The Role of FOXO3 in Mechanisms of Doxorubicin Resistance in Hepatocellular Carcinoma

By

Josiah Cox

Submitted to the graduate degree program in Microbiology, Molecular Genetics & Immunology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

Chairperson Steven A. Weinman, M.D., Ph.D.

_____

Thomas M. Yankee, Pharm.D., Ph.D.

_____

Wolfram R. Zückert, Ph.D.

_____

Udayan Apte, Ph.D.

_____

Timothy A. Fields, M.D., Ph.D.

Date Defended: December 20th, 2016

The Dissertation Committee for Josiah Cox

certifies that this is the approved version of the following dissertation:

The Role of FOXO3 in Mechanisms of Doxorubicin Resistance in Hepatocellular Carcinoma

_____

Chairperson Steven A. Weinman, M.D., Ph.D.

Date approved: January 26th, 2017

Abstract

Trans-arterial chemoembolization (TACE) with doxorubicin is commonly used to treat hepatocellular carcinoma (HCC), but has limited efficacy due to a high level of resistance. The factors that determine the sensitivity to TACE-doxorubicin are unknown. FOXO3 is a multifunctional transcription factor that plays a role in determining cell fate in response to stress. It frequently functions as a tumor suppressor but can also promote tumor pathogenesis. FOXO3 is also known to be a mediator of doxorubicin sensitivity in many types of tumor cells, while in others it can promote resistance. The role of FOXO3 in HCC and in doxorubicin resistance in HCC is unknown. FOXO3 function is largely determined by post-translational modification (PTM). Two FOXO3 PTMs, acetylation and serine 574 (S574)-phosphorylation, are known to promote its apoptotic function. Contrary to expectations, expression of FOXO3 was increased in HCC compared to surrounding liver. Cytosolic FOXO3 was significantly greater in TACE-resistant HCC as compared to treatment-naïve tumors. In Huh7 hepatoma cells, doxorubicin induced acetylation and S574-phosphorylation of FOXO3, and these modifications promoted doxorubicin-induced cell death by suppressing the pro-survival function of FOXO3. Resveratrol, an activator of SIRT deacetylase enzymes, inhibited these doxorubicin-induced PTMs and increased doxorubicin resistance. The expression of SIRT6, a known FOXO3 deacetylase, was also increased in TACE-resistant HCC tumors and correlated with cytosolic FOXO3. SIRT6 also blocked doxorubicin-induced S574-phosphorylation of FOXO3 and increased resistance to doxorubicin in Huh7 cells. Therefore, targeting SIRT6 and/or manipulating FOXO3 modifications may prove useful in enhancing the chemotherapy sensitivity of HCC.

Acknowledgements

I am sincerely grateful for the kindness, encouragement, guidance, and patience given by my mentor, Dr. Steven A. Weinman. He has helped me learn what it takes to be a scientist. I also want to thank the MD/PhD program director, Dr. Timothy A. Fields. He has constantly given time and effort for my sake. Without him, I would not be where I am today. I am also grateful for the time and guidance given by the other members of my committee: Thomas M. Yankee, Pharm.D., Ph.D. Wolfram R. Zückert, Ph.D., and Udayan Apte, Ph.D. I would also like to thank Maura O'Neil, MD, who was so helpful with the clinical part of the project. I must thank my co-workers in the lab. I could not ask for a more caring group of people to work with. They lead by example and are always willing to help others, in addition to creating such a fun environment to work in. In particular, I am extremely grateful to Drs. Anusha Vittal and Zhuan Li for the significant contributions each made to this project. Finally, I would like to thank my wife, Emilie. Her support has carried me.

Table of Contents

List of Figures

List of Tables

Chapter I: Introduction

Hepatocellular Carcinoma: Course of Disease and Therapy

Epidemiology and Clinical Course

Improved prevention strategies and the development of new therapies have led to a decline in incidence of many types of cancer world-wide over recent decades. However, the incidence of HCC in the US has been increasing for the last three decades, due in large part to a growing population with diet and infection-related risk factors, while treatment options have remained extremely limited. Liver cancer, of which HCC is the most common histologic subtype, is the second most common cause of cancer-related death in men and the sixth leading cause in women world-wide (1). Risk factors associated with HCC include chronic infection with hepatitis C virus (HCV), hepatitis B virus (HBV) infection, alcohol use, and non-alcoholic fatty liver disease (NAFLD), all of which can cause chronic hepatitis and cirrhosis. Although HCC can also develop without prior cirrhosis, approximately 90% of HCC tumors develop in the context of cirrhosis (2, 3), and patients with cirrhosis have greater risk of developing HCC, with an annual incidence between 1-8 percent depending on the cause of cirrhosis.

HCC is very difficult to diagnose early in the course of the disease because the symptoms are often masked by the symptoms of the underlying liver disease present in the vast majority of patients with HCC (2, 4). The physical findings often present in HCC, which include an enlarged liver and spleen, ascites, and jaundice, can also be present in patients with cirrhosis without HCC. Therefore, patients with chronic liver disease are entered into surveillance programs, where they undergo ultrasound imaging tests every 6 months to look for HCC. When a mass is found, the diagnosis of HCC is often confirmed using further imaging. Biopsies are only taken in cases where the imaging findings are uncertain or where the result would directly impact

treatment (5, 6). The four most important predictors of survival include severity of the underlying liver disease, the number and size of the tumor(s), tumor invasion into adjacent structures including vasculature, and the presence of metastasis (7, 8). Even with routine surveillance, given the inherent aggressiveness of HCC, the median survival after diagnosis without treatment is only 6-20 months (9). Demonstrating the high fatality rate of HCC, in 2008 the number of HCC-related deaths was 93% of the number of new cases of HCC world-wide (1). Much progress has been made over the last few decades on prevention of HCC, including the development of the HBV vaccine, improved surveillance techniques and protocols, and the recent discovery of curative anti-viral medications for HCV (10). During that same time there has been a surge of interest in discovering new therapies that can prolong life for patients with HCC. However, very little progress has been made on that front.

Overview of treatment approaches

Treatment approaches for HCC can be divided into surgical, and non-surgical, liver-directed or systemic cytotoxic therapies. The choice of therapy is determined by both the extent of the cancer and the degree of hepatic dysfunction. Surgical approaches including resection of the tumor and whole liver transplantation are the only curative therapies for HCC. However, most patients are ineligible for surgical resection because they do not have enough reserve liver function to survive the removal of a significant portion of their liver. Liver transplantation is greatly limited by the number of donor livers available. In addition to patient abstinence, there are strict criteria that specify the maximum tumor extent that can be present for a patient to be eligible for transplant. These criteria, which are based on data on recurrence and survival after transplant, are called the Milan Criteria. They state that a patient must have a solitary tumor that is no more than 5 cm in diameter, and no more than three tumors, none being more than 3 cm in

diameter, and no evidence of vascular invasion. Furthermore, as a result of the limited donor liver availability, patients are ranked on the transplant list based on how sick they are. This often leads to a situation where patients with HCC must have a low enough tumor extent to be eligible for transplant, but also have poor enough liver function to rank high on the transplant list. Systemic treatment of HCC with traditional cytotoxic chemotherapeutic agents such as cisplatin, doxorubicin, and 5-FU has been limited by systemic toxicity, poor efficacy, and acquired resistance of the tumors after exposure (11-13). Over the last two decades research efforts have been focused on discovering new drug targets and effective agents for treating HCC. After dozens of clinical trials only one drug, Sorafenib, a multi-kinase inhibitor, has shown any degree of success. However, it is only able to produce modest increases in survival of selected patients (14, 15), and it is not tolerated by patients with more advanced liver disease. Improvements in patient outcome have thus largely resulted from the use of surgical resection, local ablative techniques, and liver transplantation (2, 4).

TACE with Doxorubicin

One of the more promising developments in HCC treatment has been in targeted delivery of cytotoxic chemotherapy agents directly to the tumor. Selective injection of embolizing agents in combination with doxorubicin into arteries feeding tumors, or trans-arterial chemoembolization (TACE), has been shown to provide a survival benefit in patients with unresectable HCC (16, 17) and is now the standard of care for patients with intermediate stage HCC (4). Embolization of the tumor alone causes ischemia and can produce tumor shrinkage. However, the combination of the embolic effect with the addition of a chemotherapy agent, typically doxorubicin, has been shown in large randomized studies to increase tumor response, decrease progression, and improve overall survival (18, 19). Also, the use of embolic drug

eluting beads (DEBs) made of polyvinyl chloride (PVC) that release doxorubicin (DEBDOX) in a controlled manner has improved the TACE technique allowing for higher doses with reduced systemic exposure (20). Doxorubicin-based TACE now plays an important role in shrinking (downstaging) tumor size and number to allow eligibility for liver transplantation (16).

While TACE is highly effective in many patients, approximately 50% of tumors treated with DEBDOX show no response, and only 27% show a complete response (19, 20). Resistance to doxorubicin has thus emerged as a central problem limiting treatment of patients with HCC. Therefore, one way to drastically improve therapy for HCC is to better understand the mechanism of doxorubicin toxicity in HCC, as well as the mechanisms of cellular resistance to doxorubicin, so that new or adjunct approaches can improve the effectiveness of treatment.

Doxorubicin Mechanisms of Action

Doxorubicin is an anthracycline antibiotic that is widely used as a human antitumor therapeutic agent. Doxorubicin sensitivity is the result of delivery of the drug to the nucleus and a series of signaling events that are initiated by doxorubicin's interaction with DNA. This ultimately leads to a series of programed responses culminating in cell apoptosis. It appears to have multiple antitumor effects, but the best understood of these involves its interaction with topoisomerase IIα (TOP2A) (21). This enzyme is involved in separating entangled DNA strands, and as part of its function it transiently generates and then repairs protein-bound double-strand DNA breaks (DSBs) (22). Doxorubicin stabilizes the cleaved-strand intermediate, suppressing the completion of the process and resulting in numerous protein-bound DSBs (21). DSBs have numerous negative consequences for cells and notably trigger caspase-dependent apoptosis programs, which involve the activation of master regulators p53 and FOXO3, and suppression of pro-growth signaling pathways, which leads to changes in the ratio of anti/pro-apoptotic Bcl-2

family proteins (23). This DNA damage response is the primary factor accounting for the antitumor effect of doxorubicin, and blocking just this downstream response to DNA damage is sufficient to attenuate doxorubicin toxicity (24). Multiple other mechanisms have been observed to be involved in doxorubicin cytotoxicity as well and these include the formation of TOP2A-independent DNA adducts (25), inhibition of DNA and RNA synthesis, and mitochondrial ROS production triggering apoptosis (26). Mechanisms of tumor cell resistance to doxorubicin can be divided into three main categories: 1) reduction in the ability of the drug to accumulate in the nucleus, 2) decreased DNA damage, and 3) suppression of the downstream events that transduce the DNA damage signal into apoptosis (Figure 1).

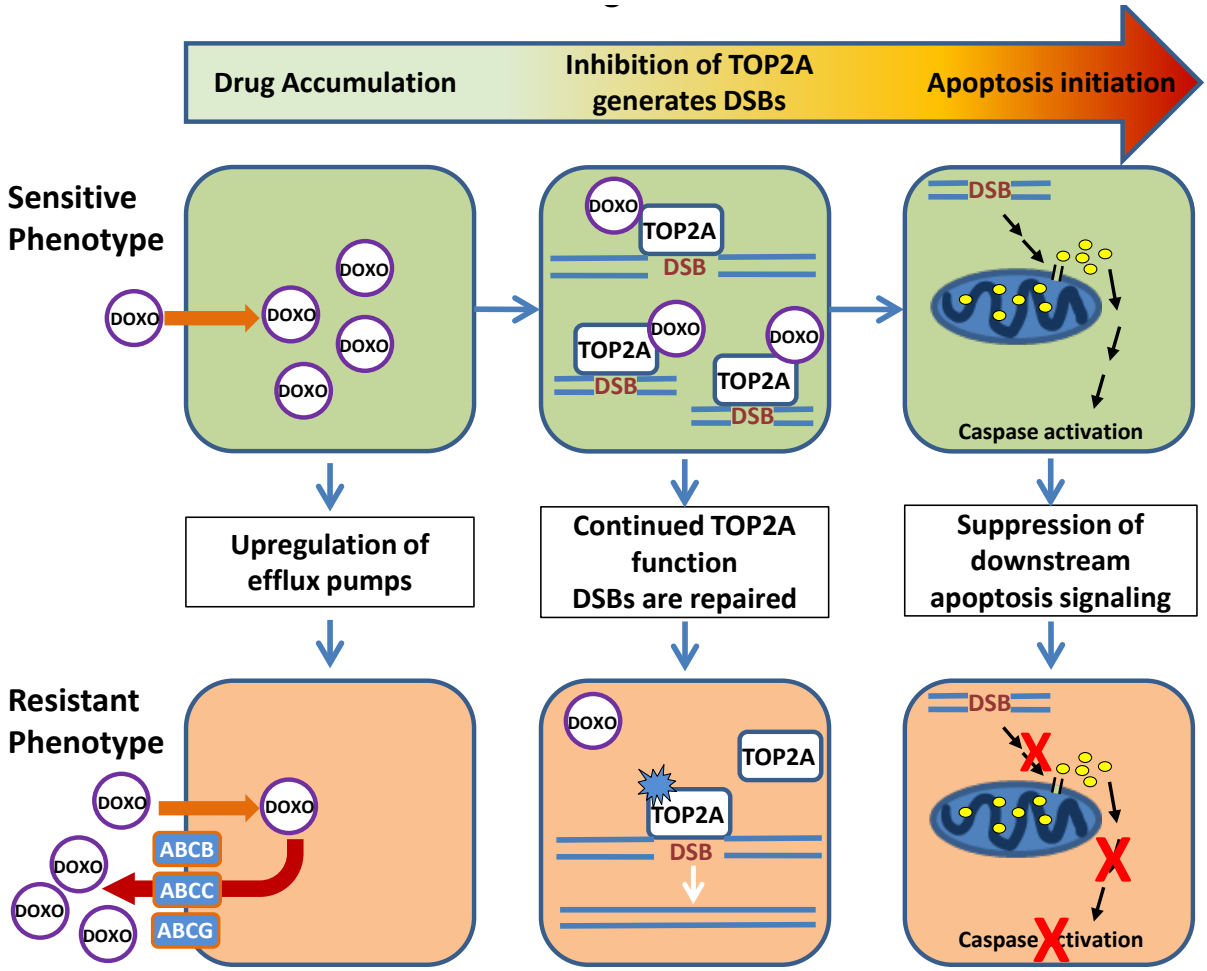

Figure 1: General mechanisms of doxorubicin resistance in hepatocellular carcinoma
Doxorubicin first must accumulate within the cell but this process is inhibited by the upregulation of ABC family efflux pumps in resistant cells. Doxorubicin then prevents the repair of TOP2A-generated DSBs in DNA, increasing TOP2A-bound DSBs. Overexpression and mutations in TOP2A allow continued TOP2A function in resistant cells. Finally, DNA damage induces apoptotic signaling pathways causing cytochorome C release from mitochondria which leads to caspase activation and cell death. Downregulation of the effectors of apoptosis and upregulation of anti-apoptotic proteins prevents the completion of apoptosis in resistant cells. ABC: ATP-binding cassette; DSB: Double-strand DNA break. Adapted from Cox et al., Hepatic Oncology. 2016 (27).

Mechanisms of Doxorubicin Resistance in HCC

Parts of this section were adapted from Cox et al., Hepatic Oncology. 2016 (27).

Upregulation of drug efflux pumps

Doxorubicin is a hydrophobic molecule that passes through cellular membranes independently of specific transporters. However, cells can fail to accumulate the drug through active drug efflux via ATP-dependent efflux transporters. This phenomenon, first described in a number of cancers and labeled "multidrug resistance," results from the expression of a group of multi-drug resistance efflux pumps. These ATP-dependent transport proteins, members of the ATP-binding cassette (ABC) transporter family, were initially identified for their pathological role in tumors before their normal physiological functions were understood. They are now known to be important components of transport in a number of tissues. Hepatocytes use multiple different ABC transporters for the transport of organic ions such as bile acids and conjugated bilirubin (28). Since these pumps are highly abundant in hepatocytes, it is not surprising that they are expressed in hepatocellular carcinoma, where increased expression results in chemotherapy resistance.

The basal expression of ABC proteins is controlled by multiple transcription factors including NF-Y and members of the Sp family (29). Additionally, p53 has been shown to repress transcription of ABC family proteins, (30) while several transcription factors including both AP-1 (31) and NF-κB (32) are capable of upregulating their expression. Activity of the enzyme COX-2 has also been implicated in the control of MDR1 expression, as the COX-2 inhibitor, celecoxib, decreases MDR1 expression in multi-drug resistant HCC cells (33, 34). In HCC, three ABC subfamilies, ABCB (the MDR proteins), ABCC (MRP proteins), and ABCG (BCRP) appear to contribute to doxorubicin resistance. Although they have different substrate

specificities in normal physiologic conditions, they have all shown an ability to transport doxorubicin (35-37). MDR1, MRP1, MRP2, and MRP3 are all expressed in HCC at the transcriptional level. MDR1 protein expression is found in 80-90% of HCC cases (35). MDR family proteins have also been found to be expressed and functionally active on mitochondrial membranes, perhaps protecting mitochondrial DNA from drug-induced damage by keeping the drug out of mitochondria or suppressing apoptosis by altering mitochondrial outer membrane permeability (38).

MDR1 expression was found to inversely correlate with response to systemic chemotherapy in one study (35), but the precise extent to which expression of ABC proteins accounts for clinical drug resistance is less clear. The situation is somewhat clearer in cell culture models of HCC. One method of generating doxorubicin resistant cultured HCC cells for study is to select for resistant HCC cells *in vitro* after exposing them to incrementally increasing doses of doxorubicin. This method consistently induces the expression of MDR1 and other ABC family members (39, 40), and the upregulation of these transporters can be shown to cause drug resistance, since inhibitors of ABC proteins such as verapamil and cyclosporine A are able to restore doxorubicin sensitivity (41). However, verapamil has not proven to be useful as a doxorubicin sensitizing agent in patients, perhaps due to the presence of other efflux transporters, pharmacological interactions between the drugs (42), the loss of important normal physiological functions (43), or the presence of unrelated resistance mechanisms. Therefore, while overexpression of MDR efflux pumps may be an important cause of drug resistance in deliberately selected HCC cell lines *in vitro*, other mechanisms are likely to be important in human disease.

Alterations in TOP2A

As mentioned above, the primary means by which doxorubicin causes cellular toxicity is by targeting the alpha isoform of topoisomerase II (TOP2A) (21), resulting in numerous protein-bound double strand DNA breaks (DSBs) and the subsequent triggering of apoptosis (44). It has been hypothesized that one mechanism of resistance to doxorubicin might be through the reduction in TOP2A expression and increased reliance on the beta isoform of topoisomerase II that is less sensitive to doxorubicin (44). Supporting this hypothesis is the finding that breast cancers with co-amplified HER2 and TOP2A genes have increased sensitivity to doxorubicin, while tumors with a TOP2A deletion have increased resistance (45, 46). Additionally, this mechanism of resistance to doxorubicin has been seen in several cancer cell lines (47). In HCC, however, TOP2A expression is increased rather than decreased. The TOP2A protein level in HCC is increased independent of gene amplification in 73% of human HCC tumors compared to adjacent non-tumor tissue (48). It is also overexpressed in several HCC cell lines with acquired doxorubicin resistance (49). Furthermore, TOP2A expression was positively correlated with histological grade, vascular invasion, and early age of onset in a tissue microarray of 172 HCC tumors, and, in contrast to breast cancers, it positively correlated with HCC doxorubicin resistance and shorter survival in 148 patients in a prospective randomized study (50). TOP2A overexpression has been found to be associated with several indices of tumor aggressiveness in many other types of cancer as well, presumably due to the role of TOP2A in facilitating DNA replication and transcription. While the association of increased TOP2A with tumor growth seems logical, it is not understood why it is also associated with doxorubicin resistance. One hypothesis is that the high expression levels are associated with the development of mutations in TOP2A that lead to its insensitivity to doxorubicin (51). Another possibility is that in order for cells to survive the high levels of TOP2A they must simultaneously suppress the downstream

apoptosis programs normally triggered by DNA strand breaks and it is the acquisition of this adaptive characteristic that confers doxorubicin resistance. At the present time this issue remains unresolved.

FOXO3

FOXO3 is a multifunctional transcription factor that was initially identified as a longevity factor responsible for antioxidant responses, cell cycle arrest, and stem cell survival (52). Under certain conditions, however, it also promotes apoptosis. This combination of functions allows it to have both tumor promoting and tumor suppressing effects depending on the tumor cell type (53). Additionally, in response to cell stress FOXO3 has been shown to be involved in apoptosis induction or cell survival depending on the context. The role of FOXO3 in HCC and its sensitivity to doxorubicin is the focus of this dissertation and will be discussed in more detail below.

P53

The tumor suppressor p53 is a frequently altered target in doxorubicin resistant HCC. It is one of the key DNA damage sensors and acts as a transcriptional activator of pro-apoptotic factors including Bax, Bak, CD95 and TRAIL receptors (54, 55). In addition, it transcriptionally represses anti-apoptotic factors including Bcl-2 and Survivin (56, 57). Doxorubicin stabilizes p53 (58) by stimulating its phosphorylation by DDR kinases. DDR-dependent phosphorylation of p53 inhibits binding to and phosphorylation by MDM2, which ordinarily promotes p53 ubiquitination and proteosomal degradation and leads to low steady-state p53 levels (59). An inhibitor of MDM2-p53 binding, Nutlin-3, has been shown to enhance p53 stabilization and activation and increase doxorubicin sensitivity in HCC cells with wild-type p53 (60). Mutation or deletion or disruption of p53 activation pathways are frequent events in HCC tumorigenesis,

providing a possible mechanism for intrinsic resistance to doxorubicin (61). The specific role of p53 in doxorubicin resistance has been illustrated by experiments showing that restoring p53 expression in HCC cells promotes doxorubicin-induced apoptosis (62).

While it thus might seem attractive to target the regulation of p53, attempts to manipulate p53 by interfering with upstream regulators have produced some unanticipated and paradoxical results. For example, one recent study showed that inhibition of the deubiquitinase USP9X, which decreased steady-state p53 levels, enhanced doxorubicin sensitivity in HCC cells. This suggests that the effects of ubiquitination inhibitors are more complex than simply causing the degradation of p53 (63). Furthermore, increasing p53 is clearly not the only way to enhance doxorubicin sensitivity, as illustrated by comparing p53 expression levels and drug sensitivity in hepatoma cell lines. HepG2 cells, which have wild-type p53, are the most resistant, and Huh7 and Hep3B, which are p53 defective, are more doxorubicin sensitive. Clearly, p53, while important, does not completely account for the phenomenon of doxorubicin resistance (64).

NF-κB

NF-κB is also a transcription factor that has multiple, sometimes opposing functions, such as tumor suppression or promotion depending on the cellular context. In HCC associated with inflammation, such as in HCV or HBV infection, NF-κB tends to have a tumor promoting effect, while in tumors induced by carcinogens such as diethylnitrosamine, NF-κB functions as a tumor suppressor (65). NF-κB signaling is activated by DNA damage and can have varying effects on subsequent apoptosis primarily through regulation of its target genes, such as Bcl-XL and XIAP (66). In general, NF-κB has an anti-apoptotic effect in response to drugs that induce DSBs in DNA such as doxorubicin, although it may be partially dependent on the cancer cell type (67). There are few studies investigating the role of NF-κB in resistance to doxorubicin in

HCC, although it has been shown to be activated in HCC cells in response to doxorubicin (68), and several studies have indicated that activation of NF-κB is a mechanism by which diverse stimuli generate an anti-apoptotic effect. For example, the anti-apoptotic gene Bcl-2-associated athanogene-1 (BAG-1) was found to enhance doxorubicin resistance by potentiating the transcriptional activity of NF-κB (69). Additionally, the HBV protein HBx has been shown to increase doxorubicin resistance through the activation of NF-κB in HCC cells (70), and reduced expression of miR-26b in HCC promotes doxorubicin resistance due to the loss of its suppression of NF-κB signaling (68).

MAP kinases

Another class of resistance mechanisms is signaling pathways that are drivers of tumor cell proliferation. These frequently inhibit apoptosis during tumorigenesis as well as after chemotherapy exposure. One such signaling pathway is the PI3K/Akt pathway. Akt is activated through phosphorylation by the second messenger PI3K following growth factor stimulation and in response to many cell stressors (71). It is negatively regulated by the phosphatase, PTEN (72). Akt then directly and indirectly regulates cell proliferation and apoptosis by phosphorylating and modulating target protein function including FOXO3, Bad, p53, and cyclin-dependent kinase inhibitors (71), as well as by activating parallel pro-growth pathways (72). This pathway is frequently activated in HCC and is correlated with decreased overall survival (73). Several studies have shown that inhibiting PI3K/Akt function using pharmacological inhibitors (74, 75) or by exogenous overexpression of an upstream inhibitor (76, 77) increases HCC cell sensitivity to doxorubicin, while activating PI3K/Akt has the opposite effect (76, 78).

The MEK/ERK signaling pathway is another important pathway that translates growth signals from the cell surface to transcription factors and other regulatory proteins to promote cell

proliferation and inhibit apoptosis (72). It promotes HCC tumor cell growth and it is frequently activated in HCC. It has also been shown to be activated by doxorubicin (79), serving as a tumor cell response that counters doxorubicin-induced toxicity. Direct inhibition of ERK activity increases doxorubicin sensitivity in HCC cells by inhibiting cell proliferation and promoting apoptosis (79). Inhibition of EGFR, an upstream activator of the MEK/ERK pathway, also increases doxorubicin sensitivity in HCC cells (80). In addition, the mechanism of action of the tyrosine kinase inhibitor, Sorafenib, which has been used as a systemic chemotherapeutic treatment for advanced HCC, also involves inhibition of the MEK/ERK pathway (81). In a randomized controlled trial of patients with advanced stage HCC, sorafenib plus systemic doxorubicin was shown to increase patient overall survival compared to doxorubicin treatment alone (82).

