METFORMIN REVERSES ABERRANT CYTOPLASMIC p21^{WAF1} EXPRESSION IN HEPATOCYTES AND PREVENTS HEPATOCELLULAR CARCINOMA DEVELOPMENT IN *Ncoa5*$^{+/-}$ MALE MICE

By

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ABSTRACT

METFORMIN REVERSES ABERRANT CYTOPLASMIC p21^{WAF1} EXPRESSION IN HEPATOCYTES AND PREVENTS HEPATOCELLULAR CARCINOMA DEVELOPMENT IN *Ncoa5*$^{+/-}$ MALE MICE

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Hepatocellular carcinoma (HCC) is a deadly disease with limited systemic therapy options and sharply increasing incidence rates in developed regions of the world. Prevention is essential to reduce HCC mortality rates, however chemopreventive agents are lacking. The first-line, type two diabetes drug, metformin, shows promise as an HCC preventive, but mechanisms of action are not fully understood. We use the *Ncoa5*$^{+/-}$ mouse model of HCC to demonstrate critical gene expression changes in the preneoplastic mouse liver that also correlate with human *NCOA5* expression in HCC and HCC-adjacent tissues. Inflammatory and p53 pathways display upregulated expression in *Ncoa5*$^{+/-}$ liver, as well as cytoplasmic p21^{WAF1}. Long term metformin treatment dramatically reduced tumor incidence in the *Ncoa5*$^{+/-}$ mouse model and reduced aberrant cytoplasmic p21^{WAF1}-positive hepatocytes. We also identified a subgroup of human HCC patients whose HCC-adjacent tissue displayed a distinct pattern of inflammation pathway enrichment and high *CDKN1A* (p21^{WAF1}) gene expression, similar to the preneoplastic *Ncoa5*$^{+/-}$ mouse liver. Gene expression changes elicited by metformin are predicted to reduce p21^{WAF1} liver expression in humans. Our study suggests a molecular mechanism underlying HCC development and uncovers new actions for metformin in the prevention of HCC.

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KEY TO ABBREVIATIONS

ADP – Adenosine diphosphate

AF2 – Activation Function 2

AFP – Alpha fetoprotein

ALT – Alanin amino transferase

AMP – Adenosine monophosphate

ATP – Adenosine triphosphate

BMI – Body mass index

Cdkn1a – Cyclin Dependent Kinase 1a

Cm – centimeter

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

DEN – Diethylnitrosamine

EdgeR – Empiracal Analysis of Digital Gene Expression Data in R

FDR – False Discovery Rate

GADD – Growth Arrest And DNA-Damage-Inducible

GAGE – Generally Applicable Gene Set Enrichment

HBV – Hepatitis B Virus

Hbx – Hepatitis B X gene

HCC – Hepatocellular Carcinoma

HCV – Hepatitis C Virus

IGF – Insulin-like growth factor

IL-6 – Interleukin 6

KEGG – Kyoto Encyclopedia of Genes and Genomes

LIHC – Liver Hepatocellular Carcinoma

MAPK – Mitogen-Activated Protein Kinase

mRNA – Messenger Ribonucleic Acid

MSIGDB – Molecular Signatures Data Base

NAFLD – Non-alcoholic fatty liver disease

NASH – Non-alcoholic steatohepatitis

Ncoa5 – Nuclear Receptor Co-Actvator 5

sNcoa5 – Short Nuclear Receptor Co-Actvator 5

NF-KB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NR – Nuclear Receptor

qRT-PCR – Quantitative Real Time Polymerase Chain Reaction

RNA – Ribonucleic Acid

T2D – Type 2 Diabetes

TNF-α – Tumor Necrosis Factor Alpha

U.S. – United States

WAF1 – Wild-Type P53-Activated Fragment 1

CHAPTER 1 INTRODUCTION

HEPATOCELLULAR CARCINOMA

Hepatocellular Carcinoma (HCC) is a malignant tumor that arises from the parenchymal cells of the liver, the hepatocytes (Mu et al., 2015). HCC makes up about 90% of all primary liver cancer diagnoses. This insidious disease is always found near atop the lists of cancer incidences and ranks among the leading causes of cancer-related deaths on a global scale. Worldwide, approximately 600,000 individuals are diagnosed with HCC each year and, due to poor prognosis, almost as many patients die annually as a result of the disease (ACS, 2017; Ferlay et al., 2015). The five year survival rate for HCC is only 17% in the U.S. and is about 10% globally (ACS, 2017; Altekruse, McGlynn, & Reichman, 2009). Therefore, the rate of HCC-related deaths for a region closely follows local incidence rates.

HCC cases are not evenly distributed among the world population and occur most frequently in second- and third-world countries, where hepatitis viral infection (hepatitis B and hepatitis C virus) is endemic. Indeed, over 80% of HCC incidences occur in sub-Saharan Africa and Eastern Asia. HCC is not equally prevalent among the sexes, with men being 2 to 4 times more likely to acquire the disease. HCC also rarely occurs in young individuals; a diagnosis age of 55-59 years is average in high risk countries, such as China, and 63-65 years in moderate to low risk regions, such as North American and Europe (El-Serag, 2012). Although the incidence rates of HCC are quite low in the developed, western world when compared with global statistics, a disturbing trend is emerging in the United States.

In the U.S., the incidence and death rates of liver cancer have risen dramatically in the last decade, despite evidence showing that cancer death rates on the whole are

declining. The incidence of liver cancer in the U.S. has increased an average of 3.7% annually in men and 3.0% in women, a rate second only to increases observed in thyroid cancer within the country. The mortality trends for HCC are also worse than that of any other cancer; from 2003 to 2012, the death rates increased by 2.8% annually for men and 2.2% annually for women (Ryerson et al., 2016). Chronic hepatitis B and hepatitis C infections remain a major cause of HCC in the U.S., with an estimated 850,000 to 2.2 million people with chronic HBV and 2.7 million individuals with chronic HCV living within U.S. borders (Denniston et al., 2014; Roberts et al., 2016). These infections are most common in individuals born between 1945 and 1965. The highest transmission rates occurred in the 1960s to 1980s, before the public became aware that the hepatitis virus could be transmitted through blood and body fluids, typically occurring through blood transfusions, sex, or the use of contaminated needles (Armstrong et al., 2006). Although chronic hepatitis viral infection remains a major cause of HCC both globally and in the United States, research suggests that the rise of metabolic disorders in the developed West are a great contributor to the rapidly increasing rates of HCC (El-Serag & Kanwal, 2014).

Metabolic disorders are epidemic and are risk factors for HCC. Approximately one-third of individuals in the U.S. are considered to be obese and only a slightly smaller percentage are estimated to have non-alcoholic fatty liver disease (Flegal, Carroll, Kit, & Ogden, 2012; Lazo et al., 2013). Diabetes is also a common metabolic disorder with about 9% of the U.S. population affected according to the American Diabetes Association. Up to 50% of U.S. HCC cases occur in the absence of hepatitis viral infection and do not have a clearly identified etiology (El-Serag, 2007). Metabolic

diseases are now known to be the main contributors to HCC development in the absence of the classic viral risk factors.

Obesity, progressive non-alcoholic fatty liver disease and type 2 diabetes contribute to HCC in the absence of viral infection and are known to further increase risk when comorbid with chronic viral hepatitis (Baffy, Brunt, & Caldwell, 2012; El-Serag & Kanwal, 2014). Multiple, large cohort studies identify the links between metabolic disorders and HCC (Bianchini, Kaaks, & Vainio, 2002; Davila, Morgan, Shaib, McGlynn, & El-Serag, 2005; El-serag, Tran, & Everhart, 2004; Larsson & Wolk, 2007). For example, a prospective study that included 900,000 U.S. adults, men that had a body mass index of greater than 35kg/m^2 were over 4.5 times more likely to die of liver cancer than people with a recommended BMI (18.5-24.9kg/m^2) (Calle, Rodriguez, Walker-Thurmond, & Thun, 2003). NAFLD on its own is not significantly linked with HCC incidence. However, about 20% of NAFLD patients also present with steatohepatitis, a progressive condition that clearly correlates with HCC occurrence (Adams et al., 2005; Baffy, et al., 2012; Ekstedt et al., 2006). The correlation between T2D and HCC is also apparent with diabetic patients having two to three times the risk of acquiring HCC (Davila, et al., 2005; El-serag, et al., 2004). These metabolic disorders are a main contributor to the increases in HCC incidences observed in the United States (El-Serag & Kanwal, 2014). It is important to understand how HCC arises in these backgrounds in order to prevent and treat this disease.

HCC is typically slow to develop and often arises in the context of inflammation and cirrhosis. Risk factors for HCC cause repeated liver injuries and inflammation. A repetitive pattern of hepatocyte damage, inflammation and regeneration leads to fibrosis

and cirrhosis. It also increases probabilities of genetic and epigenetic changes that may drive cell transformation. This process may occur for years or even decades before the occurrence of HCC and the liver cirrhosis that often precludes malignancy may be used to identify at risk individuals. It is typically recommended that these individuals undergo HCC surveillance (Hernaez & El-Serag, 2017).

HCC has a large preventive window, however current strategies are limited. Control of viral hepatitis with vaccination against HBV and antiviral treatment of patients with HBV or HCV will reduce HCC incidence. The only other preventive utilized is surveillance with the hopes of early tumor detection. Surveillance involves ultrasound imaging and measurement of serum alpha-fetoprotein levels every six months (El-Serag, 2011). Even with recommendations, HCC surveillance is underutilized and there is an urgent need to broaden preventive options for patients identified to be at risk for HCC. Upon diagnosis, there is a standard protocol to determine the disease stage and available treatment options.

Diagnosis of HCC is made predominately by imaging technologies, and biopsies are only taken if imaging results are atypical (i.e. due to very small masses) or if masses are identified in a non-cirrhotic liver (Bruix, Reig, & Sherman, 2016; Durand, Belghiti, & Paradis, 2007). The lack of conventional tumor sampling prior to treatment presents a challenge for oncologists with the advent of targeted therapies and this has limited doctors to maintain methods that have existed for decades.

Treatment for HCC is dependent on the stage. The Barcelona Clinic Liver Cancer algorithm produces a staging score based on tumor size, number, vascularity, spread beyond the liver, overall liver function and patient health (J. M. Llovet, Fuster, & Bruix,

2004). This widely used scoring system divides patients into those that have early, intermediate, advanced and end stage HCC. Early stage is a single tumor less than 5cm diameter or up to three nodules each less that 3cm in diameter. Treatment options are tumor resection, liver transplant, or local ablation. Local ablation involves inserting a probe into the tumor and physically destroying tumor cells using high energy radio waves (American Cancer Society). Intermediate stage patients display compensated cirrhosis and have larger tumor burdens but disease remains localized to the liver and there are no symptoms. A process of blocking tumor blood flow and direct administration of chemotherapeutic agents called trans-arterial chemoembolization is the primary treatment option at this stage. If symptoms such as fatigue, abdominal pain, or liver failure occur with HCC diagnosis, or if the tumor has established vascular invasion, or it has already moved beyond the liver, it is characterized as advanced. At this stage the main option is the tyrosine kinase inhibitor Sorafenib that has been shown to extend survival by several months (J. Llovet et al., 2007). End stage HCC presents with intense symptoms and the patient's disease is too far along to benefit from available treatments (Bruix, et al., 2016). HCC treatments are limited and only effective if the malignancy is identified prior to symptoms. This has placed focus on chemoprevention, and researchers have put forth considerable effort to understand how risk factors alter the liver and prime it for HCC development.

A closer look at the liver impacts of hepatitis viral infection and metabolic disorders reveals liver characteristics that promote malignancy. HBV is a partially double stranded DNA virus that binds to receptors on hepatocytes and utilizes cell machinery for replication. HBV is noncytopathic and the liver damage that results from

HBV infection is primarily due to strong inflammatory responses to the virus by the host immune system. In adults, only about 5% of HBV infections become chronic; however, if children are infected, 90% of cases become chronic (Chisari & Ferrari, 1995). Chronic HBV is due to inadequate clearance of the virus, which involves complicated mechanisms (McMahon, 2009). A chronic necroinflammatory liver disease results in repetitive cycles of inflammation, hepatocyte damage and regeneration in the liver, which then leads to fibrosis and cirrhosis in some individuals. Approximately 80% of chronic HBV patients that acquire HCC have liver cirrhosis; however, there is a minority of patients with HBV that develop HCC in the absence of cirrhosis. The HBV genome does integrate into the host hepatocyte genome, which can alter expression or function of genes important to HCC development. It is also known that the virally encoded gene product HBx is oncogenic and can regulate host genes important in growth control, which may also contribute to HCC development (Benn & Schneider, 1994; Blum & Moradpour, 2002; Di Bisceglie, 2009). Unlike HBV, HCV is a single stranded RNA virus that does not integrate into the host genome. HCV progresses to a chronic state in a majority of infected individuals regardless of age and about 10% of these chronically infected individuals will develop cirrhosis in 10 years (Farazi & DePinho, 2006; Rehermann & Nascimbeni, 2005). HCC patients with HCV almost always have advanced liver fibrosis or cirrhosis that has developed over many years. The severity of fibrosis and cirrhosis positively correlates with HCC incidence (Fattovich, Stroffolini, Zagni, & Donato, 2004; Goossens & Hoshida, 2015). As with HBV, HCV viral proteins are also thought to promote hepatocarcinogenesis through the promotion of reactive

oxygen species and also by modulating important growth and immune regulatory pathways in host cells (Hino, Kajino, Umeda, & Arakawa, 2002; Macdonald et al., 2003).

NAFLD that progresses to NASH is a newly identified risk factor for HCC that has a large impact on liver health in the Western world. Simple NAFLD, defined as triglyceride accumulation in the liver, does not significantly correlate with HCC incidence, but about 20% of individuals with NAFLD also display the specific symptoms of NASH (Baffy, et al., 2012; Spengler & Loomba, 2015; Williams et al., 2011) . NASH is defined as a hepatocyte injury due to a buildup of large lipid droplets within the cell, referred to as ballooning degeneration, which occurs with parenchymal inflammation (Brunt et al., 2009; Ekstedt, et al., 2006). NASH is also identified by the presence of fibrosis as liver fibrosis does not occur with mild forms of NAFLD (Ekstedt, et al., 2006). NASH does have potential to progress to liver cirrhosis, although patients with cirrhosis due to NASH display a lower incidence of HCC than patients with cirrhosis caused by chronic HCV infection (Sanyal et al., 2006). However, this may not be an appropriate comparison because evidence is mounting to suggest that a significant number of HCC cases caused by NAFLD/NASH occur in the absence of apparent cirrhosis (Baffy, et al., 2012; Ertle et al., 2011). Regardless of cirrhosis status, the increase in NASH cases in the Western world has made NAFLD that progresses to NASH a top etiology of HCC in the United States and Europe (El-Serag & Kanwal, 2014). With NASH, the liver is kept in a constant state of inflammation and regeneration due to the hepatocyte injury incurred. As with hepatitis viral infection, this injury, inflammation, and regeneration cycle leads cells to acquire mutations, some of which may promote cancer. These mutations are then propagated through the liver during regenerative processes.

NAFLD often occurs concurrently with other metabolic disorders, and is referred to as the hepatic manifestation of metabolic syndrome. Insulin resistance and obesity are the important pathogenic factors that contribute to NAFLD/NASH occurrence. Insulin resistance is a hallmark of T2D and about half of T2D patients have NAFLD (Gupte et al., 2004) Conversely, some level of insulin resistance is present in almost all individuals with NAFLD (Sanyal et al., 2001; Smith & Adams, 2011) .

Obesity also positively correlates with NAFLD, and BMI closely correlates with NAFLD severity and progression to NASH related fibrosis and cirrhosis (Wong et al., 2010). Obesity affects liver health by altering fatty acid storage and metabolism. Under normal weight conditions, excess lipids are stored as neutral triglycerides in the subcutaneous adipose tissue compartment. However, with obesity, the adipocytes in the subcutaneous and visceral adipose compartments swell with triglycerides and become stressed and insulin resistant. Insulin resistance leads to increased lipolysis and free fatty acids (FFAs) are released into circulation (Guilherme, Virbasius, Puri, & Czech, 2008; Reeves, Zaki, & Day, 2016). Pro-inflammatory macrophages inundate adipose compartments and secrete pro-inflammatory cytokines such as TNF-alpha and IL-6, which are thought to contribute to insulin resistance and an overall increase in low-grade systemic inflammation often observed in obese individuals (Fain, 2006; Larter, Chitturi, Heydet, & Farrell, 2010). Lipids accumulate in the hepatocytes of the liver. Mild hepatic triglyceride accumulation is not harmful because triacylglycerol is non-reactive. Severe steatosis, however, can lead to ballooning degeneration liver injury. Also, free fatty acid accumulation in hepatocytes leads to lipotoxicity, which may induce endoplasmic reticulum stress and apoptosis (Alkhouri, Dixon, & Feldstein, 2009). JNK1

pathway signaling is highly active in stressed and dying hepatocytes(Malhi, Bronk, Werneburg, & Gores, 2006). Active JNK1 signaling is known to increase insulin resistance by direct phosphorylation and inactivation of insulin receptor substrate 1 in hepatocytes (Hirosumi et al., 2002). Hepatocyte death and damage activates Kupffer cells that then secrete proinflammatory cytokines such as IL-6. IL-6 activates STAT3 signaling, which in turn activates SOCS proteins. SOCS can further disrupt insulin signaling and therefore increase insulin resistance in hepatocytes (Rui, Yuan, Frantz, Shoelson, & White, 2002). Insulin resistance results in hyperinsulinemia and hyperglycemia that then increase lipogenesis in hepatocytes. The end result is a feed-forward mechanism where NAFLD leads to insulin resistance and insulin resistance exacerbates NAFLD. Lipid hepatotoxicity from free fatty acids and damaged hepatocytes due to severe steatosis create an inflamed microenvironment that contributes to hepatocarcinogenesis (Baffy, et al., 2012; Reeves, et al., 2016).

Over 30 years ago, tumors were originally described as wounds that do not heal (Dvorak, 1986). This early observation highlights the importance of chronic inflammation and abnormal wound healing processes in the development of cancer. Approximately 15% of human cancer occurrences are thought to be linked with pre-existing infections and inflammation (Kuper, Adami, & Trichopoulos, 2000). Three of the most notable links are colon cancer - inflammatory bowel disease, gastric cancer - chronic *Helicobater pylori* infection, and hepatocellular carcinoma – chronic hepatitis B and C viral infection. Even without pre-existing inflammation, most cancers lead to massive immune cell recruitment to the tumor. This is likely due to necrosis at the core of a rapidly growing tumor that lacks oxygen and nutrients. The response by the immune system is to clear

damaged cells and promote tissue repair and regrowth. These pro-inflammatory cells promote regenerative responses by secreting various cytokines, chemokines and growth factors that further cell proliferation (Coussens and Werb 2002). Reactive oxygen and nitrogen species produced by pro-inflammatory immune cells also lead to increased DNA damage and genomic instability. These mutations activate oncogenes and disable tumor suppressors to promote tumor development (He & Karin, 2011). The result is a continued pattern of cell damage and death, followed by an inflammatory response to clean up the tissue and signal for growth, and then compensatory proliferation of surrounding tissue in response to the signals received. These chronic wound healing processes are crucial for general tumor progression and also promote initiation of some cancers, such as HCC. Although short term liver inflammation is beneficial to clear pathogens or promote regeneration after acute injury, chronic inflammation due to sustained hepatocyte damage/death or pathogen infiltration will promote maladaptive wound healing processes and contribute to hepatocarcinogenesis.

NF-KB is an important transcription factor that regulates inflammation and cell survival in the liver. NF-KB activation is a frequent, early event in hepatocarcinogenesis and is thought to be a central driver in the progression of liver injury and inflammation to HCC (He & Karin, 2011; P. Liu et al., 2002; Luedde & Schwabe, 2011). In most chronic liver diseases, such as alcohol induced liver disease, NAFLD, and hepatitis B and C viral infection, NF-KB activity is greatly elevated in the liver (Luedde & Schwabe, 2011). These diseases either damage hepatocytes directly, as in alcohol induced or non-alcoholic fatty liver diseases, or elicit strong immune responses that damage hepatocytes as is observed with hepatitis viral infection. NF-KB signaling directs

inflammatory processes to clear cell debris after cell death and also to clear pathogens from the liver, but sustained NF-KB signaling likely promotes HCC development.

The NF-KB transcription factor is composed of a pair of various subunits that are classified as the Rel family of proteins. In mammals, members of the Rel family include p50, p52, cRel, RelA(p65) and RelB. These subunits of the NF-KB complex form either homodimers or heterodimers that bind DNA and regulate transcription of genes that contain kappa B binding sites in or near their promoter regions. In non-stimulated cells, the transcriptional activity of NF-KB is prevented by binding of the IKB complex that retains NF-KB, inactive, in the cytosol. Upon stimulation, IKB is phosphorylated by the IKK complex, composed of the IKKα and IKKß catalytic subunits and the regulatory subunit IKKγ. IKB phosphorylation leads to its ubiquitylation and degradation. NF-KB is now free to translocate to the nucleus, bind target genes and direct transcription. NF-KB transcriptional targets are involved in a wide range of processes and are known to regulate inflammation, immune responses, and cell survival. NF-KB can be activated by various signals including proinflammatory molecules TNF and IL1β, as well as toll-like receptors activated by damage and pathogen associated molecular patterns. Transcriptional coregulators bind to the NF-KB complex in the nucleus to fine tune and direct specific responses, and nuclear context is extremely important to the transcriptional outcomes that result from NF-KB activation.

As discussed previously, HCC typically occurs in a background of chronic hepatic inflammation, which is reflected in the major risk factors for HCC. Mouse models of HCC also identify important roles for inflammation and NF-KB activity in hepatocytes to promote HCC development. The *Mdr2*$^{-/-}$ knockout mouse develops HCC later in life with

chronic biliary hepatitis as the main contributing factor (Mauad et al., 1994). If the IKB-super-repressor transgene is used to inhibit NF-KB signaling in later stages of tumor development, transformed hepatocytes apoptose and fail to progress to HCC. (Pikarsky et al., 2004). Another chronic inflammation based HCC mouse model that overexpresses lymphotoxinα and/or lymphotoxinβ, specifically in the liver, also supports the pro tumor role of NF-KB in HCC. In this model, hepatocyte specific knockout of IKKβ, to block NF-KB signaling, severely disrupts hepatocarcinogenesis (Haybaeck et al., 2009). Interestingly, hepatocyte specific abolishment of NF-KB signaling in the classic DEN carcinogen induced HCC animal model actually promotes HCC tumor formation. It was determined that NF-KB signaling was important for hepatocyte survival under reactive oxygen stress conditions induced by DEN treatment. Tumor formation in the DEN induced model greatly relies on hepatocyte death and turnover to propagate carcinogen induced mutations. When NF-KB signaling was prevented by hepatocyte specific deletion of IKKβ, hepatocyte turnover was increased causing tumors to form more quickly (He et al., 2010). These different mouse models highlight the importance of HCC etiology to its development and progression. In conclusion, NF-KB activation in hepatocytes contributes to HCC that develops in a background of chronic inflammation, but may be protective if pathology is predominantly due to repetitive hepatocyte death and regeneration. In truth, human HCCs likely result from a combination of both processes. Therefore, balanced NF-KB signaling in hepatocytes is necessary to maintain homeostasis.

In contrast to the varied impacts of hepatocyte NF-KB signaling to hepatocarcinogenesis, NF-KB activation in Kupffer cells has been shown to contribute

to HCC development (Maeda, Kamata, Luo, Leffert, & Karin, 2005). Activated Kupffer cells secrete a number of proinflammatory cytokines and growth factors to promote repair and regeneration of damaged tissue. If sustained, these signals perpetuate the pattern of repeated damage, inflammation, and regeneration that over time contributes to HCC formation.

The research detailed in this dissertation utilizes another genetic mouse model of HCC that will be described in the next section.

NUCLEAR RECEPTOR COACTIVATOR 5 (NCOA5)

Gene transcription is a foundational process involved in every aspect of cellular life. The intricacies of transcriptional regulation are astounding, and human knowledge is continually expanding to better understand the sophisticated mechanisms that control gene expression. Nuclear receptors are a defined class of DNA binding transcription factors that transduce signals from lipophilic endocrine hormones and cause specific changes in gene expression. The ligand bound NRs recognize and bind unique DNA sites that correspond to promoter or enhancer regions of a target gene. Most NRs have hundreds of potential target genes and the NR alters expression of bound genes through recruitment or blocking of basal transcriptional machinery at a gene's transcriptional start site. The transition from NR DNA binding to changes in target gene expression are extremely complex and involve hundreds of adapter proteins, called co-regulators. These NR co-regulators are involved in all aspects of transcription including chromatin modification, RNA Pol-II holoenzyme recruitment and initiation, elongation, and termination. The search to identify nuclear receptor coregulators is important to understand the intricate transcriptional responses to hormones and other NR ligands.

