EXPANDING EPIGENETIC MECHANISMS OF SULFORAPHANE IN

COLORECTAL CANCER: PSEUDOGENES AND PROTEIN ACETYLATION

A Dissertation

by

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ABSTRACT

Two major hallmarks of cancer cells are their ability to sustain proliferative signaling and evade growth suppressors. This can occur when the cell loses transcriptional control of key growth genes through epigenetic mechanisms, such as histone and non-histone modification, and dysregulation of non-coding RNAs. There is clinical interest in using small molecules to target these epigenetic mechanisms in order to prevent or reverse the dysregulation of the key cancer growth genes. Sulforaphane (SFN) is a dietary isothiocyanate that exhibits anticancer activity through a variety of mechanisms, such as the activation of the antioxidant response pathway, and through histone deacetylase (HDAC) inhibition. This dissertation examines two distinct mechanisms of SFN's effect on epigenetic control of gene transcription. First, SFN induces *NMRAL2P* which is the first functional pseudogene to be identified as a direct target of Nrf2, and as a downstream regulator of Nrf2-dependent *NQO1* induction. Second, SFN causes the acetylation of Cell Cycle and Apoptosis Regulator 2 (*CCAR2)* through inhibition of HDAC3. This, in turn, decreases β-Catenin nuclear localization and activity, reducing the expression of oncogenes *MYC* and *MMP7*. Also, SFN works in combination with JQ1, an inhibitor of acetylation readers, to further prevent cancer cell growth.

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1. INTRODUCTION

In the United States, colorectal cancer (CRC) is the third most common cancer in men and women, and the second leading cause of cancer deaths[1,2]. In general, the risk of CRC is linked to age, sex, ethnicity, family history and a variety of behavioral factors. About 5-6% of CRC patients have an inherited genetic mutation that leads to conditions such as Lynch syndrome and familial adenomatous polyposis (FAP)[3]. Aside from these rare hereditary conditions, about 25% of CRC patients have a family history of the disease[3]. The increased risk of CRC within families is not fully understood but is thought to be caused by genetic susceptibility controlled by multiple genes, combined with shared lifestyle factors[3,4]. The remaining 75% of CRC cases are sporadic and can be influenced by personal lifestyle behaviors, such as physical activity and diet[5]. For example, red meat consumption increases the risk of CRC due to the carcinogens that are formed during high-temperature cooking[6–9]. Conversely, diets high in fruits and vegetables lower the risk, possibly due to the fiber, nutrients, and phytochemicals in those foods[10–12].

1.1. Genetic Changes in Colorectal Cancer

There are ten hallmarks that differentiate cancer from normal tissue, including sustained proliferative signaling, resisting death, activating metastasis, and evading growth suppressors[13]. Genes that promote or counteract these traits are termed 'oncogenes' and 'tumor suppressors', respectively. During tumorigenesis, these genes tend to be mutated or dysregulated, causing dysfunctional cell activity[14]. It is tempting to categorize individual genes as either a tumor suppressor or promoter; however, many genes can have both roles, depending on tissue type, mutational status, or post translational

modifications[15]. For example, p53, a very potent tumor suppressor, has oncogenic effects with certain mutations[16].

In 1990, Vogelstein proposed a genetic model for colorectal tumorigenesis[17]. In this model, normal colonic epithelium begins hyperproliferating and progresses to adenoma and then carcinoma due to mutations in oncogenes and loss of tumor suppressor genes. This model has since been refined to include data obtained from mass sequencing efforts[18]. In CRC, loss of the *APC* gene is usually the first "gatekeeping" mutation that provides a normal epithelial cell with a growth advantage. The loss of APC function leads to the activation of the β-Catenin oncogene. This abnormal cell grows slowly until a mutation in KRAS allows the cells to hyperproliferate. Mutations in key genes, such as *PIK3CA, SMAD4,* and *TP53,* will continue to accumulate until a malignant tumor is formed[19]. The subtype of tumors that follow this model has been termed "Chromosomal Instability" (CIN) tumors[20]. CIN tumors account for about 85% of sporadic tumors and all FAP hereditary tumors[20,21].

Another less common subtype, "Microsatellite Instability" (MSI), is characterized by silencing or mutations in DNA mismatch repair genes, such as MLH1 and MSH2. MSI tumors account for 15% of sporadic tumors and for Lynch syndrome[20]. A third subtype, which can overlap with CIN and MSI, is termed "CpG island methylator phenotype" (CIMP)[20]. CIMP is associated with hypermethylation of specific genes, BRAF mutations, and microsatellite instability[22].

1.2. Epigenetic Changes in Colorectal Cancer

Epigenetic alterations are recognized as significant drivers of CRC tumorigenesis[23]. Similar to a mutation in the genetic code, dysfunction of epigenetic mechanisms can lead to the dysregulation of key tumor suppressor genes and oncogenes[23,24]. Epigenetics is defined as the heritable changes in gene expression that are not mediated by the DNA sequence[23]. These changes can be broken into three main classifications: DNA methylation, histone and non-histone modification, and expression of regulatory RNAs such as microRNA (miRNA) and long non-coding RNA (lncRNA)[20].

1.2.1. DNA Methylation

Methylation of CpG dinucleotides in DNA is a major epigenetic modification that regulates gene expression[20,23]. Clusters of CpG sequences, called CpG islands, are found in the promoters of many genes[23]. Hypermethylation of these CpG islands typically leads to the silencing of the gene. Repression is due to changes in chromatin structure and the recruitment of methyl-binding proteins which block activation factors[23].

Although hypermethylation is most common in MSI and CIMP tumors, hypermethylation and silencing of APC occurs in about 20-30% of CIN tumors[25,26]. In 85% of MSI tumors, hypermethylation silences key mismatch repair genes MLH1, MSH2, MSH6, and PMS2, which leads to DNA replication errors[23]. CIMP tumors are defined by hypermethylation of three of five genes, CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1[20]. Silencing of these genes causes CIMP tumors that are clinically unique. CIMP tumors usually appear in the proximal colon, in females, and commonly have BRAF mutations[20].

In contrast to hypermethylation silencing tumor suppressor genes, global hypomethylation can lead to chromosomal instability and activation of proto-oncogenes[23]. The hypomethylation is thought to be due to insufficient DNA methyltransferase activity but may be due to active ten-eleven translocation (TET) demethylating proteins[23].

1.2.2. MicroRNAs

MicroRNAs (miRNA) are 20-25 base pair non-coding RNA that generally target messenger RNA for degradation. The "seed sequence" of the miRNA recognizes a specific sequence on the 3' untranslated region of the mRNA and recruits proteins that degrade the transcript or inhibit its translation[27]. miRNAs are often dysregulated in cancer, which leads to the dysregulation of oncogenes and tumor suppressors[20,27]. The let-7 family members are considered tumor suppressor miRNAs because they negatively regulate oncogenic genes such as KRAS and MYC. Oppositely, the miR-17-92 cluster is considered an oncomiR, as it is activated by MYC signaling and plays a role in adenoma to adenocarcinoma progression. Likewise, miR-143~145 cluster is frequently lost in CRC, which leads to elevation in KRAS expression and carcinogenesis [28].

1.2.3. Long Non-Coding RNAs

Long non-coding RNAs (lncRNA) is a general category of transcripts that are more than 200 nucleotides in length and usually have no protein-coding ability, however, some lncRNAs can produce small functional peptides[29–31]. Pseudogenes are a class of lncRNAs that share a high percentage of homology with a protein-coding gene[32]. Once considered "junk", the importance of many lncRNA and pseudogenes in normal and cancer cells has been demonstrated in recent years[32–35]. These transcripts can function in a variety of ways,

usually acting as a scaffold to bring together protein/RNA complexes. These complexes can act to activate or repress transcription, assemble chromatin remodeling complexes, and assemble RNA splicing complexes. LncRNAs are also known to sequester miRNA so that the miRNA cannot act on its intended target[35,36].

Many lncRNAs are dysregulated in cancer, but it is difficult to predict whether the lncRNA has a "driver" or "passenger" effect on the cancer cells. To determine the function of the lncRNA, researchers first knockdown/out or overexpress the transcript. In CRC, the lncRNAs such as Colon cancer-associated transcript (CCAT1 and CCAT2), among others, are deemed functionally significant, but thousands of lncRNAs remain untested. CCAT1 and CCAT2 are highly overexpressed in colon tumors and are involved in forming activation complexes on proliferative genes such as MYC[37–41].

1.2.4. Writers, erasers, and readers of histone and non-histone proteins

In the nucleus, DNA is wrapped around histone proteins in order to package the DNA into chromatin[42]. Post-translational modifications (PTMs), such as phosphorylation, acetylation or methylation can be added or removed to the tail of histones to affect density and accessibility of the chromatin[42,43]. Specific PTMs can open chromatin and allow active transcription, while others close the chromatin and repress transcription. This has led to the proposal of a "histone code," in which the transcriptional state of a gene can be predicted by examining the PTMs of the histones near that gene[42]. For example, Histone H3 lysine di- and tri-methylation (H3K4me2/me3), and H3/H4 acetylation (H3K9Ac, H4K9Ac) are a mark of transcriptionally active genes[23]. Oppositely, H3K9me3 and H3K27me3 tend to indicate an inactive gene[23]. In this case, these histone marks recruit

the polycomb repressive complexes (PRC1 and PRC2). In CRC, components of the PRC, such as EZH1 and EZH2, are overexpressed and repress a group of tumor suppressor genes[44]. Additionally, cell cycle arrest is induced when these components are depleted by RNA interference[44].

1.2.4.1. Histone and Non-Histone Acetylation

Proteins that add and remove a histone mark are termed "writers" and "erasers", respectively. The PTMs are recognized by "reader" proteins that bind to the PTM and influence transcription[45,46]. For example, histone acetylation is written and erased by acetylases (HATs) and deacetylases (HDACs) and read by bromodomain-containing proteins[47–49]. Generally, histone acetylation is considered a mark of active gene transcription by causing chromatin to open and enhance promoter accessibility[50].

In colorectal cancer, histone deacetylases tend to be overexpressed[51–53]. This leads to decreased acetylation and inactivation of tumor suppressor genes such as p21[53]. Class I HDACs, HDAC1/2/3, are up-regulated in more than 50% of the specimens[52]. Also, HDAC expression is correlated with strong proliferation, dedifferentiated tumors, and poor patient survival[52,53]. A class III HDAC, SIRT1 is overexpressed in about 40% of CRCs and correlates to the CIMP-High and MSI-H tumor types[52].

Acetylation of non-histone proteins is also regulated by the same HATs and HDACs as histone targets[54]. Like protein phosphorylation, acetylation adds an additional regulatory mechanism which can control differential activity, localization, dimerization, and stability[54]. For example, p53 is acetylated by the HAT p300/CBP and deacetylated by HDAC1 and SIRT1[55–57]. Acetylation of p53 increases its transcriptional activity, and

deacetylation leads to its degradation[54]. Acetylation of STAT3, an important transcription factor, influences its dimerization, nuclear localization, and transcriptional activity[54,58]. Acetylation of CtIP targets the protein for degradation and subsequently inhibits the DNA damage response[59].

1.2.4.1.1. Methods of Studying Protein Acetylation

With advances in proteomics technology, acetylation sites have been identified on numerous histone and non-histone proteins, although most of such sites have unknown function[43]. The standard approach to studying specific acetylation sites has been to change Lysine to un-acetylatable Arginine or to a Glutamine acetylation mimic[60]. A major drawback of this Lys to Arg mutation is that Arg does not faithfully recapitulate the functions of unacetylated Lys, and more importantly, the mechanistic consequences of post-translational acetylation at Lys residues[60,61]. Recently, a tRNA synthetase was engineered to load acetyl-lysine onto a TAG-stop tRNA[62–64]. By mutating Lys to TAG, acetyl-lysine can be *genetically encoded* and incorporated into any protein in a site-specific manner[62]. This relatively new method vastly increases the ability to study the effects of specific histone and non-histone acetylation.

1.3. Treatment and Prevention of Colorectal Cancer

5-year survival rates for stage I, II, and III colon cancer is 92%, 87%, and 72%, respectively[65]. This relatively high survival is due to the ability to surgically resect these localized tumors. When a stage IV tumor metastasizes to a secondary location, 5-year survival drastically drops to about 12%[65]. Alongside surgery, adjuvant chemotherapy is used to treat the metastatic disease, but often has detrimental side effects[66]. This highlights

the importance of prevention and early detection of colon tumors. Cancer prevention can be divided into primary, secondary and tertiary prevention[67]. In CRC, the goal of primary prevention is to prevent the initial stages of colon tumorigenesis. This can be accomplished by avoiding carcinogens or unhealthy behaviors, and improving diet and lifestyle[68].

Secondary prevention focuses on mitigating risks in high-risk populations[67]. This includes early screening, detection, and resection of polyps. As aging is the biggest risk of CRC, patients over the age of 45 are routinely screened with colonoscopy or fecal occult tests[69]. Most FAP patients, who are genetically predisposed to have many colon polyps, will have a colectomy between the age of 16-20[70]. Sulindac, among other NSAIDs, is used as a chemopreventive measure to delay the onset of polyps and prolong the need for colectomy[70].

Tertiary prevention focuses on preventing the recurrence of cancer after treatment, or preventing symptoms or disease caused by the initial treatment[67]. After initial surgical resection of low grade tumors, about 10-30% of patients will have a recurrence within 5 years[71].

Aside from lifestyle changes, adding a chemopreventive agent could play a role in all stages of cancer prevention. There is interest in developing better chemopreventive strategies and drugs that are more efficient at targeting the disease with fewer off-target side effects.

1.3.1. Epigenetic Therapies

Epigenetic regulation is prone to dysregulation during all stages of CRC tumorigenesis, especially the early stages[23,44,52]. Unlike genetic mutations, the epigenetic

dysregulation is potentially reversible and a good target for prevention[72]. Chemopreventive drugs have been used to reverse the aberrant epigenetic modification and prevent the normal colonic epithelial from becoming hyperplastic[72]. Epigenetic therapies, such as DNMT and HDAC inhibitors, have been tested extensively in preclinical and clinical studies[53,73,74]. Newer epigenetic therapies, such as bromodomain inhibitors and non-coding RNA targeting medicines have shown promise in preclinical models[49,75,76].

DNA hypermethylation happens on the promoters of key tumor suppressor genes in many tumor types, including colon[20,26]. Therefore, the enzymes responsible for hypermethylation, DNMTs, were thought to be valid targets for treatment and prevention. DNMT inhibitors, such as Azacitidine and Decitabine show potency in pre-clinical models and in the clinic have shown efficacy in hematological malignancies, but less effect in solid tumors[74]. In colon cancer, decitabine caused a partial improvement in about 10% and a stable response in 55% of patients[77]. Due to the limited response in clinic, further research hopes to perform trials in specific tumor subtypes, such as highly methylated tumors. Also, there is evidence that DNMT inhibitors perform better in a prevention role, rather than treatment. In APC$^{Min/+}$ mice, decitabine decreases tumor formation by 82%, but only when administered early, before tumors begin to form[78].

Inhibition of HDACs has been explored in the prevention and treatment of CRC[53]. HDAC inhibition leads to the acetylation of histone and non-histone proteins, which can lead to the silencing of oncogenes and activation of tumor suppressors[53,72]. Interestingly, HDAC inhibition only results in 2-10% of activated genes, which are largely associated

with growth regulation and survival[72]. This explains why HDAC inhibition in cells results in cell cycle arrest, apoptosis, and inhibition of xenografts, but the mechanism of selective expression is still being explored[72]. In animals, butyrate, an HDACi, reduced aberrant crypt formation in AOM treated rats. Valproic acid, another HDACi, reduces adenoma formation in APC[Min] mice[53] Despite the efficacy in cell and animal models, there has been less success in the clinic. Vorinostat (SAHA) has been approved for cutaneous T-Cell lymphoma, but like DNMT inhibitors, has not shown efficacy in many solid tumors including colon[73,79]. More research is needed to understand the lack of efficacy, identify better drugs, or to identify potential patient subgroups that would benefit from HDAC inhibition.

1.3.2. Drugging Novel Epigenetic Targets

There is growing interest in discovering novel ways to "drug" the epigenome. In recent years, bromodomain inhibitors and non-coding RNA therapies have been tested in cell, animal and clinical models in various cancer types, but not always in colon[49,75,76]. Bromodomain containing protein, such as BRD3 and BRD4, recognize acetylated histones, recruit activation factors, and upregulate oncogenic genes like MYC, BCL2 and CDK6[49]. JQ1 and I-BET were the first bromodomain inhibitors discovered, but since their discovery, many other bromodomain inhibitors have been taken to phase I and II trials for a variety of cancer types[80]. In CRC, BRD4 has been identified as a major driver of proliferation and dedifferentiation[81]. Many colon cancer cell lines are sensitive to JQ1 treatment, especially those of the CIMP subtype. Another bromodomain inhibitor,

GSK525762, is currently recruiting colorectal patients in a phase I/II study, but results await to be seen[81].

Non-coding RNAs, such as miRNAs and lncRNAs are another potential, novel target for CRC prevention and therapeutics[75,76]. Numerous non-coding RNAs are dysregulated during carcinogenesis, some of which play a major role in the process[20]. RNA therapy is currently in its nascent phase, where specific targets are being identified, and targeting strategies are being developed. Strategies for targeting RNA are straightforward, either providing a mimic RNA for downregulated tumor suppressors, or administering a complementary antagonist to sequester the upregulated oncogenes[75]. For example, the miRNA let-7 family is down regulated in a variety of cancers. Administering mimic let-7 was able to reduce tumor initiation, growth, burden and metastasis in models of non-small cell lung cancer and glioblastoma. Conversely, targeting miR-10b with an antagomir prevented metastasis in a breast cancer model[75]. Some miRNA therapies have been advanced into early clinical trials, but it is too soon to determine their efficacy[82].

LncRNAs can be targeted in a similar method as miRNA. Oncogenic lncRNAs, such as CCAT1 and CCAT2 can be targeted for degradation by RNAi technology[83,84]. These therapies show promise in cell-based studies, but pre-clinical animal models of lncRNA are difficult to perform due to low homology between humans and lower organisms[76]. This has slowed down their ability to progress to clinical trials, and no lncRNA targeting drugs have yet reached clinical trials (clinicaltrials.gov).

1.4. Dietary Chemoprevention

The concept of using chemicals to prevent cancer was proposed in 1966 by Wattenberg[85]. In the 1970's, Michael Sporn coined the term "chemoprevention" and described it as using pharmacological means to control cancer during the preneoplastic stages using relatively non-toxic compounds[86,87]. Since then, numerous studies have been performed to identify and test compounds that are able to prevent cancer[88,89]. Compounds from dietary sources have proved to be promising candidates for chemoprevention. There is epidemiologic evidence that shows an inverse relationship between fruit and vegetable consumption and cancer risk. Studies indicate that people who eat five or more servings of fruits and vegetables a day have about half the risk of developing cancer compared to those who each fewer than two servings, with effects most pronounced in digestive tract cancer, including colon[88]. About 35 plant-based foods have been found to have cancer-preventive properties, including garlic, ginger, turmeric, broccoli and cauliflower. The beneficial effects of these foods may be attributed to the macro- and micro-nutrients they contain, but increased focus has been shifted to non-nutritive phytochemicals. More than a thousand phytochemicals have been identified to have anti-cancer activity, many of which have been moved into clinical chemoprevention trials[88]. Chemopreventive agents are divided into blocking and suppressing agents. Blocking agents are mainly responsible for blocking tumor initiation by preventing carcinogens from reaching target tissue, preventing bioactivation, and promoting detoxification and excretion[90]. For example, chlorophyll, from green vegetables, and its analog chlorophyllin will form a complex with carcinogens such as polycyclic aromatic hydrocarbons, heterocyclic amines, and aflatoxin

B1, which prevents absorption and facilitates excretion[91–93]. Suppressing agents act in the post-initiation stage of tumorigenesis, preventing initiated cells from becoming malignant[90]. For example, curcumin, a chemical found in turmeric, inhibits the oncogenic NF-kB and β-Catenin pathways[88,94]. Cancer chemoprevention is also mediated through induction of protective proteins such as Phase I, II, and III metabolism enzymes, or tumor suppressors such as p53 or p21[88,95].

1.4.1. Chemoprevention of Cancer by Sulforaphane

Epidemiological studies show an association between cruciferous vegetable consumption and reduced cancer risk[96]. This protective effect has been attributed to their high concentration of protective phytochemicals, such as isothiocyanates and indoles. Sulforaphane (SFN), a dietary isothiocyanate found in cruciferous vegetables, is an anti-cancer agent that acts as both a blocking and suppressing agent[97]. The chemopreventive effects of SFN are likely due to a variety of mechanisms, including the induction of phase I and II metabolism enzymes, oxidative stress response, NF-kB inhibition, HDAC inhibition, and the induction of apoptosis and cell cycle arrest[96,97].

Perhaps the most studied mechanism of SFN is the induction of the NRF2 antioxidant response pathway. When active, NRF2 induces a set of antioxidant response genes and phase II metabolizing enzymes[98]. These genes are responsible for inactivating reactive oxygen species (ROS) and detoxifying potential carcinogens. In the absence of a xenobiotic, NRF2 is sequestered in the cytoplasm by KEAP1. SFN, a strong electrophile, attacks KEAP1 and allows NRF2 to dissociate. When free, NRF2 translocates to the nucleus, binds to a specific DNA response element, and activates the response genes[98].

This NRF2 response is beneficial for blocking carcinogenesis, but active NRF2 has been shown to promote tumor progression in the post initiation stage[99].

Another major mechanism of SFN is its activity as an HDAC inhibitor, leading to cell cycle arrest and apoptosis[100,101]. In colon cancer, SFN inhibits HDAC3 and promotes its degradation. This inhibition leads to increased acetylation of histone and non-histone proteins and leads to G2/M cell cycle arrest and apoptosis[102]. Possible mechanisms include de-repression of the p21 tumor suppressor gene, and degradation of DNA repair protein CtIP[59,102]. This dissertation seeks to expand the epigenetic mechanisms of SFN and show that SFN acts through induction of *NMRAL2P* and through CCAR2 acetylation interfering with β-Catenin signaling.

2. ANTAGONISTIC DUALITY OF CCAR1 AND CCAR2: TUMOR PROMOTION VS. SUPPRESSION

2.1. Introduction

Cell Cycle and Apoptosis Regulator 2 (CCAR2) has emerged as a 'master regulator' of metabolism, aging and cancer[103]. CCAR1 is a paralog of CCAR2 with structural and functional similarities. Both CCAR1 and CCAR2 have evolved from the common ancestor *Lateral Signaling Target-3* (*LST-3*) in *C. elegans*[104]. These proteins have been implicated as key players in physiology and pathophysiology, including β-catenin/Wnt signaling, nuclear receptor function, adipogenesis, apoptosis, and the DNA damage response[105–115].

CCAR1, initially named Cell Cycle and Apoptosis Regulator Protein-1 (CARP-1), was discovered as a regulator of apoptosis signaling in breast cancer cells[112]. Later, CCAR2 gained attention as a modulator of p53 activity in response to DNA damage signaling, by inhibiting the activity of Sirtuin 1 (SIRT1)[108,116] and histone deacetylase 3[103,117] via direct protein-protein interactions. The name originally ascribed to CCAR2, Deleted in Breast Cancer 1 (DBC1), is regarded as a misnomer because this protein more typically is overexpressed in mammary cancer and other malignancies[118]. The designation 'CCAR1' and 'CCAR2' will be used here, to highlight functional aspects tied to the cell cycle and apoptosis, and to comply with HUGO Gene Nomenclature Committee (HGNC) guidelines (genenames.org).

Conflicting reports exist on the pros and cons of CCAR1 and CCAR2, and their precise roles in cancer etiology. For example, CCAR2 can trigger tumor suppressor

functions of p53[108,116,119] or serve as an oncogenic driver of Wnt/ β-catenin signaling[106]. Such a gene that has both tumor promotional and suppressive affects is said to display 'antagonistic duality'[15]. Recent reports show that these types of 'gene chameleons' are becoming more common as we understand the nuances and context in which these genes function[15,99,119]. Multiple factors contribute to these divergent activities, including genetics, tissue specificity, and dynamic regulation via post-translational modifications. This review summarizes current clinical and molecular data, and discusses the mechanisms by which CCAR family members might exhibit duality in cancer etiology. We also discuss 'friend or foe' aspects in the context of precision oncology. Since the roles of CCAR1 and CCAR2 in obesity and aging have been previously reviewed by others[103,120,121], they will not be covered extensively in the current review.

2.2. Duality of CCAR Family Members: Tumor Promotion vs Suppression

2.2.1. CCAR1

CCAR1 has been implicated in β-Catenin/Wnt Signaling[105], Nuclear receptor signaling[110,114], adipogenesis[111], and apoptosis signaling[112–115], and has a dual role in tumor promotion and suppression[122] (Fig. 1). Lu et. al. reported that in T-cell acute lymphoblastic leukemia (T-ALL) cells, CCAR1 had conflicting roles as a tumor promoter or suppressor, depending on which alternative splice variant was expressed[115]. The Par-4/THAP complex enhanced expression of the full-length protein, which exerted pro-apoptotic effects, whereas Notch3 competitively interacted with the *CCAR1* promoter and favored the formation of a shorter splice variant that had pro-survival outcomes[115].

