanisms that delay prostate cancer formation will be beneficial to facilitate the development of effective chemopreventive strategies.

An association between increased cruciferous vegetable intake and a reduced risk of developing, or being diagnosed with prostate cancer has been reported (6). Cruciferous vegetables, such as broccoli and broccoli sprouts, are a rich source of glucosinolates (7). When broccoli sprouts are chopped or chewed, the glucosinolate glucoraphanin interacts with the enzyme myrosinase, producing the phytochemical sulforaphane (SFN) (7). Broccoli sprouts and SFN have chemopreventive and cancer suppressive properties in carcinogen-induced and genetic models of prostate cancer (7-9); however, the mechanisms by which they act in vivo are not completely understood. SFN has been shown to inhibit the initiation of cancer by blocking damage caused by carcinogens through the induction of phase 2 enzymes via kelch like ECH associated
protein 1 (Keap1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling (10-13).

In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer, broccoli consumption and/or SFN treatment has been shown to slow prostate cancer growth and metastasis (8, 9, 14, 15). Several potential mechanisms have been implicated, including the induction of Nrf2 related pathways, inhibition of the cancer promoting Akt signaling cascade, suppression of a chemokine receptor (CXCR4), and through augmenting the lytic activity of natural killer cells (8, 9, 14, 15). In contrast to these results, Liu et al. did not find a significant decrease in prostate cancer in TRAMP mice feed a diet high in broccoli sprouts, highlighting a degree of controversy regarding cruciferous vegetable intake and the prevention of prostate cancer (16).

A hallmark of cancer development is the global modification of epigenetic marks (17). These marks regulate chromatin structure and thus participate in the regulation of gene expression and genome stability. Cancer cells often have dysregulated expression of genes that control epigenetics, such as upregulated histone deacetylase (HDAC) enzymes (18, 19). This contributes to cancer development and progression by turning off tumor suppressor genes, or promoting the expression of oncogenes (20). We and others have shown that SFN can alter epigenetic endpoints in cancer cell lines and tissues, including suppression of HDAC expression, changes in DNA methylation, and increased expression of epigenetically repressed genes like p21 and p16 (21-29). In an in vitro study of TRAMP C1 cells, SFN was shown to restore Nrf2 expression through epigenetic modifications and attenuated the expression of several HDAC proteins (13). While there is substantial evidence that SFN exposure can influence epigenetic endpoints in cancer cells, it has not yet been shown in an in vivo model of prostate
cancer that consumption of a whole food rich in SFN, such as broccoli sprouts, can
induce changes in epigenetic regulators and contribute to chemoprevention. We sought
to test the hypothesis that consumption of a diet high in broccoli sprouts suppresses
prostate cancer, inhibits HDAC expression, alters histone modifications, and changes
expression of genes regulated by HDACs. We show that consumption of a diet high in
broccoli sprouts decreased the incidence and severity of prostate cancer, reduced
HDAC3 protein, and altered epigenetic related endpoints.

Materials and Methods

Husbandry and Study Design

Custom AIN93G diet with 15% broccoli sprout powder and matched control diet
was prepared by Research Diets (Supplemental Table 1) (New Brunswick, NJ). This
15% broccoli sprout diet had 400 mg SFN / kg of diet, which was chosen because it is
equivalent to 1 mg SFN / day which has been used in previous studies (14, 15, 30).
Broccoli sprout powder was purchased from Natural Sprouts Company, LLC
(Springfield, MO). Diets were stored protected from the light at -20 °C. Male TRAMP
mice in C57BL/6 background were obtained from Jackson Lab and bred in the Oregon
Health & Science University (OHSU) animal facility (31-33). Animal protocol was
approved by the OHSU Institutional Animal Care and Use Committee. Animals were
housed with a 12-h light and 12-h dark cycle, in a temperature- and humidity-controlled
environment and fed standard lab chow. At 4 weeks of age the animals were placed on
either the broccoli sprout or AIN93G control diet. Food consumption was measured over
the course of the study and no difference was found in the intake of food between the
control and broccoli sprout fed groups.