Nuclear Receptor Co Activator 5 (NCOA5) was first reported by Sauvé et al. and initially given the name Coactivator independent of AF2 (CIA). Ncoa5 was discovered with a Yeast 2 Hybrid screen using the nuclear receptor RVR as bait. RVR is unique among nuclear receptors because it lacks the Activation Function 2 (AF-2) domain, a canonical site for coregulator binding. The intent was to find unknown coregulators that did not use the common AF-2 binding site. Ncoa5 was found to bind RVR, and also Rev-ErbAalpha, ERalpha, and ERbeta and was found expressed in a variety of tissue types. Ncoa5 contains an overlapping LxxLL and φxxφφ, which are motifs frequently found in nuclear receptor coactivators and corepressors respectively. A protein region rich in Arginine and Aspartate was characterized in Ncoa5 and has been known to facilitate RNA binding in other proteins. Ncoa5 localizes to the nucleus where it is thought to interact with nuclear receptors and modify their transcriptional activity in a ligand dependent manner (Sauvé et al., 2001). Ncoa5 was also discovered independently through interaction with HTATIP2 (TIP30). In this study Ncoa5 was shown to play an important cooperative role with TIP30 to inhibit ERalpha mediated c-myc expression (Jiang et al., 2004). These studies highlight the importance of Ncoa5 in regulating the transcriptional effects of NR action.

A genetic knockout mouse model where the *Ncoa5* gene was targeted for disruption has provided further information regarding Ncoa5 function. One of the first observations made about this model is that the male mice were not fertile. Thus homozygous knockout mice were not producible and heterozygous females were bred with wild type males to maintain the knockout Ncoa5 allele. Heterozygous females with one functional copy of *Ncoa5* and one knockout Ncoa5 allele ($Ncoa5^{+/-}$) were normal in

appearance and phenotype. Male *Ncoa5*$^{+/-}$ mice, however, display a wide range of phenotypes.

Male *Ncoa5*$^{+/-}$ mice display phenotypes of infertility, glucose intolerance, NAFLD/NASH characterized by fatty liver, hepatic inflammation and fibrosis, and later in life many mice acquire hepatocellular carcinoma. Infertility is due to a reduced number of viable and motile sperm. Glucose intolerance was observed at 6 weeks of age using glucose tolerance tests and insulin tolerance tests that revealed *Ncoa5*$^{+/-}$ male mice have slower clearance of glucose from the blood and are less responsive to insulin treatment. At 10 months of age NAFLD/NASH was apparent and presented with fibrosis. At this timepoint there were also an increased number of active Kupffer cells, the resident macrophages of the liver, which resulted in an increased expression of IL6 and TNFalpha in the liver. By 18 months of age, 70 to 90% of male *Ncoa5*$^{+/-}$ mice acquired HCC depending on the mouse strain used. Interestingly, partial disruption of the IL6 gene in these Ncoa5+/- male mice rescued many of the phenotypes, including fertility, glucose intolerance and fatty liver. Even though fertility was rescued, no *Ncoa5*$^{-/-}$ mice were producible. The HCC phenotype in these mice was lessened and there were fewer numbers of tumors per mouse and the tumor sizes were reduced with partial loss of IL6. This result highlighted the importance of IL6 in HCC development in these *Ncoa5*$^{+/-}$ male mice (S. Gao et al., 2013).

Other findings on Ncoa5 have pointed toward new and varied functions for this coregulator. Boser et al. found Ncoa5 to be important for pluripotent stem cell maintenance in planarians and that the mouse orthologue is also expressed in pluripotent stem cells of the mouse embryo (Böser et al., 2013). Another group has

characterized the importance of Ncoa5 in macrophage cholesterol efflux that has implications in inflammation and atherosclerosis. Gillespie and collegues used promoter enrichment-quantitative mass spectrometry to identify complexes at the Abca1 promoter. They observed that Ncoa5 acts as a corepressor of the LXR Nuclear Receptor to down regulate expression of Abca1 in macrophages. This results in a shift toward a more pro inflammatory phenotype and promotes pro atherosclerotic foam cell formation (Gillespie et al., 2015). NCOA5 likely plays various roles depending on the cell type and molecular context.

For this study, we use the Ncoa5 HCC mouse model to investigate the molecular mechanisms involved in the progression of liver pathology to HCC, with a focus on the preneoplastic stages of cancer development.

METFORMIN

N,N-dimethylbiguanide, known commonly as metformin, has recently become the most utilized therapy for treatment of type 2 diabetes (T2D). However, related compounds have been used in Europe since the middle ages and the benefits of metformin have taken almost a century to be appreciated. Extract of *Galega officinalis*, goat's rue or French lilac by common name, was used in Medieval Europe as medicine to treat polydipsia and polyurea, symptoms now known to occur with uncontrolled, elevated blood glucose levels observed with untreated diabetes (Clifford J. Bailey & Day, 1989). It was not until the late 1800's that the glucose lowering effects of *G. oficinalis* extract were attributed to the compounds guanidine and galegine. At higher concentrations, guanidine proved to be toxic, but side effects of galegine were more mild and manageable. Galegine was used briefly as an antidiabetic in the early 1920s,

but was quickly displaced by insulin, discovered by Banting and Best in 1921 (C. J. Bailey & Day, 2004). Synthetic chemists Werner and Bell first published the preparation of dimethylbiguanide in 1922, although they were unaware of its abilities to modulate blood glucose (Werner & Bell, 1922). Insulin was the standard therapeutic for T2D until side effects, such as a strong hypoglycemic response and more notably a shortened lifespan, resulted in a search for more suitable alternatives. It was not until 1957 that Jean Sterne carried out the clinical development of dimethylbiguanide, which he termed Glucophage, meaning glucose eater. Related compounds phenformin and buformin were also developed at this time and were initially favored due to stronger glucose lowering effects (Schafer, 1983). This created the drug class known as biguanides.

The biguanides buformin and phenformin were used for over a decade until they were broadly discontinued in the 1970s when the dangerous and undesirable effect of lactic acidosis became apparent (Luft, Schmülling, & Eggstein, 1978). Europe and Canada then turned to metformin, which had a weaker glucose lowering effect, but was also found to have fewer incidences of lactic acidosis. The United States lagged behind and the FDA only approved metformin for clinical use in 1994. Metformin is now taken by over 120 million people worldwide as the first line therapy for T2D and has been placed on the World Health Organization's list of essential medicines (Viollet et al., 2012).

In the United States, metformin is approved only for the management of elevated blood glucose levels in patients with T2D, but is also given off label to treat insulin resistance sometimes observed in women with polycystic ovarian syndrome. It is always taken orally either once or twice per day at doses of approximately 1000mg per day.

The dosage can slowly be increased to a total of 2000mg per day to minimize initial side effects such as nausea and diarrhea. Metformin is 50% orally bio-available, absorbed through the organic cation transporters of the small intestine. Metformin remains unbound in the serum and is not metabolized in circulation until it is excreted into the urine by the kidneys with a systemic half-life of approximately 5 hours (Graham et al., 2011). This sole route of elimination by the kidneys has led the FDA to place strict warnings that prevent metformin use by patients with compromised renal function. There is currently debate as to whether the FDA should relax standards as metformin use by patients with mild to moderate chronic kidney disease has not resulted in increased incidences of lactic acidosis (Inzucchi, Lipska, Mayo, Bailey, & McGuire, 2014).

Metformin lowers blood glucose by several general mechanisms. First and foremost, metformin reduces glucose production by the liver (Cusi, Consoli, & DeFronzo, 1996). The liver is considered the main site of action for metformin and the drug accumulates in hepatocytes due to high expression of the organic cation transporter 1 found on the hepatocyte cell membrane that facilitates cellular uptake (Shu et al., 2007; Wang et al., 2002). Metformin specifically inhibits gluconeogenesis in hepatocytes and therefore reduces overall hepatic glucose output (Hundal et al., 2000). Patients with T2D display increased gluconeogenesis, which metformin is able to directly counteract (Hundal, et al., 2000). A systemic insulin sensitizing effect has also been observed in insulin resistant patients treated with metformin. Improved insulin action results in increased glucose uptake from the blood by peripheral tissues such as liver and skeletal muscle (Tamura et al., 2008). Metformin may play a role in the gut as

well. Approximately 50% of ingested metformin is not absorbed by the small intestine and instead accumulates in the gut mucosa until it is eliminated in the feces (C. J. Bailey, Wilcock, & Scarpello, 2008). Short term experiments with delayed release formulations of metformin show that antihyperglycemic effects are maintained even when metformin bioavailability is low and the drug instead remains in the gut (Buse et al., 2016).

Metformin likely has a variety of molecular targets that influence the beneficial, physiological effects observed in T2D patients. Several main targets have been identified; however, there is debate over the regulatory details of proposed mechanisms and concern about biological relevance *in vivo* as many mechanisms were determined with extremely high metformin concentrations *in vitro*.

The predominantly accepted action of metformin is to directly inhibit mitochondrial complex I of the electron transport chain (El-Mir et al., 2000). Complex I inhibition leads to a marked reduction of ATP levels due to a decrease in cellular respiration. ATP depletion alters the ratios of AMP/ATP and ADP/ATP in the cell and activates energy conserving mechanisms directed by AMP activated protein kinase (AMPK). AMPK is activated by phosphorylation at Thr172, carried out by the upstream kinase LKB1 (Woods et al., 2003). AMPK activation leads to a shift in cell state from anabolic to catabolic metabolic processes to promote cell survival under energy-restricted conditions. Activated AMPK has a wide range of effects, including decreased expression of gluconeogenic enzymes that reduces gluconeogenesis and suppressed expression of lipogenic genes. Metformin is also known to inhibit mTORC1 and mTORC2 that play important roles in promoting protein synthesis and gluconeogenesis

respectively. This inhibition is likely mediated by AMPK-dependent and independent actions that are not fully understood (Viollet, et al., 2012).

Current evidence supports the possibility that metformin may also have cancer preventive and therapeutic potential. This was first suggested by retrospective, observational, epidemiological studies that revealed T2D patients taking metformin had lower overall cancer incidences than patients using other T2D drugs, such as sulfonylureas (DeCensi et al., 2010; Evans, Donnelly, Emslie-Smith, Alessi, & Morris, 2005). These original studies have received a large amount of criticism due to the incorporation of biases common to epidemiological studies (Suissa, 2017). Even so, these original findings generated an enormous amount of excitement and led to numerous studies *in vitro* and *in vivo* that support the anticancer properties of metformin. In many ways, a metformin anticancer property is logical as there are many similarities between a type 2 diabetic condition and cancer. Furthermore, in the case of HCC, diabetes itself is a risk factor and many other risk factors overlap between the two diseases. One major biological link between T2D and cancer is a dysregulation of insulin/IGF signaling (LeRoith, Baserga, Helman, & Roberts Jr, 1995). Hyperinsulinemia observed in type 2 diabetes is known to promote malignancy through increased tumor glucose uptake and altered insulin signaling. Both diseases are the result of large metabolic shifts as well. If metformin can normalize the diabetic condition by reducing blood glucose, decreasing insulin levels and normalizing metabolism, anticancer properties are promising (Quinn, Kitagawa, Memmott, Gills, & Dennis, 2013).

Numerous studies support metformin as a potential chemo-preventive and therapeutic for HCC. Several retrospective epidemiology studies characterize the

decreased risk of HCC in T2D patients taking metformin (Donadon, Balbi, Mas, Casarin, & Zanette, 2010; Hassan et al., 2010). A prospective study, carried out in Taiwan, which contained 800,000 individuals, found metformin treatment did reduce risk of HCC as well as pancreatic and colorectal cancers (Lee et al., 2011). Cell culture and orthotopic mouse models that use human HCC cell lines clearly show reduced growth and viability of HCC cells with metformin treatment (Qu et al., 2012). Animal models of HCC have also been used to test the ability of metformin to inhibit HCC development.

Rodent models of HCC typically display reduced cancer incidence when given metformin in food or water. The standard metformin dosing in mice is 250mg/kg. This dosing was established to match serum concentrations in patients taking metformin, which is approximately 5uM (Chandel et al., 2016). The classic DEN carcinogen induced mouse model of HCC shows a decrease in number of tumors per mouse and a reduction in tumor size with metformin treatment (Bhalla et al., 2012). A similar approach in rats produced the same results. Metformin treatment reduced the number of HCC nodules if the drug was given before the onset of liver cirrhosis (DePeralta et al., 2016). Metformin also reduced HCC incidence in a mouse model of HCC induced by a high fat diet (Tajima et al., 2013). In contrast to these previous results, metformin treatment had no effect on HCC incidence in the HBx transgenic mouse model. The HBx model of HCC was created by incorporating one copy of the Hepatitis B X gene into murine chromosome 2, which is sufficient to induce HCC by 18 months of age at a 90% penetrance (J.-H. Kim et al., 2013).

For this study the *Ncoa5*$^{+/-}$ mouse model of HCC was treated with metformin for two purposes. First, metformin was specifically tested for an ability to prevent HCC in

the Ncoa5+/- model of HCC. Second, previous evidence supports metformin as an HCC preventive; therefore, transcriptomics was used to assess changes elicited by metformin in the liver to suggest genes and pathways important to HCC development.

RATIONALE FOR DISSERTATION

Collectively, the knowledge presented summarizes the current understanding of HCC development and highlights the need for chemopreventives to reduce HCC mortality. Metformin is currently a top contender as a potential HCC chemopreventive; however, HCC preventive mechanisms of metformin remain unclear. Providing a better understanding of metformin mechanisms will clarify its use as an HCC chemopreventive, and also help identify other drugs that act on similar mechanisms and may also prevent HCC. The *Ncoa5*$^{+/-}$ mouse model of HCC is well suited to study impacts and mechanisms of metformin action on HCC development *in vivo*. With this opportunity, my dissertation research focused on using the *Ncoa5*$^{+/-}$ mouse model of HCC to better understand gene expression changes in premalignant liver and to determine how metformin treatment impacts these changes and affects later HCC incidence.

CHAPTER 2 METFORMIN REVERSES ABERRANT CYTOPLASMIC p21^{WAF1} EXPRESSION IN HEPATOCYTES AND PREVENTS HEPATOCELLULAR CARCINOMA DEVELOPMENT IN *Ncoa5*$^{+/-}$ MALE MICE

ABSTRACT

Hepatocellular carcinoma (HCC) is a deadly disease with limited systemic therapy options and sharply increasing incidence rates in developed regions of the world. Prevention is essential to reduce HCC mortality rates, however chemopreventive agents are lacking. The first-line, type two diabetes drug, metformin, shows promise as an HCC preventive, but mechanisms of action are not fully understood. We use the *Ncoa5*$^{+/-}$ mouse model of HCC to demonstrate critical gene expression changes in the preneoplastic mouse liver that also correlate with human *NCOA5* expression in HCC and HCC-adjacent tissues. Inflammatory and p53 pathways display upregulated expression in *Ncoa5*$^{+/-}$ liver, as well as cytoplasmic p21^{WAF1}. Long term metformin treatment dramatically reduced tumor incidence in the *Ncoa5*$^{+/-}$ mouse model and reduced aberrant cytoplasmic p21^{WAF1}-positive hepatocytes. We also identified a subgroup of human HCC patients whose HCC-adjacent tissue displayed a distinct pattern of inflammation pathway enrichment and high *CDKN1A* (p21^{WAF1}) gene expression, similar to preneoplastic *Ncoa5*$^{+/-}$ mouse liver. Gene expression changes elicited by metformin are predicted to reduce p21^{WAF1} liver expression in humans. Our study suggests a molecular mechanism underlying HCC development and uncovers new actions for metformin in the prevention of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a devastating disease that currently has severely limited options for systemic therapy. HCC is the fifth most frequent and second most deadly malignancy in the world, with most incidences occurring in regions endemic for hepatitis viral infection (El-Serag, 2012). However, there is an alarming trend in the developed West with a sharp increase in HCC incidences and mortality. These Western trends remain partly due to chronic hepatitis C and hepatitis B viral infection; however, metabolic diseases are HCC risk factors that have enveloped the United States and other industrialized nations (El-Serag & Kanwal, 2014). Approximately 9% of Americans have Type 2 Diabetes (T2D) and a staggering one in three individuals in the United States is obese (Control & Prevention, 2014; Flegal, et al., 2012). T2D and obesity take a great toll on many organs in the body, and the liver in particular is altered by systemic metabolic shifts and itself contributes to disease. Liver changes that occur in metabolic disorders include the development of non-alcoholic fatty liver disease (NAFLD), hepatic insulin resistance, and increased hepatic glucose output. The liver is a resilient organ and many people never experience liver complications associated with T2D and NAFLD. However, about 20% of individuals with NAFLD also develop hepatitis, termed non-alcoholic steatohepatitis (NASH) (Adams, et al., 2005; Ekstedt, et al., 2006). This hepatic inflammation results in a progressive disease that may become hepatic fibrosis and even cirrhosis that can compromise liver function. Previous studies have also established that chronic hepatic inflammation, steatosis and fibrosis lead to hepatocarcinogensis (Baffy, et al., 2012). It is currently unachievable to eliminate hepatitis viral infection and metabolic disorders, which are the main causes of hepatic

inflammation. Therefore, it is crucial to identify the molecular factors and signaling pathways that drive the progression of chronic hepatic inflammation to HCC, to open new avenues for development of HCC chemopreventives.

Epidemiological studies show that T2D is correlated with increased risk of HCC (Giovannucci et al., 2010; McGlynn & London, 2011) and patients taking the first-line T2D therapy metformin have a reduced risk of HCC incidence and mortality (Chen et al., 2013; DeCensi, et al., 2010; Donadon, et al., 2010). A commonly believed view is that activation of the insulin receptor and activation of AMP-activated protein kinase (AMPK) probably mediate the preventive effect of metformin on the risk of several types of cancers, including HCC, based on earlier studies using cultured cancer cell lines. Recently, several preclinical studies on the preventive action of metformin in rodent models of HCC have suggested a different scenario. First, a DEN carcinogen induced mouse model of HCC was used to display reduced HCC tumor multiplicity with metformin treatment, while AMPK levels in the livers were not significantly changed. Instead, suppressed *de novo* lipid synthesis in the liver was identified as a potential mechanism by which metformin prevented HCC tumor formation (Bhalla, et al., 2012). Second, a DEN induced rat model of HCC also displayed reduced HCC incidence with early and prolonged metformin treatment that was shown to inhibit hepatic progenitor cell activation (DePeralta, et al., 2016). Third, a high fat diet induced mouse model of HCC revealed reduced HCC incidence if metformin treatment began before the onset of severe NAFLD. Mechanisms proposed for lowered HCC occurrence included activation of AMPK and suppression of hepatic fat and reduced inflammation (Tajima, et al., 2013). However, metformin treatment begun after the onset of NAFLD did not provide

reduced HCC incidence. In contrast to these three reports, a fourth model, the transgenic HBX oncogene induced HCC mouse model, revealed that metformin did not suppress tumor development in these mice, suggesting that underlying etiology greatly affects the HCC inhibitory action of metformin (J.-H. Kim, et al., 2013). Nevertheless, each of these animal studies had some limitation: (1) metformin was administered to animals continually throughout the remainder times of the study; thus, the question of whether metformin treatment that is terminated before tumor initiation is sufficient to prevent HCC onset later in life, remains unknown; (2) None of these studies used high throughput transcriptomics approaches to investigate potential mechanisms mediating the tumor preventive action of metformin; (3) The effects of metformin on HCC incidence in other genetic animal models of HCC is unknown.

We previously reported that male mice carrying heterozygous deletion of the *Ncoa5* gene exhibited glucose intolerance, chronic hepatic inflammation and high incidence of HCC (S. Gao, et al., 2013). In this report, we demonstrate the preventive effect of metformin on HCC development in *Ncoa5*$^{+/-}$ mice by feeding metformin in drinking water for 40 weeks. Besides the changes in known target genes and signaling pathways, we also found that metformin reversed p53 targeted gene expression and aberrant cytoplasmic p21-expressing hepatocytes. Our study uncovers a previously undescribed action of metformin and provides further insight into the understanding of molecular pathogenesis of HCC. These concepts will aid potential strategies aimed at HCC prevention and treatment.

EXPERIMENTAL PROCEDURES

Animals and treatments.

Balb/c *Ncoa5*$^{+/-}$ female mice were backcrossed to C57BL/6J males for 7 generations to create the mice used for all experiments. See Gao et al. for generation of the *Ncoa5* knockout mouse (S. Gao, et al., 2013). All mice were housed in Optimice cages at Michigan State University animal facility on a 12:12-h light-dark cycle and fed standard rodent diet *ad libitum*. Mice in metformin experiments received metformin (Sigma Aldrich) dissolved in drinking water at a dosage of approximately 250mg/kg/day from 8 weeks of age until 48 weeks of age. At the termination of a specified experiment mice were euthanized by CO2 asphyxiation and tissues were either fixed in formalin or snap frozen in liquid nitrogen for later RNA or protein analysis. All experimental procedures on mice were approved by the Michigan State University Institutional Animal Care and Use Committee.

Tissue histology.

Formalin fixed tissues were embedded in paraffin, cut into sections and stained with hematoxylin and eosin by the Michigan State University Division of Human Pathology Investigative Histopathology Lab. Pathologic analysis was carried out by veterinary pathologist, Dr. Matti Kiupel

RNA isolation, reverse transcription, RNA sequencing, and analysis.

RNA was isolated from total liver tissue using TRIzol reagent (ThermoFisher). RNA for RT-qPCR analysis was reverse-transcribed with the SuperScript IV First-Strand Synthesis System (ThermoFisher) and amplified via a QuantStudio 3 Real-Time PCR System (ThermoFisher) with Power SYBR Green Master Mix (ThermoFisher) or

TaqMan Gene Expression Master Mix (Applied Biosystems) for respective chemistry (assay dependent). qPCR experimental design and analyses were performed according to MIQE guidelines (Bustin et al., 2009).

Liver RNA samples for RNA sequencing were further purified with the RNeasy Mini Kit (Qiagen) and assessed for quality using a Nanodrop (ThermoFisher) and a Bioanalyzer 2100 via Eukaryote total RNA Pico assay (Agilent). All RNA samples used for RNA sequencing had an RNA Integrity Number of 7.3 or higher. The 20-week old cohort RNA seq was carried out by the Michigan State University Research Technology Support Facility using a HiSeq 2500 (Illumina) with a 100bp paired-end platform, and approximately 15-50 million reads were generated for each sample. The 39-week old cohort RNA seq was carried out by Novogene using a HiSeq 4000 (Illumina) with a 150bp paired-end platform, and approximately 30 million reads were generated for each sample.

RNA sequencing reads were hard-trimmed and analyzed for quality control using Trimmomatic and FastQC (Andrews, 2010; Bolger, Lohse, & Usadel, 2014). Accepted reads were mapped to reference assemblies GRCm38 and mm10 using TopHat2 (Ensembl, 2017; D. Kim et al., 2013; UCSC, 2017). Mapped reads were then quantified for differential expression analysis by HTSeq/EdgeR and the Tuxedo suite, independently (Anders, Pyl, & Huber, 2015; Robinson, McCarthy, & Smyth, 2010; Trapnell et al., 2012).

Downstream gene set and pathway enrichment analyses were carried out with Gene Set Enrichment Analysis (GSEA), single sample GSEA via GenePattern (ssGSEA) (Reich et al., 2006; Subramanian et al., 2005), and Generally Applicable

Gene Set Enrichment (GAGE) (W. Luo, Friedman, Shedden, Hankenson, & Woolf, 2009) using MSIGDB and KEGG gene sets (Kanehisa & Goto, 2000). GAGE results were also visualized using Pathview (W. Luo & Brouwer, 2013). Hierarchical clustering analyses, including data centering and normalization, were performed using Cluster 3.0 (centered correlation and Euclidean distance algorithms) and visualized using Java TreeView (de Hoon, Imoto, Nolan, & Miyano, 2004). All other heatmaps were generated using MATLAB. Correlation analyses were calculated using Pearson coefficient (figure 6B). ANOVA was used to discern statistical difference in downstream gene and pathway expression analyses. Human translational analyses utilized the NIH TCGA-LIHC clinical HCC dataset (http://cancergenome.nih.gov/).

Immunohistochemistry (IHC) and Immunofluorescence.

