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The Role of High Glucose in Tumorigenesis Using Genome Wide Analysis

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

Selene Bobadilla

December 2017

Dissertation Committee:
Dr. Xuan Liu, Chairperson
Dr. Frances Sladek
Dr. Mei Kong

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The Dissertation of Selene Bobadilla is approved:

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This dissertation is in dedication to:

Para mi mamá, porque mi corazón no te olvida

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ABSTRACT OF THE DISSERTATION

The Role of High Glucose in Tumorigenesis Using Genome Wide Analysis

by

Selene Bobadilla

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
University of California, Riverside, December 2017
Dr. Xuan Liu, Chairperson

Altered cellular metabolism is considered a crucial hallmark of cancer, and has been an area of accelerated research. Interestingly, metabolic alterations in cancer can lead to changes in metabolite concentration, and these may play a role in tumorigenesis through functions that are not overtly metabolic. Tumor suppressor p53 plays a prominent role in cancer and much of human biology. Many functions have been attributed to p53, including roles in DNA repair and recombination through association with proteins involved in genome stability and chromatin modification. However, its broadest cellular effect is through its function as a transcription factor (TF). p53 is regulated through multiple post-

translational modifications (PTMs) in response to a variety of cellular stresses including DNA damage. One important PTM is phosphorylation on the Thr55 residue, which is mediated by TBP Associated Factor 1 (TAF1) on the p21 promoter and leads to p53 promoter dissociation and termination of p21 transcription. TAF1 is the largest subunit of transcription factor TFIID and a cell cycle regulatory protein important for G1 phase progression. Interestingly, TAF1 phosphorylates p53 in a cellular ATP level-dependent manner. Because high glucose (HG) conditions lead to increased cellular ATP levels, we tested its effect on p53 function and found that global p53 binding to target promoters is reduced under HG conditions even in the presence of DNA damage. Functional analysis of cell growth inhibition as well as cell apoptosis suggests HG is a potent inhibitor of these p53-dependent cellular responses. Further, we examined the overall HG effect on the DNA damage-dependent gene expression changes and found that HG alters the DNA damage transcriptome. Importantly, HG affected the expression of genes that have already been shown to play a role in cancer. These studies suggest that cancer cells use the dynamic nature of cell metabolism to promote transformation via metabolite concentration. Altogether, this work establishes a molecular mechanism by which hyperglycemia may indirectly contribute to malignant transformation, providing an explanation for the association between diabetes and cancer.

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Chapter 1

Introduction

1.1 Cancer Metabolism

Cancer is defined as the abnormal cell growth of previously normal cells. To meet the increased proliferation demand, growing tumors face two major metabolic challenges: (1) meet increased bioenergetics, and biosynthetic requirements of cell proliferation, and (2) learn how to survive the tumor environmental fluctuations, external nutrient and oxygen availability (Jones and Thompson, 2009). To overcome these challenges, cancer cells display changes in metabolic pathways (Garber, 2006), which culminate in highly adaptive and complex metabolic networks for cellular energy production (Phipps et al., 2015).

Cancer Bioenergetics

Besides providing chemical energy as an end product, metabolic intermediates of both glycolysis and tricarboxylic acid (TCA) cycle can be shunted into macromolecule biosynthetic processes (i.e., lipids, proteins, and nucleic acids) (Bauer et al., 2005; Hsu and Sabatini, 2008). Consequently, glucose is a critical nutrient for proliferating cells as it can be used as the primary substrate for ATP generation and it is also an essential carbon source for biosynthesis of macromolecules (Holley and Kiernan, 1974; Pardee, 1974; Hsu and Sabatini, 2008). The traditional metabolic route for glucose is glycolysis, which ends with the generation of pyruvate and a net gain of two ATP molecules. Beside glycolysis, glucose molecules can be shunted into other metabolic pathways to meet the specific needs of each cell. One of these alternate

pathways is the pentose phosphate pathway (PPP) that generates both ribose-5-phosphate (Rib-5-P), an essential intermediate for nucleic acid synthesis and NADPH, a reducing equivalent necessary for nucleic and fatty acid biosynthesis (Christofk et al., 2008a). Glycolysis can also be shunted into amino acid or lipid synthesis by donating carbon molecules to these biosynthetic pathways via glycolytic intermediate 3-phosphoglycerate (3-PG) (Jones and Thompson, 2009).

Glucose derived pyruvate, amino acids and fatty acids can feed into the tricarboxylic acid (TCA) cycle for complete oxidation to CO_2 (Palm and Thompson, 2017). TCA cycle can theoretically produce 36-38 molecules of ATP from one molecule of glucose. Newly reused protein-to-ATP ratios suggest that one molecule of glucose produces about 29.85 ATP from oxidative phosphorylation (OXPH) probably due to mitochondria inefficiency (Rich et al., 2003). Similar to glycolysis, TCA intermediates are also used for macromolecule biosynthesis. It is estimated that about 10%-50% of pyruvate is not converted to lactate in cancer cells but instead proceeds to acetyl-CoA production (Pedersen, 2007). Acetyl-CoA can be used for fatty acid synthesis, or it can enter the TCA cycle. It is speculated that cancer cells do not use the TCA cycle for energy production but rather as a biosynthetic hub (Jones and Thompson, 2009). For example, citrate, a TCA cycle intermediate, can be used for lipid biosynthesis through oxaloacetate (OAA) at the end of the cycle. Although cellular metabolism is very dynamic, normal cells are tightly regulated and only activate/deactivate metabolic pathways upon the proper signal. Due to the lack of regulation in

cancer cells, cellular metabolism can be shifted to favor proliferation. Together these findings suggest that alterations of cellular metabolic pathways are the key to cancer cell survival, given that these pathways provide both the energy and biomass necessary for proliferation and the means for tumor environment adaptation.

Bioenergetics from Alternate Fuel Sources

Recent studies in cancer metabolism have elucidated that glucose consumption is not the sole carbon source by which cancer metabolism is fueled. Recent investigations of different cancer types and micro-environments suggest that other metabolites can also provide ATP and biomass to fuel growth. In fact, some cells have been shown to be capable of obtaining their ATP requirement almost entirely from glutamine and pyruvate (Rossignol et al., 2004; DeBerardinis et al., 2007). Glutamine metabolism can generate both α-ketoglutarate, and lactate (DeBerardinis et al., 2007). The addiction to glutamine potentially allows cells to utilize glucose-derived substrates for biomass production (i.e., fatty acid synthesis). Alternatively, glutamine may also be used for the biosynthesis of amino acids via transamination reactions. In addition to glutamine, fatty acid is another metabolite that can also serve as an energy source for the cell. An example of this was found in prostate cancer, where Liu and colleagues demonstrated a preference for fatty acid oxidation despite the presence of abundant glucose (Liu et al., 2010; Liu, 2006). Finally, lactate, which was once

considered a waste product of glycolysis, is gaining interest as an important metabolite for oxidative phosphorylation, and currently, there is increasing evidence that lactate is also being consumed by cancerous cells. Interestingly, normal tissues such as the brain, skeletal and cardiac muscle consume lactate regularly, indicating a mechanism to use lactate for OXPHs exists (Kennedy et al., 2010).

Hypoxia

One of the best-characterized changes in cancer metabolism necessary for tumor cell adaptation to tumor environment fluctuations is the activation of hypoxia inducible factor 1-alpha (HIF1). Under sufficient oxygen conditions, HIF1 is regulated through interaction with the von Hippel-Lindau (VHL) tumor suppressor protein, which targets HIF1 for protein degradation (Kim and Kaelin, 2004). As the tumor begins to form and outgrow the diffusion limits of its blood supply, oxygen availability is reduced giving rise to a hypoxic state, i.e., an environment lacking oxygen (Jones and Thompson, 2009). Hypoxia leads to the stabilization of HIF1 and initiates the following two hypoxic responses. First, HIF1 shifts cell metabolism towards glycolysis, since OXPH cannot function without oxygen (Jones and Thompson, 2009). This is made possible by (a) up-regulating expression of glycolytic enzymes, glucose transporters, and inhibitors of mitochondria metabolism, such as lactate dehydrogenase A (LDH-A) (Kaelin and Ratcliffe, 2008; Semenza et al., 1994), and by (b) shunting pyruvate away from

OXPH through up-regulation of pyruvate dehydrogenase kinase 1 (PDK1) that negatively regulates pyruvate dehydrogenase (PDH), therefore inhibiting conversion of pyruvate to acetyl-CoA (Kim et al., 2006; Papandreou et al., 2006). Second, HIF1 stimulates angiogenesis, the formation of new blood vessels, by inducing gene transcription of vascular endothelial growth factor (VEGF) that functions to promote new, although disorganized and leaky, blood vessel formation in tumor cells. In this manner, oxygen and nutrient availability are restored to the tumor's inner cells (Gatenby and Gillies, 2004). These metabolic changes also shift ATP bioenergetics from OXPH to glycolysis (Jones and Thompson, 2009).

The Warburg Effect

More than 60 years ago, Otto Warburg discovered that even in the presence of ample oxygen cancer cells prefer to metabolize glucose through glycolysis rather than OXPH as observed in normal cells (Warburg, 1956). Warburg claimed that the respiratory machinery of cancer cells, the mitochondria, must be damaged, thereby causing an increased reliance upon aerobic glycolysis. Research thereafter, showed that favoring glycolysis over OXPH can occur in the presence of healthy mitochondria, implying that other factors, besides HIF1 and damaged respiratory machinery, may promote the switch to glycolysis as the primary means for ATP production in tumor cells. Perhaps this shift is one of the mechanisms cancer cells use to meet challenge two, i.e., to

achieve increased bioenergetics and biosynthetic requirements, given that mitochondria can be used as a biosynthetic hub. At first sight, the use of glycolysis over OXPH for bioenergy production seems wasteful given that one round of glycolysis produces only a net of two molecules of ATP while the same input can produce over 30 molecules of ATP through OXPH (Devlin, 2006). Interestingly, work by Guppy et al., suggests that in the presence of excess glucose, glycolysis has the potential to produce ATP in greater quantities and at a faster rate than OXPH (Guppy et al., 1993) and it is likely that much of this glucose is being consumed for proliferative purposes in cancer cells.

The Adaptive Nature of Tumor Cells

Recent studies show that tumor cells display metabolic diversity within a tumor (Berger et al., 2011; Beroukhim et al., 2010; Ding et al., 2010). Research on metabolic diversity suggest this is due to variability in oncogene activation, hypoxia levels, and local availability and utilization of carbon sources; all of which lead to variation in tumor evolution. Metabolic diversity allows tumors to develop metabolic symbiosis amongst cells as the tumor outgrows its vascularity. Metabolic symbiosis refers to a state in which lactate from hypoxic and glycolytic tumor cell populations found in the niche of tumors, fuels tumor cells from the oxygenated peripheral region of a tumor (Nakajima et al., 2013). This implies that hypoxic cancer cells import glucose as a source of fuel and export lactate, while normoxic cells import and catabolize lactate for ATP production via OXPH.

Cancer cells benefit from this symbiotic relationship to supply nutrients to the tumor as a whole and this relationship allow them to survive the harsh tumor environment. Oncogenes and tumor suppressors genes are commonly altered in carcinogenesis, and interestingly, they are also tightly involved in the regulation of many metabolic pathways (Hsu and Sabatini, 2008), suggesting that genetic alterations to these genes may promote the metabolic flexibility necessary for the consumption of available nutrients.

Oncogenes and Tumor Suppressors in Metabolism

Not surprisingly, activation of oncogenes and inactivation of tumor suppressors are directly related to altered metabolism in cancer cells. Recent work has demonstrated that oncogenes are involved in the up-regulation of glycolysis (Hsu and Sabatini, 2008). Among these oncogenes are RAS, a small GTPase protein involved in transmitting signals within cells that promote glycolysis (Dang and Semenza, 1999; Ramanathan et al., 2005); Akt kinase, a serine/threonine-specific protein kinase and an effector of insulin signaling that can promote glucose uptake (Manning and Cantley, 2007); and Myc, an oncogenic transcription factor that upregulates the expression of several metabolic genes (Gordan et al., 2007). On the other hand, tumor suppressors have been shown to function to inhibit glycolysis and promote OXPH (Hsu and Sabatini, 2008). For example, loss-of-function mutations in both tumor suppressor metabolic enzymes succinate dehydrogenase (SDH) and fumarate

hydratase (FH) promote HIF1 stabilization under normoxic conditions (Isaacs et al., 2005; Selak et al., 2005). Tumor suppressor p53 is also involved in the regulation of several metabolic genes. One of p53 target genes, TIGAR (TP53-induced glycolysis and apoptosis regulator), for instance, negatively regulates glycolysis by inhibiting fructose-2,6-bisphosphate, a potent stimulator of glycolysis through allosteric modulation of phosphofructokinase 1 (PFK-1) (Bensaad et al., 2006). p53 also regulates the synthesis of cytochrome c oxidase (SCO2), a protein involved in assembly of the cytochrome c oxidase (COX) complex, a complex important in maintaining the function of mitochondrial respiration (Matoba et al., 2006). Putting all these observations together, it is evident that deregulation of oncogenes, and tumor suppressors induce metabolic changes and their cellular outcome in tumor cells is more complicated than previously anticipated in the field and potentially much more involved in cancer progression. Interestingly, the effect that altered metabolites have on oncogenes, tumor suppressors and both cancer initiation and progression has been only minimally explored.

1.2 Diabetes and Cancer

According to the American Diabetes Association (ADA) guidelines, diabetes can be divided into type 1 (T1) and type 2 (T2) (ADA, 2014). T1 diabetes is a condition characterized by high blood glucose levels due to complete lack of insulin, the hormone required for glucose uptake by muscle cells and adipose

tissue. T1 diabetes occurs when the body's immune system attacks the insulin-producing β cells and destroys them. T2 diabetes is a condition characterized by high blood glucose due to either the relative lack of insulin or the body's inability to use insulin efficiently because of alterations in the insulin receptors or downstream effectors that make up insulin signaling pathways in muscle or adipose tissue. T2 occurs due to different risk factors, including age, obesity, genetic predisposition or lack of physical activity. In the United States, T2 diabetes accounts for 90 to 95% of the diabetes cases reported. Table 1.1 summarizes both T1 and T2 diabetes.

	Type 1 Diabetes	**Type 2 Diabetes**
Onset	Sudden	Gradual
Age of Onset	Mostly in youth	Mostly in adulthood
Prevalence	5 to 10% of diagnosed diabetes	90 to 95 % of diagnosed diabetes
Endogenous Insulin	Low or absent	Normal, decreased, or increased
Genetic Predisposition	Moderate	Very strong
Defect or deficiency	Destruction of β cells, eliminating insulin production	Inability of β cells to produce appropriate quantities of insulin; insulin resistance; other defects

Table 1.1 Comparison of type 1 and 2 diabetes.

Also, according to the ADA guidelines, fasting blood glucose (FBG) concentration for a healthy individual should be below 5.6 mM (100mg/dL), and diabetes is diagnosed when FBG is 7mM (126mg/dL) or higher. However, the degree of hyperglycemia, or high blood glucose, varies among each individual, and it is often above 7mM depending on the stage and individual management of

the disease (ADA, 2014). Therefore, fluctuating glucose levels are commonly observed in diabetic individuals and the degree of hyperglycemia is unique to each individual.