The p38 MAPK pathway is also activated by doxorubicin and may play a role in regulating doxorubicin-induced apoptosis. Its activation is necessary for the phosphorylation of FOXO3 responsible for its nuclear translocation following doxorubicin treatment in breast cancer cells (83). MK5, a downstream target of p38, is upregulated in HCC cells and downregulated by doxorubicin. Overexpression of MK5 decreased doxorubicin-induced apoptosis (84).

Sirtuins

The sirtuin family of NAD-dependent deacetylases is also known to play a crucial regulatory role in the cellular response to stress, apoptosis, metabolism, and aging (85). There are seven members of the sirtuin family (SIRTs) in humans, SIRT1-7, and the expression of several SIRTs are altered in HCC, some with pro-tumorigenic and some with anti-tumorigenic effects (85-88). SIRT1 is consistently found to be overexpressed in HCC (89), and was shown to inhibit

doxorubicin-induced apoptosis in HCC cells (85). The mechanism for SIRT1-mediated inhibition of doxorubicin sensitivity in HCC is unknown but it may involve the deacetylation of p53 (90), FOXO3 (91), or YAP2 (92), where deacetylation of each of these factors has been shown to inhibit its apoptotic activity. Additionally, in breast cancer cells with acquired resistance to epirubicin, a doxorubicin homolog, SIRT4, 5, 6 and 7 were found to be upregulated, particularly SIRT6, which was shown to mediate epirubicin resistance by deacetylation and inhibition of FOXO3 (93).

FOXO3 introduction

Parts of this section are adapted from Tikhanovich et al., Journal of Gastroenterology and Hepatology. 2013 (94).

The O branch of the large forkhead family of transcription factors is ubiquitously expressed and highly conserved evolutionarily (52). The prototype of the FOXO family was first described in *C. elegans* as daf16, a factor that is required for formation of a long-lived dormant form of the organism called the dauer larval stage. Subsequently, FOXO factors were shown to play a similar role in higher organisms and function to prevent cellular proliferation, induce antioxidant and stress response genes, and modify insulin sensitivity (52, 95). In mammals there are 4 FOXO proteins, FOXO1, FOXO3, FOXO4 and FOXO6. While FOXO6 is largely specific to neurons, the other 3 factors are widely distributed and are present in most tissues. There appears to be considerable overlap in the transcriptional targets of the three, but the consequences of knock outs in mice are very different. FOXO1 knock out is embryonically lethal due to failure of angiogenesis, FOXO3 knock out produces premature ovarian failure, and FOXO4 knock out exhibits no obvious phenotype (53). Numerous, specific functions have been identified for each of these FOXO proteins, but several reports have described a prominent role

for FOXO3 in tumor biology and cellular adaptation to stress. Thus, FOXO3 may be particularly relevant in HCC and doxorubicin response.

Similar to its homolog in invertebrates, FOXO3 is an important longevity factor in humans as three FOXO3 single nucleotide polymorphisms in the FOXO3 gene have been associated with centenarians in Japanese (96), German, and French populations (97). The specific cellular functions of FOXO3 have been investigated in many cell types and tissues. In stem cells, FOXO3 plays an important role in maintaining quiescence and self-renewal capability (98), which has also been shown to carry over into several types of cancer stem cell-like cells (99-102). In general, FOXO3 is involved in the cellular response to stress, including nutrient deprivation, oxidative stress, hypoxic stress, and DNA damage. It plays a significant role in determining cell fate, promoting apoptosis or cell survival when cells are exposed to these conditions in normal physiologic or in pathologic scenarios.

The cellular response to stress can involve many different types of processes and can vary greatly depending on the type and extent of the stress and the cell type and conditions. One cellular response to stress, particularly nutrient deprivation, is the upregulation of autophagy. FOXO3 has been shown to stimulate autophagy by increasing the expression of autophagy-related proteins in response to metabolic stress, promoting cell survival (103). However, FOXO3 seems to have the opposite role, inhibiting autophagy, in many cancer cell lines (104). Another cellular response to stress, particularly DNA damage, is to inhibit cell cycle progression to allow cells time to repair damage. Several target genes for FOXO3 are involved in cell cycle control including cyclin-dependent kinase inhibitors p15, p21, and p27 (71). In the liver, FOXO3 has been shown to limit cellular proliferation in a model of liver regeneration in mice (105). FOXO3 also directly promotes DNA repair by interacting with ATM, a vital component of the DNA

repair pathway, promoting its phosphorylation and downstream signaling in response to DNA DSBs (106). Furthermore, during the G2-M checkpoint FOXO3 promotes DNA repair through its transcriptional control of GADD45a, a growth arrest and DNA damage response protein (107). Hypoxia is another type of cell stress for which FOXO3 has been shown to play a role in the cellular response. In breast cancer cells FOXO3 was upregulated by hypoxia and protected cells from HIF-1-mediated apoptosis (108). FOXO3 can also promote resistance to hypoxic stress through c-Myc inhibition leading to decreased expression of mitochondrial proteins, respiratory complexes, and respiratory activity (109, 110). TACE is known to induce tumor hypoxia (111), however the role of FOXO3 in the HCC cell response to TACE-induced hypoxia is unknown. In response to oxidative stress, cells can respond by upregulating antioxidant enzymes and ROS scavenging systems and/or induce apoptosis if the damage becomes too great. FOXO3 has been shown to promote cell survival in response to oxidative stress by increasing the expression of antioxidant enzymes, SOD2 and catalase (112). However, FOXO3 has also been shown to promote apoptosis in many different cell types by controlling expression of target genes involved in both the intrinsic and extrinsic apoptosis pathways (113-116). It is likely that cell type and conditions have significant influence on FOXO3 function in response to oxidative stress. For example, it has been shown that in low glucose, FOXO3 promoted cell survival in response to oxidative stress, while in high glucose it promoted apoptosis (117). Furthermore, the mechanism by which cell type and conditions regulate FOXO3 function is likely to be through upstream enzyme-mediated post-translational modifications on FOXO3, which have been shown to be crucial to determining FOXO3 function.

A complete understanding of the role of FOXO3 in normal cellular function and disease requires a more detailed understanding of the many upstream events that affect its function.

Multiple post translational modifications of FOXO3 have been described including phosphorylation, acetylation, methylation, and ubiquitination (118). These PTMs can alter nuclear import and export steps, modify the DNA binding affinity, and alter the pattern of transcriptional activity for specific target genes (52, 119).

The first layer of regulation of FOXO3 is a series of modifications that controls the translocation between nucleus and cytosol. These FOXO3 PTMs can be divided into two groups. The first group promotes nuclear export, polyubiquitination and proteosomal degradation. These include phosphorylation by Akt (the main pathway of FOXO3 degradation) (120), ERK (121), IKKβ (122) and CDK2 (123). Sites for all those modifications have been described, and activation of the respective kinases typically correlates with loss of nuclear FOXO3. The second group of PTMs that control the nuclear-cytosolic distribution are those that promote nuclear localization and are associated with an increase in transcriptional activity. These include phosphorylation by JNK (124), p38 (83), AMPK (125), CDK1 (126), and MST1 (127), as well as monoubiquitination by unknown enzymes (118), and arginine methylation by PRMT1 (128). The latter modification, arginine methylation, prevents FOXO3 nuclear export by inhibition of AKT phosphorylation due to the proximity of methylated arginine residues to the AKT sites (128).

Notably, there are potential cross-regulatory interactions among the PTMs that regulate FOXO3 localization. For example, FOXO3 contains numerous phosphorylation ‘SP’ motifs that are substrate targets for JNK, p38 and ERK. Phosphorylation on these sites has been detected following oxidative stress and other stimuli. FOXO3, for example, contains p38 phosphorylation sites on Ser7, Ser12, Ser294, Ser344, and Ser425 that can be also targeted by JNK (Ser294 and Ser425) and ERK (Ser294, Ser344, and Ser425) (83). While p38 and JNK are known to promote

nuclear localization, ERK modification has an opposite effect (121). One can speculate that these modifications can happen consecutively by different enzymes and various combinations throughout the FOXO3 sequence create unique protein conformations that define its localization. The balance between these two groups of modifications in the liver creates an environment that defines the amount of FOXO3 in the nucleus. Complete loss of nuclear FOXO3 undoubtedly leads to deregulation of above mentioned pathways controlled by FOXO3 transcriptional activity.

The second layer of regulation includes a series of modifications that regulate FOXO3 transcriptional activity by changing DNA binding and promoter binding specificity. This group includes acetylation by the redox activated acetyl transferase, p300 (129), deacetylation by SIRT1 (91, 118), SIRT2 (130), and SIRT3 (131), lysine methylation (132), and glycosylation (133). Lysine methylation at K270 of FOXO3 promotes loss of DNA binding and reduces FOXO3-mediated apoptosis (132). Deacetylation by SIRT1 has been shown to differentially alter DNA binding affinity, so that more highly acetylated forms of FOXO3 favor expression of pro-apoptotic genes, (Bim, TRAIL and FasL), while the more deacetylated forms favor expression of antioxidant and cytoprotective genes (91). SIRT2 also deacetylates FOXO3 and increases its DNA-binding activity (130). The binding of CBP/p300 to FOXO3 is essential for transactivation of target genes (129). However, the acetylation itself attenuates FOXO3 transcriptional activity. Acetylation may inhibit the ability of the DNA binding domain of FOXO3 to interact with its consensus binding motif (106).

Several lysines were reported to be acetylated in FOXO3. Brunet et al. found that FOXO3 is acetylated at K242, K259, K271, K290 and K569 in the presence of stress stimuli (91). O-glycosylation is another modification that does not affect the nuclear/cytosolic

distribution of FOXO3, but results in the up-regulation of specific gene expression such as G6Pase21 and other gluconeogenic genes (134). Recent studies show that some of these effects involve the ability of specific PTMs, such as GlcNAcylation to produce differential binding of FOXO3 to cofactors such as PGC-1α with a subsequent increase in specific transcriptional activities (133).

Phosphorylation events can also regulate functionally relevant transcriptional specificity in addition to FOXO3 localization. For example, JNK-stimulated phosphorylation of FOXO3 at serine-574 is translocated to the nucleus where it selectively binds to pro-apoptotic promoters and induces cell death. In the absence of this phosphorylation, FOXO3 initiates an antioxidant and cell protective transcriptional program (114). Thus, whether FOXO3 serves as a pro-apoptotic or pro-survival factor likely depends on the state of its modification by upstream enzymes. This phenomenon may be particularly relevant in HCC sensitivity to chemotherapy and will be addressed below and in further detail in Chapters 4 and 5.

This second layer of modifications gives an idea of how FOXO3 transcriptional activity can be regulated. However, the question of how FOXO3 decides which transcriptional program is activated in any given condition is still unclear. Since FOXO3 recognizes a conserved consensus motif TTGTTTAC (135, 136) present in multiple genes, the promoter binding patterns may be defined more by differential binding to various cofactors. FOXO3 has been shown to interact with a large number of binding partners resulting in changes in transcriptional activity of both proteins. The list includes a number of nuclear hormone receptors, other transcription factors such as β-catenin, RUNX3, SMADs and histone modifying enzymes such as acetylases and methyltranferases (137). In addition to being binding partners, these modifying enzymes can

directly affect the PTMs of FOXO3 itself, as well as histone modifications, providing an additional level of complexity to the activation of FOXO3 target genes.

FOXO3 function in cancer

FOXO3 as a tumor suppressor

The ability of FOXO proteins to function as tumor suppressors has been well documented. Firstly, mice with conditional triple knockout of FOXO1/3/4 genes are cancer prone, developing thymic lymphomas and hemangiomas (53). In cell culture, Akt or ERK-mediated degradation of FOXO proteins are required for Ras and Myc-induced oncogenic transformation respectively as FOXO proteins were shown to suppress transformation by inhibiting cell cycle progression and blocking induction of Myc target genes (121, 138-140). Further evidence for the tumor suppressive role of FOXO3 specifically is its frequent inactivation in human tumors. It has been found to be inactivated by the PI3K/AKT pathway (58), and in the absence of AKT activation, IKK-β activation has also been shown to inactivate FOXO3 (122). In fact, many studies have shown an association between the down-regulation of FOXO3 and poor prognosis including in gastric adenocarcinoma (141), urothelial carcinoma (142), colon adenocarcinoma (143), serous ovarian carcinoma (144), mantel cell lymphoma (145), and breast adenocarcinoma (122). The tumor suppressive function of FOXO3 has primarily been attributed to its ability to inhibit cell proliferation through its control of cell cycle proteins (122), however other mechanisms have also been described. In lung cancer, FOXO3 transcriptionally represses a DNA methyltransferase, DNMT3B, which when overexpressed promotes tumorigenesis by increasing the methylation status of tumor suppressor genes (146). And in prostate cancer, FOXO3 inhibits β-catenin-mediated tumor promotion by two mechanisms: decreasing its expression by upregulating a β-catenin specific miRNA, and by

suppressing β-catenin-mediated transcription by directly binding to β-catenin, competing with its transcriptional co-factor, TCF (147).

FOXO3 as a tumor promoter

Although it seems clear that FOXO3 often suppresses tumor growth by playing an anti-proliferative role as it tries to control cell cycle progression, FOXO3 also has a well-documented role in promoting cell survival in response to various types of cell stress. Therefore, it should not be surprising that FOXO3 has also been found to function as a tumor promoter in specific contexts, in sub-populations of cancer cells, and in certain types of cancer in general. For example, FOXO3 seems to function as a tumor suppressor in colon cancer as it is frequently inactivated by increased PI3K/AKT activation, and FOXO3 can induce apoptosis when its activity is increased through the use of a PI3K/AKT inhibitor. However, when FOXO3 is activated in cells with high nuclear β-catenin activity, instead of causing apoptosis, it acts in concert with β-catenin to promote metastasis. Nuclear colocalization of FOXO3 and β-catenin was significantly increased in patients with metastatic colon cancer and shorter survival (148). Additionally, in cytogenetically normal acute myelogenous leukemia (CN-AML), high FOXO3 expression is associated with lower patient survival (149), and in other types of AML it seems to protect leukemia-initiating cells from DNA damage and inhibit their differentiation (101, 150). In chronic myelogenous leukemia, FOXO3 acts as a tumor suppressor in a majority of cells as the BCR-ABL translocation creates a constitutively active tyrosine kinase which activates AKT and inactivates FOXO3. However, it also seems to be essential for the maintenance of a small population of leukemia initiating cells that are resistant to BCR-ABL inhibitor treatment, have high nuclear FOXO3, and are dependent on FOXO3 for their tumor initiating ability (151). High FOXO3 expression was also found in most patients with Hodgkin's Lymphoma, especially in

cancer stem cell-like cells, but not in Non-Hodgkin's Lymphoma (99). Finally, FOXO3 is overexpressed at the mRNA and protein levels in anaplastic thyroid carcinoma (ATC) tissue and cell lines and it increases proliferation of ATC cells through transcriptional upregulation of cyclin A1 (152).

The mechanisms by which FOXO3 promotes a malignant phenotype have been further studied in several cancer cell models which seem to point to the importance of FOXO3 in helping tumor cells adapt to changing environmental conditions. For example, FOXO3 transcriptionally activates IDH1, an enzyme that generates NADPH, an essential co-factor for ROS scavenging systems, and converts isocitrate to alpha-ketoglutarate (α-KG) which promotes differentiation and tumor suppression through α-KG-dependent dioxygenases in untransformed cells. However, when mutated as it is in several types of cancer, IDH1 is able to further convert α-KG to 2-hydroxyglutarate, a recently discovered "oncometabolite", able to drive tumorigenesis. These cancer cells are then dependent on FOXO3-driven mutated IDH1 expression (153). Nutrient deprivation is another type of stress that tumor cells encounter as tumor mass increases. In response to serum starvation, FOXO3 can promote cell survival by activating NF-κB through transcriptional upregulation of BCL10, an upstream regulator of IKK (154). It has also been shown to induce expression of MMP-9 and MMP-13 in response to serum starvation, leading to increased tumor cell invasion (155). Finally, exposure to chemotherapy, such as doxorubicin, is another extreme type of stress that tumor cells encounter and frequently survive. As will be discussed in more detail below, FOXO3 is able to promote apoptosis of tumor cells treated with chemotherapy; however it has also been shown to promote resistance to chemotherapy.

FOXO3 in HCC

While the role of FOXO3 in many types of cancer has been well studied, strikingly little investigation has been done on the role of FOXO3 in HCC. Two previous studies have suggested FOXO3 may play a tumor suppressor role in HCC. One study found that the tumor promoter, upregulator of cell proliferation (URGCP), is overexpressed in many HCC tumors and cell lines. When URGCP was knocked down in HCC cells, proliferation decreased, and this was shown to be dependent on FOXO3 when its expression was knocked down and the effect was lost (156). Another study found miR-96 to be overexpressed in HCC cells and demonstrated that it promotes HCC cell proliferation as well as decreased FOXO1 and FOXO3 expression, Knock-down of FOXO1 and FOXO3 expression was shown to slightly decrease cell proliferation (157). However, evidence has also been given for tumor promotion role for FOXO3 under certain conditions. When HCC cells were cultured in serum-free medium, FOXO3 expression was downregulated and this downregulation was shown to be responsible for the decreased cellular proliferation in this condition. When FOXO3 expression was restored, HCC cell proliferation was restored. Additionally, serotonin, which can signal through 5-HT2B receptors expressed on hepatocytes, was also able to increase proliferation in HCC cells cultured in serum-free medium by increasing expression and activation of FOXO3 (158).

The only previous study reporting on FOXO3 expression in HCC used 91 samples from untreated surgical resections done in China, 86% of which were positive for HBV and only 44% had cirrhosis [only 10-15% of HCC patients in the U.S. are positive for HBV and the vast majority have cirrhosis (159)]. They performed immunohistochemistry for FOXO3 and divided the samples according to their FOXO3 expression, classifying 46 samples as having “high” FOXO3 and 45 having “low” FOXO3. The low FOXO3 group had a statistically significant worse histologic grade, but the high FOXO3 group had a statistically significant higher

percentage of tumors in that were greater than 5cm in size and were more likely to be negative for cirrhosis. Additionally, they found that the high FOXO3 group had greater survival out to 5 years compared to the low FOXO3 group (160). However, they did not see any relationship between tumor size and survival, which is concerning as tumor size has been found repeatedly to have great influence on HCC recurrence and survival after transplant (161). There is clearly a need for more investigation into the role of FOXO3 in HCC, and this need is addressed in Chapters 3 and 4 of this dissertation.

FOXO3 function in chemotherapy

FOXO3 has been shown to mediate apoptosis induction for several types of chemotherapy in several types of cancer. Resistance to cisplatin, a DNA cross-linking agent, occurs in breast and colon cancer through IKK-β (162) and Akt-mediated (163) FOXO3 cytoplasmic sequestration, respectively. Sensitivity to paclitaxel, a microtubule inhibitor, in breast cancer is dependent on JNK phosphorylation of AKT and activation of FOXO3 (164). Other compounds shown to be cytotoxic to HCC cells include bortezomib, a proteasome inhibitor (165), and melatonin (166), both of which cause apoptosis in a FOXO3-dependent manner.

FOXO3 has also been shown to mediate doxorubicin-induced apoptosis in a number of different tumor cell types. Doxorubicin increases nuclear accumulation of FOXO3 in breast cancer (83), lung cancer, neuroblastoma (116), and osteosarcoma cells (167). Additionally, pharmacological approaches that inhibit Akt or otherwise increase FOXO3 nuclear accumulation work synergistically with doxorubicin to enhance apoptosis (145, 168). The mechanisms by which FOXO3 mediates doxorubicin-induced apoptosis include transcriptional repression of miR-21 which represses translation of Fas-L (116), transcriptional upregulation of Bim, a pro-

apoptotic Bcl-2 homolog (169), and transcriptional repression of Bcl-2 (114) and survivin, an anti-apoptotic Bcl-2 family member (170).

The role of FOXO3 in doxorubicin sensitivity of HCC, whether it promotes apoptosis or resistance, has not been studied previously. This is a knowledge gap that will be addressed in Chapter 4 of this dissertation. Furthermore, as has been discussed previously, FOXO3 can be responsible either for enhanced cell survival or enhanced apoptosis. While it has been shown that FOXO3 frequently promotes apoptosis through its transcriptional program in response to doxorubicin treatment, there has been no mechanism put forth to explain how and why FOXO3 promotes apoptosis rather than cell survival. Changes in FOXO3 that cause a loss of its apoptotic function or an enhancement of its survival function may promote doxorubicin resistance. In fact, this scenario has been demonstrated previously in breast cancer and certain leukemias that have developed doxorubicin resistance. High nuclear FOXO3 expression was observed in doxorubicin-resistant breast cancer where it promoted AKT activation and cell survival (171). In leukemic cells with acquired doxorubicin-resistance, FOXO3 was shown to contribute to this resistance by transcriptionally activating MDR1 (172). In breast cancer cells, doxorubicin-mediated nuclear accumulation of FOXO3 was found to be dependent on p38-induced phosphorylation of FOXO3 on Serine-7 (83). However, this PTM was not shown have an effect on FOXO3 function. Recently, S574-phosphorylation of FOXO3 was shown to be necessary for its ability to cause apoptosis in response to ethanol in HCC cells (114), and deacetylated FOXO3 was shown to promote cisplatin-resistance in urothelial carcinoma cells (173). To date, no FOXO3-PTMs have been reported to regulate FOXO3 function in response to doxorubicin, and this is another knowledge gap that will be addressed in Chapter 4.

Project Aims

The function of FOXO3 in HCC pathogenesis, including the response to chemotherapy, remains unknown. Investigations of FOXO3 function in other malignancies suggest a possible role as a tumor suppressor and chemo-sensitizing factor. However, FOXO3 can also promote cell survival and resistance to cell stress. The conditions and mechanisms that determine how FOXO3 will function and the influence it will have on cell fate have not been fully elucidated. The purpose of the research described in this dissertation was to evaluate the hypothesis that HCC sensitivity to doxorubicin is mediated through changes in FOXO3 PTMs. Furthermore, this work seeks to correlate expression of SIRT6, an upstream modifier of FOXO3, with doxorubicin resistance in HCC. The experiments presented in the following chapters have combined both human HCC tumor samples and *in vitro* cell culture modes to critically evaluate this hypothesis.

Chapter 3 of this dissertation examines the expression and cellular distribution of FOXO3 in human HCC tumor samples. I also examine the correlation of FOXO3 expression with tumor behavior.

Chapter 4 investigates the FOXO3 PTM changes induced by doxorubicin exposure in Huh7 cells, specifically acetylation and S574-phosphorylation. I examined the significance of these PTM changes in doxorubicin sensitivity by blocking there induction and determining if resistance is increased.

Chapter 5 examines the expression of SIRT6 in TACE-doxorubicin-resistant and treatment-naïve human HCC tumors. I also examine the effect of SIRT6 overexpression on doxorubicin-induced FOXO3 PTM changes and cell death.

These studies are the first to show that FOXO3 expression correlates with HCC tumor behavior including response to chemotherapy. Furthermore, they point to SIRT6 overexpression and blocking of doxorubicin-induced changes in FOXO3 PTMs as a possible mechanism of resistance to TACE-doxorubicin in human HCC.

Chapter II: Methods

Materials

General materials were purchased from VWR International (Randor, PA), Sigma-Aldrich (St. Louis, MO), or Fisher Scientific (Pittsburgh, PA). Dulbecco's modified Eagle medium (DMEM) was purchased from VWR. Fetal bovine serum (FBS) was purchased from ATCC (Manassas, VA). MEM nonessential amino acids, FluoroBrite™ DMEM, Opti-MEM, Pierce™ LDH Cytotoxicity Assay Kit, Dynabeads M-280 Sheep anti-rabbit IgG, and X-tremeGENE™ HP DNA Transfection Reagent were purchased from Fisher. The Dako LSAB$^+$ System-HRP immunohistochemistry visualization kit was purchased from Dako (Carpinteria, CA, K0679). Protease inhibitor cocktail (Sigma-Aldrich, P8340) was used at 1:100 dilution. Doxorubicin hydrochloride suitable for fluorescence, resveratrol, Duolink® In Situ PLA® Probe and In Situ Detection kits were purchased from Sigma-Aldrich. Geneticin and puromycin were purchased from Invitrogen (Carlsbad, CA).

Plasmids

The plasmid pECE-HA-FOXO3a-WT was provided by M. Greenburg (Addgene (Cambridge, MA, plasmid # 1787). The pECE-HA-FOXO3-S574A and S574D plasmids were generated using the Q5® Site-Directed Mutagenesis Kit, purchased from New England Biolabs (Ipswitch, MA). All sequences were confirmed by DNA sequencing analysis. The

pcDNA3.1+SIRT6-flag was provided by E. Verdin (Addgene plasmid # 13817). The plasmid pcDNA3.1 SIRT6_H133Y-flage was provided by K. Chua (Addgene plasmid # 53149). It has a single point mutation which inactivates its ability to catalyze acetyl group transfer, and was previously described (174). The lentiviral vector coding for human FOXO3 3'UTR-specific shRNA (MISSION® TRC shRNA TRCN0000040100) and the control vector pLKO.1 were obtained from Sigma-Aldrich and used to make sh-FOXO3 and sh-non-target (sh-NT) lentivirus. These plasmids contain a puromycin resistance gene for selection in mammalian cells. The pMD2.G VSV-G envelope-expressing plasmid for lentivirus production was provided by D. Trono (Addgene plasmid # 12259). The lentiviral packaging plasmid, psPAX2, was a gift from Didier Trono (Addgene plasmid # 12260)

Cell Culture

Huh7 cells (provided by Dr. Charles Rice) are a hepatoma cell line derived from the HCC tumor of a Japanese male (175). They have a mutated p53 gene and have a high degree of karyotypic variability and instability (64). Huh7 cells used for experiments were between passages 7-12. They were maintained in DMEM with 10% FBS, and 0.1mM MEM nonessential amino acids. The 293FT cell line (ATCC) is a clonal isolate from HEK cells that have been transformed with SV40 large T antigen, controlled by the cytomegalovirus (CMV) promoter. They are fast growing and highly transfectable and were used for the production of lentivirus. 293FT cells were cultured in DMEM with 10% FBS, 0.1mM MEM nonessential amino acids, and 500 μg/ml geneticin. All cells were incubated in 37°C, 5% CO2.