IHC was performed according to the protocol supplied by Vector Laboratories. P21 (F-5, SC-6246, Santa Cruz Biotechnology), P16 (Orb97580, Biorbyt), Epcam (ab71916, Abcam), Krt19 (10712-1-AP, Proteintech) antibodies were used. Quantification of Epcam-positive or Krt19-positive cells and IHC staining score of P21 and P16 were performed in five random high-power-fields per slide. Immunofluorescent staining was performed on the liver sections of 20-week old mice with primary antibody P21and Epcam described above, and Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (F6057, Sigma).0.1% Sudan black B (3545-12, Sigma) in 70% ethanol was used to reduce the autofluorescence of the tissue.

RESULTS

Ncoa5$^{+/-}$ male mice display altered liver gene expression in inflammation and p53 mediated pathways and *NCOA5* expression correlates with differentially expressed gene homologs in human HCC and HCC-adjacent human tissue.

We previously reported that *Ncoa5* haploinsufficiency enhances expression of IL-6 and TNF-α in Kupffer cells, which in turn promotes hepatic inflammation, steatosis and HCC development (S. Gao, et al., 2013). To gain further insight into the molecular mechanisms that contribute to HCC development, high throughput RNA sequencing and differential gene expression analyses were carried out on early preneoplastic livers of C57BL/6, *Ncoa5*$^{+/-}$, male mice compared to *Ncoa5*$^{+/-}$ control livers at 20 weeks of age (n=4). EdgeR identified 25 differentially regulated genes, with a FDR ≤ 0.05, in *Ncoa5*$^{+/-}$ vs. *Ncoa5*$^{+/+}$ male, mouse livers (Figure 1A; table 1). Several differentially regulated genes and other potentially important genes were validated with qRT-PCR analysis (Figure 1B). Manual investigation revealed that many of these genes are downstream targets of NF-KB and are involved in inflammatory processes. Several p53 target genes were also observed to be upregulated, including *Cdkn1a* (p21^{WAF1}) and *Gadd45β*. The correlations between human *NCOA5* expression and expression of human homologs of these differentially expressed murine genes were examined in liver hepatocellular carcinoma (LIHC) datasets using Cancer Genome Atlas data (http://www.cbioportal.org) (J. Gao et al., 2013). The heat-map correlation matrix was used to characterize associations between members of the *Ncoa5*$^{+/-}$-altered gene set and *NCOA5* expression in humans. Results revealed that expression of 15 out of 25 mouse-human homologs were significantly correlated ($p < 0.05$) with *NCOA5*

expression in human HCC tumor and adjacent-tissue specimens, and indicates similarity between mice and humans (Figure 1C). An alternative differential expression algorithm was also used to evaluate the same 20 week old *Ncoa5*$^{+/-}$ vs *Ncoa5*$^{+/+}$ mouse liver expression data. Interestingly, the Cuffdiff differential expression analysis tool of the Tuxedo pipeline produced 252 genes with significant differential expression (FDR<0.05) in *Ncoa5*$^{+/-}$ vs. *Ncoa5*$^{+/+}$ livers.

Generally Applicable Gene set Enrichment (GAGE) was used to determine signaling pathway changes in *Ncoa5*$^{+/-}$ mouse livers at 20 weeks. GAGE pathway analysis revealed that expression changes were upregulated in 21 and downregulated in 16 KEGG pathway gene sets in *Ncoa5*$^{+/-}$ mouse livers when compared to wildtype livers, using a cutoff of FDR<0.25 (Table 2A and B). Many KEGG gene sets with upregulated expression in the 20 week *Ncoa5*$^{+/-}$ livers are associated with inflammation and cancer, including p53, NF-KB, and MAPK signaling pathways (highlighted in table). These results provide a snapshot of altered gene and pathway expression at an early stage of hepatocarcinogensis.

To test whether these genes and pathways remain altered at a later preneoplastic stage of hepatocarcinogenesis, RNA sequencing analysis was carried out in 39 week old mice. As before, *Ncoa5*$^{+/-}$ male mouse liver gene expression was compared to gene expression of *Ncoa5*$^{+/+}$ livers. The Cuffdiff program calculated 236 differentially expressed genes (FDR<0.05). Twenty significantly altered genes are shared between 20 and 39 week Cuffdiff results, having a log2Fold Change>1 and a similar direction in expression change (Figure 1D). GAGE analysis revealed 17 pathways with upregulated expression in 39 week *Ncoa5*$^{+/-}$ vs *Ncoa5*$^{+/+}$ livers (FDR

<0.25, N=5 for *Ncoa5*$^{+/}$, N=4 for *Ncoa5*$^{+/+}$) (Table 3). Of these pathways, the p53 signaling pathway and MicroRNAs in cancer are also found with upregulated expression in the earlier 20 week cohort. Pathview analysis revealed individual gene expression changes within the p53 pathway. Interestingly, *Cdkn1a* (p21^{WAF1}) and *Gadd45*, two canonical p53 downstream targets, were seen with highly elevated expression in the livers of *Ncoa5*$^{+/-}$ male mice at 39 weeks of age. These results are consistent with Cuffdiff individual gene expression analysis. These findings identify upregulation of *Cdkn1a* (p21^{WAF1}) expression and other p53 targeted genes as critical changes in early and late preneoplastic stages of hepatocarcinogenesis in *Ncoa5*$^{+/-}$ male mice.

Figure 1. Differential expression analysis reveals gene expression changes induced by Ncoa5 deficiency that also correlate to *NCOA5* expression in human HCC and HCC-adjacent tissue

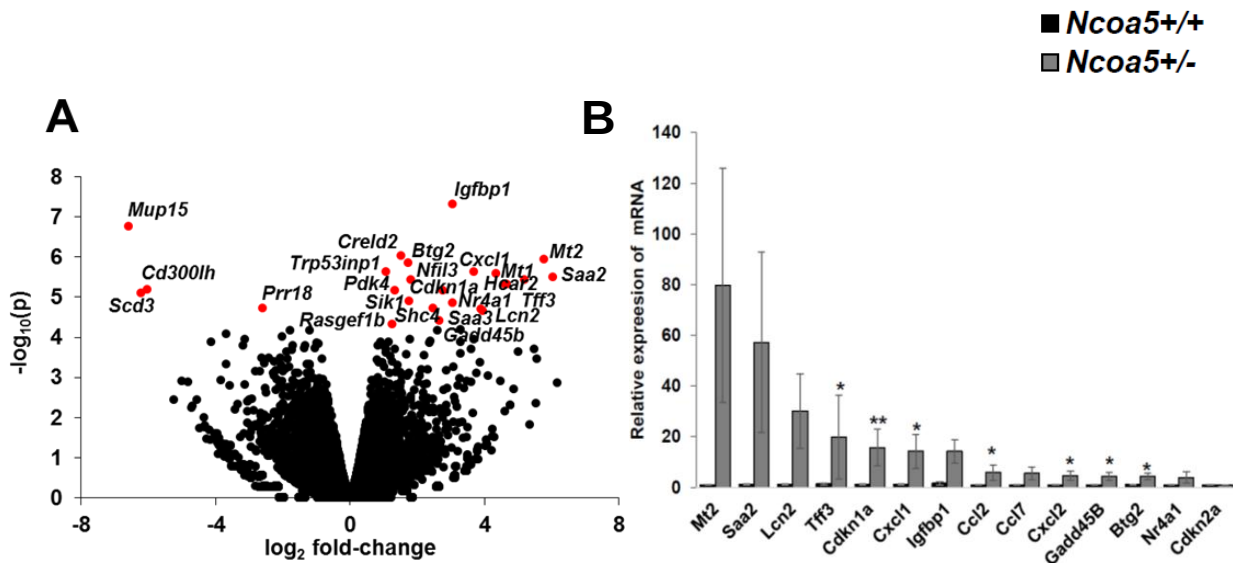
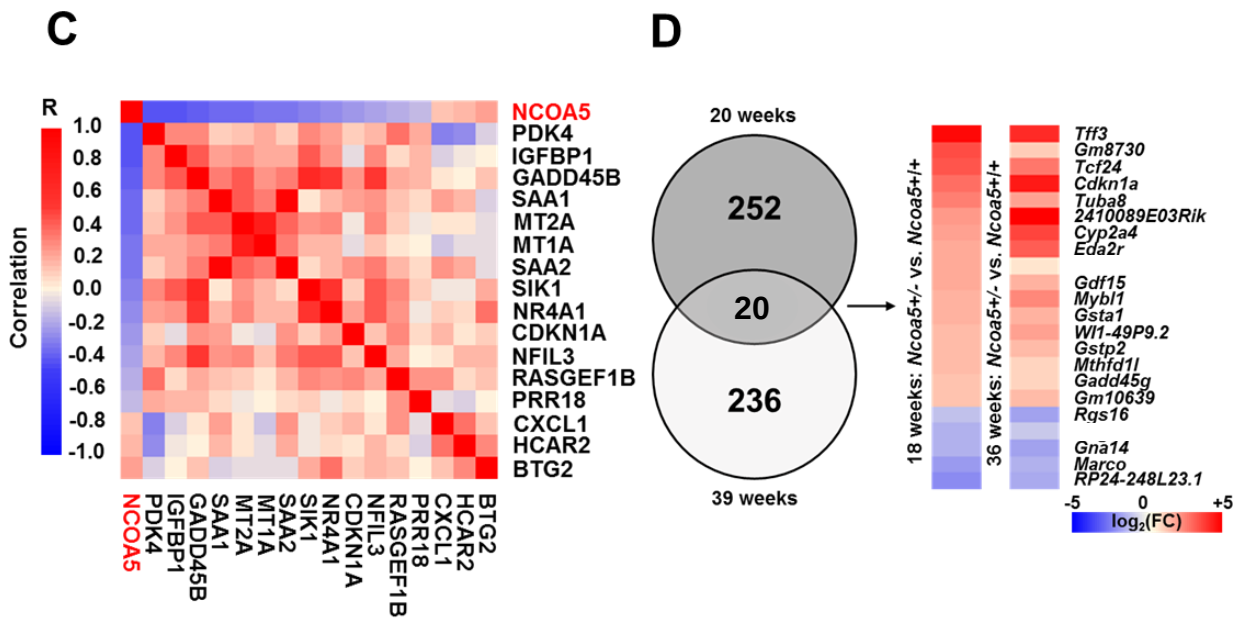


Figure 1 (cont'd)



(A) Volcano plot displaying EdgeR differential expression analysis results from 20 week old male *Ncoa5*$^{+/-}$ vs *Ncoa5*+/+ mouse liver RNA sequencing (N=4). Genes with significant changes in expression (FDR<0.05) are red and labeled with the gene name. (B) qRT-PCR validation of differentially expressed genes in 20 week old *Ncoa5*$^{+/-}$ mouse liver (N=8). All data is represented as mean ± SEM. * <0.05, ** <0.01. (C) Correlation heatmap of significant human homologs of differentially expressed murine genes vs. *NCOA5* expression in HCC tumor and adjacent tissue (TCGA-LIHC data). (D) Significant differentially expressed genes in *Ncoa5*$^{+/-}$ vs. *Ncoa5*$^{+/+}$ liver tissue identified in both 20 week and 39 week old cohorts. There are 20 significant genes common to both groups with a log2 fold change > 1, and a similarly altered direction.

Table 1. EdgeR Differential Genes: 20 weeks *Ncoa5*+/- vs. *Ncoa5*+/+ (FDR < 0.05)

Symbol	Full Name	Log2 FC	p	FDR
Igfbp1	Insulin-like growth factor binding protein 1	3.056	4.92E-08	0.0012
Mup15	Major urinary protein 15	-6.58	1.73E-07	0.0021
Creld2	Cysteine-rich with EGF-like domain 2	1.51	9.46E-07	0.0066
Mt2	Metallothionein 2	5.76	1.16E-06	0.0066
Btg2	B-cell translocation gene 2	1.73	1.38E-06	0.0066
Trp53inp1	Transformation-related protein 53 inducible nuclear protein 1	1.08	2.33E-06	0.0077
Cxcl1	C-X-C motif chemokine ligand 1	3.69	2.38E-06	0.0077
Mt1	Metallothionein 1	4.35	2.57E-06	0.0077
Saa2	Serum amyloid A2	6.03	3.14E-06	0.0083
Nfil3	Nuclear factor, interleukin 3 regulated	1.81	3.69E-06	0.0083
Saa1	Serum amyloid A1	5.20	3.79E-06	0.0083
Tff3	Trefoil factor 3	4.60	4.78E-06	0.0089
Hcar2	Hydroxycarboxylic acid receptor 2	4.67	4.82E-06	0.0089
Cd300lh	CD300 antigen like family member H	-6.03	6.53E-06	0.0103
Pdk4	Pyruvate dehydrogenase kinase 4	1.34	6.74E-06	0.0103
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	2.76	6.82E-06	0.0103
Scd3	Stearoyl-coenzyme A desaturase 3	-6.22	8.00E-06	0.0113
Sik1	Salt-inducible kinase 1	1.75	1.26E-05	0.0168
Nr4a1	Nuclear receptor subfamily 4 group A member 1 (*NUR77*-homolog)	3.04	1.37E-05	0.0174
Prr18	Proline-rich 18	-2.60	1.86E-05	0.022
Shc4	Src homology 2 (SH2) domain-containing family member 4	4.48	1.92E-05	0.022
Saa3	Serum amyloid A3	3.90	2.02E-05	0.0221
Lcn2	Lipocalin 2	3.95	2.26E-05	0.0237
Gadd45b	Growth and arrest DNA-damage inducible beta	2.66	3.90E-05	0.0391
Rasgef1b	RasGEF-domain family member 1B	1.26	4.87E-05	0.0469

Table 2A. GAGE: 20 weeks *Ncoa5*+/- vs. *Ncoa5*+/+ (upregulated, FDR < 0.25)

KEGG Pathways	p	FDR
mmu04141 Protein processing in endoplasmic reticulum	2.30E-11	6.28E-09
mmu00190 Oxidative phosphorylation	1.55E-07	2.12E-05
mmu05012 Parkinson's disease	2.87E-05	0.002613
mmu05034 Alcoholism	0.000192	0.013077
mmu04010 MAPK signaling pathway	0.000317	0.017296
mmu04260 Cardiac muscle contraction	0.000615	0.024656
mmu05010 Alzheimer's disease	0.000632	0.024656
mmu03060 Protein export	0.001604	0.052840
mmu05203 Viral carcinogenesis	0.001742	0.052840
mmu05016 Huntington's disease	0.003498	0.095489
mmu04668 TNF signaling pathway	0.003962	0.098318
mmu03050 Proteasome	0.005120	0.110992
mmu04978 Mineral absorption	0.005667	0.110992
mmu04064 NF-kappa B signaling pathway	0.005692	0.110992
mmu04932 Non-alcoholic fatty liver disease (NAFLD)	0.006294	0.114548
mmu05206 MicroRNAs in cancer	0.011871	0.193805
mmu05134 Legionellosis	0.012143	0.193805
mmu05031 Amphetamine addiction	0.012778	0.193805
mmu05322 Systemic lupus erythematosus	0.015933	0.219327
mmu04115 p53 signaling pathway	0.016068	0.219327
mmu05169 Epstein-Barr virus infection	0.018008	0.234098

Table 2B. GAGE: 20 weeks *Ncoa5*+/- vs. *Ncoa5*+/+ (downregulated, FDR < 0.25)

KEGG Pathways	p	FDR
mmu02010 ABC transporters	4.53E-05	0.012372
mmu00280 Valine, leucine and isoleucine degradation	0.000145	0.019793
mmu00100 Steroid biosynthesis	0.000439	0.039911
mmu00640 Propanoate metabolism	0.000937	0.043468
mmu01212 Fatty acid metabolism	0.000957	0.043468
mmu00310 Lysine degradation	0.000997	0.043468
mmu01040 Biosynthesis of unsaturated fatty acids	0.001261	0.043468
mmu00650 Butanoate metabolism	0.001274	0.043468
mmu04146 Peroxisome	0.003344	0.101430
mmu04976 Bile secretion	0.004282	0.107494
mmu00630 Glyoxylate and dicarboxylate metabolism	0.004331	0.107494
mmu04152 AMPK signaling pathway	0.006969	0.158544
mmu03430 Mismatch repair	0.009100	0.191092
mmu04144 Endocytosis	0.011360	0.219948
mmu00770 Pantothenate and CoA biosynthesis	0.012246	0.219948
mmu00900 Terpenoid backbone biosynthesis	0.012891	0.219948

Table 3. GAGE: 39 weeks *Ncoa5*+/- vs. *Ncoa5*+/+ (upregulated)

KEGG Pathways	p	FDR
mmu05340 Primary immunodeficiency	3.37E-05	0.008157
mmu04115 p53 signaling pathway	8.85E-05	0.008157
mmu03010 Ribosome	8.93E-05	0.008157
mmu00980 Metabolism of xenobiotics by cytochrome P450	0.000154	0.01052
mmu05214 Glioma	0.000375	0.020553
mmu04110 Cell cycle	0.00049	0.022397
mmu05219 Bladder cancer	0.001128	0.044147
mmu05215 Prostate cancer	0.001884	0.064538
mmu05218 Melanoma	0.002884	0.087806
mmu00982 Drug metabolism - cytochrome P450	0.003407	0.09335
mmu04068 FoxO signaling pathway	0.004516	0.104072
mmu00480 Glutathione metabolism	0.004587	0.104072
mmu05204 Chemical carcinogenesis	0.004938	0.104072
mmu04974 Protein digestion and absorption	0.007356	0.143971
mmu05220 Chronic myeloid leukemia	0.011757	0.214755
mmu05206 MicroRNAs in cancer	0.013049	0.22346
mmu04514 Cell adhesion molecules (CAMs)	0.014668	0.23642

Cytoplasmic p21^{WAF1}-positive hepatocytes are progressively increased in preneoplastic livers of *Ncoa5*$^{+/-}$ male mice and are located adjacent to expanded hepatic progenitor cell populations.

p21^{WAF1} was previously demonstrated to have anti-proliferative or anti-apoptotic activity dependent on the nuclear or cytoplasmic location, respectively. Moreover, although loss of p21^{WAF1} was reported to promote HCC development in some mouse models of HCC, while other studies suggest increased p21^{WAF1} is promotive of HCC development (Ehedego et al., 2015; Marhenke et al., 2014). The aforementioned elevated mRNA levels of *Cdkn1a* (p21^{WAF1}) and *Gadd45β* in the livers of *Ncoa5*$^{+/-}$ male mice were confirmed with qRT-PCR analysis. Both gene transcript levels were progressively elevated in the livers of *Ncoa5*$^{+/-}$ male mice at the ages of 8, 20, and 39 weeks compared to age matched *Ncoa5*$^{+/+}$ livers (Figure 2A). In contrast, expression of *Cdkn2a* (p16^{INK4A}), another cell cycle kinase inhibitor, was not significantly changed in

the livers of *Ncoa5*$^{+/-}$ male mice compared to the control wild type livers (Figure S1A). Immunohistochemistry (IHC) staining for p21^{WAF1} in the liver confirmed increased expression at 8, 20, and 39 weeks of age compared to *Ncoa5*$^{+/+}$ control mice of the same age. Surprisingly, immunostaining revealed that p21^{WAF1} was predominantly localized to the cytoplasm of hepatocytes (Figure 2C and D). Intriguingly, the majority of cytoplasmic p21-positive hepatocytes were also positive for Ki-67 staining, indicating that these cells are proliferative. Elevated p21^{WAF1} and *Gadd45β* were accompanied by progressively increased expression of several cytokines and chemokines including IL-6, CCL2, CCL8, and CXCLl1 in the livers of *Ncoa5*$^{+/-}$ male mice (Figure 2E). These results prompted further investigation into the effects of cytoplasmic p21^{WAF1} positive cells on hepatocarcinogenesis. These factors may influence hepatic stem/progenitor cell differentiation and proliferation, early cancer-initiating events. Therefore, hepatic cells were examined for expression of Epcam and Keratin 19 (CK-19), common cell markers expressed in both hepatic progenitor cells and cholangiocytes. In agreement with the observation of increased cytokines and chemokines, immunostaining analysis showed an increase in Epcam and CK-19 positive cells (Figure 2F and G). These positively stained cells were specifically found in the areas surrounding p21^{WAF1}-positive cells in the livers of *Ncoa5*$^{+/-}$ mice and were frequently found adjacent to portal tracts (Figure H). These data suggest that upregulation of p21^{WAF1} in hepatocytes may stimulate proliferation and differentiation of hepatic progenitor cells, which plays a critical role in promoting HCC development in *Ncoa5*$^{+/-}$ mice.

Figure 2. Cytoplasmic p21^{WAF1} positive hepatocytes are progressively increased in preneoplastic livers of *Ncoa5*$^{+/-}$ male mice

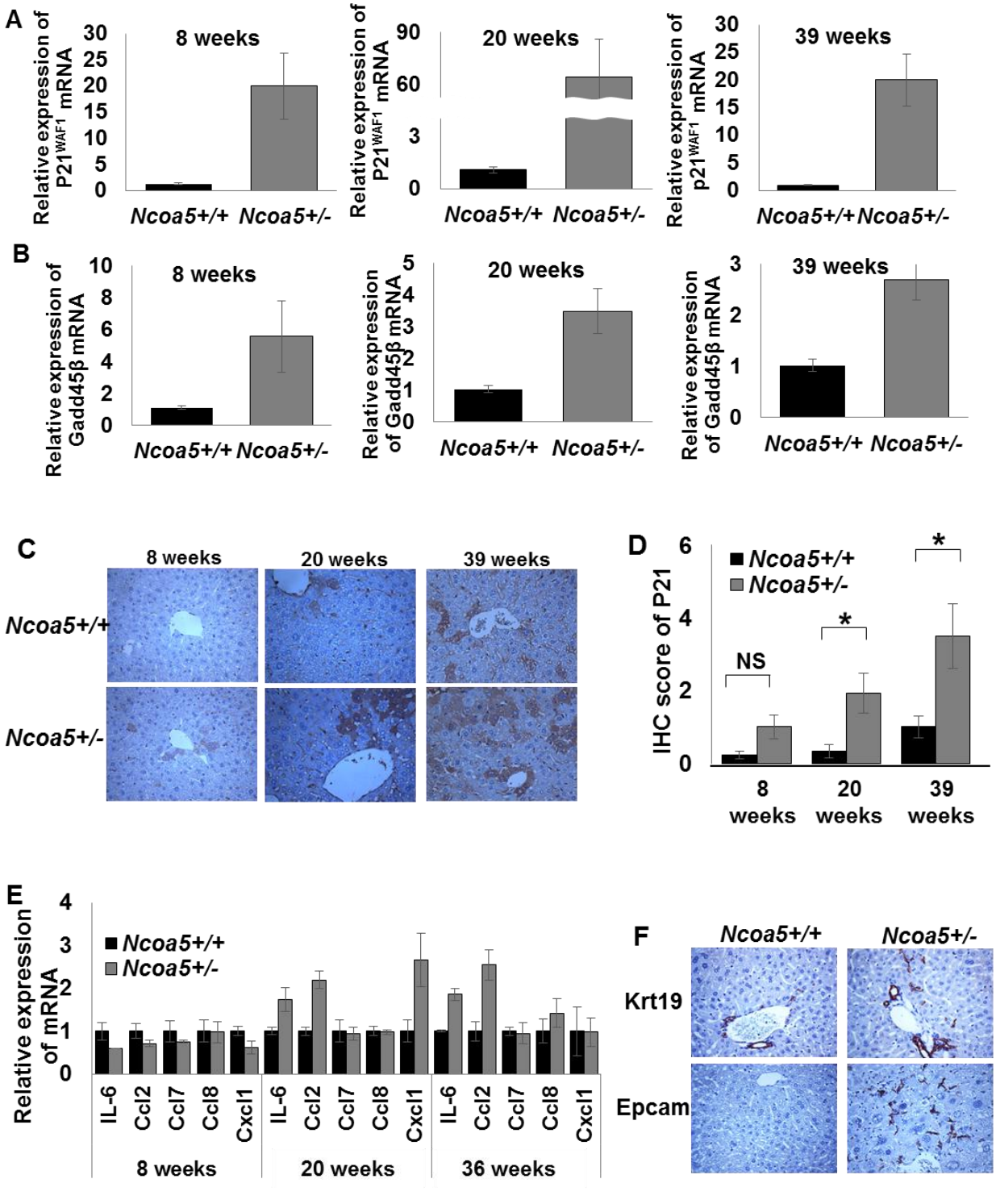
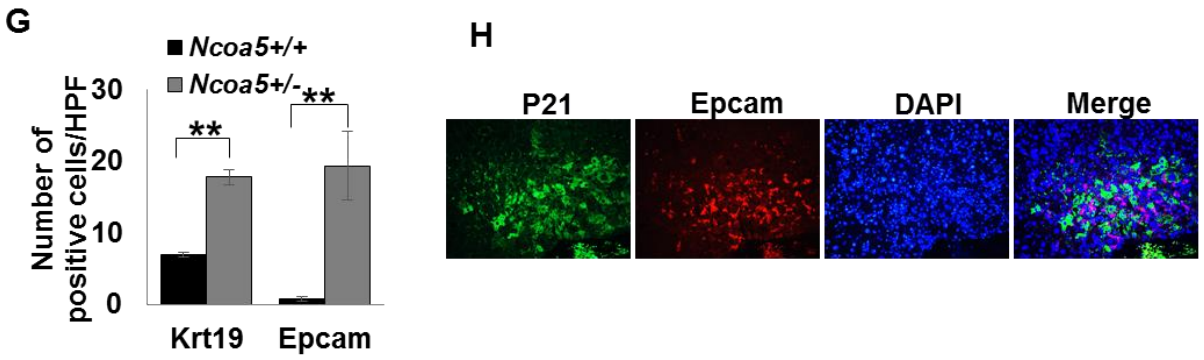


Figure 2 (cont'd)



mRNA expression of (A) *Cdkn1a* ($p21^{WAF1}$) and (B) *Gadd45β* in the livers of male mice at the ages of 8, 20, and 39 weeks. (C) Representative images of immunohistochemical staining of $P21^{WAF1}$ in the livers of $Ncoa5^{+/+}$ and $Ncoa5^{+/-}$ male mice at 8, 20, and 39 weeks of age. (D) Quantification of IHC staining score for $P21^{WAF1}$ in the livers of $Ncoa5^{+/+}$ and *Ncoa5+/-* male mice. (E) mRNA expression of several chemokine and cytokine factors in the livers of male mice. (F) Representative images of immunohistochemical staining of Krt19- or Epcam-positive hepatic progenitor cells in the livers of $Ncoa5^{+/+}$ and $Ncoa5^{+/-}$ mice. (G) Quantification of Krt19- or Epcam-positive hepatic progenitor cells in livers of $Ncoa5^{+/+}$ and $Ncoa5^{+/-}$ male mice. (H) Immunofluorescence double staining of $P21^{WAF1}$ (green) and Epcam (red) in the liver of a 20 week old male $Ncoa5^{+/-}$ mouse. Cell nuclei are stained with DAPI.