Association between Cancer and Diabetes

A series of epidemiological studies have addressed the association between cancer and both T1 and T2 diabetes, both of which have been associated with a higher cancer incidence and mortality (Figure 1.1 and 1.2) (Harding et al., 2015). Given that T1 diabetes association with cancer is limited and variable, more associations have been discovered for T2 diabetes. However, there is evidence to suggest an increased risk for cancers of the pancreas, liver, and stomach for T1 diabetes (Gordon-Dseagu et al., 2013). The consensus report by the American Cancer Society (ACS) and the ADA states that the relative risk imparted by T2 diabetes is most significant (about two-fold or higher) for cancers of the liver, pancreas, and endometrium, and lesser (about 1.2– 1.5-fold) for cancers of the colon, rectum, breast, and bladder (Giovannucci et al., 2010). Similarly, Vigneri et al. also compiled a series of studies that confirm the risk for several cancers in the presence of T2 diabetes. These studies conclude that T2 diabetic patients are at a higher risk for cancer of the liver, pancreas, colorectal, kidney, bladder, endometrial, breast and non-Hodgkin's lymphoma (Table 1.2) (Vigneri et al., 2009). As suggested by Table 1.2, the

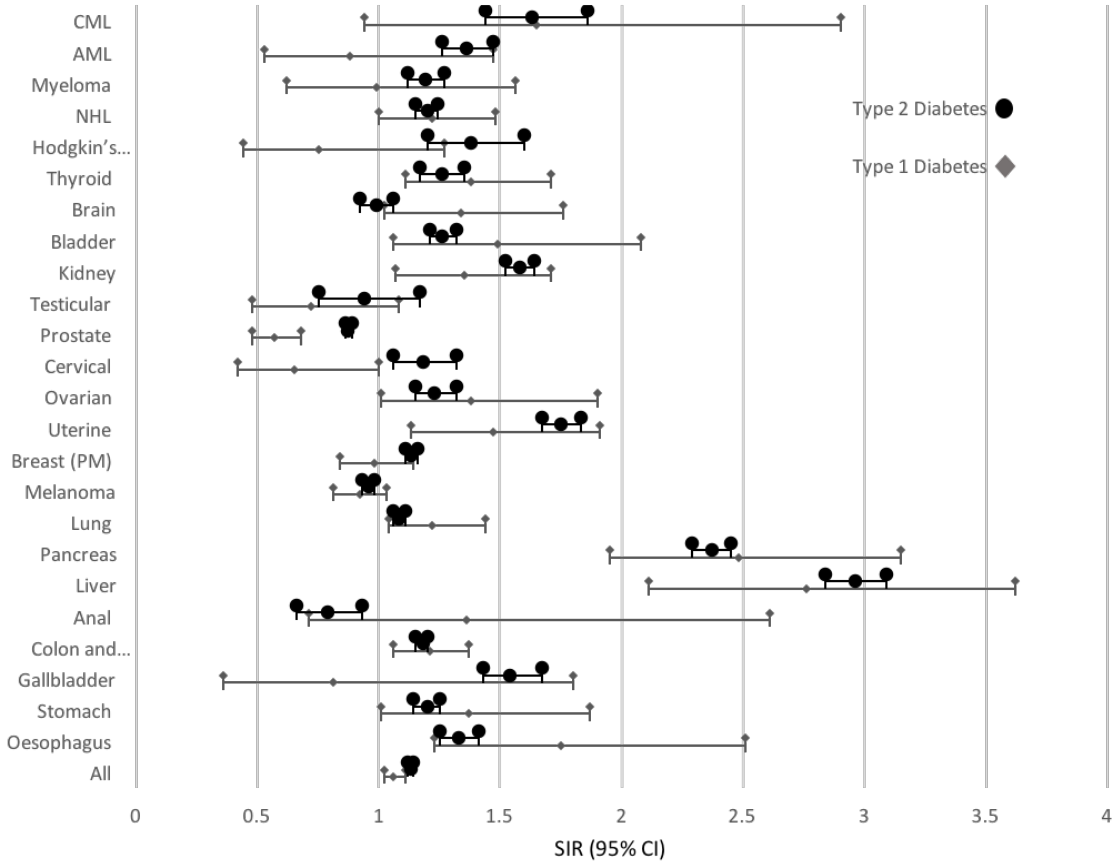

Figure 1.1. Cancer incidence ratios in type 1 and type 2 diabetes. Standardized incidence ratios (SIRs) in type 1 and type 2 diabetes compared with the general population, 1997–2008. Data gathered from Harding et al., 2015. (CI: Confident Interval).

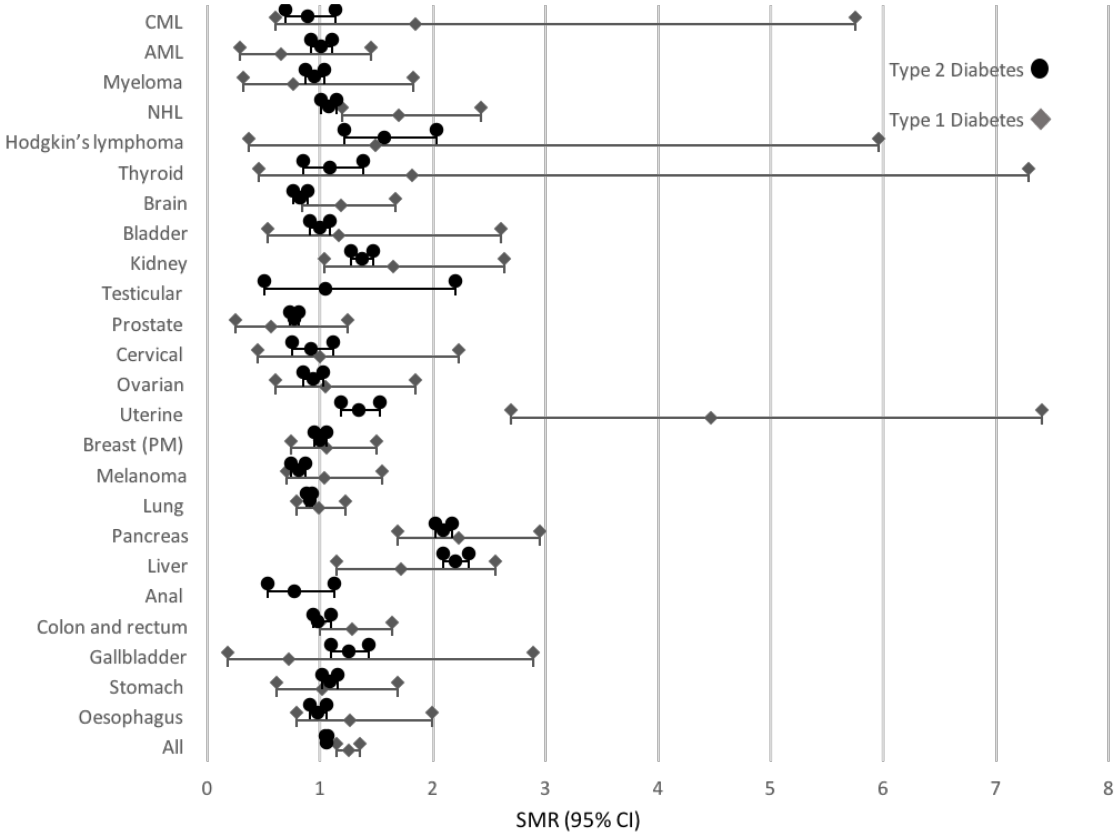

Figure 1.2. Cancer mortality ratios in type 1 and type 2 diabetes. Standardized mortality ratios (SIRs) in type 1 and type 2 diabetes compared with the general population, 1997–2008. Data gathered from Harding, et al., 2015. (CI: Confident Interval).

Cancer		RR (95% CI)
Liver (El-Serag et al., 2006)	13 case-control studies 7 cohort studies	2.50 (1.8–3.5) 2.51 (1.9–3.2)
Pancreas (Huxley et al. 2005)	17 case-control studies 19 cohort studies	1.94 (1.53–2.46) 1.73 (1.59–1.88)
Kidney (Lindblad et al., 1999, Washio et al., 2007)	1 cohort study 1 cohort study	1.50 (1.30–1.70) 2.22 (1.04–4.70)
Endometrium (Friberg et al., 2007)	13 case-control studies 3 cohort studies	2.22 (1.80–2.74) 1.62 (1.21–2.16)
Colon–rectum (Larsson et al., 2005)	6 case-control studies 9 cohort studies	1.36 (1.23–1.50) 1.29 (1.16–1.43)
Bladder (Larsson et al., 2006)	7 case-control studies 3 cohort studies	1.37 (1.04–1.80) 1.43 (1.18–1.74)
Non-Hodgkin's lymphoma (Mitri et al., 2008)	5 cohort studies 11 case-control studies	1.41 (1.07–1.88) 1.12 (0.95–1.31)
Breast (Larsson et al., 2007)	5 case-control studies 15 cohort studies	1.18 (1.05–1.32) 1.20 (1.11–1.30)
Prostate (Kasper & Giovannucci, 2006)	9 case-control studies 10 cohort studies	0.89 (0.72–1.11) 0.81 (0.71–0.92)

Table 1.2. Meta-analyses on the relative risk (RR) of cancer in different organs of diabetic patients
Data on kidney cancer were not obtained from meta-analysis.
Data obtained from Vigneri et al., 2009.

strongest association is for cancer of the pancreas and liver, similar to what was reported in the ACS and the ADA report (Giovannucci et al., 2010). Likewise, another meta-analysis by Pothiwala et al., also indicates that women with a history of T2 diabetes have an approximately 16% higher risk of developing breast cancer than nondiabetic women (Pothiwala et al., 2009).

The molecular links between cancer and diabetes remain somewhat elusive given that both diseases are characterized by metabolic alterations. It remains to be discovered whether the relationship may be due to a combination of the metabolic changes common to both diabetes and cancer (i.e., hyperglycemia, hyperinsulemia, obesity, insulin resistance) affecting all tissues; or site-specific mechanisms, due to one of the aforementioned alterations, altering a specific organ's cellular mechanisms (Vigneri et al., 2009). Obesity is most often classified as the most reasonable connection between both diseases. Plenty of research indicates that obesity is associated with a higher incidence and higher mortality in cancer, independent of T2 diabetes (Adami & Trichopoulos, 2003; Vigneri et al., 2009). A tight correlation has also been observed among obesity, circulating estrogen levels, and increased breast cancer risk (Key et al., 2003; Cleary & Grossmann, 2009). Interestingly, insulin resistance and hyperinsulinemia are much more prominent in T2 than T1 diabetes, while hyperglycemia is more similar between the two, suggesting hyperglycemia may be more important than previously anticipated. Hyperglycemia has also been linked to cancer in recent years, independent of

body mass index (BMI) (Pothiwala et al., 2009), suggesting it may be a factor that increases cancer incidence in diabetic individuals. Interestingly, hyperglycemia is a hallmark of both T1 and T2 diabetes and remains the most widely studied metabolic change in diabetes (Ryu et al., 2014).

Hyperglycemia

Hyperglycemia or high blood glucose is a condition that may exist in individuals with diabetes, obesity, pancreatitis, chronic stress, and cancer (Dobbs et al., 1975; Martyn et al., 2008; Fogar et al., 1998; Mechanick et al., 2006). Studies, as those summarized in Table 1.3, have evaluated the relationship between hyperglycemia and cancer and suggest that a high intake of sugar and elevated blood glucose levels are strongly associated with the risk of cancer. As shown in Table 1.3, hyperglycemia may increase cancer susceptibility and mortality, suggesting that a state of hyperglycemia might be a contributing factor that increases cancer incidence, prognosis, and mortality in diabetic individuals.

Little is known about the molecular mechanisms by which hyperglycemia promotes cancer. There are, however, some speculations suggesting hyperglycemia may promote cancer cell proliferation and metastasis (Vigneri et al., 2009; Suh et al., 2011; Johnson et al., 2012).

First, the Warburg effect suggests high glucose (HG) availability can provide a fuel source for cancer cells to support rapid proliferation (Warburg, 1956). Cancer cell studies indicate that high concentrations of glucose and

Study	Reference	Results
The connection between insulin and fasting glucose and risk of recurrent colorectal adenomas.	Flood A et al., 2007	Patients with increased insulin and glucose are at higher risk of adenoma recurrence, and for those with increased glucose, the increase in risk for recurrence of advanced adenomas is even greater.
The association between post-load plasma glucose concentration and risk of pancreatic cancer mortality among persons without self-reported diabetes.	Gapstur et al., 2000	The risk was 2.2-fold higher for participants whose post-load plasma-glucose level was at least 11.1 mM/L (200 mg/dL) at baseline compared with those whose level was less than or equal to 6.6 mM/L (119 mg/dL). This association was independent of other known and suspected pancreatic cancer risk factors (i.e., age, race, cigarette smoking, BMI, serum uric acid concentration).
The relationship between post-load plasma glucose and 12-year cancer mortality was studied in 11,521 white men and 8591 white women aged 35–64 years.	Levine W et al., 1990	Hyperglycemia was related to cancer mortality in men, but not in women, after controlling for age, BMI, smoking, serum cholesterol, systolic blood pressure, education, and antihypertensive treatment.
Effects of blood sugar level on mammary tumor tolerance on murine models. Three glucose levels were tested (approximately 120, 100, and 80 mg/dL).	Santisteban GA et al., 1985	Murine models showed that susceptibility to an aggressive mammary tumor was significantly influenced by blood glucose. With average glucose elevated only by 15% above normal, the mortality due to the tumor was increased 200% above the mortality in normoglycemic animals. In addition, in hypoglycemic animals, with average glucose decreased by 21% of normoglycemic, mortality was only one-sixth of the control mortality.

Table 1.3. Studies investigating the association between hyperglycemia and the risk of cancer susceptibility or mortality.

insulin change overall RNA and protein levels involved in tumor cell proliferation (Masur et al., 2011). Second, some pancreatic cancer cell lines, including BXPC-3 and Panc-1, have shown to induce epidermal growth factor (EGF) levels and subsequently activate its receptor, the epidermal growth factor receptor (EGFR) upon HG treatment (Han et al., 2011). The EGF pathway is a well-characterized oncogenic pathway, suggesting HG can promote transformation via this receptor. Finally, studies in MCF-7 breast cancer cells revealed that HG stimulated levels of protein kinase C and consequently induced a more aggressive cancer phenotype (Okamur et al., 2002; Ways et al., 1995).

Hyperglycemia has also been associated with invasion, primarily via induction of epithelial-mesenchymal transition (EMT), a multifaceted process that is critical for the acquisition of migration, invasiveness and pluripotent stem cell-like phenotype (Iwatsuki et al., 2010). EMT plays an essential role in the metastatic process. Studies in basal luminal breast carcinoma suggested that HG induced the EMT phenotype, which resulted in the consequent reduction of reactive oxygen species (ROS) generation and increased cell survival (Dong et al., 2013). Indisputably, elucidating the molecular mechanisms by which hyperglycemia promotes cell proliferation and metastasis may offer new therapeutic avenues.

1.3 Tumor Suppressor p53

Cancer development and progression is a very complicated process modulated by many changes in cellular biological pathways that result in a transformed phenotype. Much of cancer investigation has been explicitly focused on (1) alterations of essential genes that result in activation of oncogenes or inactivation of tumor suppressor genes, and (2) the environmental and genetic factors responsible for such alterations. One significantly altered gene, tumor suppressor p53, plays a critical role in our understanding of cancer progression. Since its discovery, p53 research has been devoted to key mutations that lead to tumorigenesis and post-translational modifications necessary for its tumor suppressor activity.

Tumor suppressor p53 is directly involved in the maintenance of genetic integrity as it can become activated upon any kind of cellular stress, i.e., ultraviolet radiation, chemical mutagens, reactive oxygen species, among many. p53 is most commonly studied in its well-established role in deciding between life and death. As a transcription factor p53 can regulate cell cycle arrest through activation of target genes such as cyclin-dependent kinase inhibitor p21 (El-Deiry et al., 1993), or apoptosis through activation of apoptotic genes such as Bax (Miyashita and Reed, 1995). Interestingly, p53 has been shown to be a versatile regulator given that p53-regulated genes encompass a wide variety of other cellular processes including senescence, metabolism, autophagy, angiogenesis and DNA repair depending on the severity and type of cellular stress.