Transfection

For transfection of Huh7 cells with FOXO3, SIRT6, or mutants, cells were plated at a density of 15 x 10^4 cells/well in a 6-well plate or 2.5 x 10^4 cells/well in a 24-well plate. For

transfection of cells in a 6-well plate, the following day the medium was replaced with 1.5ml Opti-MEM 1 hour before transfection. For each well, 1.5ug of plasmid DNA and 1.5ul of transfection reagent were diluted in 100ul Opti-MEM and incubated for 15-30 minutes. For transfection of cells in a 24-well plate, the same ratios were used but the amounts were reduced by a factor of 4. The transfection complexes were added and incubated with cells for 4 hours. The medium was then replaced with fresh Opti-MEM. The next day the medium was replaced with complete medium and experiments were begun.

Lentivirus Production and Transduction

For lentivirus production, 7 x 10^5 293FT cells were seeded on 6cm tissue culture dishes in 5 mL of media. The following day the medium was replaced with antibiotic-free medium and transfection was performed. The psPAX2, pMD2.G and either pLKO.1 sh-RNA plasmid were co-transfected using a ratio of 0.75/0.25/1µg ratio and X-tremeGENE™ HP DNA Transfection Reagent using a 3µl/µg of DNA ratio. The following day, the medium was changed to remove the transfection reagent and 5ml of fresh medium was added. The next day, lentivirus-containing medium was harvested and stored at 4°C. Fresh media was again added to the cells. The next day, the medium was again harvested and pooled with the medium from the previous day. The medium was spun at 1,250 rpm for 5 minutes to pellet any 293FT cells that were inadvertently collected during harvesting. Virus was aliquoted and then frozen at -80°C for long-term storage.

For transduction of lentivirus and sh-RNA mediated FOXO3 knock-down, Huh7 cells were seeded a T-25 flask at 1.5 x 10^6 cells per flask. The next day, the sh-FOXO3 and sh-NT viruse-containing media were thawed on ice. 2µl of polybrene from 8mg/ml stock was added to 2ml of virus-containing medium and incubated at room temperature for 15min at room temperature. Virus was added to the cells and incubated for 5-6 hours before adding 2ml of fresh

medium. The next day, media were replaced with fresh medium. Pools of shRNA-transduced cells were selected the following day by adding 3μg/ml puromycin to the medium. The following day, the media were replaced with fresh puromycin medium. The following day, the media were replaced with normal medium without antibiotics and the cells were allowed to recover for 3-5 days before checking the level of FOXO3 knock-down by western blot. Sh-FOXO3 and sh-NT Huh7 cells were only used in experiments for two passages.

Human Samples

At the University of Kansas Hospital, approximately 150 liver transplants are done each year, of which approximately 30 have a diagnosis of HCC. When liver transplantations for patients with HCC are done the explant livers are dissected and gross and microscopic analyses are performed to determine the number, size, and histologic grade of the tumor(s), which are used to help determine the clinical stage, prognosis, and risk of recurrence. We developed collaborations with nurses, technicians, residents, surgeons, and pathologists, so that we could have access to samples of tumor and adjacent tissue from the explant livers that would have been discarded. We also collected corresponding clinical data including patient gender, age, etiology of liver disease, treatment prior to transplantation, tumor size, histologic grade of the HCC, presence or absence of tumor metastasis and vascular invasion, and we periodically followed patients on our list to watch for tumor recurrence after transplant. Tumor size was measured during gross dissection of the liver after transplant. The differentiation or histologic grade of each tumor was determined by a pathologist and given a qualitative score of well, moderate to well, moderate, moderate to poor, or poor. For measuring correlations with staining, each state was assigned a numerical value (1-5), with 1 being well differentiated and 5 being poorly differentiated. All human tissues were obtained with informed consent from each patient,

according to ethical and institutional guidelines. The study was approved by the Institutional Review Board at the University of Kansas Medical Center.

Immunohistochemical Staining of Human Liver Tissue

Human liver samples were fixed in 4% paraformaldehyde for 24 hours and were then paraffin embedded. 5μm tissue sections were made and put on slides for immunohistochemical (IHC) staining. To begin IHC staining, sections were deparaffinized and hydrated by three washes with xylene for 15 minutes each, followed by three washes with 100% ethanol for five minutes each, followed by one was in 70% ethanol for ten minutes and two washes in distilled H_2O for five minutes each. Antigen unmasking in the tissue sections was done by boiling the slides in a pressure cooker in 10mM sodium citrate buffer with 0.5% Tween 20, pH 6.0 for ten minutes, and allowed to cool down for 30 minutes. The slides were then washed twice in distilled H_2O for five minutes each. Blocking of the slides was then done using 3% hydrogen peroxide for five minutes, followed by a rinse in distilled H_2O for five minutes. Slides were then washed twice in tris buffered saline with 0.1% Tween 20 (TBS-T) for three minutes each. The reagents for visualization including blocking reagent, dilution reagent, linker reagent, streptavidin peroxidase, and substrate-chromogen solution were provided in the Dako LSAB$^+$ System-HRP kit. Slides were blocked using the serum-free blocking reagent for five minutes. The primary antibody was diluted 1:100 in the blocking reagent and was incubated on the tissue sections overnight at 4^0C in a humidified chamber. The primary antibody was removed and the slides were washed twice in TBS-T for 15 seconds each time. The tissue sections were covered with the link buffer and incubated for 30 minutes at room temperature. The slides were then washed twice in TBS-T for 15 seconds each time. The sections were then covered with 1-3 drops of streptavidin peroxidase for 30 minutes at room temperature, followed by two washes with TBS-T

for 15 seconds each time. Substrate-Chromgen Solution was applied to slides and incubated 5-10 minutes. Sections were counterstained with hematoxylin for 0.5-1 min, followed by washing with tap water twice for three minutes each. Slides were then dehydrated and a coverslip was applied.

We enlisted the help of a pathologist, Dr. Maura O'Neil, to examine the IHC stained slides with us and distinguish areas of HCC from dysplastic and regenerative nodules. After staining the samples, we used the Aperio® AT2 digital pathology slide scanner (Leica Biosystems, Buffalo Grove, IL) to acquire digital images of each slide. We then used Aperio ImageScope software and algorithms, licensed by the University of Kansas Hospital Pathology Department to quantitate the amount of FOXO3 and SIRT6 expression in selected areas of cirrhosis and HCC. Briefly, this software uses hematoxylin staining to identify cell nuclei and quantifies the amount of HRP staining in each nucleus. It also measures cytoplasmic staining by quantifying the amount of HRP within a chosen radius around each nucleus. The software measures the intensity of each pixel. Intensity, which is proportional to the amount of light transmitted through the slide, ranges from zero (black) to 255 (bright white), so that a large intensity value means there is less staining present. The average intensity of all of the objects (nuclei or cytoplasm) within a chosen area on a slide is given on a scale from 0 (low) to 255 (high). We subtracted these values from 255 in order to present the data on a numerical scale where a low value represents low staining and a high value represents high staining. We verified the data by having two people provide a semi-quantitative score of the nuclear and cytoplasmic FOXO3 staining in the samples while blinded to all clinical data. We found it consistent with the Aperio quantitative measurements.

Western Blotting

Whole cell lysates were prepared from cells lysed in RIPA buffer (20mM Tris [pH 7.4], 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM $Na_4P_2O_7$, protease inhibitors). Lysates were centrifuged at 20,000 x g for 20 min, supernatants collected, and protein concentration measured using the Bio-Rad protein assay kit. Cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P membranes; Millipore, Billerica, MA, USA). Membranes were blocked with blocking buffer (5% bovine serum albumin, 0.1% Tween-20 in TBS) for 1 h at room temperature. After incubation with the appropriate primary antibodies overnight at 4 °C, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, detected using the WesternBright ECL system from Advansta (Menlo Park, CA) and the ODYSSEY Fc, Dual-Mode Imaging system (Li-COR, Lincoln, NE, USA). Primary antibodies used were rabbit polyclonal anti-p-FOXO3-S574 polyclonal antibody generated by our lab through Abcam (Burlingame, CA, 1:500) (Li, 2016), rabbit anti-GAPDH (FL-335, Santa Cruz Biotechnology, Dallas, TX, 1:2000), rabbit anti-HA antibody (Santa Cruz, 1:1000), anti-rabbit FOXO3 (75D8) (Cell Signaling Technologies, 1:500), and rabbit anti-P-JNK antibody (Cell Signaling Technologies, 1:1000).

Immunoprecipitation

Huh7 cells were seeded on 100mm dishes at a density of 5 x 10^6 cells/dish and were treated as indicated. Following treatment, cells were washed with ice-cold PBS, lysed in 400-500ul cell RIPA buffer containing protease inhibitors, and kept on ice for 15min. Lysates were centrifuged for 15min at 16,000g at 4^0C and protein concentration was measured in the supernatant. 400μg of total protein was used for each immunoprecipitation (IP). Samples were diluted in IP Buffer (1%NP-40, 25mM Tris-HCl pH7.4, 0.2%SDS, 50mM NaCl, 0.5% sodium

deoxycholate with protease inhibitors). The immunoprecipitation antibody was added (~4ug or 1:100) and incubated overnight at 4°C with rotation. 20µl of Dynabeads M-280 Sheep anti-rabbit IgG (Fisher) were added to each sample and incubated at 4°C with rotation for 3-4 hours. Samples were washed 5 times with IP buffer, rotating for 10 min at room temperature. Magnetic beads were pelleted with a magnetic separator and the liquid was discarded. Samples were re-suspended in 10µl of new prepared 3X Western sample buffer and incubated at room temperature for 10 min, followed by incubation at 95 °C for at 3 min before proceeding to western blot. Primary antibodies used were anti-rabbit FOXO3 (75D8) (Cell Signaling Technologies, 1:500), and rabbit anti-Acetyl-Lysine (Cell Signaling Technologies, 1:00)

Immunofluorescence

Huh7 cells grown on coverslips were washed in PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. The fixed cells were then incubated in IF buffer (1% BSA, 2.5 mM EDTA in PBS) for 1 hr at room temperature. Cells were then incubated in primary antibody diluted in IF buffer for 1 h at room temperature or overnight at 4^0C. After washing with PBS, the coverslips were incubated with AlexaFluor conjugated goat secondary antibody (1: 500; Molecular Probes, Waltham, MA, USA) for 1 h in the dark at room temperature. Nuclei were counterstained by incubation for 5 min with 1.0 µg/ml DAPI and the cover slips were mounted in Fluorsave mounting medium (Invitrogen). Primary antibodies used were rabbit anti-FLAG antibody (F7425) (Sigma-Aldrich, 1:200), rabbit polyclonal p-FOXO3-S574 polyclonal antibody, and mouse anti-FOXO3 clone 15F7.2 (EMD Millipore, Darmstadt, Germany, 1:100). Images were acquired by using Nikon Eclipse Ti microscope (Nikon Americas Inc., Melville, NY, USA).

Proximity Ligation Assay

Proximity ligation assays (PLA) were carried out using Duolink® In Situ PLA® Probe and In Situ Detection kits according to the manufacturer's instructions. Before detection Huh7 cells were seeded in 6-well plates on coverslips treated at a density of 15 x 10^4 cells/well and were treated as indicated. After treatment, cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed with PBS, permeabilized with 1% Triton in PBS, blocked with supplied PLA blocking buffer, and incubated with mouse anti-FOXO3 antibody clone 15F7.2 (EMD Millipore, 1:100) and rabbit anti-Acetyl-Lysine (Cell Signaling Technologies, 1:100) overnight at 4^0C. Interactions were visualized using detection reagent supplied in the kit. The PLA assay omitting one or both primary antibodies was used as a negative control. Counterstaining for flag-positive cells after the Duolink® In Situ assay was performed according to the manufacturer's protocol using the DYKDDDDK Tag Alexa Fluor® 488 Conjugate antibody (Cell Signaling, 1:100). Images were acquired by using Nikon Eclipse Ti microscope (Nikon Americas Inc., Melville, NY, USA).

LDH Release and TUNEL Assays

To determine doxorubicin-induced cytotoxicity, Huh7 cells were seeded in 24-well plates at a density of 5 x 10^4 cells/well. The next day, cells were treated with doxorubicin. The concentrations of doxorubicin used for experiments were 0.5 or 1μM. doxorubicin treatment was continued for 72 hours before LDH release and total LDH were determined using the Pierce™ LDH Cytotoxicity Assay Kit according to manufacturer's instruction. When determining the effect of resveratrol treatment on doxorubicin cytotoxicity, resveratrol was added at a concentration of 50μM 4 hours before doxorubicin treatment and was continued for 24 hours during doxorubicin treatment. If cells were to be transfected before doxorubicin exposure, they

were seeded at a density of 2.5 x 10^4 cells/well and were transfected 24 hours before beginning doxorubicin treatment.

The DeadEND Fluorometric TUNEL System was also used to determine the effect of overexpressing SIRT6 or the SIRT6-H133Y on doxorubicin cytotoxicity in Huh7 cells. Cells were seeded in 6-well plates on coverslips at a density of 15 x 10^4 cells/well 24 hours before transfection. The next day, the medium was replaced with fresh medium containing 1μM. doxorubicin, and this was continued for 36 hours. The cells were then fixed with 4% paraformaldehyde at room temperature for 30 min. After a PBS rinse, cells were stained using the DeadEND Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Following TUNEL staining, immunofluorescence was performed, counter staining for flag to determine which cells were transfected. The percentage of TUNEL positivity was quantified in untransfected (no flag expression), and SIRT6 and SIRT6-H133Y-transfected cells (positive for flag expression) by examining 5 randomly selected 10X fields.

Statistics

Results are expressed as the mean ± standard error (SE). Student's t-test, Mann-Whitney rank sum test, and Spearman's rank correlation were used for statistical analyses using SigmaPlot version 11.0 (Systat Software, Inc., San Jose, CA). $p < 0.05$ was considered significant.

Chapter III: Expression of FOXO3 in Human Hepatocellular Carcinoma and Correlation with Clinical Behavior

Background

FOXO3 plays an important role in liver diseases, including HCV infection and alcoholic liver disease (114, 128). This transcription factor plays a role in the pathogenesis of several types of cancer, as significant correlations between increased or decreased FOXO3 expression and clinical outcome have been reported in colorectal cancer (143), breast cancer (122, 171), acute myeloid leukemia (176), and prostate cancer (177), to name a few. However, there has been little investigation of FOXO3 in the pathogenesis of HCC. To date, only one study has examined FOXO3 expression in HCC tumors. These data are incomplete, though, because the majority of these samples were from patients with HBV infection without cirrhosis (160), while most HCC patients in the U.S. have both HCV infection and cirrhosis (2, 4). Since FOXO3 function can drastically vary depending on the cell context, it is possible that the role of FOXO3 in HCC will also vary depending on tumor and patient characteristics such as the presence of cirrhosis.

To address this gap in knowledge, this chapter provides a detailed characterization of FOXO3 expression in HCC in samples that are representative of the population of HCC cases in the US. The strategy for this study was to begin by determining the pattern of FOXO3 expression in human HCC samples, including the overall expression level in both tumor and surrounding cirrhotic tissue, as well as the cellular localization. As discussed in Chapter 1, FOXO3 localization can provide some insight into its function. The first layer of regulation of FOXO3 is through PTMs that control its translocation between the nucleus, where it is able to activate or repress gene transcription, and the cytosol, where it may carry out other functions or be degraded. This level of regulation has been reported to occur in other cancers such as breast

cancer, where FOXO3 activity is frequently suppressed by cytoplasmic sequestration and degradation (122).

Since both levels of expression and localization can potentially affect FOXO3 function, we also compared expression to clinical parameters, such as tumor size, risk for recurrence, and treatment effects. These analyses revealed significant increases in FOXO3 expression in tumor compared to non-tumor, as well as positive correlation of FOXO3 expression with markers of aggressive behavior. In addition, this analysis revealed an unexpected effect of doxorubicin treatment on the expression pattern of FOXO3, which may have implications for clinically relevant drug resistance.

Results

FOXO3 expression in HCC compared to cirrhotic tissue

To characterize FOXO3 expression in human HCC tumors, we performed IHC staining in HCC samples and surrounding adjacent cirrhotic tissue from 35 different patients. We measured nuclear, cytoplasmic, and total FOXO3 expression. In cirrhotic tissue, there was quite a bit of variation from sample to sample, but, when present, FOXO3 was typically found in the nucleus, with very little cytoplasmic protein (Figure 2A-D). The expression of FOXO3 in HCC was extremely variable, particularly the localization (Figure 2E-H). Most tumors had a high level of nuclear FOXO3, while some also had a very high level of cytosolic FOXO3. Comparing the total FOXO3 expression in each of the HCC samples to adjacent cirrhotic tissue, we found the mean value to be significantly higher in HCC (Figure 3B). A comparison of either the nuclear or cytosolic expression of FOXO3 in HCC samples with adjacent cirrhotic tissue revealed that the mean nuclear FOXO3 expression in HCC is significantly higher than in cirrhotic tissue (Figure 3C). Due to the high variation in cytosolic FOXO3 in the tumor samples, the Shapiro-Wilk

normality test failed, so we were unable to use a t-test to compare tumor to cirrhosis. Instead, we used a Mann-Whitney Rank Sum Test to compare the median value of cytosolic FOXO3 expression in HCC compared to cirrhosis, and we found it to be significantly higher in HCC (Figure 3D). Collectively these data indicate that the total, nuclear, and cytosolic FOXO3 expression is higher in HCC compared to adjacent cirrhotic tissue.

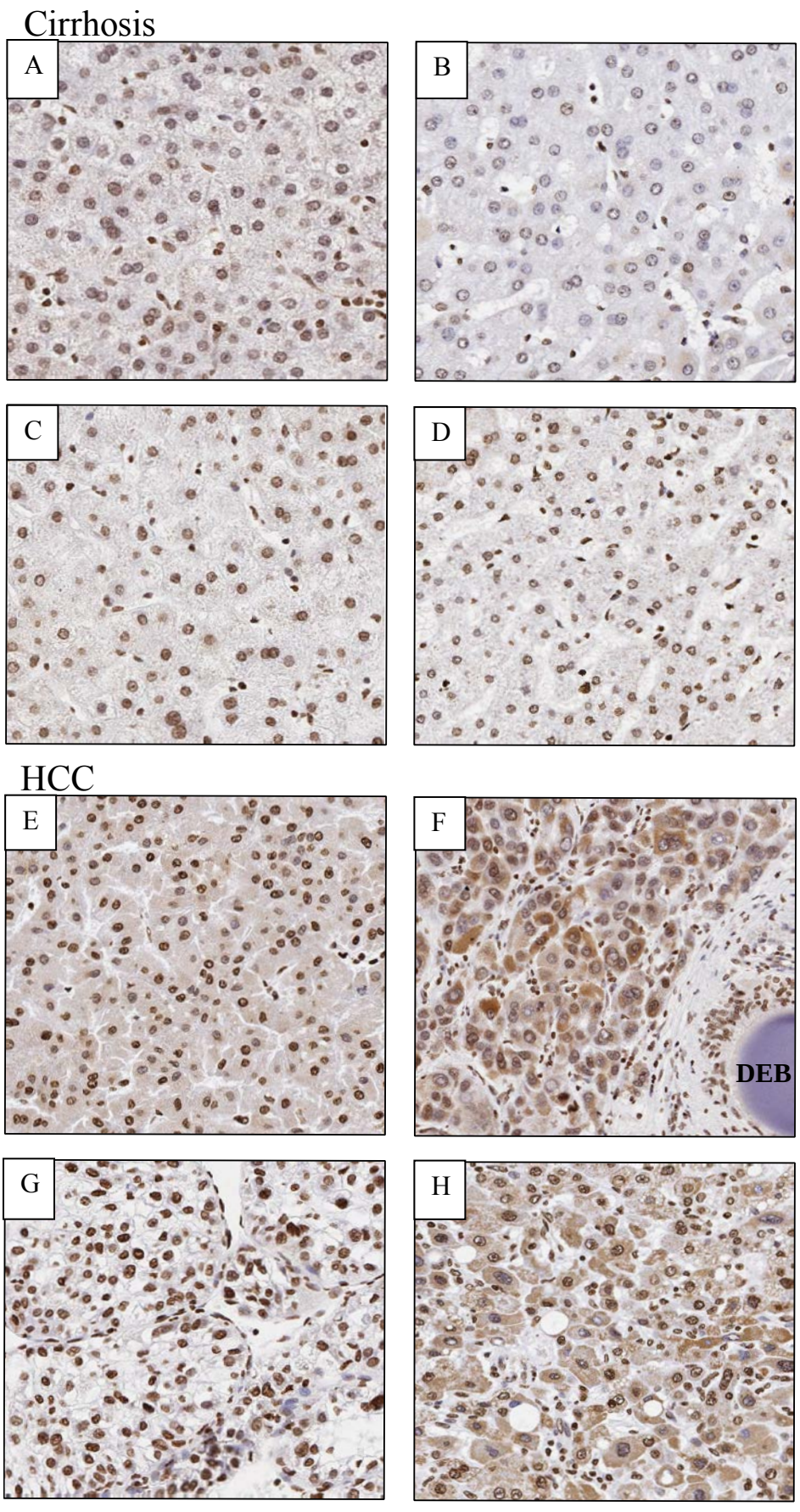

Figure 2: FOXO3 expression in HCC and adjacent cirrhotic liver. IHC staining for FOXO3 (brown) and hematoxylin (blue). **(A-D)** Typical examples of FOXO3 expression in tumor-adjacent, non-malignant hepatocytes. **(E-F)** Typical examples of FOXO3 expression in HCC, drug eluting bead (DEB) labeled

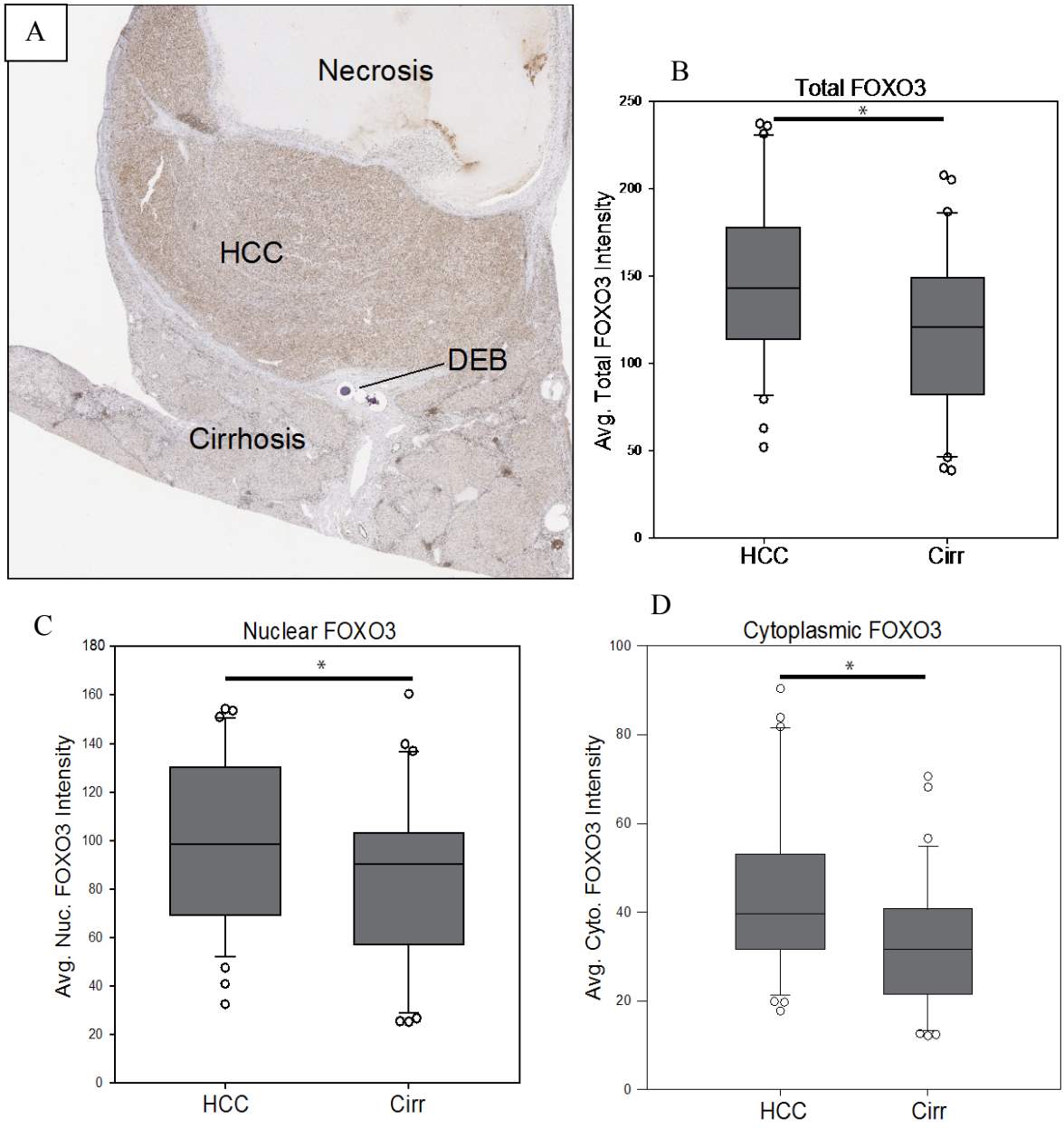

Figure 3: Quantification of FOXO3 expression in HCC and cirrhosis.
(A) Picture of IHC staining for FOXO3 and hematoxylin with DEB and HCC, cirrhosis, and necrosis areas labeled. **(B)** Total FOXO3 expression (nuclear and cytosolic) in HCC (left) and cirrhosis (right). **(C)** Nuclear FOXO3 expression in HCC and cirrhosis. **(D)** Cytoplasmic FOXO3 expression in HCC and cirrhosis. Box plot upper boundary represents 75th percentile, lower boundary represents 25th percentile, and the line within the box represents the median. The upper and lower error bars represent the 90th and 10th percentile respectively. For HCC samples n=35, and for cirrhosis samples n=33. Values for each sample in B-D represent the average staining intensity of all the objects (nuclei or cytoplasm) within a selected area subtracted from 255 (maximum intensity) so that a higher value equals increased expression. * indicates $p \leq 0.05$

Correlation of FOXO3 expression with clinical characteristics

Since the overall, nuclear, and cytosolic FOXO3 expression was higher in HCC compared to adjacent cirrhotic tissue, but yet there was marked variability in expression from tumor sample to sample, we hypothesized that FOXO3 expression patterns may correlate with clinical and pathologic behavior. To test this we compared expression with a number of variables, including tumor size, histologic grade, vascular invasion, prior treatment, and recurrence after transplant. In this initial analysis, we decided to include only the 22 tumors that were treatment-naïve because we wished to investigate the significance of variation in FOXO3 expression in HCC apart from its role in response to treatment. FOXO3 expression in treated tumors is analyzed separately (see below).