Metformin lowers serum ALT, reduces macrophage liver infiltration, and decreases HCC incidence in *Ncoa5*$^{+/-}$ male mice.

To investigate potential causal relationships between dysregulated signaling pathways and HCC development, a cohort of male $Ncoa5^{+/-}$ and $Ncoa5^{+/+}$ C57BL/6 mice were given drinking water with or without metformin for 40 weeks. Treatment began when

mice were post pubertal at 8 weeks of age and metformin treatment was stopped at 48 weeks of age. Mice were then observed for development of preneoplastic lesions and HCC until 18 months (78 weeks) of age. A dramatic reduction in HCC incidence was observed in male *Ncoa5*$^{+/-}$ mice treated with metformin versus *Ncoa5*$^{+/-}$ untreated mice (Figure 3A). Chronic inflammation, cell death, and regeneration play crucial roles in the early stages of HCC development in human patients and mouse models of HCC, including *Ncoa5*$^{+/-}$ male mice in a C57BL/6/129SVJ mixed and BALB/c genetic background (S. Gao, et al., 2013). Therefore, impacts of metformin on preneoplastic liver lesions were assessed in a cohort of 39 week old, male, C57BL/6 mice, dosed with metformin in the water for 31 weeks compared to mice given plain drinking water. Serum ALT levels were markedly increased in *Ncoa5*$^{+/-}$ male C57BL/6 mice compared to *Ncoa5*$^{+/+}$ male C57BL/6 mice, but were reduced in *Ncoa5*$^{+/-}$ male C57BL/6 mice dosed with metformin (Figure 3B). Serum levels of AFP were also increased in *Ncoa5*$^{+/-}$ mice versus control wildtype mice; however, metformin did not significantly reduce serum AFP in *Ncoa5*$^{+/-}$ mice (Figure 3C). *Ncoa5*$^{+/-}$ male mice of the BALB/c strain were previously shown to have increased macrophage infiltration in the liver, and increased macrophage infiltration is also observed in *Ncoa5*$^{+/-}$ livers of the C57BL/6 strain used here. Interestingly, metformin treatment reduced the number of macrophages in the *Ncoa5*$^{+/-}$ C57BL/6 livers (Figure 3D and E). Gross histological analysis of 39 week old livers did not reveal any statistically significant differences in liver morphology and lesions between *Ncoa5*$^{+/+}$ and *Ncoa5*$^{+/-}$ mice, treated with metformin or not. This is likely due to spontaneous age associated lesions that frequently arise in the livers of C57BL/6 mice (Thoolen et al., 2010). In addition, no differences in body weight or liver to body

weight ratio were observed for 39 or 78 week old cohorts, without tumors (Figure S2). Tumor bearing mice expectedly show a decrease in overall body weight and a large increase in liver to body weight ratio (Figure S2). Consistently, the serum AFP and ALT levels in the 78 week old cohort were statistically unchanged by metformin treatment status (Figure S2). Also, there was not a statistically significant difference in NAFLD severity between metformin treated and untreated mice at 72 weeks of age; this is possibly due to recurrence, as metformin treatment stopped when mice were 48 weeks old (Figure S2). These results indicate that metformin treatment may not have a significant impact on the ultimate NAFLD progression if treatment is terminated.

Figure 3. Metformin treatment reduces serum ALT, macrophage liver infiltration and HCC incidence in *Ncoa5+/-* male mice

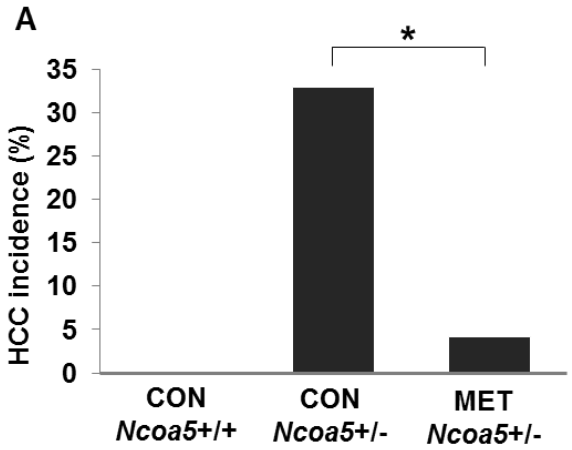
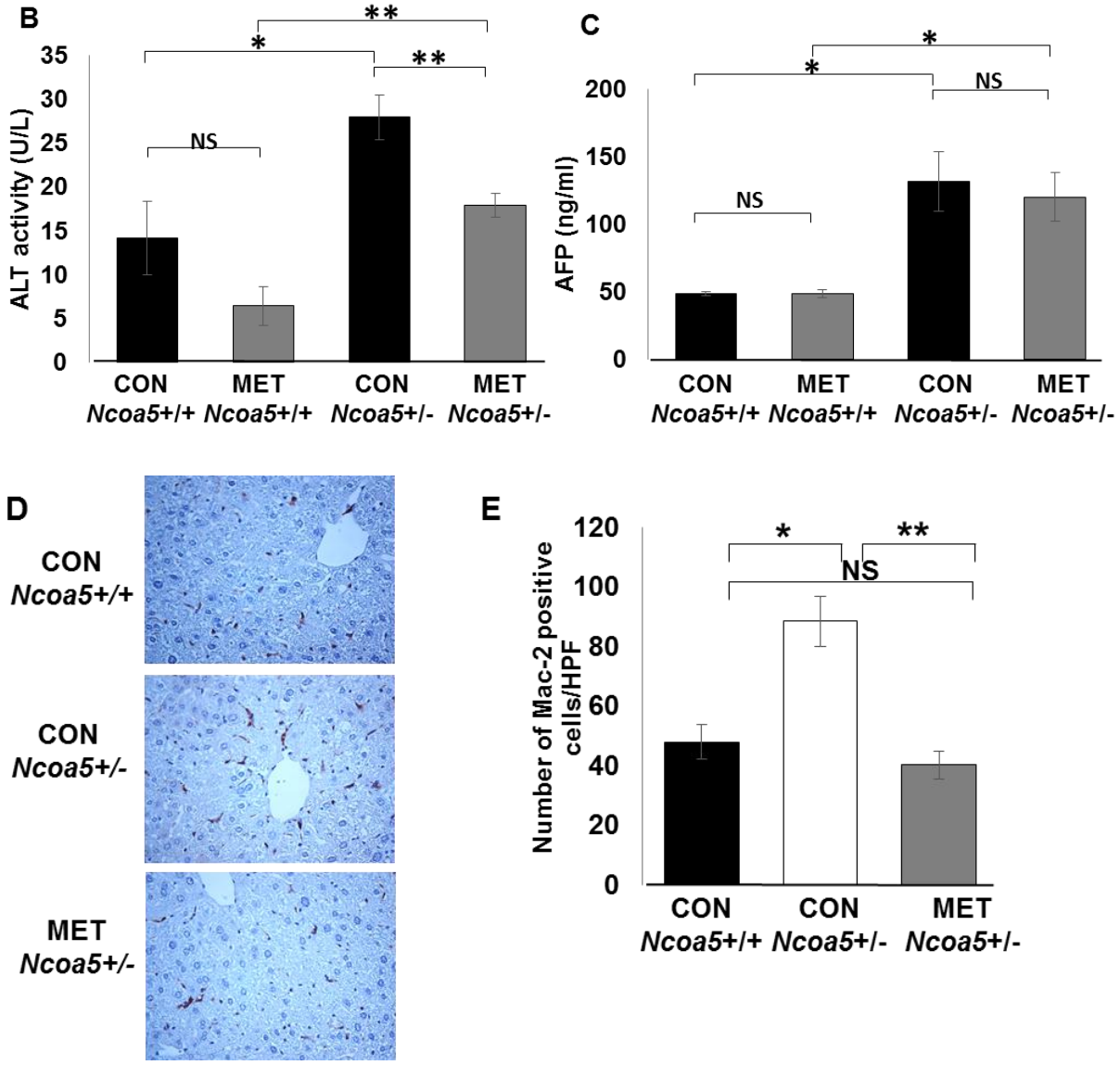


Figure 3 (cont'd)



(A) Percent HCC incidence in C57BL/6 *Ncoa5*$^{+/-}$ male mice at 78 weeks of age either untreated (CON) or dosed with metformin (MET) from 8 to 48 weeks of age. Fisher Exact Probability Test was used to determine significance (n=24; * $p<0.05$). (B) Serum alanine aminotransferase (ALT) activity and (C) Serum Alpha fetoprotein (AFP) levels in 39 week old male mice either untreated or treated for 31 weeks beginning at 8 weeks of age. (D) Representative images of immunohistochemical staining of Mac-2 in 39 week

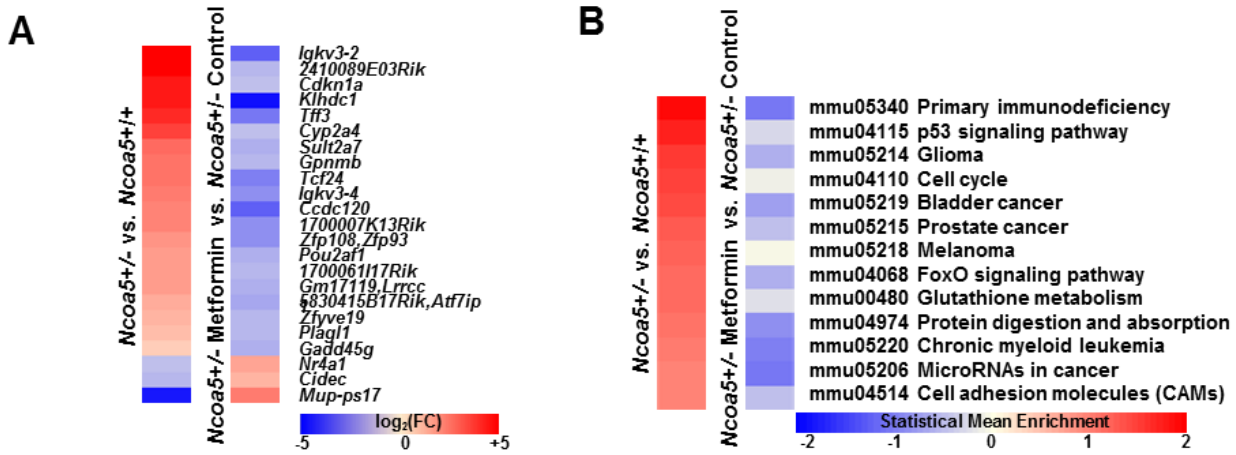
Figure 3 (cont'd)

old mice. (G) Quantification of Mac-2 positive cells in 39 week old mice (n=4-5; *p<0.05; **p<0.01).

Metformin reduces p53 pathway and p21^{WAF1} gene expression in *Ncoa5*$^{+/-}$ male mice.

In view of the HCC preventive effects observed with metformin treatment, the next goal was to identify impacts of metformin on gene expression, to identify potential molecular mechanisms that underlie metformin mediated HCC prevention. Total liver RNA samples were acquired from 39 week old *Ncoa5*$^{+/+}$ and *Ncoa5*$^{+/-}$ mice that had received metformin treatment for 31 weeks or not. This is the same 39 week cohort described previously. RNA sequencing was carried out to generate a gene expression profile for each sample. The main comparison was between *Ncoa5*$^{+/-}$ metformin treated and *Ncoa5*$^{+/-}$ untreated groups, with intent to determine if expression of genes and pathways found altered in *Ncoa5*$^{+/-}$ untreated vs *Ncoa5*$^{+/-}$ untreated groups, were reversed by metformin treatment. Cuffdiff analysis identified that *Cdkn1a* (p21^{WAF1}) mRNA expression was significantly reversed with metformin treatment along with expression of 22 other genes (Figure 4A). GAGE analysis revealed that several pathways, including p53 and primary immunodeficiency pathways that had elevated expression in *Ncoa5*$^{+/-}$ mice, displayed a reduced trend with metformin treatment to be more like *Ncoa5*$^{+/+}$ control mice (Figure 4B).

Figure 4. Metformin reverses a group of genes altered in *Ncoa5*$^{+/-}$ male mouse liver



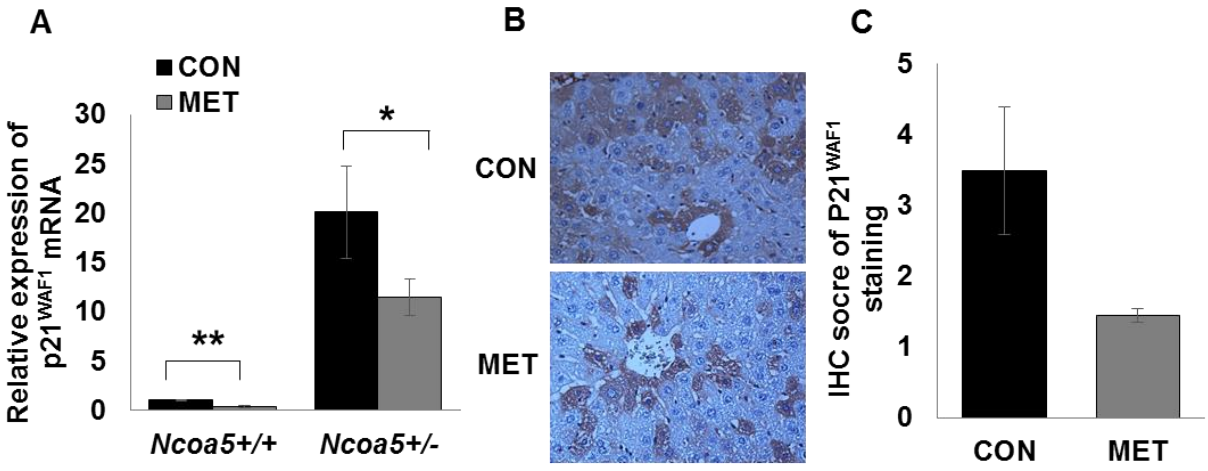
(A) Genes significantly altered in 39 week old *Ncoa5*$^{+/-}$vs *Ncoa5*$^{+/+}$ that are significantly reversed by metformin treatment as determined by Cuffdiff. Significance threshold is FDR<0.05. (B) KEGG pathways with significant upregulated (FDR<0.25) expression in 39 week old *Ncoa5*$^{+/-}$vs *Ncoa5*$^{+/+}$ that displayed a reversed trend with metformin treatment as determined by GAGE. No reversed pathways displayed FDR<0.25.

Metformin reduces cytoplasmic p21^{WAF1}-positive hepatocytes and hepatic progenitor cell numbers.

Quantitative RT-PCR analysis confirmed that *Cdkn1a* (p21^{WAF1}) mRNA levels, that were dramatically increased in 39 week old *Ncoa5*$^{+/-}$ vs *Ncoa5*$^{+/+}$ mouse liver, were reduced by 43% with metformin treatment (Figure 5A). Consistent with the reduced expression of *Cdkn1a* (p21^{WAF1}) mRNA, IHC staining score was reduced, reflecting the reduced number of cytoplasmic p21^{WAF1}-positive hepatocytes with metformin treatment (Figure 5B,C). Since hepatic progenitor cells were seen elevated in *Ncoa5*$^{+/-}$ liver, the

hepatic progenitor markers Epcam and CK-19 were checked in livers of mice treated with metformin. Both Epcam and CK-19 positive hepatic progenitor cells were dramatically decreased in metformin treated *Ncoa5*$^{+/-}$ mice compared to the untreated *Ncoa5*$^{+/-}$ group (Figure 5D,E). These data indicate that metformin treatment over 31 weeks suppresses the appearance of aberrant cytoplasmic p21^{WAF1}-positive hepatocytes and reduces the expansion of hepatic progenitor cells in livers of *Ncoa5*$^{+/-}$ male mice, thereby reducing initiation of HCC. Previous studies have shown metformin action via activation of AMP kinase (Shaw et al., 2005; Zhou et al., 2001). AMPK activation, detected by Thr172 phosphorylation, was seen in 39 week old *Ncoa5*$^{+/-}$ male mice treated with metformin for 31 weeks (Figure 5F).

Figure 5. Metformin reduces cytoplasmic p21^{WAF1}-positive hepatocytes and hepatic progenitor cell number, and increases pAMPK in *Ncoa5*$^{+/-}$ male mouse liver



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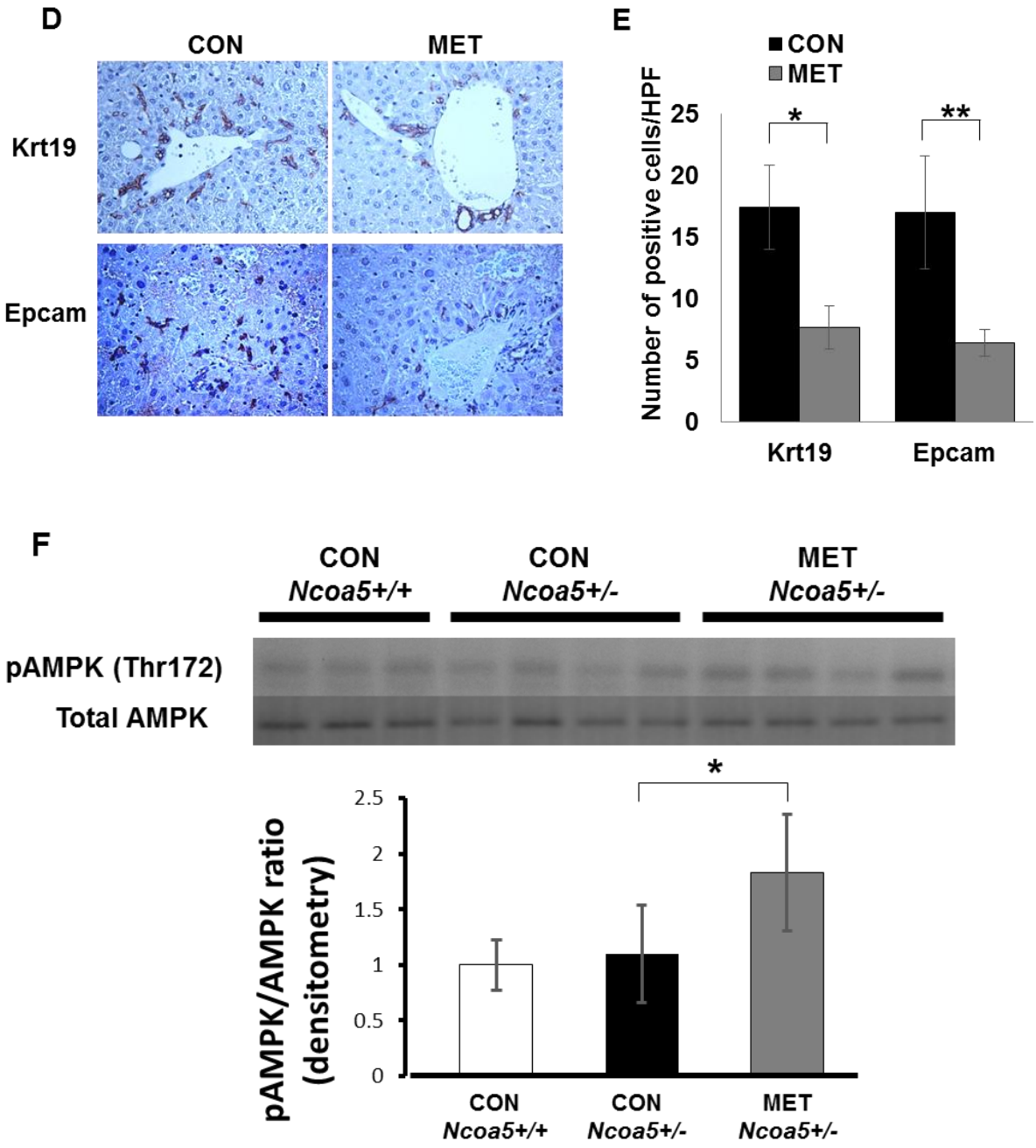


(A) mRNA expression of liver $p21^{WAF1}$ in *Ncoa5*$^{+/+}$ and *Ncoa5*$^{+/-}$ untreated (CON) or metformin treated (MET) mice at 39 weeks of age (n=4-5; * $p<0.05$; ** $p<0.01$). (B) Representative images and (C) quantification of immunohistochemical staining of $p21^{WAF1}$ in 39 week old *Ncoa5*$^{+/-}$ male mice that were untreated (CON) or metformin

Figure 5 (cont'd)

treated (MET) (n=5; * p<0.05). (D) Representative images and (E) quantification of Immunohistochemical staining of Krt19 and Epcam in 39 week old *Ncoa5*$^{+/-}$ male mice at 39 weeks of age that were untreated (CON) or metformin treated (MET) (n=5; *p<0.05; ** p<0.01). (F) Immunoblotting and quantification by densitometry of pAMPK (Thr172) and total AMPK in 39 week old male mice that were untreated (CON) or metformin treated (MET) (n=3,4; * p<0.05).