Structure of Human p53

Human p53 protein consists of 393 amino acids that are divided into three functional domains: N-terminal (a.a. 1-97), central core (a.a. 98-292) and C-terminal domain (a.a. 300-393) (Bode and Dong, 2004). The N-terminal domain of p53 contains the acidic transactivation domain (TAD), which is subdivided into two sub-domains, TAD1 (residues 1–40) and TAD2 (residues 41–61) (Bode and Dong, 2004; Wong, 2009). The TAD domain is required for transactivation activity, and it is highly post-transcriptionally modified during stressed and unstressed conditions. In addition, the interaction between p53 and Mouse Double Minute 2 (MDM2) is mediated through the N-terminal domain of p53 (Bode and Dong, 2004). MDM2 remains the principal endogenous RING finger-containing E3-ligase with high specificity for p53 and regulates p53 through ubiquitin-mediated proteasome degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Moreover, the N-terminal domain also contains a proline-rich Scr homology 3-like (SH3) domain. The SH3 domain is required for interaction of p53 with SIN3, a protein that protects p53 from degradation (Bode and Dong, 2004). p53 also contains two leucine-rich nuclear export signals (NES), and one is found on the N-terminus (Zhang et al., 2001). p53 nuclear export is mediated through the nuclear export factor CRM1 (Lohrum, 2001).

The central core domain of the p53 protein contains the sequence-specific DNA-binding domain. The canonical p53 response element (RE) contains two inverted pentameric sequences with the pattern 5′-RRRC(A/T)|(A/T)GYYY-3′

where R denotes an adenine (A) or guanine (G), and Y denotes a cytosine (C) or a thymine (T) (Kruse et al., 2009). In addition, this motif contains two repeats separated by a spacer of 0 to 13 base pairs (bp). Although still a mystery, much attention has been paid to both the mechanisms by which p53 selects its target genes and the degree of induction for a given gene. X-ray crystallographic analyses have revealed that four p53 core domains in the active p53 tetramer bind as a dimer of dimers to the two cognate half sites in DNA (Kitayner et al., 2006). However, the contacts made between the p53 protein surfaces and DNA half-sites have been shown to have DNA-sequence-specific differences, suggesting these differences could translate into the degree of induction for a given target gene (Kitayner et al., 2006). Interestingly, the vast majority of tumor-associated p53 mutations occur within its DNA-binding domain (Hainaut and Hollstein, 2000). This underscores the importance of the sequence-specific DNA binding activity. This also suggests that the genes under p53-mediated transcriptional regulation are essential for inhibiting a cancer phenotype (Cho et al., 1994; Russo et al., 2002).

The C-terminal domain of p53 contains several sub-domains necessary for p53 regulation, including the nuclear localization (a.a. 300-323) and export signals (a.a. 351-362) (NLS and NES, respectively), the tetramerization domain (a.a. 324-355), and the regulatory domain (a.a. 363-393) (Bode and Dong, 2004). C-terminal domain regulation is mainly mediated through a set of basic amino acids that have been found to be the site for many post-translational

modifications (PTMs). Among these basic amino acids are six lysine residues (Lys305, Lys320, Lys370, Lys372/373, Lys382, Lys386) whose PTM combination plays a significant role in the state of p53 in terms of stabilization and activity (Bode and Dong, 2004). Work in this field suggests that the C-terminus can regulate p53's ability to bind its RE, and two models, the conformation and competition model, have been proposed for such regulation (Göhler et al., 2002; Ho et al., 2006). The conformation model states that the p53 protein exists in two different conformations, an inactive and active conformation. In the latent conformation, p53 is DNA-binding inactive as a result of direct interaction between the C-terminal and the core domain, which inhibits sequence-specific DNA binding. In the activated conformation, p53 becomes DNA-binding active when the allosteric inhibition is relieved as the C-terminal is post-transcriptionally modified, which allows p53 to bind DNA (Hupp et al., 1992). On the other hand, the competition model states that the C-terminal indirectly inhibits sequence-specific DNA-binding due to its own ability to bind DNA un-specifically, therefore competing for DNA binding. Again, PTMs on the C-terminus may displace un-specifically bound DNA and allow p53 to bind with more specificity (Anderson et al., 1997).

Function of Human p53

p53 tumor suppressor activation requires three steps (1) p53 stabilization mediated by a series of PTMs followed by tetramerization (2) nuclear localization

and specific-DNA binding and (3) transcription activation/repression of target genes through interaction with the transcriptional machinery (Kruse et al., 2009). Among some of the cellular stresses that induce activation of p53 are the response to DNA damage, oncogene activation, telomere erosion and hypoxia (Vousden and Prives, 2009). However, p53 activation and its implication in cellular fate are extremely complicated since many different factors are involved. For example, target gene activation is dependent on p53's consensus sequence, affinity to the sequence, p53 protein conformation, and the relative location of the proximal promoter (Wahl and Carr, 2001). Moreover, the aforementioned factors are further dependent on the different signaling pathways activated by various types of stress, different combinations of p53 PTMs each signaling pathway promotes, and cofactor association with the p53 complex due to both the specific conformation of the p53-tetramer and the type of core promoter (Wahl and Carr, 2001; Zhao et al., 2000). Nonetheless, as a result of activation p53 subsequently orchestrates transient cell cycle arrest to fix each specific cellular stress, for example, increasing DNA repair or enhancing the expression of enzymes to detoxify ROS (Riley et al. 2008). Alternatively, p53 can also mediate apoptotic cell death, if the damage to the cell is unrepairable (Yee and Vousden 2005; Riley et al. 2008). Other cellular processes also regulated by p53 transcription factor activity include senescence (Yang et al., 2006), autophagy (Feng et al., 2007) and metabolism (Li et al., 2012).

Regulation of Cellular p53 Levels Under Normal Homeostasis

Given that p53 can exert a magnitude of cellular processes, p53 is under tight regulation under unstressed conditions. This is largely through its interaction with MDM2, as aforementioned, allowing p53 to be maintained at low protein levels. Interestingly, it has been shown that p53 can undergo protein degradation in the absence of MDM2 (Ringshausen et al., 2006), suggesting the existence of alternative pathways for p53 degradation. Indeed, several E3-ligases such as COP1 (Dornan et al., 2004), Pirh2 (Leng et al., 2003), and Arf-BP1 (Chen et al., 2005), have been shown to be a factor in the efficient control of p53 levels in both tissue culture and *in vitro* experiments. p53-MDM2 interaction can be inhibited by phosphorylation of on both p53 and MDM2 and by acetylation on p53 (Lu et al., 2007; Gu and Roeder, 1997). Interestingly, tumor suppressor ARF is a physiological regulator of MDM2 as it interferes with the MDM2-p53 interaction, thereby acting to stabilize and activate p53 (Lowe and Sherr, 2003). Upon DNA damage or cellular stress, p53 undergoes a plethora of PTMs, which leads to p53 dissociation from MDM2 and protein stabilization.

Regulation of p53 via Post-Translational Modifications

Upon stress, it is generally accepted that regulation of p53 occurs mainly through alterations in the p53 protein, while changes in the rate of transcription of the *Tp53* gene plays a minor role (Oren, 1999). PTMs play a pivotal role in increasing the half-life of the p53 protein. PTMs alter both p53 protein-protein

and protein-DNA interactions. The most commonly reported PTMs of p53 include phosphorylation of serine and/or threonine residues, as well as acetylation, ubiquitination and sumoylation of lysine residues (Bode and Dong, 2004). This array of PTMs can occur both during normal homeostasis and in stress-induced responses and each result in a particular combination of PTMs on p53.

Phosphorylation

Phosphorylation of p53 serine or threonine residues generally results in protein stabilization and has been shown to increase its sequence-specific DNA binding affinity in the presence of stress. For example, in human cells following DNA damage p53 is phosphorylated on all three domains: N-terminal, central core, and C-terminal domains (Bode and Dong, 2004). However, some residues, i.e., Thr55, Ser376, and Ser378, are also constitutively phosphorylated in unstressed cells (Gatti et al., 1997; Waterman et al., 1998). Furthermore, Thr55 and Ser376 are known to be dephosphorylated in the presence of ionizing radiation, indicating that not all phosphorylation sites result in p53 protein stability. Interestingly, some p53 residues are redundantly phosphorylated by different kinases, and the same kinases can phosphorylate several residues on p53. It is believed this redundancy serves as a 'fail-safe' mechanism to ensure proper response to cellular stress and suggests the importance of p53 tumor suppressor activity (Bode and Dong, 2004).

Acetylation

Similar to phosphorylation, cellular stress increases acetylation of p53 lysine residues, which induces stabilization and protein activation, although occasionally previous phosphorylation is required for subsequent acetylation (Luo et al., 2000; 2001; Vaziri et al., 2001; Ito et al., 2001; Knights et al., 2006). Histone acetyltransferases (HATs) are the proteins responsible for adding one or several acetyl groups to p53 and are important regulators of p53 (Brooks and Gu, 2003). During transcription activation, for example, histone acetyltransferase CBP/p300 can acetylate p53. This PTM is critical for recruitment of cofactors and the general transcription machinery and in turn for the efficient activation of p53 target genes (Goodman and Smolik, 2000; Iyer et al., 2004). Acetylation also enhances p53's ability to bind DNA (Gu and Roeder, 1997). Tip60 and hMof are other cofactors with HAT activity that can be recruited by p53 and subsequently acetylate p53 and histones (Tang et al., 2006b; Sykes et al., 2006).

Interestingly, both hyperphosphorylation and hyperacetylation have been observed in cancer cell lines. For instance, untreated human pulmonary epithelial type II (A549) cells, which express wild-type p53, showed a profile of hyperphosphorylation at Ser6 and Ser9 that increased approximately ten-fold after exposure to either ionizing radiation or ultra-violet (UV) light (Higashimoto et al., 2000). Furthermore, it has been shown that expression of hepatitis C virus (HCV) core protein, a virus commonly associated with carcinogenesis, can induce hyperacetylation of Lys373 and Lys382 on p53 (Kao et al., 2004).

Although the significance of these observations is still unclear, it is believed that this phenomenon may affect wild-type p53 in a similar manner as mutant p53, i.e., by altering p53's protein-protein interaction, protein-DNA interaction, or p53 protein stability.

Ubiquitination

Unlike phosphorylation and acetylation, ubiquitination of p53 is most active during normal growth conditions. The six C-terminal lysine residues of p53 are the predominant sites for MDM2-mediated ubiquitination (Lohrum et al., 2001). Given that acetylation of these lysine residues occurs in response to stress, this suggests that acetylation inhibits p53 MDM2-mediated degradation (Ito et al., 2001; Wang et al., 2004). Ubiquitination and acetylation are therefore mutually exclusive modifications, and competition between these modifications is thought to affect p53 stability.

Furthermore, the number of ubiquitin molecules on each lysine plays a significant role in the regulation of p53. Work by Li et al., have shown that p53 localization and degradation status is modulated by mono- versus poly-ubiquitination (Li et al., 2003). Li et al. were able to show that mono-ubiquitination of p53 was associated with low cellular MDM2 levels and promoted p53 nuclear retention, whereas polyubiquitination of p53 was associated with high cellular MDM2 levels and promoted nuclear-delocalization, rapid deactivation, and subsequent degradation of p53.

1.4 Loss of Function of p53 in Cancer

It is no surprise that plenty of research has been devoted to p53 mutagenesis, as p53 mutations are the most frequently observed genetic alterations in human cancer (Hainaut and Hollstein, 2000; Kandoth et al., 2013). While p53 mutations occur in all coding exons of the *TP53* gene, there is substantial predominance in exons 4-9 which corresponds to the DNA-binding domain of the protein, resulting in loss of p53 DNA-interaction (Cho et al., 1994; Donehower, 1996). Within the DNA-binding domain mutations, about 30% fall within 6 "hotspot" residues (R175, G245, R248, R249, R273, and R282). Interestingly, these are frequent in almost all types of cancer. However, other mechanisms also exist for inactivating p53 tumor suppressor function, such as the nuclear exclusion of wild-type p53, mutations affecting p53 interacting proteins, or viral oncoproteins that bind and inhibit p53 activity. For example, p53 can be rendered inactive if it is sequestered in the cytoplasm due to either improper tetramerization (Stommel et al., 1999) or by association with overexpressed inhibitors such as MDM2 (Kubbutat et al., 1997). The latter can achieve both the nuclear exclusion of wild-type p53 and down-regulate p53 protein abundance. In this manner, MDM2 acts as an oncogene resulting in the constitutive inhibition of wild-type p53. In fact, MDM2 has been shown to be over-amplified in multiple tumor types (Momand et al., 1998). Many viral oncoproteins have also evolved mechanisms to inhibit p53 activity; among these is the SV40 large T-antigen and the E6 protein from the Human Papillomavirus. The protein SV40 large T-antigen, for example, can bind

p53 and prevent it from binding to DNA, (Sheppard et al., 1999) while the E6 protein from the Human Papillomavirus leads to p53 degradation upon host infection (Scheffner et al., 1990).

Thus far, research on p53 has advanced our knowledge in mechanisms for p53 inactivation, which can be summarized by (a) direct mutations on the p53 protein, (b) lesions in proteins that associate with and regulate p53, or (c) virus proteins that associate with and inactivate p53. These mechanisms have taught us that while direct mutations on p53 remain the primary cause for p53 inactivation, alternative mechanisms exist and may also lead to p53 down-regulation.

1.5 Regulation of p53 Function via TAF1

TAF1

TATA-binding protein (TBP) associated factor 1 (TAF1) is the largest subunit of general transcription factor TFIID. TFIID is itself composed of TBP and 13 TAFs (Burley and Roeder, 1996; Tora, 2002). TAF1 contains two tandem bromodomain modules (double bromodomain, DBrD), a 110-residue structural module with a hydrophobic acetyllysine binding pocket, that bind to both diacetylated histone H4 peptides (Jacobson et al., 2000) and p53 (Li et al., 2007). The crystal structure of the TAF1 DBrD revealed that the acetyllysine binding pockets span a linear distance equivalent to about eight or more residues in an extended oligopeptide (Jacobson et al., 2000). At the molecular level p53

acetyllysine residues can be recognized by bromodomains (Dhalluin et al., 1999; Jacobson et al., 2000). Interestingly, TAF1 DBrD is capable of directly binding to acetyl-K373 and –K382 residues on the C-terminal domain of p53, which are separated by eight residues (Li et al., 2004). These stabilizing acetylation marks can recruit TAF1 to p53-binding sites on promoter DNA. In the event of stress, this mechanism has been shown to activate transcription on p21. Importantly, these studies showed that TAF1 is initially recruited to the p53 binding site (-1.4kb site from the p21 proximal promoter (aka. 3' site)) on the p21 promoter and then looped to the TATA-box containing core promoter under stressed conditions, unlike other general TFs (i.e., TBP, TAF4 and TAF5) which are first recruited to the core promoter (Li et al., 2007).

In addition, human TAF1 has intrinsic protein kinase activity (Dikstein et al., 1996), histone acetyltransferase activity (HAT; Mizzen et al., 1996), and ubiquitin-activating and conjugating activity (E1/E2; Pham and Sauer, 2000). TAF1 is a bipartite kinase, which consists of N- and C-terminal domains. TAF1 kinase has been involved in autophosphorylation, phosphorylation of the 74 kDa subunit (RAP74) of transcription factor IIF, and phosphorylation of p53. Further, TAF1 is important for progression through the G1 phase (Wang and Tjian, 1994), which is believed to be kinase activity-dependent (O'Brien and Tijan, 1998). Indeed, it has been shown that the retinoblastoma protein (RB) interacts directly with TAF1 and inhibits the kinase activity of TAF1 (Siegert and Robbins, 1999).

Phosphorylation of p53 on Thr55

TAF1 kinase has been shown to phosphorylate p53 at Thr55, and this phosphorylation leads to p53 inactivation and protein degradation as it increases interaction of p53 with both CRM1 and MDM2 (Li et al., 2004; Cai and Liu, 2008). Unlike most other p53 phosphorylations, Thr55 phosphorylation occurs under cell growth conditions, and this is important for TAF1 in promoting G1 progression. As mentioned before, upon DNA damage acetyl-p53 is responsible for recruitment of TAF1 to the core promoter for transcriptional activation of target gene p21 (Li et al., 2007). This work revealed that TAF1 plays a dual role in the regulation of p53 by (a) facilitating p53 target gene activation upon stress conditions and (b) inactivating p53-mediated transcription by marking p53 for inactivation via Thr55 phosphorylation. Importantly, the molecular switch for this observed TAF1 dual function to both activate and terminate p53-mediated transcription is due to fluctuations in cellular ATP levels (Wu et al., 2014).