Importantly, tumor size, vascular invasion, and histologic grade are reported to be strong predictors of recurrence after transplant (178), and we found our samples to be consistent with this finding, as tumor size had a significant correlation with recurrence, and the presence of vascular invasion correlated with recurrence as well, although not quite statistically significant (Table 1). Evidence of vascular invasion by the tumor was found in four patients, two of which to this date have had tumor recurrence, and there were two more patients who have had tumor recurrence, totaling four recurrences to this date. We also found that both nuclear and cytosolic FOXO3 expression had significant positive correlations with tumor size, and that nuclear FOXO3 significantly correlated with tumor grade as well.

Table 1: FOXO3 Clinical Correlations

		Avg. Cyto. FOXO3	Avg. Nuc. FOXO3	Tumor Diameter	Differentiation (Grade)	Vascular invasion	Recurrence
Avg. Cyto. FOXO3	Pearson Correlation	1	.710**	.506*	.390	.269	.283
	P. value		.000	.016	.072	.227	.202
Avg. Nuc. FOXO3	Pearson Correlation	.710**	1	.438*	.548**	.287	.285
	P. value	.000		.042	.008	.195	.198
Diameter	Pearson Correlation	.506*	.438*	1	.262	.526*	.554**
	P. value	.016	.042		.238	.012	.007
Differentiation	Pearson Correlation	.390	.548**	.262	1	.640**	.442*
	P. value	.072	.008	.238		.001	.040
Vascular invasion	Pearson Correlation	.269	.287	.526*	.640**	1	.389
	P. value	.227	.195	.012	.001		.074
Recurrence	Pearson Correlation	.283	.285	.554**	.442*	.389	1
	P. value	.202	.198	.007	.040	.074	

Table 1: FOXO3 clinical correlations.
Values for cytosolic and nuclear FOXO3 in 22 treatment-naïve HCC samples represent the average optical density (O.D.) of all the objects (nuclei (N) or cytoplasms (C)) within a selected area subtracted from 240 (maximum O.D.) so that a higher value equals increased expression. Pearson correlation coefficients between these values and clinical variables were determined using SPSS. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

Nuclear/Cytosolic Distribution of FOXO3 in TACE-treated HCC Compared to Treatment-naïve

Many patients with HCC are treated with TACE doxorubicin as a "bridging" therapy while on the waiting list for transplant, or as means of "down-staging" a patient's tumor in order to fit within Milan criteria for transplant eligibility (179). Of the 35 patients in this study, 13 had received prior treatment using TACE with doxorubicin at least once, some of whom had been treated multiple times, and still had viable tumor cells remaining on histology at the time of transplant. On histology, we could frequently see DEBs within the vasculature in or around the tumor remaining from prior TACE treatment in these samples (as seen in Figure 2F and Figure 3A). Surprisingly, several of the tumors that had been treated with TACE had very high cytosolic FOXO3 (as seen in Figure 2F, H). When compared to treatment-naïve tumors, the cytosolic expression of FOXO3 was significantly higher in the TACE-treated group (Figure 4C), while the total and nuclear FOXO3 expression were not significantly different between these two groups (Figure 4A, B). When these data were expressed as nuclear to cytosolic ratio (N/C) of FOXO3 expression, the analysis demonstrated a striking reduction in the TACE-treated compared to the treatment-naïve group (Figure 4D).

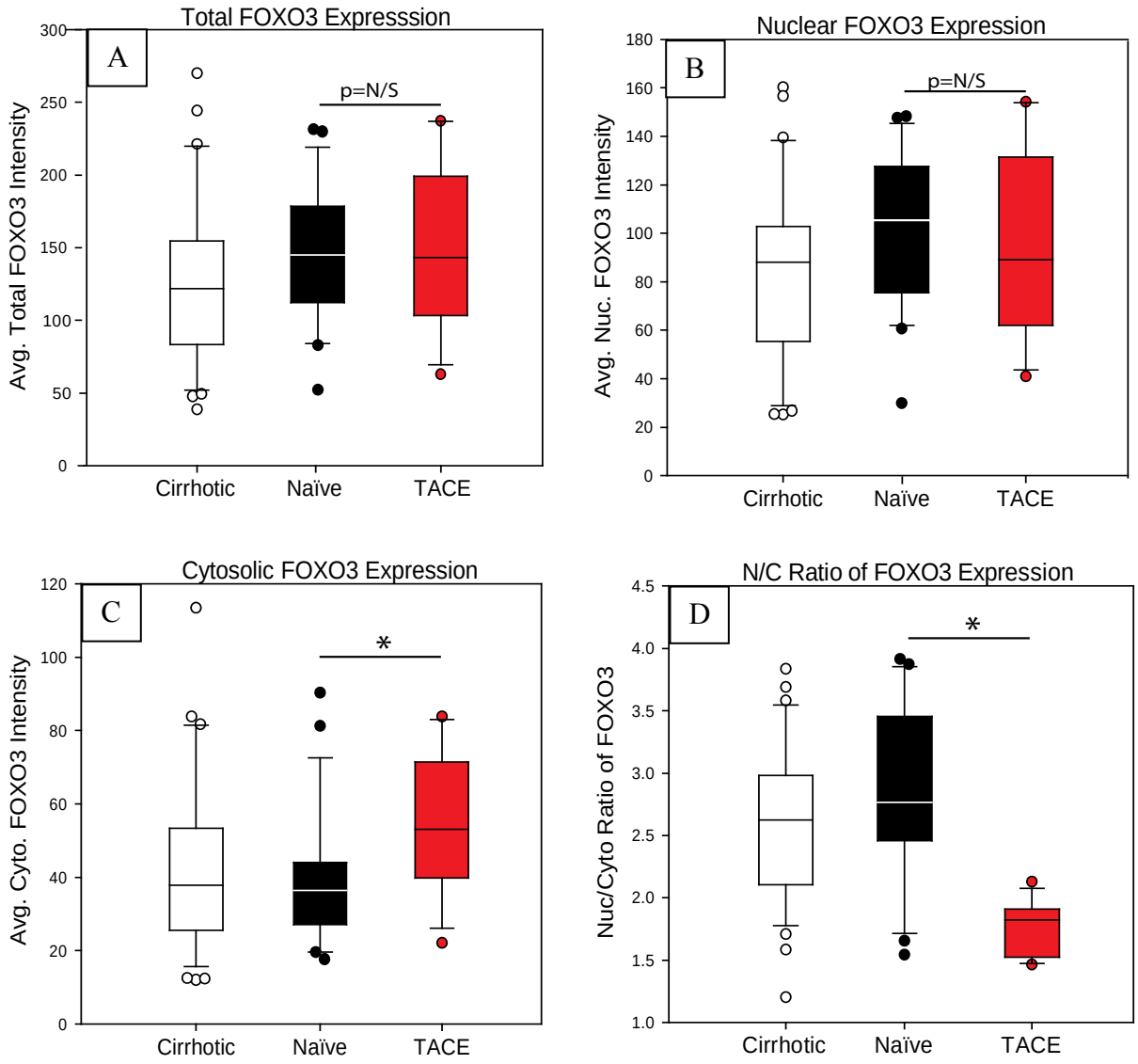

Figure 4: Quantification of FOXO3 expression in treatment-naïve and TACE-doxorubicin treated HCC.

(A) Total FOXO3 expression (nuclear and cytosolic) in cirrhosis (left), treatment-naïve HCC (middle), and TACE-doxorubicin-treated HCC (right). **(B)** Nuclear FOXO3 expression. **(C)** Cytoplasmic FOXO3 expression. **(D)** Nuclear/cytosolic (N/C) ratio of FOXO3 in each sample. Box plot upper boundary represents 75th percentile, lower boundary represents 25th percentile, and the line within the box represents the median. The upper and lower error bars represent the 90th and 10th percentile respectively. Circles represent samples with values outside the 10th and 90th percentiles. For cirrhosis samples n=33, for treatment-naïve HCC samples n=22, and for TACE-doxorubicin-treated HCC n=13. Values for each sample in A-C represent the average intensity of all the objects (i.e. nuclei) within a selected area subtracted from 255 (maximum intensity) so that a higher value equals increased expression. Values for each sample in D. p=N/S indicates $p \geq 0.05$. * indicates $p \leq 0.05$

Discussion

The level of FOXO3 expression has been reported for several types of human cancer and has been found to correlate with clinical behavior in some. In colon cancer, suppression of FOXO3 expression was found to correlate significantly with worsening pathologic stage, and patients with low-FOXO3-expressing tumors had significantly shorter survival (143). However, in the CN subtype of AML, high FOXO3 is correlated with worse patient survival (149). The only other report on FOXO3 expression in HCC found that it correlated inversely with histologic grade and tumor size and those patients with high FOXO3-expressing tumors had increased survival (160). However, this study looked at tumors in predominantly non-cirrhotic livers with HBV infection. In contrast, I have found in our samples, which all had cirrhosis and a majority had HCV infection, that FOXO3 expression is higher in HCC compared to adjacent cirrhotic tissue, and correlates positively with worse histologic grade, and greater tumor size. Clearly, much more investigation is needed to understand the role of FOXO3 in HCC. However, this evidence suggests that FOXO3 may be promoting tumor cell survival in HCC.

It is possible that FOXO3 can play a tumor-suppressing role in certain contexts or at certain times during tumor progression, while in other contexts and at other times it may be promoting tumor cell survival and growth. One such context that may help determine what role FOXO3 plays is the metabolic environment. A recent study found FOXO3 to promote apoptosis in response to oxidative stress when cells were grown in medium with 10% serum, however, when cells were grown in 5% serum and were exposed to oxidative stress, FOXO3 inhibited apoptosis (117). Cirrhosis alters stiffness and blood flow in the liver and thus hugely affects hepatocyte function. For example, in a cell culture of cirrhosis using HepG2 hepatoma cells and a collagen matrix, increasing stiffness of the hepatoma cells caused decreased glucose

metabolism and suppressed AKT activation (180). Cirrhosis, present in all the livers in our study, likely altered the metabolic environment of both normal and malignant hepatocytes, potentially influencing the role FOXO3 plays in HCC. The exact role of FOXO3 in HCC initiation and progression is an important area for future study. However, given the significant change in the FOXO3 expression pattern in TACE-doxorubicin-treated HCC, and the potential clinical impact of gaining further understanding of mechanisms of doxorubicin resistance in HCC, we decided to focus our investigation on the role of FOXO3 in determining doxorubicin sensitivity in HCC.

The data presented in figure 3 demonstrate that FOXO3 expression in tumors that had been treated with TACE-doxorubicin, and still had remaining viable cells, had higher cytosolic FOXO3 and a lower N/C ratio of FOXO3 expression in their cells compared to treatment-naïve HCC. Resistance to TACE-doxorubicin in HCC is frequently seen and can be inherent to the tumor cells or it can represent an acquired property (27). In these samples, there were often large areas of necrosis, indicating partial effectiveness of the treatment, although some samples showed no signs of treatment effectiveness. Tumor cells in these samples likely represent cells which were either inherently resistant to TACE-doxorubicin or acquired resistance which allowed them to survive. The fact that the pattern of FOXO3 expression in these samples is significantly altered points to the possibility that FOXO3 is playing a role in doxorubicin-resistance in HCC.

As discussed in chapter 1, phosphorylation of FOXO3 by AKT (120), ERK (121), or IKK-β (122) can lead to its nuclear export, polyubiquitination, and proteosomal degradation. Accordingly, the increased cytosolic FOXO3 in TACE-doxorubicin resistant cells could indicate a suppression of FOXO3 activity. Additionally, FOXO3 has been shown to mediate doxorubicin-induced apoptosis in several types of cancer cells in which suppression of FOXO3 promotes

doxorubicin resistance (93). Therefore, one potential explanation for why TACE-doxorubicin-resistant HCC cells have significantly higher cytosolic FOXO3 is that suppression of FOXO3 activity is one mechanism by which these cells mediate resistance. One flaw with this explanation, however, is that neither the nuclear or total FOXO3 in these cells was significantly different from that in the treatment-naïve HCC.

Another potential explanation for increased cytosolic FOXO3 in TACE-doxorubicin-resistant HCC is that FOXO3 is serving a function in the cytosol which is increased in these cells. Although transcription factors are typically thought of as "active" in the nucleus where they can increase gene transcription, many transcription factors, including p53 (181) and FOXO1 (182), have been shown to have transcription-independent functions in the cytosol. FOXO3 as well, has been shown to exist in mitochondria where it can physically interact with and be deacetylated by the mitochondrial sirtuin, SIRT3. SIRT3-mediated deacetylation of FOXO3 was shown to increase FOXO3 DNA-binding, increase expression of some FOXO3-dependent genes, and decrease intracellular ROS (183). Therefore, more investigation needs to be done to determine whether FOXO3 has a cytosolic function in HCC and particularly in TACE-doxorubicin-resistant HCC cells.

Finally, an altered cellular localization of FOXO3 in TACE-doxorubicin-resistant HCC cells almost certainly represents a change in the PTMs on FOXO3. As discussed in chapter 1, PTMs including phosphorylation and acetylation have been shown to regulate FOXO3 localization. PTMs on FOXO3 by upstream enzymes do not occur in a vacuum, but rather, PTMs which have opposing effects on FOXO3 are likely occurring simultaneously, and the balance of these modifications determine the ultimate pattern of FOXO3 localization in cells. For example, AKT-mediated phosphorylation of FOXO3 causes its cytoplasmic translocation and degradation

(120), however, SIRT1-mediated deacetylation of FOXO3 has been shown to be able to override AKT and maintain FOXO3 nuclear localization (184).

In addition to altering FOXO3 localization, PTMs are known to regulate the transcriptional program of FOXO3 (91). Accordingly, the PTMs which are altering FOXO3 localization in TACE-doxorubicin-resistant HCC cells may also be altering FOXO3 function in these cells. Although FOXO3 has been shown to mediate doxorubicin-induced apoptosis in other cancer cell types, specific PTMs necessary for this function have not been identified. Additionally, FOXO3 has been shown to promote resistance to oxidative stress and promote resistance to DNA-damage, like that caused by doxorubicin, in certain cell types and contexts. Therefore, the role of FOXO3 in determining the doxorubicin sensitivity of HCC cells requires further investigation into the FOXO3 PTM changes caused by doxorubicin and how these affect FOXO3 function.

Chapter IV: The Role of FOXO3 and Specific Post-translational Modifications of FOXO3 on Doxorubicin Sensitivity in Huh7 Cells

Background

FOXO3 is capable of both activating and suppressing transcription, and these functions, as well as the pattern of target genes affected, allow FOXO3 to promote many different, sometimes opposing, cellular functions. For example, FOXO3 is capable of both inhibiting cell cycle progression, by increasing transcription of its target genes p21 and p27 (71), and increasing cell proliferation, through transcriptional upregulation of cyclinA1 (152). One mechanism by which FOXO3 can achieve these diverse effects is through post-translational modifications (PTMs). As discussed in chapter 1, FOXO3 PTMs can affect function by influencing cellular localization. In addition, these PTMs can influence FOXO3 transcriptional regulatory activity directly.

The effects of specific PTMs on FOXO3 transcriptional activity have not been as extensively investigated as their effect on FOXO3 localization. However, several studies have examined upstream pathways that affect FOXO3 transcriptional activity and function (52), and have implicated two classes of PTMs in regulating FOXO3 function: acetylation and phosphorylation. Depending on the site and context, these PTMs have been linked to induction of both FOXO3-mediated stress-resistance and FOXO3-promoted apoptosis. For example, nutrient deprivation in 293T cells was shown to induce AMPK-dependent phosphorylation of FOXO3 at two sites, Ser413 and Ser588. These phosphorylation events promoted increased expression of FOXO3 target genes that facilitate resistance to injury and stress, including GADD45 (185).

Oxidative stress (91) and DNA damaging agents (142) have been shown to stimulate FOXO3 acetylation, and this PTM also influences FOXO3 transcriptional function. Acetylation of FOXO3 apparently has opposing effects relative to AMPK-stimulated phosphorylation, since deacetylation of FOXO3 has been shown to promote resistance to stress. In particular, deacetylation of FOXO3 by Sirtuin family deacetylases (SIRTs) promoted FOXO3-dependent resistance to cell stress (91) by enhancing both FOXO3-dependent expression of genes involved in DNA repair (GADD45) (91) and oxidative stress (SOD2) (186), and FOXO3-dependent suppression target genes involved in apoptosis, including BIM (187) and Fas-L (91). Additionally, the SIRT-activating compound resveratrol, which is produced in plants in response to stress (188), increases FOXO3 expression (189) and FOXO3-dependent transcription of SOD2 and catalase in response to oxidative stress. The mechanism of this effect was attributed to activation of both SIRT1 and AMPK (190). Thus, AMPK-dependent phosphorylation and deacetylation of FOXO3 both promote FOXO3-dependent resistance to stress and inhibition of apoptosis.

In contrast, a recent study demonstrated that JNK-dependent phosphorylation of FOXO3 at Ser574 is necessary for FOXO3-mediated induction of apoptosis. At baseline, very little FOXO3 is S574-phosphorylated. In response to LPS or ethanol, FOXO3 became S574-phosphorylated in hepatoma and macrophage cells respectively. S574-P-FOXO3 specifically bound to promoters and increased expression of the apoptosis-inducing TRAIL, while suppressing transcription of anti-apoptotic BCL-2. On the other hand, FOXO3 unphosphorylated at S574 bound to the promoters and increased the expression of BCL-2 and antioxidant genes PrxIII and SOD2 (114).

The functional effects of FOXO3 acetylation and phosphorylation may be relevant in a clinically important role for FOXO3, i.e., cellular sensitivity to the chemotherapeutic agent doxorubicin. FOXO3 has been shown to be capable of promoting doxorubicin-induced cell death (116, 169) or resistance to doxorubicin (171), depending on the cancer cell type and condition. Many studies have shown that doxorubicin induces nuclear translocation of FOXO3 (Chapter 1), and both phosphorylation and acetylation have been shown to affect FOXO3 nuclear localization. (91, 114, 191, 192) Whether doxorubicin induces these PTMs and whether these PTMs influence sensitivity to the drug are unknown.

To address these questions in this Chapter, we investigated the effects of doxorubicin on S574-phosphorylation and acetylation of FOXO3 in an HCC cell line Huh7. These analyses revealed that doxorubicin induces both of these PTMs. Further, our analyses indicate that these PTMs are critical for doxorubicin sensitivity in HCC cells. These data link for the first time specific FOXO3 PTMs and doxorubicin sensitivity and may provide potential strategies for decreasing doxorubicin resistance in HCC.

Results

The Effect of Doxorubicin on S574-phosphorylation and Acetylation of FOXO3

To determine the effect of doxorubicin on S574-phosphorylation of FOXO3 in HCC cells, we treated Huh7 cells with 1μM doxorubicin for 0, 4, 8 hours and performed a western blot probing for S574-P-FOXO3, using an antibody that specifically recognizes the S574-phosphorylated form of FOXO3. Doxorubicin induced phosphorylation of FOXO3 at S574 without changing the expression of total FOXO3 (Figure 5A). After exposure of Huh7 cells to doxorubicin, FOXO3 S574 phosphorylation increased with time up to 24 hours (Figure 5A-B).

Immunofluorescence microscopy demonstrated that the S574-P-FOXO3 form is largely nuclear in location (Figure 5B).

To determine the effect of doxorubicin on the acetylation status of FOXO3, we treated cells with 1μM doxorubicin and immunoprecipitated FOXO3 and probed the samples for acetyl-lysine. Similar to S574 phosphorylation, doxorubicin treatment also induced acetylation of FOXO3 (Figure 5C, D). Proximity ligation assay (PLA) using acetyl-lysine and FOXO3 antibodies confirmed these findings, with FOXO3 acetylation increasing with time up to 24 hours after doxorubicin exposure. Also as with S574-P-FOXO3, Ac-FOXO3 was also shown to be predominantly nuclear in location (Figure 5E).

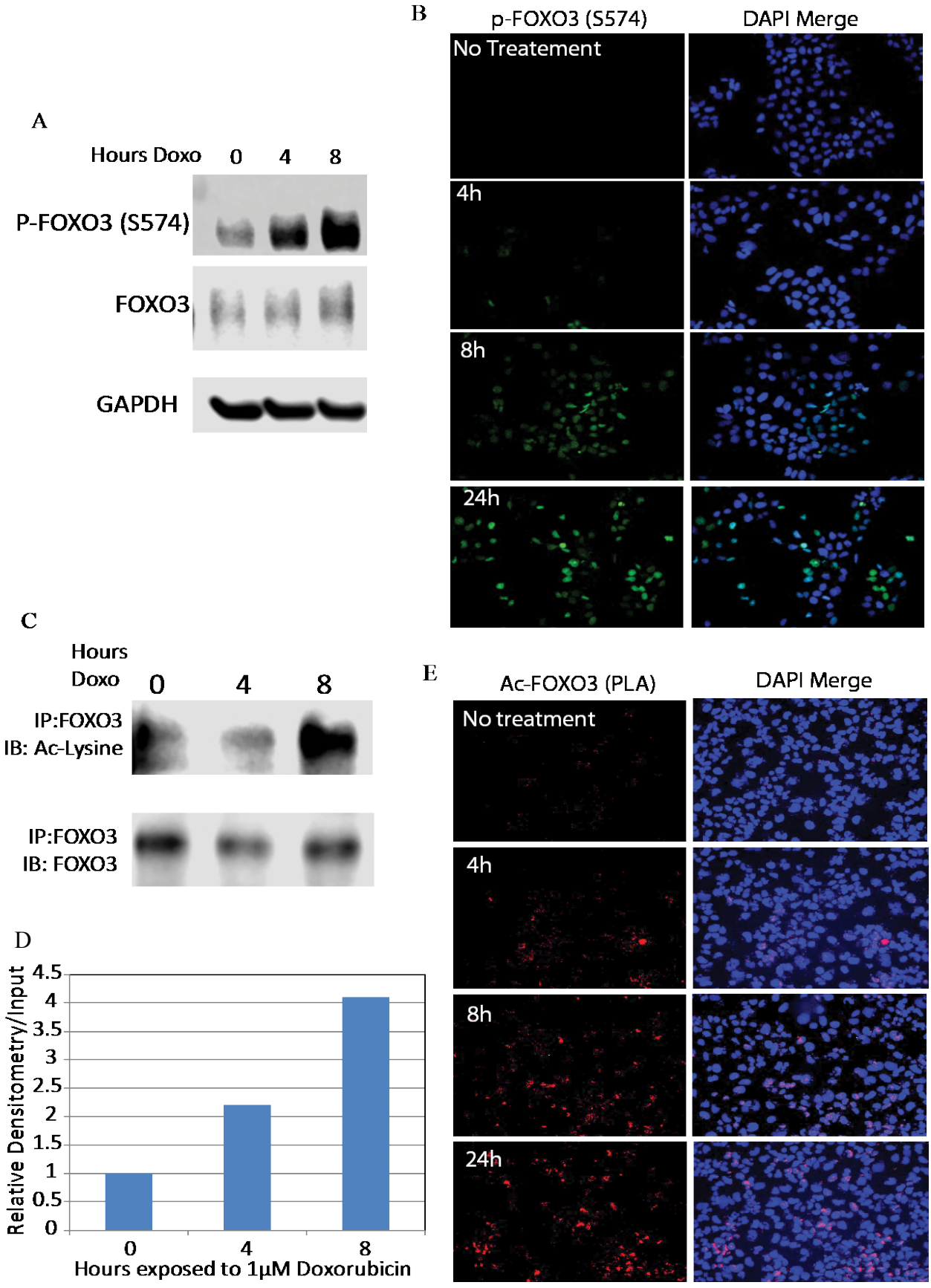
A
Hours Doxo 0 4 8
P-FOXO3 (S574)
FOXO3
GAPDH
B
p-FOXO3 (S574)
DAPI Merge
No Treatement
4h
8h
24h
C
Hours Doxo 0 4 8
IP:FOXO3
IB: Ac-Lysine
IP:FOXO3
IB: FOXO3
D
Relative Densitometry/Input
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 4 8
Hours exposed to 1μM Doxorubicin
E
Ac-FOXO3 (PLA)
DAPI Merge
No treatment
4h
8h
24h

Figure 5: Doxorubicin induces S574-phosphorylation and acetylation of FOXO3.
(A) Western blot of lysates from Huh7 cells treated with 1μM doxorubicin for 0, 4, or 8 hours, probing for P-S574-FOXO3, total FOXO3, and GAPDH as a loading control. **(B)** Immunofluorescence staining for P-S574-FOXO3 (green) and DAPI (blue) in Huh7 cells fixed after treating with 1μM doxorubicin for 0, 4, 8, or 24 hours. **(C)** Western blot using lysates from Huh7 cells treated with 1μM doxorubicin for 0, 4, or 8 hours, immunoprecipitated with FOXO3 antibody, blotting for Acetyl-Lysine (Ac-Lysine) and FOXO3 as input control. **(D)** Densitometry of Ac-Lysine western blot bands in the upper portion of figure 5C, relative to densitometry of corresponding FOXO3 bands in the lower portion of figure 5C. **(E)** Proximity ligation assay for acetylated FOXO3 (Ac-FOXO3, red) and staining for DAPI (blue) in Huh7 cells fixed after treating with 1μM doxorubicin for 0, 4, 8, or 24 hours.