2.2.1.1. CCAR1 and Tumor Promotion

In the reported literature, immunohistochemistry data for CCAR1 expression in patient samples appears to be sparse and incomplete. In hepatocellular carcinoma, CCAR1 levels were correlated with unfavorable overall survival and recurrence free survival[123]. Few, if any reports have examined the reverse scenario.

CCAR1 was found to interact with and activate β-Catenin. Depletion of CCAR1 in colon cancer cell line inhibits expression of β-Catenin target genes and suppresses anchorage independent growth[105]. Furthermore, CCAR1 is a key regulator of nuclear receptor signaling[114]. This is accomplished by recruiting the Mediator complex and p160, which turns on the expression of key proliferation genes. CCAR1 was found to be a regulator of the androgen receptor by binding to and stabilizing AR and GATA2, which promote their activity[110]. This shows that CCAR1 may act in an oncogenic manner in AR driven cancers.

2.2.1.2. CCAR1 and Tumor Suppression

CCAR1 overexpression can cause an induction of apoptosis, especially exemplified in breast cancer cells. CCAR1 overexpression causes p21 induction and apoptosis[112]. This is accomplished by sequestering 14-3-3 and reducing proliferative gene expression, such as c-Myc and Cyclin B1. CCAR1 has also been shown to co-activate p53, but this interaction has not been fully explored[114].
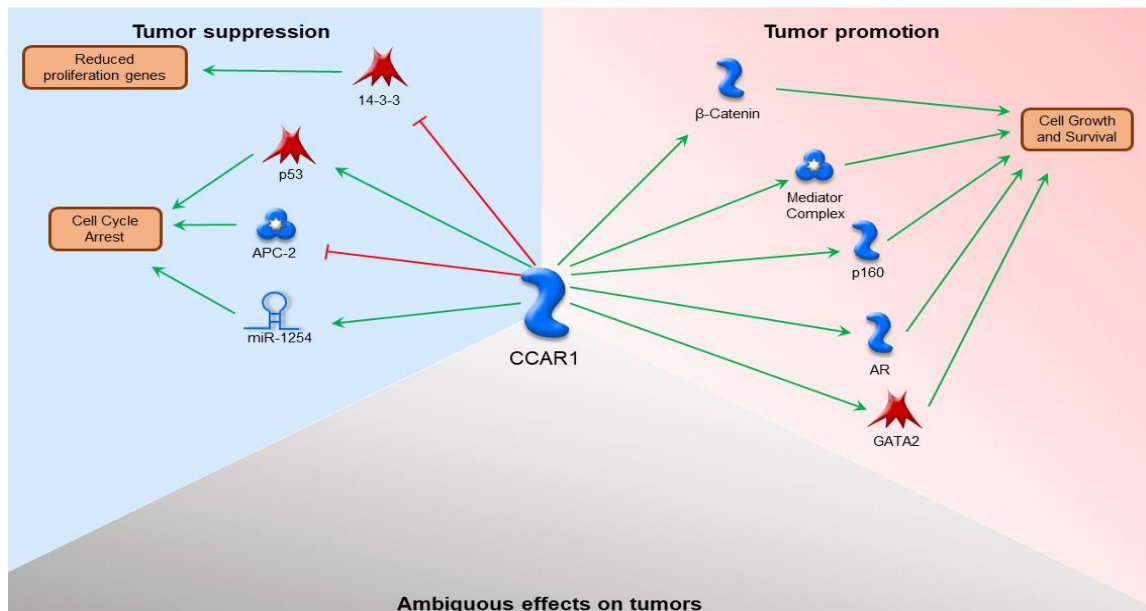
Figure 2.1 Protein interactions of CCAR1 leading to tumor suppression (blue), tumor promotion (red), or yet undetermined (grey) effects.

Because CCAR1 expression is low in breast cancer, there have been attempts to induce CCAR1 expression and/or function. A screen has identified CCAR1 functional mimics (CFMs) that seek to duplicate the binding of CCAR1 to Anaphase promoting complex 2 (APC-2), to halt cell cycle and promote apoptosis[124].

Recently, the 5' UTR sequence of CCAR1 was shown to stabilize and increase the activity of miR-1254, a microRNA that is highly downregulated in breast cancer. The researchers are employing this 5'UTR to target miR-1254[125].

Withaferin A, a bioactive compound from the Withania somnifera medicinal plant upregulates CCAR1 in mesothelioma. Knockdown of CCAR1 causes Withaferin A to lose some of its anti-cancer activity[126].

2.2.2. CCAR2

CCAR2 was first identified in a genetic screen carried out for identifying potential tumor suppressor genes located on a region of human chromosome 8 that is frequently deleted in breast cancer[118]. However, the authors found that CCAR2 was not deleted in breast, nor other cancer types[118]. This was in stark contrast to DBC2, a similarly named gene detected in the same chromosomal locus, that was identified as a bona fide tumor suppressor gene[118].

Subsequently, multiple studies have examined CCAR2 expression patterns in various human cancers and their associated clinical outcomes, which support an oncogenic role for CCAR2 in certain instances and a tumor suppressor role in certain other cases (Table 2.1). Possibly, one reason for the discrepancy is related to the different cancer types that were studied, although conflicting observations have been noted even within the same cancer type[127–131]. Similarly, detailed analysis of molecular pathways also suggested that CCAR2 might have both an oncogenic or tumor suppressive role. In the following subsections, we have tried to explain the apparent duality in CCAR2 roles, by reviewing mechanistic, preclinical, and clinical studies that have described a role for CCAR2, either as an oncogene or as a tumor suppressor.

2.2.2.1. CCAR2 and Tumor Suppression

2.2.2.1.1. Human Clinical Studies

Although CCAR2 was originally considered a putative tumor suppressor in breast cancer the majority of evidence shows it is overexpressed and promotional in breast cancer[118]. However, studies in other tissue types have shown that it still has suppressive

functions. In colon, and in head and neck cancers, CCAR2 has been shown to be a potential tumor suppressor, based on a loss of heterozygosity[132,133]. More clinical studies are warranted to confirm these observations.

In gastric cancer, high CCAR2 expression has been associated with lower disease stage, less lymph node invasion and metastasis, and overall better prognosis and survival rate[130,131]. Similarly, CCAR2 was associated with better clinicopathological variables and better overall survival in gall bladder carcinoma patients[134]. Likewise, CCAR2 was associated with favorable clinical factors, such as reduced lymph node metastasis and tumor differentiation in laryngeal and hypopharyngeal carcinoma[135]. In pancreatic ductal adenocarcinoma, the majority of tumors showed high CCAR2 expression, and this was associated with better overall survival, while the tumors expressing less CCAR2 tended to be poorly differentiated[136].

Additionally, supporting evidence was found when we probed The Cancer Genome Atlas (TCGA) for CCAR2 "high" and CCAR2 "low" expression in patient tissues. Based on the TCGA data, CCAR2 expression was correlated with overall patient survival (OS) and recurrence free patient survival (RFS). As shown in Fig. 2.2, the CCAR2 "high" group correlated with increased OS in breast and colon cancer (Fig. 2.2, top row). High CCAR2 gene expression also correlated with increased RFS in prostate cancer (Fig. 2.2, top row).

Table 2.1 Correlation of CCAR2 expression and outcome in human clinical studies

Cancer Type	Sample Type	Method	Outcome	Reference
Tumor Suppressor Role				
Colorectal Cancer	51 sporadic CRCs	SNP Array	LOH on 8p (containing CCAR2) in 40 microsatellite stable sporadic colon cancers patients.	Reid 2009
Gall Bladder Carcinoma	104 gallbladder carcinoma	IHC	CCAR2 is associated with better OS and other clinicopathologic variables.	Won 2015
Gastric Cancer	452 gastric cancer	IHC	CCAR2 is associated with lower stage, less lymphatic invasion, and better OS.	Kang 2012
Gastric Cancer	557 cohort gastric cancer	IHC	CCAR2 is related to lower stage, lymph node metastasis, and better prognosis.	Noguchi 2014
Laryngeal and Hypopharyngeal Carcinoma	120 LSCC or HSCC	IHC	CCAR2 is correlated to lymph node metastasis and tumor differentiation.	Yu 2013
Laryngeal and Hypopharyngeal Carcinoma	41 HNSCC Cell Lines	SNP Array	LOH on 8p22-p21.3 (containing CCAR) (88.7%).	Ye 2007
Pancreatic Ductal Adenocarcinoma	104 stage I and II PDAC	IHC	CCAR2 is associated with better survival and with more differentiated tumors	Pinho 2016
Tumor Promoter Role				
Breast Cancer	48 breast core-needle biopsy	IHC	CCAR2 is associated with tumor nuclear grade.	Hiraike 2011
Breast Cancer	122 breast core-needle biopsy	IHC	CCAR2 is associated with distant metastatic relapse, increased tumor stage, poor OS and RFS.	Lee 2011
Breast Cancer	202 ER-negative breast cancer. 128 ER-negative/HER2-positive	IHC	CCAR2 is related to lower RFS in ER(-) and ER(-)/HER2(+) cancers.	Kim 2015
Clear Cell Renal Carcinoma	200 CRCC	IHC	CCAR2 expression correlates with shorter OS, RFS and CSS.	Noh 2013
Colorectal Cancer	186 CRC	IHC	CCAR2 is overexpressed in tumor compared to adjacent normal, and associated with tumor grade, TNM stage, metastatic status, and poor OS.	Zhang 2014
Colorectal Cancer	200 CRC	IHC	CCAR2 expression correlates with lower RFS	Yu 2015
Diffuse Large B Cell Lymphoma	101 DLBCL	IHC	CCAR2 is associated with high clinical stage, elevated serum lactate dehydrogenase levels, prognostic index score, shorter OS and RFS.	Park 2013
Esophageal Sqamous cell Carcinoma	165 ESCC & 34 normal	IHC	CCAR2 is overexpressed in ESCC, and associated with poor prognosis.	Kim 2012
Gastric Cancer	142 gastric adenocarcimoa	IHC	CCAR2 is overexpressed in tumors, and associated with TNM stage, lymph node metastasis, and lower OS.	Huan 2015
Gastric Cancer	187 gastric carcinoma	IHC	Phosphorylated CCAR2 is associated with higher tumor grade, poor OS and RFS.	Bae 2015
Gastric Cancer	177 gastric cancer	IHC	CCAR2 is associated with tumor stage, lymph node metastasis, tumor invasion, shorter OS and RFS.	Cha 2009
Hepatocellular Carcinoma	158 HCC	IHC	CCAR2 is associated with poor RFS and OS	Li 2017
Hepatocellular Carcinoma	55 patient matched HCC and Normal Liver	IHC	CCAR2 is overexpressed in HCC and associated with size, stage and differentiation	Li 2017
Osteosarcoma	35 osteosarcoma	IHC	CCAR2 is associated with shorter OS, RFS, and higher clinical stage.	Wagle 2015
Ovarian Carcinoma	104 Ovarian carcinomas	IHC	CCAR2 is overexpressed in tumor, and associated with stage, metastasis, platinum resistance, CA12 levels, histological grade, poor OS and RFS.	Cho 2015
Soft Tissue Sarcoma	104 Soft tissue sarcoma	IHC	CCAR2 is associated with stage, grade, mitotic counts, distant metastasis, lower OS and EFS.	Kim 2013

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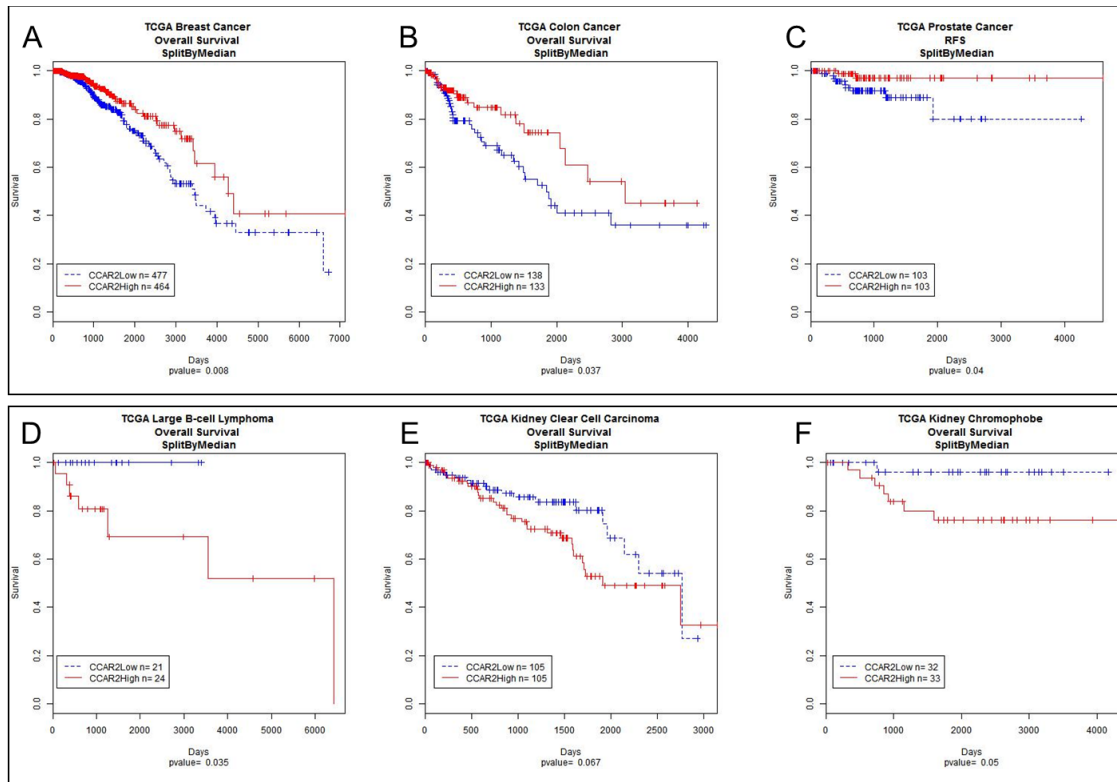
Figure 2.2 Kaplan-Meier Survival curves separated by high (red) or low CCAR2 expression (blue).
A-C: High CCAR2 expression has favorable survival outcome in (A) breast, (B) Colon, and (C) Prostate cancer; and poor survival outcome in cancers such as (D) Large B-Cell Lymphoma, (E) Kidney Clear cell, and (F) Kidney Chromophobe.

2.2.2.1.2. Preclinical Studies

Perhaps the most important and convincing evidence for CCAR2 function in cancer was provided by gene knockout studies. In fact, the CCAR2 knockout C57BL/6 mice were susceptible to spontaneous lymphomas, liver tumors, lung tumors, and teratomas[119]. The knockout mice had poor survival (OS) compared to their wild type counterpart, and this was related to increased tumorigenesis[119]. Further, Mouse Embryonic Fibroblasts (MEFs) from the knockout mice showed increased cell proliferation and colony formation, and re-introduction of CCAR2 reversed this phenotype[119]. Notably, this

was attributed to the tumor suppressor role of CCAR2, because of CCAR2's regulatory role on p53 stability[119]. CCAR2 binds to p53 and competes with MDM2, the key p53 inhibitory protein[119], thus increasing p53 stability[119]. Remarkably, CCAR2 mutants lacking the p53 binding site were unable to inhibit cell proliferation and colony formation of MEFs[119].

Although the C57BL/6 CCAR2 knockout mice developed tumors[119], the 129/J x C57BL/6J CCAR2 knockout mice did not develop tumors[137]. In addition to the different methodologies that were employed for gene knockout in the two knockout models[119,137], the different genetic background of the mouse strains used[138] may also explain the observed differences between the two knockout models. Notably, hematopoietic tumors, lymphoma, and histiocytic sarcoma were less common in 129S4/SvJae (a related substrain), than in B6 or B6/129 mice, while lung tumors and Harderian gland tumors were more common than in B6 or B6/129 mice[138]. The C57BL/6 mice that developed spontaneous tumors upon CCAR2 knockout used a gene trap inserted between exons 6 and 7, leaving a 162 amino acid peptide that could theoretically be produced[119], unlike the 129/J knockout model, where an insertion of GFP at the ATG start site, ablated the whole protein[137]. Notably, the mouse models[119,137] focused on CCAR2's role during spontaneous tumorigenesis, and results could be different if the mice are exposed to mutagens or carcinogens.

2.2.2.1.3. Cell Based Mechanistic Studies

Studies have shown that depleting CCAR2 decreases apoptosis in response to DNA damaging agents, such as etoposide or radiation[108,116,139,140]. Accordingly,

overexpression of CCAR2 leads to increased sensitivity upon exposure to DNA damaging agents[140–142], directly via CCAR2's role in DNA damage[143,144], or indirectly through its ability to activate p53[108,116,119] (Fig. 2.3). Thus, CCAR2 has been shown to possess tumor suppressor functions in cell based studies, and in such studies, the role for CCAR2/SIRT1 protein interactions has been suggested as a possible mechanism. The inhibition of SIRT1 by CCAR2 allows p53 to be acetylated and activated, triggering apoptosis[108,116], whereas in cells that lack endogenous SIRT1/CCAR2 interactions, no such changes were observed[145]. Similarly, ATM/ATR phosphorylates CCAR2 at Thr454 following DNA damage, which increases SIRT1 binding[139,142]. Recent studies have shown that additional protein partners, long noncoding RNAs (lncRNA), and post-translational modifications regulate SIRT1 binding[141,144,146–148]. For instance, acetylation regulates CCAR2/SIRT1 interactions. Under non-DNA damage conditions, CCAR2 is acetylated by hMOF at lysines K112 and K215[140]. These acetylation sites disrupt CCAR2/SIRT1 binding and increase SIRT1 activity[140,149]. Subsequent to DNA damage, phosphorylation of CCAR2 causes hMOF to dissociate and CCAR2 is deacetylated, leading to inhibition of SIRT1[140]. The lncRNA, MALAT1, can activate SIRT1 activity by binding to and sequestering CCAR2[146].

Besides inhibiting SIRT1, CCAR2 is also an endogenous inhibitor of HDAC3[150], a class I histone deacetylase that regulates gene expression by deacetylation of histones and non-histone proteins. The N-terminus of CCAR2 has been shown to bind to the C-terminus of HDAC3, thereby inhibiting HDAC3 activity and subcellular distribution[150]. Therefore, knockdown of CCAR2 in cells and/or mouse tissues has been shown to increase

HDAC3 deacetylase activity[150]. Together, these results identify CCAR2 as a potential inhibitor of two key deacetylases that are associated with oncogenesis, SIRT1[114,116] and HDAC3[150], highlighting its role as a tumor suppressor.
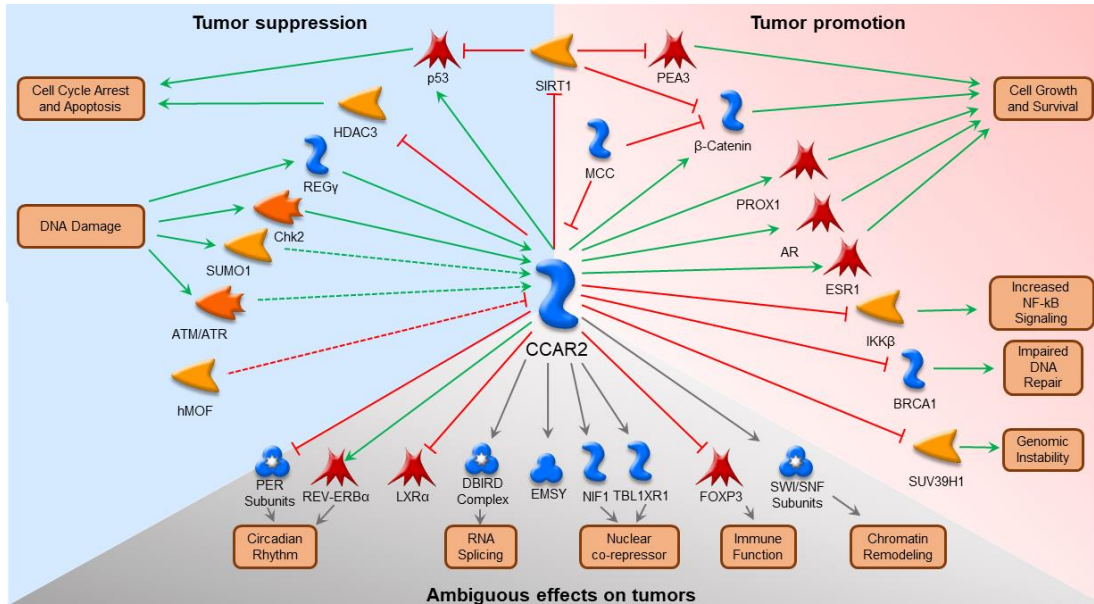
Figure 2.3 Protein interactions of CCAR2 leading to tumor suppression (blue), tumor promotion (red), or yet undetermined (grey) effects.

2.2.2.2. CCAR2 and Tumor Promotion

2.2.2.2.1. Human Clinical Studies

CCAR2 is overexpressed in many different human tumors, and this has been associated with poor prognosis and unfavorable clinical outcomes. In fact, there is ample evidence in the literature pointing towards CCAR2 possessing a tumor promoting role, as compared to its tumor suppressive role. For example, high CCAR2 expression is associated with shorter patient survival (both OS and RFS) in osteosarcoma, soft tissue

sarcoma, clear cell renal carcinoma, colorectal cancer, diffuse large B-cell lymphoma, breast cancer, hepatocellular carcinoma and esophageal squamous cell carcinoma[106,151–159]. Moreover, higher tumor stage/grade, lymph node metastasis, or distant metastasis were associated with high CCAR2 expression in many of the above mentioned cancer types[128,155,157–160]. A careful analysis of the TCGA database provides supporting evidence to the fact that large B-cell lymphomas and clear cell renal carcinomas with high CCAR2 expression correlate with poor patient survival (Fig. 2.2, bottom row). In addition, kidney cancers and mesothelioma also show poor survival (OS) in patients having high CCAR2 expression (Fig. 2.2, bottom row).

A dual role for CCAR2 has been noticed in certain gliomas, gastric, and breast cancer studies, wherein CCAR2 expression has been correlated either with better survival in some studies, or with poor survival in certain other studies (Table 2.1). This certainly points to the duality of CCAR2 function, and a potential role for additional or associated factors that are critical in determining whether CCAR2 would possess a tumor suppressive or promoting role in cancer. An example that has been widely cited in this context is the mutational status of p53. It is known that wild type and mutant p53 have opposite effects during tumorigenesis, and we know that both forms of p53 can be stabilized by CCAR2, through SIRT1 inhibition and resulting p53 acetylation[119]. Since CCAR2 can interact with both wild type and mutant p53[119], it is conceivable that these protein interactions may be important in deciding if CCAR2 functions as a tumor suppressor or a tumor promoter. For instance, analysis of the TCGA database for gliomas indicated that, neither p53 (Fig. 2.4A) nor CCAR2 (Fig. 2.4B) expression was an independent prognostic marker of patient

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survival (RFS). However, when patient data was segregated based on the expression of wild-type or mutant p53 (Fig. 2.4C-D), wild-type p53 bearing patients who also had high CCAR2 expression had significantly (p = 0.002) increased RFS, when compared to wild-type p53 bearing patients who had low CCAR2 expression (Fig. 2.4C). On the contrary, in patients with p53 mutations, high CCAR2 expression significantly (p = 0.005) decreased RFS, when compared to patients with low CCAR2 expression. These observations were not only restricted to glioma patients; similar interpretations hold true in other cancers, such as breast cancer. For example, although p53 mutation status was not an independent prognostic marker in breast cancer patients, it was noteworthy that patients with high CCAR2 expression had significantly (p = 0.008) better survival (OS), than patients with low CCAR2 expression. As noted earlier, the tumor suppressor function of CCAR2 was retained in patients with wild type p53 (p = 0.039), whereas it was offset attenuated in patients who had p53 mutations (based on TCGA database).