A summary of the events elucidated by Wu et al. is shown in Figure 1.3. During the initial stages of DNA damage, cellular ATP is depleted by poly (ADP-ribose polymerase-1 (PARP-1)), a nuclear enzyme that catalyzes the covalent attachment of ADP-ribose units on the y-carboxyl group of glutamate (Glu) residues of acceptor proteins, which depletes cellular ATP levels (Schreiber et al., 2006). PARP-1 activation is due to DNA double-strand breaks (DSBs), and this effect has been observed under both UV irradiation and bleomycin treatment. Exhaustion of cellular ATP levels will suppress TAF1 kinase activity. Notably, a

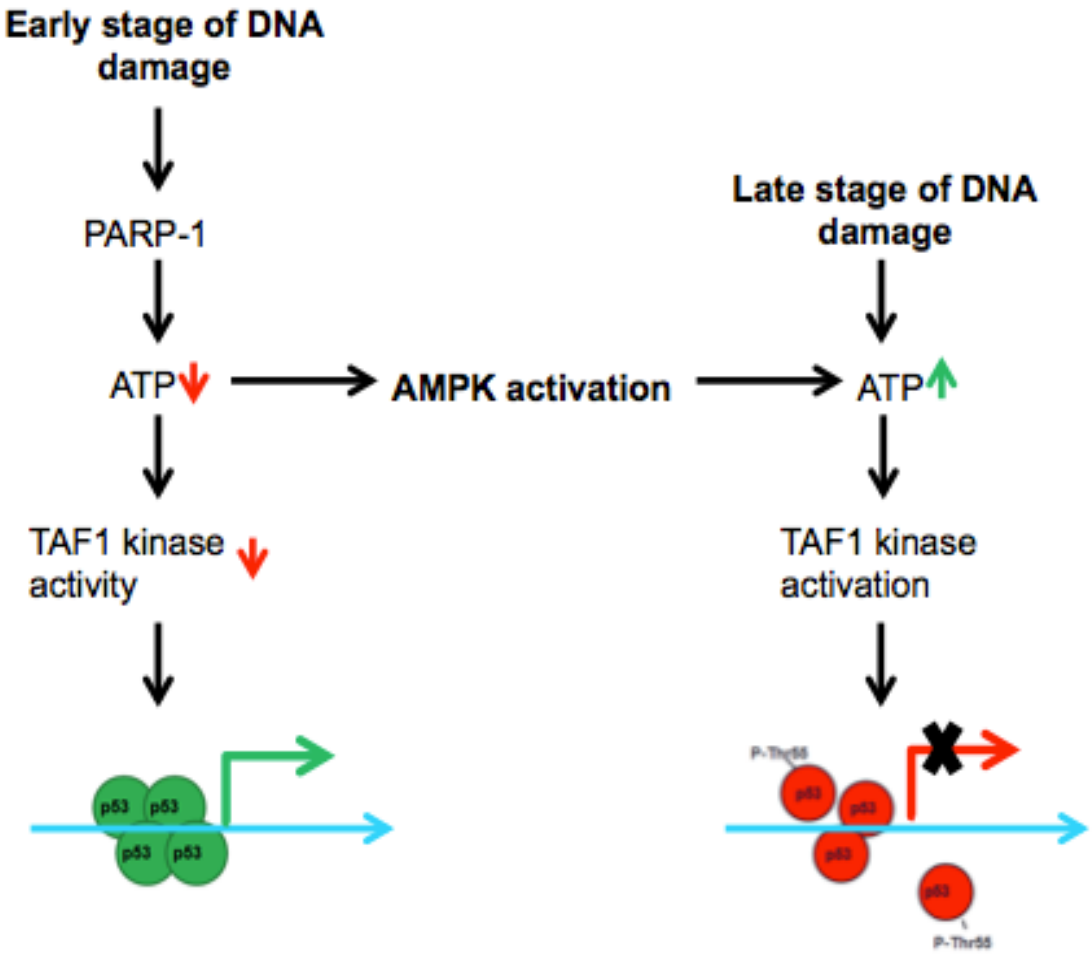

Figure 1.3. TAF1 kinase regulates p53 transcription factor activity in the event of DNA damage. p53 DNA damage response regulation dependent on fluctuating cellular ATP levels and TAF1 kinase activity. (Legend: PARP-1, poly (ADP-ribose polymerase-1); ATP, adenosine triphosphate; AMPK, AMP-activated protein kinase; TAF1, TBP associated factor 1). Information gathered from Wu et al., 2014.

decrease in Thr55 phosphorylation is observed initially after DNA damage, likely due to the combinatory effect of lower cellular ATP levels and activation of PP2A, which has been shown to be the phosphatase responsible for dephosphorylation of Thr55 (Li et al., 2007b). Meanwhile, p53 is post-transcriptionally modified on other residues, including acetylation of residues K373 and K382, which allows for TAF1 recruitment by p53 to the p21 promoter. Together these events enable proper p53 transcription activation. Cellular ATP exhaustion also leads to a shift in the cellular AMP-to-ATP ratio resulting in the activation of AMP-activated protein kinase (AMPK). One of AMPK's cellular responses is to enhance ATP-production by stimulating catabolic pathways (Hardie et al., 2007). Subsequently, during the later stages of DNA damage cellular ATP levels are restored, resulting in an enhancement of TAF1 kinase activity, Thr55 phosphorylation, p53 promoter dissociation, and consequently down-regulation of p53 transcription. Hence, as cells recover from DNA damage, cellular ATP fluctuation acts as a molecular switch through Thr55 phosphorylation for termination of p21 transcription. Furthermore, genome-wide analysis of p53-DNA binding during the course of DNA damage response revealed that p53 undergoes promoter dissociation at a global level. Importantly, p53 acetylation mutants that fail to bind to TAF1 have been shown to lack TAF1 Thr55 phosphorylation suggesting that this unique p53-TAF1 interaction is important for keeping TAF1 in close proximity to p53, thus allowing for Thr55 phosphorylation to occur on the promoter to turn off gene transcription (Li et al., 2007a; Wu et al., 2014).

Overall, this research suggested that TAF1 kinase activity may be regulated via cellular ATP availability. Of interest, glucose is a critical nutrient for proliferating cells as it can be used as the primary substrate for ATP generation (Hsu and Sabatini, 2008), and it is generally accepted that cancer cells consume more glucose than normal cells do (Jones and Thompson, 2009). This would suggest that a nutrient-rich environment, such as is the case of people with diabetes who often experience hyperglycemia (HG), would result in more cancer cell cellular ATP production. Cellular ATP fluctuations in mammalian cells range from 1-5 mM (Gribble et al., 2000). Interestingly, most kinases K_m for ATP are not within the mammalian range of 1-5 mM (Knight and Shokat, 2005), suggesting cellular ATP fluctuation does not affect their kinase activity. For example, ATM has a Km for ATP (K_m, ATP) of 29 uM, and thus ATM-mediated p53 Ser15 phosphorylation is not affected by cellular ATP fluctuations. Alarmingly, the calculated K_m, ATP for TAF1 is 1.9 mM, suggesting that fluctuations in cellular ATP can affect TAF1 kinase activity due to its poor K_m. Hence, growth-promoting TAF1 kinase activity may be deleterious to people with diabetes as cellular ATP can continuously fluctuate above basal levels due to HG.

1.6 Summary of Chapters

Given that glucose is a primary substrate for cellular ATP production and that cancer cells can uptake more glucose than normal cells do, suggests that when

glucose is readily available altered metabolism may promote tumorigenesis. Thus far, our research has shown TAF1 kinase is modulated by cellular ATP levels, but we have yet to investigate how a HG microenvironment might affect p53 activity via TAF1 regulation. To this end, Chapter 2 investigates a glucose oversupplied environment and the regulation of p53. These studies revealed that HG indeed elevated ATP levels and consequently induced TAF1 kinase activity, leading to both p53 Thr55 phosphorylation and p53 inhibition. The studies in this chapter also indicate that HG inhibition on p53 activity poses a significant threat to the genome integrity especially in the presence of DNA damage. Further in Chapter 3, we investigate the overall effect of HG on the DNA damage-mediated transcriptome change. Our analysis elucidated that HG alters the RNA levels of genes that have already been shown to play a role in cancer pathways and some have alterations in human cancer samples. In summary, these studies provide an important connection between high glucose and cancer.

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Chapter 2

High glucose inhibits p53 tumor suppressive function via Thr55 phosphorylation

Chapter 2 is intended to become a publication. Work presented in this chapter is a collaboration of all co-authors for the future publication. Dr. Yong Wu is responsible for the work shown in Figure 2.1A, C; Figure 2.2; Figure 2.3 B (p53 Chip), C; Figure 2.5C; Figure S2.1 A, C, G; Figure S2.2; Figure S2.4 C. Dr. Yumiko Nobumori is responsible for the work presented in Figure 2.6 B, C. Dr. Michael Reid is responsible for the work shown in Figure S2.1 I.

2.1 Introduction

The p53 tumor suppressor plays a critical role in guarding cell genomes against DNA damage. As a DNA-specific transcription factor, p53 controls a plethora of genes with a diverse range of cellular functions including cell cycle arrest, apoptosis, and metabolism (Menendez et al., 2009). Among the genes induced by p53 is the cyclin-dependent kinase inhibitor p21, an important cell cycle arrest gene that binds and inhibits all currently known cyclin-dependent protein kinases (Cazzalini et al, 2010).

Previously, we reported that TBP associated factor 1 (TAF1), phosphorylates p53 at Thr55 on the p21 promoter, and this phosphorylation leads to p53 dissociation from the promoter (Wu et al., 2014). TAF1 is the largest subunit of general transcription factor TFIID, which consists of the TATA box-binding protein (TBP) and 14 TBP-associated factors (TAFs) (Burley and Roeder, 1996; Tora, 2002; Thomas and Chiang, 2006; Wright et al., 2006) and possesses intrinsic protein kinase activity (Dikstein et al., 1996). Interestingly, phosphorylation of p53 by TAF1 is modulated by cellular ATP levels (Wu et al., 2014), indicating that cellular ATP level could potentially affect p53 activity via TAF1-mediated p53 phosphorylation.

Given that ATP is an essential by-product of cellular metabolism; we directed our attention to altered metabolism, a known and crucial hallmark of cancer (Hanahan and Weinberg, 2011). Recent work suggests that when nutrients, such as glucose, are abundant, metabolic reprogramming direct

cancerous cells to enhance their nutrient acquisition. Enhanced nutrient consumption facilitates the production of both bioenergetics and biosynthetic requirements necessary for cell growth and proliferation (Jones and Thompson, 2009). Interestingly, excess nutrient uptake may also lead to changes in metabolite concentrations, which affects protein post-translational modifications (PTMs) and function in the cell (Ward and Thompson, 2012). While it is well accepted that cancer cells uptake more nutrients than normal cells do, the effect a glucose-oversupplied microenvironment has on tumor suppressors has yet to be explored. To address this question, we sought to investigate whether, under high glucose (HG) conditions, increased cellular ATP and subsequent TAF1 phosphorylation inhibit p53 tumor suppressor activity.

In this study, we report that upon HG exposure, elevated ATP levels induced the kinase activity of TAF1, which leads to p53 Thr55 phosphorylation. The phosphorylation dissociates p53 from the p21 promoter and results in the reduction of p21 transcription. Importantly, genome-wide analysis of p53 promoter binding revealed a global dissociation of p53 from its target genes, indicating that HG inhibits overall p53 activity after DNA damage. Inhibition of TAF1-mediated p53 Thr55 phosphorylation abolished those events, supporting the role of Thr55 phosphorylation in regulating p53 function under HG conditions. Functional analysis of cell growth inhibition as well as cell apoptosis suggests HG is a potent inhibitor of these p53-dependent cellular responses. These data

indicate that HG acts as a tumor promoter through, at least in part, modulating p53 Thr55 phosphorylation and activity.

2.2 Results

High Glucose Down-Regulates p53 Through Thr55 Phosphorylation

To establish the effect of HG on cellular ATP levels and the subsequent effect on p53 activity we first treated U2OS cells with HG for different times (Figure 2.1A) and at various concentrations (Figure S2.1A). Our results indicated that cellular ATP levels, as well as cellular ATP/ADP ratio (Figure S2.1B), were induced in a dose and time-dependent manner. Importantly, correlated increase in p53 Thr55 phosphorylation, but not other phosphorylation, was also observed. To exclude osmotic effect of HG treatment, we treated cells with the same concentration of mannitol and increased cellular ATP levels or Thr55 phosphorylation was not observed (Figure 2.1B).

The protein serine/threonine kinase Akt, a pro-survival kinase activated upon serum stimulation, has been shown to inhibit p53 through activation of MDM2 (Gottlieb et al., 2002). Because cell proliferation is dependent on both growth factors and availability of nutrients (Dang, 2013), we questioned whether HG treatment might induce Akt and lead to p53 inhibition. To investigate this, we assayed the Akt activation marker S473 phosphorylation (Sarbassov et al., 2005; Bayascas and Alessi, 2005) as well as its target MDM2 S166 phosphorylation (Gottlieb et al., 2002) upon HG treatment. Our results indicated that the

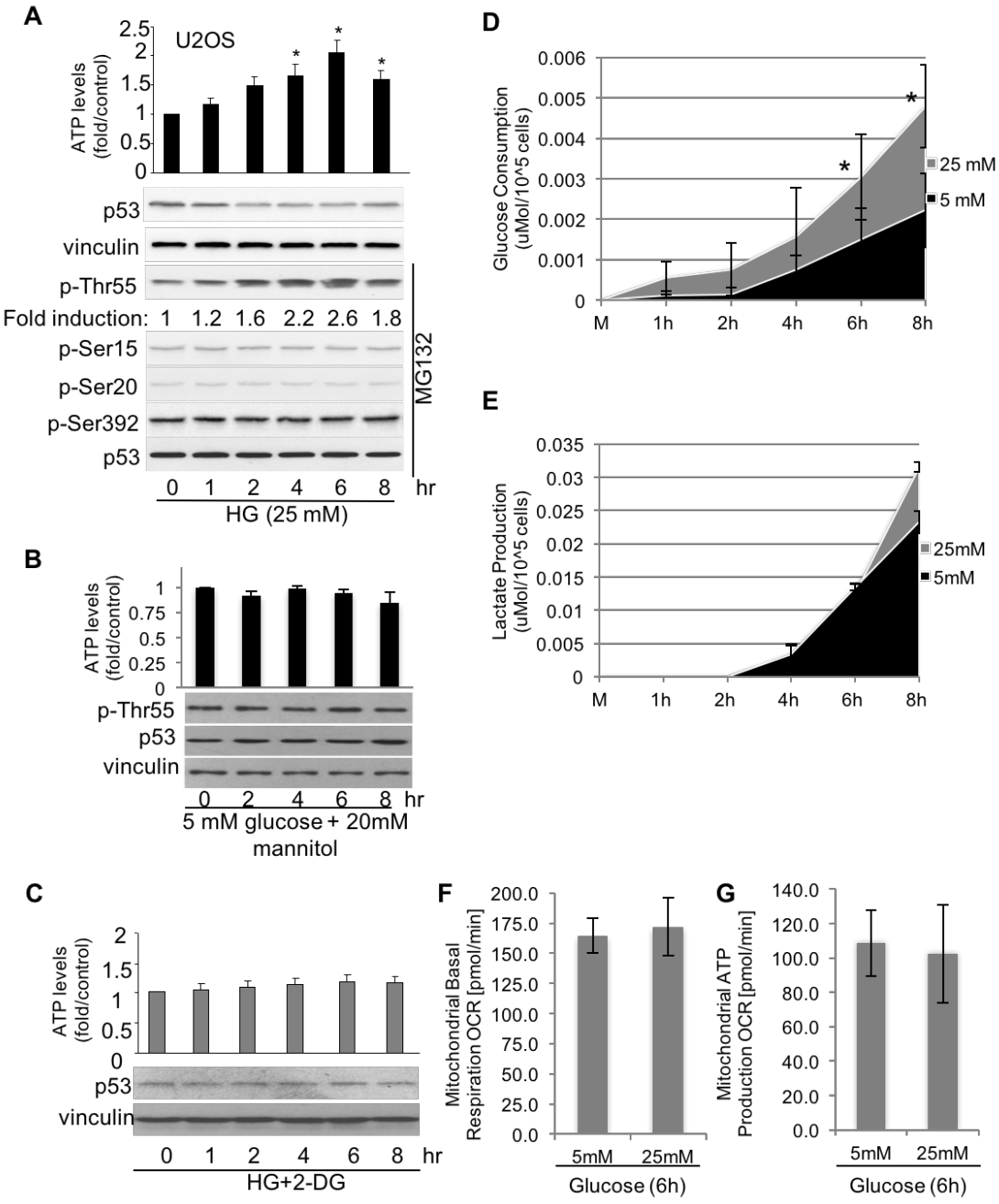
A
U2OS
ATP levels (fold/control)
p53
vinculin
p-Thr55
Fold induction: 1 1.2 1.6 2.2 2.6 1.8
p-Ser15
p-Ser20
p-Ser392
p53
MG132
0 1 2 4 6 8 hr
HG (25 mM)
B
ATP levels (fold/control)
p-Thr55
p53
vinculin
0 2 4 6 8 hr
5 mM glucose + 20mM mannitol
C
ATP levels (fold/control)
p53
vinculin
0 1 2 4 6 8 hr
HG+2-DG
D
Glucose Consumption (uMol/10^5 cells)
25 mM
5 mM
M 1h 2h 4h 6h 8h
E
Lactate Production (uMol/10^5 cells)
25mM
5mM
M 1h 2h 4h 6h 8h
F
Mitochondrial Basal Respiration OCR [pmol/min]
5mM 25mM
Glucose (6h)
G
Mitochondrial ATP Production OCR [pmol/min]
5mM 25mM
Glucose (6h)