FOXO3 S574-phosphorylation Increases Doxorubicin Sensitivity in Huh7 Cells

To examine the impact of FOXO3 S574-phosphorylation on doxorubicin sensitivity in Huh7 cells, we engineered FOXO3 mutations at Serine-574 to prevent (S574A) or mimic (S574D) its phosphorylation. The wild-type (WT) and mutant forms of FOXO3 were expressed in Huh7 cells in which native FOXO3 had been knocked down. Delivery of lentiviral short hairpin RNA directed against the 3'UTR of native FOXO3 mRNA resulted in ~80% knockdown of FOXO3 as assessed by western blot (Figure 6B). FOXO3 knockdown or cells transfected with empty vector (EV), FOXO3-WT, S574A, or S574D, were treated with 1μM doxorubicin for 72 hours and cell death measured by LDH release. In control Huh7 cells with native FOXO3 expression [i.e., cells transduced with non-targeted lentivirus (sh-NT)], overexpression of FOXO3-WT, S574A, or S574D had no effect on doxorubicin sensitivity (Figure 6A). Surprisingly, knockdown of FOXO3 alone did not significantly reduce doxorubicin sensitivity. Rescue of FOXO3 expression in sh-FOXO3 cells with WT or S574D did not affect doxorubicin sensitivity. However, rescue of FOXO3 with S574A significantly decreased doxorubicin sensitivity (Figure 6A).

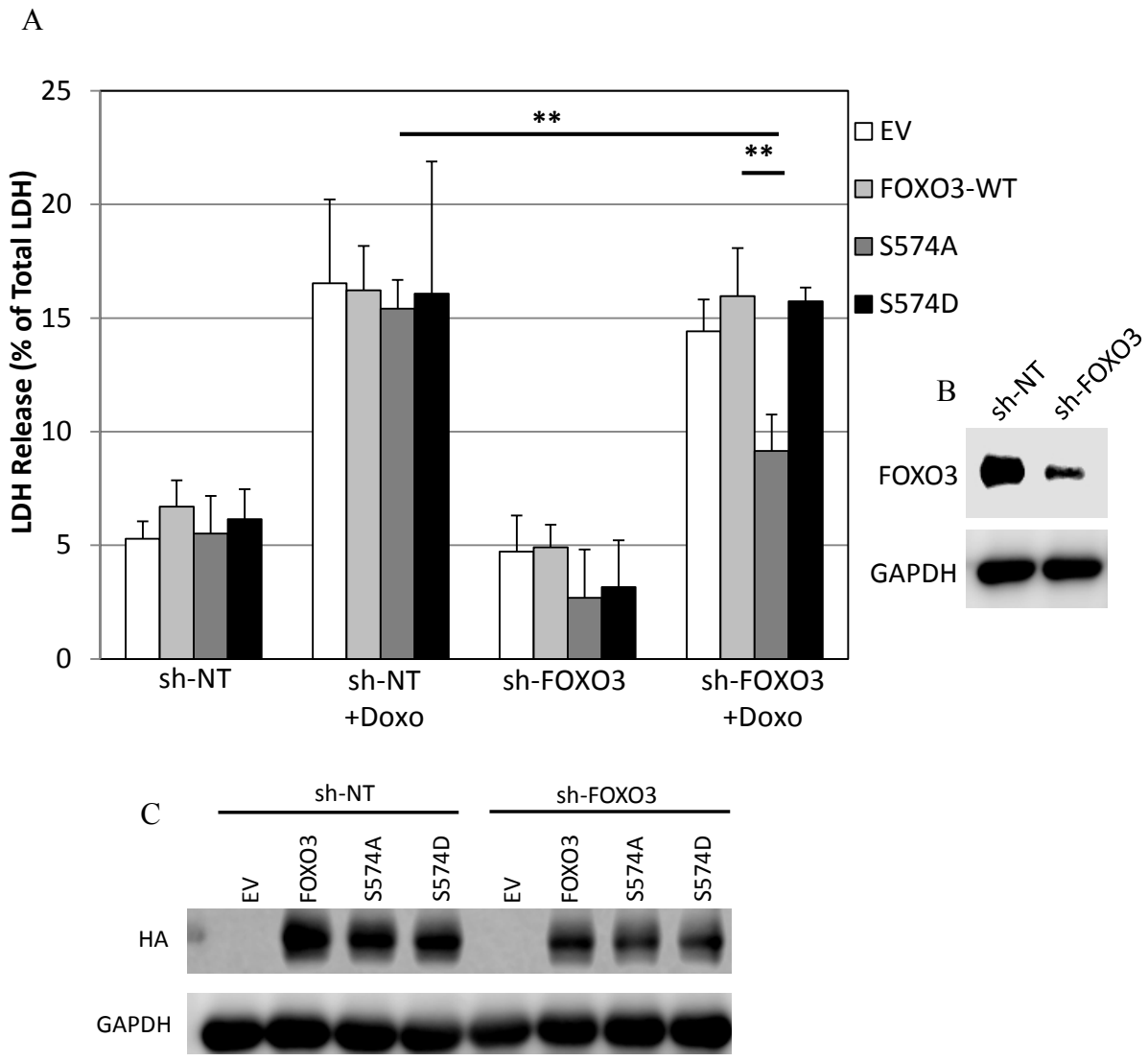

Figure 6: Effect of blocking S574-phosphorylation of FOXO3 on doxorubicin sensitivity in Huh7 cells.

(A) Huh7 FOXO3 knock down cells (sh-FOXO3) and control cells (sh-NT) were transfected with empty vector (EV), or HA-tagged wild-type FOXO3 (FOXO3-WT), S574A-FOXO3 (S574A), or S574D-FOXO3 (S574D). Cells were untreated or treated with 1μM doxorubicin for 72 hours. Cell death was evaluated by LDH release. Data is presented as the mean of four independent experiments ± SE, and represents the amount of LDH release as a percent of total LDH. ** indicates $p \leq 0.01$, Student's t-test. **(B)** Western blot to assess sh-RNA-mediated knock-down of FOXO3 expression. **(C)** Western blot to assess relative HA protein expression

Resveratrol Blocks Doxorubicin-induced FOXO3 PTMs and Promotes Resistance to Doxorubicin-induced Cell Death

To determine the impact of doxorubicin-induced FOXO3 acetylation, we used the SIRT deacetylase activator, resveratrol. Huh7 cells were treated with 100μM resveratrol followed by 1μM doxorubicin for 8 hours. Immunoprecipitation and western blot analysis of FOXO3 revealed that treatment of Huh7 cells with resveratrol significantly reduced doxorubicin-induced FOXO3 acetylation (Figure 7B). Interestingly, resveratrol also increased total FOXO3 expression, while significantly suppressing induction of S574-P-FOXO3 (Figure 7A). We also found that resveratrol blocked doxorubicin-induced phosphorylation of JNK, the kinase known to target FOXO3 S574 (Figure 7B). FOXO3 immunlocalization studies showed that doxorubicin increased nuclear translocation of FOXO3, consistent with previous studies (83). Moreover, resveratrol, while not completely blocking FOXO3 nuclear localization, significantly increased the amount of FOXO3 in the cytoplasm (Figure 7C).

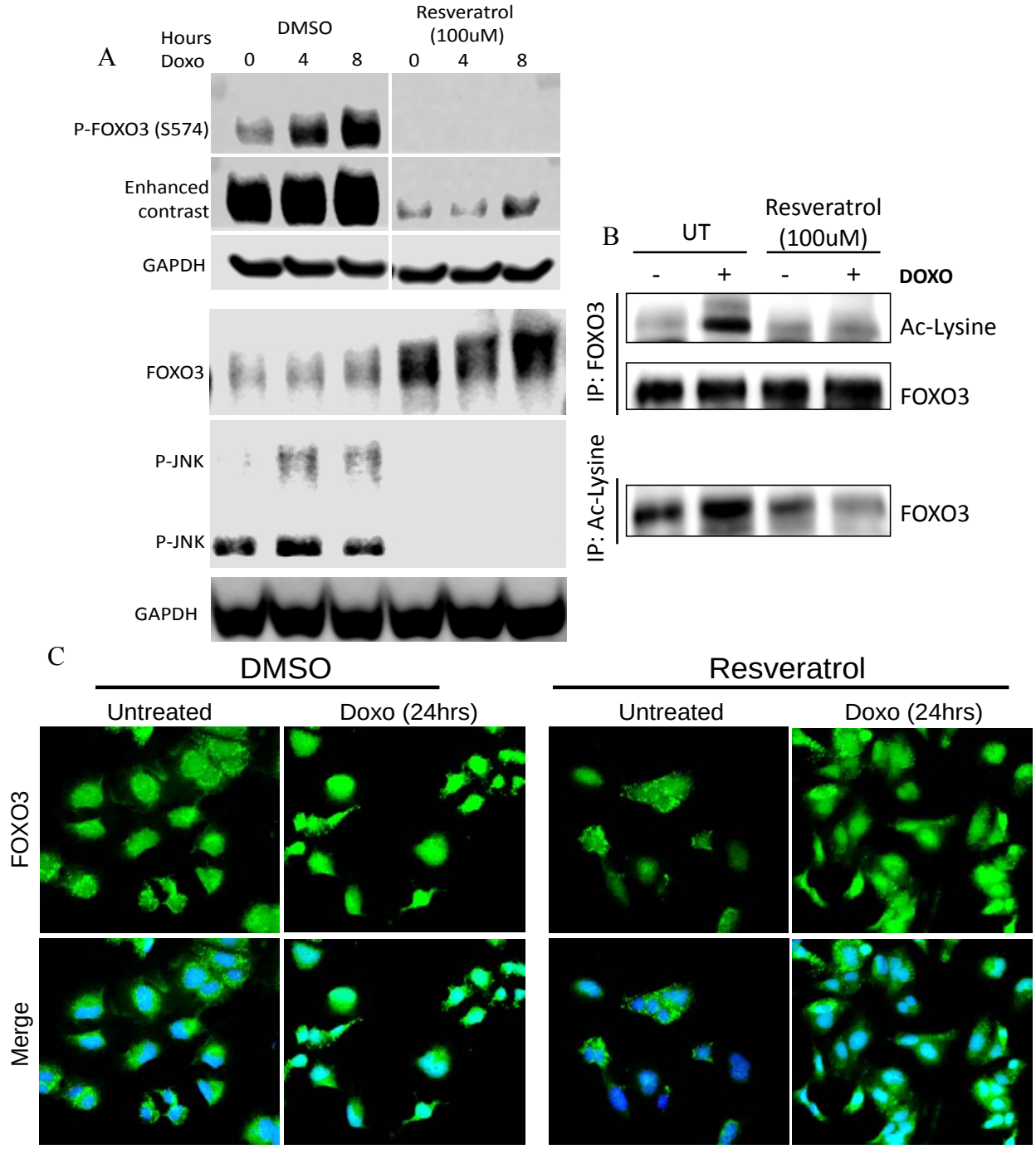

Figure 7: Resveratrol alters FOXO3 PTMs, expression, and localization.
(A) Western blot of lysates from Huh7 cells treated with DMSO or 100μM resveratrol for 4 hours before adding 1μM doxorubicin for 0, 4, or 8 hours, probing for P-S574-FOXO3, total FOXO3, phosphor-JNK (P-JNK), and GAPDH as a loading control. **(B)** Western blot of lysates from Huh7 cells treated with DMSO or 100μM resveratrol for 4 hours before adding 1μM doxorubicin for 0 or 8 hours, immunoprecipitated with FOXO3 antibody, blotting for Acetyl-Lysine (Ac-Lysine) and FOXO3 as input control. **(C)** Immunofluorescence staining for FOXO3 (green) and DAPI (blue) in Huh7 cells fixed after treating with DMSO or resveratrol for 4 hours before adding 1μM doxorubicin for 0 or 24 hours.

Since resveratrol blocked doxorubicin induction of FOXO3 acetylation and S574 phosphorylation, both pro-apoptotic PTMs, the SIRT activator would be predicted to decrease sensitivity to doxorubicin. To test this directly, Huh7 cells were pre-treated with resveratrol followed by increasing concentrations of doxorubicin. As expected, resveratrol significantly suppressed doxorubicin-induced cell death (Figure 8A). To confirm that resveratrol-mediated doxorubicin resistance is dependent on FOXO3, the experiment was repeated in FOXO3 knockdown Huh7 cells. In the absence of FOXO3, resveratrol did not have a protective effect (Figure 8B). In fact, resveratrol may have slightly increased doxorubicin sensitivity in these cells. The difference between the resveratrol effect in sh-NT cells and sh-FOXO3 cells (black bars) shows a positive trend after two independent experiments, but did not quite reach statistical significance ($p=0.056$)

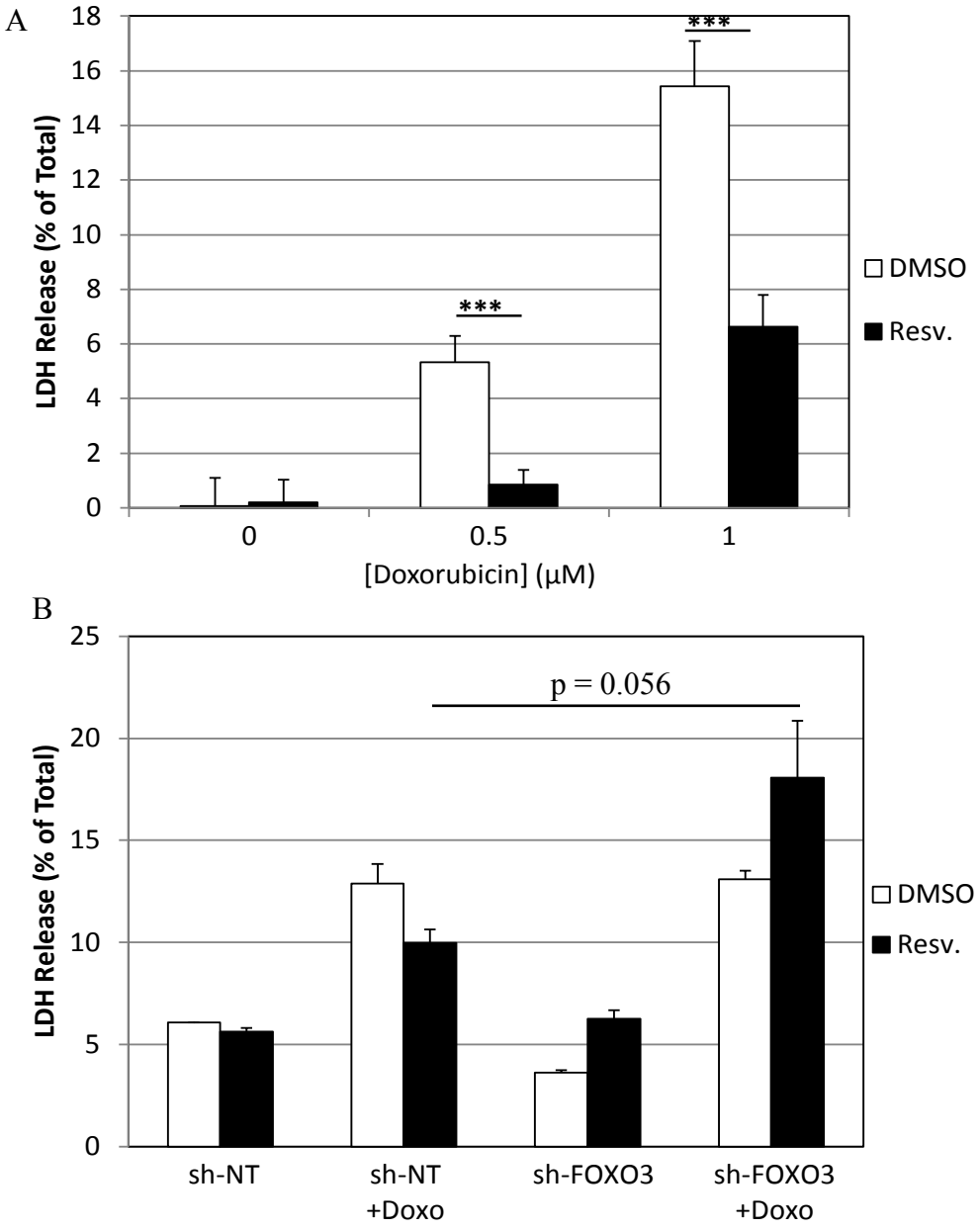

Figure 8: Resveratrol decreases doxorubicin sensitivity in Huh7 cells.
(A) Huh7 cells were treated with DMSO or 50μM resveratrol before adding 0, 0.5 or 1μM doxorubicin for 72 hours, removing resveratrol treatment after 24 hours while continuing doxorubicin treatment. Cell death was evaluated LDH **(B)** Huh7 sh-NT or sh-FOXO3 cells were treated with DMSO or resveratrol as above before adding 1μM doxorubicin for 72 hours. Cell death was evaluated by LDH release. Data is presented as the mean of four (A) or two (B) independent experiments ± SE, and represents the amount of LDH release as a percent of total LDH. ** indicates $p \leq 0.001$, Student's t-test.

Discussion

Doxorubicin is capable of inducing apoptosis in many types of cancer cells, but the exact mechanism by which it does so varies among different cell types (193). FOXO3 has been shown to play a pivotal role in doxorubicin-induced apoptosis in several cancer cell types (116, 145, 146). However, the role of FOXO3 in doxorubicin sensitivity in HCC has not been previously studied. In chapter 3, I demonstrated that FOXO3 is overexpressed in many HCC tumors, and that its cellular localization is significantly altered in TACE-doxorubicin-resistant HCC. This suggests that FOXO3 may be playing an important role in doxorubicin sensitivity in HCC.

Cellular localization of FOXO3 is regulated by upstream signaling pathways through PTMs. Thus, the altered cellular localization in TACE-doxorubicin-resistant HCC tumors also suggests that FOXO3 PTMs are altered in these cells. In addition to localization, the transcriptional program of FOXO3 is also regulated through PTMs. FOXO3 is a multifunctional transcription factor that can promote many different, sometimes opposing, cellular functions. Hence, it may be difficult to understand the role of FOXO3 in a given scenario simply by increasing or decreasing its expression. In fact, FOXO3 consistently undergoes nuclear translocation in response to doxorubicin (83, 116, 167), but it has been shown to be capable of both inducing apoptosis and promoting cell survival (116, 171). Therefore, in order to investigate the role of FOXO3 in doxorubicin sensitivity in HCC, we decided to examine the induction of important FOXO3 PTMs by doxorubicin, and attempt to determine their functional significance.

Two PTMs have been shown to be vital for allowing FOXO3 to induce apoptosis: acetylation and S574-phosphorylation. FOXO3 acetylation in response to cisplatin in urothelial carcinoma cells is important for its ability to induce apoptosis (173). S574-phosphorylation as well, is necessary for FOXO3 to induce apoptosis in hepatoma cells in response to ethanol

treatment (114). The data in Figure 5 demonstrate that FOXO3 becomes acetylated and S574-phosphorylated in response to doxorubicin in Huh7 cells. However, as seen in Figure 6, knock-down of FOXO3 expression alone did not reduce doxorubicin sensitivity. This could indicate that FOXO3 does not play a significant role in doxorubicin sensitivity in Huh7 cells. Another possibility is that FOXO3 is capable of playing an anti-apoptotic role in response to doxorubicin treatment in doxorubicin-sensitive cells, but that this function is suppressed by doxorubicin-induced PTMs on FOXO3, or when we artificially knock-down its expression. In support of this hypothesis, when we rescued FOXO3 knock-down with S574A-FOXO3 we saw a decrease in doxorubicin sensitivity (Figure 6A). These results suggest that one possible means for HCC cells to achieve resistance to doxorubicin is by suppressing the S574-phosphorylation of FOXO3.

Our strategy to determine the significance of doxorubicin-induced acetylation of FOXO3 was to use a SIRT activator which would increase SIRT-mediated deacetylation of FOXO3, and examine the effect on doxorubicin sensitivity. Resveratrol is a known SIRT activator that has also been shown to promote resistance to oxidative stress in a FOXO3-dependent manner (190). In Figure 7 we demonstrate that resveratrol is able to block doxorubicin-induced FOXO3 acetylation. Interestingly, we also found that resveratrol blocks doxorubicin-induced S574-phosphorylation of FOXO3. This could indicate that FOXO3 acetylation and S574-phosphorylation are inter-dependent. Another possibility is that resveratrol is altering multiple cell signaling pathways, those responsible for FOXO3 acetylation, as well as those responsible for FOXO3 S574-phosphorylation. Resveratrol suppresses oxidative stress-induced JNK activation (194), and JNK has previously been shown to be required for S574-phosphorylation of FOXO3 (114). In Figure 7, we also demonstrate that resveratrol suppresses doxorubicin-induced JNK phosphorylation. Additionally, we show that resveratrol increases FOXO3 expression and it

alters doxorubicin-induced changes in FOXO3 cellular distribution. Interestingly, the cellular distribution of FOXO3 in Huh7 cells treated with resveratrol and doxorubicin appears similar to that seen in TACE-doxorubicin-resistant HCC tumors (see Figure 2 in chapter 3).

Very little is known about the effect of resveratrol on doxorubicin sensitivity in HCC. Resveratrol has been shown to have anti-tumor effects in many types of tumor cells including HCC, both *in vitro* and *in vivo* (195, 196). Furthermore, resveratrol has been shown to promote doxorubicin sensitivity and doxorubicin resistance depending on the cell type (197, 198). The only published report on the effect of resveratrol on doxorubicin sensitivity in HCC used Hep3B cells overexpressing the hepatitis B virus X protein and showed that resveratrol increased doxorubicin sensitivity in these cells (199). However, the data we present in figure 8 demonstrate that resveratrol significantly reduces doxorubicin sensitivity in Huh7 cells. We are also attempting to determine whether this effect depends on FOXO3. In two independent experiments done thus far, resveratrol increased doxorubicin sensitivity in FOXO3 knock-down cells, in contrast to decreasing doxorubicin sensitivity in control cells. However, the difference between the control and FOXO3 knock-down cells is not statistically significant with a p value of 0.056. We plan on repeating this experiment to see if this trend continues. Clearly, resveratrol causes many changes in FOXO3, and further studies are required to determine the mechanism behind these changes and their impact on FOXO3 function.

Taken together, the data is this chapter suggests that the pro-survival function of FOXO3 is suppressed in doxorubicin sensitive cells exposed to doxorubicin by the induction of its acetylation and S574-phosphorylation. Treatment of doxorubicin sensitive cells with resveratrol causes cellular changes that appear to preserve and maximize the pro-survival function of FOXO3 by increasing its expression, while blocking its acetylation and S574-phosphorylation.

This, along with the data presented in chapter 3, raises the possibility that cellular changes similar to those caused by resveratrol occur in doxorubicin resistant HCC cells, providing a potential mechanism for doxorubicin resistance in HCC.

Chapter V: Expression of SIRT6 in Human HCC and its Effect on FOXO3 Post-translational Modifications and Doxorubicin Sensitivity in Huh7 Cells

Background

The data presented in Chapter 3-4 indicate that suppression of FOXO3 acetylation and S574-phosphorylation preserves its pro-survival function and is one potential mechanism of resistance to doxorubicin in HCC. This hypothesis is consistent with other studies showing that SIRT-mediated deacetylation of FOXO3 is able to tip the balance of FOXO3 function away from apoptosis induction towards DNA damage repair and oxidative stress resistance (200), both of which are caused by doxorubicin. Since resveratrol, a SIRT deacetylase activator, was able to protect Huh7 HCC cells from doxorubicin (Chapter 4), this suggests the possibility that SIRT-mediated FOXO3 deacetylation may play a central role in TACE-doxorubicin-resistant HCC tumors.

Of the seven mammalian SIRT enzymes, SIRT1 (91), 2 (130), and 3 (183) have been shown to interact with and suppress acetylation of FOXO3 in certain conditions. SIRT6 has not been shown to directly deacetylate FOXO3, but it does interact with FOXO3 (201)). Also, the *C. elegans* SIRT6/7 homolog, SIR-2.4, was shown to interact with and suppress acetylation of the FOXO3 homolog, DAF-16 (202). SIRT6 (203-206), along with SIRT1 (207), is an important factor involved in repair of DNA damage, including DSBs, and both promote resistance to oxidative stress by increasing FOXO3-dependent transcription of antioxidant genes (91, 202). While studies have identified SIRT1 overexpression in HCC (85, 89, 208, 209) and have linked SIRT1 to chemoresistance (85, 173), the role of SIRT6 in HCC pathogenesis is not well established. Two studies have reported SIRT6 to have a tumor suppressive role in HCC (87, 210), but two separate studies have reported it to have tumor promoting activity (211, 212).

Importantly, SIRT6 was shown to promote resistance to epirubicin, a doxorubicin homolog, in breast cancer cells. Furthermore, the mechanism was shown to involve deacetylation of FOXO3 (93). However, there have been no previous reports on the role of SIRT6 in doxorubicin sensitivity in HCC nor have studies been performed in human samples.

In this Chapter, I examined the role of SIRT6 in HCC, especially with respect to a potential function in doxorubicin resistance. SIRT6 expression in treatment-naïve and TACE-doxorubicin-resistant HCC patient samples was compared, and SIRT6 overexpression was used to confirm a protective effect from doxorubicin in HCC cells and identify the mechanism of this protection. These analyses revealed a significant increase in SIRT6 expression in TACE-doxorubicin-resistant HCC and suggest that regulation of FOXO3 acetylation by SIRT6 is a critical mechanism by which HCC tumors become resistant to doxorubicin.