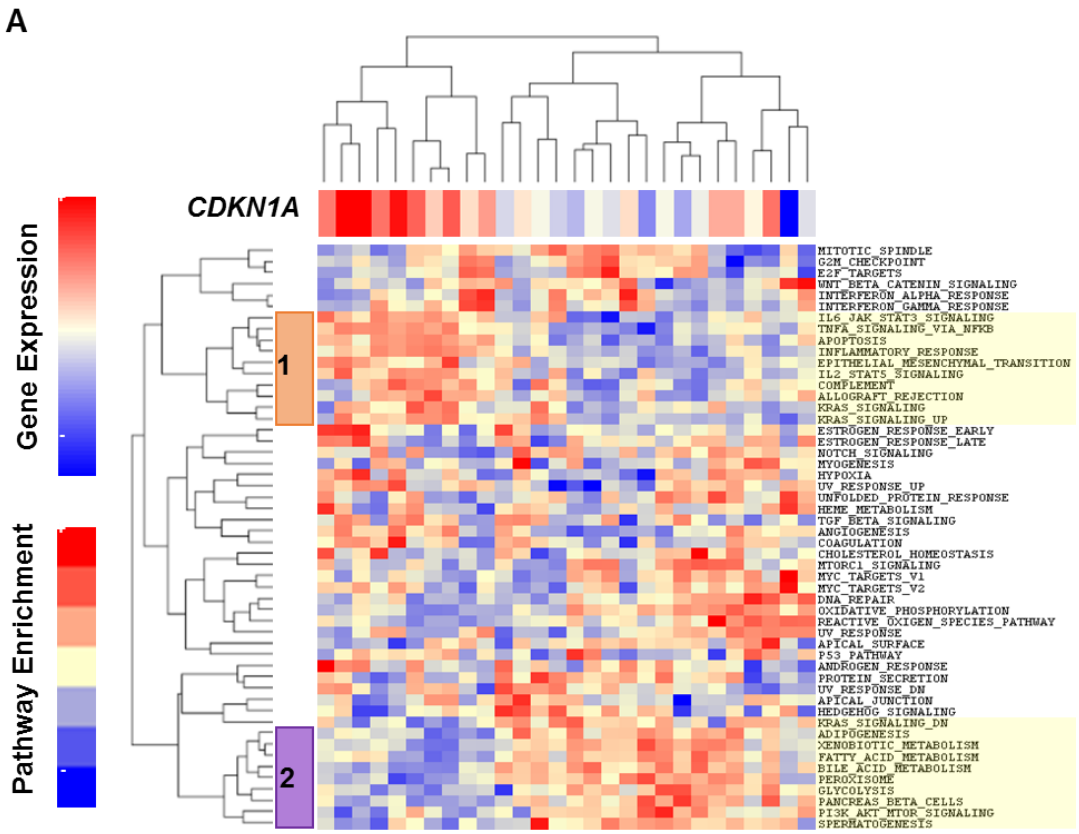
ssGSEA identifies a p21^{WAF1} high expressing subgroup of human HCC-adjacent tissues, and p21^{WAF1} expression negatively correlates with enrichment of the metformin-treated *Ncoa5*$^{+/-}$ gene signature.

To assess the relevance of increased p21^{WAF1} expression to human HCC, non-cancerous HCC-adjacent samples from a cohort of human male patients were analyzed for Hallmark Pathway enrichment with ssGSEA, and results were used to carry out unbiased clustering (Figure 6A). When *CDKN1A* expression level was displayed, above the cluster heatmap, for each sample, a high p21^{WAF1} expressing subgroup was apparent. These high p21^{WAF1} expressing samples show a distinct pattern of enrichment in several Hallmark Pathway gene signatures, particularly in the pathways labeled group 1 and 2. Similar to the observations in preneoplastic liver tissues in *Ncoa5*$^{+/-}$ mice, IL6/JAK/STAT3, TNFA/NF-KB and KRAS signaling pathways were positively enriched in high p21^{WAF1} HCC-adjacent samples.

Next, a metformin-treated gene set was created by converting the genes found differentially expressed in metformin treated *Ncoa5*$^{+/-}$ mouse liver to human homologs.

HCC-adjacent samples were scored for enrichment in the metformin-treated gene set and plotted vs. *CDKN1A* (p21WAF1) expression (Figure 6B). A significant negative regression coefficient was determined, indicating that sample expression similarity to the metformin-treated gene set correlates with reduced *CDKN1A* expression. This correlation is consistent with what is seen in liver of *Ncoa5*$^{+/-}$ male mice treated with metformin. These results suggest that a high p21^{WAF1} preneoplastic niche may impact risk of HCC development and also be responsive to metformin treatment.

Figure 6. Male HCC-adjacent tissue clustering based on Hallmark Pathway enrichment identifies *CDKN1A* (p21^{WAF1}) as a segregating factor, and gene set enrichment associated with metformin-treated preneoplastic tissue is inversely correlated with *CDKN1A* (p21^{WAF1}) expression in human HCC-adjacent tissue



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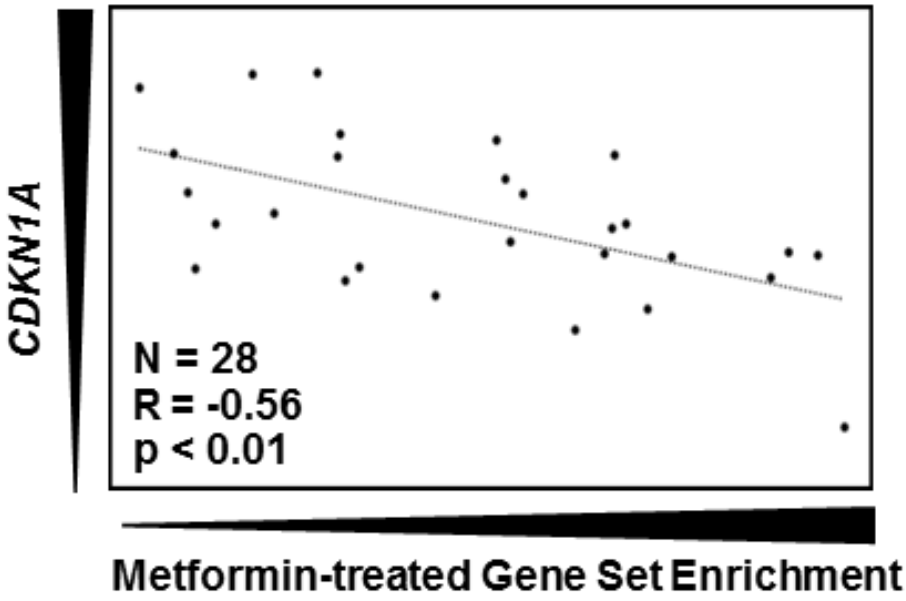


(A) Unbiased clustering of Hallmark Pathways was carried out in human, male HCC-adjacent tissues (TCGA-LIHC, n=28) analyzed by ssGSEA. Each row and column represents clustered Hallmark Pathways and HCC-adjacent patient samples, respectively. *CDKN1A* ($p21^{WAF1}$) expression is displayed for each sample above the ssGSEA clustered heatmap. Pathway clusters labeled 1 and 2 correspond to groups of Hallmark Pathways that display a distinct pattern of enrichment, which aligns with a high *CDKN1A* expressing subgroup of the HCC-adjacent male human samples.

(B) A metformin-treated gene set was created by converting the genes found differentially expressed in metformin treated *Ncoa5*$^{+/-}$ mouse liver and converting the gene names to human homologues.

DISCUSSION

Prevention and treatment options for HCC are presently limited, underscoring the need to understand molecular mechanisms of HCC development and identify new therapeutic targets. We demonstrate that altered expression of a set of genes, including expression of $p21^{WAF1}$ in inflammatory and p53 pathways, correlates with NCOA5 deficiency in the preneoplastic livers of the *Ncoa5*$^{+/-}$ mouse model of HCC and a subgroup of the adjacent non-tumorous and tumorous tissues of human HCC. Long-term Metformin treatment, exclusively during a preneoplastic period, dramatically reduced tumor incidence in the *Ncoa5*$^{+/-}$ mouse model of HCC at 78 weeks. Metformin also reversed increases in p53 pathway gene member expression in the liver and specifically reduced aberrant cytoplasmic $p21^{WAF1}$-positive hepatocytes after 31 weeks of treatment. An increase in a myeloid-derived suppressor cell expression signature was also significantly reduced with metformin. Our study suggests a molecular mechanism underlying HCC and uncovers new actions for metformin in the prevention of HCC.

Activation of p53 and NF-KB signaling pathways play a critical role in the early stages of hepatocarcinogenesis.

Accumulating evidence has suggested there are common pathogenic pathways and processes important to HCC development among the various mechanisms of hepatocarcinogenesis (Farazi & DePinho, 2006). Specifically, inactivation of p53 signaling is a consistent event in HBV-,HCV-, and aflatoxin-B1-induced HCC, whereas, activation of NF-KB and MAPK signaling pathways, leading to increased secretion of many chemokines and cytokines are also frequently detected in HBV-, HCV-, and alcohol-induced hepatocarcinogenesis. A recent comprehensive and integrative

genomic analysis of human HCC cases revealed that HCCs can be segregated according to p53 pathway activation status. A large group of tumors displayed relative increases in functional p53 pathway activation, whereas some HCC cases, even with wildtype p53, had decreased p53 target gene expression in tumors (TCGA-Research-Network, 2017). Furthermore, a comprehensive human and mouse model transcriptomics analysis of cirrhotic livers identifies inflammation factors, including *TNF* and *IL6* as important dysregulated genes that contribute to HCC development (Nakagawa et al., 2016). Taken together, the evidence strongly supports a theory that changes in p53 signaling and inflammation-associated pathways fundamentally contribute to HCC development.

In agreement with this theory, we previously reported enhanced expression of IL-6 and TNF-α in Kupffer cells of the *Ncoa5*$^{+/-}$ mouse model of HCC indicating increased hepatic inflammation (S. Gao, et al., 2013). In this current study, we extend our understanding of the NCOA5 deficient liver at both early and late preneoplastic stages by identifying changes in MAPK, TNF, NF-KB and p53 signaling pathways in early stages of hepatocarcinogenesis in the *Ncoa5*$^{+/-}$ mouse model of HCC. Results suggest that cytoplasmic hepatocyte expression of p21^{WAF1}, a key p53 target gene, is critical to HCC development.

At the earlier, 20 week time point, EdgeR differential gene expression analysis produced small list of 25 genes with significantly altered expression. Interestingly, the human homologues of many of these differentially regulated genes correlate with human *Ncoa5* expression, indicating that transcriptional regulatory mechanisms involving Ncoa5 may be similar between mice and humans. Upon further observation of

the 25 differentially expressed genes, we noticed many of the upregulated genes were NF-KB signaling targets. Increased NF-KB activity in the *Ncoa5*$^{+/-}$ mouse livers is consistent with reports in humans that describe increased NF-KB activity in most liver diseases that increase HCC risk.

To improve our interpretation of biological meaning from differential gene expression results, we used the gene set analysis tool, GAGE. Gene set analysis is a powerful approach to analyze expression data based on previous pathway knowledge. Gene set analysis results reflect more systems level changes within an experimental group, which is often more informative and more systematic than determining gene relationships manually as was done for NF-KB targets above. GAGE was specifically selected for its superior ability to handle analyses with a small number of biological replicates, as is the case here. GAGE analysis corroborated our initial observations about upregulated NF-KB pathway activity, and also found twenty other gene sets with upregulated expression in *Ncoa5*$^{+/-}$ liver. Important cancer pathways found upregulated in 20 week old *Ncoa5*$^{+/-}$ livers include the MAPK, TNF, NF-KB and p53 signaling pathways. We wanted to identify expression changes that are most important for driving hepatic inflammation to HCC, therefore we also investigated *Ncoa5*$^{+/-}$ livers at 39 weeks, a later, preneoplastic timepoint. GAGE analysis revealed that the p53 signaling pathway expression not only remained increased at 39 weeks, but was even more significantly elevated compared to the 20 week timepoint. In the liver context, progressively increasing p53 pathway activity is likely indicative of increasing cell stress and DNA damage. We also identified 20 individual genes, with Cuffdiff, that had similarly dysregulated expression at both early and late time points. Of these common,

altered genes, Cdkn1a ($p21^{WAF1}$) and Gadd45 were of interest, being known targets of p53. Thus, our findings suggest that activation of p53 signaling pathway is a fundamental process that precedes HCC development. Given the fact that DNA damage, cell death and regeneration are the common processes in hepatocarcinogenesis induced by diverse risk factors, it is conceivable that activation of p53 signaling pathway plays an important role in early HCC development.

Hepatocytes with cytoplasmic p21 expression possibly contribute to the establishment of a HCC tumorigenic niche.

Previous studies have demonstrated that $p21^{WAF1}$ in cellular functions are largely dependent on $p21^{WAF1}$ subcellular localization and interactions with other cellular proteins. When localized to the cytoplasm, p21 appears to act as an oncoprotein by inhibiting apoptosis or possibly promoting cell proliferation (Abbas & Dutta, 2009). Using HBx transgenic mice (Xg) and HBx-transfected hepatoma cell lines, Yano and colleagues demonstrated that overexpression of cytoplasmic p21 plays an important role in hepatocarcinogenesis. cytoplasmic $p21^{WAF1}$ induced by HBx is suggested as a cell cycle promoter through pRb inactivation mediated by increased cyclin D1 levels (Yano et al., 2013). In addition, Bearss et al. reported that $p21^{WAF1}$ deficiency had minimal effects on the rate of mammary tumors in the context of Myc overexpression, but accelerated tumor development with overexpression of the Ras oncogene (Bearss, Lee, Troyer, Pestell, & Windle, 2002). These results point to a strong cell context dependent effect on $p21^{WAF1}$ function. Consistent with the findings in mammary tumors, $p21^{WAF1}$ ablation also differentially affects HCC incidence in different mouse models. Hepatocyte specific IKKγ knockout mice, which lose NF-KB signaling and develop HCC

due to continued hepatocyte cell death and regeneration, display increases in DNA damage and HCC incidence with knockout of *Cdkn1a* ($p21^{WAF1}$) (Ehedego, et al., 2015). However, the *Mdr2*$^{-/-}$ knockout mouse, which develops HCC in the context of chronic inflammatory liver injury, exhibits delayed HCC formation when combined with $p21^{WAF1}$ knockout (Marhenke, et al., 2014). In human cancer development, p21 is frequently reported as having a tumor suppressor role; however, increased $p21^{WAF1}$ expression in liver cirrhosis has been reported to correlate with risk of HCC occurrence (Wagayama et al., 2001). Moreover, as HCCs developed and progressed, increased cytoplasmic expression of $p21^{WAF1}$ was observed (Shiraki & Wagayama, 2006). Together, these results suggest that $p21^{WAF1}$ action is highly context dependent, and under specific conditions, increased $p21^{WAF1}$ facilitates HCC formation.

To investigate $p21^{WAF1}$ further in *Ncoa5*$^{+/-}$ mice, we first used qRT-PCR to confirm elevated $p21^{WAF1}$ mRNA in *Ncoa5*$^{+/-}$ liver at 8, 20, and 39 weeks of age; thus, this elevated expression is sustained throughout early adulthood. Immunohistochemistry staining, using an antibody against $p21^{WAF1}$, displays increasing cytoplasmic $p21^{WAF1}$ protein expression in the liver as *Ncoa5*$^{+/-}$ mice age. As discussed previously, cytoplasmic $p21^{WAF1}$ is associated with an oncogenic function. The $p21^{WAF1}$ IHC staining clearly displays cytoplasmic subcellular localization. In addition, some of these $p21^{WAF1}$-positive cells are also positive for Ki-67, indicating they are proliferative; therefore, we propose that hepatocytes with cytoplasmic $p21^{WAF1}$ expression may play a protumorigenic role in *Ncoa5*$^{+/-}$ mice by providing a tumorigenic niche for the tumor initiating cells.

Consistent with the idea of a tumorigenic niche, we showed increased expression of known cytokines and chemokines, Il-6, Ccl2, Ccl8, and Cxcl1 using qRT-PCR. These factors are known to induce hepatic progenitor cell differentiation and proliferation, and hepatic progenitor cell expansion is implicated in hepatocarcinogenesis. In fact, experiments staining for progenitor cells did reveal an increased hepatocyte progenitor cell number. Interestingly, these cells were found in high density regions intermixed with, but always distinct from p21-positive cells. A relationship between p21-positive hepatocytes and hepatic progenitor cells seems likely. A simple possibility is that the p21-positive hepatocytes are secreting cytokines and chemokines and stimulating progenitor differentiation and proliferation. In agreement with this possibility, previous studies have demonstrated that increased $p21^{WAF1}$ expression is an immediate early response to differentiation inducers, and is uncoupled from G1 arrest in the presence of deregulated c-myc (Steinman et al., 1994). Importantly, in support of our findings, transgenic mice that specifically overexpress p21WAF1/CIP1 in both the nucleus and cytoplasm of hepatocytes exhibited increased number of hepatic oval cells, which are bipotential progenitors of hepatocytes and bile ductal cells, and increased formation of nodular foci of hepatocytes in the liver. Interestingly, most proliferative hepatocytes in the nodule foci also did not contain detectable expression of p21WAF1/CIP1 (Wu et al., 1996). Given the finding that increased hepatocyte progenitor cells are implicated as the origin of tumor initiating cells (Finkin et al., 2015; He et al., 2013; X. Luo et al., 2016; Sia, Villanueva, Friedman, & Llovet, 2017), we, therefore, propose that hepatocytes with cytoplasmic p21WAF1/CIP1 expression may play a protumorigenic role by providing a protumorigenic niche for promoting transformation of tumor initiating cells, although its

overexpression alone is not sufficient to cause malignant transformation of hepatocytes (Wu, et al., 1996). However, we have not shown increased inflammatory cytokines and chemokines specifically in $p21^{WAF1}$-positive hepatocytes, and alternatively, these inflammatory cytokines and chemokines may also be secreted by stimulated immune cells such as Kupffer cells. Even so, the close proximity of p21-positive hepatocytes and progenitor cells suggests an important relationship that may impact the course of HCC development.

Early preneoplastic metformin treatment is sufficient to prevent later HCC development, possibly by disrupting the HCC protumorigenic niche.

Animal models of HCC have determined the importance of early metformin treatment to observe HCC preventive effects. Metformin was able to reduce HCC incidence in a high fat induced HCC mouse model only if treatment began prior to the onset of liver pathology (Tajima, et al., 2013). Consistent with this finding, DePeralta et al. found that metformin was not able to protect against DEN induced HCC in rats if given after the first signs of cirrhosis, although it was effective if given earlier. Interestingly, this group proposed activation of the hepatic progenitor cell compartment as a crucial step that segregates effective and ineffective metformin preventive abilities (DePeralta, et al., 2016). These results suggest and important early role in metformin prevention of HCC. However, both of these studies maintained metformin treatment throughout carcinogenesis and HCC progression; therefore, it remains unknown if metformin treatment during an exclusive preneoplastic period is sufficient to prevent HCC development later in life. We propose that metformin is able to prevent HCC by specifically disrupting the formation of a protumorigenic niche in the liver.

The experiments described above clearly show that aberrant cytoplasmic expression of p21 in hepatocytes, along with changes in a number of cancer related pathways correlate with the development of HCC in *Ncoa5*$^{+/-}$ male mice. These results are consistent with the prooncogenic roles of these changes suggested by previous studies (Abbas & Dutta, 2009; Marhenke, et al., 2014); however, they do not address whether these alterations indeed lead to HCC development in *Ncoa5*$^{+/-}$ mice. Therefore, given the previous observations that metformin inhibited HCC development in mouse models of HCC, we examined whether metformin prevented HCC development in *Ncoa5*$^{+/-}$ mice and carried out an unbiased experimental approach to determine which altered factors may be improved when HCC development was inhibited.

Our studies demonstrate that HCC incidence at 78 weeks of age was dramatically reduced when metformin is given between 8 and 48 weeks of age. Hepatocyte damage and regeneration are known to contribute to HCC incidence, and increased serum levels of Alanine Amino Transferase (ALT) are an indicator of hepatocyte damage. Serum ALT levels are increased in *Ncoa5*$^{+/-}$ mice compared to wildtype mice at 39 weeks of age. Metformin was able to reduce serum ALT levels in *Ncoa5*$^{+/-}$ male mice. This indicates that Metformin is able to protect *Ncoa5*$^{+/-}$ livers from damage. Metformin did not impact serum levels of Alpha Feto Protein (AFP), a common biomarker that is elevated in the serum with the occurrence of HCC. HCC tumors were not observed at this 39 week time point, so it is not surprising that metformin does not reduce serum AFP levels. The increased macrophage infiltration observed in the *Ncoa5*$^{+/-}$ liver was completely reversed to be similar with wildtype mice. This likely indicates that metformin reduces inflammation in the *Ncoa5*$^{+/-}$ liver. Although there are

indications of inflammation and liver damage in 39 week old *Ncoa5*$^{+/-}$ mice, there was no statistically significant gross impact of metformin on the liver at this 39 week time point. These experiments were carried out in C57BL/6 mice, unlike our previous results that used the Balb/c strain. The Balb/c *Ncoa5*$^{+/-}$ male mice displayed marked histological changes at a similar age, including widespread NAFLD/NASH and even mild fibrosis. The milder phenotype at 39 weeks in C57Bl/6 *Ncoa5*$^{+/-}$ male mice was also reflected in a lower overall incidence of HCC in this mouse strain. Indeed, only one-third of the C57BL/6 *Ncoa5*$^{+/-}$ males acquired HCC at 18 months of age, versus three-quarters of mice acquiring HCC in the Balb/c strain. Mouse strain differences are known to greatly impact phenotype severity. It seems that C57BL/6 mice are more resistant to liver pathology induced by *Ncoa5* disruption, although the mechanisms for this are currently unknown. Importantly, metformin was still shown to improve HCC incidence in these C57BL/6 mice even with the lower overall incidence. Consistent with previous results in mice and humans, metformin did not significantly alter overall body weight or liver to body weight ratios at 39 weeks of age or in non-tumorigenic 79 week old mice. Mice with tumors become cachectic and lose overall body weight while the liver to bodyweight ratio increases rapidly. This is a common occurrence with many cancers. NAFLD/NASH was frequently observed in *Ncoa5*$^{+/-}$ male mouse livers at 79 weeks of age, and metformin had no impact on NAFLD severity. Previous studies have identified a reduced severity of NAFLD with metformin treatment in animal models. However, the mice in this study stopped taking metformin at 48 weeks of age. If metformin did initially reduce NAFLD severity, the protection may have been lost after metformin treatment ceased and NAFLD likely recurred by 79 weeks of age.

To understand how metformin affects gene expression, we included a metformin treatment group in the RNA sequencing of the 39 week old mouse cohort for both wildtype and *Ncoa5*$^{+/-}$ experimental groups. Metformin treatment began at 8 weeks of age and continued until mice were harvested at the 39 week time point. Our main focus was to understand how metformin reduces HCC incidence in *Ncoa5*$^{+/-}$ male mice on the level of gene and pathway expression. Therefore, we focused on results comparing the *Ncoa5*$^{+/-}$ metformin treated group vs. the *Ncoa5*$^{+/-}$ untreated group and the *Ncoa5*$^{+/-}$ untreated group vs. the *Ncoa5*$^{+/+}$ untreated group. In other words, we want to know which genes have altered expression in the *Ncoa5*$^{+/-}$ mice that is then corrected by metformin treatment. Twenty-three individual genes were found to be significantly reversed by metformin treatment using Cuffdiff for differential gene expression analysis. Our previously identified gene of interest, *Cdkn1a* (p21^{WAF1}), that was found elevated in *Ncoa5*$^{+/-}$ mouse liver, was significantly reduced by metformin treatment according to Cuffdiff results. Decreased p21^{WAF1} by metformin is consistent with an *in vitro* finding that metformin was able to inhibit high-glucose induced p21^{WAF1} expression in Hek293 cells (Molnar, Millward, Tse, & Demaine, 2014). No elevated pathways in *Ncoa5*$^{+/-}$ livers, as determined by GAGE, were reduced with an FDR <0.25; however, the previously discussed p53 pathway that was elevated in *Ncoa5*$^{+/-}$ liver did trend reduced with metformin treatment.

We verified that metformin reversed the increases in p21^{WAF1} mRNA by qRT-PCR. IHC staining score confirmed that cytoplasmic p21^{WAF1} protein expression was reduced by metformin treatment in 39 week old *Ncoa5*$^{+/-}$ mice. A corresponding reduction in Krt19 and Epcam positive cells indicates that metformin treatment also

reduced the number of hepatic progenitor cells in the *Ncoa5*$^{+/-}$ liver at 39 weeks. We propose that metformin may disrupt an HCC protumorigenic niche by preventing aberrant p21^{WAF1} expression in hepatocytes and reducing the differentiation and proliferation of hepatic progenitor cells, thereby preventing later HCC formation.

As a counter argument to our proposed importance of p21^{WAF1} reduction by metformin, it may be suggested that increased p21^{WAF1} in the liver is simply a protective response to genomic stress induced by inflammation observed in the liver. It may be said that metformin has been shown to reduce inflammation, and for this reason, a reduced p21^{WAF1} expression is just a passive indicator of reduced inflammation-mediated DNA stress. In response, it is true that that metformin may reduce p21^{WAF1} by direct and/or indirect mechanisms such as reduced inflammation. However, it is important to emphasize current findings of the oncogenic potential of p21^{WAF1} in certain contexts, especially with regard to cytoplasmic localization. We favor the concept that increased p21^{WAF1} expression is likely promotive of HCC development and that metformin disrupts tumorigenesis by indirect and possibly more direct impacts on p21^{WAF1} expression.