Figure 2.1. High glucose down-regulates p53 through Thr55 phosphorylation. (A-C) U2OS cells were treated with (A) high glucose (25 mM), (B) mannitol (20mM), or (C) high glucose (25 mM) in combination with 2-DG (20 mM). Cell lysates underwent SDS-PAGE followed by Western blotting with various primary antibodies as indicated and visualized by the enzyme-linked chemiluminescence system. The blot is representative of 3 separate experiments. To normalize the p53 protein level, cells were treated with MG132 before harvesting them. Phosphorylated p53-Ser15, Ser20, and S392 were detected by Western blotting. The level of Thr55-p was detected by immunoprecipitation with Thr55 phospho-specific antibody (202 IP) and immunoblotting with anti-p53 antibody. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (D-E) U2OS cells were cultured for 48 h in complete medium followed by 5 or 25mM glucose treatment. (D) Glucose uptake and (E) lactate production measurements were taken using the Nova Bioprofile 100 analyzer for each time indicated. Shaded area under the curve represents consumption/production over time (±SD) from 3 independent experiments (*$p < 0.05$). (F-G) Representative graphs of OCR measured in U2OS cells using the XF-24 Seahorse system. (F) Mitochondrial basal respiration and (G) ATP mitochondrial production in 5mM or 25mM glucose treated cells for 6h. Data presented are mean ± SD of 5 independent experiments.

Akt/MDM2 pathway is not involved in down-regulation of p53 in U2OS cells (Figure S2.1C).

To investigate whether HG inhibits p53 by altering cellular ATP levels in other cell lines, we tested HG treatment in colorectal (HCT116), breast (MCF7), liver (SK-HEP1), and lung cancer cells (A549) (Figure S2.1) and examined cellular ATP and p53 protein levels. Our findings verify that the HG effect is not cell specific.

To assess whether the observed effect on cellular ATP and p53 is glucose metabolism dependent, we first treated cells with HG in the presence of the glucose metabolism inhibitor 2-deoxyglucose (2-DG) (Figure 2.1C). The efficient inhibition of cellular ATP induction and resultant p53 down-regulation was evident upon 2-DG treatment. Next, we assayed glucose consumption of HG-treated U2OS cells. As shown in Figure 2.1D, HG-treated cells indeed display more glucose consumption over the control treated cells post HG treatment, consistent with the early time where the higher cellular ATP level was observed. After 6h of HG exposure, however, HG no longer increased cellular ATP level even though glucose consumption remained active. This result suggests that glucose metabolism may be re-directed away from ATP producing pathways, i.e., glycolysis and oxidative phosphorylation (OXPH), after 6h of HG exposure. Nevertheless, these results indicate that p53 inhibition is glucose-metabolism dependent.

Next, we addressed the role of glycolysis and OXPH in HG-induced ATP enhancement in U2OS cells. First, we speculated that perhaps HG inhibits pyruvate dehydrogenase (PDH) complex, which is responsible for importing pyruvate into the mitochondria for the tricarboxylic acid cycle (TCA cycle) (Gillies et al., 2008). To test this possibility, we assayed the phosphorylation marker of deactivated PDH, S293, and showed that HG had no effect on PDH phosphorylation (Figure S2.1H). Interestingly, HG treatment did not significantly induce lactate production (Figure 2.1E), basal mitochondrial respiration (Figure 2.1F), or mitochondrial cellular ATP production (Figure 2.1G, Figure S2.1I). Given that glucose can be diverted into multiple metabolic pathways, we were not entirely surprised with this outcome. Our studies show that, although not statistically significant, HG-treated cells display more lactate production over time, suggesting that either (a) multiple pathways may be active in this cell line, preventing us from observing significant changes in either ATP-producing pathway (i.e., lactate production or mitochondrial OXPH) or (b) U2OS cells are further utilizing lactate carbon skeletons for downstream catabolic pathways. The latter speculation is supported by recent work suggesting there exists an intracellular lactate shuttle where lactate formed during glycolysis can be continuously used as an energy source within the same cell, implying the continuous formation and oxidation of lactate can occur (Kane et al., 2014). Nevertheless, while the source of ATP remains to be fully understood, altogether

these results suggest increased glucose metabolism contributes to the observed induction of cellular ATP level upon HG treatment.

TAF1 Kinase Activity is Responsible for HG-Induced p53 and p21 Inhibition

Our previous work has elucidated that Thr55 phosphorylation by TAF1 kinase leads to p53 inactivation (Li et al., 2004; Cai and Liu, 2008). More recently, we discovered that TAF1 kinase activity is cellular ATP level dependent (Wu et al., 2014). We speculated that perhaps HG-induced cellular ATP activates TAF1 kinase activity, hence the observed Thr55 phosphorylation and p53 down-regulation. To test this, we treated cells with HG with or without apigenin, a TAF1 kinase inhibitor, and assayed for Thr55 phosphorylation and p21 protein levels. As shown in Figure 2.2A, treatment with apigenin successfully blocked Thr55 phosphorylation and subsequent p21 protein inhibition. While apigenin has been shown to inhibit TAF1 kinase activity both *in vivo* and *in vitro* (Li et al., 2004), apigenin may also have nonspecific effects that affect Thr55 phosphorylation. To exclude this possibility, we also knocked down TAF1 using small interfering RNA (siRNA) and showed inhibition of Thr55-p and subsequent rescue of p21 protein levels upon HG treatment (Figure 2.2B). Importantly, when wild-type (WT) TAF1 was re-introduced, to re-express the fully functional protein, we once again observed the HG-effect on p53 inhibition, however, when we reintroduced a dominant-negative TAF1 kinase-dead mutant (KD(A2)), rescue of p53 inhibition was no longer observed. To prove the role of

TAF1 in Thr55 phosphorylation directly, we assayed HG-induced cellular ATP effect on TAF1 kinase activity in an *in vitro* phosphorylation reaction using TAF1 immunoprecipitated from cells as kinase and baculovirus expressed and purified p53 as substrate. As shown in Figure 2.2C, TAF1 immunoprecipitated from mock-treated U2OS cells display a low but specific autophosphorylation, as well as p53 Thr55 phosphorylation. Interestingly, when TAF1 was purified from HG-treated cells, both TAF1 autophosphorylation and p53 Thr55 phosphorylation were significantly enhanced. Importantly, treating cells with either 2-DG or apigenin blocked enhanced TAF1 kinase activity. Additionally, treating cells with 2-DG successfully blocked both HG-induced TAF1 autophosphorylation and p53 phosphorylation in a time-dependent manner, further establishing the importance of glucose metabolism in TAF1 kinase activation (Figure S2.2A).

It is known that under certain circumstances p21 induction can be a p53-independent event. For example, in the colorectal cancer cell line HCT116, it has been shown antioxidants can induce p21 expression via the CCAAT/enhancer binding protein family of transcription factors in a p53-independent manner (Chinery et al., 1997). This prompted us to investigate whether the observed p21 inhibition by HG was a p53-dependent event (Figure S2.2B). This assay showed p21 inhibition by HG is p53-dependent as HG treatment in p53 null cells failed to show inhibition of p21. To further validate this, we showed p53 knockdown eliminated the HG effect on p21 expression, confirming p53 is necessary for the observed p21 inhibition (Figure S2.2C).

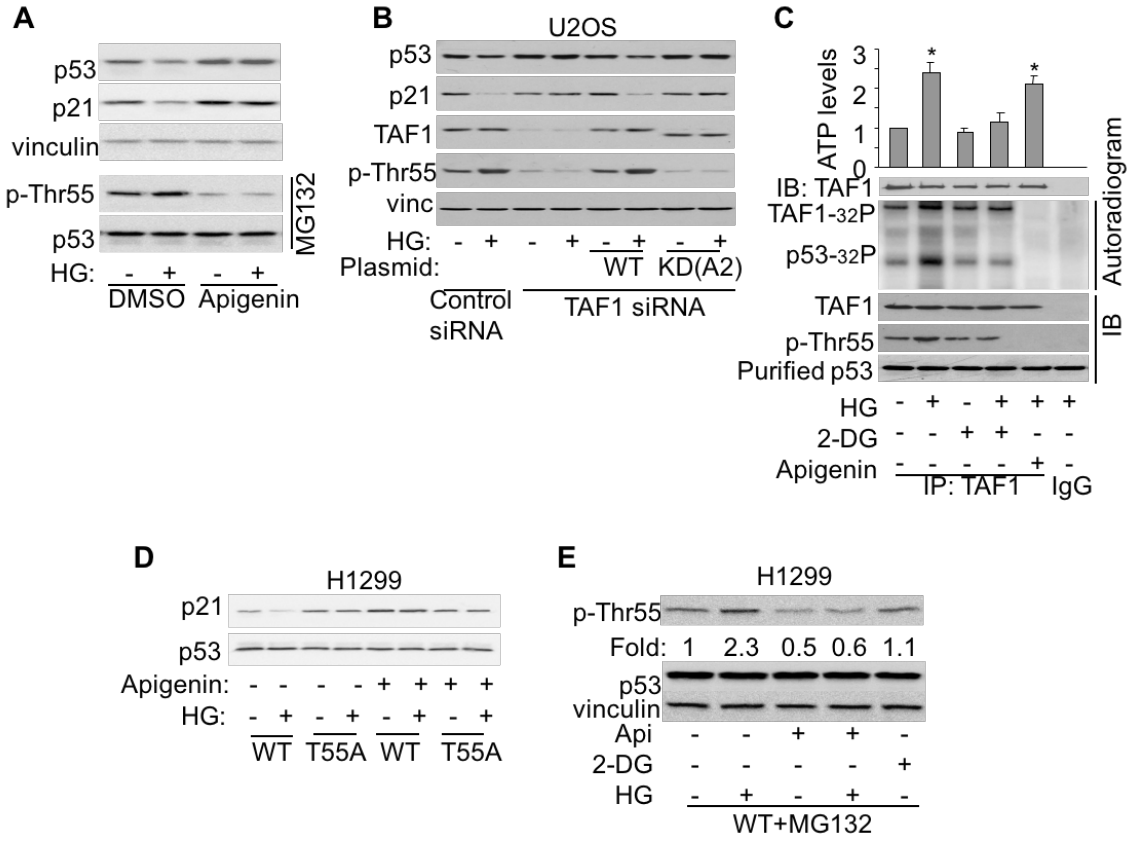
A
p53
p21
vinculin
p-Thr55
p53
MG132
HG: - + - +
DMSO Apigenin
B
U2OS
p53
p21
TAF1
p-Thr55
vinc
HG: - + - + - + - +
Plasmid: WT KD(A2)
Control siRNA
TAF1 siRNA
C
ATP levels
3
2
1
0
*
*
IB: TAF1
TAF1-32P
p53-32P
Autoradiogram
TAF1
p-Thr55
Purified p53
IB
HG - + - + + +
2-DG - - + + - -
Apigenin - - - - + -
IP: TAF1 IgG
D
H1299
p21
p53
Apigenin: - - - - + + + +
HG: - + - + - + - +
WT T55A WT T55A
E
H1299
p-Thr55
Fold: 1 2.3 0.5 0.6 1.1
p53
vinculin
Api - - + + -
2-DG - - - - +
HG - + - + -
WT+MG132

Figure 2.2. TAF1 kinase activity is responsible for HG-induced p53 and p21 inhibition. (A) U2OS cells were incubated with HG for 6 h in the presence of dimethylsulfoxide (DMSO) or apigenin (40 μM). Aliquots of cell lysates were subjected to p53, p21 or vinculin protein detection. Thr55 phosphorylation was assayed using the phosphospecific antibody as described in Method. (B) U2OS cells treated with individual TAF1–specific siRNA or control siRNA were then transfected with or without wild-type TAF1 construct (WT) or kinase-dead mutant (A2) as indicated. The transfected U2OS cells were then treated with or without high glucose (25 mM) for 6 h. Aliquots of cell lysates were subjected to p53, p21 or TAF1 protein detection. Thr55 phosphorylation was assayed using phosphospecific antibody as described in Method. The blot is a representative of 3 separate experiments. Note that the transfected A2 migrated faster because of the TAF1 CTK truncation. (C) U2OS cells were treated with HG for 6 h in the presence or absence of 2-DG and apigenin, and TAF1 was immunoprecipitated using Ab1230 antibody. In vitro TAF1 kinase assay was performed using immunoprecipitated TAF1 along with baculovirus-expressed and purified p53. TAF1 and p53 phosphorylation were detected by either autoradiography or immunoblotting as indicated. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (D) H1299 cells transfected with wild-type p53 (WT) or T55A were treated with HG or apigenin alone or their combinations. p21 and p53 protein levels were analyzed by Western blotting. (E) H1299 cells were transfected with wild-type p53 (WT) and treated with HG in the presence and absence of 2-DG and apigenin. To normalize the p53 protein level, cells were treated with MG132 before harvest. Thr55 phosphorylation was assayed using the phosphospecific antibody as described in Method.

To further confirm the dependence of p53 Thr55 phosphorylation on p53 and p21 inhibition, we compared the effect of HG on WT p53 and the Thr55 phosphorylation mutant T55A with or without apigenin (Figure 2.2D). In the presence of the WT, we clearly observed p21 inhibition with HG treatment, however, when we overexpressed the T55A mutant, p21 protein levels were rescued. Importantly, treatment with apigenin successfully blocked both p53 and p21 protein reduction even in the presence of overexpressed WT *p53*. Thr55 phosphorylation status of transiently overexpressed WT *p53* was verified in the presence of HG (Figure 2.2E). Indeed, our data suggests TAF1 kinase-dependent Thr55 phosphorylation is essential for the HG-induced p21 inhibition.

Given that AMP-activated protein kinase (AMPK) senses changes in cellular energy levels (Hardie, 2007), it's possible AMPK may phosphorylate and activate TAF1, leading to Thr55 phosphorylation. To exclude this possibility, we showed that treating cells with the AMPK inhibitor Compound C (Zhou et al., 2001), or with the AMPK activator AICAR (Narkar et al., 2008) did not affect Thr55 phosphorylation, suggesting Thr55 phosphorylation is independent of AMPK activity (Figure S2.2D). Together these results indicate that TAF1 kinase activity is responsible for HG-induced p53 and p21 inhibition.