Results

SIRT6 Expression in Treatment- naïve vs. TACE-doxorubicin-resistant HCC and Correlation with Nuclear/Cytosolic Distribution of FOXO3

To characterize SIRT6 is expression in TACE-doxorubicin-resistant HCC, tumors from 15 different patients were immunostained for SIRT6. Of the 15 tumors, 8 were treatment-naïve and 7 had viable tumor cells remaining after TACE-doxorubicin treatment. All 15 tumors were positive for SIRT6 expression, although, similar to FOXO3, there appeared to be significant variation in the amount and localization of expression between the samples. Overall, there was a significant increase in SIRT6 expression in TACE-doxorubicin resistant compared to the treatment-naïve HCC tumors (Figure 9; Figure 10D). In the treatment-naïve tumors, SIRT1 expression was typically nuclear in distribution (Figure 9A-D); there was no significant difference in the nuclear SIRT6 expression between naïve and treated tumors (Figure 10C).

However, there was more cytosolic SIRT6 in the TACE-doxorubicin-resistant tumors (Figure 9E-H), and both the total and cytosolic levels of SIRT6 expression were significantly higher in the TACE-doxorubicin-resistant tumors (Figure 10B, D). Interestingly, the FOXO3 N/C expression ratio was inversely correlated with the level of cytosolic SIRT6 expression in this same group of 15 tumors (Figure 10A), indicating that as cytosolic SIRT6 expression increases, the distribution of FOXO3 expression becomes more cytosolic in location.

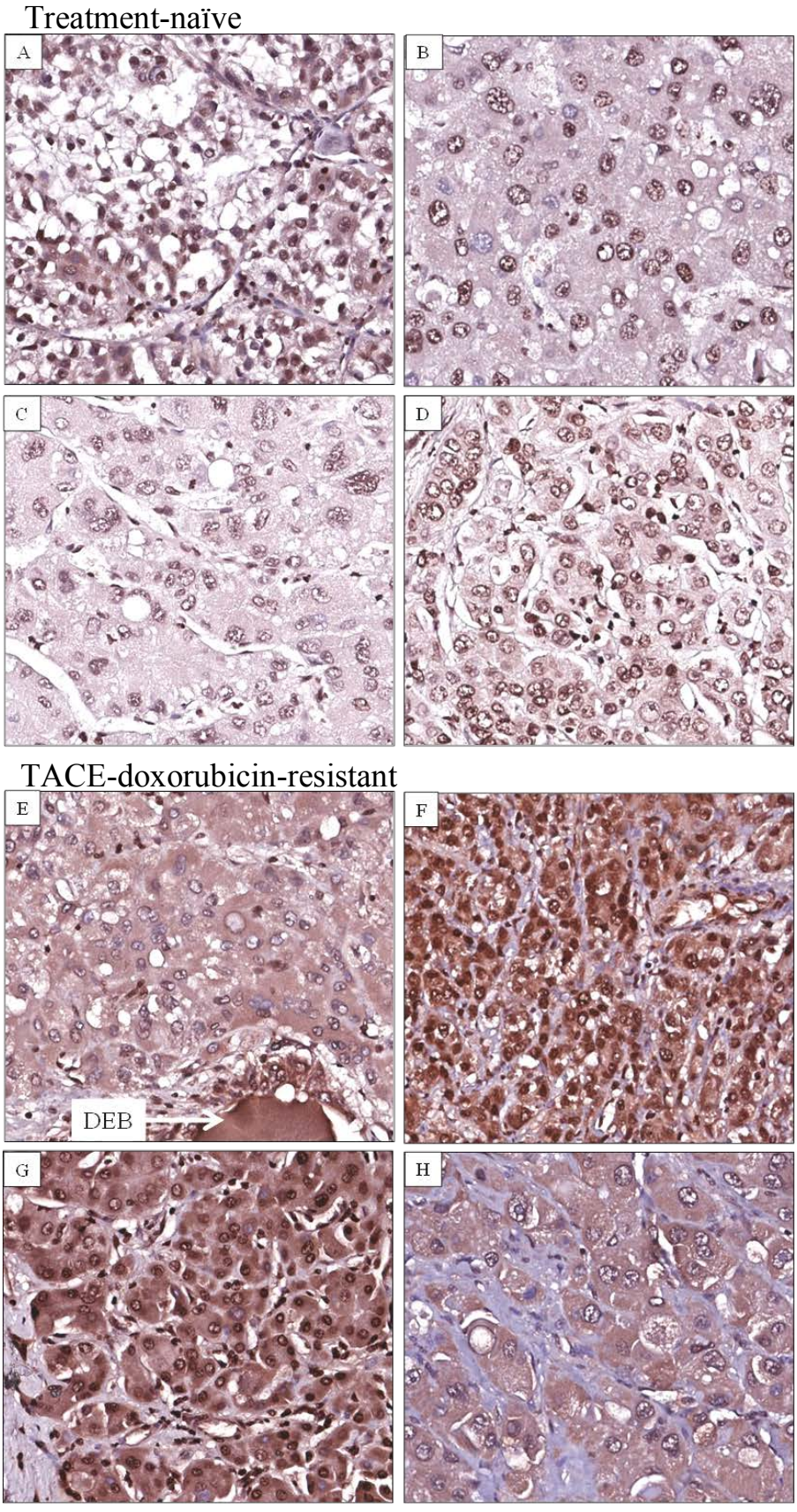

Figure 9: SIRT6 expression in treatment-naïve and TACE-doxorubicin-resistant HCC.
IHC staining for SIRT6 (brown) and hematoxylin (blue). **(A-D)** Typical examples of SIRT6 expression in treatment-naïve HCC tumors. **(E-F)** Typical examples of SIRT6 expression in TACE-doxorubicin-resistant tumors, drug eluting bead (DEB) labeled

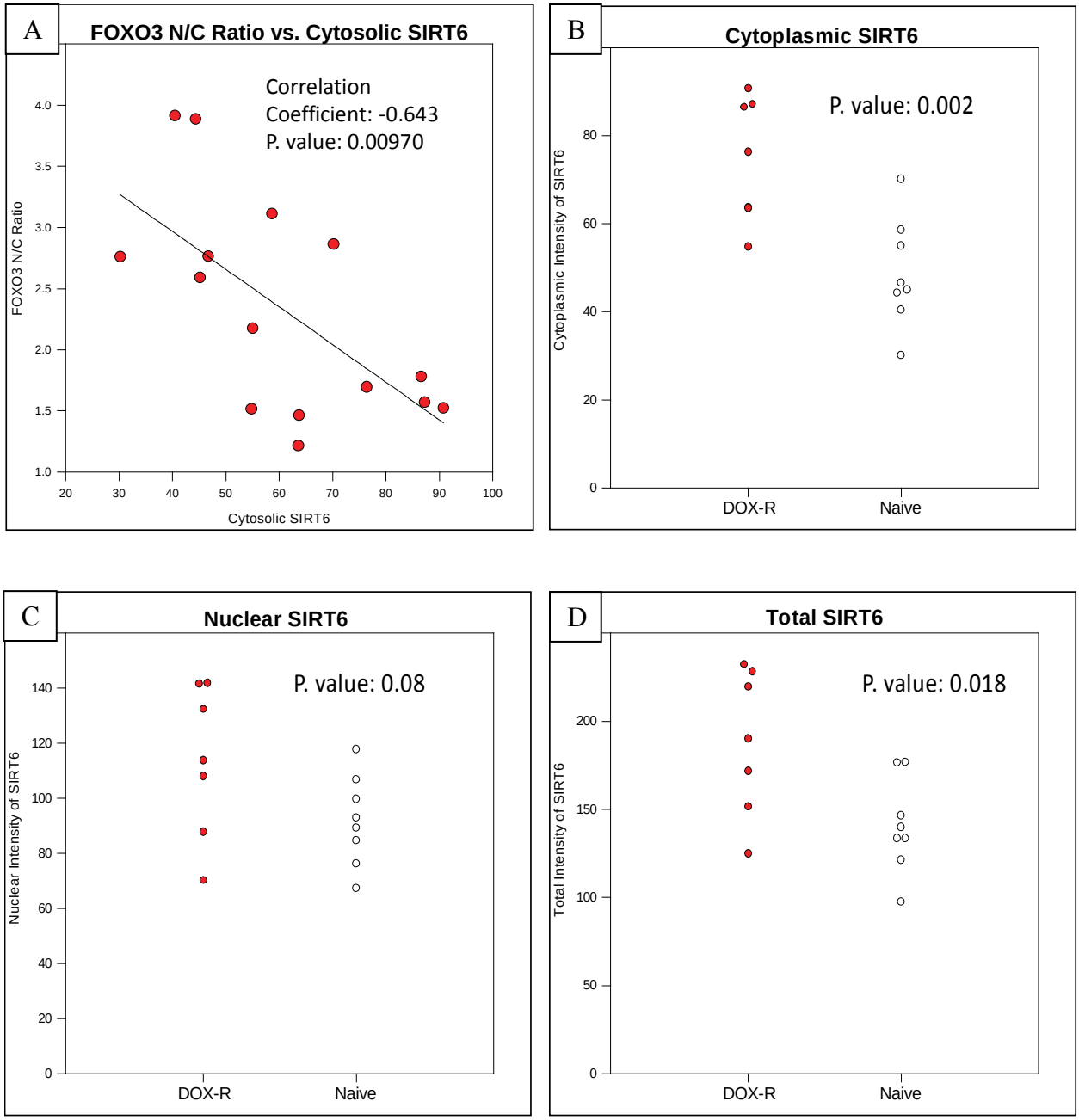

Figure 10: SIRT6 expression is altered in TACE-doxorubicin-resistant HCC tumors. **(A)** Correlation between N/C ratio of FOXO3 expression and cytosolic SIRT6 expression in HCC tumors, n=15. **(B)** Cytosolic SIRT6 expression in TACE-doxorubicin resistant tumors (DOX-R, left), n=7, and treatment-naïve tumors (Naïve, right), n=8). **(C)** Nuclear SIRT6 expression in DOX-R and Naïve tumors. **(D)** Total SIRT6 expression in DOX-R and Naïve tumors. Values for each sample in B-D represent the average optical density (O.D.) of all the objects (nuclei (N), cytoplasms (C), or N+C (Total)) within a selected area subtracted from 240 (maximum O.D.) so that a higher value equals increased expression. P-value was determined Student's t-test.

SIRT6 Overexpression Inhibits Doxorubicin-induced S574-phosphorylation of FOXO3

The correlation of increased SIRT6 expression levels and FOXO3 cytosolic localization (Figure 10A) suggests possible effects on FOXO3 PTMs. To determine whether SIRT6 overexpression is able to block doxorubicin-induced S574-phosphorylation of FOXO3, similar to resveratrol, we transfected Huh7 cells with SIRT6 or a previously characterized catalytically inactive SIRT6 mutant, SIRT6-H133Y. We then treated the cells with 1μM doxorubicin for 24 hours and examined FOXO3 S574-phosphorylation by immunofluorescence. In SIRT6 overexpressing cells, we found significant reduction in FOXO3 S574-phosphorylation (Figure 11). We also found that this suppression was dependent on SIRT6 deacetylase activity, as we did not find any suppression of FOXO3 S574-phosphorylation in SIRT6-H133Y overexpressing cells (Figure 11).

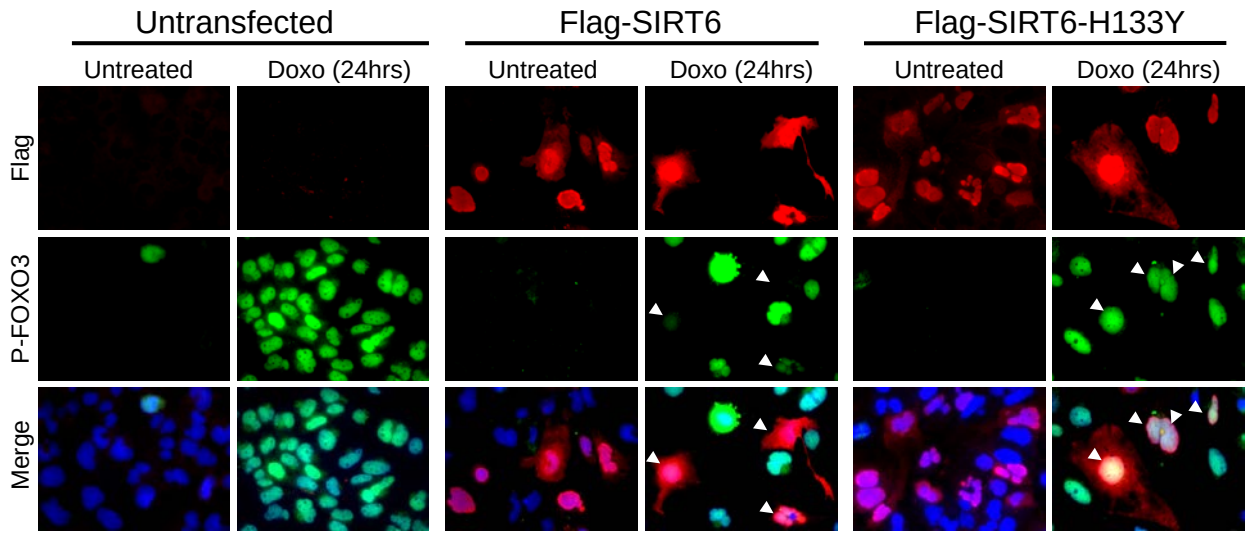

Figure 11: SIRT6 overexpression blocks doxorubicin-induced S574-phosphorylation of FOXO3 through its deacetylase activity.
Huh7 cells were mock transfected or transfected with flag tagged SIRT6 or the catalytically inactive mutant, SIRT6-H133Y. Cells were then treated with 1μM doxorubicin for 24 hours before fixing and staining for flag peptide (red) S574-P-FOXO3 (P-FOXO3, green) and DAPI (blue). White arrows indicate cells positive for SIRT6 transfection.

SIRT6 Overexpression Protects from Doxorubicin-induced Apoptosis

To directly measure effects of SIRT6 overexpression on doxorubicin sensitivity, we transfected Huh7 cells with SIRT6 or the catalytically inactive mutant and treated the cells with 1μM doxorubicin. As expected, doxorubicin treatment of untransfected cells resulted in abundant apoptotic cells (near 25%), as measured by TUNEL assay (Figure 12, left). Overexpression of SIRT6, however, significantly inhibited doxorubicin-induced apoptosis (Figure 12, middle), while expression of catalytically inactive SIRT6 had no significant effect on doxorubicin sensitivity (Figure 12, right). Thus, SIRT6 overexpression suppressed doxorubicin-induced cell death, and this function required deacetylase activity.

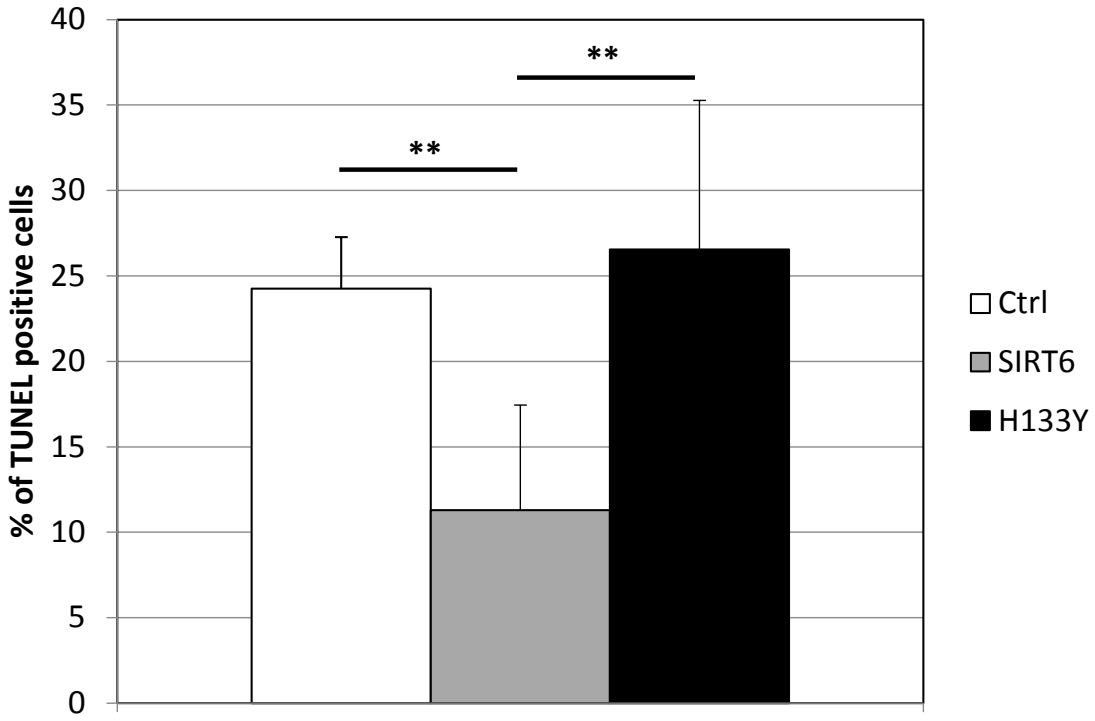

Figure 12: SIRT6 protects against doxorubicin-induced cell death
Huh7 cells were transfected with SIRT6 or catalytically inactive SIRT6 (H133Y) before treatment with 1μM doxorubicin for 36 hours. Cell death was measured by TUNEL assay. The percentage of untransfected (white), and transfected cells positive for TUNEL staining were counted. Data represent the mean of 5, 10X fields counted for each group, ± SE. ** indicates $p \leq 0.01$, Student's t-test.

Discussion

SIRT6 has been previously shown to be an upstream modifier of FOXO3, suppressing its acetylation and altering its function (202). Furthermore, overexpression of SIRT6 and the subsequent deacetylation of FOXO3 were shown to be an important mechanism of resistance to epirubicin in breast cancer cells (93). Given that the acetylation and S574-phosphorylatio of FOXO3 appear to play an important role in mediating doxorubicin sensitivity in Huh7 cells, we hypothesized that upregulation of the deacetylase, SIRT6, might be an *in vivo* mechanism of resistance to doxorubicin in human HCC tumors.

In order to test this hypothesis we first determined whether SIRT6 is overexpressed in human TACE-doxorubicin-resistant tumors relative to treatment-naïve tumors. The data in figure 10 show that SIRT6 expression is significantly increased in TACE-doxorubicin-resistant tumors. Interestingly, we found that cytosolic SIRT6 was especially increased, and that there was no difference in the nuclear SIRT6 level between the two groups. SIRT6 is best known as a nuclear protein, carrying out its function through histone deacetylation and repression of gene transcription (213). However, there is emerging evidence for SIRT6 activity outside the nucleus and deacetylation of non-histone targets. For example, in non-small cell lung carcinoma (NSCLC), SIRT6 was found to be overexpressed, particularly in the cytoplasm, where it was suggested to be involved in promoting autophagy and resistance to paclitaxel chemotherapy. Additionally, cytosolic SIRT6 correlated with aggressive tumor behavior and shorter survival (214). Another cytosolic function of SIRT6 is the assembly of stress granules, an important pro-survival mechanism in the cellular response to stress. SIRT6 localizes to cytoplasmic stress granules during cellular stress, and catalytically inactive SIRT6 disrupts and delays the formation of stress granules (215). Therefore, it is possible that the increased cytosolic SIRT6 is playing a

role in TACE-doxorubicin-resistant HCC, but further research is needed to confirm this hypothesis and determine the exact mechanism.

Another interesting finding from figure 10 is the correlation between cytosolic SIRT6 and the nuclear/cytosolic distribution of FOXO3 in the same group of tumors. Given that FOXO3 localization is regulated by PTMs such as acetylation, this suggests that cytosolic SIRT6 may be directly or indirectly mediating the redistribution of FOXO3 in TACE-doxorubicin-resistant HCC. Another possibility is that SIRT6 and FOXO3 are translocating to the cytoplasm in order to carry out a common function, potentially involved in mediating resistance to TACE-doxorubicin. Further research is required to determine the function and significance of cytosolic SIRT6 and FOXO3.

The data presented in chapter 4 demonstrate that suppression of doxorubicin-induced acetylation and S574-phosphorylation of FOXO3 is a potential mechanism of resistance to doxorubicin. Resveratrol, a SIRT activator, blocked doxorubicin-induce acetylation, as well as S574-phosphorylation of FOXO3. Given that SIRT6 has been linked to deacetylation of FOXO3 and that SIRT6 is overexpressed in TACE-doxorubicin-resistant HCC, we hypothesized that SIRT6 overexpression may be contributing to doxorubicin resistance through is suppression of doxorubicin-induced FOXO3 PTMs. It is unclear whether acetylation is necessary for S574-phosphorylation of FOXO3, or vice versa, so we decided to investigate the role of SIRT6 in regulating both PTMs, by overexpressing SIRT6 in Huh7 cells before treating them with doxorubicin. The data in figure 11 demonstrates that SIRT6 overexpression in Huh7 cells significantly blocks doxorubicin-induced S574-phosphorylation of FOXO3, while the catalytically inactive mutant of SIRT6 is unable to do so, indicating that its deacetylase activity is required. We have not yet directly examined the effect of SIRT6 overexpression on

doxorubicin-induced acetylation of FOXO3, but we plan to do so by using PLA to measure FOXO3 acetylation.

Finally, SIRT6 has been previously reported to be involved in mediating resistance to both paclitaxel (214) and epirubicin (93) in NLSCLC and breast cancer respectively. However, its potential role in chemotherapy resistance in HCC, doxorubicin or otherwise, has not been investigated. The data in figure 12 demonstrate that SIRT6 overexpression in Huh7 cells is able to suppress doxorubicin-induced cell death and that this requires its deacetylase activity. This finding is of notable clinical relevance as it suggests that SIRT6 overexpression is mediating TACE-doxorubicin-resistance in human HCC tumors.

Chapter VI: Conclusions

Background

HCC is the third leading cause of cancer related death. It ranks 16th in absolute causes of death world-wide (216), and its incidence has increased 62% in the last 20 years (217). Surgical resection or liver transplantation are the only curative therapies for HCC, but both are unavailable options for most patients, and there are still significant rates of recurrence for those who do receive them (217, 218). For patients with intermediate stage HCC, TACE with doxorubicin provides a modest survival benefit and can help patients maintain or achieve eligibility for liver transplantation (111, 179). However, TACE-doxorubicin treatment rarely achieves a complete tumor response, and a significant proportion of tumors show no response at all (19, 20). Furthermore, the factors and mechanisms involved in resistance of HCC to TACE-doxorubicin remain elusive. There is a clearly evident need for investigation into the critical drivers of HCC tumor progression and response to treatment. The transcription factor FOXO3 has proven to have a pivotal role in the pathogenesis of many types of cancer, including response to chemotherapy (71, 147, 162, 163, 171-173). However, very little is known about the function of FOXO3 in the pathogenesis of HCC. The research presented in this dissertation was undertaken to address these clinically relevant gaps in scientific knowledge.

FOXO3 as a Potential Driver of HCC Pathogenesis

One way by which FOXO3 is regulated is through control of its level of expression. If it is not expressed it cannot function and if its expression is increased then its function may be enhanced. As discussed in chapter 1, another important level of FOXO3 regulation is the control of its cellular localization. As a transcription factor, its primary function is served in the nucleus through gene transcription. Therefore, a first step in understanding the role of FOXO3 in the

pathogenesis of HCC is to determine its expression and cellular localization in human HCC tumors. We found that nuclear and cytosolic FOXO3 expression is increased in HCC tumors compared to adjacent cirrhotic tissue. Additionally, its expression correlated with aggressive tumor behavior. This was a somewhat surprising finding given the well-characterized function of FOXO3 as a tumor suppressor in many other types of cancer (121, 122, 143, 177). However, the ability of FOXO3 to promote survival and resistance to cellular stress is also well characterized, and it has been found to promote growth and survival in other types of tumors and in certain contexts (148, 152, 171). Furthermore, HCC is a unique tumor in many respects. For example, a vast majority of tumors arise in the context of extensive fibrosis and inflammation. Therefore, it is certainly possible that FOXO3 is a previously unrecognized tumor promoter in HCC. Future studies investigating the function, rather than just the expression, of FOXO3 are needed to determine its role in HCC. Ultimately, if FOXO3 is proven to be a driver of HCC pathology, therapies that target FOXO3 and suppress or alter its function may be clinically useful. Additionally, comparing FOXO3 function in HCC to its tumor suppressive function in other types of cancer could help elucidate the conditions that determine whether FOXO3 functions as a tumor suppressor or tumor promoter. This knowledge could have broad implications for the treatment of other types of cancer as well.

Altered FOXO3 Distribution in TACE-doxorubicin-resistant HCC

The tumor cells that had survived TACE-doxorubicin treatment had a significantly lower nuclear/cytosolic ratio of FOXO3 expression compared to treatment-naïve tumor cells. At first glance, this could suggest that FOXO3 is being inactivated in these tumors through cytosolic sequestration. However, there was no significant difference in nuclear or total FOXO3 between the treatment-naïve and resistant tumors. Given that FOXO3 localization and function are largely

controlled by PTMs, one interpretation of this finding is that the FOXO3 PTM pattern and therefore its function in the resistant tumors may be altered. We were not able to test this hypothesis, but instead began investigating the function of FOXO3 in doxorubicin-mediated cell death *in vitro.* Very little is known about extra-nuclear functions of FOXO3. One previous report demonstrated that FOXO3 can be found in mitochondria and activate transcription of mitochondrial encoded genes (183). Directly investigating FOXO3 PTMs in TACE-doxorubicin resistant tumors in the future may provide an indication as to why there is more FOXO3 in the cytoplasm of these tumor cells. It may also provide the knowledge necessary to interrogate its function *in vitro* and understand how FOXO3 may be functioning in the cytoplasm. Our data suggest that we will find decreased FOXO3 acetylation and S574-phosphorylation in resistant tumors.