Animals were sacrificed in the morning during a 3-4 hour window at 12 and 28
weeks of age. Lung, liver, spleen, kidney, colon and urogenital tract were removed.
Weights of the urogenital tract and prostate lobes were recorded. The prostate lobes
were then formalin fixed, paraffin embedded, sectioned and stained with hematoxylin
and eosin (H&E) and scored for cancer incidence and severity by multiple pathologists
(CVL from Oregon Veterinary Diagnostic Laboratory at Oregon State University and GT
from Oregon Health & Science University). Some prostates were further dissected to
separate the anterior, dorsolateral, and ventral lobes and analyzed separately because
in this TRAMP model the cancer is driven by the T antigen oncoprotein primarily in the
ventral and dorso-lateral lobes (reviewed in (34)). Individual lobes were snap frozen or
put into RNA later for subsequent molecular assays.

HPLC-MS/MS analysis

The methods for evaluating levels of SFN metabolites in mouse tissues via HPLC-
MS/MS analysis were performed as previously described (35). Briefly, approximately 50
mg of frozen tissue was homogenized using a mortar and pestle in liquid nitrogen. An
internal standard (5 µL of 100 µM deuterated SFN-NAC) and 50 µL of 10% TFA (v/v) in
water was added to the sample and vortexed vigorously. The homogenate was then
frozen at −80°C. Later samples were thawed, vortexed, centrifuged (11,600 × g, 5 min,
4 °C) and the supernatant was filtered through a 0.2 µm pore size filter. Ten µL of
filtered sample were separated on a Shimadzu Prominence HPLC (Shimadzu, Kyoto,
Japan) using a reversed-phase Phenomenex Kinetex PFP 2.6 µm 100Å 100 × 2.6 mm HPLC column. The LC eluent was analyzed by an API triple quad mass spectrometer 3200 (Applied Biosystems, Foster City, CA) with electrospray ionization in positive mode. Tandem mass spectrometry using multiple reaction monitoring was used to detect the analytes with the following precursor and product ions: SFN (178>114), SFN-glutathione, (SFN-GSH, 485>114), SFN-cysteinlyglycine (SFN-CG, 356>114), SFN-cysteine (SFN-Cys, 299>114), SFN-N-acetylcysteine (SFN-NAC, 341>114). Spike and recovery experiments using the internal standard confirmed that >80% of all compounds were recovered. Quantification was performed by using a standard curve ranging from 0.16 to 25 µM.

**Immunohistochemistry**

Immunohistochemical staining was performed on an autostainer (Dako Autostainer Universal Staining System; Dako, Carpinteria, CA) following standard operating procedures of the Oregon Veterinary Diagnostic Laboratory. In brief, paraffin sections were high-temperature antigen retrieved with BDTM Retrieval A solution (Dako) pH 9.0 (HDAC6) or pH 6.0 (all others). Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide (10 min). Primary rabbit anti-human antibodies for HDAC3 (Abcam ab32369), HDAC6 (Abcam ab1440), H3K18ac (need info from OHSU), H3K9ac (Cell Signaling 9671), and H3K9me3 (Abcam ab8898) were applied for 30 minutes at room temperature. MaxPoly-One Polymer HRP Rabbit Detection solution (MaxVision Biosciences, Bothell, WA) was applied (7 min, RT). Nova Red (SK-4800; Vector Labs, Burlingame, CA) was used as chromagen and Dako hematoxylin (S3302) as counterstain. Serial sections of neoplastic tissue incubated with
Dako Universal negative serum served as negative controls. Images were visualized on a Nikon system that included Eclipse E400 microscope, DS-Fi2 camera, and NIS-Elements BR software package. For HDAC3, HDAC6, histone H3 lysine 9 acetylation (H3K9ac), and H3K9me3 five images were captured for each prostate lobe for each individual mouse. HDAC3 and H3K9ac images were taken with 400x magnification with a correction for white balance and the mean intensity was calculated by the software for 25 nuclei in each of the images, and then averaged for each individual. HDAC6 images were captured with 200x magnification and the intensity of cytoplasmic staining was calculated by the software for three regions per image. HDAC6 intensity values in each image were then corrected for differences in white balance, and then averaged for each mouse. For H3K9me3, images were captured with 1000x magnification and staining intensity was measured with the software in 1) punctate regions of the nucleus with high intensity staining that we refer to as foci, and 2) the whole nucleus. Each H3K9me3 image had between 20 and 70 nuclei that were fully in focus and quantified. There were an average of ~3 foci of H3K9me3 staining analyzed per nuclei. The staining intensity results were then averaged for each mouse. The number and size of foci with H3K9me3 staining was also captured. The staining intensity data for HDAC3, HDAC6, H3K9ac, H3K9me3 are expressed as mean staining intensity, subtracted from the intensity of true white, and expressed as a percentage of all possible color. Positive staining for histone H3 lysine 18 acetylation (H3K18ac) was defined as intensity of red chromagen precipitate in the nuclei and was scored blindly on a scale from 0-3.
Quantitative Real-Time PCR (qPCR)