Although we propose a metformin impact on p21^{WAF1} expression to reduce HCC incidence, there are likely multiple mechanisms of action by metformin that contribute to its cancer inhibitory effects. These different actions probably disable tumor development differently at the various stages of hepatocarcinogenesis. For example, metformin was shown to reduce expression of lipogenic enzymes in the livers of a DEN induced mouse model of HCC that displayed decreased tumor multiplicity with metformin treatment (Bhalla, et al., 2012). *De novo* lipogenesis is a common feature of liver tumors

compared to normal adjacent tissue and is especially important for rapidly dividing cells of a growing tumor (Yahagi et al., 2005). A metformin decrease in liver lipogenic enzymes may prevent HCC formation by limiting damage induced by NAFLD, or by other signaling mechanisms but it will more likely interfere with stages of rapid tumor expansion.

There is a consensus that one major mechanism of metformin action for inhibition of cancer relies on AMPK activation, which will lead to downstream inhibition of mTOR signaling that leads to decreased protein synthesis and reduced proliferation. Increased activation of AMPK is known to impact a broad range of pathways and functions that could feasibly impact every essential step of carcinogenesis. For example, AMPK activation has been shown to reduce expression of key enzymes in *de novo* lipogenesis such as fatty acid synthase and acetyl-CoA carboxylase and thus could lead to an effect as seen in the mouse model mentioned previously. Metformin has also been shown to reduce chronic inflammation, a finding corroborated by our results (Grisouard et al., 2011). Reduced inflammation by metformin is also likely to be at least partly due to AMPK activation (Salminen, Hyttinen, & Kaarniranta, 2011). Indeed, our results do show that AMPK Thr172 phosphorylation was increased in *Ncoa5*$^{+/-}$ liver with metformin treatment. In conclusion, metformin exerts its beneficial effects by many different mechanisms including activation of AMPK; we propose a reversal of increased cytoplasmic p21^{WAF1} expression in hepatocytes as another potential mechanism by which metformin prevents HCC development.

ssGSEA of Hallmark gene sets identifies human liver tissue similarities to *Ncoa5*$^{+/-}$ liver and may be similarly responsive to metformin treatment.

Previous studies have identified increased p21^{WAF1} expression livers of patients with HCC risk factors. As mentioned previously Wagayama and collegues identified that increased p21^{WAF1} expression in human liver is positively correlated with HCC risk in HBV and HCV infected individuals (Wagayama, et al., 2001). This finding indicates there may be a human population with liver expression that displays similarities to *Ncoa5*$^{+/-}$ mice.

In order to investigate p21^{WAF1} expression in human liver, we carried out single sample GSEA (ssGSEA) using expression data from HCC-adjacent samples supplied by the Cancer Genome Atlas. Clustering by Hallmark pathways revealed the interesting finding that a subgroup of samples consistently had a high *CDKN1A* (p21^{WAF1}) expression while two other subgroups generally had low *CDKN1A* (p21^{WAF1}) expression. The high *CDKN1A* (p21^{WAF1}) subgroup correspond to an increased Hallmark Pathway enrichment score in group 1 and a decreased enrichment score in group 2. This result suggests that p21 expression segregates pathways in HCC-adjacent tissue, and the high p21^{WAF1} subgroup displays similarities to *Ncoa5*$^{+/-}$ mouse liver with positive enrichment in IL6/JAK/STAT3, TNFA/NF-KB and KRAS signaling pathways.

We also assessed the ability of the metformin-treated expression signature to predict p21 expression in HCC-adjacent tissue. The metformin-treated expression signature is simply the list of genes identified differentially expressed in liver of *Ncoa5*$^{+/-}$ mice treated with metformin versus untreated *Ncoa5*$^{+/-}$ mouse liver. We do see a significant correlation between our metformin-treated gene signature and *CDKN1A*

(p21^{WAF1}) expression in human HCC-adjacent samples. This correlation indicates that patients who display expression similar to our metformin treated mice tend to have lower p21 mRNA expression. If metformin action on gene expression is generally similar between mice and humans, then metformin treatment may correlate with reduced *CDKN1A* (p21^{WAF1}) expression in human liver.

In summary, the above indicates that metformin is able to prevent HCC in *Ncoa5*$^{+/-}$ male mice in part by reversing aberrant cytoplasmic p21^{WAF1} expression in hepatocytes and likely disrupting an early HCC tumorigenic niche. Our findings highlight the importance of early prevention and may prompt further research regarding the effect of metformin on p21^{WAF1} expression in HCC development.

AUTHOR CONTRIBUTIONS

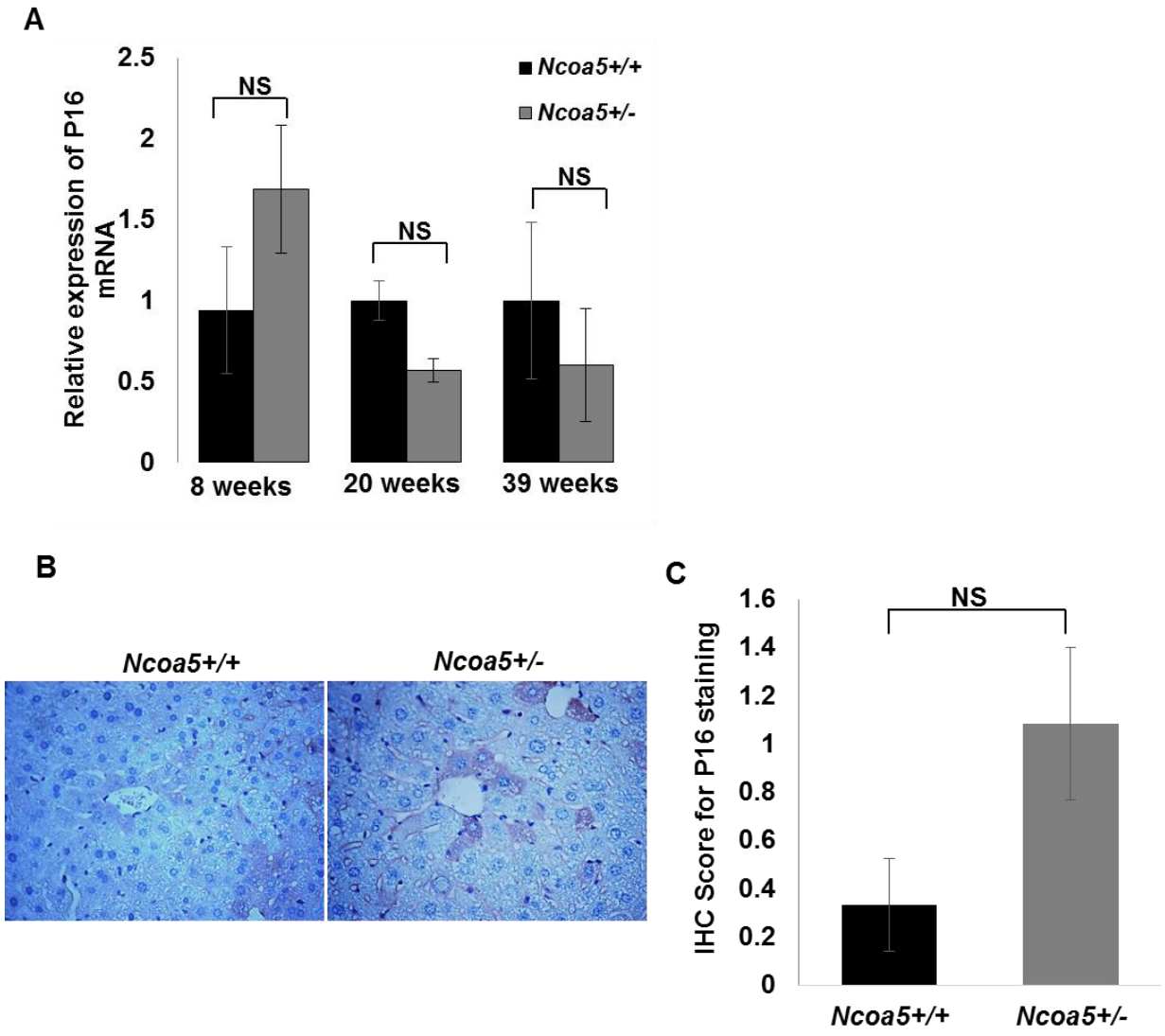
This work was a highly collaborative effort among the authors. Authors of this work include me (Mark Williams), Xinhui Liu, Jake Reske, Elliot Ensink, Matti Kiupel, and Hua Xiao. I am the lead author of this work carried out with Hua Xiao as the principal investigator. I was responsible for mouse breeding, metformin treatment and active animal monitoring with the help of Michigan State University Veterinary staff. At indicated ages, I was responsible for euthanizing the mice and collecting tissue for later analysis. Analysis of collected tissue was a collaborative effort between me, Xinhui Liu, and Jake Reske. One of my roles in sample processing was preparation and quality analysis of RNA for sequencing. RNA sequencing was carried out by indicated organizations and raw read data was received in return. Processing of RNA sequencing data was a collaborative effort between me, Jake Reske, and Elliot Ensink. I and Jake Reske collaborated to further analyze RNA sequencing results to determine key

findings. I focused on identifying altered pathways in mice using GAGE, and Jake Reske focused on external databases and bioinformatics tools to apply findings in mice to human samples. Matti Kiupel analyzed tissue histology as our collaborating veterinary pathologist. Further mouse tissue analysis was carried out as collaboration between me, Xinhui Liu and Jake Reske. RT-qPCR, immunoblotting, and serum marker analysis was carried out as a combined effort. Xinhui Liu provided further expertise in carrying out immunostaining procedures. Hua Xiao supervised the project as principal investigator.

APPENDIX

SUPPLEMENTAL INFORMATION

Figure S1. P16 is not significantly increased in preneoplastic livers of *Ncoa5*$^{+/-}$ mice



(A) mRNA levels of p16 in the livers of male mice at the ages of 8,20, and 39 weeks of age. (B) Immunohistochemistry of P16 in the liver of 20 week old male mice. (C) Quantification of IHC staining score of p16 in the liver of *Ncoa5*$^{+/+}$ or *Ncoa5*$^{+/-}$ male mouse liver.

Figure S2. Liver and body weight is unchanged by metformin

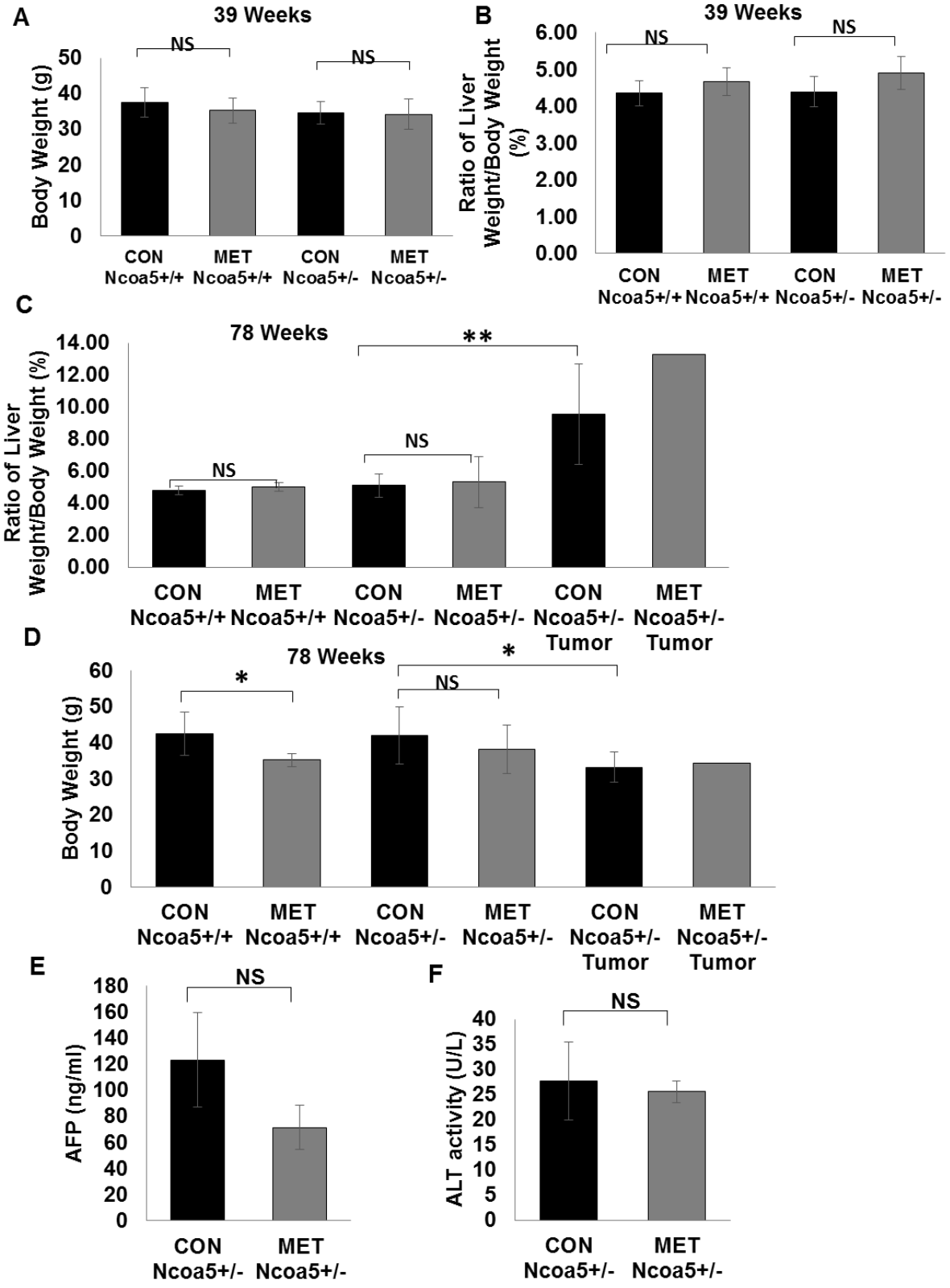


Figure S2 (cont'd)

(A) Body weight and (B) liver/body weight ratio of 39 week old mice. (C) Liver/body weight ratio and (D) body weight of 78 week old mice. (E) AFP and (F) ALT serum concentrations in non-tumorous mice at 78 weeks.

CHAPTER 3 PROSPECTIVE STUDIES

FUTURE STUDIES

In the previous chapter, we have shown that *Ncoa5*$^{+/-}$ male mice display critical gene expression changes at early and late preneoplastic stages of hepatocarcinogenesis, including increased cytoplasmic expression of p21^{WAF1} in hepatocytes along with p53 and inflammatory pathway dysregulation. We demonstrate that metformin treatment during the preneoplastic stage in adult *Ncoa5*$^{+/-}$ male mice is sufficient to prevent HCC later in life, and that metformin treatment may disrupt a protumorigenic niche in the liver, in part by reducing cytoplasmic p21^{WAF1} expression in hepatocytes, decreasing numbers of hepatic progenitor cells and modulating liver inflammation.

Our study supports an elevated p21^{WAF1} expression mechanism of HCC and uncovers a new action of metformin in the prevention of HCC. Even so, many questions remain that require further experimental analysis. Possible future studies to address important remaining questions are proposed.

Contribution of individual cell types to HCC in the *Ncoa5*$^{+/-}$ mouse model of HCC.

The current *Ncoa5*$^{+/-}$ mouse model of HCC was produced by systemic targeting of *Ncoa5* for disruption. Therefore, many different cell types likely contribute to the recognized mouse phenotypes. With regard to HCC incidence, questions remain regarding which cell type initiates the progression to liver pathology observed, and specifically, what are the mechanisms by which p21^{WAF1} expression is elevated in hepatocytes. Two probable possibilities are proposed. First, Ncoa5 deficiency may cause intrinsic changes to hepatocytes. A role for NCOA5 function in hepatocytes is suggested by *in vitro* experiments, as induced overexpression of NCOA5 in human

HCC cell lines slows growth and promotes senescence. $P21^{WAF1}$ was observed to increase with NCOA5 overexpression (X. Liu et al., 2017). It is possible that the change in $p21^{WAF1}$ expression observed in *Ncoa5*$^{+/-}$ male mouse hepatocytes is due to an intrinsic effect of targeted Ncoa5 disruption. For example, if NCOA5 is involved in p21 repression through direct promoter binding, partial NCOA5 reduction could result in increased $p21^{WAF1}$ expression. This idea could be tested through chromatin immunoprecipitation assays by pulling down NCOA5 and carrying out PCR for regions of the p21 promoter. Luciferase assays could also be carried out to identify direct impacts of NCOA5 on regulation of the *Cdkn1a* promoter. Secondly, liver pathology and increased $p21^{WAF1}$ could be due to indirect effects on hepatocytes. Gillespie et al. determined that NCOA5 plays a central role in mediating crosstalk between pro-inflammatory and anti-inflammatory pathways in bone marrow derived macrophages (Gillespie, et al., 2015). These results indicate that perturbed *Ncoa5* expression in macrophages may impact inflammatory processes. It is possible that partial Ncoa5 disruption promotes liver inflammation by directly altering inflammatory activity in Kupffer cells. Improperly active Kupffer cells may affect hepatocyte signaling in a paracrine manner and also initiate downstream inflammatory cascades that could lead to hepatocyte damage and increased $p21^{WAF1}$ expression in hepatocytes.

To address these questions, a conditional knockout mouse has been produced to dissect cell-type specific functions of *Ncoa5 in vivo* in the Xiao laboratory. Cre mediated deletion of *Ncoa5* in either hepatocytes or the myeloid lineage will determine each cell compartment contribution to liver pathology and also assess whether increased $p21^{WAF1}$ expression in hepatocytes is a cell autonomous or non-cell autonomous effect.

Dissect the relationship between cytoplasmic p21^{WAF1}-positive hepatocytes and hepatic progenitor cells.

We observe a close spatial relationship between p21^{WAF1}-positive hepatocytes and hepatic progenitor cells in 39 week old *Ncoa5*$^{+/-}$ male mouse liver, as staining for these cells occurs in discrete periportal areas of the liver. Of note, p21^{WAF1} staining was not detected to be co-localized with progenitor cell markers, indicating the presence of two, distinct, intermingled cell populations. Marhenke et al. revealed an important role for p21^{WAF1} in hepatic progenitor cell expansion, as *Cdkn1a* (p21^{WAF1}) deletion in the *Mdr2* knockout mouse model of HCC severely impaired hepatic progenitor cell expansion during liver injury (Marhenke, et al., 2014). To genetically confirm the role of p21 in Ncoa5 deficiency induced HCC, *Ncoa5*$^{+/-}$ mice could be crossed with p21^{WAF1} knockout mice to create a double knockout, and the importance of p21^{WAF1} to hepatic progenitor cells could be confirmed in a different genetic mouse model of HCC. Better though, would be to investigate potential signaling mechanisms between these two groups of cells with cell specific deletion of p21^{WAF1}.

We propose that chemokine and cytokine factors may be produced by p21^{WAF1} hepatocytes to stimulate progenitor cell expansion and possibly progression to HCC. A similar idea has been investigated in the DEN induced model of HCC, where cell clusters determined to be HCC progenitor cells were shown to acquire autocrine IL-6 signaling to stimulate growth and malignant progression (He, et al., 2013). Ideally, we could use a similar approach to isolate these cell clusters and analyze them *in vitro* and in orthotopic transplantation studies. However, it is currently unknown whether these high p21^{WAF1} hepatocyte regions would remain intact with standard liver cell isolation

procedures. Instead of isolation, we could carry out immunostaining of tissue sections to detect chemokine and cytokine factor expression in these $p21^{WAF1}$-positive hepatocytes. Microdissection techniques could also be used to acquire a tissue sample consisting only of these hepatic progenitor/$p21^{WAF1}$-positive hepatocyte regions that then could be analyzed for levels of gene expression. Another simple option is to observe the development of these regions over time.

A reasonable hypothesis is that these $p21^{WAF1}$-positive hepatocytes are initially required to support hepatic progenitor cell populations until the progenitor cells develop pathological autocrine growth signaling mechanisms described by He et al. Analysis of liver tissue from 12 month *Ncoa5*$^{+/-}$ mice may reveal HCC progenitor cell nodules in the absence of $p21^{WAF1}$-positive hepatocytes. Results from the proposed experiments would better characterize the exact mechanisms of HCC development and progression in the *Ncoa5*$^{+/-}$ mouse model.

The role of cytoplasmic vs nuclear $p21^{WAF1}$ expression may also be tested. IFN-β treatment has been shown to shift cytoplasmic $p21^{WAF1}$ to the nucleus in HBx transgenic mice (Yano, et al., 2013). Interestingly, IFN-β administration, from three to six months of age, prevented HCC incidence at 18 months (Yamazaki et al., 2008). It would be interesting to test the ability of IFN-β to alter cytoplasmic localization of $p21^{WAF1}$ to the nucleus of $p21^{WAF1}$-positive hepatocytes in *Ncoa5*$^{+/-}$ mice. If this transition was confirmed, then impact of $p21^{WAF1}$ subcellular localization on hepatic progenitor cell expansion could be studied.

Determine possible mechanisms of metformin impact on p21^{WAF1}.

We report a decreased expression of *Cdkn1a* (p21^{WAF1}) in *Ncoa5*$^{+/-}$ liver with metformin treatment compared to untreated livers. However, specific mechanisms are currently unknown. As discussed in the previous chapter, metformin likely modifies p21^{WAF1} expression by direct and indirect actions. *In vitro* results indicate that a direct impact of metformin on p21^{WAF1} expression is highly context dependent. For example, metformin has been shown to decrease elevated p21 levels in Hek293 cells treated with high-glucose (Molnar, et al., 2014). However, metformin decreases proliferation in many cancer cell lines and increased p21^{WAF1} expression was reported in these cases (Cai et al., 2013; Yi et al., 2013). Metformin affect transformed cells differently than non-cancerous cells and may also have an impact on a specific population of cells within a cell line, such as the EpCAM+ population (Saito et al., 2013). A luciferase reporter assay could identify impacts of metformin treatment on the *Cdkn1a* promoter in various *in vitro* cell lines and conditions. It would also be interesting to isolate p21^{WAF1}-positive cells from *Ncoa5*+/- livers and perform metformin treatment *in vitro*, to test effects of metformin on p21^{WAF1} expression, in the absence of other liver cells. AMPK activators/inhibitors may also suggest if p21^{WAF1} expression changes occur through metformin ability to activate AMPK.

If NCOA5 was shown to inhibit p21^{WAF1} expression by localizing to the *Cdkn1a* promoter in hepatocytes, then partial depletion of NCOA5 could explain the observed increase in p21^{WAF1} expression, as stated previously. Chromatin immunoprecipitation could then be used to test metformin ability to modulate NCOA5 recruitment to the *Cdkn1a* promoter to reduce p21^{WAF1} expression.

Characterize the immune cell contribution to hepatic inflammation observed in *Ncoa5*$^{+/-}$ male mice and determine how metformin impacts these populations in the liver.

Ncoa5$^{+/-}$ male mice display increased expression of inflammatory pathways in the liver at a young age. We have previously identified an increased recruitment of pro-inflammatory macrophages to the *Ncoa5*$^{+/-}$ liver in male mice that likely play an important role in HCC formation (S. Gao, et al., 2013). However, information regarding other immune cell types in the inflamed *Ncoa5*$^{+/-}$ liver is lacking. We plan to identify immune cell populations and that are increased in *Ncoa5*$^{+/-}$ livers. This is done by isolating non-parenchymal cell populations in the liver and then using cell surface markers to enrich for the different general cell populations. Flow cytometry of surface-marker stained cells is carried out to count the numbers of various immune cell types and to indicate their inflammation status. It is expected that there will be increased numbers of various inflammation associated cells, such as CD8+ T cells, in the *Ncoa5*$^{+/-}$ vs. *Ncoa5*$^{+/-}$ livers. Metformin has been shown to reduce chronic inflammation systemically and specifically in the liver (Saisho, 2015; Woo et al., 2014). It would also be an appropriate next step to assess how metformin treatment impacts specific immune cell populations in the *Ncoa5*$^{+/-}$ mouse liver. This will highlight immune cell compartments critical for development of HCC in *Ncoa5*$^{+/-}$mice and also identify potential mechanisms of metformin action in the inflamed liver.