Yet another factor that can contribute to the functional duality of CCAR2, based on clinical studies, is post-translational modifications (PTMs). For example, in one gastric cancer study, it was found that phosphorylated CCAR2 (pCCAR2), but not the unphosphorylated form, contributed to its tumor promoting role, as pCCAR2 expression was associated with poor survival (OS and RFS) and higher tumor grade[127]. Since CCAR2 can have many PTMs[43], namely phosphorylation and acetylation, it is conceivable that such PTMs can play a key role in the recruitment of binding partners that modulate the overall function of CCAR2 in cancer.
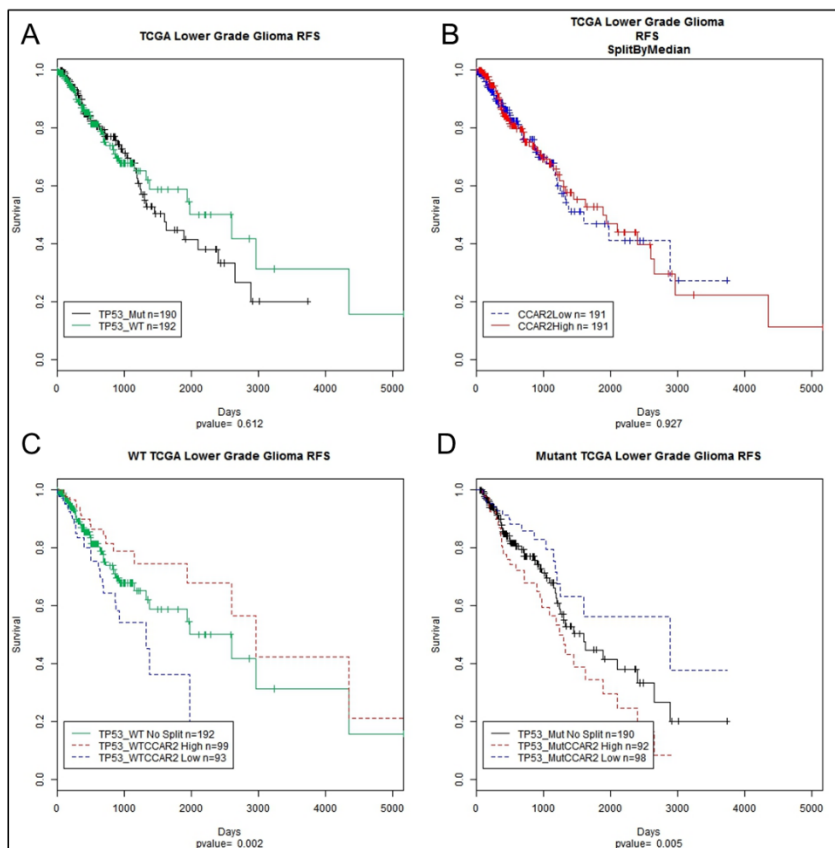
Figure 2.4 Combined effect of CCAR2 expression and P53 mutation status in Glioma. Survival curves comparing (A) wildtype P53 (green) vs. mutant (black) P53; (B) high CCAR2 (red) vs. low (blue) CCAR2 expression; (C) high CCAR2 (red) vs. low (blue) CCAR2 expression in wildtype P53 samples, where the green line indicates the survival of wildtype P53 only; and (D) high CCAR2 (red) vs. low (blue) CCAR2 expression in mutant P53 samples, where the black line indicates the survival of mutant P53 only.

2.2.2.2.2. Cell Based Mechanistic Studies

The first molecular function attributed to CCAR2 was its ability to bind and stabilize unliganded Estrogen Receptor α (ERα), and promote hormone-independent breast cancer cell survival[107]. Subsequently, knockdown and overexpression of CCAR2 demonstrated its role in the promotion of cell survival, and the inhibition of cell death[106,127,151,155,158,161–164] (Fig. 2. 3). For example, CCAR2 depletion in colon cancer cells

has been shown to decrease cell growth, colony formation and xenograft growth[106]. Likewise, CCAR2 depletion in breast cancer cells has been shown to decrease cell growth[151], cell migration/invasion[151], xenograft formation[151], and lead to cell death by apoptosis[107,162,164]. Furthermore, CCAR2 deficient lung cancer cells are more sensitive to etoposide and radiation[165], indicating a role in chemosensitization.

As mentioned before, besides CCAR2 expression, PTMs on CCAR2 can modify its functional role in oncogenesis. For example, phosphorylation of CCAR2 by CK2α caused an upregulation of EMT related genes such as MMPs, SMAD, and N-Cadherin[127]. Moreover, CCAR2 can influence the PTMs on other key proteins. For example, CCAR2 was found to promote the activity of β-Catenin in the colon[106,166], by binding to β-Catenin and promoting Lys49 acetylation of β-Catenin *via* SIRT1 inhibition[166], leading to increased β-Catenin signaling[106]. Also, MCC (Mutated in Colorectal Cancer), a gene that is commonly mutated and inactivated in colon cancers, keeps β-Catenin under check by sequestering the CCAR2/ β-Catenin complex in the cytosol, and maintaining β-Catenin in the deacetylated form[166]. However, when MCC is mutated (R506Q), it is not able to re-localize CCAR2 to the cytosol, and the brake on β-Catenin is released promoting oncogenesis[166].

The transcriptional activity of PEA3, an oncogenic ETS family transcription factor, is inhibited by SIRT1 mediated deacetylation[151]. Here, inhibition of SIRT1 by CCAR2 activated PEA3 and enhanced tumorigenic phenotypes in ER-negative breast cancer[151].

In another case, SIRT1 mediated deacetylation is required for promoting the activity of SUV39H1, a methyltransferase responsible for maintaining genomic stability[167]. Inhibition of SIRT1 by CCAR2 causes dissociation of SUV39H1 from SIRT1 and loss of activity, which leads to genomic instability[167,168].

CCAR2 also has effects on tumor promoting pathways that are not dependent on its SIRT1 inhibition. For example, in addition to CCAR2 activating β-Catenin through acetylation, Yu et al. have shown that CCAR2 can also regulate β-Catenin signaling by co-activating the transcriptional activity of PROX1, a β-Catenin target gene[106]. Other examples demonstrate that CCAR2 can activate NF-κB through interaction with IKK-β[129,162], activate AKT signaling[163], repress BRCA1 through direct binding[169], and stabilize the androgen receptor[158,170]; all of which happens independent of SIRT1 inhibition.

2.3. Discussion

CCAR1 and CCAR2 are dynamically regulated proteins that have roles in tumor suppression and tumor promotion. Understanding their roles during cancer depends on understanding the functions of their numerous protein partners. Although some protein partners of CCAR2 are well-studied proteins, such as p53 and β-catenin, we still do not fully understand the functional roles of many other CCAR2 protein partners. The role of CCAR2 also depends on knowing when and where CCAR2 interacts with each of its partners. The majority of studies show CCAR2 only interacting with a few proteins at a time, but according to recent proteomic studies, CCAR2 can interact with hundreds of different proteins, many of which are novel interactions with unknown functional consequences on tumorigenesis (Fig. 2.3). For example, Giguère et al. found that

CCAR2 binds to five components of SWI/SNF, a chromatin modifying complex that is commonly mutated in cancer[117], but the outcome of these interactions is unknown. They also showed that CCAR2 binds to components on the Period Complex (PER) and is involved in regulation of the circadian rhythm[117]. This supports a previous report showing that CCAR2 is involved in the circadian rhythm, although they found that CCAR2 binds and stabilizes REV-ERBα. There is a growing interest in understanding the relation between the circadian rhythm and cancer[171], and studying CCAR2 in the circadian rhythm may provide useful insights.

CCAR2 has other biological functions whose effect on tumorigenesis is currently unknown. CCAR2 interacts with and destabilizes FOXP3, which diminishes its immunosuppressive role[172]. This has implications in autoimmune diseases and cancer as FOXP3 is a master regulator of T regulatory cell. CCAR2 may also play a role during cancer immunotherapy, such as anti-CTLA-4, which targets FOXP3-positive T-regulator cells.

CCAR2 plays a role in alternative splicing, by interacting with ZIRD and hnRNPA1 to form the DBIRD complex[173]. This complex targets A/T rich regions of the DNA and splices nascent mRNA[173]. Validated target genes of the ZIRD complex included RAD50, which is a DNA damage repair protein and has been identified as a cancer susceptibility gene[173].

In conclusion, the main question of whether CCAR2 is a tumor suppressor or tumor promotor still persists. If anything, it should be dubbed a gene chameleon[15]. As this review discusses, the role of CCAR2 is context dependent and complicated. In

clinical studies and cell based mechanistic studies, CCAR2 predominantly acts like a tumor promoter in a variety of tissues, cells and it plays a role in many tumorigenic pathways. On the contrary, the fact that C57/BL6 CCAR2 knockout mice develop spontaneous tumors suggests that CCAR2 can also act as a tumor suppressor. Furthermore, the well-known role of CCAR2 in activating p53 signaling also favors the tumor suppressor paradigm. Therefore, CCAR2 has seemingly conflicting or contrasting functions due to the fact that it can bind/interact with several different protein partners. We hope that this contradiction can be clarified with more research in the coming years that will carefully tease out the context under which CCAR2 is acting, the function of its binding partners, and the regulation of CCAR2 itself.

3. ACETYLATION OF CCAR2 ESTABLISHES A BET/BRD9 ACETYL SWITCH IN RESPONSE TO COMBINED DEACETYLASE AND BROMODOMAIN INHIBITION[*]

3.1. Abstract

There continues to be interest in targeting epigenetic 'readers, writers and erasers' for the treatment of cancer and other pathologies. A mechanistic understanding is frequently lacking, however, for the synergy observed when combining deacetylase and bromodomain inhibitors. Here we identify cell cycle and apoptosis regulator 2 (CCAR2) as an early target for acetylation in colon cancer cells treated with sulforaphane (SFN). N-terminal acetylation of CCAR2 diminished its interactions with histone deacetylase 3 (HDAC3) and β-catenin, interfering with Wnt coactivator functions of CCAR2 including in cells harboring genetically encoded CCAR2 acetylation. Protein domain arrays and pull-down assays identified acetyl 'reader' proteins that recognized CCAR2 acetylation sites, including BRD9 and members of the bromodomain and extraterminal domain (BET) family. Treatment with the BET inhibitor JQ1 synergized with SFN in colon cancer cells and suppressed tumor development effectively in a preclinical model of colorectal cancer. Studies with SFN+JQ1 in combination implicated a BET/BRD9 acetyl switch and a shift in the pool of acetyl 'reader' proteins in favor of BRD9-regulated target genes. **Significance:** These results highlight the competition that exists among the 'readers' of

acetylated histone and non-histone proteins and provide a mechanistic basis for potential new therapeutic avenues involving epigenetic combination treatments.

3.2. Introduction

Cell cycle and apoptosis regulator 2 (CCAR2), also known as DBC1/KIAA1967, has gained attention as a 'master regulator' of metabolism, aging, and cancer[108,117,174,175]. This designation derives from the interactions of CCAR2 with protein partners that exert critical roles in physiology and pathophysiology, including Sirtuin 1 (SIRT1) and CHK2, linking CCAR2 to p53 function and DNA repair[106,108,117,140,150,174,175]. Less is known about the N-terminal region of CCAR2 that associates with, and inhibits, histone deacetylase 3 (HDAC3), while also interacting with b-catenin to stabilize b-catenin/Tcf complexes in the nucleus[106,150]. In so doing, CCAR2 serves as a coactivator of Wnt signaling, a well-studied pathway in disease and development[176].

Our attention was drawn to CCAR2 based on two converging observations. First, when CCAR2 is overexpressed in colon tumors, the corresponding patients exhibit significantly reduced survival[106]. Second, as reported here, CCAR2 was identified as an early target for acetylation by sulforaphane (SFN), an agent that causes inhibition and turnover of HDAC3 in colon cancer cells[59,101,102,177]. Notably, when SFN was combined with JQ1, an inhibitor of the bromodomain and extraterminal domain (BET) family[49,178,179], CCAR2 no longer served as an effective coactivator of Wnt/β β-catenin signaling *in vitro* and *in vivo*.

There is growing interest in targeting epigenetic 'readers, writers, and erasers' deregulated in cancer and other pathologies[49,178–180]. This investigation combined

SFN+JQ1 to affect CCAR2 acetylation, and in so doing provided new mechanistic insights into the competition that exists among the 'readers' of acetylated histone and non-histone proteins that are regulated during epigenetic combination therapies.

3.3. Materials and Methods

3.3.1. Cells and Treatments

HCT116, SW480 (human colon cancer cells) and CCD841 (non-transformed colonic epithelial cells) were from ATCC (Manassas, VA, USA), and used within 10-15 passages from receipt. Each cell line was confirmed independently to be of human origin, with no mammalian inter-species contamination, and with the correct genetic profile based on allele-specific markers (Idexx Radil, Columbia, MO[181,182]). Cells were cultured in McCoy's 5A media (Invitrogen) or EMEM (Invitrogen), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at 37^0C in a humidified chamber with 5% CO_2. All cells were tested routinely for mycoplasma by DAPI staining, and by using a PCR-based methodology[183].

JQ1 was purchased from MedChem Express (Monmouth Junction, NJ), whereas the other test agents were from the sources noted elsewhere [59]. Nominal concentrations were as follows: 15 μM SFN, 6-methylsulfinylhexyl isothiocyanate (6-SFN), 9-methylsulfinylnonyl isothiocyanate (9-SFN), and allyl isothiocyanate (AITC); 1 μM trichostatin A (TSA); 10 mM sodium butyrate (NaB); and 1 mM valproic acid (VPA). For combination index (CI) experiments, SFN, JQ1, and suberoylanilide hydroxamic acid (SAHA) were tested in the range 2-17 μM, 1-60 μM and 0.1-2 μM, respectively, with dimethylsulfoxide (DMSO) as vehicle. In most experiments, cells were treated with test

agents 24 h after seeding[59,102], except in HDAC3 siRNA knockdown assays, which were conducted according to a published methodology[59,177]. HDAC3 siRNA (Trilencer-27) and control siRNA were procured from Origene, and cells were transfected with RNAiMAX reagent (Invitrogen) for 24–48 h, using the manufacturer's protocol. Two of the target siRNAs, designated as siRNA(1) and siRNA(3), produced the most efficient knockdown of HDAC3, and the data are shown in the corresponding figures. Unless indicated otherwise, whole cell lysates or nuclear and cytoplasmic fractions[102] were harvested 6 h after treatment with test agents, followed by RNA or protein expression analyses.

Additional experiments involved CCAR2 deletion from colon cancer cells via CRISPR/Cas9 genome editing[184,185]. The PX459 Vector control (Addgene) included a non-targeting gRNA sequence integrated into the vector. For re-introduction of CCAR2 into CCAR2-null cells, transient transfection was conducted using expression constructs for WT protein or acetylation mutants. In the latter case, a Q5 Site-Directed Mutagenesis kit (New England BioLabs, Ipswich, MA) was used to convert Lys to Arg, starting with CCAR2 plasmid pcDNA Myc DBC1 (Addgene plasmid #35096)[169], with confirmation by direct sequencing.

3.3.2. Genetically Encoded Acetylation of CCAR2

A system for genetically encoded Lys acetylation on histones[62] was adopted for CCAR2. In brief, CCAR2 and 3xHA were PCR amplified and sub-cloned into pGEM-9Zf(-) (Promega # P2391) to generate HA-CCAR2. HA-CCAR2 was restriction cloned into pE337, replacing H3.3-HA. Q5 Site-Directed Mutagenesis was used to convert Lys to TAG stop codons at defined sites in CCAR2. Plasmids pE312 and pE337-HA-CCAR2

WT and Lys mutants were stably expressed in CCAR2 null HCT116 cells using Super PiggyBac Transposase vector (SBI #PB210PA-1) and selected with puromycin and neomycin. Cells were treated for 24 h with 10 mM *N*-acetyl-L-lysine to express acetylated CCAR2 (Sigma #A4021).

3.3.3. Immunoblotting (IB) and Immunohistochemistry (IHC)

IB used published procedures for whole cell lysates, nuclear/cytoplasmic fractions, and tissue lysates of colon tumors or normal colon biopsies[59,101,102,181,186]. Antibody to CCAR2 was from Bethyl Labs (Montgomery, TX), whereas acetyl-lysine (Ac-Lys), histone H3, histone H4, histone H4K12-acetylated (H4K12ac), 14-3-3, RAD54, HDAC3, β-catenin, c-Myc, cyclin D1, matrix metalloproteinase 7 (MMP7), poly(ADP-ribose)polymerase (PARP), Caspase-3, Pin1, Lamin, and β-actin primary antibodies were from sources reported[59,101,102,181,186]. IHC followed the general procedures described elsewhere[181,186].

3.3.4. Proximity Ligation Assays (PLA)

Protein-protein interactions were examined *in situ*, in cell-based assays and tissue sections, using the Duolink PLA Fluorescence Protocol (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's recommendations.

3.3.5. Pulldown Assays

Immunoprecipitation (IP) methodologies were as reported for endogenous proteins[59,102], or Myc-, GST- and HA-tagged proteins[140,187–189].

3.3.6. Mass Spectrometry

Acetylation sites on CCAR2 were identified following the general approach reported[140]. In brief, 24 h after seeding, HCT116 cells were treated with SFN or DMSO, and 6 h later the cell lysates were subjected to IP using CCAR2 antibody. Following SDS-PAGE separation, the CCAR2 band was excised from the gel and digested overnight with trypsin prior to extraction and analysis on an Eksigent cHiPLC™ with nanoLC linked via a nanoflex to an ABSCIEX TripleTOF 5600™ mass spectrometer (Mass Spectrometry-Proteomics Core, Baylor College of Medicine, Houston, TX). Peaks Studio version 7.0 (Bioinformatics Solutions Inc.) was used to match spectra to peptides using the NCBI non-redundant database, including consideration of lysine acetylation. Modified peptides were verified by manual inspection of MS/MS data.

3.3.7. RNA Analyses

RNA-seq sequencing (RNA-seq) and bioinformatics analyses were as reported[190] for adenomatous colon polyps from familial adenomatous polyposis (FAP) patients (GSE88945, GSE106500) and the polyposis in rat colon (Pirc) preclinical model[191]. Library preparation via a NEBNext® Ultra™ Directional RNA Library Prep Kit was followed by Illumina® sequencing on a NextSeq 500/550 instrument (Illumina, La Jolla, CA). Real-time reverse transcription quantitative PCR (RT-qPCR) used a reported methodology[190].

3.3.8. Docking *in silico*

After multiple sequence alignment[192], docking of BRD2, BRD3, BRD4 and BRD9 was performed using AutoDock Vina[193], on CCAR2 structures predicted via SWISS-

MODEL[194].   Ligand-protein interactions were analyzed using PDBePISA[195,196] and LPC/CSU[197]. Initial work-up confirmed that the docking of JQ1 with BRD2, BRD3, and BRD4 corresponded favorably with the reported orientations[49].

3.3.9. Chromatin Immunoprecipitation (ChIP)

The ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA) was used, as reported[198].   Following drug treatment, HCT116 cells were cross-linked with formaldehyde and homogenized in order to isolate the nuclear fraction.   DNA fragmentation was performed using a Biorupter for 15 cycles of 20 secs each.   Ten microliters of fragmented chromatin was kept as input, while the remainder was subjected to IP with anti-CCAR2 (Cell Signaling), BRD9 (Active Motif), or BRD3 (Active Motif) antibodies. After reversing the cross-linking, and proteinase treatment, DNA was purified using the QIAquick PCR Purification kit (Qiagen). PCR was run on a Roche Light Cycler 480 II with pre-incubation for 5 min at 95 °C, then 55 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Each experiment was repeated at least twice. Primer sequences were as follows:

E1 (Forward Primer) TTGTCGCAGGTATGCTGAGTC

E1 (Reverse Primer) TGTGATTACCCAGGCACACT

E2 (Forward Primer) TCCTGAGTCACGGAGTTGTCT

E2 (Reverse Primer) TGCGATCTTCAGAGGGCCTA

E3 (Forward Primer) GAGATTACAGGGAGTGGCAGTG

E3 (Reverse Primer) TGGAAACTCAGATACTCCTGGG

E4 (Forward Primer) CTCCCGAGGGCGATAAAAGG

E4 (Reverse Primer) GGATGTTTGCTGGAACGCTG

Promoter (Forward Primer) TGCATGACCGCATTTCCAATA

Promoter (Reverse Primer) CGGACAAACCGGACGTTTAATTC

3.3.10. Preclinical Experiments

All studies were approved by the Institutional Animal Care and Use Committee.
For xenograft experiments, $5x10^6$ cells (SW480 CCAR2 CRISPR/Cas9 knockout or
vector controls) were injected into either flank of male athymic nude mice (Envigo,
Somerset, NJ). After 10 d, animals were randomized as follows (n=5 mice/group): SFN,
100 mg/kg body weight (BW) via daily oral gavage; JQ1, 50 mg/kg BW, twice weekly
i.p. injection; SFN+JQ1, at doses of the individual compounds, or vehicle. Tumor volumes
were measured twice/week using calipers. In rat experiments, Pirc males[191] at 5 months
of age were assigned to study groups (3-4/group), and 2 months later occluding colon
polyps were resected[199]. Rats were then treated for 5 weeks with test agents, as follows:
SFN, 400 parts per million (p.p.m.) in AIN93 diet; JQ1, 12.5 mg/kg BW via twice weekly
i.p. injection; SFN+JQ1, at the doses of the individual compounds, or vehicle. The study
was terminated 2 months after polypectomy, and GI lesions were enumerated prior to IB
and RNA-seq, as reported[190]. To our knowledge, this is the first report to examine
secondary prevention in a murine model of FAP, following surgical intervention.

3.3.11. Statistics

Results are representative of findings from at least three independent experiments,
expressed as mean±SE, unless indicated otherwise. Student's *t*-test was used for paired
comparisons, whereas multiple groups were subjected to analysis of variance and

Bonferroni's test (GraphPad Prism™ v5.04, La Jolla, CA, USA). Statistical significance was shown in the corresponding figures, as follows: *P<0.05, **P<0.01, ***P<001, ****P<0.0001.

3.4. Results

3.4.1. Novel Acetylation Sites Are Produced on CCAR2 by SFN

Whole cell lysates were prepared from HCT116 colon cancer cells incubated with SFN for 6 h, and anti-acetyl-lysine (Ac-Lys) antibody was used to IP endogenous acetylated proteins. IB confirmed the increased histone acetylation after treatment with pan-HDAC inhibitors NaB and TSA, but not by SFN at 6 h (Fig. 3.1*A*, red arrow). Under the same conditions, robust acetylation of CCAR2 was detected in SFN-treated cells (Fig. 3.1*A*, red box), comparable to that of NaB and TSA. None of the test agents caused 14-3-3 acetylation. Similar observations were made when SW480 cells were treated with SFN, TSA, SAHA, NaB, or VPA (Appendix Fig. A.1); all of the test agents except SFN (arrow) caused increased histone acetylation at 6 h, and all of the compounds – including SFN (box) – produced a marked increase in CCAR2 acetylation without affecting the acetylation status of its paralog, CCAR1. Acetylation of CCAR2 at 6 h occurred in both the cytoplasmic and nuclear compartments (Fig. 3.1*B*), whereas SFN had no effect on the acetylation status of cyclin D1. Structural analogs of SFN that also were reported to inhibit HDAC activity and to turnover HDAC3 protein[59], namely 6-SFN and 9-SFN, similarly increased the acetylation status of CCAR2 without affecting a negative control, RAD51 (Fig. 3.1*C*). Increased CCAR2 acetylation was not observed for allyl isothiocyanate (AITC), which lacks HDAC inhibitory activity in colon cancer cells[59]. No HDAC3

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acetylation was detected under conditions in which HDAC3 protein levels were reduced by SFN/6-SFN/9-SFN at 6 h (Fig. 3.1*C*, dashed box). SiRNA-mediated knockdown of HDAC3 recapitulated the induction of CCAR2 acetylation in colon cancer cells (Fig. 3.1D and Appendix Fig. A.2), without changing the acetylation status of β-catenin (Fig. 3.1*D*). Thus, CCAR2 acetylation can occur in colon cancer cells in the absence of similar changes to other non-histone proteins, including β-catenin and HDAC3.
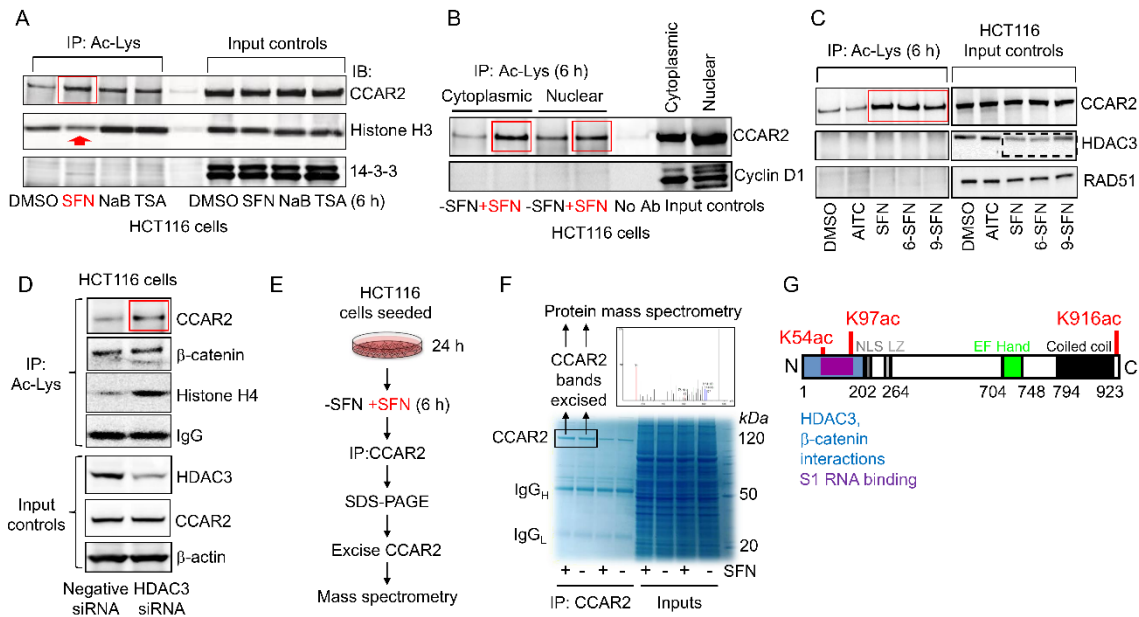
Figure 3.1 CCAR2 is an early target for acetylation in SFN-treated colon cancer cells.
A, HCT116 cells were treated with test agents, and 6 h later cell lysates were subjected to IP with Ac-Lys antibody. **B,** Protocol from A, applied to nuclear and cytoplasmic extracts. **C,** Protocol from A, repeated with SFN analogs 6-SFN, 9-SFN and AITC. **D,** siRNA-mediated knockdown of HDAC3 and IP/IB of cell lysates, as indicated. For HDAC3 knockdown in SW480 cells, refer Appendix Fig. A.2. **E,F,** After IP and SDS-PAGE, CCAR2 was excised from the gel, digested with trypsin, and analyzed by protein mass spectrometry. **G,** Positions of SFN-induced acetylation sites. NLS, nuclear localization signal; LZ, leucine zipper. The IP and IB data shown in each figure panel are from a single experiment in each case, and are representative of the findings from three or more independent experiments.