Total RNA was collected from indicated prostate lobes in 12-13 week old animals using a standard Trizol extraction method (Life Technologies). cDNA was synthesized using 1 µg of total RNA and SuperScript III First-Strand Synthesis SuperMix (Life Technologies). Real time PCR was done using primers that amplify all known transcript isoforms of each mouse gene as a single product of expected size, between 140 and 300bp, with the exception of p16 where the primers where designed to only amplify p16 and not the isoform of CDKN2A that codes for ARF (alternate open reading frame). Primer sequences were as follows: 18S, (forward) 5'-CCGCAGCTAGGAATAATGGAAT-3' and (reverse) 5'-CGAACCTCCGACTTTTCGTTCT-3'; CtIP (also known as RBBP) (forward) 5'-GACCCAGGAGCAGACCTTTC-3' and (reverse) 5'-CATCTGGTACCTGGGAGAAGC-3'; heme oxygenase 1 (HO1) (forward) 5'-GACACCTGAGGTCAAGCACA-3' and (reverse) 5'-CTAGCAGGCCTCTGACGAAG-3'; NAD(P)H dehydrogenase, quinone 1 (NQO1) (forward) 5'-TAGCCTGTAGCCAGCCCTAA-3' and (reverse) 5'-GCCTCCTTTCATGGCGTAGTT-3'; p16 (forward) 5'-AACTCGAGGAGGAGCCATCTG-3' and (reverse) 5'-GGGGTACGACCGAAAGAGTT-3'; Serpinb5 (forward) 5'-CCGGAATCAGAACAAGAGATGT-3' and (reverse) 5'-CTTGGGGAGCACAATGAGCA-3'; signal transducer and activator of transcription 3 (Stat3) (forward) 5'-AGTTCTGGCACCCTTGGATT-3' and (reverse) 5'-CGATCCGGGCAATTCCATT-3'. Reactions were performed using Fast SYBR Green Mastermix (Life Technologies) on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR conditions were programmed as follows: 95°C for
20 s, followed by 40 cycles of denaturing at 95°C for 1 s, annealing and extension at 58°C for 20 s, followed by a dissociation curve at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. A dilution series of template DNA served as internal standard for quantification (36). Data represent the transcript level of the gene of interest (as expressed as a copy number) normalized to the copy number of the housekeeping gene 18s.

**Immunoblot analysis**

Protein was harvested from prostate lobes using a hand held pestle (Thermo Fisher, Waltham, MA, USA), and radioimmunoprecipitation assay (RIPA) protein lysis buffer (Thermo Fisher) supplemented with protease inhibitor cocktail (Thermo Fisher) and processed as previously described (37). Equal amounts of protein were separated on NuPage Bis-Tris SDS-PAGE gels (Thermo Fisher) and blotted to a PVDF or nitrocellulose membrane (Bio-Rad, Hercules, CA) in accordance with the manufacturer’s protocol (Thermo Fisher). Membranes were blocked overnight with 5% powdered milk in PBST at 4 °C and then probed for the indicated proteins following standard protocols using anti- p16 (10883, Proteintech, Rosemont, IL), and β-actin (A5441) (Sigma-Aldrich, St. Louis, MO) antibodies at 1:200 and 1:10,000 dilutions, respectively. Goat anti-rabbit (1:1,000 dilution), or goat anti-mouse (1:50,000 dilution) secondary antibodies were also used (Santa Cruz Biotechnology) using standard conditions. Membranes were incubated in SuperSignal West Femto Reagent (Thermo Fisher) and developed on the ChemiDoc MP imaging system for visualization (Bio-Rad). Densitometric analyses were performed on the native membrane image using Image Lab 4.0 software (Bio-Rad). The relative densitometric value of each replicate for p16...
was normalized to the corresponding relative level of β-actin and expressed relative to the mean amount found in mice fed a control diet.