FOXO3 Acetylation and S574-phosphorylation: Turning Off FOXO3 Anti-apoptotic Function in HCC

Doxorubicin is a potent chemotherapeutic agent causing numerous DNA DSBs (21), and mitochondrial depolarization and formation of ROS (17), culminating in cell apoptosis. FOXO3 is a key player in determining cell fate in response to DNA damage and oxidative stress, so it is not surprising that it has been found to have a prominent role in doxorubicin-induced cell death in several types of cancer cells (83, 116, 167). However, the function of FOXO3 in the response to doxorubicin is not universal to all types of cancer cells, but rather varies depending on cell type and context. Additionally, there are no previous studies on the role of FOXO3 in doxorubicin-induced cell death in HCC.

The data in chapter 4 demonstrate that FOXO3 becomes acetylated and S574-phosphorylated after exposure to doxorubicin in Huh7 cells. Rather than producing a gain of pro-

apoptotic function, these modifications appear to produce a loss of pro-survival function. When FOXO3-S574A is expressed in place of endogenous FOXO3, it is able to suppress doxorubicin-induced cell death. Furthermore, resveratrol treatment increases FOXO3 expression, inhibits doxorubicin-induced FOXO3 acetylation and S574-phosphorylation, and increases resistance to doxorubicin-induced cell death. Previous studies have shown that both deacetylated FOXO3 and FOXO3 that is unphosphorylated at S574 promote transcription of antioxidant and anti-apoptotic genes (91, 114). However, further investigation is needed to understand the mechanism behind the differential transcriptional programs of specific FOXO3 species. Additionally, in the absence of doxorubicin, FOXO3 is primarily deacetylated and unphosphorylated at S574 in Huh7 cells. Therefore, these studies may also provide insight into the function of FOXO3 in the pathogenesis of HCC apart from its role in the response to doxorubicin (Figure 13).

SIRT6 as a Mediator of Resistance to TACE-doxorubicin in HCC

There is an urgent need to understand the mechanisms underlying resistance to TACE-doxorubicin in human HCC. Our *in vitro* studies suggest that blocking doxorubicin-induced acetylation and S574-phosphorylation of FOXO3, and thereby maintaining its anti-apoptotic function, is one potential mechanism of doxorubicin resistance in HCC. To explore the relevance of this potential mechanism *in vivo*, we returned to the human HCC tumors to examine the expression of upstream modifiers of FOXO3. A previous study showed that SIRT6 overexpression promotes resistance to epirubicin, a doxorubicin homolog, in breast cancer cells, and suggested that the mechanism may involve SIRT6-mediated deacetylation of FOXO3 (93). The data in chapter 5 demonstrate that SIRT6 expression is significantly increased in TACE-doxorubicin-resistant compared to treatment-naïve HCC. Furthermore, cytosolic SIRT6 expression is significantly correlated with the nuclear/cytosolic distribution of FOXO3. These

results suggest that SIRT6 overexpression may be increasing resistance to TACE-doxorubicin, possibly by modifying FOXO3 and promoting its anti-apoptotic function. Indeed, when overexpressed in Huh7 cells, SIRT6 blocked doxorubicin-induced S574-phosphorylation of FOXO3 and increased resistance to doxorubicin. Further studies are needed to determine whether SIRT6 is also capable of suppressing doxorubicin-induced acetylation of FOXO3. In addition, further investigation is needed to determine the level of FOXO3 acetylation in treatment-naïve and TACE-doxorubicin-resistant tumors. If FOXO3 acetylation negatively correlates with SIRT6 expression and resistance, it would provide important evidence for the relevance of this mechanism *in vivo*.

The next step in determining the significance of SIRT6 overexpression in mediating doxorubicin resistance in HCC is to investigate whether inhibiting SIRT6 in doxorubicin-resistant HCC increases doxorubicin sensitivity. Small molecule inhibitors of other SIRT enzymes have already undergone clinical trials in other diseases (219), and selective SIRT6 inhibitors have also been identified (220). *In vitro* models of doxorubicin resistance have been developed in many cancer cell lines including the hepatoma cell line, HepG2 (221) and could be used to answer this question. However, the ideal experiment would be to acquire fresh, viable samples of HCC tumors, including TACE-doxorubicin-resistant tumors, and investigate the effect of SIRT6 inhibition in doxorubicin sensitivity *ex vivo*.

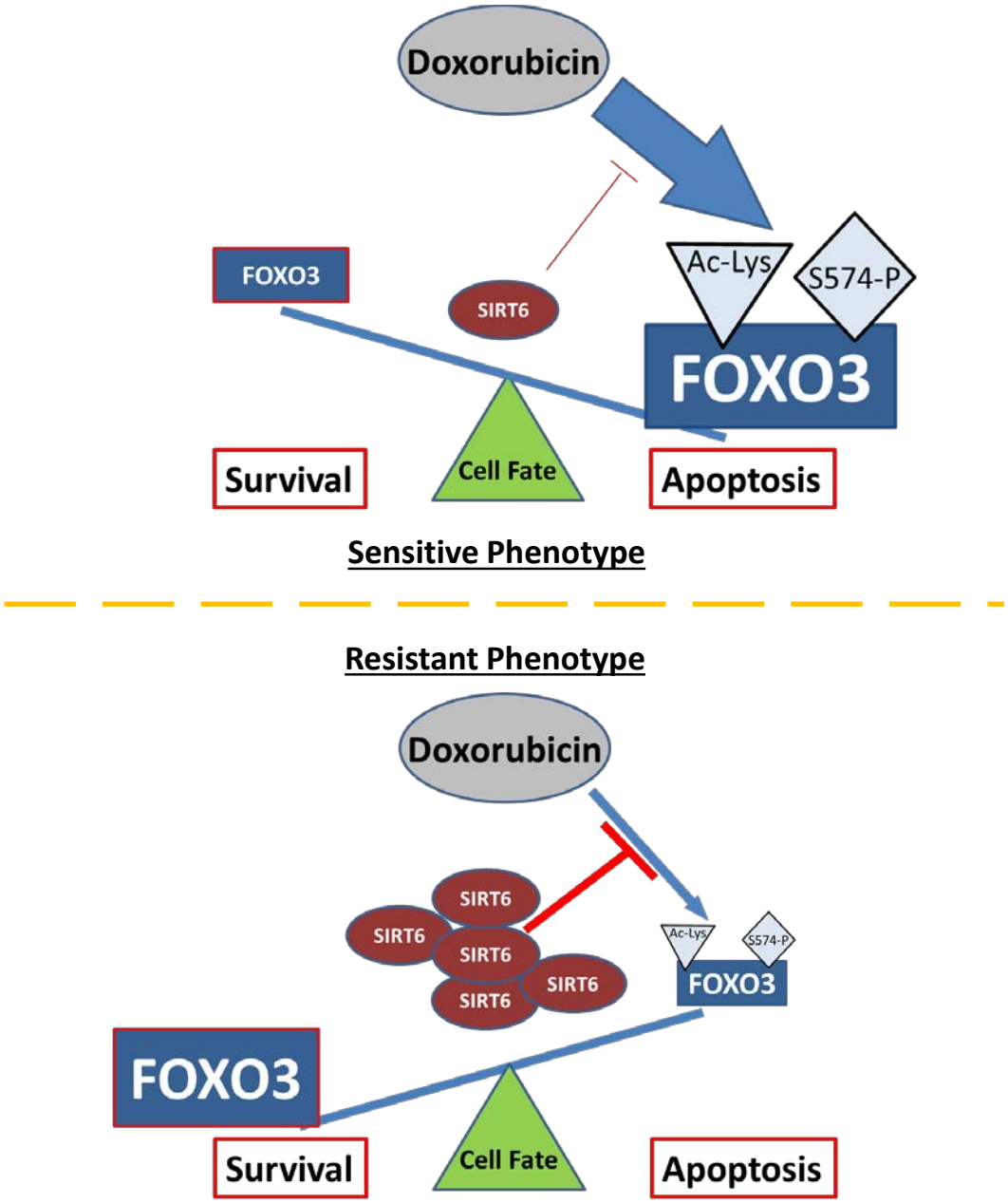

Figure 13: Potential model of doxorubicin resistance mechanism in HCC.
SIRT6 overexpression in TACE-doxorubicin-resistant HCC tumors cells blocks doxorubicin-induced FOXO3 acetylation and S574-phosphorylation, tipping the balance of FOXO3 function towards cell survival.

The Effect of Tumor Embolization Alone

One caveat to the implications of the *in vitro* aspects of this study is that they did not account for the non-doxorubicin-mediated effects of TACE, namely the hypoxia and nutrient deprivation caused by tumor embolization. These conditions *in vivo* may be significantly affecting the role of FOXO3 in the development of resistance to TACE-doxorubicin. The nutrient sensing-kinase, AMPK, was found to be activated in response to hypoxia in osteosarcoma cells (222). Moreover, activation of AMPK increased resistance to doxorubicin; however, the downstream mechanism was not explored (222). Resveratrol has been shown to activate AMPK (190), and AMPK-mediated phosphorylation of FOXO3 at Ser413 and Ser588 increases expression of FOXO3 and FOXO3-dependent expression of genes promoting resistance to cell stress (185). These studies suggest the possibility that the role of FOXO3 in promoting resistance to TACE-doxorubicin in HCC may be two-fold: promoting resistance to doxorubicin and embolization-induced cell death. To test this hypothesis, future studies could investigate the level of AMPK activation and FOXO3 S413 and S588 phosphorylation in TACE-doxorubicin-resistant tumors. *In vitro* models of hypoxia and nutrient deprivation, as well as animal studies of trans-arterial embolization with or without chemotherapy could also be used to determine the role of FOXO3.

In conclusion, we have shown that the expression of FOXO3 is increased in HCC and it correlates with tumor behavior, including resistance to TACE-doxorubicin treatment. Doxorubicin induces a dramatic change in FOXO3 PTMs, including increased acetylation and S574-phosphorylation. Blocking the induction of these PTMs increased resistance to doxorubicin in Huh7 cells. Furthermore, SIRT6 overexpression was demonstrated in TACE-doxorubicin-resistant HCC tumors, and it was shown *in vitro* to block doxorubicin-induced S574-

phosphorylation of FOXO3 and increase resistance to doxorubicin. These novel insights into the mechanisms of resistance to TACE-doxorubicin in HCC could have therapeutic potential and provide a foundation for future studies that will reveal new details of HCC pathogenesis.

Bibliography

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
2. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet 2012;379:1245-1255.
3. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907-1917.
4. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology 2011;53:1020-1022.
5. Bialecki ES, Ezenekwe AM, Brunt EM, Collins BT, Ponder TB, Bieneman BK, Di Bisceglie AM. Comparison of liver biopsy and noninvasive methods for diagnosis of hepatocellular carcinoma. Clin Gastroenterol Hepatol 2006;4:361-368.
6. Crippin JS. Biopsy of suspicious liver nodules: does it change management? Clin Gastroenterol Hepatol 2006;4:296-298.
7. Prospective validation of the CLIP score: a new prognostic system for patients with cirrhosis and hepatocellular carcinoma. The Cancer of the Liver Italian Program (CLIP) Investigators. Hepatology 2000;31:840-845.
8. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, Nakajima Y, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer 1985;56:918-928.
9. Galo C, Peronne F, Capuano G, Daniele B, Gaeta GB, Pignata S. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. Hepatology 1998;28:751-755.
10. Singal AG, El-Serag HB. Hepatocellular Carcinoma From Epidemiology to Prevention: Translating Knowledge into Practice. Clin Gastroenterol Hepatol 2015;13:2140-2151.
11. Beaugrand M, N'Kontchou G, Seror O, Ganne N, Trinchet JC. Local/regional and systemic treatments of hepatocellular carcinoma. Semin Liver Dis 2005;25:201-211.
12. Burroughs A, Hochhauser D, Meyer T. Systemic treatment and liver transplantation for hepatocellular carcinoma: two ends of the therapeutic spectrum. Lancet Oncol 2004;5:409-418.
13. Ganne-Carrie N, Trinchet JC. Systemic treatment of hepatocellular carcinoma. Eur J Gastroenterol Hepatol 2004;16:275-281.
14. Kalyan A, Nimeiri H, Kulik L. Systemic therapy of hepatocellular carcinoma: current and promising. Clin Liver Dis 2015;19:421-432.
15. Llovet JM, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. Nat Rev Clin Oncol 2015;12:408-424.
16. Fong ZV, Tanabe KK. The clinical management of hepatocellular carcinoma in the United States, Europe, and Asia: a comprehensive and evidence-based comparison and review. Cancer 2014;120:2824-2838.
17. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. Hepatology 2003;37:429-442.
18. Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. Lancet 2002;359:1734-1739.
19. Malagari K, Pomoni M, Kelekis A, Pomoni A, Dourakis S, Spyridopoulos T, Moschouris H, et al. Prospective randomized comparison of chemoembolization with doxorubicin-eluting

beads and bland embolization with BeadBlock for hepatocellular carcinoma. Cardiovasc Intervent Radiol 2010;33:541-551.
20. Lammer J, Malagari K, Vogl T, Pilleul F, Denys A, Watkinson A, Pitton M, et al. Prospective randomized study of doxorubicin-eluting-bead embolization in the treatment of hepatocellular carcinoma: results of the PRECISION V study. Cardiovasc Intervent Radiol 2010;33:41-52.
21. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 1984;226:466-468.
22. Chen SH, Chan NL, Hsieh TS. New mechanistic and functional insights into DNA topoisomerases. Annu Rev Biochem 2013;82:139-170.
23. Roos WP, Kaina B. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett 2013;332:237-248.
24. Wilson TR, Johnston PG, Longley DB. Anti-apoptotic mechanisms of drug resistance in cancer. Curr Cancer Drug Targets 2009;9:307-319.
25. Swift LP, Rephaeli A, Nudelman A, Phillips DR, Cutts SM. Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death. Cancer Res 2006;66:4863-4871.
26. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 1999;57:727-741.
27. Cox J, Weinman S. Mechanisms of doxorubicin resistance in hepatocellular carcinoma. Hepat Oncol 2016;3:57-59.
28. Bonin S, Pascolo L, Croce LS, Stanta G, Tiribelli C. Gene expression of ABC proteins in hepatocellular carcinoma, perineoplastic tissue, and liver diseases. Mol Med 2002;8:318-325.
29. Scotto KW. Transcriptional regulation of ABC drug transporters. Oncogene 2003;22:7496-7511.
30. Johnson RA, Ince TA, Scotto KW. Transcriptional repression by p53 through direct binding to a novel DNA element. J Biol Chem 2001;276:27716-27720.
31. Osborn MT, Chambers TC. Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. J Biol Chem 1996;271:30950-30955.
32. Kuo MT, Liu Z, Wei Y, Lin-Lee YC, Tatebe S, Mills GB, Unate H. Induction of human MDR1 gene expression by 2-acetylaminofluorene is mediated by effectors of the phosphoinositide 3-kinase pathway that activate NF-kappaB signaling. Oncogene 2002;21:1945-1954.
33. Fantappie O, Solazzo M, Lasagna N, Platini F, Tessitore L, Mazzanti R. P-glycoprotein mediates celecoxib-induced apoptosis in multiple drug-resistant cell lines. Cancer Res 2007;67:4915-4923.
34. Mazzanti R, Platini F, Bottini C, Fantappie O, Solazzo M, Tessitore L. Down-regulation of the HGF/MET autocrine loop induced by celecoxib and mediated by P-gp in MDR-positive human hepatocellular carcinoma cell line. Biochem Pharmacol 2009;78:21-32.
35. Ng IO, Liu CL, Fan ST, Ng M. Expression of P-glycoprotein in hepatocellular carcinoma. A determinant of chemotherapy response. Am J Clin Pathol 2000;113:355-363.
36. Nies AT, Konig J, Pfannschmidt M, Klar E, Hofmann WJ, Keppler D. Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. Int J Cancer 2001;94:492-499.

37. Silverman JA, Thorgeirsson SS. Regulation and function of the multidrug resistance genes in liver. Prog Liver Dis 1995;13:101-123.
38. Solazzo M, Fantappie O, D'Amico M, Sassoli C, Tani A, Cipriani G, Bogani C, et al. Mitochondrial expression and functional activity of breast cancer resistance protein in different multiple drug-resistant cell lines. Cancer Res 2009;69:7235-7242.
39. Cheung ST, Cheung PF, Cheng CK, Wong NC, Fan ST. Granulin-epithelin precursor and ATP-dependent binding cassette (ABC)B5 regulate liver cancer cell chemoresistance. Gastroenterology 2011;140:344-355.
40. Ueda K, Cardarelli C, Gottesman MM, Pastan I. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. Proc Natl Acad Sci U S A 1987;84:3004-3008.
41. Shiraga K, Sakaguchi K, Senoh T, Ohta T, Ogawa S, Sawayama T, Mouri H, et al. Modulation of doxorubicin sensitivity by cyclosporine A in hepatocellular carcinoma cells and their doxorubicin-resistant sublines. J Gastroenterol Hepatol 2001;16:460-466.
42. Kerr DJ, Graham J, Cummings J, Morrison JG, Thompson GG, Brodie MJ, Kaye SB. The effect of verapamil on the pharmacokinetics of adriamycin. Cancer Chemother Pharmacol 1986;18:239-242.
43. Ferry DR, Traunecker H, Kerr DJ. Clinical trials of P-glycoprotein reversal in solid tumours. Eur J Cancer 1996;32A:1070-1081.
44. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer 2009;9:338-350.
45. Jarvinen TA, Tanner M, Rantanen V, Barlund M, Borg A, Grenman S, Isola J. Amplification and deletion of topoisomerase IIalpha associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. Am J Pathol 2000;156:839-847.
46. Press MF, Sauter G, Buyse M, Bernstein L, Guzman R, Santiago A, Villalobos IE, et al. Alteration of topoisomerase II-alpha gene in human breast cancer: association with responsiveness to anthracycline-based chemotherapy. J Clin Oncol 2011;29:859-867.
47. Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 2000;11:265-283.
48. Panvichian R, Tantiwetrueangdet A, Angkathunyakul N, Leelaudomlipi S. TOP2A amplification and overexpression in hepatocellular carcinoma tissues. Biomed Res Int 2015;2015:381602.
49. Pang E, Hu Y, Chan KY, Lai PB, Squire JA, Macgregor PF, Beheshti B, et al. Karyotypic imbalances and differential gene expressions in the acquired doxorubicin resistance of hepatocellular carcinoma cells. Lab Invest 2005;85:664-674.
50. Wong N, Yeo W, Wong WL, Wong NL, Chan KY, Mo FK, Koh J, et al. TOP2A overexpression in hepatocellular carcinoma correlates with early age onset, shorter patients survival and chemoresistance. Int J Cancer 2009;124:644-652.
51. Okada Y, Tosaka A, Nimura Y, Kikuchi A, Yoshida S, Suzuki M. Atypical multidrug resistance may be associated with catalytically active mutants of human DNA topoisomerase II alpha. Gene 2001;272:141-148.
52. Calnan DR, Brunet A. The FoxO code. Oncogene 2008;27:2276-2288.

53. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, Miao L, et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 2007;128:309-323.
54. Bartke T, Siegmund D, Peters N, Reichwein M, Henkler F, Scheurich P, Wajant H. p53 upregulates cFLIP, inhibits transcription of NF-kappaB-regulated genes and induces caspase-8-independent cell death in DLD-1 cells. Oncogene 2001;20:571-580.
55. Ryan KM, Phillips AC, Vousden KH. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 2001;13:332-337.
56. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002;277:3247-3257.
57. Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM. Negative regulation of bcl-2 expression by p53 in hematopoietic cells. Oncogene 2001;20:240-251.
58. Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 1999;104:263-269.
59. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 2001;27:247-254.
60. Zheng T, Wang J, Song X, Meng X, Pan S, Jiang H, Liu L. Nutlin-3 cooperates with doxorubicin to induce apoptosis of human hepatocellular carcinoma cells through p53 or p73 signaling pathways. J Cancer Res Clin Oncol 2010;136:1597-1604.
61. Guan YS, La Z, Yang L, He Q, Li P. p53 gene in treatment of hepatic carcinoma: status quo. World J Gastroenterol 2007;13:985-992.
62. Zhao HC, Zhang Q, Yang Y, Lu MQ, Li H, Xu C, Chen GH. p53-expressing conditionally replicative adenovirus CNHK500-p53 against hepatocellular carcinoma in vitro. World J Gastroenterol 2007;13:683-691.
63. Liu H, Chen W, Liang C, Chen BW, Zhi X, Zhang S, Zheng X, et al. WP1130 increases doxorubicin sensitivity in hepatocellular carcinoma cells through usp9x-dependent p53 degradation. Cancer Lett 2015;361:218-225.
64. Lee TK, Lau TC, Ng IO. Doxorubicin-induced apoptosis and chemosensitivity in hepatoma cell lines. Cancer Chemother Pharmacol 2002;49:78-86.
65. Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF-kappaB as the matchmaker. Nat Immunol 2011;12:715-723.
66. Fan Y, Dutta J, Gupta N, Fan G, Gelinas C. Regulation of programmed cell death by NF-kappaB and its role in tumorigenesis and therapy. Adv Exp Med Biol 2008;615:223-250.
67. Perkins ND. The diverse and complex roles of NF-kappaB subunits in cancer. Nat Rev Cancer 2012;12:121-132.
68. Zhao N, Wang R, Zhou L, Zhu Y, Gong J, Zhuang SM. MicroRNA-26b suppresses the NF-kappaB signaling and enhances the chemosensitivity of hepatocellular carcinoma cells by targeting TAK1 and TAB3. Mol Cancer 2014;13:35.
69. Ni W, Chen B, Zhou G, Lu C, Xiao M, Guan C, Zhang Y, et al. Overexpressed nuclear BAG-1 in human hepatocellular carcinoma is associated with poor prognosis and resistance to doxorubicin. J Cell Biochem 2013;114:2120-2130.
70. Liu Y, Lou G, Wu W, Zheng M, Shi Y, Zhao D, Chen Z. Involvement of the NF-kappaB pathway in multidrug resistance induced by HBx in a hepatoma cell line. J Viral Hepat 2011;18:e439-446.

71. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. Biochim Biophys Acta 2011;1813:1978-1986.
72. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, et al. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul 2006;46:249-279.
73. Schmitz KJ, Wohlschlaeger J, Lang H, Sotiropoulos GC, Malago M, Steveling K, Reis H, et al. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. J Hepatol 2008;48:83-90.
74. Cusimano A, Puleio R, D'Alessandro N, Loria GR, McCubrey JA, Montalto G, Cervello M. Cytotoxic activity of the novel small molecule AKT inhibitor SC66 in hepatocellular carcinoma cells. Oncotarget 2015;6:1707-1722.
75. Simioni C, Martelli AM, Cani A, Cetin-Atalay R, McCubrey JA, Capitani S, Neri LM. The AKT inhibitor MK-2206 is cytotoxic in hepatocarcinoma cells displaying hyperphosphorylated AKT-1 and synergizes with conventional chemotherapy. Oncotarget 2013;4:1496-1506.
76. Kessler SM, Pokorny J, Zimmer V, Laggai S, Lammert F, Bohle RM, Kiemer AK. IGF2 mRNA binding protein p62/IMP2-2 in hepatocellular carcinoma: antiapoptotic action is independent of IGF2/PI3K signaling. Am J Physiol Gastrointest Liver Physiol 2013;304:G328-336.
77. Wei W, Chua MS, Grepper S, So SK. Soluble Frizzled-7 receptor inhibits Wnt signaling and sensitizes hepatocellular carcinoma cells towards doxorubicin. Mol Cancer 2011;10:16.
78. Yang CL, Jiang FQ, Xu F, Jiang GX. ADAM10 overexpression confers resistance to doxorubicin-induced apoptosis in hepatocellular carcinoma. Tumour Biol 2012;33:1535-1541.
79. Choi J, Yip-Schneider M, Albertin F, Wiesenauer C, Wang Y, Schmidt CM. The effect of doxorubicin on MEK-ERK signaling predicts its efficacy in HCC. J Surg Res 2008;150:219-226.
80. Huether A, Hopfner M, Sutter AP, Schuppan D, Scherubl H. Erlotinib induces cell cycle arrest and apoptosis in hepatocellular cancer cells and enhances chemosensitivity towards cytostatics. J Hepatol 2005;43:661-669.
81. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, Wilhelm S, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851-11858.
82. Abou-Alfa GK, Johnson P, Knox JJ, Capanu M, Davidenko I, Lacava J, Leung T, et al. Doxorubicin plus sorafenib vs doxorubicin alone in patients with advanced hepatocellular carcinoma: a randomized trial. JAMA 2010;304:2154-2160.
83. Ho KK, McGuire VA, Koo CY, Muir KW, de Olano N, Maifoshie E, Kelly DJ, et al. Phosphorylation of FOXO3a on Ser-7 by p38 promotes its nuclear localization in response to doxorubicin. J Biol Chem 2012;287:1545-1555.
84. Zhou J, Wan B, Liu XM, Li R, Wang Y, Yu L. MK5 is degraded in response to doxorubicin and negatively regulates doxorubicin-induced apoptosis in hepatocellular carcinoma cells. Biochem Biophys Res Commun 2012;427:581-586.
85. Chen HC, Jeng YM, Yuan RH, Hsu HC, Chen YL. SIRT1 promotes tumorigenesis and resistance to chemotherapy in hepatocellular carcinoma and its expression predicts poor prognosis. Ann Surg Oncol 2012;19:2011-2019.