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CCAR2 was pulled down from colon cancer cells (Fig. 3.1*E*), and after protein separation the CCAR2 band was excised from the gel (Fig. 3.1*F*) and subjected to tandem mass spectrometry. Following SFN treatment, three novel acetylation sites were identified on CCAR2 at K54, K97, and K916 (Fig. 3.1*G* and Appendix Fig. A.3). N-terminal acetylation sites were within a region that interacts with HDAC3 and β-catenin, whereas C-terminal acetylation was adjacent to a coiled coil domain (Fig. 3.1*G*).

3.4.2. CCAR2 Acetylation Interferes with Wnt Coactivator Functions

To examine the functional consequences of SFN-induced CCAR2 acetylation, we first deleted CCAR2 from colon cancer cells using CRISPR/Cas9 (Fig. 3.2*A* and 3.2*B*). Clones that lacked CCAR2 protein had reduced growth rates compared with the vector controls *in vitro* (Fig. 3.2*A*), and when injected into nude mice (Fig. 3.2*C*). CCAR2 was then re-introduced back into CCAR2-null cells via transient transfection of the corresponding expression constructs, either as wild type (WT) CCAR2 or as acetylation mutants. IP with Ac-Lys antibody revealed low basal acetylation for WT CCAR2 in the vehicle controls, which was increased after SFN treatment, but was less marked for the acetylation mutants K97R and K916R (Fig. 3.2*D*). Starting with CCAR2 null cells, re-introduction of acetylation mutants K54R, K97R, and K916R, or the double-mutant K54R/K97R, had no effect on *MYC* expression, whereas reintroduction of WT CCAR2 increased *MYC* levels significantly (Fig. 3.2*E*). Similar results were obtained for *MMP7* (Appendix Fig. A.4*A*), and while this was reversed by SFN treatment following transient transfection of WT CCAR2, acetylation mutants such as K54R were resistant to SFN (Appendix Fig. A.4*B*).
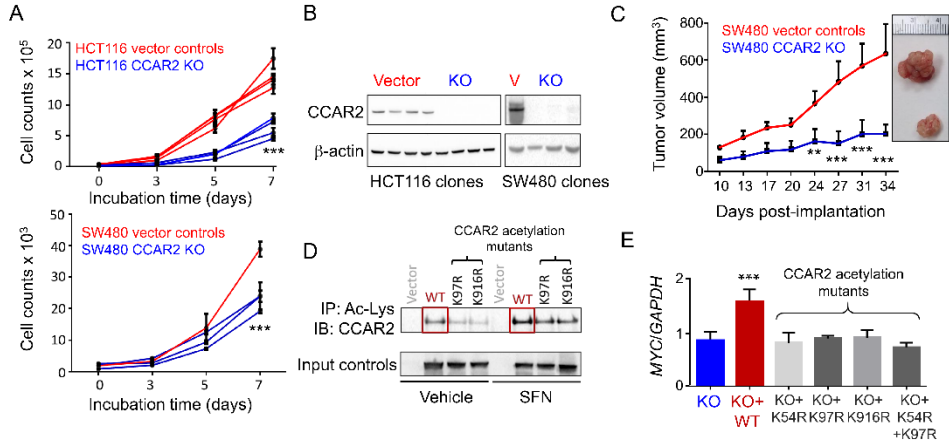
Figure 3.2 CCAR2 acetylation lowers oncogene expression in colon cancer cells.
A, Deletion of CCAR2, with each line signifying a different clone, and each data-point representing mean±SD (n=3). **B,** Confirmation by IB of CCAR2 loss after CRISPR/ Cas9 genome editing. V, vector; KO, knockout. **C,** Xenograft studies in mice. Each data-point represents mean±SD (n=5). **D,** CCAR2 null colon cancer cells transiently transfected with vector control, or expression constructs for wild type (WT) or acetylation mutants of CCAR2. After 24 h, cells were treated with SFN or vehicle, and 6 h later cell lysates were subjected to IP/IB, as indicted. **E,** After treatment of colon cancer cells as indicated in *D*, total RNA was isolated and RT-qPCR was performed for *MYC* normalized to *GAPDH*. Data bars = mean±SD (n=3). **P<0.01 and ***P<0.001 significant difference from the corresponding vector/KO control. The data shown in each figure panel are from a single experiment in each case, and are representative of the findings from two or more independent experiments.

In addition to *MYC* and *MMP7*, RNA-seq revealed a suit of Wnt/β-catenin target genes downregulated in SFN-treated colon cancer cells (Fig. 3.3*A*). Next, we examined direct interactions between endogenous CCAR2 and β-catenin proteins via PLA[200,201]. In CCD841 non-transformed colonic epithelial cells, CCAR2/β-catenin interactions were detected at low levels, whereas numerous interactions were observed in HCT116 and SW480 cells (Fig. 3.3*B*, red dots). After SFN treatment, fewer CCAR2/β-catenin

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interactions were detected, especially in the nucleus. Using the same approach, we also detected diminished CCAR2/HDAC3 nuclear interactions (Fig. 3.3*C*).

To corroborate these findings, we pulled-down endogenous β-catenin or HDAC3 from nuclear extracts of colon cancer cells (Fig. 3.3*D*), and confirmed that interactions with CCAR2 were reduced markedly after SFN treatment (red boxes). Peptidyl-prolyl cis/trans isomerase 1 (Pin1), which interacts with HDAC3 in the nuclear compartment[102], was used as a control in some experiments. We conclude that CCAR2/HDAC3/β-catenin interactions are disrupted in SFN-treated colon cancer cells, interfering with the Wnt coactivator role of CCAR2 (Appendix Fig. A.5). Next, a system for genetically encoding lysine modifications on histones[62] was used, for the first time, to engineer acetylation sites on a non-histone protein, CCAR2. Starting with CCAR2 null colon cancer cells, stable clones were generated containing HA-tagged CCAR2 or HA-tagged CCAR2-K54-TAG (abbreviated hereafter as HA-CCAR2 and HA-K54-TAG) – the 'TAG' premature stop codon preventing protein expression in the absence of acetyl-lysine (Ac-Lys) reagent[62]. Twenty-four hours after the addition of Ac-Lys, nuclear and cytoplasmic extracts were subjected to IP with an antibody to the HA-tag on CCAR2, followed by IB with the same antibody (Fig. 3.3*E*). In cells stably transfected with HA-K54-TAG, no band was detected until the addition of Ac-Lys reagent, consistent with the formation of genetically encoded CCAR2-K54 acetylated protein (Fig. 3.3*E*, dotted boxes). In the presence of Ac-Lys reagent, IP with HA antibody followed by IB for β-catenin revealed a strong band in the nuclear compartment of cells stably transfected with HA-CCAR2 but not HA-K54-TAG (Fig. 3.3*E*, solid boxes). Thus, acetylation of Lys 54 on CCAR2 was sufficient to block

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its interactions with β-catenin in the nuclear compartment. Colon cancer cells also were generated containing stably transfected HA-K97-TAG; no CCAR2-K97ac band was detected after Ac-Lys treatment (Appendix Fig. A.6). The K97ac site may be destabilizing to CCAR2 under the conditions used, in the absence of K54ac.
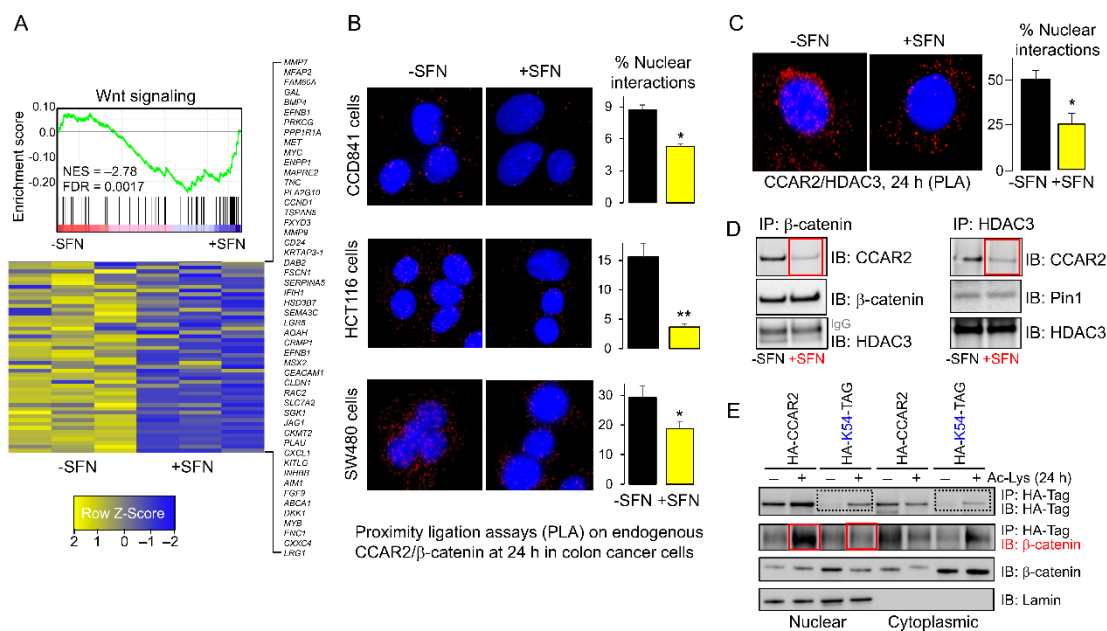
Figure 3.3 CCAR2 protein interactions are disrupted by SFN treatment, and genetically encoded acetylation of CCAR2 Lys 54 blocks β-catenin interactions.
A, RNA-seq data from HCT116 colon cancer cells, 6 h after treatment with SFN or vehicle. Each column is a biological replicate (n=3). Wnt signaling was among the top five cancer-related pathways altered by SFN [202], and gene set enrichment analysis (GSEA) prioritized 118 Wnt-related genes among 22727 genes in the dataset. **B,** PLA identified endogenous interactions of CCAR2 and β-catenin proteins. Cells were imaged 24 h after treatment with SFN or vehicle. **C,** The approach in *B*, used to examine endogenous CCAR2/HDAC3 interactions. Data bars designate mean±SD (n=3); *P<0.05, **P<0.01, compared with vehicle. **D,** 24 h after treating HCT116 cells with SFN or vehicle, nuclear extracts were subjected to IP/IB. **E,** A system for genetically encoding lysine modifications on histones [62] was used to engineer K54 acetylation on CCAR2. Nuclear and cytoplasmic extracts were subjected to IP/IB with the antibodies shown, 24 h after addition of Ac-Lys, to trigger the designed acetylation on CCAR2. The data shown in each figure panel are from a single experiment in each case, and are representative of the findings from two or more independent experiments.

3.4.3. CCAR2 Acetyl 'Readers' Include BET Family Members and BRD9

Based on the CCAR2 acetylation sites observed after SFN treatment (Fig. 3.1*G*), we turned our attention to the acetyl 'reader' proteins. Biotin-tagged peptide mimetics of CCAR2 were screened[187–189] via protein arrays comprising all known acetyl readers (Fig. 3.4*A*), followed by GST-pulldown assays for validation (Fig. 3.4*B*). CCAR2-K97ac peptide was recognized by bromodomains of ASH1L and BAZ1A, whereas CCAR2-K916ac peptide interacted with bromodomains of ASH1L, BRDT, BRD2, BRD3 and BRD9. The arrays also implicated BRD7 interacting with CCAR2-K916ac peptide, but this was not corroborated in pulldown experiments (Fig. 3.4*B*, right panel). Protein arrays did not recognize a peptide mimetic for CCAR2-K54ac (Fig. 3.4*A*, left panel).

The BET members BRD2, BRD3, and BRDT, along with BRD4, interact with high specificity on the arrays with JQ1 (M.T. Bedford, manuscript in preparation). However, CCAR2 peptide mimetics did not interact with BRD4 on the arrays, despite favorable docking scores *in silico* (Appendix Fig. A.7). Docking scores supported the preferred interactions of BET members and BRD9 with CCAR2K916ac *versus* H4K16ac, suggesting a scenario in which acetylated CCAR2 competes with acetylated histones for the binding of acetyl readers and their inhibitors, such as JQ1.
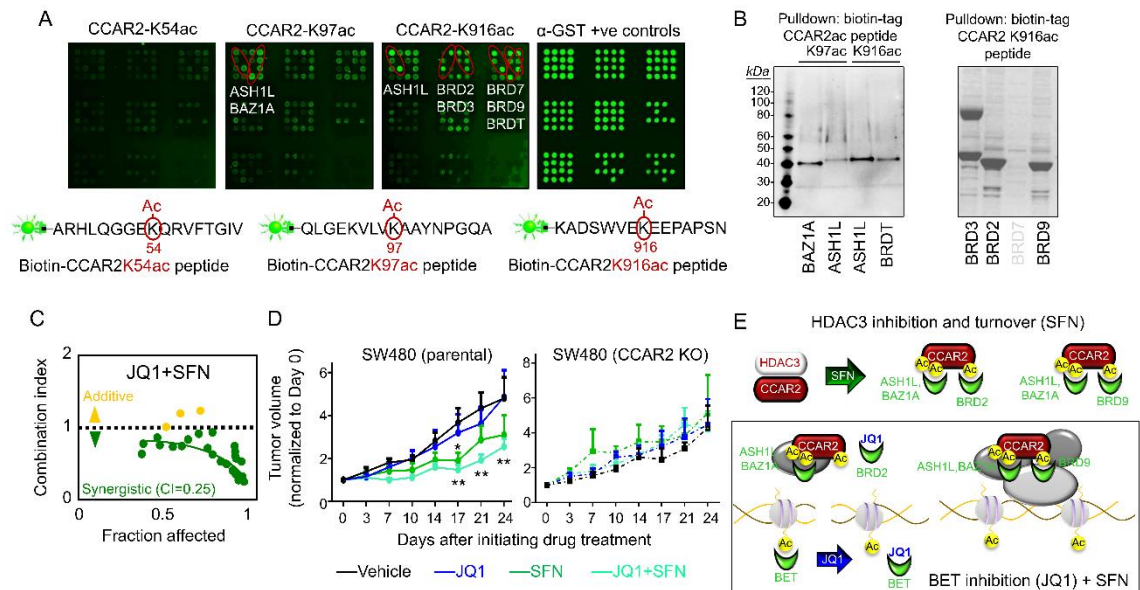
Figure 3.4 Acetylation sites on CCAR2 are recognized by acetyl 'reader' proteins.
A, Protein arrays comprising all BROMO/ PHD/PWWP/YEATS domains were screened using peptide mimetics of acetylated CCAR2, followed **B,** by GST-pulldown assays for validation. **C,** Viability of colon cancer cells; CI, combination index. **D,** Mice were injected in either flank with parental SW480 cells or SW480 CCAR2 KO cells, and 10 d later animals were treated with test agents (see Methods). Data-points, mean±SD (n=5); **P<0.01, *P<0.05 compared with vehicle time-point. **E,** Working model of acetyl readers and CCAR2 interactions. The data shown in each figure panel are from a single experiment in each case, and are representative of the findings from two or more independent experiments.

JQ1 exhibited strong synergy with SFN in colon cancer cells, the combination index of 0.25 (Fig. 3.4*C*) being comparable to 0.13 and 0.33, respectively, for JQ1 plus SAHA or 6-SFN (Appendix Fig. A.8*A*). Co-treatment with JQ1 plus SFN or 6-SFN increased the levels of cleaved PARP and cleaved Caspase-3, indicating enhanced apoptosis in colon cancer cells (Appendix Fig. A.8*B*, dotted boxes), consistent with prior studies using SFN and 6-SFN alone (10-12). Next, we took SW480 cells that are reported

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to be resistant to JQ1[203], but also exhibited reduced CCAR2/β-catenin interactions after SFN treatment (Fig. 3.3*B*), and examined their growth in nude mice. As expected for a JQ1 resistant cell line[203], JQ1 alone had no effect, but JQ1 enhanced the tumor suppressive actions of SFN *in vivo* (Fig. 3.4*D*, left panel, JQ1+SFN, **P<0.01) despite an apparent lack of synergy under the conditions employed. When mice were injected with SW480 CCAR2-null cells, as before (Fig. 3.2*C*), no inhibition was observed for SFN, JQ1, or SFN+JQ1 (Fig. 3.4*D*, right panel). Thus, SFN required the presence of CCAR2, and circumvented resistance mechanisms in JQ1 resistant colon cancer cells[203,204]. Our working model (Fig. 3.4*E*) proposes a shift to increased BRD9/CCAR2-containing chromatin complexes as a basis for the synergistic interactions of SFN+JQ1 in colon cancer cells, supported by bioinformatics data – see below.

3.4.4. Cooperative Inhibition by SFN+JQ1 in a Genetic Model of Colorectal Cancer

We next re-examined RNA-seq data from a recent study[190] and observed the stratification of CCAR2 in colon adenomas of FAP patients (Fig. 3.5*A*). Subjects with high CCAR2 levels in colon adenomas also had high CCAR2 expression in normal-looking tissues, whereas patients with lower CCAR2 levels in colon adenomas had reduced CCAR2 expression in normal-looking tissues. Normal-looking colon in FAP patients, and in preclinical models of FAP, is rarely 'normal' due to the presence of microadenomas and other preneoplastic lesions.
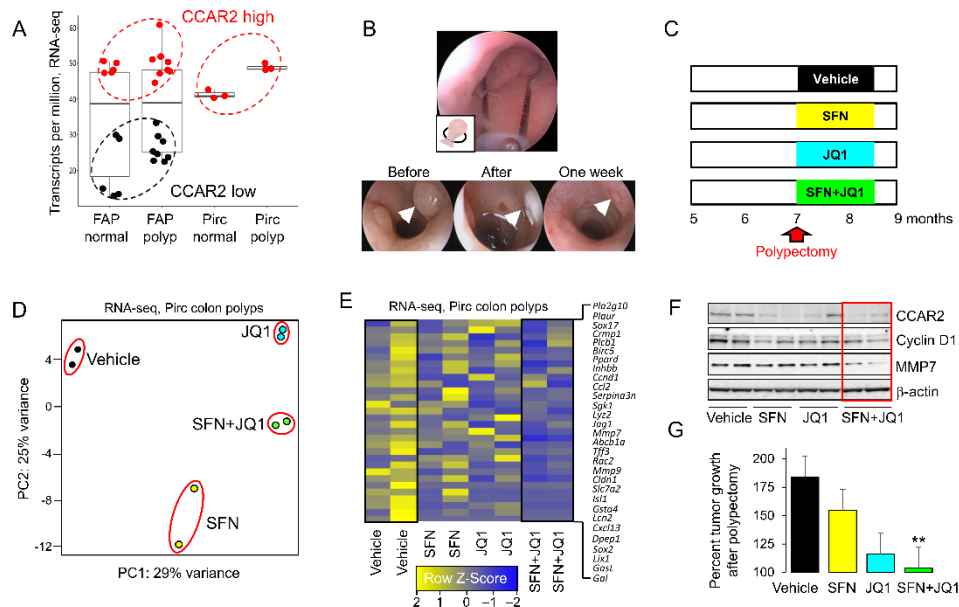
Figure 3.5 SFN+JQ1 protect in a murine model of FAP.
SFN+JQ1 protect in a murine model of FAP. **A,** RNA-seq data[190] mined for *CCAR2* levels in adenomatous colon polyps from FAP patients and the Pirc rat[191]. Each data-point designates an individual polyp or a normal-looking colonic mucosa sample. **B,** Polypectomy in the Pirc model [199]. **C,** At 5 months of age, Pirc males (3-4/ group) were assigned to different arms of the study, and two months thereafter occluding colon polyps were resected. Rats were then treated for 5 wks with SFN, JQ1, SFN+JQ1, or vehicle (see Methods). The study was terminated 2 months after polypectomy, and duplicate lesions in each group provided data for **D,E,** RNA-seq and **F** IB. The IB data are from a single experiment, and are representative of the findings from two independent experiments. D, Principle component analysis of 1436 differentially-expressed genes (DEGs) identified E, Wnt targets downregulated by JQ1+SFN. **G,** Compared with vehicle, JQ1+SFN inhibited the growth of colon polyps significantly (**P<0.01).

Interestingly, the 'CCAR2 high' molecular phenotype also was detected (Fig. 3.5*A*) in the Pirc model of FAP[191]. We resected occluding polyps in the rat (Fig. 3.5*B*), as reported[199], and animals were then treated with SFN, JQ1, SFN+JQ1, or vehicle (Fig. 3.5*C*). When the study was terminated, 2 months after polypectomy, paired colon polyps in each group were subjected to RNA-seq and IB. RNA-seq segregated the groups based

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on principle component analyses of 1436 genes in the dataset (Fig. 3.5*D*). Notably, RNA-seq recapitulated findings from cell-based assays with respect to Wnt genes downregulated by SFN (Fig. 3.3*A*), and these observations were extended to JQ1 and SFN+JQ1 groups (Fig. 3.5*E*). IB of tissue lysates from Pirc colon tumors also showed reduced expression of target proteins such as CCAR2, cyclin D1, and MMP7, especially for SFN+JQ1 in combination (Fig. 3.5*F*, red box). Consistent with these molecular changes, SFN+JQ1 suppressed colon tumor growth significantly, exceeding the inhibition observed for SFN or JQ1 alone (Fig. 5*G*, **$P<0.01$).

Based on the working model (Fig. 3.4*E*), we took the entire RNA-seq dataset (Fig. 3.6*A*), and prioritized 104 combination-specific 'cooperativity/synergy' candidates among 324 genes in the SFN+JQ1 group (green circle, Fig. 3.6*B*). In addition to Wnt, top cancer-specific pathways included hypoxia, p53, inflammation, reactive oxygen species (ROS), KRAS, RB, and apoptosis (Fig. 3.6*C*), and the most upregulated and downregulated genes were identified (Fig. 3.6*D*). Notably, when all 104 SFN+JQ1 'cooperativity/synergy' genes were interrogated together with available ChIP-seq data for BRD9 (GSM2092891), BRD9 was localized at the corresponding transcription start sites (Fig. 3.6*E*, red line). No corresponding BRD9 signal was detected at transcription start sites of 104 randomly selected genes (Fig. 3.6*E*, green line), implicating BRD9 enrichment on the promoters of SFN+JQ1 cooperativity/synergy genes as being mechanistically relevant.
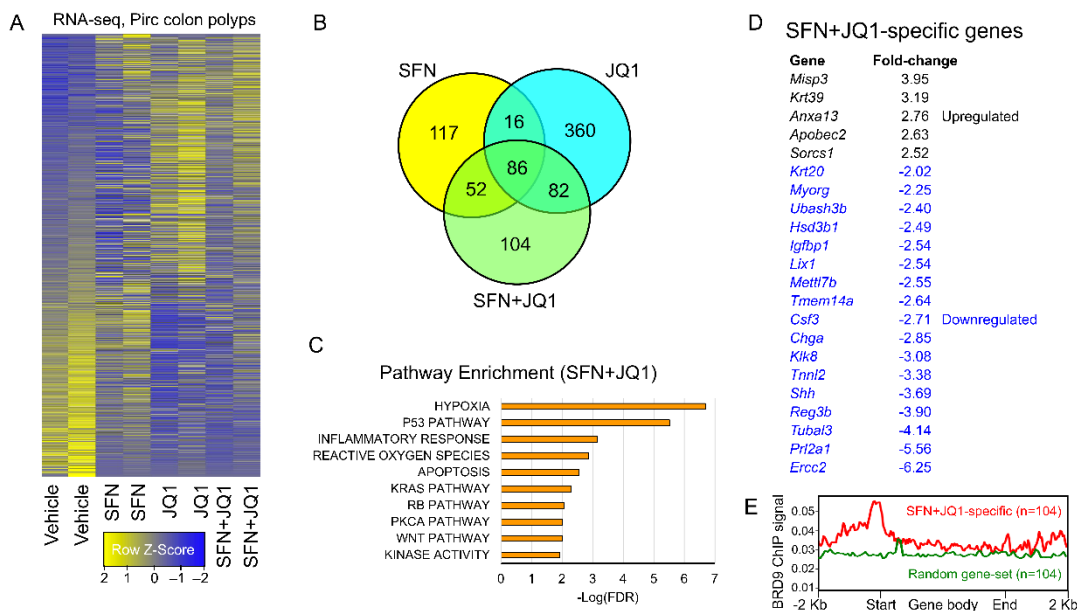
Figure 3.6 RNA-seq prioritizes HDAC+BET 'cooperativity' genes in Pirc colon tumors.
A, Heatmaps for groups analyzed in duplicate (1436 DEGs, no cutoff applied). **B,** Number of DEGs compared to vehicle controls. **C,** Pathway enrichment analysis for SFN+JQ1 DEGs. **D,** Highly upregulated and downregulated SFN+JQ1-specific genes. **E,** Human genome reference GRCh38 was interrogated using BRD9 ChIP-seq data downloaded from GSM2092891. The profile was plotted using Mmint, with the red line representing the average BRD9 signal for SFN+JQ1 'synergy/cooperativity' genes. The corresponding BRD9 signal also was examined for a set of 104 randomly selected genes, not among the 'synergy/cooperativity' candidates (green line). Start, transcription start sites; End, transcription stop sites (n=104).