HG Abrogates p53 DNA Binding in an ATP/TAF1/Thr55-p Dependent Manner

Next, we investigated whether HG-induced Thr55 phosphorylation leads to dissociation of p53 from the p21 promoter and inhibition of p53 transcriptional activity. As shown in Figure 2.3A, HG treatment significantly reduced p21 transcript levels. To confirm whether p21 transcript inhibition was p53-dependent, we assayed binding of p53 to both p53-binding sites (3' and 5') on the p21 promoter (Figure 2.3B). The assay shows HG treatment reduced both p53 and TAF1 binding to the p21 promoter, further supporting our hypothesis. To firmly establish the role of Thr55 phosphorylation in the dissociation of p53 from the promoter, we compared binding of WT p53 and T55A on the p21 promoter upon HG treatment (Figure 2.3C). Our results clearly indicate that, under HG treatment where cellular ATP levels are significantly induced, p53 binding is abrogated, whereas binding of T55A remained unchanged. Importantly, both 2-DG and apigenin blocked the effect of HG on p53 promoter binding and p21 transcription, suggesting glucose metabolism/TAF1 kinase-dependent Thr55 phosphorylation increase. Together, these results support the hypothesis that HG increases ATP levels and this, in turn, increases TAF1-mediated p53 Thr55 phosphorylation on the p21 promoter, which results in dissociation of p53 from the promoter.

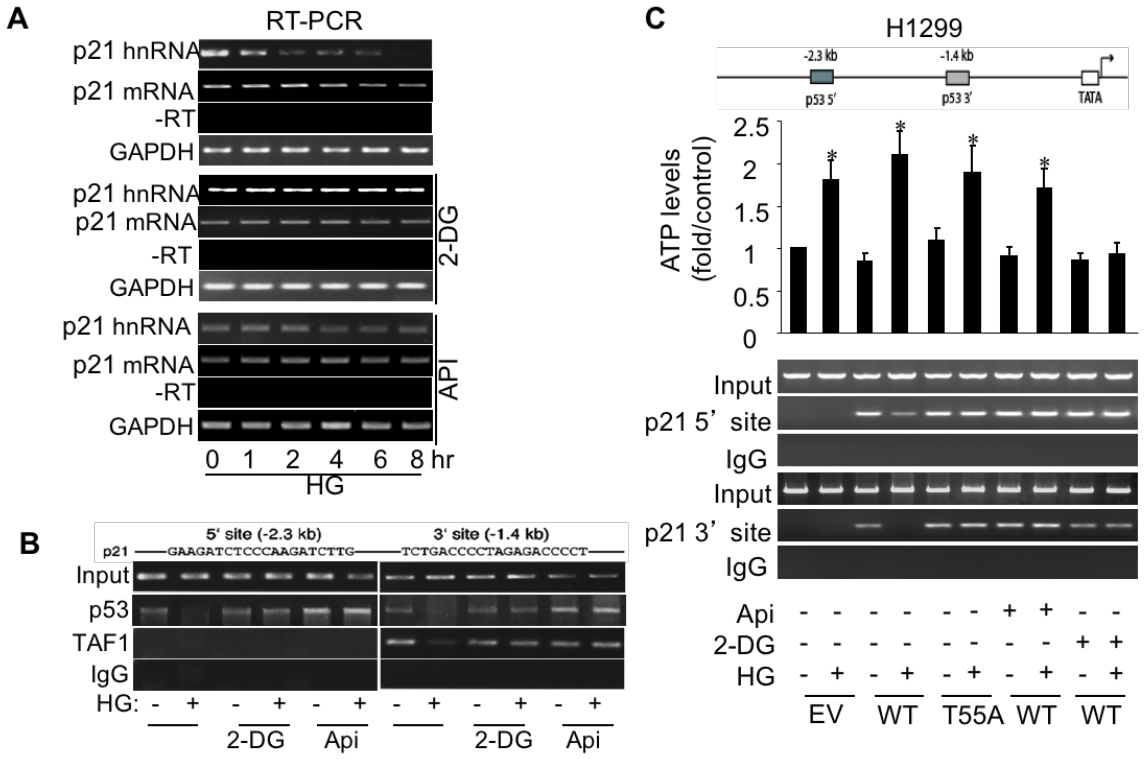

Figure 2.3. HG abrogates p53 DNA binding in an ATP/TAF1/Thr55-p regulated manner. (A) U2OS cells were treated with high glucose (25 mM), UV irradiation (20J/m^2), or apigenin as indicated. Cells were harvested and total RNA was extracted. The level of p21 hnRNA and mRNA was analyzed by RT-PCR. (B) U2OS cells were treated with high glucose (25 mM) in the presence or absence of 2-DG or apigenin. Protein extracts were prepared and subjected to ChIP assays using the indicated antibodies. PCR was performed to test for enrichment of the indicated p21 promoter regions. PCR products were resolved by agarose-gel electrophoresis and visualized by ethidium bromide staining. (C) H1299 cells were transfected with either empty vector (EV) wild-type p53 (WT) or T55A and treated with HG, 2-DG, apigenin and their combinations. Protein extracts were prepared from H1299 with different treatments and subjected to ChIP assays using anti-p53 antibody. PCR was performed to test for enrichment of the indicated p21 promoter regions. PCR products were resolved by agarose-gel electrophoresis and visualized by ethidium bromide staining. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$).

HG Inhibits p53 Activation Upon DNA Damage

DNA damage stress is known to induce PTMs that are essential for p53 transcription activation. Under DNA damage conditions Ser15 phosphorylation reduces the interaction between p53 and its negative regulator MDM2 (Bode and Dong, 2004), leading to p53 stability. Similarly, acetylation regulates the interaction of p53 with coactivators and corepressors and affects its transcription activity (Knights et al., 2006). First, we decided to investigate what effect (if any) does HG treatment have on p53 stabilization upon DNA damage (Figure 2.4A). Our results show that HG has no effect on several PTMs that have been shown to stabilize p53 (i.e., p-Ser15, p-Ser37, and Ac-Lys373). Interestingly, we observed a clear induction of Thr55 phosphorylation with HG treatment under DNA damage conditions, and more importantly, apigenin treatment blocked this effect. These data suggest PTMs that induce stabilization of p53 have no effect on HG-induced Thr55 phosphorylation. To exclude the possibility that HG inhibits p53 in a UV specific manner, we also treated cells with bleomycin, a DNA-damaging alkylating agent that generates double-strand DNA breaks (DSBs) (Dong et al., 2010) (Figure S2.3A-B). The assay shows significant increased Thr55 phosphorylation and reduced p53 protein levels in the presence of bleomycin, indicating that the effect of HG is not specific to UV DNA damage.

The observation that Thr55 phosphorylation was also induced by HG treatment in the presence of DNA damage prompt us to investigate whether

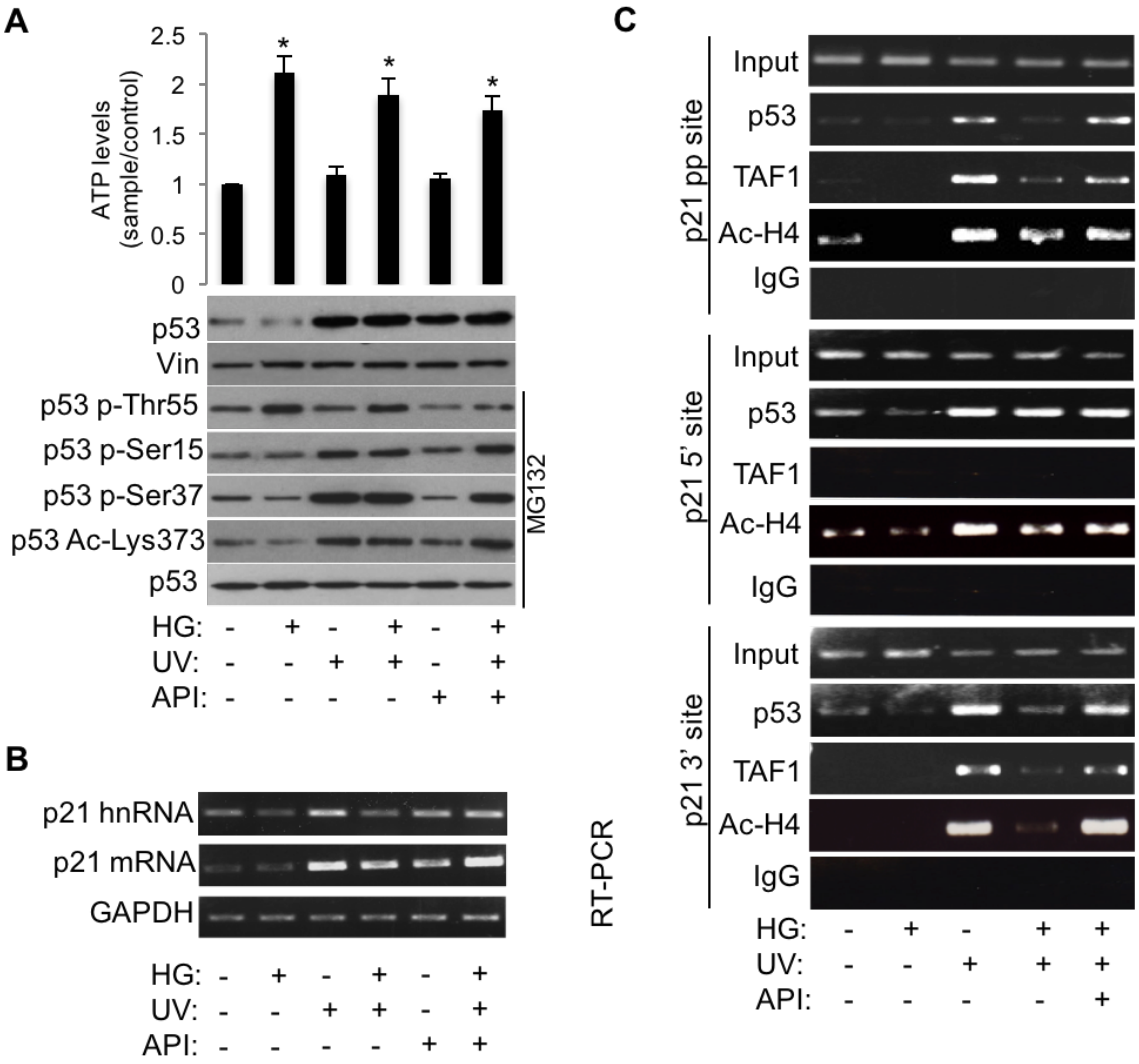
A
ATP levels (sample/control)
2.5
2
1.5
1
0.5
0
*
*
*
p53
Vin
p53 p-Thr55
p53 p-Ser15
p53 p-Ser37
p53 Ac-Lys373
p53
MG132
HG: - + - + - +
UV: - - + + - +
API: - - - - + +
B
p21 hnRNA
p21 mRNA
GAPDH
HG: - + - + - +
UV: - - + + - +
API: - - - - + +
C
RT-PCR
p21 pp site
Input
p53
TAF1
Ac-H4
IgG
p21 5' site
Input
p53
TAF1
Ac-H4
IgG
p21 3' site
Input
p53
TAF1
Ac-H4
IgG
HG: - + - + +
UV: - - + + +
API: - - - - +

Figure 2.4. HG inhibits p53 stress response in the presence of DNA damage. (A-C) U2OS cells were treated with high glucose (25 mM), UV irradiation (20J/m^2), or apigenin as indicated. (A) Cell lysates underwent SDS-PAGE followed by Western blotting with various primary antibodies as indicated and visualized by the enzyme-linked chemiluminescence system. To normalize the p53 protein level, cells were treated with MG132 before harvest. Phosphorylated p53-Ser15, Ser37, and K373 were detected by Western blotting. Thr55 phosphorylation was assayed using the phosphospecific antibody as described in Method. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (B) Cells were harvested, and total RNA was extracted. The level of p21 hnRNA, mRNA and GAPDH mRNA was analyzed by RT-PCR. (C) Protein extracts were prepared and subjected to ChIP assays using the indicated antibodies. PCR was performed to test for enrichment of the indicated p21 promoter regions. PCR products were resolved by agarose-gel electrophoresis and visualized by ethidium bromide staining.

Thr55 phosphorylation is sufficient to inhibit p53 activation upon stress. As shown in Figure 2.4B, p21 RNA levels, which clearly increased upon DNA damage, were inhibited upon HG treatment. To ensure the observed inhibition on p21 transcription was due to p53 dissociation from the p21 promoter we used a ChIP assay to analyze whether HG affects p53 DNA binding in response to DNA damage (Figure 2.4C). As expected, DNA damage significantly increased p53 binding to both p53-binding sites (3' and 5') and proximal promoter site (pp site) on the p21 promoter. Importantly, HG reduced DNA damage-induced p53 binding to the p21 promoter. To further verify this, we also showed that HG inhibits DNA damage-induced p53 binding on the p21 promoter in HCT116 cells (Figure S2.3C). More intriguingly, TAF1 ChIP showed that TAF1 binding to the p21 pp and 3' site was also reduced by HG treatment upon DNA damage. This finding provides additional evidence that TAF1 is involved in the HG-dependent p53 dissociation from the p21 promoter. Together, these data suggest that HG-induced Thr55 phosphorylation is sufficient to abolish p53 DNA damage response.

Genome-Wide Analysis Reveals Global Inhibition of p53 DNA Binding by HG in Response to DNA Damage

To investigate the effect of HG on overall p53 DNA binding in response to DNA damage, we carried out a genome-wide analysis using a ChIP-seq approach to assess global p53 occupancy. Upon DNA damage, our analysis

identified 1722 chromatin regions with significant p53 enrichments above the background. Upon HG, however, only 1073 chromatin regions were detected with DNA damage (Figure S2.4A).

Compared to the untreated control (mock, M) from our previous analysis (Wu et al., 2014), we identified 700 regions out of the 1,722 with significant enriched p53 occupancy. Interestingly, out of those 700 enriched occupancy regions, 286 displayed significantly reduced occupancy in the presence of HG treatment, suggesting HG inhibits a widespread of p53 chromatin binding sites (Figure 2.5A). Consistent with previous results, the most affected motif identified at sites bound by p53 was the well-characterized p53-binding motif (Figure S2.4B).

To verify our ChIP-seq results we assayed p53 binding on seven p53-activated genes using individual ChIP assay, six (*FADS2, GPX1, GADD45A, XPC, DHRS3, and TRAF4*) of which displayed inhibition of p53 binding by HG, and one (*LGALS7/PIG1*) of which exhibited no significant inhibition by HG (Figure 2.5B). Our results showed similar trends between ChIP-seq and individual ChIP for all seven analyzed gene promoters tested, validating our ChIP-seq and bioinformatics analysis.