86. Kim HS, Vassilopoulos A, Wang RH, Lahusen T, Xiao Z, Xu X, Li C, et al. SIRT2 maintains genome integrity and suppresses tumorigenesis through regulating APC/C activity. Cancer Cell 2011;20:487-499.
87. Marquardt JU, Fischer K, Baus K, Kashyap A, Ma S, Krupp M, Linke M, et al. Sirtuin-6-dependent genetic and epigenetic alterations are associated with poor clinical outcome in hepatocellular carcinoma patients. Hepatology 2013;58:1054-1064.
88. Wang JX, Yi Y, Li YW, Cai XY, He HW, Ni XC, Zhou J, et al. Down-regulation of sirtuin 3 is associated with poor prognosis in hepatocellular carcinoma after resection. BMC Cancer 2014;14:297.
89. Chen J, Zhang B, Wong N, Lo AW, To KF, Chan AW, Ng MH, et al. Sirtuin 1 is upregulated in a subset of hepatocellular carcinomas where it is essential for telomere maintenance and tumor cell growth. Cancer Res 2011;71:4138-4149.
90. Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, et al. Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 2001;107:137-148.
91. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004;303:2011-2015.
92. Mao B, Hu F, Cheng J, Wang P, Xu M, Yuan F, Meng S, et al. SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. Oncogene 2014;33:1468-1474.
93. Khongkow M, Olmos Y, Gong C, Gomes AR, Monteiro LJ, Yague E, Cavaco TB, et al. SIRT6 modulates paclitaxel and epirubicin resistance and survival in breast cancer. Carcinogenesis 2013;34:1476-1486.
94. Tikhanovich I, Cox J, Weinman SA. Forkhead box class O transcription factors in liver function and disease. J Gastroenterol Hepatol 2013;28 Suppl 1:125-131.
95. Burgering BM. A brief introduction to FOXOlogy. Oncogene 2008;27:2258-2262.
96. Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, et al. FOXO3A genotype is strongly associated with human longevity. Proc Natl Acad Sci U S A 2008;105:13987-13992.
97. Flachsbart F, Caliebe A, Kleindorp R, Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S, et al. Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc Natl Acad Sci U S A 2009;106:2700-2705.
98. Wang Y, Tian C, Zheng JC. FoxO3a contributes to the reprogramming process and the differentiation of induced pluripotent stem cells. Stem Cells Dev 2013;22:2954-2963.
99. Ikeda J, Tian T, Wang Y, Hori Y, Honma K, Wada N, Morii E. Expression of FoxO3a in clinical cases of malignant lymphoma. Pathol Res Pract 2013;209:716-720.
100. Renault VM, Rafalski VA, Morgan AA, Salih DA, Brett JO, Webb AE, Villeda SA, et al. FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell 2009;5:527-539.
101. Santos MA, Faryabi RB, Ergen AV, Day AM, Malhowski A, Canela A, Onozawa M, et al. DNA-damage-induced differentiation of leukaemic cells as an anti-cancer barrier. Nature 2014;514:107-111.
102. Sunayama J, Sato A, Matsuda K, Tachibana K, Watanabe E, Seino S, Suzuki K, et al. FoxO3a functions as a key integrator of cellular signals that control glioblastoma stem-like cell differentiation and tumorigenicity. Stem Cells 2011;29:1327-1337.
103. Ni HM, Du K, You M, Ding WX. Critical role of FoxO3a in alcohol-induced autophagy and hepatotoxicity. Am J Pathol 2013;183:1815-1825.

104. Zhu WL, Tong H, Teh JT, Wang M. Forkhead box protein O3 transcription factor negatively regulates autophagy in human cancer cells by inhibiting forkhead box protein O1 expression and cytosolic accumulation. PLoS One 2014;9:e115087.
105. Kurinna S, Stratton SA, Tsai WW, Akdemir KC, Gu W, Singh P, Goode T, et al. Direct activation of forkhead box O3 by tumor suppressors p53 and p73 is disrupted during liver regeneration in mice. Hepatology 2010;52:1023-1032.
106. Tsai WB, Chung YM, Takahashi Y, Xu Z, Hu MC. Functional interaction between FOXO3a and ATM regulates DNA damage response. Nat Cell Biol 2008;10:460-467.
107. Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ, Jr., DiStefano PS, Chiang LW, et al. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science 2002;296:530-534.
108. Bakker WJ, Harris IS, Mak TW. FOXO3a is activated in response to hypoxic stress and inhibits HIF1-induced apoptosis via regulation of CITED2. Mol Cell 2007;28:941-953.
109. Ferber EC, Peck B, Delpuech O, Bell GP, East P, Schulze A. FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. Cell Death Differ 2012;19:968-979.
110. Jensen KS, Binderup T, Jensen KT, Therkelsen I, Borup R, Nilsson E, Multhaupt H, et al. FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. EMBO J 2011;30:4554-4570.
111. Lencioni R. Chemoembolization for hepatocellular carcinoma. Semin Oncol 2012;39:503-509.
112. Yin L, Huang L, Kufe D. MUC1 oncoprotein activates the FOXO3a transcription factor in a survival response to oxidative stress. J Biol Chem 2004;279:45721-45727.
113. Hou J, Chong ZZ, Shang YC, Maiese K. FOXO3a governs early and late apoptotic endothelial programs during elevated glucose through mitochondrial and caspase signaling. Mol Cell Endocrinol 2010;321:194-206.
114. Li Z, Zhao J, Tikhanovich I, Kuravi S, Helzberg J, Dorko K, Roberts B, et al. Serine 574 phosphorylation alters transcriptional programming of FOXO3 by selectively enhancing apoptotic gene expression. Cell Death Differ 2016;23:583-595.
115. Nakamura T, Sakamoto K. Forkhead transcription factor FOXO subfamily is essential for reactive oxygen species-induced apoptosis. Mol Cell Endocrinol 2008;281:47-55.
116. Wang K, Li PF. Foxo3a regulates apoptosis by negatively targeting miR-21. J Biol Chem 2010;285:16958-16966.
117. Poulsen RC, Carr AJ, Hulley PA. Cell proliferation is a key determinant of the outcome of FOXO3a activation. Biochem Biophys Res Commun 2015;462:78-84.
118. van der Horst A, Burgering BM. Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol 2007;8:440-450.
119. Daitoku H, Sakamaki JI, Fukamizu A. Regulation of FoxO transcription factors by acetylation and protein-protein interactions. Biochim Biophys Acta 2011.
120. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857-868.
121. Yang JY, Zong CS, Xia W, Yamaguchi H, Ding Q, Xie X, Lang JY, et al. ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. Nat Cell Biol 2008;10:138-148.

122. Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, et al. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 2004;117:225-237.
123. Huang H, Regan KM, Lou Z, Chen J, Tindall DJ. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science 2006;314:294-297.
124. Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, Shoji I, et al. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 2008;82:10375-10385.
125. Chiacchiera F, Simone C. Inhibition of p38alpha unveils an AMPK-FoxO3A axis linking autophagy to cancer-specific metabolism. Autophagy 2009;5:1030-1033.
126. Yuan Z, Becker EB, Merlo P, Yamada T, DiBacco S, Konishi Y, Schaefer EM, et al. Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. Science 2008;319:1665-1668.
127. Lehtinen MK, Yuan Z, Boag PR, Yang Y, Villen J, Becker EB, DiBacco S, et al. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. Cell 2006;125:987-1001.
128. Tikhanovich I, Kuravi S, Campbell RV, Kharbanda KK, Artigues A, Villar MT, Weinman SA. Regulation of FOXO3 by phosphorylation and methylation in hepatitis C virus infection and alcohol exposure. Hepatology 2014;59:58-70.
129. Nasrin N, Ogg S, Cahill CM, Biggs W, Nui S, Dore J, Calvo D, et al. DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. Proc Natl Acad Sci U S A 2000;97:10412-10417.
130. Wang F, Nguyen M, Qin FX, Tong Q. SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. Aging Cell 2007;6:505-514.
131. Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J Clin Invest 2009;119:2758-2771.
132. Calnan DR, Webb AE, White JL, Stowe TR, Goswami T, Shi X, Espejo A, et al. Methylation by Set9 modulates FoxO3 stability and transcriptional activity. Aging (Albany NY) 2012;4:462-479.
133. Housley MP, Udeshi ND, Rodgers JT, Shabanowitz J, Puigserver P, Hunt DF, Hart GW. A PGC-1alpha-O-GlcNAc transferase complex regulates FoxO transcription factor activity in response to glucose. J Biol Chem 2009;284:5148-5157.
134. Housley MP, Rodgers JT, Udeshi ND, Kelly TJ, Shabanowitz J, Hunt DF, Puigserver P, et al. O-GlcNAc regulates FoxO activation in response to glucose. J Biol Chem 2008;283:16283-16292.
135. Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J 2000;349:629-634.
136. Xuan Z, Zhang MQ. From worm to human: bioinformatics approaches to identify FOXO target genes. Mech Ageing Dev 2005;126:209-215.
137. van der Vos KE, Coffer PJ. FOXO-binding partners: it takes two to tango. Oncogene 2008;27:2289-2299.
138. Bouchard C, Marquardt J, Bras A, Medema RH, Eilers M. Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. EMBO J 2004;23:2830-2840.

139. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 2000;404:782-787.
140. Yang JY, Chang CJ, Xia W, Wang Y, Wong KK, Engelman JA, Du Y, et al. Activation of FOXO3a is sufficient to reverse mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor chemoresistance in human cancer. Cancer Res 2010;70:4709-4718.
141. Yang XB, Zhao JJ, Huang CY, Wang QJ, Pan K, Wang DD, Pan QZ, et al. Decreased expression of the FOXO3a gene is associated with poor prognosis in primary gastric adenocarcinoma patients. PLoS One 2013;8:e78158.
142. Shiota M, Song Y, Yokomizo A, Kiyoshima K, Tada Y, Uchino H, Uchiumi T, et al. Foxo3a suppression of urothelial cancer invasiveness through Twist1, Y-box-binding protein 1, and E-cadherin regulation. Clin Cancer Res 2010;16:5654-5663.
143. Bullock MD, Bruce A, Sreekumar R, Curtis N, Cheung T, Reading I, Primrose JN, et al. FOXO3 expression during colorectal cancer progression: biomarker potential reflects a tumour suppressor role. Br J Cancer 2013;109:387-394.
144. Levanon K, Sapoznik S, Bahar-Shany K, Brand H, Shapira-Frommer R, Korach J, Hirsch MS, et al. FOXO3a loss is a frequent early event in high-grade pelvic serous carcinogenesis. Oncogene 2014;33:4424-4432.
145. Obrador-Hevia A, Serra-Sitjar M, Rodriguez J, Villalonga P, Fernandez de Mattos S. The tumour suppressor FOXO3 is a key regulator of mantle cell lymphoma proliferation and survival. Br J Haematol 2012;156:334-345.
146. Yang YC, Tang YA, Shieh JM, Lin RK, Hsu HS, Wang YC. DNMT3B overexpression by deregulation of FOXO3a-mediated transcription repression and MDM2 overexpression in lung cancer. J Thorac Oncol 2014;9:1305-1315.
147. Liu H, Yin J, Wang H, Jiang G, Deng M, Zhang G, Bu X, et al. FOXO3a modulates WNT/beta-catenin signaling and suppresses epithelial-to-mesenchymal transition in prostate cancer cells. Cell Signal 2015;27:510-518.
148. Tenbaum SP, Ordonez-Moran P, Puig I, Chicote I, Arques O, Landolfi S, Fernandez Y, et al. beta-catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer. Nat Med 2012;18:892-901.
149. Santamaria CM, Chillon MC, Garcia-Sanz R, Perez C, Caballero MD, Ramos F, de Coca AG, et al. High FOXO3a expression is associated with a poorer prognosis in AML with normal cytogenetics. Leuk Res 2009;33:1706-1709.
150. Sykes SM, Lane SW, Bullinger L, Kalaitzidis D, Yusuf R, Saez B, Ferraro F, et al. AKT/FOXO signaling enforces reversible differentiation blockade in myeloid leukemias. Cell 2011;146:697-708.
151. Naka K, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T, Kondo Y, Nakao S, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. Nature 2010;463:676-680.
152. Marlow LA, von Roemeling CA, Cooper SJ, Zhang Y, Rohl SD, Arora S, Gonzales IM, et al. Foxo3a drives proliferation in anaplastic thyroid carcinoma through transcriptional regulation of cyclin A1: a paradigm shift that impacts current therapeutic strategies. J Cell Sci 2012;125:4253-4263.
153. Charitou P, Rodriguez-Colman M, Gerrits J, van Triest M, Groot Koerkamp M, Hornsveld M, Holstege F, et al. FOXOs support the metabolic requirements of normal and tumor cells by promoting IDH1 expression. EMBO Rep 2015;16:456-466.

154. Li Z, Zhang H, Chen Y, Fan L, Fang J. Forkhead transcription factor FOXO3a protein activates nuclear factor kappaB through B-cell lymphoma/leukemia 10 (BCL10) protein and promotes tumor cell survival in serum deprivation. J Biol Chem 2012;287:17737-17745.
155. Storz P, Doppler H, Copland JA, Simpson KJ, Toker A. FOXO3a promotes tumor cell invasion through the induction of matrix metalloproteinases. Mol Cell Biol 2009;29:4906-4917.
156. Xie C, Song LB, Wu JH, Li J, Yun JP, Lai JM, Xie DY, et al. Upregulator of cell proliferation predicts poor prognosis in hepatocellular carcinoma and contributes to hepatocarcinogenesis by downregulating FOXO3a. PLoS One 2012;7:e40607.
157. Xu D, He X, Chang Y, Xu C, Jiang X, Sun S, Lin J. Inhibition of miR-96 expression reduces cell proliferation and clonogenicity of HepG2 hepatoma cells. Oncol Rep 2013;29:653-661.
158. Liang C, Chen W, Zhi X, Ma T, Xia X, Liu H, Zhang Q, et al. Serotonin promotes the proliferation of serum-deprived hepatocellular carcinoma cells via upregulation of FOXO3a. Mol Cancer 2013;12:14.
159. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557-2576.
160. Lu M, Ma J, Xue W, Cheng C, Wang Y, Zhao Y, Ke Q, et al. The expression and prognosis of FOXO3a and Skp2 in human hepatocellular carcinoma. Pathol Oncol Res 2009;15:679-687.
161. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 1996;334:693-699.
162. Tezil T, Bodur C, Kutuk O, Basaga H. IKK-beta mediates chemoresistance by sequestering FOXO3; a critical factor for cell survival and death. Cell Signal 2012;24:1361-1368.
163. Fernandez de Mattos S, Villalonga P, Clardy J, Lam EW. FOXO3a mediates the cytotoxic effects of cisplatin in colon cancer cells. Mol Cancer Ther 2008;7:3237-3246.
164. Sunters A, Madureira PA, Pomeranz KM, Aubert M, Brosens JJ, Cook SJ, Burgering BM, et al. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. Cancer Res 2006;66:212-220.
165. Yang Z, Liu S, Zhu M, Zhang H, Wang J, Xu Q, Lin K, et al. PS341 inhibits hepatocellular and colorectal cancer cells through the FOXO3/CTNNB1 signaling pathway. Sci Rep 2016;6:22090.
166. Carbajo-Pescador S, Steinmetz C, Kashyap A, Lorenz S, Mauriz JL, Heise M, Galle PR, et al. Melatonin induces transcriptional regulation of Bim by FoxO3a in HepG2 cells. Br J Cancer 2013;108:442-449.
167. Dieudonne FX, Marion A, Marie PJ, Modrowski D. Targeted inhibition of T-cell factor activity promotes syndecan-2 expression and sensitization to doxorubicin in osteosarcoma cells and bone tumors in mice. J Bone Miner Res 2012;27:2118-2129.
168. Wang JG, Zheng XX, Zeng GY, Zhou YJ, Yuan H. Purified vitexin compound 1 induces apoptosis through activation of FOXO3a in hepatocellular carcinoma. Oncol Rep 2014;31:488-496.
169. Hagenbuchner J, Kuznetsov A, Hermann M, Hausott B, Obexer P, Ausserlechner MJ. FOXO3-induced reactive oxygen species are regulated by BCL2L11 (Bim) and SESN3. J Cell Sci 2012;125:1191-1203.

170. Obexer P, Hagenbuchner J, Unterkircher T, Sachsenmaier N, Seifarth C, Bock G, Porto V, et al. Repression of BIRC5/survivin by FOXO3/FKHRL1 sensitizes human neuroblastoma cells to DNA damage-induced apoptosis. Mol Biol Cell 2009;20:2041-2048.
171. Chen J, Gomes AR, Monteiro LJ, Wong SY, Wu LH, Ng TT, Karadedou CT, et al. Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. PLoS One 2010;5:e12293.
172. Hui RC, Gomes AR, Constantinidou D, Costa JR, Karadedou CT, Fernandez de Mattos S, Wymann MP, et al. The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression. Mol Cell Biol 2008;28:5886-5898.
173. Shiota M, Yokomizo A, Kashiwagi E, Tada Y, Inokuchi J, Tatsugami K, Kuroiwa K, et al. Foxo3a expression and acetylation regulate cancer cell growth and sensitivity to cisplatin. Cancer Sci 2010;101:1177-1185.
174. Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, et al. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature 2008;452:492-496.
175. Nakabayashi H, Taketa K, Yamane T, Miyazaki M, Miyano K, Sato J. Phenotypical stability of a human hepatoma cell line, HuH-7, in long-term culture with chemically defined medium. Gan 1984;75:151-158.
176. Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N, Coombes KR. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. Clin Cancer Res 2010;16:1865-1874.
177. Shukla S, Shukla M, Maclennan GT, Fu P, Gupta S. Deregulation of FOXO3A during prostate cancer progression. Int J Oncol 2009;34:1613-1620.
178. Yao FY, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, Ascher NL, et al. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. Hepatology 2001;33:1394-1403.
179. Yao FY, Kinkhabwala M, LaBerge JM, Bass NM, Brown R, Jr., Kerlan R, Venook A, et al. The impact of pre-operative loco-regional therapy on outcome after liver transplantation for hepatocellular carcinoma. Am J Transplant 2005;5:795-804.
180. Sun S, Song Z, Cotler SJ, Cho M. Biomechanics and functionality of hepatocytes in liver cirrhosis. J Biomech 2014;47:2205-2210.
181. Follis AV, Llambi F, Merritt P, Chipuk JE, Green DR, Kriwacki RW. Pin1-Induced Proline Isomerization in Cytosolic p53 Mediates BAX Activation and Apoptosis. Mol Cell 2015;59:677-684.
182. Zhao Y, Yang J, Liao W, Liu X, Zhang H, Wang S, Wang D, et al. Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. Nat Cell Biol 2010;12:665-675.
183. Jacobs KM, Pennington JD, Bisht KS, Aykin-Burns N, Kim HS, Mishra M, Sun L, et al. SIRT3 interacts with the daf-16 homolog FOXO3a in the mitochondria, as well as increases FOXO3a dependent gene expression. Int J Biol Sci 2008;4:291-299.
184. Liang R, Rimmele P, Bigarella CL, Yalcin S, Ghaffari S. Evidence for AKT-independent regulation of FOXO1 and FOXO3 in haematopoietic stem and progenitor cells. Cell Cycle 2016;15:861-867.

185. Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem 2007;282:30107-30119.
186. Chen IC, Chiang WF, Chen PF, Chiang HC. STRESS-responsive deacetylase SIRT3 is up-regulated by areca nut extract-induced oxidative stress in human oral keratinocytes. J Cell Biochem 2014;115:328-339.
187. Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, et al. Mammalian SIRT1 represses forkhead transcription factors. Cell 2004;116:551-563.
188. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006;444:337-342.
189. Hori YS, Kuno A, Hosoda R, Horio Y. Regulation of FOXOs and p53 by SIRT1 modulators under oxidative stress. PLoS One 2013;8:e73875.
190. Ido Y, Duranton A, Lan F, Weikel KA, Breton L, Ruderman NB. Resveratrol prevents oxidative stress-induced senescence and proliferative dysfunction by activating the AMPK-FOXO3 cascade in cultured primary human keratinocytes. PLoS One 2015;10:e0115341.
191. Frescas D, Valenti L, Accili D. Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J Biol Chem 2005;280:20589-20595.
192. Senf SM, Sandesara PB, Reed SA, Judge AR. p300 Acetyltransferase activity differentially regulates the localization and activity of the FOXO homologues in skeletal muscle. Am J Physiol Cell Physiol 2011;300:C1490-1501.
193. Tacar O, Dass CR. Doxorubicin-induced death in tumour cells and cardiomyocytes: is autophagy the key to improving future clinical outcomes? J Pharm Pharmacol 2013;65:1577-1589.
194. Liu C, Zhang R, Sun C, Zhang H, Xu C, Liu W, Gao W, et al. Resveratrol prevents cadmium activation of Erk1/2 and JNK pathways from neuronal cell death via protein phosphatases 2A and 5. J Neurochem 2015;135:466-478.
195. Bishayee A, Politis T, Darvesh AS. Resveratrol in the chemoprevention and treatment of hepatocellular carcinoma. Cancer Treat Rev 2010;36:43-53.
196. Carter LG, D'Orazio JA, Pearson KJ. Resveratrol and cancer: focus on in vivo evidence. Endocr Relat Cancer 2014;21:R209-225.
197. Fulda S, Debatin KM. Sensitization for anticancer drug-induced apoptosis by the chemopreventive agent resveratrol. Oncogene 2004;23:6702-6711.
198. Yang W, Park IJ, Yun H, Im DU, Ock S, Kim J, Seo SM, et al. AMP-activated protein kinase alpha2 and E2F1 transcription factor mediate doxorubicin-induced cytotoxicity by forming a positive signal loop in mouse embryonic fibroblasts and non-carcinoma cells. J Biol Chem 2014;289:4839-4852.
199. Srisuttee R, Koh SS, Kim SJ, Malilas W, Boonying W, Cho IR, Jhun BH, et al. Hepatitis B virus X (HBX) protein upregulates beta-catenin in a human hepatic cell line by sequestering SIRT1 deacetylase. Oncol Rep 2012;28:276-282.
200. Giannakou ME, Partridge L. The interaction between FOXO and SIRT1: tipping the balance towards survival. Trends Cell Biol 2004;14:408-412.
201. Tao R, Xiong X, DePinho RA, Deng CX, Dong XC. FoxO3 transcription factor and Sirt6 deacetylase regulate low density lipoprotein (LDL)-cholesterol homeostasis via control of the

proprotein convertase subtilisin/kexin type 9 (Pcsk9) gene expression. J Biol Chem 2013;288:29252-29259.
202. Chiang WC, Tishkoff DX, Yang B, Wilson-Grady J, Yu X, Mazer T, Eckersdorff M, et al. C. elegans SIRT6/7 homolog SIR-2.4 promotes DAF-16 relocalization and function during stress. PLoS Genet 2012;8:e1002948.
203. Lombard DB. Sirtuins at the breaking point: SIRT6 in DNA repair. Aging (Albany NY) 2009;1:12-16.
204. Lombard DB, Schwer B, Alt FW, Mostoslavsky R. SIRT6 in DNA repair, metabolism and ageing. J Intern Med 2008;263:128-141.
205. McCord RA, Michishita E, Hong T, Berber E, Boxer LD, Kusumoto R, Guan S, et al. SIRT6 stabilizes DNA-dependent protein kinase at chromatin for DNA double-strand break repair. Aging (Albany NY) 2009;1:109-121.
206. Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, Gellon L, Liu P, et al. Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 2006;124:315-329.
207. Martinez-Pastor B, Mostoslavsky R. Sirtuins, metabolism, and cancer. Front Pharmacol 2012;3:22.
208. Bae HJ, Noh JH, Kim JK, Eun JW, Jung KH, Kim MG, Chang YG, et al. MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. Oncogene 2014;33:2557-2567.
209. Choi HN, Bae JS, Jamiyandorj U, Noh SJ, Park HS, Jang KY, Chung MJ, et al. Expression and role of SIRT1 in hepatocellular carcinoma. Oncol Rep 2011;26:503-510.
210. Zhang ZG, Qin CY. Sirt6 suppresses hepatocellular carcinoma cell growth via inhibiting the extracellular signalregulated kinase signaling pathway. Mol Med Rep 2014;9:882-888.
211. Feng XX, Luo J, Liu M, Yan W, Zhou ZZ, Xia YJ, Tu W, et al. Sirtuin 6 promotes transforming growth factor-beta1/H2O2/HOCl-mediated enhancement of hepatocellular carcinoma cell tumorigenicity by suppressing cellular senescence. Cancer Sci 2015;106:559-566.
212. Ran LK, Chen Y, Zhang ZZ, Tao NN, Ren JH, Zhou L, Tang H, et al. SIRT6 Overexpression Potentiates Apoptosis Evasion in Hepatocellular Carcinoma via BCL2-Associated X Protein-Dependent Apoptotic Pathway. Clin Cancer Res 2016.
213. Sebastian C, Zwaans BM, Silberman DM, Gymrek M, Goren A, Zhong L, Ram O, et al. The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. Cell 2012;151:1185-1199.
214. Azuma Y, Yokobori T, Mogi A, Altan B, Yajima T, Kosaka T, Onozato R, et al. SIRT6 expression is associated with poor prognosis and chemosensitivity in patients with non-small cell lung cancer. J Surg Oncol 2015;112:231-237.
215. Jedrusik-Bode M, Studencka M, Smolka C, Baumann T, Schmidt H, Kampf J, Paap F, et al. The sirtuin SIRT6 regulates stress granule formation in C. elegans and mammals. J Cell Sci 2013;126:5166-5177.
216. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2095-2128.
217. Llovet JM, Hernandez-Gea V. Hepatocellular carcinoma: reasons for phase III failure and novel perspectives on trial design. Clin Cancer Res 2014;20:2072-2079.
218. Rude MK, Crippin JS. Liver transplantation for hepatocellular carcinoma. Curr Gastroenterol Rep 2015;17:11.

219. Sussmuth SD, Haider S, Landwehrmeyer GB, Farmer R, Frost C, Tripepi G, Andersen CA, et al. An exploratory double-blind, randomized clinical trial with selisistat, a SirT1 inhibitor, in patients with Huntington's disease. Br J Clin Pharmacol 2015;79:465-476.
220. Parenti MD, Grozio A, Bauer I, Galeno L, Damonte P, Millo E, Sociali G, et al. Discovery of novel and selective SIRT6 inhibitors. J Med Chem 2014;57:4796-4804.
221. Zhang CG, Zhu WJ, Liu Y, Yuan ZQ, Yang SD, Chen WL, Li JZ, et al. Novel polymer micelle mediated co-delivery of doxorubicin and P-glycoprotein siRNA for reversal of multidrug resistance and synergistic tumor therapy. Sci Rep 2016;6:23859.
222. Zhao C, Zhang Q, Yu T, Sun S, Wang W, Liu G. Hypoxia promotes drug resistance in osteosarcoma cells via activating AMP-activated protein kinase (AMPK) signaling. J Bone Oncol 2016;5:22-29.