3.5. Discussion

Acetylation of CCAR2 by hMOF at K112/K215 sites is known to displace SIRT1[140], and we speculated that novel N-terminal acetylation sites identified here might similarly interfere with β-catenin interactions. Consistent with this idea, we observed reduced nuclear CCAR2/β-catenin interactions coinciding with downregulation of multiple Wnt targets. Expression of a genetically encoded K54ac site on CCAR2 was

sufficient to block its interactions with β-catenin, indicating a key role for this post-translational modification in regulating β-catenin associations. Further, the K54 acetylation mutant interfered with the ability of SFN to reduce downstream targets of β-catenin, such as *MMP7* (Appendix Fig. A.4*B*). Xenograft studies in mice indicated that CCAR2 was required for tumor growth inhibition by SFN+JQ1 *in vivo*, and we extended these observations to the Pirc model, showing suppression of adenomatous colon polyps by SFN+JQ1 in the rat. In Pirc colon polyps and in a subset of adenomas from FAP patients (Fig. 3.5*A*), as well as in adenomatous polyps from a screening colonoscopy trial (Appendix Fig. A.9), a 'CCAR2 high' molecular phenotype was observed, which is noteworthy given that CCAR2 overexpression is associated with poor prognosis in colorectal cancer patients[106]. 'CCAR2 high' adenomatous polyps from the screening colonoscopy trial had elevated expression of β-catenin and its downstream targets, such as, MMP7, c-Myc, and cyclin D1, and increased β-catenin/CCAR2 interactions were detected by PLA (Appendix Fig. A.9).

The C-terminal K916 CCAR2 acetylation site appears to be distant from N-terminal K54/K97 acetylation sites that overlap with HDAC3/β-catenin interacting domains (Fig. 3.1*G*). However, as a protein with structural flexibility[104], circumstances might dictate that the ends become aligned, for example after binding lysine methyltransferase ASH1L, which interacted with acetylated peptides from N- and C-terminal regions of CCAR2 (Fig. 3.4*B*), or BAZ1A. BAZ1A is a non-catalytic ISWI subunit that associates relatively weakly with acetylated histones, but is critical for DNA damage recovery[205], which is a key function of CCAR2[143,144]. An intriguing question is

whether BAZ1A and ASH1L interact preferentially with acetylated non-histone proteins such as CCAR2, affecting gene expression changes as members of specific chromatin remodeling complexes in response to SFN+JQ1 treatment. In this context, competition between BET members and BRD9 for the K916ac site on CCAR2 would shift in favor of CCAR2/BRD9 complexes after SFN+JQ1 treatment (Fig. 3.4*E*).

The latter working model derives from three interrelated observations: (i) like BRD2 and BRD3, BRD9 interacts favorably with CCAR2-K916ac (Fig. 3.4*B*); (ii) unlike BRD2 and BRD3, BRD9 is not subject to inhibition by JQ1; and (iii) BRD9 is a required subunit of SWI-SNF complexes[206]. We speculate that SFN-induced acetylation sites on CCAR2 might exert distinct functions, with K54ac for β-catenin displacement, K97ac for ASH1L/BAZ1A-mediated chromatin interactions, and K916ac as an acetyl switch between BET *vs.* BRD9 functions. This does not preclude JQ1 also inhibiting BET acetyl readers on histones (Fig. 3.4*E*) to affect changes in gene expression[49,178–180]. The possibility that SFN and JQ1 might interact synergistically at the level of *MYC* transcription was investigated via ChIP assays, with the following observations: (i) CCAR2 interactions were confirmed on promoter and superenhancer regions, (ii) these interactions were almost completely inhibited by the combination of SFN+JQ1 (Appendix Fig. A.10*A*), and (iii) BRD3 interactions on superenhancer regions also were reduced, to a lesser degree, by JQ1 alone (Appendix Fig. A.10*B*).

Finally, as an HDAC3-interacting protein, CCAR2 might be targeted using HDAC3-selective inhibitors[207,208], although these agents have yet to enter clinical trials. One approach to enhancing efficacy might involve modifying SFN as a lead

compound[202,209], and combining with improved, second-generation bromodomain inhibitors[210,211]. This strategy could provide further insights into the 'cooperativity/synergy' candidate genes prioritized here, and the associated regulatory pathways to be targeted in future clinical trials. We conclude that JQ1+SFN interferes with the Wnt coactivator role of CCAR2, and shifts the pool of acetyl readers in favor of BRD9-regulated genes, providing a mechanistic basis for new therapeutic avenues combining HDAC3+BET inhibition.

3.6. Acknowledgements

4. A FUNCTIONAL PSEUDOGENE, NMRAL2P IS REGULATED BY NRF2 AND SERVES AS A CO-ACTIVATOR OF NQO1 IN SULFORAPHANE TREATED COLON CANCER CELLS[*]

4.1. Abstract

Scope: The anticancer agent sulforaphane (SFN) acts via multiple mechanisms to modulate gene expression, including the induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-dependent signaling and the inhibition of histone deacetylase activity. Transcriptomics studies were performed in SFN-treated human colon cancer cells and in non-transformed colonic epithelial cells in order to pursue new mechanistic leads.

Methods and results: RNA-sequencing corroborated the expected changes in cancer-related pathways after SFN treatment. In addition to *NAD(P)H quinone dehydrogenase 1* (*NQO1*) and other well-known Nrf2-dependent targets, SFN strongly induced the expression of *Loc344887*. This non-coding RNA was confirmed as a novel functional pseudogene for NmrA-like redox sensor 1 (*NMRAL1*), and was given the name NmrA-like redox sensor 2 pseudogene (*NMRAL2P*). Chromatin immunoprecipitation experiments corroborated the presence of Nrf2 interactions on the *NMRAL2P* genomic region, and interestingly, *NMRAL2P* also served as a co-regulator of *NQO1* in human colon cancer cells. Silencing of *NMRAL2P* via CRISPR/Cas9 genome-editing protected against SFN-mediated inhibition of cancer cell growth, colony formation, and migration.

Conclusion: *NMRAL2P* is the first functional pseudogene to be identified both as a direct transcriptional target of Nrf2, and as a downstream regulator of Nrf2-dependent *NQO1* induction. Further studies are warranted on *NMRAL2P*-Nrf2 crosstalk and the associated mechanisms of gene regulation.

4.2. Introduction

Sulforaphane (SFN) is a dietary agent that exerts anticancer effects against various malignancies, including colorectal cancer[212,213]. Chemopreventive outcomes of SFN have been attributed to multiple mechanisms[213–220]. SFN regulates antioxidant activity and the detoxification of carcinogens through induction of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway[221,222]. Under normal conditions, Nrf2 is sequestered in the cytosol by protein partner Kelch-like ECH-associated protein 1 (Keap1). Upon treatment with SFN, Nrf2 dissociates from Keap1, translocates to the nucleus, and dimerizes with small musculoaponeurotic fibrosarcoma (MAF) proteins on the antioxidant response element (ARE) sequences of target genes[220,223]. Target genes activated by Nrf2 include *NAD(P)H quinone dehydrogenase 1* (*NQO1*), *heme oxygenase 1* (*HMOX1*), and various *glutathione S-transferases* (*GST*s). The response of these genes is influenced by nuclear/cytoplasmic trafficking of Nrf2, and by post-translational modifications that affect its interactions with small MAF proteins, chromatin remodeling factors, histone deacetylase (HDAC) enzymes, and other transcriptional regulators[177,224–227].

Among the epigenetic mechanisms implicated in human colon and prostate cancer cells, SFN has been shown to act via HDAC inhibition/turnover and changes in DNA methylation[59,100,102,228]. Acetylation of histone and non-histone proteins was linked to de-

repression of tumor suppressor genes and the activation of apoptotic and G_2/M cell arrest pathways, most notably in cancer cells as compared with the corresponding non-transformed cell lines[54,59,102,229].

Dietary isothiocyanates also alter the expression of various noncoding RNAs (ncRNAs), including microRNAs (miRNAs)[230] such as mir-155, mir-23b, and mir-27b in colonic epithelial cells[231], mir-155 in macrophages[232], miR-200c in bladder cancer cells[233], mir-21 in glioblastoma[234], and let-7 family members in pancreatic ductal adenocarcinoma cells[235]. Other ncRNAs, such as long noncoding RNAs (lncRNAs) and pseudogenes, also have been identified with roles in gene regulation, genome stability, cancer cell survival, and drug resistance[236–242]. There is a general lack of information on how these various ncRNAs might be impacted by diet and lifestyle factors.

While performing transcriptomics studies in SFN-treated colon cancer cells and in non-transformed colonic epithelial cells, we identified a ncRNA, *Loc344887*, that was directly regulated by Nrf2, and that served as a coactivator for *NQO1*. Localized on chromosome 3q27.2 and sharing 62% homology with the protein-coding gene NmrA-like redox sensor 1 (*NMRAL1*) on chromosome 16p13.3, the novel functional pseudogene was assigned the name *NMRAL2P*, and was pursued in mechanistic studies of cell viability, colony formation, and cell migration.

4.3. Materials and Methods

4.3.1. Cell Culture and Treatments

Cell lines were obtained from ATCC and grown at 37℃ in 5% CO_2 with 1% penicillin/streptomycin. Human colon cancer cell lines HCT116 and HT29 were

maintained in McCoy's 5A media (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS), whereas Caco2 cells and CCD841 non-transformed colonic epithelial cells were maintained in EMEM supplemented with 20% FBS. Treatments were performed when cells were ~70% confluent. Unless indicated otherwise, cells were incubated with 15 μM SFN or with the corresponding volume of dimethylsulfoxide (DMSO) vehicle. Allyl isothiocyanate (AITC), 6-methylsulfinylhexyl isothiocyanate (6-SFN), 9-methylsulfinylnonyl isothiocyanate (9-SFN), Oltipraz, *tert*-butyl hydroquinone (TBHQ), trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), valproic acid (VPA), and sodium butyrate were used in some experiments. Concentrations were based on pre-determined IC_{50} values and prior reports[59,101,102].

4.3.2. RNA Isolation and Sequencing (RNA-Seq)

RNA was isolated using the miRNeasy kit (Qiagen), according to the manufacturer's protocol. For RNA-seq, RNA was isolated with Trizol (Life Technologies) and was purified and processed as reported[243]. In some experiments, the PARIS kit (ThermoFisher) was used to isolate nuclear and cytosolic RNA. Enrichment of nuclear RNA was confirmed by reference to *metastasis associated lung adenocarcinoma transcript 1* (*MALAT1*). For a comprehensive list of the primers used, see Appendix Table B.1.

RNA-seq data quality was examined using Fastqc, and low quality reads (>50% bases with Q<30) were filtered out. Bowtie2 with default parameters was used to map reads to hg19 reference genome. Uniquely mapped genes were used to calculate the RPKM for each gene, and DESeq2 (R package) was used to identify significant differentially

expressed genes with a threshold of fdr<0.05 and fold change >4. GOstats (R package) was used to perform KEGG pathway enrichment analysis, with a threshold of p<0.05. TCGA data for *NMRAL2P* were downloaded from the colon adenocarcinoma (COAD) dataset. Nrf2 target genes were selected from the ChIP-seq data of Chorley et al[244], whereas Wnt signaling genes were identified from the gene list generated by Yu et al[106].

4.3.3. Quantitative PCR (qPCR)

SuperScript III First-Strand Synthesis Master Mix (Invitrogen) was used on 1 μg of RNA to synthesize cDNA. qPCR was performed using SYBR Green I dye (Roche), cDNA, and gene-specific primers. Assays were run in a Light Cycler 96 or 480 (Roche) and normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)* using the $2^{-\Delta\Delta CT}$ method. Each experiment was repeated at least twice, and data were normalized to vehicle controls.

4.3.4. CRISPR-Cas9 Genome Editing

A small guide RNA (sgRNA NMRAL2P [upstream1], Appendix Table B.1), specific to the promoter region of *NMRAL2P*, was designed by CRISPR Design (crispr.mit.edu). Restriction cloning was used to insert the sgRNA into plasmid pSPCas9(BB)-2A-GFP (PX458), kindly provided by Dr. Feng Zhang (Addgene plasmid #48138)[245]. In brief, oligos were annealed by temperature ramp-down, phosphorylated, and ligated into the BbsI site of PX458 which contained sgRNA expression, Cas9 protein, and eGFP selection marker. PX458 plasmid (2.5 μg) was transfected into cells in a 6-well dish for 24 h. Green fluorescing cells were sorted individually into 96-well plates on a BD Biosciences FACSFusion Cell Sorter. Primers flanking exon 1 of *NMRAL2P* (see

Appendix Table B.1) were used to screen genomic DNA of individual colonies, and PCR products were confirmed by sequencing.

4.3.5. siRNA Transfection

Gene specific siRNAs (Sigma-Aldrich) or a Universal Control were transfected into cells using RNAiMax transfection reagent (Invitrogen), according to the manufacturer's protocol. Unless stated otherwise, siRNA incubations were for 24-48 h. For siRNA primer sequences, see Appendix Table B.1.

4.3.6. MTT Assays

Each treatment was performed in triplicate on *NMRAL2P* knockout cells, or the corresponding vector controls, plated at 1×10^4 cells per well. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added at 500 μg/ml and incubated at 37°C for 2 h. Resulting formazan dye was solubilized in DMSO and absorbance was measured at 562 nm (OD562).

4.3.7. Soft Agar Colony Formation Assays

Six-well plates were pre-coated with 0.6% agarose Type III-A (Sigma). Cells were mixed with 0.4% top agar and added to pre-coated plates at 3×10^4 cells/ well. After solidifying, 2 ml of liquid media containing SFN or DMSO was added to each well. Each treatment was performed in triplicate. Cells were incubated at 37°C for 2 weeks, and stained with 0.5% crystal violet in 6% formaldehyde. Two independent experiments were performed as biological replicates.

4.3.8. Transswell Assays

To the upper chamber of a transwell insert was added 3×10^4 cells in serum-free media (Costar #3422), and serum-containing media without cells was added to the bottom chamber. After 24 h, inserts were fixed with 6% formaldehyde, the top of the membrane was swabbed, and then stained with 0.2% crystal violet. Membranes were washed and mounted onto glass slides. The number of cells that migrated through the insert was counted for ten 10X fields per treatment, for three wells per treatment. The experiment was repeated three times.

4.3.9. Immunoblotting

Cells were suspended in IP lysis buffer and lysed by freeze-thawing. Immunoblotting used the methodology described previously[59,102], with primary antibodies to Keap1 (Cell Signaling #4617), Nrf2 (Cell Signaling #12721), NQO1(Cell Signaling #3187), and β-Actin (Sigma #A1978).

4.3.10. Chromatin Immunoprecipitation (ChIP)

The ChIP-IT Express Sonication kit (Active Motif) was used according to the manufacturer's protocol. Cells in 150 mm dishes were formaldehyde cross-linked, harvested, and then sonicated in a Bioruptor using 10-s intervals. Chromatin was immunoprecipitated with Nfr2 (Cell Signaling #3187) or Mafk antibodies (Abcam #ab50322) and pulled-down with Protein G magnetic beads (Active Motif). The DNA was reverse cross-linked and purified via the chromatin IP DNA purification kit (Active Motif). qPCR was performed using primers that flanked the AREs of *HMOX1* and *NQO1*. Three putative AREs on the *NMRAL2P* locus were found by sequence analysis using the

consensus sequence from Chorley et al[244]. For the primer sequences, see Appendix Table B.1.

4.4. Results

4.4.1. *NMRAL2P* Is Highly Upregulated in SFN-Treated Colon Cancer Cells

RNA-seq was performed in human HCT116 colon cancer cells and CCD841 non-transformed colonic epithelial cells treated with vehicle or 15 µM SFN for 6 h, in triplicate. Principal component analysis confirmed that the two colonic epithelial cell lines had significantly different endogenous gene expression profiles, which became even more marked after SFN treatment (Appendix Fig. B.1A). Approximately 50% of ~12,000 differentially expressed genes (DEGs) were upregulated and 50% were downregulated in HCT116 cells compared to CCD841 cells (Appendix Fig. B.1B). These DEGs likely reflect "cancer vs. non-cancer" differences, as well as genetic variation between the two cell lines. In HCT116 cells, 4846 genes were altered by SFN treatment ("SFN effect in cancer"), compared with 1691 genes in CCD841 cells ("SFN effect in non-transformed cells", Appendix Fig. B.1B). The distribution and fold-changes of DEGs following incubation with SFN revealed a larger spread in HCT116 cells than in CCD841 cells (Appendix Fig. B.1C, D). Thus, not only were more genes altered in HCT116 cells, but the DEGs were changed by a larger fold-difference after SFN treatment.

Hierarchical clustering segregated between vehicle- and SFN-treated CCD841 and HCT116 cell lines (Fig. 4.1A). KEGG analysis showed enrichment of cancer-related pathways, including upregulation of p53 signaling and downregulation of Wnt/β-catenin signaling in SFN-treated HCT116 cells (Fig. 4.1B,C). Cell cycle targets included

upregulated G_2/M-related genes and downregulated G_1/S-related genes, consistent with the reported role of SFN in G_2/M arrest[102]. As anticipated, multiple Nrf2 target genes were upregulated in HCT116 cells following SFN treatment (Appendix Fig. B.2). However, among the genes most highly *reversed* by SFN treatment, eleven that were under-expressed in HCT116 cells compared with CCD841 cells were strongly upregulated by SFN, and fifty-six constitutively overexpressed genes in HCT116 cells compared with CCD841 cells were markedly downregulated by SFN (Appendix Fig. B.3). Notably, Loc344887 (*NMRAL2P*) was detected at low constitutive levels in HCT116 cells and was the most dramatically induced target of SFN (Appendix Fig. B.3 and Figs. 4.1D,E).

Under identical SFN treatment conditions, *NMRAL2P* was induced more significantly in colon cancer cells than in non-transformed colonic epithelial cells (p<0.001), namely, 1.65-fold in CCD841 cells, 7.8-fold in Caco2 cells, 12.6-fold in HT29 cells, and 35-fold in HCT116 cells (Fig. 4.1F). Mining of The Cancer Genome Atlas (TCGA) (cancergenome.nih.gov) revealed that human colorectal cancers expressed significantly lower *NMRAL2P* levels than the corresponding normal tissues from patients, earmarking *NMRAL2P* as a potential new tumor suppressor biomarker (Fig. 4.1G, p<0.001).
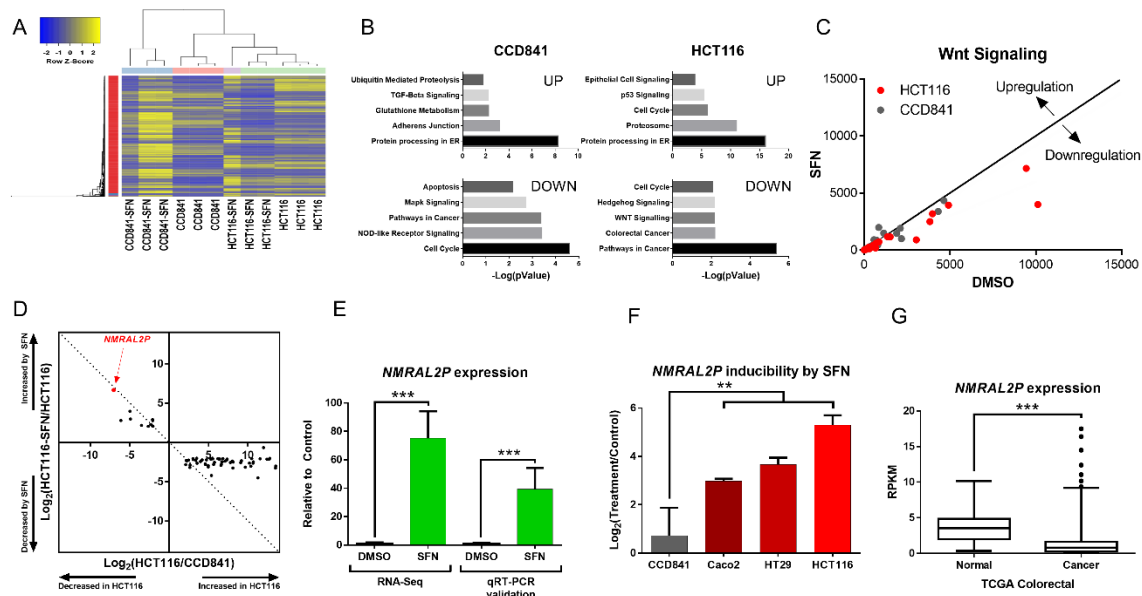
Figure 4.1 Identification of *NMRAL2P* as a novel target of SFN.
(A) Heatmap of differentially expressed genes (DEGs) showed distinct clustering of HCT116 vs. CCD841 cells, in the presence and absence of SFN treatment. Each column represents a separate biological replicate for RNA-seq analysis. (B) Top five cancer-related pathways significantly upregulated and downregulated for each cell line ($p < 0.05$). (C) DEGs associated with the Wnt signaling pathway, with each point designating the RPKM of a gene after treatment with SFN vs. vehicle control. The diagonal line represents a fold-change of zero. (D) *Loc344887*, renamed as *NMRAL2P* (HUGO Gene Nomenclature Committee, HGNC ID 52332), was identified as the most highly altered transcript in SFN-treated HCT116 cells (see also Appendix Fig. B.3). (E) qRT-PCR validation of *NMRAL2P* inducibility by SFN in HCT116 cells; mean±SD, n=3 (***$p < 0.001$), representative data from an experiment repeated three times. (F) *NMRAL2P* induction was significantly greater in Caco2, HT29, and HCT116 colon cancer cells than in non-transformed CCD841 cells treated with SFN; mean±SD, n=3 (*$p < 0.05$), from an experiment repeated three times. (G) Data from The Cancer Genome Atlas (TCGA) revealed significant *NMRAL2P* downregulation in human colorectal cancers (n=380) compared with normal colon (n=50, ***$p < 0.001$).

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4.4.2. *NMRAL2P* Silencing Protects Colon Cancer Cells from SFN-Mediated Inhibition of Cell Growth, Colony Formation, and Migration

An sgRNA with complementary sequence to the *NMRAL2P* promoter (see NMRAL2P[upstream1], Appendix Table B.1) was used to target Cas9 protein to the corresponding genomic region, seeking to disrupt transcription initiation. Screening of the genomic DNA of individual colonies, using PCR primers flanking exon 1 of *NMRAL2P,* identified a clone with a 390-bp deletion (Appendix Fig. B.4, clone 5). Sequencing confirmed that the deletion was localized to the promoter region of *NMRAL2P* (Fig. 4.2A). The corresponding *NMRAL2P* knockout cells had similar overall growth and viability characteristics as the vector and mock controls (Appendix Fig. B.5). No *NMRAL2P* expression was detected in the knockout cells before or after SFN treatment, in contrast to the parental HCT116 line under the same experimental conditions (Fig. 4.2B). In the MTT assay, *NMRAL2P* knockout cells were significantly less responsive than vector controls to low concentrations of SFN that attenuated cell viability (Fig. 4.2C). Inhibition in the colony formation assay at 7.5 μM SFN was partially rescued by *NMRAL2P* silencing (Fig. 4.2D), although this was not observed at 15 μM SFN, a concentration known to trigger autophagy and apoptosis in HCT116 cells[59,102]. A similar trend was noted in the transwell assay, with the inhibition of cell migration by SFN being partially rescued in *NMRAL2P* knockout cells incubated with 7.5 μM SFN (Fig. 4.2E, p<0.01).
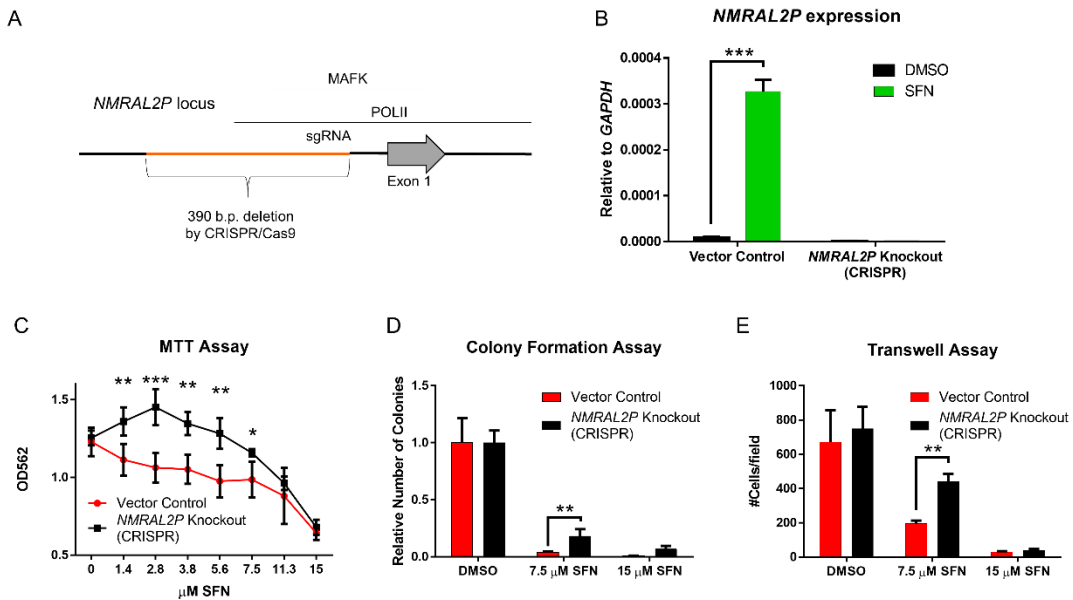
Figure 4.2 Phenotypic changes in colon cancer cells after *NMRAL2P* silencing.
(A) In HCT116 cells, CRISPR/Cas9 genome-editing was used to delete a 390-base pair region in the *NMRAL2P* promoter (orange), interfering with RNA polymerase II and Mafk interactions, based on ENCODE Chip-seq data (ENCODE Project Consortium 2012). (B) Removal of the target sequence introduced the expected 390-bp deletion (see Appendix Fig. B.4) and completely abrogated *NMRAL2P* inducibility by SFN; mean±SD, n=3 (***p<0.001), from an experiment repeated three times. (C) Enhanced viability of cells lacking *NMRAL2P* expression, 24 h after SFN treatment; actual absorbance readings at 562 nm in the MTT assay. (D) Colony formation and (E) transwell assays with *NMRAL2P* knockout and vector control cells. Data for colony formation are indicative of two independent experiments, whereas three separate experiments were conducted for MTT and transwell assays. In (C)-(E), data = mean±SD, n=3; *<0.05, **p<0.01, ***p<0.001.