**Statistical Analysis**

All data were graphed in GraphPad Prism 5 software (La Jolla, CA) with bars indicating the mean ± the SEM. To determine if there were statistically significant differences between groups for continuous variables, both two-way ANOVA and unpaired t-tests were performed. For categorical variables, we used either Fisher's exact or Chi-square tests to compare groups. Cochran–Armitage tests were conducted for trend analysis. Bonferroni corrections (also known as Bonferroni post-tests) were used to account for multiple comparisons. A statistically significant difference between groups was noted for p-values less than 0.05.

**Results**

Broccoli sprout consumption slowed prostate cancer formation and decreased cancer severity.

Animals fed the 15% broccoli sprout diet had detectable levels of SFN metabolites in liver, kidney, colon, and prostate tissues (Figure 1). SFN-cysteine was the most abundant metabolite in the kidney and prostate, while SFN conjugated to glutathione was highest in the liver. SFN-N-acetylcysteine was the most abundant SFN metabolite in the colon. The parent compound, and SFN-cysteinylglycine were not detected in any samples tested. The average total SFN metabolites were 1.1, 8.7, 5.8 and 0.6 pmol SFN / mg tissue for the liver, kidney, colon and prostate, respectively. SFN metabolites were not detected in control animals (data not shown).
In the TRAMP model, the SV40 transgene expression is turned on at sexual maturity between 8 and 10 weeks of age. Following this, mouse prostatic intraepithelial neoplasia (mPIN) lesions are seen at 12 weeks of age. By 28 weeks adenocarcinomas and metastasis can occur (31-33). As expected both the urogenital tract and prostate weights increased with age in control mice (Figure 2A and 2B). At the 12 week point, urogenital tract weight and prostate weight in animals on the high broccoli sprout diet were 2.8-fold and 2.3-fold, respectively, lower than in mice on the control diet (Figure 2A and 2B). At 28 weeks of age the effect of broccoli sprout diet was not as apparent on urogenital tract and prostate weights, and there was no significant differences between the animals on control and broccoli sprout diet (Figure 2A and 2B).

Prostate cancer incidence and severity was significantly reduced in the broccoli sprout groups at both the 12 and 28 week time points (Figure 2C-E). At 12 weeks of age all control mice developed at least early neoplastic lesions (mPIN), while seven out of 20 of the broccoli sprout fed animals had normal prostates (Figure 2C and 2D). Furthermore, only one broccoli sprout mouse developed an adenocarcinoma while 10 out of 18 control animals had adenocarcinomas at 12 weeks of age (Figure 2C and 2D). By 28 weeks of age, 16 out of 18 control animals had an adenocarcinoma, while only 7 out of 19 broccoli sprout mice had cancer that had advanced to this state (Figure 2C and 2E). It is also worth noting that at the point of sacrifice, two of the broccoli sprout fed animals never developed any prostate lesions (Figure 2C). Importantly, consumption of a diet high in broccoli sprouts significantly reduced the incidence of invasive prostate cancer by 11- and 2.4-fold, at the 12 and 28 week time points, respectively (Figure 2F).
Broccoli sprout consumption decreased HDAC3 expression in prostate epithelium

To determine if HDAC protein expression was altered in prostate epithelium we performed immunohistochemistry using antibodies against HDAC3 and HDAC6. We focused on these HDACs because HDAC3 is highly expressed in prostate cancer and HDAC6 regulates androgen receptor signaling, and both HDACs are decreased by SFN treatment in *in vitro* models of cancer (22, 23, 28, 38-40). A significant decline in HDAC3 protein expression was detected in the ventral and anterior lobes of the prostate of mice fed broccoli sprout diet (Figure 3A and 3B). The broccoli sprout-induced decline in HDAC3 protein was more apparent at the 12-week time point (Figure 3B). We did not detect a significant change in HDAC6 protein abundance in the prostates of broccoli sprout fed mice at 12 or 28-weeks of age (Figure 4A and 4B). It is worth noting that HDAC6 protein appeared lower with broccoli sprouts at the 28-week time point in the ventral lobe of the prostate, and a t-test confirmed a trend for decreased HDAC6 with broccoli sprouts in this lobe (Figure 4B, t-test, p=0.056).