To assess the role of glucose metabolism and Thr55 phosphorylation in global inhibition of p53-chromatin binding we assayed global p53 chromatin occupancy and Thr55-p status in the presence of HG (Figure S2.4C). This assay supports HG treatment significantly inhibits p53 DNA binding in response to DNA

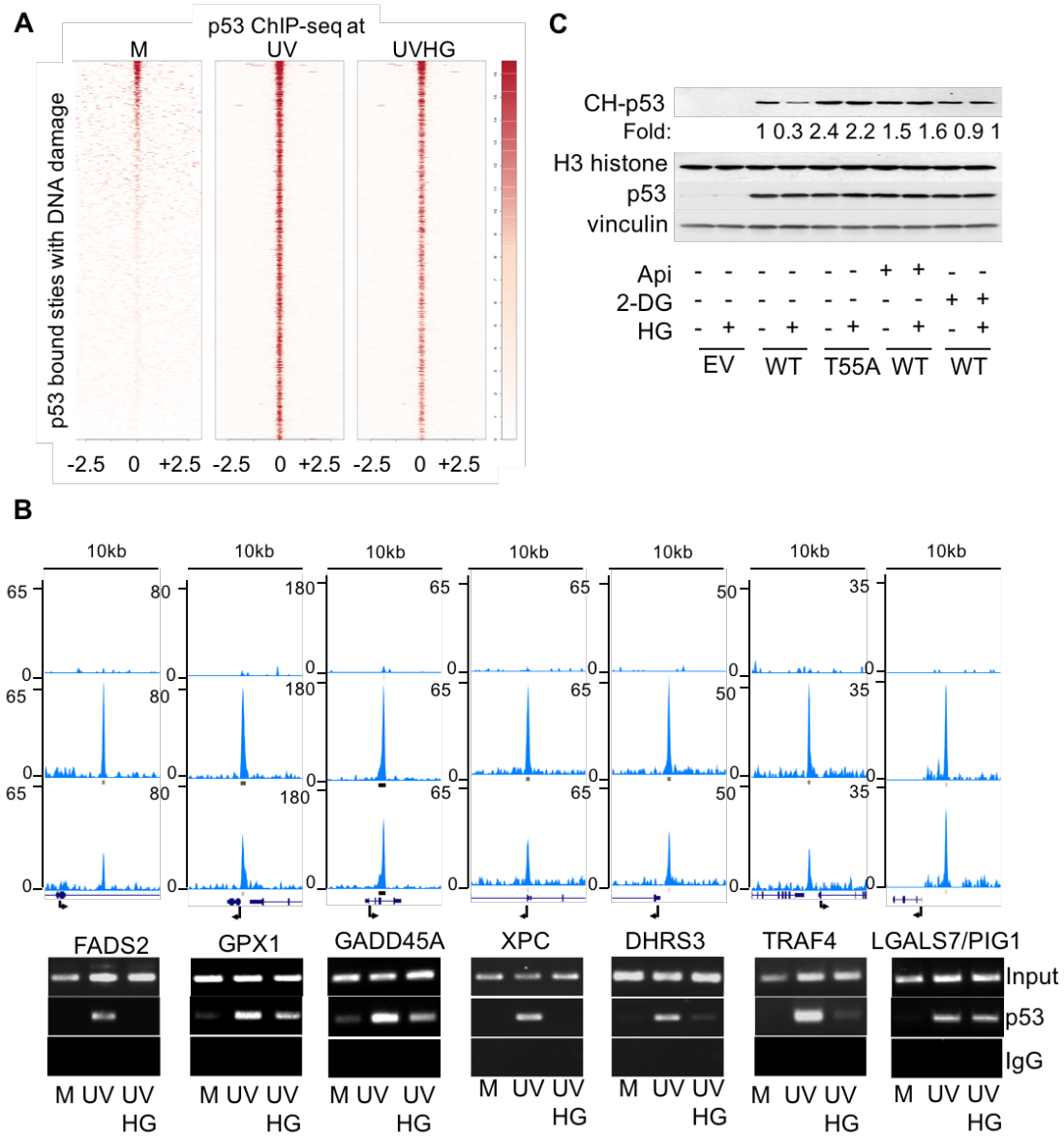
A
p53 ChIP-seq at
M
UV
UVHG
p53 bound sties with DNA damage
-2.5 0 +2.5 -2.5 0 +2.5 -2.5 0 +2.5
C
CH-p53
Fold:
1 0.3 2.4 2.2 1.5 1.6 0.9 1
H3 histone
p53
vinculin
Api
2-DG
HG
EV
WT
T55A
WT
WT
B
10kb
FADS2
GPX1
GADD45A
XPC
DHRS3
TRAF4
LGALS7/PIG1
Input
p53
IgG
M UV UV HG

Figure 2.5. Genome-wide analysis reveals global p53 inhibition by HG from its target promoters in DNA damage response. (A) Heatmaps showing p53 ChIP-seq levels of 5 kb regions centered on the summit of the peaks at mock-treated cells (M) (left), UV-irradiation (20J/m^2) (UV) (middle), and UV-irradiation (20J/m^2) with 25mM D-glucose treatment (UVHG) (right). The heatmap is ranked according to the enrichment of p53 occupancy after DNA damage. (Red represents enriched; white represents not enriched). (B) Individual gene tracks of p53 binding at the FADS2, GPX1, GADD45A, XPC, DHRS3, TRAF4, and LGALS7/PIG1 promoters at M (top), HG (middle), and UVHG (bottom). The x-axis shows genomic position. The y-axis shows signal per million reads for fragment pileup profiles generated by MACS2. Verification of p53 binding on the corresponding genes by individual ChIP was shown below. (C) U2OS cells treated with HG in the presence or absence of 2-DG or apigenin were fractionated to chromatin-bound and unbound fractions and analyzed for p53 and histone H3 protein levels by immunoblotting of chromatin-bound fraction.

damage, and more importantly, this effect was blocked by 2-DG. To prove that the effect of HG on p53 global DNA binding is Thr55-p dependent, we compared WT p53 and T55A chromatin occupancy in the presence of HG (Figure 2.5C). This assay shows that unlike WT p53, T55A mutant failed to dissociate from DNA when HG was present. Importantly, treatment with 2-DG or apigenin blocked HG-induced global dissociation of p53 from chromatin. Those results further confirm that HG-induced global dissociation of p53 from chromatin in the event of DNA damage is Thr55 phosphorylation dependent.

Annotation analysis of p53 enriched regions revealed 144 characterized RefSeq genes after DNA damage, and 62 of them showed reduced p53 occupancy in the presence of HG (Figure S2.4A). Strikingly, functional analysis of those HG-inhibited chromatin regions revealed inhibition of p53-dependent cellular pathways, including response to DNA damage, cell cycle regulation, apoptosis, etc. (Figure S2.4D). Altogether, our data indicates that HG globally inhibits p53 DNA damage response via ATP induced TAF1 phosphorylation on Thr55.

HG Acts as a Tumor Promoter by Hindering p53-Dependent Growth Arrest and Apoptosis.

Given that our results indicated HG inhibits p53 chromatin binding we decided to investigate the HG effect on p53 transcriptional regulation. We carried out RT-PCR analysis in the presence of DNA damage with or without HG

treatment for two additional p53 targets (*GPX1*, and *TRAF4*) on which HG affected p53 binding (Figure 2.6A). Similar to p21, both transcript levels increased with DNA damage and were inhibited by HG.

Our finding that HG can affect p53 transcriptional activity prompted us to investigate the biological significance of the HG effect. It is well known that overexpression of p53 inhibits cell proliferation. We first employed a cell proliferation assay to evaluate the effect of HG on p53-dependent inhibition of cell proliferation. As shown in Figure 2.6B, overexpression of WT p53 led to approximately 40% decrease in cell growth compared to the control, however, in the presence of HG, cell growth only decreased by approximately 20%. Importantly, when we overexpressed p53 T55A mutant, a similar level of cell growth inhibition was observed compared to the control, however, HG treatment failed to reduce p53-dependent growth inhibition, once again providing additional evidence that Thr55 phosphorylation is essential for HG inhibition of p53 function.

We also investigated the effect of HG on the cell's ability to undergo apoptosis in the presence of DNA damage. As shown in Figure 2.6C, HG treatment caused a 40% reduction in cells undergoing apoptosis compared to control (5 mM glucose). Importantly, treating cells with apigenin abolished the effect of HG on apoptosis, providing additional evidence for the involvement of TAF1 kinase activity.

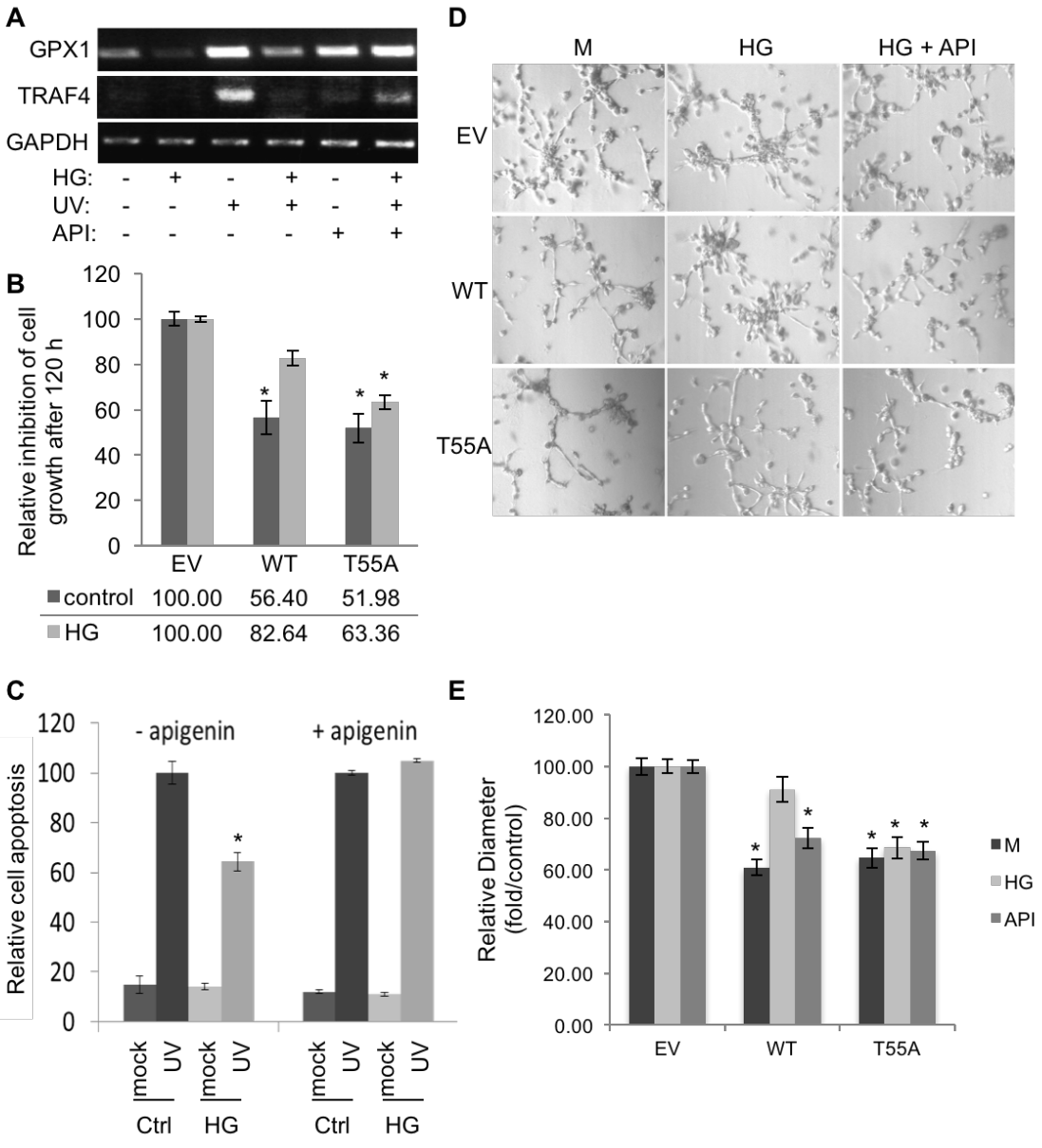
A
GPX1
TRAF4
GAPDH
HG: - + - + - +
UV: - - + + - +
API: - - - - + +
B
Relative inhibition of cell growth after 120 h
120
100
80
60
40
20
0
EV
WT
T55A
control 100.00 56.40 51.98
HG 100.00 82.64 63.36
C
Relative cell apoptosis
120
100
80
60
40
20
0
- apigenin
+ apigenin
mock
UV
Ctrl
HG
D
M
HG
HG + API
EV
WT
T55A
E
Relative Diameter (fold/control)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
EV
WT
T55A
M
HG
API

Figure 2.6. HG acts as a tumor promoter by hindering p53-dependent cellular stress response. (A) U2OS cells were treated with high glucose (25 mM), UV irradiation ($20J/m^2$), or apigenin as indicated. Cells were harvested, and total RNA was extracted. The level of GPX1, TRAF4, and GAPDH mRNA was analyzed by RT-PCR. (B) HCT116 $p53^{-/-}$ cells were transfected with *WT*-p53, T55A, or a control cytomegalovirus empty vector, seeded in triplicate and counted at 120h post seeding. For HG, glucose was added to the media, so the glucose concentration is 25 mM. The presence of overexpressed p53 protein at the analysis was verified by immunoblotting. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (C) U2OS untreated or treated with 25 mM D-glucose, UV-irradiated ($20\ J/m^2$) for 30 h, or apigenin (20 μM) 30 min prior to any other treatment as indicated. Cells were fixed in 70% ethanol and stained with propidium iodine. Sub-G1 percentage of cells was analyzed by FACs. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (D) 3D/Matrigel cell culture with *WT-p53* or Thr55A overexpression, with or without HG treatment or apigenin as indicated. (E) Graphical representation of colony size (diameter) formation for 3D/Matrigel cell culture for each condition indicated. Diameters were quantified from over 70 colonies from each condition from triplicate experiments using image analysis software (*$p < 0.05$).

To provide more evidence, we assayed 3D/matrigel cell cultures as this system can better mimic the natural microenvironment for tumor growth analysis. As shown in Figure 2.6D-E, overexpression of WT p53 clearly led to reduced colony growth, however, HG significantly reduced the p53 effect. Interestingly, when we overexpressed the T55A mutant, HG failed to inhibit p53's ability to lessen colony growth. Altogether, our results implicate HG as a tumor promoter via inhibiting p53-dependent cellular stress response.

2.3 Discussion

The findings described above suggest a novel model for p53 regulation, i.e. to regulate p53 activity via glucose availability, a nutrient that is essential to all living cells, normal and cancerous alike. Current view on cancer metabolism tends to focus on the role of tumor suppressors and oncogenes in metabolic regulation. However, the effect that altered metabolites have on tumor suppressors and both cancer initiation and progression has yet to be explored. Not surprisingly, nutrients have already been shown to modify the epigenome via metabolic intermediates such as acetyl-CoA, S-adenosylmethionine, nicotinamide adenine dinucleotide (NAD+), and α-ketoglutarate (α-KG) (Katada et al., 2012). This suggests that nutrients can alter gene expression via metabolite abundance.

In this study, we expand on the role metabolites play in the regulation of gene expression. We show that excess glucose results in elevated cellular ATP levels, which inhibits p53 activity by altering TAF1 kinase activity. TAF1, a

transcription factor and component of the general transcription machinery (Thomas and Chiang, 2006), is a cell cycle regulatory protein involved in G1 progression (Sekiguchi et al., 1991; Wang and Tjian, 1994; Li et al., 2004). Perhaps in normal cells, under a nutrient-rich environment, enhanced cellular ATP serves as a signal for cell growth via induction of TAF1 kinase activity and consequent inhibition of p53. Conversely, under a nutrient-poor environment, where both ATP levels and TAF1 kinase activity remain low, more p53-dependent transcription can occur. Indeed, activation of p53 has been shown to be critical for cell survival following glucose depletion (Jones et al., 2005), supporting the notion that in a poor nutrient environment lack of p53 Thr55 phosphorylation may lead to p53 activation.

Altered metabolism is a common disorder in a variety of diseases, including both diabetes and cancer. Interestingly, diabetes is associated with several types of cancer incidence and mortality (Giovannucci et al., 2010; Vigneri et al., 2009). The diabetes-cancer relationship is complicated given that both diseases are characterized by several metabolic alterations. While more than one common metabolic change (i.e., hyperglycemia, hyperinsulemia, obesity) are likely involved in the diabetes-cancer connection, our study implicates hyperglycemia as a molecular link for increased cancer risk in a nutritional oversupplied environment (Figure 2.7).