4.4.3. *NMRAL2P* is Regulated Directly by Nrf2 in Response to SFN Treatment

In time-course experiments, *NMRAL2P* was upregulated within 1 h of SFN treatment and peaked at 8 h, similar to the well-known Nrf2 target gene *HMOX1* and ahead of a second Nrf2-regulated gene, *NQO1* (Fig. 4.3A). Several SFN analogs (AITC, 6-SFN, 9-SFN), Nrf2 activators (Oltipraz, TBHQ), and pan-HDAC inhibitors (TSA, SAHA, VPA, sodium butyrate) were compared as inducers of *NMRAL2P* expression. Surprisingly, pan-

HDAC inhibitors either had no effect or reduced the expression of *NMRAL2P* at 6 and 24 h*,* whereas SFN analogs and Nrf2 activators induced the target gene, implicating a role for Nrf2 (Fig. 4.3B).

The repressive partner of Nrf2, Keap1, was knocked down via siRNA-mediated inhibition, which resulted in highly significant induction of *NMRAL2P*, consistent with *NMRAL2P* activation by Nrf2 (Fig. 4.3C). In the reverse scenario, siRNA-mediated knockdown of Nrf2 interfered with the ability of SFN to activate *NMRAL2P* (Fig. 4.3D). Reduced expression of the corresponding siRNA targets, Keap1 and Nrf2, was confirmed both at the mRNA and protein level in these experiments (Fig. 4.3E).

In ChIP assays, three putative AREs were interrogated on the *NMRAL2P* locus (Fig. 4.3F). Upon SFN treatment, Nrf2 and its transcriptional coactivator partner, Mafk, were co-localized to ARE2, upstream of exon 2, and Mafk also interacted with ARE1, upstream of exon 1 (Fig. 4.3F). Neither Nrf2 nor Mafk were detected on ARE3, upstream of exon 4. As additional controls for the ChIP assays, Nrf2 and Mafk interactions were confirmed on *HMOX1* and *NQO1* (Fig. 4.3F). These findings supported the hypothesis that, like *HMOX1* and *NQO1*, *NMRAL2P* was a direct transcriptional target of Nrf2.
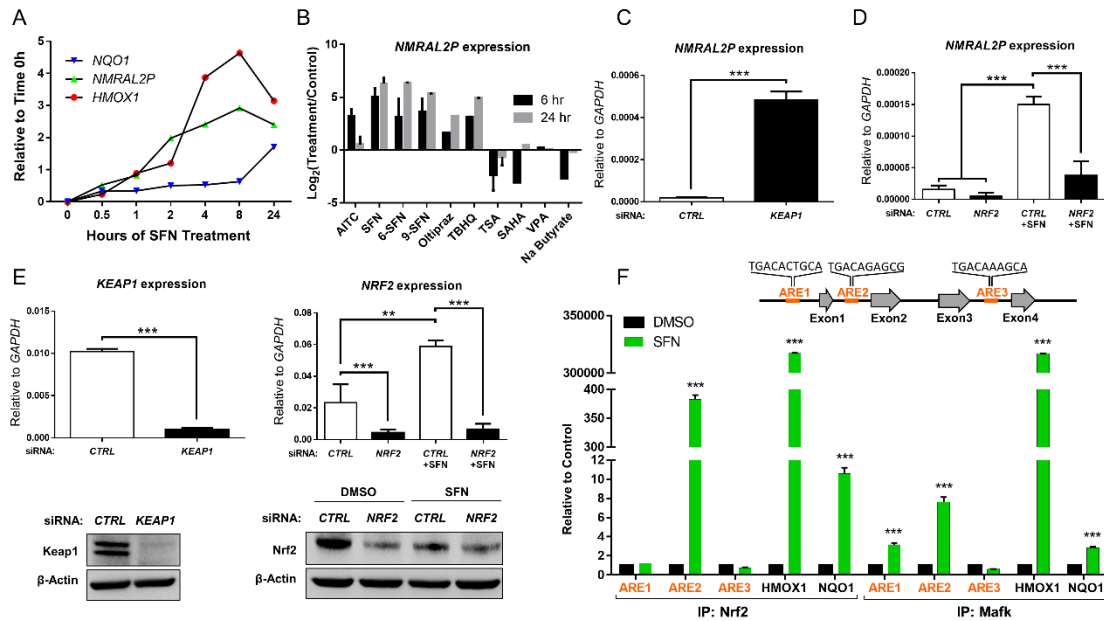
Figure 4.3 *NMRAL2P* is directly regulated by Nrf2.
(A) Time-course for induction by SFN of *NMRAL2P*, *HMOX1,* and *NQO1*. (B) Changes in *NMRAL2P* expression in HCT116 cells treated with SFN and its analogs, Nrf2 activators, and pan-HDAC inhibitors at 6 and 24 h. (C) Induction of *NMRAL2P* following siRNA-mediated knockdown of Keap1, a negative regulator of Nrf2. (D) siRNA-mediated knockdown of Nrf2 interferes with *NMRAL2P* induction by SFN. (E) Confirmation by qRT-PCR and immunoblotting of siRNA targets from experiments shown in panels (C) and (D). (F) Chromatin immunoprecipitation (ChIP) assays using antibodies to Nrf2 and Mafk, with PCR primers recognizing known AREs in *HMOX1* and *NQO1* as positive controls. Three putative AREs were interrogated on *NMRAL2P* (ARE1, ARE2, ARE3). In panels (A)-(F), data = mean±SD from two or more independent experiments; **p<0.01, ***p<0.001.

NMRAL2P, both in the nuclear and cytosolic compartments (Fig. 4.4A, green bars). Future studies should seek to corroborate these findings, for example using fluorescence *in situ* hybridization, but we elected to focus on the nuclear aspects related to gene regulation.

From the time-course experiments (Fig. 4.3A), it was postulated that early induction of *NMRAL2P* might serve as an upstream regulator of *NQO1*. To test this hypothesis, siRNAs were targeted to two different regions of the *NMRAL2P* transcript (siRNAs #1 and #2), which resulted in significantly reduced *NMRAL2P* induction following SFN treatment (Fig. 4.4B). In these experiments, *NQO1* mRNA and protein induction by SFN was attenuated significantly compared with the siRNA controls (Fig. 4.4C,D). Keap1 knockdown also was used to induce Nrf2; following siRNA-mediated silencing of *NMRAL2P,* a significant reduction was observed in the inducibility of both *NMRAL2P* (Fig. 4.4E) and *NQO1* (Fig. 4.4F,G). Similar results were obtained in colon cancer cells lacking *NMRAL2P* expression due to CRISPR/Cas9 genome editing, with the inducibility of *NQO1* being attenuated significantly after treatment with SFN (Fig. 4.4H).

Finally, no effect was seen on *NMRAL1* after *NMRAL2P* knockdown or SFN treatment (Appendix Fig. B.6), indicating that the siRNAs and PCR primers were specific for the functional pseudogene, rather than the protein-coding gene sharing 62% homology.

4.5. Discussion

Plasma SFN metabolites have been detected at 2 μM in people consuming broccoli sprouts[246], and chemopreventive outcomes in a mouse model of intestinal tumorigenesis were associated with tissues levels in the gastrointestinal tract of ~3-30 μM total SFN[247]. Although lower SFN concentrations might be considered in future RNA-seq experiments, minimizing apoptosis end-points[59,102], we sought to parallel the prior transcriptome
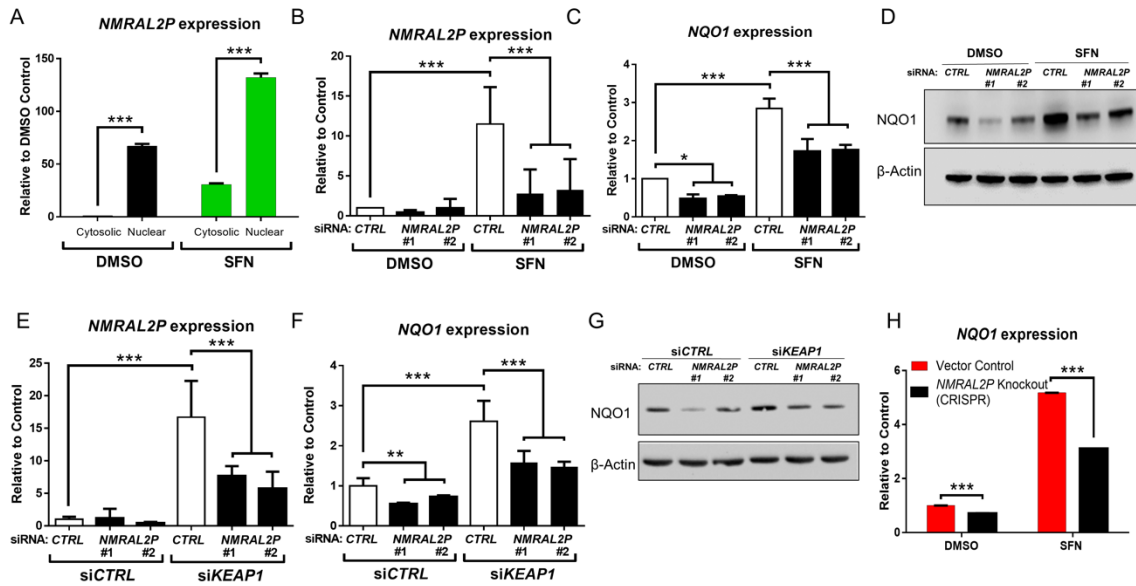
Figure 4.4 *NMRAL2P* is a non-coding RNA that influences *NQO1* expression.
(A) Higher expression of *NMRAL2P* in the nuclear vs. cytoplasmic compartment, before and after SFN treatment in HCT116 cells. (B,C) siRNAs targeting two different regions of *NMRAL2P* (siRNA #1 and #2) interfered with both *NMRAL2P* and *NQO1* inducibility after SFN treatment. (D) Immunoblotting confirmed that NQO1 protein induction was abrogated in cells treated with *NMRAL2P* siRNAs. (E) *NMRAL2P* and (F) *NQO1* expression following partial knockdown of both *NMRAL2P* and *KEAP1*. (G) Immunoblotting of NQO1 protein expression from the double knockdown experiments shown in panels (E) and (F). (H) *NQO1* inducibility by SFN is attenuated in cells lacking *NMRAL2P* due to CRISPR/Cas9-mediated genome editing of the promoter region (see Fig 4.2A). In panels (A), (B), (C), (E), (F) and (H), data = mean±SD from two or more independent experiments; n=3; *p<0.05, **p<0.01, ***p<0.001.

profiling in prostate cancer cells treated with 15 μM SFN, which implicated multiple cancer-related pathways[243]. In the current investigation of colon cancer cells treated with 15 μM SFN, multiple cancer-related pathways also were implicated, including cell cycle, hedgehog signaling, p53 signaling, Wnt signaling, colorectal cancer, and protein processing in ER (Fig 4.1B). Several Nrf2-dependent targets were upregulated by SFN in colon cancer cells (Appendix Fig. B.2). However, our attention was drawn to Loc344887

(*NMRAL2P*) as a transcript with low constitutive levels in colon cancer cells that was dramatically induced by SFN (Appendix Fig. B.3). Importantly, this ncRNA was identified as significantly downregulated in human colorectal cancer (Fig. 4.1G), suggesting a possible tumor suppressor function in the colon, and the potential to serve as a clinical biomarker in patients at risk of developing malignancy of the large intestine.

We observed that *NMRAL2P*, but not the protein-coding gene *NMRAL1*, was strongly induced by SFN in colon cancer cells, and corroborated the presence of *bona fide* Nrf2/Mafk binding sites in the corresponding genomic region of *NMRAL2P* (Fig. 4.3F). The ChIP assays focused on Nrf2 and Mafk, but we cannot rule out contributions from other MAF family members, or associated coactivators. Knockdown experiments that targeted either Keap1 or Nrf2 further supported the role of Nrf2 in regulating *NMRAL2P*, and loss of *NMRAL2P* implicated the ncRNA as a downstream activator of *NQO1*. We noted, however, that *NQO1* inducibility was partially retained in SFN-treated colon cancer cells even after *NMRAL2P* silencing via siRNA treatment or CRISPR/Cas9 genome-editing (Figs 4.4C and 4.4H). This suggests that *NMRAL2P* probably cooperates with other factors in regulating *NQO1* gene activity.

This is the first report to identify a functional pseudogene that is both a direct transcriptional target of Nrf2, and a downstream regulator of Nrf2-dependent *NQO1* induction. Polymorphisms in *NQO1* have been linked to increased risk for human colorectal cancer[248], and an anticancer role for *NQO1* also has been identified in preclinical models. For example, in a rat colon carcinogenesis model, Oltipraz treatment resulted in reduced colonic aberrant crypt foci and tumor formation associated with *NQO1*

induction, whereas *NQO1* knockout mice were more susceptible to radiation-induced myeloproliferative disease[249–251]. Thus, a change in *NQO1* activity has potential implications for cancer susceptibility in the colon and in other tissues.

It is unlikely, however, that *NMRAL2P* serves as a master regulator of all Nrf2-dependent target genes. This point is perhaps best exemplified by the well-known Nrf2-dependent gene *HMOX1*. Thus, *HMOX1* was induced rapidly by SFN (Fig. 4.3A), but *HMOX1,* like *NFE2L2* (the gene coding for Nrf2), was unaffected by *NMRAL2P* knockdown (Appendix Fig. B.7). Prior reports noted that *HMOX1* was upregulated at an earlier time-point than other Nrf2-dependent target genes, including *NQO1*[252,253], and implicated multiple factors in the dynamic regulation of *HMOX1*[227,254]. For example, Bach1 can inhibit *HMOX1* induction by antagonizing Nrf2 binding[254], whereas the SWI/SNF chromatin remodeling factor BRG1 interacts with Nrf2 to selectively induce *HMOX1*[227]. TET-dependent DNA methylation changes and post-translational modifications to Nrf2 also influence Keap1/Nrf2 interactions, and the extent of Nrf2 nuclear-cytoplasmic trafficking[224,226,255,256]. These mechanisms would likely dictate the degree to which Nrf2, and perhaps Mafk, interact with *NMRAL2P* in the nuclear compartment.

Several colon cancer-related lncRNAs have emerged as potential prognostic biomarkers[23,41,257]. Mechanisms that have been implicated include lncRNA-miRNA associations, lncRNA-protein interactions, and actions as miRNA precursors or pseudogenes[36,83]. Pseudogene *PTENP1* can serve as a miRNA "decoy" for the protein-coding gene phosphatase and tensin homolog (*PTEN*)[258], mutations in high-mobility

group-1 pseudogenes (e.g., *HMGA1P6* and *HMGA1P7*) alter their decoy functions that regulate *HMGA1*[33], and pseudogene *MYLKP1* regulates the mRNA stability of smooth muscle myosin light chain kinase, altering cell proliferation in cancer cells[34]. Considering the influence of SFN in regulating intracellular redox status, it is tempting to speculate on the role(s) of *NMRAL2P* as a functional pseudogene. One intriguing possibility centers on the emerging evidence for functional short peptides (sPEPs) encoded by minimal open reading frames[31]. Allowing for start codons besides ATG, the *NMRAL2P* transcript has several putative open reading frames for sPEPs; one of the hypothetical candidates based on conceptual translation, hCG2041270, was listed in prior studies with TBHQ[259]. Thus, we do not formally discriminate here between possible ncRNA and/or sPEP roles of *NMRAL2P*. Given the diverse actions of Nrf2 in cancer etiology[99,220,222,260], we conclude that further studies are warranted on *NMRAL2P*/Nrf2 crosstalk and the associated direct versus indirect mechanisms of gene regulation (Fig 4.5).

4.6. Acknowledgements
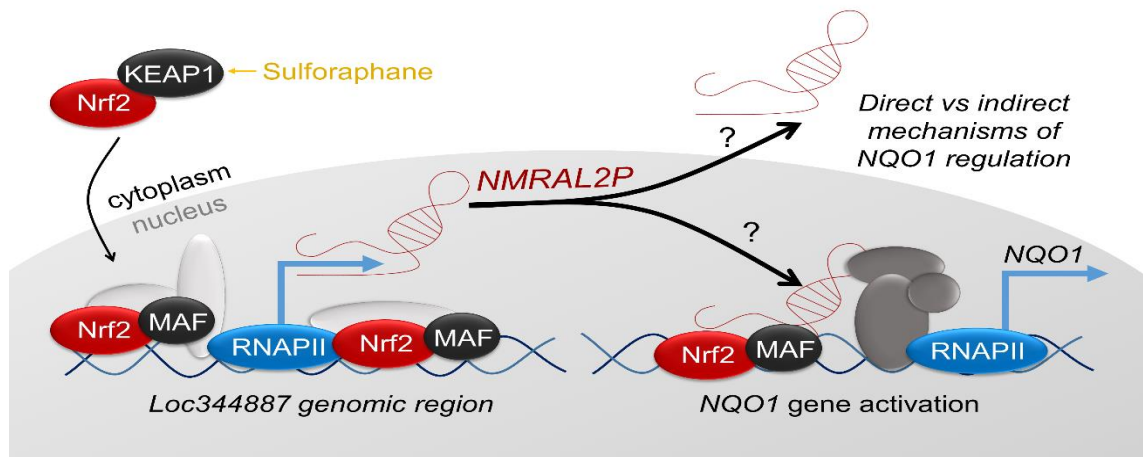
Figure 4.5 Working model for the induction of *NMRAL2P*, and its role as a downstream coactivator of *NQO1* in SFN-treated colon cancer cells.

5. CONCLUSIONS AND DISCUSSION

5.1. Conclusions

There is much interest in targeting epigenetic mechanisms for the treatment and prevention of cancer. Sulforaphane (SFN) has been shown to affect different aspects of epigenetic control of gene expression, such as DNA methylation, post-translational modifications (protein acetylation/phosphorylation), and non-coding RNA regulation, all of which contribute to its anti-cancer effects[102,228,261,262]. This dissertation sought to expand the understanding of epigenetic mechanisms regulated by SFN. This work began in 2012, inspired by the newly discovered class of functional long non-coding RNAs(lncRNA)[35,76]. Once viewed as transcriptional 'noise', lncRNAs have important functions in gene regulation and chromatin assembly. Using RNA sequencing, ~30 novel lncRNAs were identified as being dysregulated in CRC and normalized by SFN treatment. The focus was directed towards *Loc344887,* one of the most upregulated transcripts by SFN. This thesis work identified *Loc344887* as more than a basic lncRNA, and rather as a novel, *functional pseudogene* that acts via the Nrf2 pathway. In discussion with the Hugo Gene Nomenclature Committee (HGNC), *Loc344887* was renamed to *NmrA Like Redox Sensor 2, Pseudogene (NMRAL2P),* due to its homology with the protein coding gene, *NMRAL1*.

The dissertation work also clarified, for the first time, SFN mechanisms involving deacetylase inhibition and *acetylation of a non-histone protein,* Cell Cycle and Apoptosis Regulator 2 (CCAR2). Traditional Lys to Arg mutations were initially employed to modify and study specific CCAR2 acetylation sites. Next, this thesis adapted, for the first

time, a genetically-encoded acetylation system for histones to generate 'designed' acetylation sites on CCAR2 (see below). Concurrently, we became interested in combined HDAC+BET inhibition, based on a publication by Mazur et al[178]. In pancreatic ductal adenocarcinoma, they showed synergistic effects by combining SAHA and JQ1, an HDACi and BETi, respectively. The thesis thus adopted a similar approach in CRC, for the HDAC3 inhibitor SFN and BETi JQ1.

5.1.1. CCAR2 Acetylation

SFN, an anticancer agent from the human diet, has been shown to preferentially target HDAC3 for inhibition and turnover in human colon cancer cells, via dissociation of the HDAC3/SMRT corepressor complex[102]. However, direct targeting of HDAC3 was implicated in the rapid and early acetylation of CCAR2, a non-histone protein, at three specific lysine residues, two in the N-terminus and one in the C-terminus. A literature search sought to understand the known functions of CCAR2, and how those roles might be impacted by novel SFN-induced acetylation sites. CCAR2 is considered to be a highly flexible protein, and serves as an 'adaptor' molecule that can activate or repress its binding partners. According to clinical outcome correlations and cell-based experiments, CCAR2 shows both a tumor suppressor and promoter role, depending on its binding partners, cancer type, mutational status, and post-translational modifications on CCAR2 itself (Chapter 2). A major conclusion from the thesis is that CCAR2 acts mainly as an oncogenic factor in CRC. This was supported by IHC experiments that associate high CCAR2 expression with poor regression-free survival, poor overall survival, increased tumor grade/stage, and metastatic status[106,159]. The molecular mechanisms behind these

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clinical associations are likely multifactorial, but a major contributor is that CCAR2 is a co-activator of β-Catenin/Wnt signaling[106], a powerful oncogenic driver commonly dysregulated in CRC.

We hypothesized that CCAR2 acetylation might affect CCAR2 binding with its protein partners. Traditionally, in order to study specific acetylation sites, researchers employ the mutation of lysine to un-acetylatable arginine, or to a glutamine acetylation 'mimic'. This method is suboptimal, as arginine and glutamine do not faithfully recapitulate the function of an un-acetylated or acetylated lysine, respectively[61]. Recently, a novel, cutting-edge technology was created that can genetically-encode histone proteins with acetylations at specific lysine residues[62]. This technology was adapted, for the first time, to genetically-encode acetylation on a non-histone protein, CCAR2. The successful outcome of this assay provided empirical evidence that SFN-induced Lys54 acetylation was sufficient to disrupt CCAR2/β-Catenin interactions, especially in the nucleus. This resulted in downregulation of multiple β-Catenin/Wnt target genes. In general, direct targeting of β-Catenin in the clinical setting has proven problematic, for example in patients with genetic or sporadic CRC, but this thesis supports an alternative strategy involving interference with Wnt *coactivators*, such as CCAR2.

We used a protein acetyl 'reader' array to identify bromodomains that recognize the three SFN-induced acetylation sites (M.T. Bedford, manuscript in preparation) (Table 5.1) Lys54 was not recognized by any bromodomain readers, supporting its role as a β-Catenin 'evictor', rather than a 'reader' mark. Lys97 was read by ASH1L and BAZ1A,

and Lys916 by ASH1L, BRD2, BRD3, and BRD9, implicating these acetylation sites as 'reader' marks.

Table 5.1 Acetyl Domain Reader Array

Acetyl Domain	Accession #	Acetyl Domain	Accession #
BROMO		**BROMO**	
ASH1L	Q9NR48	TAF1L	Q8IZX4
ATAD2A	NP_054828.2	MLL1	Q03164
ATAD2B	Q9ULI0	PBRM1/PB1	Q86U86
BAZ1A	Q9NRL2	PHIP	Q8WWQ0
BAZ2A/BMZF-2	Q9UIF9	KIAA2026	Q5HYC2
BAZ2B	Q9UIF8	GCN5/KAT2A	Q92830
CECR2	Q9BXF3	PCAF/KAT2B	Q92831
BRD1	O95696	**BROMO/PHD**	
BRD2	P25440	CBP/CREBBP	Q92793
BRD3	Q15059	FALZ/BPTF	Q12830
BRD4	O60885	EP300	Q09472
BRD7	Q9NPI1	TRIM24	O15164
BRD8	Q9H0E9	TRIM33/TIF1G	Q9UPN9
BRD9	Q9H8M2	TRIM66	O15016
BRDT	Q58F21	DPF3	Q92784
BRPF1/Peregrin	P55201	**BROMO/PHD/PWWP**	
BRPF3	Q9ULD4	ZMYND11	Q15326
BRWD1/WDR9	Q9NSI6	**YEATS**	
BRWD3	Q6RI45	YEATS4/GAS41	NP_006521.1
SMARCA2	P51531	ENL/MLLT1	NP_005925
SMARCA4	P51532	AF9/MLLT3	NP_004520.2
TAF1 (1)	P21675	YEATS2	NP_060493

Next, we examined combination effects of SFN with the bromodomain inhibitor JQ1. In a xenograft model, JQ1+SFN combination slowed tumor growth significantly when CCAR2 was present. Importantly, when CCAR2 was knocked out, via CRISPR/Cas9 genome editing, JQ1+SFN treatment had no effect on tumor growth,

indicating that CCAR2 is an important and necessary target for these combination agents. In CRC cells, JQ1+SFN treatment markedly downregulates β-Catenin/Wnt target genes.