Broccoli sprout-induced changes in histone modifications in TRAMP mouse prostates

HDACs regulate gene expression by removing acetylation marks from histones (41). Since histone H3 lysine 18 (H3K18) and histone H3 lysine 9 (H3K9) acetylation have been shown to be regulated by HDAC3, we tested if broccoli-sprout mediated decrease in HDAC3 expression resulted in alterations in H3K18 and H3K9 acetylation levels in the prostate (42). Surprisingly, broccoli sprout diet induced a significant 2 fold decline in H3K18 acetylation levels in all prostate lobes at the 28 week time point (Figure 5A and B). There was no significant change in H3K18ac at 12 weeks when
HDAC3 protein was significantly decreased, and thus no correlation between HDAC3 and H3K18 acetylation was detected (Figure 3 and 5B). We did not detect a significant change in acetylation of H3K9 residues in the prostate of mice fed broccoli sprouts, but we did find a significant age effect in the anterior lobe of the prostate (Supplemental Figure 1). Since we did not find the expected changes in acetylation of histones, we next examined if consumption of broccoli sprouts altered the tri-methylation status of histone H3 lysine 9 (H3K9me3), which has been previously reported to decrease in vitro following SFN treatment in PC-3 prostate cancer cells and is altered with HDAC3 deletion (26, 43). More specifically, because the anterior lobe of the prostate exhibited a marked loss of HDAC3, we examined the area, intensity, and number of H3K9me3 foci, and noted H3K9me3 punctate staining in the nucleus. A significant decline in H3K9me3 also occurred with age, but there was no apparent effect of diet (Supplemental Figure 2A). We next focused our examination of H3K9me3 levels in the ventral lobe at the 12 week time point, because this was when HDAC3 was significantly downregulated. A significant 13% decrease in the mean area / foci for H3K9me3 staining was found with broccoli sprout consumption in the ventral lobe (Supplemental Figure 2B).

Effect of broccoli sprouts diet on the expression on HDAC3 target genes

To gain further insights into how decreases in HDAC3 by broccoli sprouts could slow prostate cancer progression we evaluated the expression of several genes which are known to be regulated by HDAC3. We examined the mRNA expression of p16 (also known as cyclin dependent kinase inhibitor 2A (CDKN2A)), signal transducer and activator of transcription 3 (STAT3), retinoblastoma binding protein 8, endonuclease (RBBP8 also known as CTIP), and serpin family B member 5 (SERPINB5) (38, 44, 45).
This work was done in TRAMP prostates of 12 week old animals when HDAC3 was decreased with broccoli sprouts. As a positive control we examined the expression of a known target of SFN, the NAD(P)H quinone dehydrogenase 1 gene (NQO1), and show that its mRNA level was significantly upregulated with broccoli sprout consumption in the dorsolateral lobe of the prostate (Figure 6A, and (46)). We found significant differences in the expression of mRNA in all 5 genes when compared among the different prostate lobes (Figure 6, and Supplemental Figure 3). No significant effect of broccoli sprout consumption on the mRNA levels of RBBP8 and SerpinB5 were detected although there was a trend for increased expression of STAT3 with broccoli sprout consumption (Supplemental Figure 3A-C). Unexpectedly, the tumor suppressor gene p16 was significantly decreased at the mRNA level with broccoli sprout consumption in all prostate lobes (Figure 6B). This coincided with the time point when HDAC3 was decreased. Western blotting revealed no change in the amount of p16 at the protein level (Figure 6C). We also examined p21 protein expression but it was not detectable in the prostate lobes (data not shown).

Discussion

Given the high incidence and mortality associated with prostate cancer worldwide, reducing prostate cancer incidence and slowing progression is of great importance. The World Health Organization has identified that between 30-50% of the current global cancer burden could be prevented, and indicate an unhealthy diet and low fruit and vegetable intake is a key modifying risk factor for cancer development (47).
Here we show in a preclinical model that consumption of a diet high in broccoli sprouts results in detectable levels of SFN metabolites in the prostate and reduced prostate cancer incidence and severity. We showed for the first time that a diet high in cruciferous vegetables can decrease HDAC expression, primarily HDAC3, in the prostate epithelial cells at a time when prostate cancer is developing. We also showed that broccoli sprout-diet caused significant changes in some epigenetic marks, with broccoli-induced declines in the acetylation of histone H3 lysine 18 being the most notable.