CHAPTER 4 NCOA5 DISRUPTION AND SNCOA5 OVEREXPRESSION RESULT IN REDUCED CELL PROLIFERATION AND INHIBITED G2/M CELL CYCLE PROGRESSION

ABSTRACT

Nuclear Receptor Coactivator 5 (NCOA5) is transcriptional coregulator with varied functions that depend on context. The *Ncoa5*$^{+/-}$ mouse model identifies NCOA5 as a haploinsufficient tumor suppressor in hepatocellular carcinoma (HCC). In order to better understand the function of NCOA5 in the context of HCC, we carry out CRISPR mediated knockout of NCOA5 in human HCC cell lines. Interestingly, NCOA5 disruption results in decreased cell proliferation likely due to inhibited cell cycle progression at the G2/M phase, and increased cellular senescence. This phenotype is very similar to the phenotype observed with NCOA5 inducible overexpression reported previously.

NCOA5 is also known to have splice variants, and the most prominent protein that results from alternatively splicing in humans is sNCOA5. sNCOA5 overexpression within HCC cell lines indicates that this shorter protein maintains at least a partial function of the full length NCOA5 and displays a similar albeit seemingly more mild phenotype than full length NCOA5 overexpression. Our work identifies a dose dependent requirement of NCOA5 for optimal proliferation and cell cycle progression within human HCC cell lines.

INTRODUCTION

Great scientific effort has been put forth to identify genes and to understand their functions. Various genetic and biochemical techniques are commonly used to elucidate gene function, including knockout and overexpression studies, which are often followed by phenotypic characterization and biochemical techniques such as Western blot and protein-protein interaction assays. CRISPR technology has allowed for simple protein coding gene knockout experiments to be performed both *in vivo* and *ex-vivo*. These knockout studies are easily carried out in immortalized cell lines and have become a foundational approach to understand gene functions.

The functions of Nuclear Receptor Coactivator 5 (Ncoa5) are not fully understood and are likely varied. However, the work by Sauvé et al. has provided an introductory understanding of potential Ncoa5 functions. They identify NCOA5 as a nuclear protein with transcriptional co-regulator abilities that interacts with and modifies transcriptional effects of several nuclear receptors such as ER-α, ER-β, RVR, and REV-ERB-α (Sauvé, et al., 2001). These nuclear receptors carry out a wide range of functions including impacts on cell cycle progression. For example, NCOA5 was found to interact with HTATIP2 to regulate transcription of cell cycle regulator c-Myc (Jiang, et al., 2004). These studies highlight the important role of NCOA5 as a nuclear receptor coregulator; however, NCOA5 likely has many other uncharacterized functions. Unknown NCOA5 actions are indicated by a genetic mouse knockout of NCOA5. This work determined that NCOA5 is a haploinsufficient tumor suppressor and that heterozygous *Ncoa5* deletion results in Hepatocellular Carcinoma (HCC) development in male mice (S. Gao, et al., 2013).

Recent work to understand NCOA5 tumor suppressor function has utilized an inducible overexpression system in human HCC cell lines. Liu and colleagues inducibly overexpressed either wild type NCOA5 or a mutant form of NCOA5 found in HCC patients, NCOA5T445A. This work identifies NCOA5 as an important regulator of cell cycle by inducing G2/M phase cell cycle arrest, and this is likely an important part of NCOA5 tumor suppressing ability. Interestingly, the single amino acid change, T445A, impairs NCOA5 ability to inhibit HCC cell proliferation (X. Liu, et al., 2017).

Alternative splicing of mRNAs is known to provide a greater diversity in the proteins produced by a protein coding gene and can greatly impact protein function. NCOA5 is no exception and one predominant alternatively spliced form has been identified in humans. This alternative splicing results in an extended exon 7, a codon frame shift, and an early stop codon resulting in a shortened protein called shortNCOA5 (sNCOA5). Interestingly, sNCOA5 was found to be elevated in HCC tumors while full length NCOA5 was seen reduced in many HCC tumors compared to adjacent tissue. This may indicate that NCOA5 deficiency, possibly by increasing alternative splicing to enhance expression of the sNCOA5 splice form, may contribute to human HCC development (S. Gao, et al., 2013).

CRISPR technology has paved the way for easy loss of function analysis both *in vitro* and *in vivo*. CRISPR knockout provides a means of creating specific indels within a targeted region and is now commonly used to mutate specific genes within cell lines to carry out knockout studies. One large benefit as opposed to older RNA interference approaches is the ability to achieve a complete loss of protein expression.

In this study, we carry out CRISPR mediated knockout of Ncoa5 to determine the impacts of complete NCOA5 loss on human cells, and we also perform sNCOA5 overexpression studies to investigate the functions of *NCOA5* in human HCC cell lines and determine possible NCOA5 mediated contributions to HCC development.

EXPERIMENTAL PROCEDURES

Plasmids and cell lines.

CRISPR knockout clonal cell lines were established using the protocol from the Zhang lab (Cong et al., 2013). Briefly, gRNAs were cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid using the BbsI sites and correct orientation was confirmed with Sanger sequencing. Correct plasmids were transfected into target cell lines, HepG2 and PLC/PRF5, using Lipofectamine 300 (Thermo Fisher Scientific). Twenty-four hours post transfection, cells were selected with puromycin, which continued for another 72hrs. Surviving cells were resuspended and plated singly in 96 well plates for expansion. Clonal lines were then confirmed for NCOA5 knockout using Western blot. gRNAs targeting NCOA5 were chosen using the bioinformatic selection approach carried out by the Church laboratory (Mali et al., 2013). *NCOA5* targeting gRNAs used are as follows: Exon 2 of *NCOA5* was targeted with gRNA 5'-GGGTACTTTACCTTCGTGTG-3'; Exon 3 of *NCOA5* was targeted with gRNA 5'-GCAGGAGTGTGCGCGACGTT-3'.

pSIN-EF2-sNCOA5-HA-EGFP was generated by cloning PCR amplified sNCOA5 sequence into the pSIN-EF2-OCT4-EGFP plasmid using the restriction sites SpeI and EcoRI. SpeI was added to 5-prime end of sNCOA5 and an HA tag/EcoRI site was added to the 3-prime end of the sNCOA5 sequence during the PCR. The pSIN-EF2-sNCOA5-HA-EGFP plasmid was used along with the pMd2.G envelope plasmid and the

pCMV=dR8.2 dvpr packaging plasmid to create lentiviral particles using standard methods. Briefly, Hek293t cells were transfected with all three plasmids simultaneously using Lipofectamine 3000 (Thermo Fisher Scientific). Lentiviral particles were collected at 24 and 48hrs post transfection and pooled. Human HCC cell lines HepG2, PLC/PRF5, and Hep3b (ATCC) were then transduced with the lentiviral particles, changing the media 24hrs post transduction. At 48hrs post transduction, cells were resuspended and flow sorted for EGFP on a BD Influx sorter. Cells were then cultured according to the suppliers guidelines (DMEM high glucose + glutamine + 10%FBS).

Cell proliferation, sphere formation and soft agar colony formation assays.

For hemocytometer based cell counting assays, 150,000 cells were plated onto 6cm cell culture treated dishes (Sigma Aldrich) and allowed to proliferate. Cells were resuspended using 0.25% trypsin and estimated total cell counts were acheived using a standard hemocytometer. Trypan blue identified dead cells, which were excluded from the count. Three counts were carried out per dish and were averaged. Experiments were carried out in triplicate and cells were counted at 24hrs, 72hrs, and 120hrs post plating. Media was changed at 48hrs and 96hrs.

For sphere formation assays, 5000 individual HepG2 cells were placed into low adherence 6-well cell culture dishes (Sigma Aldrich) and allowed to grow for 14 days under established growth conditions. Spheres were then stained with crystal violet and entire wells were imaged and counted. Results are expressed as the Average Number of Spheres per well with three wells counted per sample.

Soft agar colony formation assays were carried out by suspending 5000 individual PLC/PRF5 cells in 0.3% agar containing standard media using 3.5cm dishes

and allowing colonies for form for twenty-one days. Colonies were then stained with crystal violet, imaged, and counted. Experiment was performed in triplicate for each sample group. Results are expressed as an average number of colonies per dish.

Cell cycle assay.

HepG2 and PLC/PRF/5 cells were plated onto 10cm dishes, grown to 50% confluence, and then harvested by trypsinization. After centrifugation, the cell cycle assay was performed according to manufacturer directions (propidium iodide Sigma P4170) and at least 10,000 cells were analyzed per sample.

Cell senescence assay using β-galactosidase.

2 x 10^5 cells were plated onto 6-well plates. For β-galactosidase staining, cells were stained using the Senescence β-Galactosidase Staining Kit (CST, #9860) on the fifth day after cell plating as described by the manufacture's protocol. The β-galactosidase positive cells were counted according to the development of blue color. Results are expressed as the percentage of β-galactosidase positive cells out of total cells counted.

Western blot.

Proteins were extracted from HepG2 and PLC/PRF/5 cells and blotted with primary antibodies NCOA5 (1:1000; ab70831, Abcam), β-actin (1:1000; sc-47778, Santa Cruz Biotechnology), and HA-tag (1:1000; cst-3724, Cell Signaling Technology). All membranes were then incubated with IRDye 800CW Goat anti-Rabbit IgG (H + L) or 680RD Donkey anti-Mouse IgG (H + L). Protein bands were visualized with the Odyssey scanner and the Odyssey 2.1 software (LI-COR Biosciences, Lincoln, NE, USA).

RESULTS

NCOA5 CRISPR knockout results in decreased proliferative and sphere forming abilities in human HCC cell lines with an observed delay in the G2/M cell cycle phase and increased senescence.

Previous results indicate that loss of Ncoa5 results in HCC development in mice (S. Gao, et al., 2013). To better understand the role of NCOA5 in cell cycle and proliferation, CRISPR technologies were used to fully disrupt NCOA5 protein expression in human HCC cell lines, HepG2 and PLC/PRF5. Exon 2 and Exon 3 were each targeted for mutation (Figure 7A). Individually isolated cell clones were screened using Ncoa5 immunoblotting to identify clones that lacked Ncoa5 expression (Figure 7B). Sanger sequencing was then carried out on each individual clone to identify the specific indels acquired that result in a codon frame shift and early stop to produce a nonfunctional NCOA5 protein (not shown). Individual knockout clones were then selected for further study. Knockout clones displayed a more epithelial appearance than the control parental cell line (Figure 7C). Knockout clones also tended to grow more slowly than the wild type cell line (Figure 7D). Propidium Iodide staining and flow cytometry revealed an increase in the percentage of cells within the G2/M cell cycle phase and a corresponding decrease in the portion of cells in phase G1 within the Ncoa5 knockout cell populations (Figure 7E). Non-adherent sphere forming ability was also assessed in the HepG2 cell line, in which the Ncoa5 knockout clones display a reduced ability to form non-adherent spheres than wildtype control clones (Figure 7F). The initial experimental comparisons used the mixed parental cell line as a control. However, individual wild type clones were later isolated and used as control samples to

correspond to the individual knockout clones. The results for both mixed parental control and individual clone controls were consistent. Contrary to initial hypotheses, these results indicate that NCOA5 expression is beneficial for growth and adherence independent survival of human HCC cancer cell lines *in vitro*. A follow up study was carried out to assess the senescence state of the PLC/PRF5 cell line. Beta galactosidase staining reveals that NCOA5 knockout clonal populations had higher percentages of β galactosidase positive cells, indicating a higher percentage of senescent cells within the knockout population compared to wild type clonal populations (Figure 7G). These results indicate that loss of NCOA5 expression reduces proliferative abilities, inhibits G2/M cell cycle phase transition, and increases senescence in human HCC cell lines.

Figure 7. NCOA5 CRISPR knockout reduces proliferation and increases senescence in human HCC cell lines

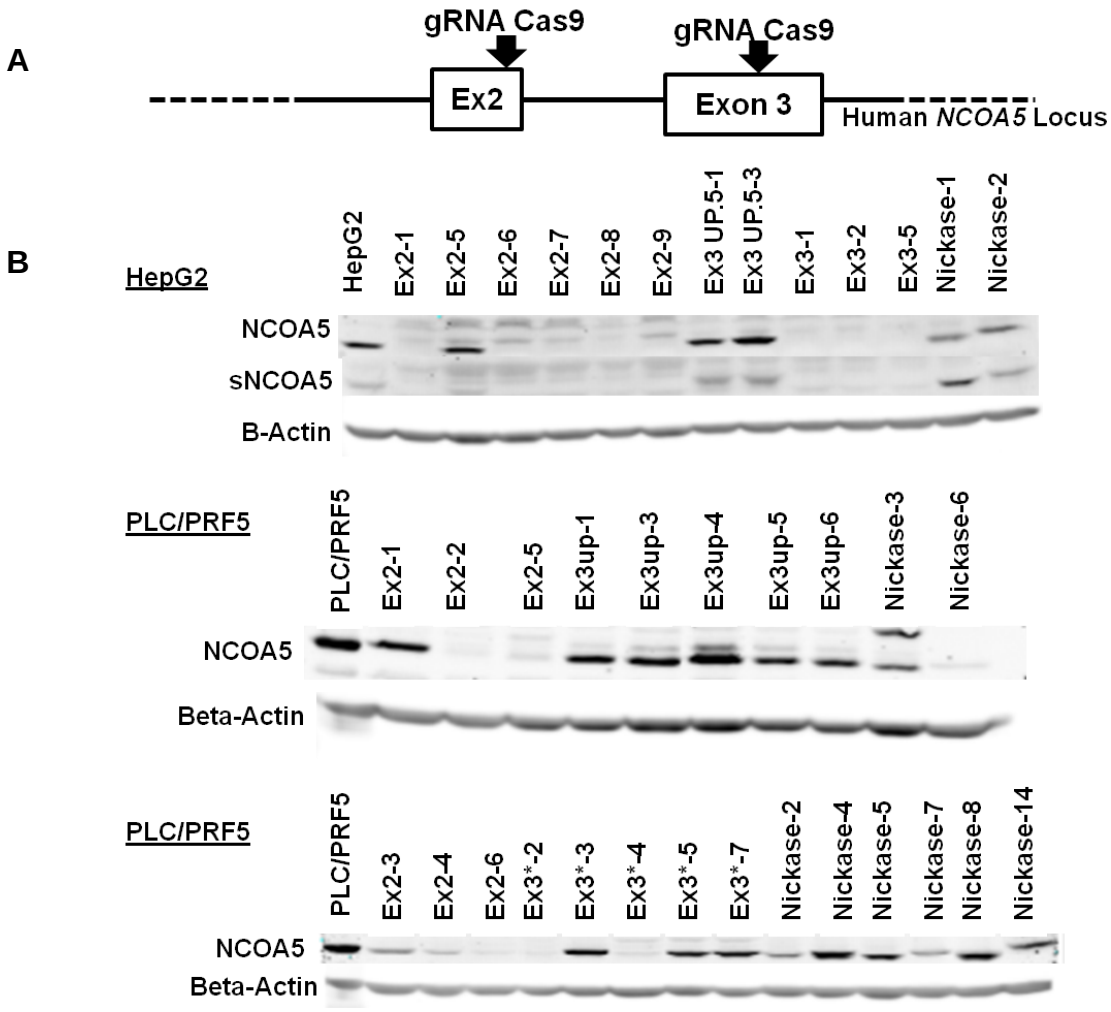
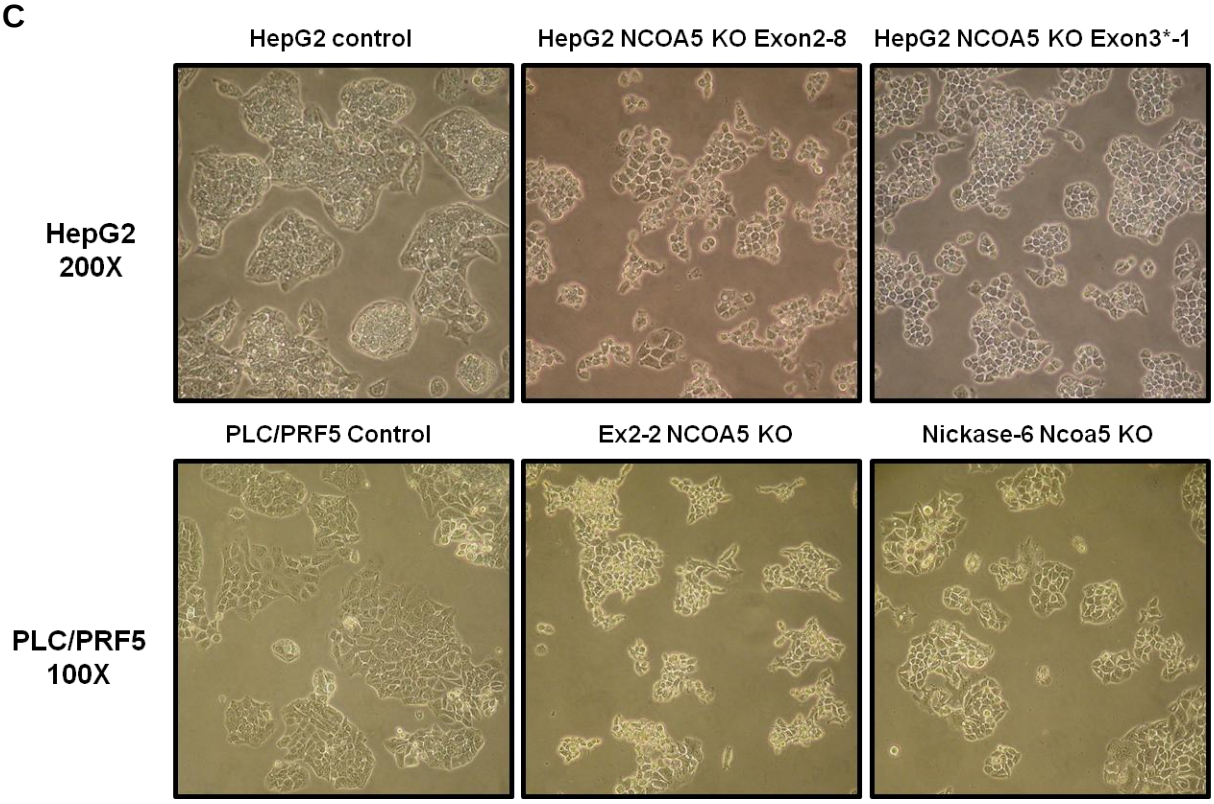


Figure 7 (cont'd)

C



D

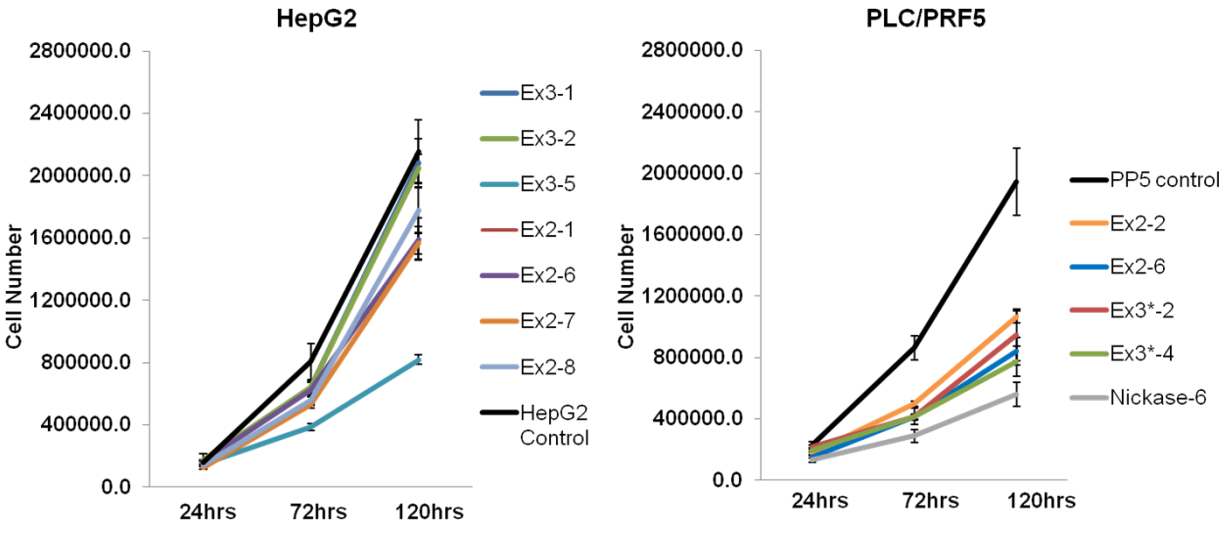


Figure 7 (cont'd)

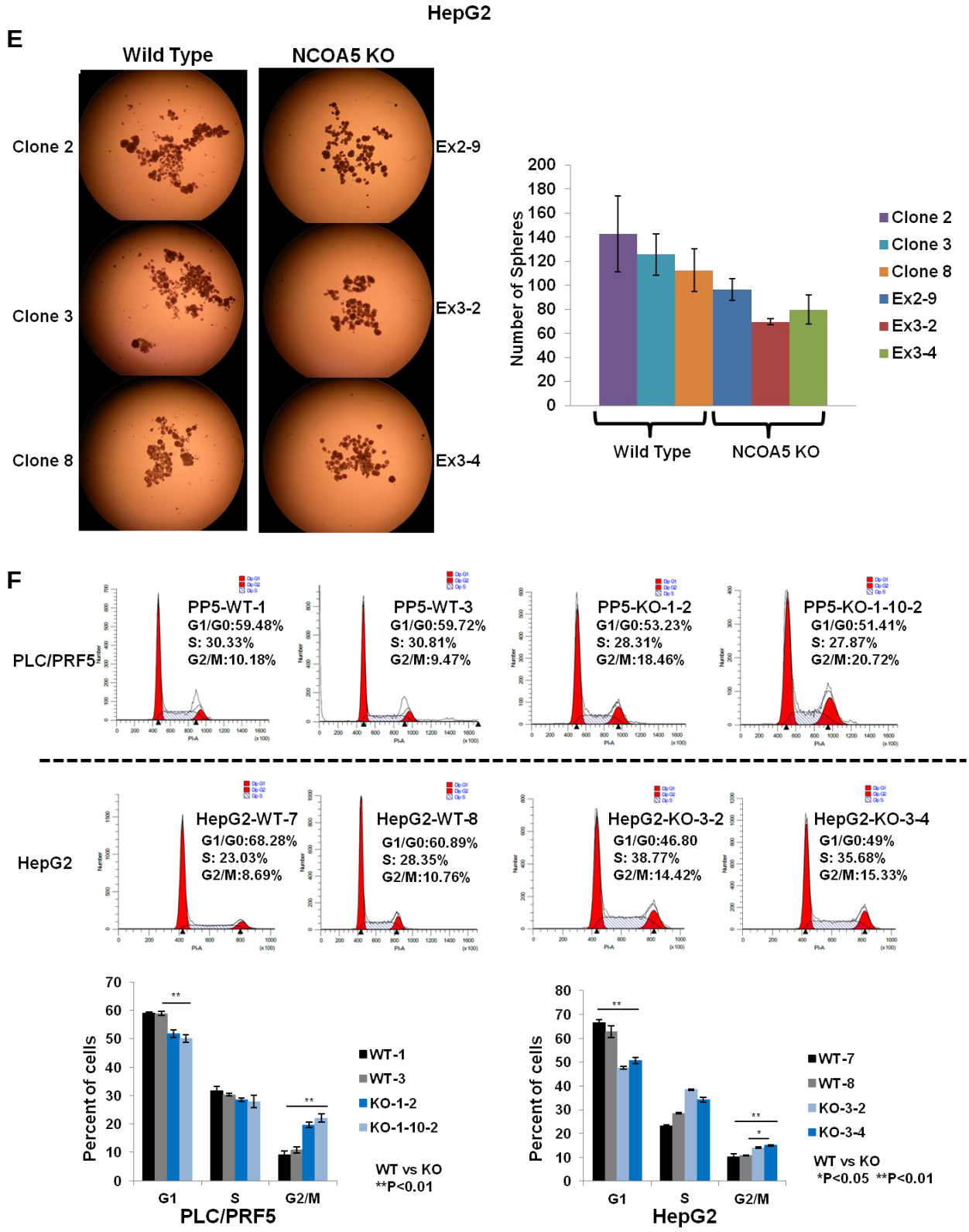
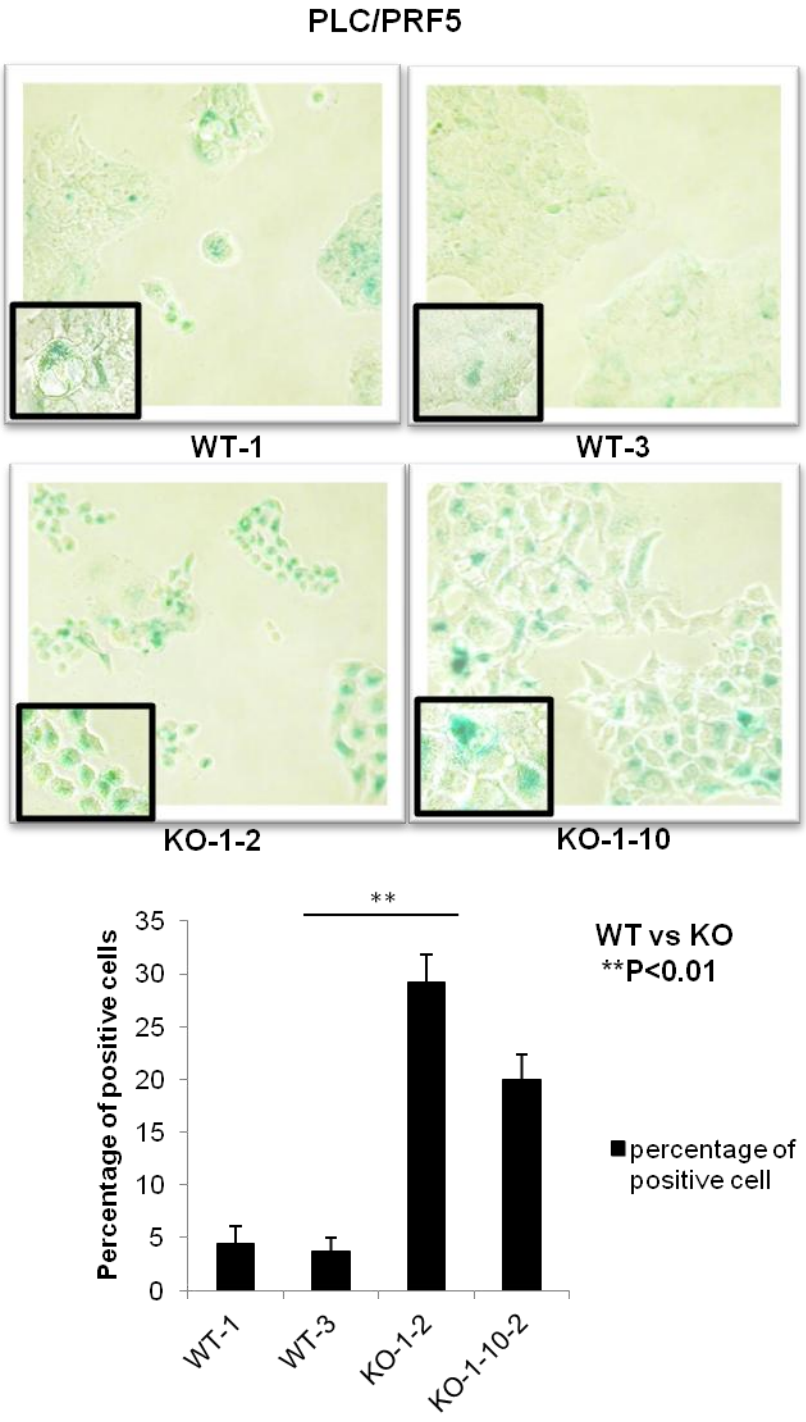