We expanded the latter combination strategy into the Apc-mutant polyposis in rat colon (Pirc) model of FAP, showing that JQ1+SFN significantly reduced tumor growth in the GI tract. In follow-up RNA-seq and bioinformatics analyses, BRD9 is implicated rather than BET-regulated genes in the colon polyps. Interestingly, accessing online ChIP –seq data identified BRD9 on the promoter of genes that were specifically differentially expressed in the SFN+JQ1 combination treatment. This led to the hypothesis that Lys916 acetylation may be 'read' by BRD2/BRD3/BRDT as well as BRD9 (a non-BET protein), but during JQ1+SFN combination treatment, JQ1 inhibits BET members, but not BRD9, allowing BRD9 to preferentially bind to Lys916 without competition from BET members. Thus, an acetyl reader pool 'switch' is envisioned (Fig. 5.1)

This thesis project also showed, for the first time, that CCAR2 interacts with DNA under endogenous conditions, as exemplified by ChIP assays on the *MYC* promoter. JQ+SFN combination treatment acted synergistically, and caused CCAR2 to dissociate from *MYC* promoter and super enhancer regions.
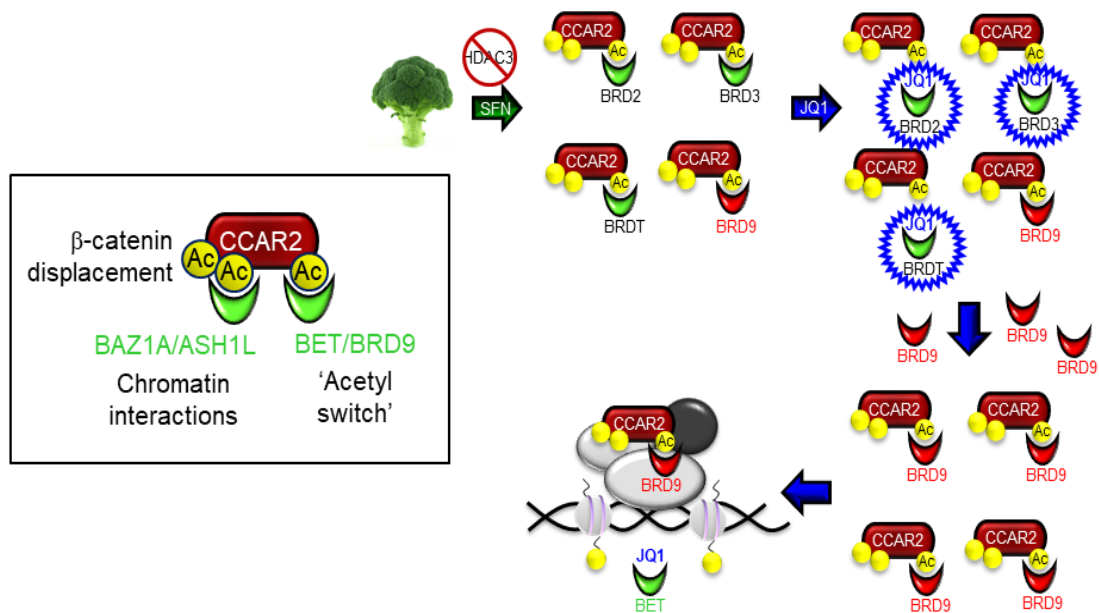
Figure 5.1 Working Model for the BET/BRD9 reader switch caused by JQ1+SFN combination treatment.

5.1.2. *NMRAL2P*

In SFN-treated CRC cells, the most highly upregulated transcript corresponded to the pseudogene *NMRAL2P,* which before this work had no known function. Using knockout and knockdown experiments, evidence was generated supporting *NMRAL2P* as a tumor suppressor. Interestingly, *NMRAL2P* was identified as a direct target gene of Nrf2, with *bona fide* Nrf2/Maf binding sites localized in the promoter, while also acting downstream to co-activate a well-established target of Nrf2 signaling, *NQO1.* Knockout experiments revealed that *NQO1* was attenuated but was still expressed in the absence of *NMRAL2P.* This indicates that while *NMRAL2P* is a co-activator of *NQO1,* it is not absolutely essential for *NQO1* expression. Additionally, other Nrf2 target genes, such as

HMOX1, were not affected by depletion of *NMRAL2P*, highlighting the complexity of the Nrf2 signaling network.

5.2. Discussion

Typically, cancer-related genes are classified as either oncogenes or tumor suppressors, depending on function[14]. However, there is increasing evidence that many genes can act as *both* promoter and suppressor, depending on the context[15]. For example, Nrf2 has been described as having a 'friend or foe' role in cancer chemoprevention[99]. This paradoxical activity between suppression and promoter is called "antagonistic functional duality", and such genes are dubbed "gene-chameleons."[15] The function of a gene-chameleon is determined by the genetic network present in the cell. According to Heng, Stevens, and Stepanenko, "The overall changes in karyotype may result in alterations of the role and function of the same genes and pathways within different genetic backgrounds."[15,263–266]. Although this thesis work has identified CCAR2 as a tumor promoter in CRC, based on its role as a β-Catenin/Tcf co-factor, it is likely a gene-chameleon in other tumor tissues, for example when Wnt signaling is less critical. In contrast, *NMRAL2P* has tumor suppressive roles in CRC (Chapter 3), and oncogenic effects in gallbladder[267] and lung[268], but more studies are required to further characterize the underlying mechanisms.

Targeting a gene-chameleon, such as CCAR2, presents potential benefits and difficulties. With one target, there is the ability to inactivate an oncogenic pathway, while activating a tumor suppressor function, which would produce a beneficial double 'hit' to the cancer. CCAR2 is known to interact with and activate tumor suppressor proteins such

as p53[119]. Thus, the 'friend/foe' read-out might depend critically on the mutation status of p53, and whether WT or mutant p53 subsequently exert beneficial or deleterious outcomes. Just as Lys54 acetylation interferes with β-Catenin interactions, other PTMs at Lys112 and Lys215 of CCAR2 displace Sirt1 and influence the role of CCAR2 as a regulator of p53 function[140]. Ideally, we would be able to target CCAR2 in such a way that β-Catenin interactions are disrupted, but p53 interactions are enhanced. This could be accomplished by altering specifically the acetyltransferases and deacetylases that regulated the corresponding PTM on CCAR2.

One major concern for targeting these genes is the potential for off target effects in other tissues[15]. For example, if we target the oncogenic aspects of CCAR2 in the colon, we may inadvertently disrupt suppressive effects in the pancreas. These difficulties could be subverted by targeting specific tissues, or by altering more selectively protein-protein interactions or PTMs and by focusing on selected patient subpopulations, e.g. high risk FAP patients. In each of these cases, it is imperative to understand the underlying context in which CCAR2 acts as a suppressor or promoter. With advances in proteomics, transcriptomics, and tumor microarrays, it is becoming easier for researchers to determine the 'friend or foe' role of CCAR2 in various tissue types.

In order to study protein-protein interactions of CCAR2 and its binding partners, this thesis work utilized two techniques: immunoprecipitation followed by immunoblotting (IP-IB) and proximity ligation assay (PLA). These methods each have their pros and cons, but both provide a level of confidence regarding endogenous protein-protein interactions, without the necessity for overexpression of tagged proteins. IP-IB is

a relatively easy and robust method for detecting protein complexes. Because the IP-IB procedure lyses cells, some detected interactions may artificially form after the proteins are released from their cellular compartments and can 'contaminate' other subcellular localizations. This effect can be minimized by using sub-cellular fractionation, although this introduces additional technical steps. On the other hand, PLA relies on the close proximity (<40 nm) of two fixed epitopes in their native form[201]. This allows the localization of interactions to be determined, without worrying about sub-cellular contamination. For CCAR2, this technique was instrumental in first determining that CCAR2/β-Catenin interactions are shifted to the cytosol following SFN treatment. PLA is limited by whether anti-mouse *and* anti-rabbit high quality antibodies are available for the targets of interest. PLA also can be technically challenging, relying on high-magnification and high-throughput microscopy. However, advances in image acquisition technology and image analysis software are minimizing these challenges.

One hurdle is that both IP-IB and PLA reveal relatively few interactions at one time. In the age of '-omics', it is now possible to use IP followed by mass spectrometry to study the entire 'interactome' of CCAR2 and its presumed binding partners. Recently, Giguere et al. published that CCAR2 interacts with ~3000 different proteins in untreated HEK 293 and CEM T cells[117]. Using bioinformatics pathway analysis software, they reported that most interactions could be categorized into roles related to gene regulation, chromatin organization/modification, and cell cycle control. Additionally, they identified novel interactions that related to cancer progression, although these interactions need to be further validated. This method creates great potential in being able to understand how

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the binding partners of CCAR2 affect its promotional or suppressive effects in various cancer types. It also allows the investigation of how targeting compounds, such as SFN, can dynamically regulate many interactions at the same time. Unfortunately, these interactome experiments can be costly and technically challenging. Identifying true interacting proteins can be difficult, due to contaminants that interact with the solid-phase support, affinity reagents or epitope tags[269]. Potential 'hits' need to be validated using other methods such as IP-IB. Also, interactome experiments are prone to false positives when artificially overexpressing the 'bait' protein to levels that are not found endogenously in the cell. New techniques are being developed to circumvent this problem. For example, Liu et al. developed a method called CRISPR affinity purification *in situ* of regulator elements (CAPTURE)[270]. CAPTURE utilizes a biotinylated dCAS9 and a sgRNA specific to the genome area of interest. Streptavidin is used to pulldown the dCas9 and simultaneously pulls out the endogenous proteins, DNAs, and RNAs that are associated with that gene region. Proteomics can be used to study the interactome of regulatory proteins at their endogenous location. Furthermore, long-range DNA interactions and chromatin-associated RNAs can be analyzed[270].

Interactome experiments, as well as –omic experiments in general, can be bioinformatically challenging, producing a large amount of data that may be difficult to interpret[271]. Using pathway analysis software can be useful in understanding this complex data, but the pathways may be incomplete and are only as trustworthy as the validation experiments that were used to produce them[272].

Animal models are an underutilized tool in studying the antagonistic duality of CCAR2. Currently, there are two CCAR2 knockout mice models, exhibiting disparate cancer outcomes. In one model, mice develop spontaneous lymphoma, teratoma, lung tumors, and liver tumors late in life, while the other model had no tumor phenotype. In order to study the role of CCAR2 in specific tissues, future studies might cross the CCAR2 knockout mouse with animals predisposed to specific tumor types, or treat knockout mice with tissue specific carcinogens. For example, knockout animals could be crossed with the APC$^{Min/+}$ mouse, or treated with standard chemical carcinogens such as AOM or PhIP.

This thesis work took place during the beginning of the CRISPR technology revolution[184,185,245]. This technology was crucial for studying the effects of SFN in CRC etiology, and will continue to be a valuable tool in the future. As early as 2013, we utilized CRISPR-Cas9 genome editing to either knockout CCAR2, or to delete Nrf2 binding sites in the *NMRAL2P* promoter region, each approach providing useful mechanistic leads in human colon cancer cells. Future studies might utilize the homologous recombination capability to create acetylation mutants (Lys to Arg, or Lys to TAG stop) *in vivo*, at the endogenous gene locus. This would more closely mimic the effect of SFN-induced acetylation, rather than producing CCAR2 from an exogenous transcript. CRISPR will facilitate the generation of gene-specific knockouts and mutations in animal models. CRISPR could be used to knockout CCAR2 in the Pirc rat in order to evaluate the role of CCAR2 in colon tumorigenesis. This rat model would more accurately model CCAR2-related *colon* tumorigenesis than the APC$^{min/+}$ mouse, which mainly develops small intestine tumors.

5.2.1. CCAR2 Acetylation

In this thesis project, efforts were directed towards characterizing the function of the Lys54 acetylation site in CCAR2, and implicated a β-Catenin 'eviction' role, rather than an acetyl 'reader' function *per se.* By acetylating CCAR2, we demonstrated an approach towards disruption CCAR2/β-Catenin interactions and down regulating Wnt signaling. This is a proof-of-principle which can be utilized to develop better inhibitors with more potent effects.

Recently, it has been shown that HDAC+BET inhibition can act synergistically in treating pancreas cancer[178]. Subsequent reports supported this 'combination epigenetic therapeutics' idea in lymphoma, melanoma and rhabdomyosarcoma[273–275]. This is promising, as it may provide a way to circumvent resistance mechanisms of mono-agents, such as JQ1. At the outset, HDAC+BET inhibition might seem counterintuitive, using one drug to enhance protein acetylation and another to block the very 'acetyl reader' functions that were generated. HDAC+BET inhibition has been proposed to target distinct or cross-talking pathways, such as MYC and p57[178], or AKT and Hippo signaling[273].

In this thesis, a novel 'acetyl switch' and a shift in the pool of acetyl readers was identified, for the first time, and provided insight into the synergy that arises from HDAC+BET inhibition. Thus, a non-histone protein, CCAR2, was defined as having a Lys916 acetyl site that set up competition between BET and other BRD bromodomain readers, extending the current paradigm beyond chromatin modification toward non-chromatin protein complexes. SFN, via HDAC3 inhibition and turnover, facilitated CCAR2 acetylation, and bromodomain arrays and pull-down assays strongly supported

bromodomain protein binding at Lys97 and Lys916 of CCAR2. Lys97 was shown to bind to ASH1L and BAZ1A, while Lys916 binds to BRD9, ASH1L, and the BET family members, BRD2, BRD3 and BRDT. We hypothesize that JQ1, a BET specific inhibitor, causes an 'acetyl reader switch,' shifting the Lys916 reader pool from BET family members (BRD2/BRD3/BRDT to BRD9. The increased BRD9/CCAR2 complexes would enhancer the corresponding cadre of BRD9-regulated genes, possibly via new protein-protein interactions that are dependent on acetyl functionalities on CCAR2 and other non-histone protein, acting via gene expression or other roles (e.g. DNA repair). The idea of an 'acetyl reader switch' mechanism is relatively unexplored. There has been a lot of interest in developing specific inhibitors to bromodomain proteins[49,81,276,277], but most of these studies only study the effects on the bromodomain of interest, ignoring how other bromodomain proteins may 'fill in' to the vacated acetylation sites. When cells are treated with a BETi, the acetylation marks that were previously 'read' by BET proteins are free to interact with other bromodomain readers, which may carry out different functional effects. For example, Histone H3 Lysine 74 can be read by both BRD2 and ASH1L[278]. If cells are treated with a BRD2 inhibitor, then ASH1L would have preferential binding on H3K74ac. This thesis has opened new avenues to explore in the future, while clarifying some of the questions pertaining to the synergy that arises from combined deacetylase plus bromodomain inhibition.

5.2.2. Experimental Approaches – Future Perspectives

Hypotheses proposed here can be tested by using IP, ChIP, or the CAPTURE methodology noted above. Using published ChIP data, we found that BRD9 is enriched

on the promoters of differentially expressed genes specific to JQ1+SFN combination. ChIP-seq experiments are needed to further show how BRD2/BRD3, and BRD9 occupancy acetyl 'switches' on these promoters during JQ1+SFN treatment. Using CAPTURE, specific promoters can be pulled down in the context of 3D chromatin interactions, and the ratio of BET to BRD9 can be compared before and after BET inhibition.

Prior to this study, CCAR2 had never been ChIPed and there was no information about its genomic occupancy. We found that CCAR2 binds to *MYC* promoter and super enhancer regions. In the future, ChIP-seq experiments for CCAR2 could identify other target genes, and potential new mechanisms by which CCAR2 acts as a 'master regulator' of metabolism, aging, and cancer[103]. ChIP-seq would performed under endogenous conditions and during JQ1+SFN treatment. Additionally, ChIP-seq using the genetically encoded acetylation will shed light on the effect of acetylation on CCAR2/DNA binding.

Acetylation is an important mechanism of protein regulation[54,279,280]. Most acetylation sites in the proteome have no known function. In order to study CCAR2 acetylation, we utilized a method of genetically encoding lysine acetylation[62]. This approach utilizes an engineered tRNA synthetase that loads an Nε-Acetyl-Lysine onto a $tRNA_{CUA}$[62,63]. When these components are present in the cell, the ribosome uses the $tRNA_{CUA\,to}$ read through a TAG stop codon that has been mutated at the site of interest. As noted above, we did not invent this exciting and cutting-edge technology, but the thesis evolved naturally toward its successful adoption for CCAR2, a non-histone protein. More generally, by demonstrating the possibility to apply this methodology to non-histone

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proteins, the current dissertation project should encourage others to follow suit, with other protein acetylation targets that may be of interest.

In addition to faithfully recapitulating protein acetylation, another benefit of the genetically encoded acetylation system is that it provides for site-specific acetylation without the use agents that might exert more pleiotropic, less discrete actions on target proteins. Individual acetylation sites, for example on CCAR2, can be studied under endogenous conditions, or in combination with a bromodomain inhibitor.

This genetically-encoded acetylation system theoretically can be applied to any post-translational modification, assuming a tRNA synthetase can be engineered accordingly onto a tRNA$_{CUA}$[281]. Specialized tRNA synthetases have been made for phospho-serine[282,283], -tyrosine[284], and –threonine[285], and a similar methodology has been developed for lysine di-methylation[286]. Thus, there is much potential in future research using the genetically-encoded system; in PhosphoSitePlus, CCAR2 has >80 different PTM sites, including acetylation, phosphorylation and ubiquitination[43]. Utilizing and developing this method will help advance our understanding of the roles of various PTMs.

Because this system uses read-through of a TAG stop codon, one common concern is its effect on other TAG stop codons in the cell. The TAG stop codon was selected because it is relatively rare compared to the TAA and TGA stop codons. In bacteria, where this technology was first developed, the TAG codon is used in less than 1% of all coding sequencing[287]. In *E.coli*, Lajoie et al[288] replaced all TAG stop codons with a TAA codon to eliminate the off target effects of this system. The TAG codon makes up about 20% of all stop codons in mammalian cells, and is not as easily dispensed with as in

bacteria[289]. This is the rarest stop codon, but a potential exists nonetheless for off target effects. In this case, careful controls will need to be utilized when validating the acetylation system[62].

One known imperfection of the genetically-encoded acetylation system is the inability to express certain residues, thought to be due to the inability of the ribosome to run through certain nucleic acid sequences surrounding the TAG stop codon. We were unable to express CCAR2 acetylated at Lys97, despite stably engineering cells with the corresponding constructs, and using ribosomal inhibitors known to increase stop codon read-through rates (Appendix Fig. A.6). It is hypothesized that acetylation at Lys97 is dependent for stability on the adjacent acetylation site of CCAR2 at Lys54. Preliminary data supports this idea, showing expression of the Lys54/Lys97 double acetylation (Fig. 5.2). To our knowledge, this is the first time that the genetically-encoded system has been used to generate paired acetylation sites on a target protein. This highlights the concept of crosstalk among PTMs, which can act in concert to affect regulatory systems in cells, perhaps best exemplified by the acetyl/methyl 'marks' on histone tails. Ideally, it would be feasible to generate and study the Lys54/Lys97/Lys916 triple acetylation sites, in order to fully understand the role of SFN-induced acetylation of CCAR2, for example, after flexibility of the CCAR2 protein and its associated protein partners at either end.
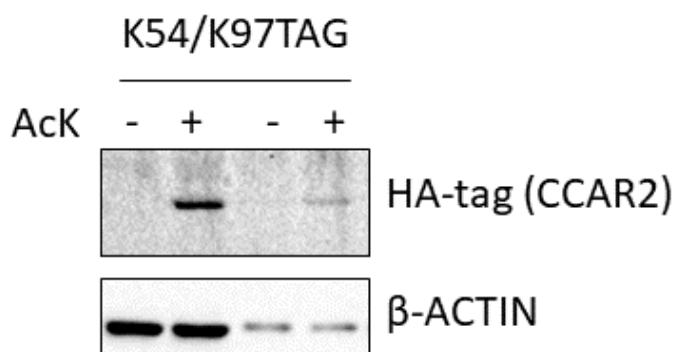
Figure 5.2 Genetically encoded acetylation in CCAR2 K54/K97TAG double mutant
HA-Tag antibody shows expression of acetylated CCAR2 in AcK treated cells in two different clones of HCT116 CCAR2 KO cells.

This thesis also highlighted future perspectives for other deacetylase and bromodomain inhibitors. Would the mechanisms proposed hold true with HDAC3-specific inhibitors? This would help to circumvent off-target 'pleiotropic' effects of dietary agents, such as SFN. Also, due to resistance related concerns, improvements of JQ1 are being proposed and tested[204]. One such interesting improvement involves proteolytic targeting via chimera agents (PROTACs), which link JQ1 to a ubiquitin ligase, and promotes the degradation of bromodomain proteins, such as BRD4[290]. These 'BET degraders' offer improvements over JQ1, but they have yet to be validated adequately in preclinical models or in patients.

5.2.3. *NMRAL2P* and Future Perspectives

This thesis identified *NMRAL2P* as a novel, functional pseudogene that is an Nrf2-regulated target and a co-activator of *NQO1*, a tumor suppressor linked to cancer risk outcomes in preclinical and clinical studies of CRC[248–251].

'Pseudogenes' are non-coding transcripts that share a high degree of homology with a protein coding gene. These transcripts derive from protein coding genes that are duplicated and lose their protein coding potential through a frame shift or premature stop codon[291]. These were once thought of as transcriptional 'junk' or replicative errors, but recently, some pseudogenes have been found to play a role in gene regulation[292] and cell proliferation[34]. Additionally, pseudogenes can act as miRNA 'sponges' and can be processed into endogenous miRNAs[291]. Because these share a high homology with their parent gene, it is common for the pseudogene to regulate its homologous coding gene. This appears not to be the case for *NMRAL2P*, as knockout/knockdown of *NMRAL2P* did not affect the levels of the homologous protein-coding counterpart, *NMRAL1*.

The standard Nrf2 paradigm is that upon oxidative stress, Nrf2 is released from Keap1 in the cytoplasm and translocates to the nucleus where it binds to antioxidant response elements (AREs) on the DNA and induces antioxidant response genes. The current thesis work supports the idea that this view is too simplistic, as Nrf2 may dynamically regulate different genes, utilizing a variety of co-activators. For example, recently it was reported that binding to RPA1 switches Nrf2 from an activator to repressor on a set of genes different from the classical ARE genes[293]. Another example, BRG1, a

SWI/SNF chromatin remodeling factor, can interact with Nrf2 to selectively induce *HMOX1,* but not other Nrf2 target genes[227].

NMRAL2P is directly transcriptionally regulated by Nrf2 and acts as a co-activator of a fellow Nrf2 target, *NQO1.* Future studies are needed to examine the mechanism by which *NQO1,* but not *HMOX1* or other Nrf2 targets*,* is co-activated by *NMRAL2P.* For example, *NMRAL2P* may serve as a 'scaffold' for Nrf2 and its protein partners on the promoter of *NQO1* but not HMOX1. This can be accomplished using RNA immunoprecipitation, pulling down *NMRAL2P* and identifying its potential binding partners, such as Nrf2 or Maf proteins. Additionally, Chromatin Isolation by RNA Purification (ChIRP) can be used to identify the potential promoters or enhancers that are regulated by *NMRAL2P*[294,295].

This thesis was the first to define a function of the pseudogene *NMRAL2P.* Since publication in 2017, our published report has been cited by other researchers in the field[267,296–298]. One study showed that *NMRAL2P* is amplified in gallbladder cancer and promotes epithelial to mesenchymal transition, an oncogenic effect[267]. In lung cancer, high *NMRAL2P* is associated with advanced stage, poor differentiation, and lymph node metastasis[268]. These results are opposite to the suppressor effects of *NMRAL2P* in CRC, again highlighting that *NMRAL2P* may be a gene chameleon[15]. This duality is not particularly surprising, as *NMRAL2P* is an Nrf2-regulated gene, and Nrf2 itself has been shown to have dual effects, acting as a 'friend of foe' depending on the circumstances[99]. Other studies that have cited this thesis work have validated that *NMRAL2P* is indeed regulated by Nrf2 in other cancer types[298]. They have also discussed the importance of

Nrf2 signaling networks, and support the idea that Nrf2 target genes can be dynamically

regulated by various co-activators[297].
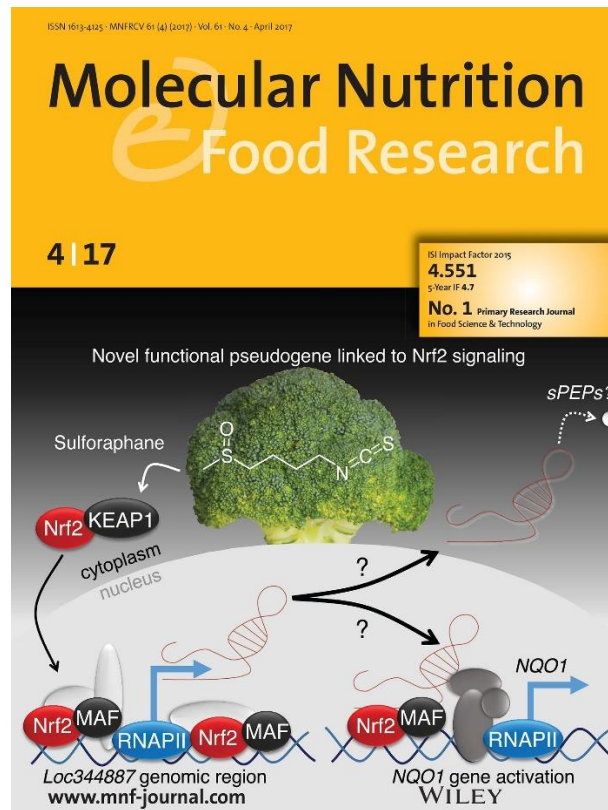
Figure 5.3 Cover Image of Molecular Nutrition and Food Research featuring the *NMRAL2P* thesis work[202].