The TRAMP model of prostate cancer was utilized because the tumors occur in the prostate epithelium and the tumor tissue histopathology closely mimics human disease. Additional advantages include that the tumors arise spontaneously and appear in ~100% of mice (31-33). The cancer is driven by the oncoprotein SV40 T antigen which binds to p53 and retinoblastoma proteins, disrupting their tumor suppressor function and the normal signaling circuitry that controls cell cycle (48). Our data are in agreement with several studies in TRAMP mice where a diet high in broccoli sprouts, or treatment with SFN, suppressed prostate cancer development and/or metastasis (8, 14, 15, 49). Overall this literature suggests that broccoli sprouts (and/or SFN) are acting through multiple mechanisms to decrease prostate cancer development, including inhibition of cell cycle, inhibition of the chemokine receptor CXCR4, and increased apoptosis via mechanisms like inhibition of the Akt signaling pathway (8, 14, 49). In contrast to these studies, Liu et al. did not find a significant effect of broccoli sprout diet on prostate cancer, although they used a lower amount of broccoli sprouts (10%...
broccoli sprout powder), and started the animals on the diet at a later age than our study (16).

Our results of broccoli-induced decrease in HDAC3 protein is significant because HDAC3 is highly expressed in carcinomas of prostate cancer patients, and upregulation of class I HDACs are thought to be an early event in prostate carcinogenesis (40). Our results are consistent with previous work showing inhibition of HDAC3 with broccoli related supplements in preclinical models of colon and skin cancer, and in clinical studies looking at human breast tissue and blood cells (27-29). Together, these studies show that HDAC3 is suppressed following broccoli sprout consumption across multiple tissue types and species. The mechanism by which SFN induces HDAC3 degradation has been previously described in colon cancer cells and is likely similar in prostate tissue, involving disruption of corepressor interactions and increased nuclear-cytoplasmic trafficking (23). We did not see a significant decrease of HDAC3 when the prostate cancer was more advanced. It is not clear why this effect was lost, and future work will have to explore this phenomenon. One issue that may contribute to the loss of some of the expected effects is the adaption of the organism and/or cancer to a high broccoli sprout diet that was consumed over the majority of the animals’ life. HDAC6 has been observed to be inhibited and/or decreased by SFN in cultured prostate cancer cells (22, 39). We saw only a trend of decreasing HDAC6 protein with broccoli sprout consumption. It will be interesting for future work to determine if HDAC3 or HDAC6 expression is suppressed in prostate biopsies of men who have consumed broccoli sprout or related supplements.
We encountered limitations with the TRAMP model when we found a diet high in broccoli sprouts significantly decreased H3K18ac, and p16 mRNA levels. Inhibition of HDAC3 is generally thought to increase histone acetylation, and increased expression of p16 has previously been observed in human PBMCs following consumption of broccoli sprout extracts, and in colon tumors of wild-type mice treated with SFN (28).

We cannot rule out effects of the dietary treatments on histone acetyltransferases (HATs), which coordinate with HDACs to regulate overall histone acetylation status. Previous changes in epigenetic targets reported with SFN may be different than what is observed with a whole food approach tested here, as the food has added components that could cause differences in downstream molecular mechanisms. It is important to note though that in the TRAMP model the large T antigen is known to upregulate p16 expression and promote global H3K18 hypoacetylation through interactions with the histone acetyltransferases p300 and CBP, and this is likely effecting the epigenetic targets studied here (50-53). Our study cannot directly confirm large T antigen effects on P16 or H3K18ac because we did not follow these endpoints over a continuum of prostate cancer development. Nevertheless, it is encouraging that the broccoli-induced alterations in p16 mRNA and H3K18ac levels we observed were correcting for changes that are thought to contribute to cancer promotion in this model (52, 53). The changes in cell signaling induced by the large T-antigen are also the likely mechanism for why no changes in p16 protein abundance was found (50, 51). Interestingly, p16 overexpression has been found in human benign tumors, high-grade malignancies, and in a specific mouse model of colon cancer, where SFN treatment decreased p16 protein levels when the animals were heterozygous for the gene Nrf2 (28, 54, 55).
Broccoli sprout consumption also decreased the area of H3K9 tri-methylation in the ventral prostate lobe at the same time when HDAC3 was decreased. This decline in H3K9 tri-methylation is consistent with a previous report in our lab showing SFN decreased global H3K9me3 by modifying the histone methyltransferase SUV39H1 (26). Taken together, the data from this study support that broccoli-induced alteration of the epigenetic landscape is likely one important mechanism by which a diet high in broccoli sprouts contributes to prostate cancer chemoprevention. The study also highlights that the cellular context in which a chemopreventive treatment is given is critical in determining the expected molecular endpoints, and point to the need to conduct studies using human clinical samples when possible.