Figure 7 (cont'd)

G



(A) A diagram of CRISPR targeted regions within the *NCOA5* gene locus. (B) Anti-NCOA5 antibody immunoblots of CRISPR targeted clones with either successful or

Figure 7 (cont'd)

unsuccessful NCOA5 disruption. (C) Morphological appearance of control parental populations and NCOA5 disrupted clonal populations at the listed magnification levels. (D) Cell proliferation of HepG2 and PLC/PRF5 control parental and NCOA5 disrupted clonal populations using hemocytometer based cell counting. (E) Sphere formation assay to display the ability of HepG2 control clonal cell lines and NCOA5 disrupted clonal cell lines to grow under non-adherent conditions. (F) Cell cycle analysis of HepG2 and PLC/PRF5 control clonal cell lines and NCOA5 disrupted clonal cell lines using propidium iodide. (G) β galactosidase assay to identify senescent cells within PLC/PRF5 control clonal cell lines and NCOA5 disrupted clonal cell lines.

Splice variant sNCOA5 overexpression delays human HCC cell line proliferation and inhibits colony formation in soft agar.

Previous results identified a prominent alternative *Ncoa5* protein product in humans. This short NCOA5 protein is caused by an alternative mRNA splicing event characterized by an alternative 5-prime donor site within intron 7. The result is an early stop codon found at the 5-prime end of exon 8, a loss of 195 amino acids from the full length protein product, and the presence of 22 new amino acids at the C-terminus of the protein (S. Gao, et al., 2013). Interestingly, the sNCOA5 splice variant product was found more highly expressed within many human HCC specimens when compared to the corresponding adjacent tissue. It is currently not known if increased sNCOA5 expression confers an advantage to HCC tumors. Therefore, stable overexpression of sNCOA5 was carried out in human HCC cell lines to investigate its function.

Immunoblotting was used to confirm the presence of sNCOA5 overexpression, both by an anti-Ncoa5 antibody and an anti-HA antibody that will exclusively detect the HA tag located at the C-terminus of overexpressed sNCOA5 (Figure 8A). Proliferation analysis based on cell counting revealed that sNCOA5 overexpressing HCC cell lines grew more slowly than empty vector control lines (Figure 8B). These results were repeatable and were confirmed by alternative methods such as MTT assay (Not shown). An adherence independent soft agar colony formation assay was also carried out for the PLC/PRF5 cell line, and sNCOA5 overexpressing cells formed a severely reduced number of colonies in soft agar compared to empty vector controls (Figure 8C). These results indicate that sNCOA5 retains function that is detrimental to proliferation and colony formation in human HCC cell lines. We previously reported that full length NCOA5 overexpression resulted in a strong inhibition of proliferation due to a G2/M phase transition block as evidenced by an increased percentage of cells at the G2/M phase in NCOA5 overexpressing cells compared to control cells (X. Liu, et al., 2017). This same cell cycle analysis was used to assess sNCOA5 overexpressing PLC/PRF5 cells, and consistent with previous results, the percentage of cells within the G2/M phase of the cell cycle was increased in the sNCOA5 overexpressing cell line compared to the empty vector control line (Figure 8D). This result indicates that sNCOA5 likely retains at least some of the full length NCOA5 function and decreases proliferation due to decreased G2/M cell cycle phase transition.

Figure 8. sNCOA5 overexpression inhibits human HCC cell proliferation and colony formation in soft agar

A

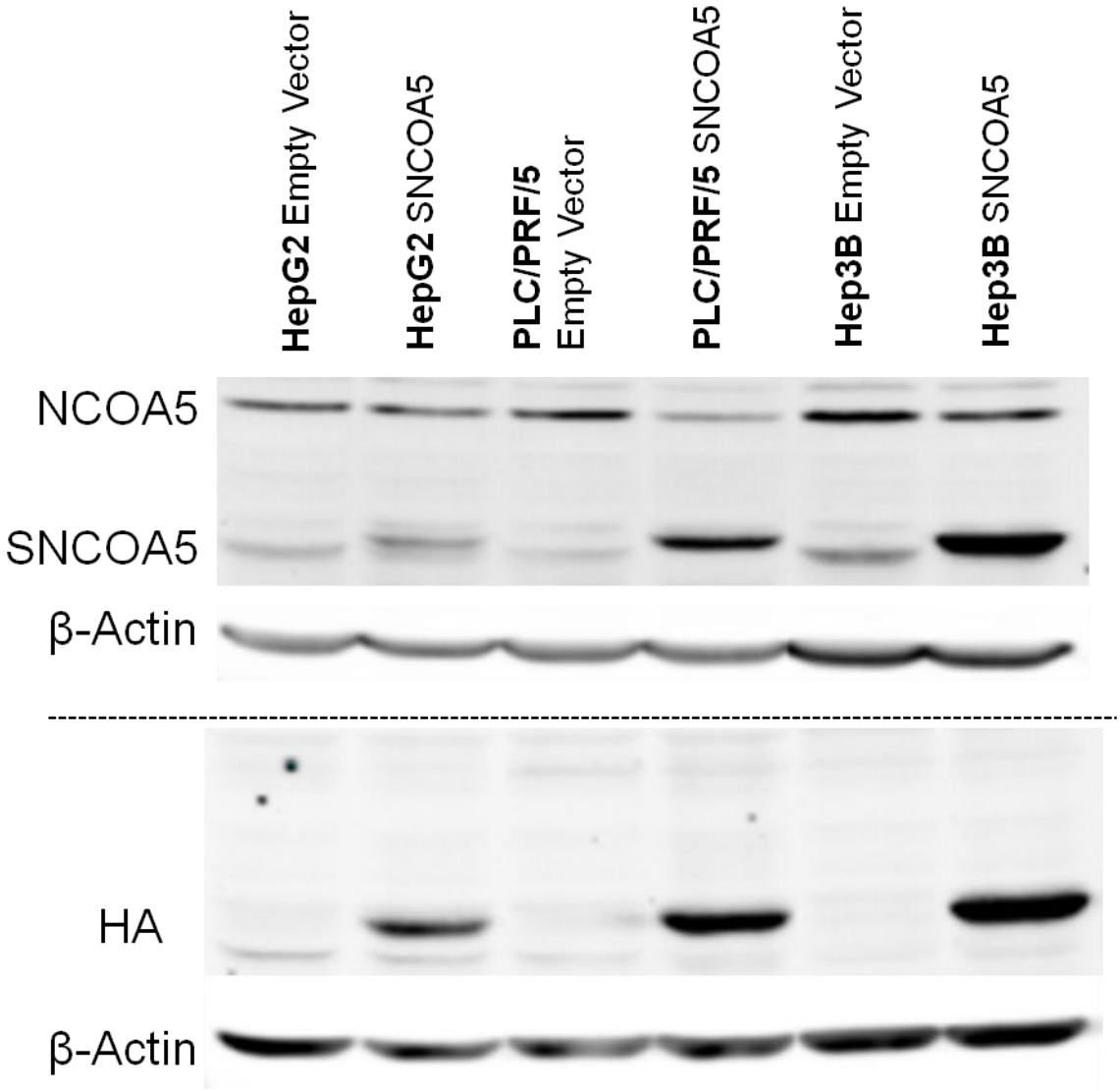


Figure 8 (cont'd)

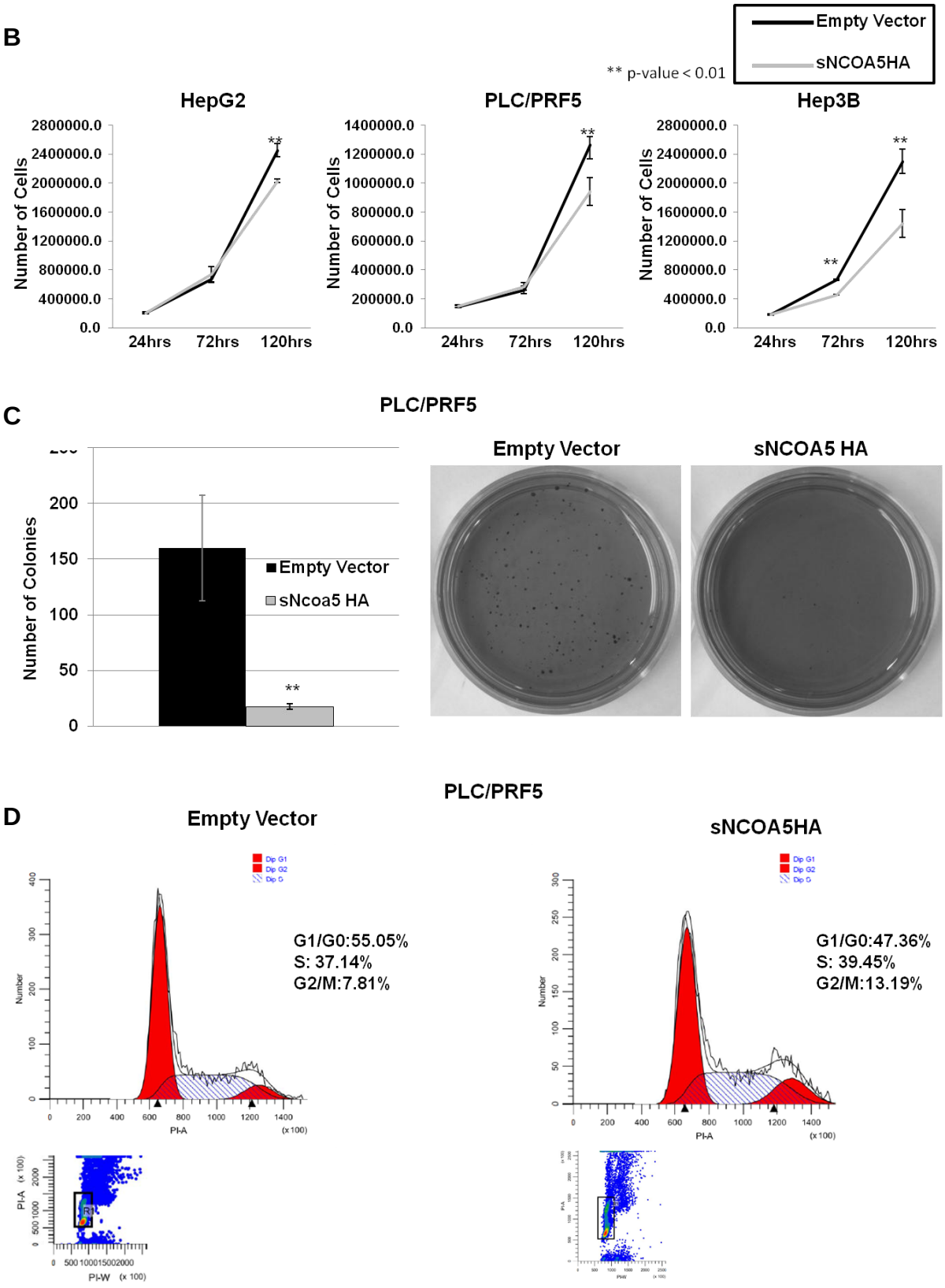


Figure 8 (cont'd)

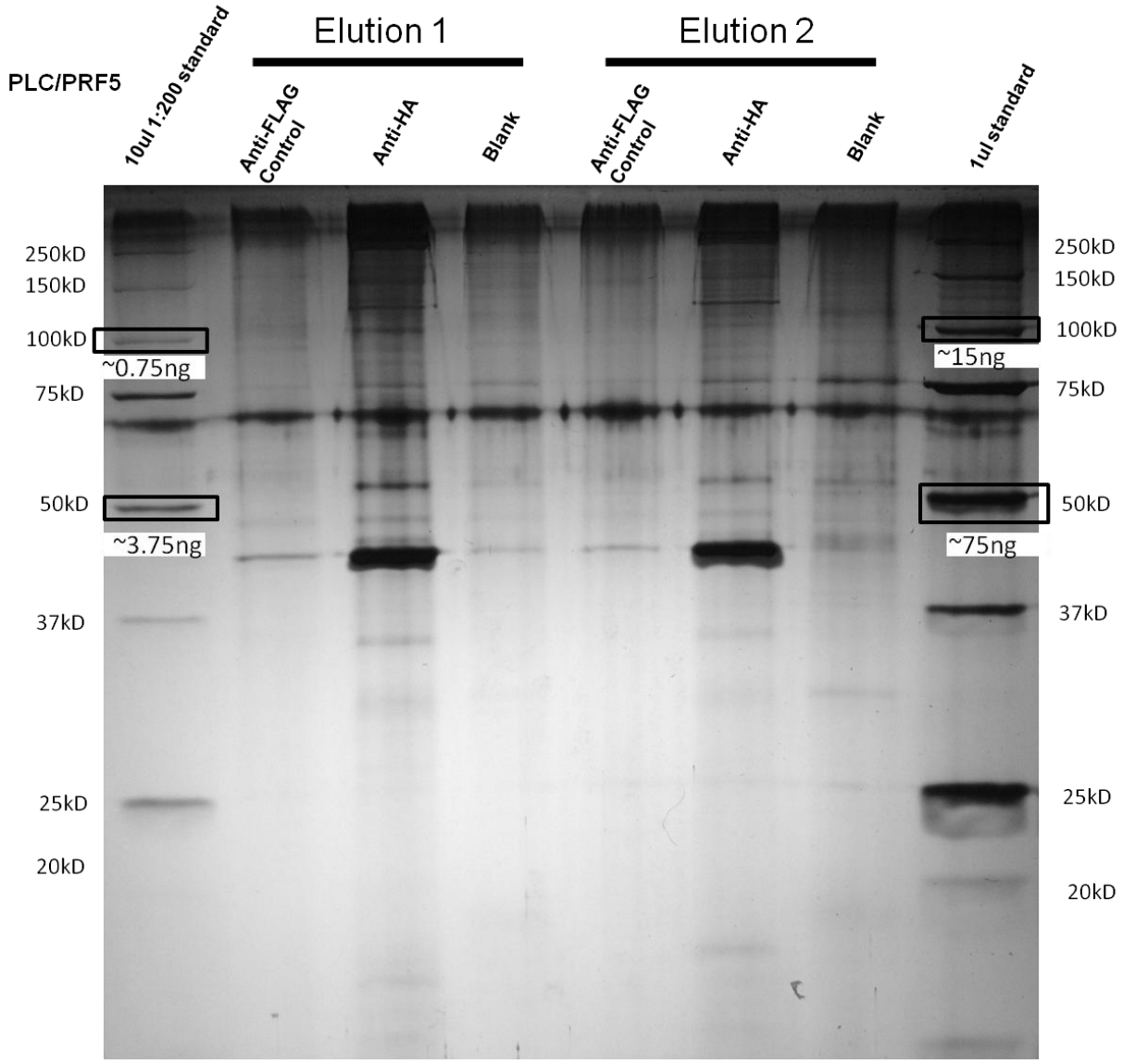
(A) Immunoblot with anti-NCOA5 and anti-HA tag antibodies to visualize sNCOA5 overexpression in HepG2, PLC/PRF5, and Hep3B HCC cell lines. (B) Cell proliferation of empty vector control and sNCOA5 overexpressing cell populations. (C) Soft agar colony formation assay to characterized adherent independent growth of PLC/PRF5 cells overexpressing sNCOA5. (D) Cell cycle analysis of PLC/PRF5 empty vector cells and sNCOA5 overexpressing cells using propidium iodide.

sNCOA5 co-immunoprecipitation indicates the presence of bound proteins.

Nuclear receptor coregulators commonly act in complexes to exert cellular function. Therefore, in order to detect the presence of possible sNCOA5 binding proteins, sNCOA5 was co-immunoprecipitated utilizing the HA tag present at the C-terminus of the overexpressed protein. Silver staining reveals a strong protein band that correctly corresponds to the HA-tagged sNCOA5 at approximately 46kDa. Also present are several protein bands that were pulled down with the overexpressed sNCOA5 protein that were not pulled down by anti-flag control beads (Figure 9A). These bands indicate possible protein binding partners of sNCOA5, however; further work is needed to identify and confirm these sNCOA5 binding proteins.

Figure 9. CoImmunoprecipitation of HA tagged sNCOA5 reveals potential sNCOA5 binding proteins

A



(A) Silver stained polyacrylamide gel loaded with protein eluate that bound to anti-HA antibody sepharose beads or anti-FLAG sepharose control beads.

DISCUSSION

The function of NCOA5 and its splice variants are only beginning to be determined. NCOA5 functions are likely multiple and varied in the context of different conditions, and more work is needed to characterize its role in specific contexts. A genetically-engineered mouse model of *Ncoa5* deficiency identifies NCOA5 as a haploinsufficient tumor suppressor (S. Gao, et al., 2013). However, the tumor suppressor functions of NCOA5 are not fully understood. To gain further insight into the function of NCOA5 in cancer, knockout and overexpression studies were carried out *in vitro*. We demonstrate the complete loss of NCOA5 or the overexpression of splice variant sNCOA5 inhibits cancer cell proliferation and anchorage independent growth with an observed decrease in G2/M phase transition of the cell cycle. Our work identifies a dose dependent requirement of NCOA5 in optimal proliferation and cell cycle of human HCC cell lines and highlights cell cycle related functions that will require further work to elucidate.

NCOA5 plays an important dose dependent role in human HCC cell line proliferation.

Accumulating evidence suggests an important role for NCOA5 in cell cycle arrest, as slight increases in wild type full length NCOA5 expression results in DNA damage responses and HCC cell senescence (X. Liu, et al., 2017). These results are consistent with the idea that NCOA5 acts as a tumor suppressor. Intriguingly, we show here that total loss of NCOA5 expression also results in reduced cell cycle progression at the G2/M transition and increased senescence, that may lead to the observed decreased cell proliferation. These findings are quite similar to overexpression of wild

type full length NCOA5 and introduce a new potential mechanism where NCOA5 dosage is essential to cell cycle progression. Either an increase or decrease in NCOA5 expression results in cell cycle arrest. These finding confound the role of NCOA5 as a classical tumor suppressor and display the complex nature of NCOA5 function. Further work is necessary to uncover the role of NCOA5 in cell cycle control and to investigate possible dosage effects observed. One possible explanation for an NCOA5 dosage dependence could be due to important interacting proteins. For example, NCOA5 may act in a protein complex to exert function and the proportions of all interacting proteins may be crucial to complex action. An increase in the amount of Ncoa5 proteins may squelch the complex and prevent its function, whereas a loss of NCOA5 may cause a loss of complex function due to NCOA5 absence. It will be important to identify Ncoa5 binding proteins and to characterize whole protein complex action while determining the role for individual proteins within the complex by altering their expression.

NCOA5 splice variant sNCOA5 retains at least partial function of wild type NCOA5.

Alternative protein products due to splice variant mRNAs have become an important area of research focus. Splicing provides further protein diversity within a single copy of a gene. Alternatively spliced protein products may have similar or widely variable functions. sNCOA5 was found more highly expressed than full length NCOA5 in many HCCs compared to adjacent tissue and could possibly be contributing to tumor development and progression (S. Gao, et al., 2013). However, this work indicates at least a partial maintenance of normal NCOA5 function within sNCOA5. Indeed, human HCC cell line proliferation was reduced with overexpression of sNCOA5 and a G2/M

phase cell cycle arrest was observed as with wild type full length induced expression. Although sNCOA5 and wild type full length NCOA5 function were not directly compared, there is some evidence that sNCOA5 retains only partial function. For example, in order to overexpress wild type NCOA5, and inducible overexpression system was required because cells failed to adequately proliferate in culture after overexpression. However, with sNCOA5 overexpression, special inducible systems were not required and the cell populations still grew albeit at a slower rate than the control cells. This likely indicates that sNCOA5 retains some functions of full length NCOA5, but sNCOA5 seems to lack the full potency of the full length protein. Further work is required to directly compare these proteins and confirm the partial loss of function of sNCOA5. A partial loss of NCOA5 function could be important for HCC development and or progression. Indeed, Liu and colleagues recently published work characterizing a full length NCOA5 missence mutation that significantly reduced the ability of NCOA5 to suppress HCC tumor growth *in vivo*. Similarly, sNCOA5 may also have a reduced function that inhibits full tumor suppressor abilities as seen in NCOA5. It is possible that certain NCOA5 functions are beneficial to cancer cell survival and proliferation, but that the wild type full length protein also maintains a strong antitumor activity. Partial loss of NCOA5 function, either by mutation as Liu et al. describe or by alternative splicing to a shorter sNCOA5 form as described here, may provide essential survival functions to the tumor with minimized antitumor effects. It is also important to acknowledge that these studies relied on overexpression of this protein, which may not reflect normal functions at a physiological level. It is possible that the growth inhibitory effects of sNCOA5 are only due to the high overexpression of sNCOA5, and in fact may not be deleterious to

tumors at observed physiological levels. Even so, sNCOA5 clearly displays some similar functions as full length NCOA5, but these effects are likely impaired.

In summary, this work identifies a dosage dependent effect of NCOA5, with loss of NCOA5 and overexpression of sNCOA5 resulting in a decreased proliferation phenotype within human HCC cell lines possibly due to an apparent delay at the G2/M transition of the cell cycle. These findings may prompt further research into the dosage dependence of NCOA5 and how its dysregulation contributes to HCC formation and progression.

AUTHOR CONTRIBUTIONS

The completion of this work was a collaborative effort. Authors of this chapter include me (Mark Williams), Su Rila, and Hua Xiao. I am the lead author of the work with guidance provided by Hua Xiao as the principal investigator. I was responsible for completion of most experiments with help from Su Rila to complete the cell cycle analysis and β-galactosidase staining. Thank you to Su Rila for your assistance and thank you to Hua Xiao for your support and supervision as principal investigator.

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