5.2.4. Summary

The studies in this dissertation help to understand the mechanisms of SFN in colorectal

cancer prevention. They also elucidate the function of CCAR2 acetylation and *NMRAL2P*

induction in response to SFN. In clinical studies, CCAR2 acetylation could be a potential

biomarker of HDAC3 inhibition or SFN consumption. Likewise, *NMRAL2P* induction could be a biomarker of Nrf2 activation or SFN consumption.

Both of these genes were found to be putative 'gene chameleons' playing a dual role in tumor promotion and suppression (Fig. 5.3). Although a direct mechanistic link between *NMRAL2P* and CCAR2 acetylation was not evident from the current thesis work, this possibility warrants future investigation. CCAR2 interactions with lncRNAs have not yet been investigated, but it is feasible that CCAR2 function could also rely on protein-RNA interactions, as well as protein-protein interactions.
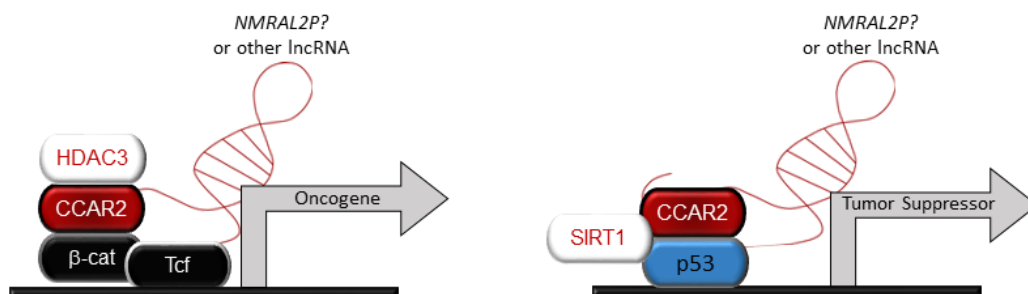
Figure 5.4 CCAR2 and *NMRAL2P* have oncogenic or tumor suppressive function, depending on the gene promoter being regulated.

Due to its multiple binding interactions, CCAR2 has been called a master regulator of metabolism, aging and cancer[103] and plays a role in diverse pathways, such as immune function[172], splicing[173], and DNA damage repair[143]. While these studies were in CRC, our studies on CCAR2 demonstrate the importance of acetylation in regulating its protein-

protein interaction. These may have implications on the function of CCAR2 in other cancer types, obesity, aging, or immunotherapy.

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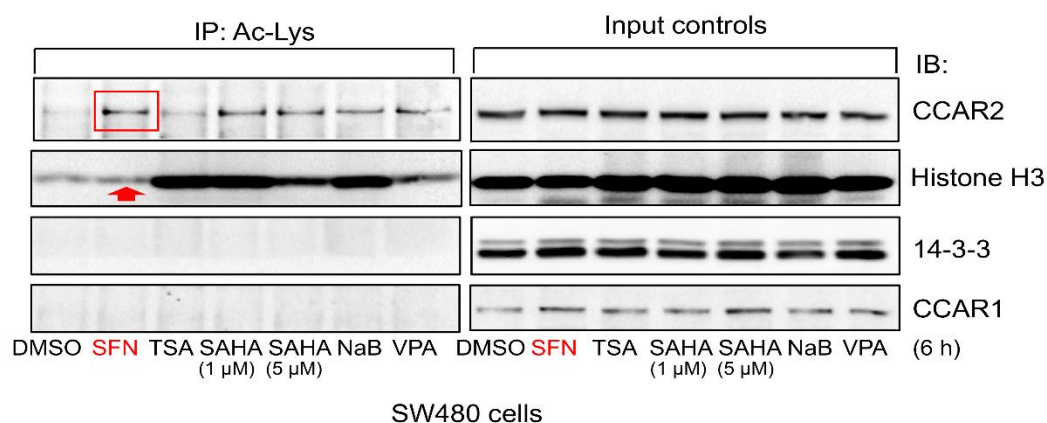
Figure A.1 CCAR2 acetylation in SW480 colon cancer cells treated with SFN and other deacetylase inhibitors.
Following incubation with the test agents for 6 h, as described in HCT116 cells (Fig. 3.1*A*), cell lysates were subjected to IP with Ac-Lys antibody and then IB for targets of interest.
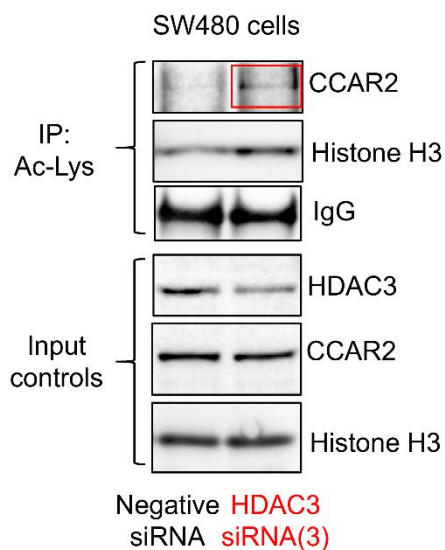

Figure A.2 CCAR2 acetylation in SW480 cells after HDAC3 Knockdown
SW480 cells were treated with Negative control siRNA or HDAC3 siRNA(3), and cell lysates were subjected to Ac-Lys IP followed by IB, as indicated.

Figure A.3 Amino acid sequence of CCAR2, and peptide maps of novel acetylation sites conserved among human rat and mouse, from protein mass spectrometry studies in SFN-treated colon cancer cells.
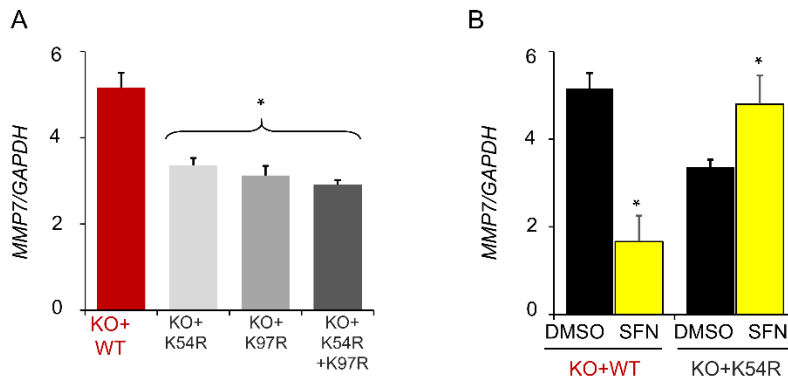

Figure A.4 CCAR2 acetylation mutants were less effective than WT CCAR2 at increasing *MMP7* expression.
WT CCAR2 (red) or the acetylation mutants K54R, K97R, or K54R/K97R (gray) were transiently transfected into CCAR2 null HCT116 cells for 24 h followed by detection of *MMP7* by RT-qPCR. **B,** WT CCAR2 or the K54R acetylation mutant was transiently transfected into CCAR2 null HCT116 cells, followed by treatment with SFN or vehicle (DMSO) for 24 h. *MMP7* expression was determined by RT-qPCR (*P<0.05, mean±SD, n=3).
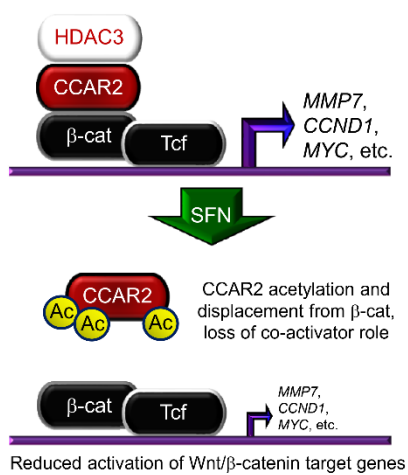
Figure A.5 Working model that integrates HDAC3 inhibition/turnover by SFN, increased CCAR2 acetylation, displacement of CCAR2 from β-Catenin, and reduced Wnt/β-Catenin gene activation.
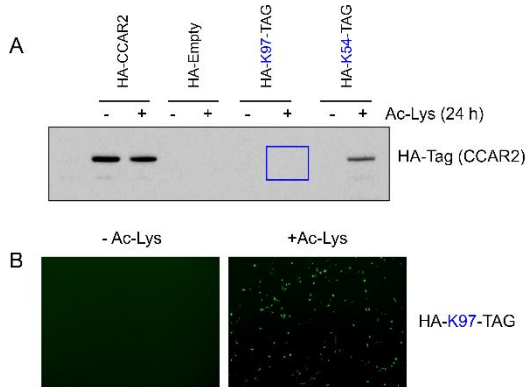

Figure A.6 Genetically encoding K54 and K97 of CCAR2.
A, IB of HA-tagged CCAR2 in HCT116 colon cancer cells containing stably transfected HA-K54-TAG or HA-K97-TAG. Upon addition of Ac-Lys, a band was detected for CCAR2-K54, implicating inducible acetylation at the Lys 54 target site (right lane), whereas no such band was detected for CCAR2-K97 (blue box). **B,** Dendra2 expression (green dots) in HA-K97-TAG-containing HCT116 cells treated with Ac-Lys, indicating that the requisite components of the assay were functioning according to the reported methodology[62].
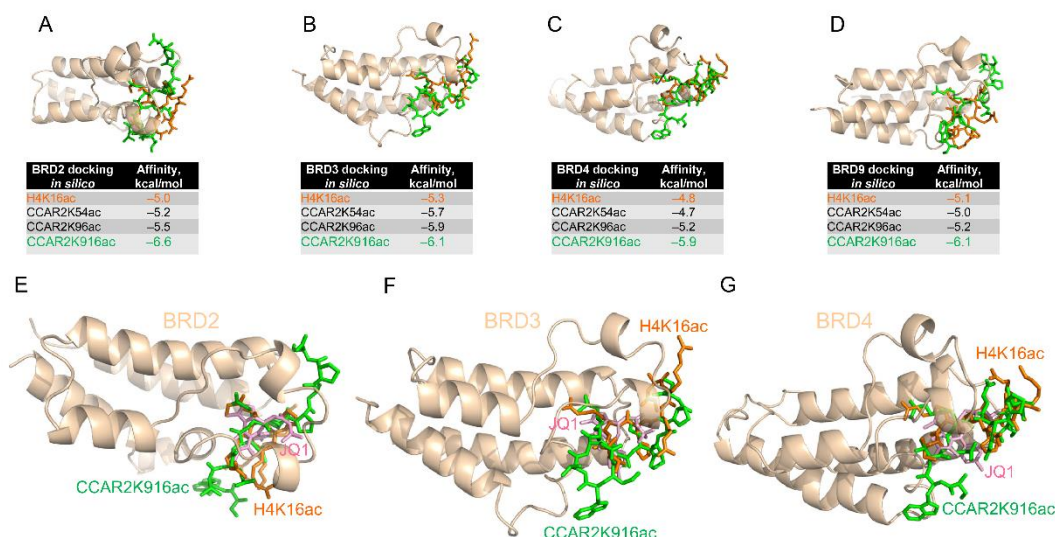
135

Figure A.7 Docking studies *in silico*
A, BRD2; **B,** BRD3; **C,** BRD4; and **D,** BRD9 were predicted to interact favorably with CCAR2K916ac peptide (in green) compared with acetylated histone H4K16 (in orange). Docking scores (kcal/mol) were assessed as outlined in Methods. **E-G,** CCAR2K916ac peptide in relation to H4K16ac and JQ1 binding to BRD2, BRD3, and BRD4, respectively.
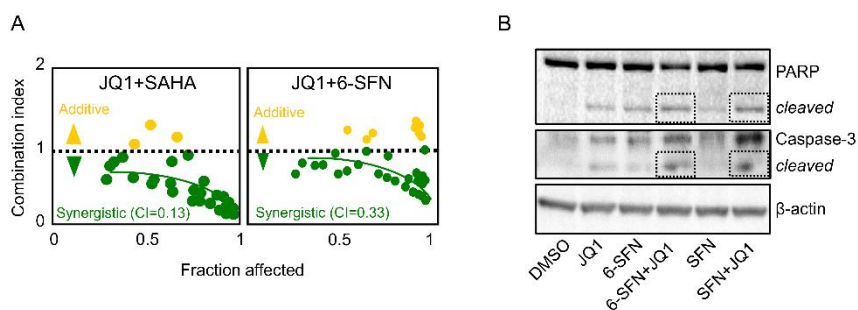

Figure A.8 JQ1 combination with isothiocyanates.
A, Combination index (CI) data for JQ1+SAHA and JQ1+6-SFN in HCT116 human colon cancer cells, determined using CompuSyn software (CompuSyn, Inc.). For the corresponding data on JQ1+SFN, refer to Fig. 3.4*C*. **B,** HCT116 cells were treated with test agents, and 24 h later cell lysates were subjected to IB for PARP, Caspase-3, and β-actin.
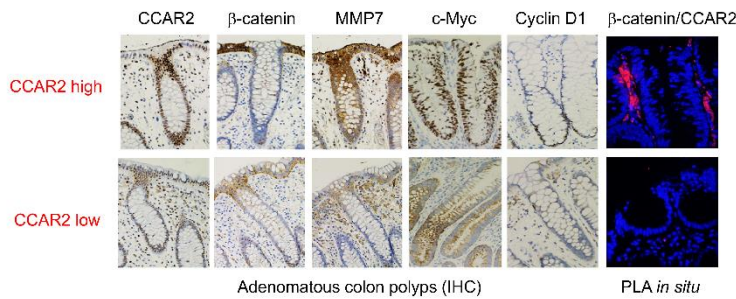
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Figure A.9 CCAR2/β-Catenin interactions in human adenomatous colon polyps.
IHC was used to stratify archived tissues from a screening colonoscopy trial (12) into 'CCAR2 high' and 'CCAR2 low' groups, along with the corresponding expression of β-catenin, MMP7, c-Myc and cyclin D1. β-Catenin/CCAR2 interactions in the same tissue sections were detected using PLA *in situ* (36,37). The images shown are representative findings from five or more separate tissue sections.
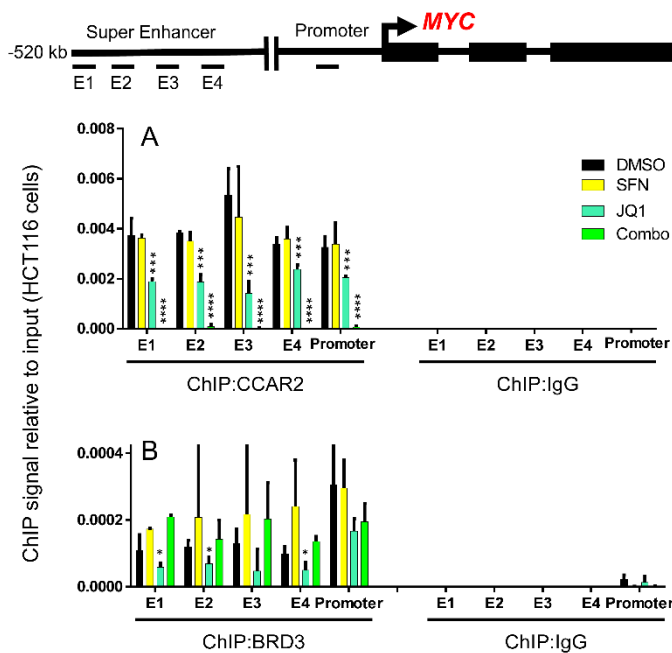

Figure A.10 The combination of SFN+JQ1 decreases CCAR2 interactions on *MYC* promoter and superenhancer regions.
Primers were designed around Promoter and Superenhancer regions, E1-E4, as illustrated in the figure (top). **A,** CCAR2 and **B,** BRD3 interactions on *MYC* were interrogated using ChIP assays in HCT116 colon cancer cells. Non-specific IgG served as a negative control. Data = mean±SD, and are representative of the findings from two independent experiments; *P<0.05, ***P<0.001, ****P<0.0001.

APPENDIX B

SUPPLEMENTARY TABLE AND FIGURES FOR SECTION 4

Table B.1 Comprehensive listing of primers used for qRT-PCR, ChIP, sgRNA and siRNA experiments.

| qRT-PCR primers	Forward	Reverse
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
HMOX1	CTTCTTCACCTTCCCCAACA	GCTCTGGTCCTTGGTGTCAT
KEAP1	GGCCAAGCAAGAGGAGTTCT	GGGGTTGTAACAGTCCAGGG
MALAT1	GGATCCTAGACCAGCATGCC	AAAGGTTACCATAAGTAAGTTCCAGAAAA
NMRAL1	AGAAGTAGTGCAGGGAGACCA	CTCAGGAGTCATCTTGGCATCG
NMRAL2P	CTGGAGAACGTCAAGCGACT	GGGATGCCAATGGACCAGAA
NQO1	AAAGGACCCTTCCGGAGTAA	AGGCTGCTTGGAGCAAAATA
NRF2	GCTTGGAGGCTCATCTCACA	TGCCATCTCTTGTTTGCTGC
ChIP primers		
ARE1	GCTGTGACTGTGACATTGCA	GCCTTTTATTGTGCCCCTCC
ARE2	AGACCACCGTGAAACCCG	CCCATTTCAAAGTCTTTCACAGT
ARE3	CCACAGCACACTCTAGCC	CACGTTGGCCAGGCATCC
HMOX1	CCCTGCTGAGTAATCCTTTCCCGA	ATGTCCCGACTCCAGACTCCA
NQO1	CCCTTTTAGCCTTGGCACGAAA	TGCACCCAGGGAAGTGTGTTGTAT
sgRNA sequence		
NMRAL2P [upstream1]	GTAAGTGGACTAGTGGTAAC	
NMRAL2P [screening primers]	TTGGGTCTTCAGGCCAACTG	CCCACACTTGCAGGCTCTAA
siRNA sequences		
siNMRAL2P #1	GCCAAUAUGUGUCGUUUCU	
siNMRAL2P #2	GUAUCUCUGUUGCUGAUAU	
siNRF2	CAAACAGAAUGGUCCUAAA	
siKEAP1	GUGUUACGACCCAGAUACA	

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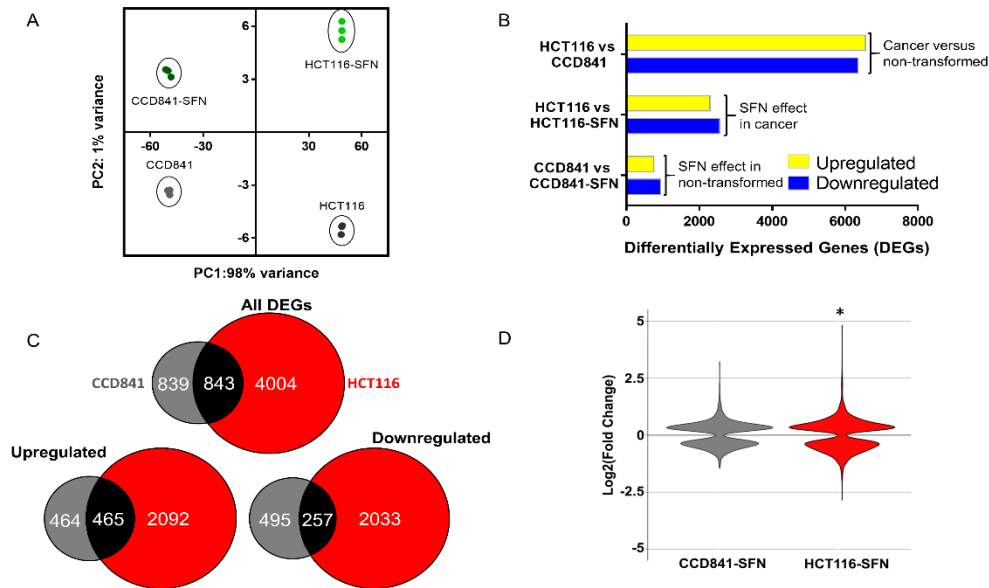
Figure B.1 Transcriptome analyses in SFN-treated human colonic epithelial cells.
RNA-seq was performed in HCT116 colon cancer cells and CCD841 non-transformed colonic epithelial cells incubated with 15 μ**M** SFN or DMSO vehicle for 6 h. (A) Principle component analysis and (B) number of differentially expressed genes (DEGs) under the treatment conditions employed. (C) Venn diagrams illustrating the number of overlapping DEGs in CCD841 and HCT116 cells. (D) Violin plot showing the distribution of fold changes caused by SFN treatment in each cell line (*p<0.05).
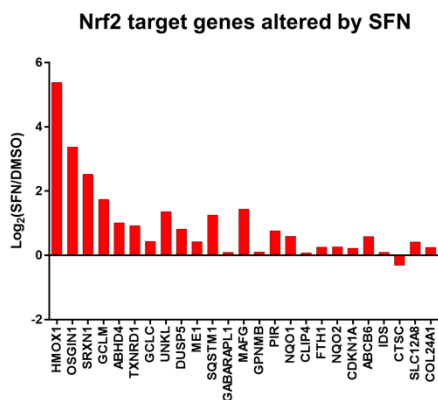

Figure B.2 RNA-seq analyses in SFN-treated colon cancer cells identified the induction of multiple Nrf2-regulated genes.

Figure B.3 RNA-seq prioritized gene targets that were either constitutively under or over expressed in colon cancer cells, compared with CCD841, and reversed by SFN treatment.

Loc344887 (NMRAL2P) was identified as the most highly upregulated transcript of SFN.

Figure B.4 CRISPR/Cas9 genome-editing was used to delete a 390-bp region of the NMRAL2P promoter, disrupting PolII-mediated transcription initiation.

PCR primers flanking exon 1 of *NMRAL2P* were used to screen genomic DNA of individual colonies, and identified a clone containing a 390-bp deletion (see asterisk). Two percent agarose gel stained with ethidium bromide. For PCR primer sequences, see Appendix Table B.1.
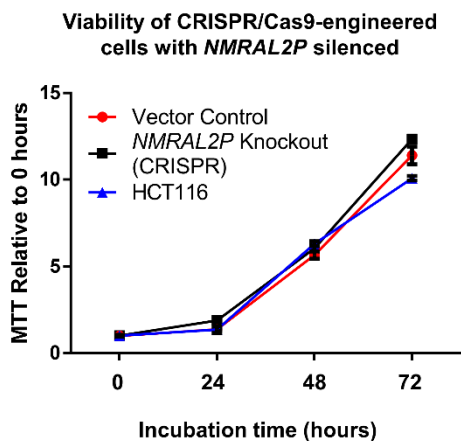
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Figure B.5 No change in the overall growth rate or viability of *NMRAL2P* knockout cells compared to vector or mock controls (parental HCT116 cells)
Data indicate mean±SD, n=3 replicates, from an experiment that was repeated three times. All cells were plated at 1×10^4 cells per well.
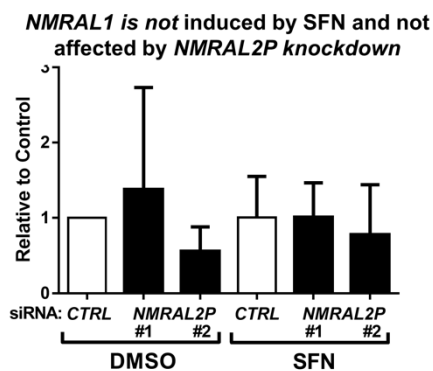

Figure B.6 *NMRAL1* was unaffected by *NMRAL2P* knockdown or SFN treatment.
Data indicate mean±SD, n=3 replicates, from an experiment that was repeated three times. The siRNA conditions were exactly as shown in Fig. 4.4B.

HMOX1 and **NFE2L2** are unaffected
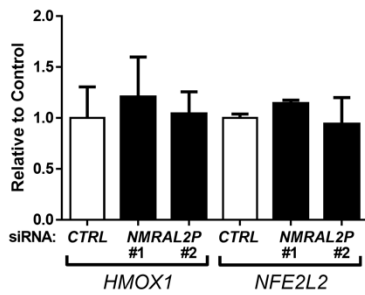by **NMRAL2P** knockdown

**Figure B.7 Silencing of *NMRAL2P* in colon cancer cells had no effect on *HMOX1* or
NFE2L2 gene expression.**
The siRNA conditions were exactly as shown in Fig. 4.4B. Data = mean±SD, n=3, from
an experiment that was repeated twice.