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References


51. Morey Kinney SR, Smiraglia DJ, James SR, Moser MT, Foster BA, Karpf AR. Stage-specific alterations of DNA methyltransferase expression, DNA hypermethylation,


**Figure Legends**

**Figure 1: SFN metabolites were detectable in mice fed a diet rich in broccoli sprouts.** SFN-cysteine (SFN-Cys), SFN-glutathione (SFN-GSH), and SFN-N-acetylcysteine (SFN-NAC) were detected in the indicated organs of mice on 15% broccoli sprout diet at 12 weeks of age (n=3-6). SFN metabolites were not detected in control animals.

**Figure 2: Broccoli sprout consumption suppressed prostate cancer development.** TRAMP mice were fed the indicated control or 15% broccoli sprout diet and evaluated for A) urogenital (UG) tract weight, B) prostate weight, and C-F) the presence and severity and lesions associated with prostate cancer, at 12 and 28 weeks of age (n= 17-20). A-B) Statistical significance was calculated using a t test where * and ** indicate significant differences between the groups where p < 0.05 and p < 0.01, respectively. C) Colors indicate the number and distribution of mice with the indicated tumor grade level by dietary intake and age (n=18-20). Cochrane-Armitage Trend test was used to evaluate differences in tumor grade level between control and broccoli sprout groups where at 12 weeks *** indicate p < 0.0001 and 28 weeks * indicate p = 0.019. D-E) H&E stained sections of prostate tissue at D) 12, and E) 28 weeks of age; left column 100x; right column 200x. D) Mouse prostatic intraepithelial neoplasia (mPIN) is evident in the anterior prostate lobe of both control and broccoli sprout mice. However, cribriform mPIN is diffuse and pronounced in control mice in contrast to the multifocal distribution of early mPIN in broccoli sprout mice. Note, mitotic figures are more frequent in the latter, but cellular and nuclear atypia more prominent in control prostates. E) Well-differentiated, cribriform to tubular adenocarcinoma in the prostate of a broccoli sprout
mouse is smaller and has low cellular atypia compared to moderately-differentiated adenocarcinoma with numerous mitotic figures and noticeable cellular atypia in a control prostate. F) Bars indicate the percentage of mice with an invasive tumor at the indicated age and diet. Mice with normal prostates, and mice with mPIN and advanced mPIN were considered non-invasive. Early, moderate and poorly differentiated adenocarcinomas were grouped as invasive tumors. Statistical significance was calculated using a Fisher exact test where ** and *** indicate significant differences between the groups where \( p < 0.01 \) and \( p < 0.001 \) respectively.

**Figure 3: Broccoli sprouts decreased HDAC3 protein in prostate epithelial cells.**

A-B) HDAC3 was detected in the prostate lobes of TRAMP mice fed control, or 15% broccoli sprout diet using immunohistochemistry. Chromagen Nova Red; counterstain hematoxylin. A) Representative images of HDAC3 staining in anterior and ventral prostate lobes at 12 weeks of age taken with 400x magnification. B) HDAC3 staining intensity quantification, where staining is expressed as a percentage of all possible color. Significant differences between samples were calculated using two-way ANOVAs with results detailed in individual tables for each prostate lobe (n=9-15, except for dorsal-lateral with broccoli sprout where n=4-7). Bonferroni post-tests were used to determine differences between control and broccoli sprout groups where ** indicates a significant differences between the groups where \( p < 0.01 \).

**Figure 4: Trend of decreased HDAC6 protein in the ventral prostate lobe with broccoli sprouts.**

A-B) HDAC6 was detected using immunohistochemistry in the prostate lobes of TRAMP mice fed control, or 15% broccoli sprout diet. Chromagen Nova Red; counterstain hematoxylin. A) Representative images of HDAC6 staining in
ventral prostate lobe at 28 weeks of age taken with 400x magnification. B) HDAC6 staining intensity quantification where staining is expressed as a percentage of all possible color. Significant differences between samples were calculated using two-way ANOVAs with results detailed in individual tables for each prostate lobe (n=6-16).

**Figure 5: Broccoli sprouts decreased histone H3 lysine 18 acetylation in prostate epithelial cells.** A-B) H3K18ac was detected using immunohistochemistry in the prostate lobes of TRAMP mice fed control, or 15% broccoli sprout diet. Chromagen Nova Red; counterstain hematoxylin. A) Representative images of H3K18ac staining in indicated prostate lobes at 28 weeks of age taken with 400x magnification. B) H3K18 acetylation was quantified on a scale from 0-3. Significant differences between samples were calculated using two-way ANOVAs with results detailed in individual tables for each prostate lobe (n=9-18). Bonferroni post-tests were used to determine differences between mice fed a control and broccoli sprout diet where *** indicates a significant differences between the groups where p < 0.001.

**Figure 6: Broccoli sprouts decreased p16 mRNA levels but did not significantly alter p16 protein abundance.** Bars represent mean A) mRNA levels of NQO1, B) mRNA levels of p16, or C) p16 protein levels in prostate lobes of mice fed a control diet (white bars), or 15% broccoli sprout diet (green bars). Tissue was collected at 12 weeks of age. A-B) Significant differences between groups was calculated using two-way ANOVAs with results detailed in individual tables for each gene. Bonferroni post-tests were used to determine differences between control or broccoli sprout groups where * and *** indicate significant differences between the groups where p < 0.05 and p < 0.001 respectively and n=4-7. C) Images are representative Western blots of mouse nutrition.
prostate tissue analyzed for p16 protein abundance, with corresponding densitometry results were no significant differences between control and broccoli sprout groups was detected (t-test p > 0.05).
Figure 3

(A) Images of tissue sections showing the effect of broccoli sprouts on HDAC3 expression in the anterior and ventral lobes.

(B) Bar charts representing the HDAC3 percent change over time in the anterior, dorsal-lateral, and ventral lobes. The tables show the p-values for the effect of diet, age, and interaction.
Figure 5

A

Anterior

Dorsal-Lateral

Ventral

B

Anterior lobe

Dorsal-lateral lobe

Ventral lobe

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### Supplemental Table 1

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Supplemental Figure 1: Broccoli sprouts did not change histone H3 lysine 9 acetylation in prostate epithelial cells. A-C) H3K9ac was detected by immunohistochemistry in the indicated prostate lobes of TRAMP mice fed control, or 15% broccoli sprout diet. H3K9 acetylation was quantified and data are mean staining intensity expressed as a percentage of all possible color. Significant differences between samples were calculated using two-way ANOVAs with results detailed in individual tables for each prostate lobe (n=8-16 except for dorsal lateral lobe 28 week broccoli sprout diet where n=5). Bonferroni post-tests indicated no significant differences between control and broccoli sprout groups.
Supplemental Figure 2: Broccoli sprouts decreased the area of histone H3 lysine 9 tri-methylation foci in the ventral lobe of the prostate. A-B) H3K9me3 was detected by immunohistochemistry in the indicated prostate lobes of TRAMP mice fed control, or 15% broccoli sprout diet. A) The intensity of staining for H3K9me3 in foci in intranuclear of the anterior lobe was quantified and data are presented as mean staining intensity expressed as a percentage of all possible color. Significant differences between samples were calculated using two-way ANOVAs with results shown in the table (n=5-9) B) Quantification of the mean area / foci for H3K9me3 staining in the ventral lobe of the prostate at 12 weeks of age. A t-test was used to determine significant differences between samples, n= 6-7 and *** indicates p < 0.001.
Supplemental Figure 3: Broccoli sprouts did not change STAT3, RBBP8, and SERPINB5 mRNA levels. Bars represent mean normalized mRNA levels of indicated genes in prostate lobes of mice feed a control diet, or 15% broccoli sprout diet. Tissue was collected at 12 weeks of age and n=4-7. Significant differences between treatments was calculated using two-way ANOVAs with results detailed in individual tables for each gene. Bonferroni post-tests indicated there was no significant difference in mRNA levels between control or broccoli sprout groups.