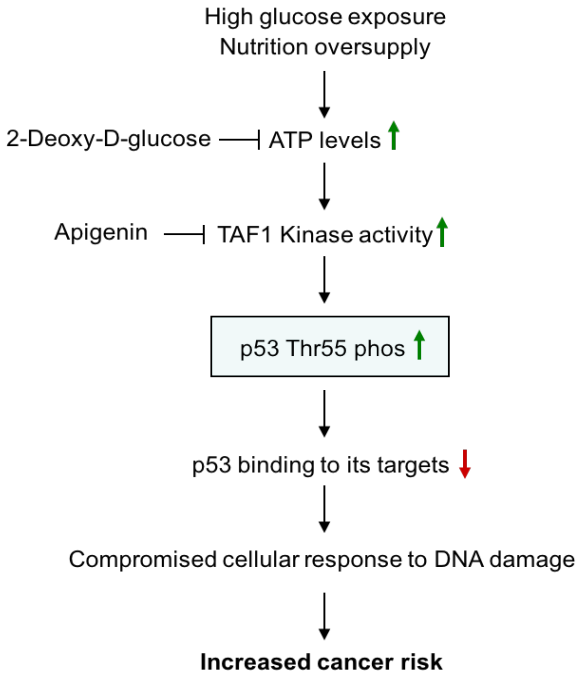

Figure 2.7. A proposed model for increased cancer risk by high glucose exposure.

Whereas in normal cells, it is possible that TAF1 kinase activity acts as a sensor of nutrient sufficiency for growth, in cells where cellular ATP can continuously fluctuate above basal levels due to hyperglycemia, TAF1 kinase may act as an oncogene by continuously promoting growth.

Apigenin has also been shown to possess anti-inflammatory, antioxidant and anticancer properties (Shukla and Gupta, 2010). Interestingly, apigenin anti-cancer properties have been correlated with protection against breast, digestive tract, prostate and hepatological malignancies—cancers that also happen to be associated with diabetes. Furthermore, some reports suggest that apigenin's protective effect involves stabilization of p53 and p21 and consequent cell cycle

arrest—making the case that this effect may be due to inhibition of TAF1 kinase activity.

Our results also suggest that increased cellular ATP is sufficient to inhibit p53 DNA damage response under HG conditions. Indeed, our ChIP-seq revealed that HG treatment inhibits p53 binding to stress response transcriptional targets (such as *p21* and *GADD45A, TRAF4,* and *XPC)* suggesting that under HG conditions, cellular response to DNA damage may be compromised. In addition, our ChIP-seq analysis also revealed that HG inhibits p53 promoter binding to antioxidant genes (such as *GPX1* and *DHRS3*) suggesting that HG treatment may also inhibit cellular response to reactive oxygen species (ROS). Alarmingly, if HG stimulates more cellular metabolism, in turn, it may also promote more ROS. ROS are known to be potent cancer promoters comprising of H_2O_2, superoxide O2-, and hydroxyl radical OH- (Finkel, 2011). This suggests that through inhibition of p53 activity, HG may promote the mutagenic effect of ROS via inhibition of antioxidants. Lastly, our ChIP-seq results revealed HG inhibits p53 promoter binding to metabolic genes (such as *FADS2*). Evidence to date supports the concept that cellular metabolism is reprogrammed in transformed cells to meet the bioenergetic and biosynthetic needs of a proliferating cell. The resulting high rate of glycolytic metabolism leads to the conversion of mitochondria into synthetic organelles that support glucose-dependent lipid synthesis and non-essential amino acid production (Bauer et al., 2005; Lum et al., 2007). Indeed, the loss of p53 in tumors can enhance glycolysis and anabolic

synthesis from glycolytic intermediates (Bensaad et al., 2006; Kondoh et al., 2005; Matoba et al., 2006). These observations, in addition to the evidence reported here, supports the notion that p53 inhibition is necessary for HG tumor promoter activity.

Indisputably, our results indicate that HG plays a role in tumor promotion, but not initiation as it is well established that mammalian cells acquisition of nutrients is tightly regulated by growth factors (Palm et al., 2017). Cell-extrinsic regulation of nutrient consumption constitutes a fundamental barrier to cellular transformation. This implies HG alone may not be sufficient to induce proliferation in normal cells. We speculate that perhaps under a more physiological environment, other diabetic alterations work in conjunction with HG to promote tumor initiation. Actually, one of the markers of diabetes is insulin insensitivity, which can stimulate the production of both insulin and insulin-like growth factor-1 (IGF-1) from pancreatic β cells (Khandekar et al., 2011). Interestingly, recent studies suggest that hyperinsulinemia can enhance IR/IGF-IR and Akt phosphorylation in mammary tissue and promote mammary tumor growth (Novosyadlyy et al., 2010). This suggests that hyperglycemia (which inhibits p53 activity) and IGF-1 secretion may work together to promote proliferation and, in this manner, hyperglycemia may act as a tumor initiator.

Interestingly, in a mutational landscape across twelve major cancer types report by the Cancer Genome Atlas (TCGA) Pan-Cancer effort, TAF1 was one of the most significantly mutated genes with a mutation rate of 2.3%, which is

comparable to the mutation rate of several well-established tumor suppressors and oncogenes (such as *ATM*, *ATR, BRCA1*, *BRCA2*) suggesting that TAF1's involvement in carcinogenesis may be significant (Kandoth et al., 2013). It is tempting to hypothesize that, similar to regulating p53, TAF1 may also regulate other transcription factors to promote growth. It will be interesting to further identify other TAF1 kinase targets to reveal a better picture of TAF1 as a nutrition sensor in carcinogenesis.

2.4 Methods

Reagents and Western Blot Analysis

For DNA damage, U2OS and HCT116 $p53^{-/-}$ cells were subjected to UV radiation (20 J/m^2) or 30 mg/ml bleomycin (Sigma). For inhibition of TAF1, U2OS cells were treated with 40 uM apigenin (Sigma). Antibodies used in western blot analysis were anti-p53 (DO-1, Santa Cruz), anti-phospho-Ser15 (9284, Cell Signaling Technology), anti-phospho-Ser20 (9287, Cell Signaling Technology), anti-phospho-Ser37 (9289, Cell Signaling Technology), anti-phospho-Ser46 (2521, Cell Signaling Technology), anti-acetyl-Lys373 (Upstate), anti-p21 (C-19; Santa Cruz), anti-TAF1 (Ab1230), anti-TAF4 (Ab4A6, a gift of Dr. E. Wang), anti-TAF5 (a gift of Dr. R.G. Roeder), anti-TBP (SI-1, Santa Cruz), anti-TAF9 (TAF II p32 (C-19), Santa Cruz), anti-Thr55-Phos (Ab202), anti-vinculin (VIN-11-5, Sigma), anti-AMPK (Ab23A3, Cell Signaling Technology), anti-phospho-AMPK (40H9, Cell Signaling Technology), anti-AKT (9272, Cell Signaling Technology),

anti-phospho-Ser473-AKT (9271, Cell Signaling Technology), anti-phospho-Ser166-MDM2 (3521, Cell Signaling Technology) and anti-histone H3 (Ab24834, Abcam) antibodies.

Cellular ATP Level and ATP/ADP Ratio

ATP level was measured by the luciferin/luciferase method using the ATP Determination Kit according to manufacturer's protocol (Molecular Probes). Cellular ATP concentration was determined by comparing to a standard dilution curve of fresh ATP (Cell Signaling Technologies). The cell volume of 4 pl was used for calculation of cellular ATP concentration in U2OS cells, as reported in Beck et al. (2011). For measuring total ATP+ADP, ADP was first converted into ATP by mixing 2.3 units pyruvate kinase (Lee Biosolutions) to 100 ml cell lysate and incubating at room temperature for 15 min. ADP levels were then calculated by subtracting ATP from the total ATP+ADP. A standard curve was generated from known concentrations of ATP and ADP in each experiment and used to calculate the concentration of ATP and ADP in each sample. The results were expressed as fold (means ± SE) over untreated cells (mock) from 3 independent experiments.

Bioprofile Plus 100 Metabolite and Doubling Time Measurements

Glucose consumption and lactate production were measured using a Bioprofile Plus 100 metabolite analyzer by Novus biological. Measurements were

conducted over the time period indicated and normalized to cell number area under the curve, as previously described (Lee et al., 2014). Briefly, metabolite consumption was defined as $v = V(x_{(medium)\ control} - x_{(final)})/A$, where v is metabolite consumption/ production, V is culture volume, x is metabolite concentration, and A is cell number area under the curve. A was calculated as $N(T)d/\ln 2(1 - 2^{-T/d})$, where N(T) is the final cell count, d is doubling time, and T is the time of experiment. Doubling time was calculated as $d = (T)[\log(2)/\log(Q2/Q1)]$, where Q1 is starting cell number, and Q2 is final cell number, as determined by manual counting using a hemocytometer.

ChIP Analysis

ChIP analysis was carried out as described previously (Li et al., 2007a). For U2OS and HCT116 *p53*$^{+/+}$ cells, nuclear extracts were collected at indicated time points after mock or 20 J/m^2 UVC treatment and sonicated to generate chromatin fragments of 300 bp. Antibodies and p21 primer sets used in PCR were as described (Li et al., 2007a). Primer set for LGALS7 are 5'-GTCC ACAAAAAGAAAAGACACTCCT and 5'-TACAGGAAAGGAGCCAGCCT; for FADS2, 5'-CCTACTTTGGTAAGGTGCTGG and 5'-CCTACTGACCTATAATCCGTGACTC; for GPX1, 5'-CCTAACTCAGGAACCTCTGAGAAA and 5'- CAGGAAAAGGCTGGAGAGTG; for GADD45A, 5'-CCGAGATGTGCTAGTTTCATCACC and 5'-CCCTGCTAAAGGAATTAGTCACG; for XPC, 5'-CCTTGGATTTCTGGCTGCG

and 5’-CGAAGTGGAATTTGCCCAG; for TRAF4, 5’-CTTTGGAGCAGGGCAAGCCAG and 5’-GGATGAAAGTGTAGGGGAGGTGG; and for DHRS3, 5’-CTGGCTCCCCTCTCTGCAAG and 5’-GGCAAGGAGGAACGTCTCG. All binding sites were amplified with 30–35 cycles of PCR. The PCR products were electrophoresed by agarose gels and visualized by ethidium bromide.

Library Generation and Illumina Sequencing

ChIP experiments were performed as described previously (Li et al., 2007). Briefly, chromatin from fixed cells was sheared with Diagenode Bioruptor 300 to a size range approximately 200-400 bp. Clarified nuclear lysate was pre-cleared with BSA pre-blocked Protein A agarose (Pierce) beads to remove nonspecific binding. 5 µg of the pre-cleared lysate was set aside as Input DNA while 45 µg of the lysate was immunoprecipitated with anti p53 polyclonal antibody (FL-393, Santa Cruz) and pulled down by the A beads, washed and eluted. After reversal of cross-link and RNase A treatment (Qiagen), six independent IPs were combined, ethanol precipitated and purified using QIAquick PCR Purification Kit. The purified ChIP DNA was quantified by Qubit 2.0 Fluorometer (Invitrogen). Purified ChIP-DNA was used to prepare Illumina multiplexed sequencing libraries. Libraries for Illumina sequencing were prepared following the Illumina-compatible NEXTflex ChIP-seq Kit (Bioo Scientific). In brief, 10 ng of ChIP DNA was end-repaired, 3' end-adenylated and ligated to

barcoded adapters. DNA insert fragments between 300-400 bp were selected for using Agencourt AMPure XP beads (Beckman Coulter). The purified ligated inserts were enriched by 14 cycles of PCR using the same primers complementary to the adaptors and purified using same beads. The resulting DNA libraries were validated and quantified by checking the size, purity, and concentration of the amplicons on the Agilent Bioanalyzer High Sensitivity DNA chip. The libraries were pooled in equimolar amounts and sequenced on an Illumina HiSeq 2000 instrument to generate 50 base reads. Image deconvolution and quality values calculation were performed using the modules of the Illumina pipeline.

Bioinformatics Data Analysis

All ChIP-seq datasets were aligned using Bowtie (version 1.1.1) (Langmead et al., 2009) to the human reference genome (GRCh37/hg19). The alignment files were analyzed with MACS2 v. 2.1.0.20140616 using a 0.001q value cutoff (Zhang et al., 2008) to identify the p53 binding peaks. To determine differential regions among each condition we used the MACS2 function bdgdiff which compares pileup tracts of two condition using C2 cutoff. The peaks identified by ChIP-seq were analyzed with the RBioconductor package, ChIPpeakAnno (Zhu et al., 2010), to retrieve the nearest Ensembl gene (10 kb around TSS).

The Multiple EM for Motif Elicitation (MEME) algorithm was used to identify enriched sequence motifs in p53 binding data with sequences ± 300 bp around the summit of the peaks at M, UV, and UV+HG (Bailey and Elkan, 1994). Since p53 is known to bind a 20-mer sequence, we ran MEME with parameters to allow for discovery of motifs between 18 and 24 bp in length. We used the k-means clustering function of the Cistrome ‘‘Heatmap" tool (Liu et al., 2011) to display p53 ChIP-seq levels on heatmaps. In this analysis, the signal profiles from M, UV, and UV+HG were entered into Cistrome along with a BED file containing the genomic regions centered at the summits of p53 peaks after DNA damage to generate heatmaps. In the heatmap representation, each row represented the ±2.5 kb centered on the summit of p53 enriched peak and ranked according to the enrichment of p53 occupancy at after DNA damage.

Functional Classification of p53 Target Genes

The genes where p53 binding increased more than 2-fold at 8 h after DNA damage were functionally annotated with DAVID (Huang et al., 2009). This algorithm measures the similarities among GO terms or KEGG pathways based on the extent of their associated genes and assembles the similar terms or pathways into annotation clusters. Each cluster is assigned an enrichment score to rank its biological significance. The resulting clusters were further curated to keep only GO terms with p-values < 0.05.

RT-PCR

Total RNA was extracted using TRIzol reagent (Sigma) according to manufacturer's protocol. RT-PCR was performed using SuperScript One-Step RT-PCR kit (Invitrogen). Primer sets for amplification of p21 mRNA are 5'-CGACTGTGATGCGCTAATGG and 5'- GGCGTTTGGAGTGGTAGAAATC; for p21 hnRNA, they are 5'- GACACAGCAAAGCCCGGCCA and 5'-CAACTCATCCCGGCCTCGCC; for GPX1 mRNA, they are 5'-CAACCAGTTTGGGCATCAG and 5'- CGATGTCAATGGTCTGGAAG; for TRAF4 mRNA, they are 5'- AGGAGTTCGTCTTTGACACCATC and 5'-CTTTGAATGGGCAGAGCACC.

Cell Apoptosis Analysis U2OS

U2OS cells were cultured in DMEM with 10 mM glucose. At the time of UV treatment (20 J/m^2), 25 mM glucose was added for HG. For apigenin treatment, cells were treated with 20 μM apigenin 30 min prior to UV. Cells were harvested 30 h after UV and stained with propidium iodide (Invitrogen). Cells were analyzed on an LSRII instrument (BD Bioscience), followed by data analysis using Modfit (Verity Software).

Cell Proliferation Assay

HCT116 $p53^{-/-}$ cells were cultured in McCoy's 5A. To generate proliferation curves, cells were transfected with wild-type p53, T55A, or a control

cytomegalovirus empty vector using BioT, seeded in triplicate and counted at 120h post seeding. For HG, glucose was added to the media, so the glucose concentration is 25 mM. The presence of overexpressed p53 protein at the analysis was verified by immunoblotting.

3D-Cell Growth/Matrigel Assay

H1299 $p53^{-/-}$ cells were cultured in RPMI 1640 (10-043 Corning). Cells were transfected with wild-type p53, T55A, or a control cytomegalovirus empty vector using BioT (Bioland). For 3D/Matrigel cultures, a base layer of Matrigel (BD Bioscience CB-40230; 200ul/well) was overlaid in triplicate wells of a four well dish with $1.0*10^4$ cells suspended in 1ml of complete medium. Images were captured 72 hours post plating using bright field illumination on a Nikon Eclipse TE2000U inverted microscope and analyzed on NIS-Elements AR 2.30 image analysis software. For diameter analysis, the image analysis software was used to measure the diameter of colonies in the same experiments with a minimum of 40 colonies analyzed for each diameter for each experimental condition. The data reported as the fold-change in diameter for each experimental condition relative to the appropriate control.

TAF1 Kinase Assay

Nuclear extract was prepared from U2OS cells as described previously, and TAF1 was immunoprecipitated with TAF1 antibodies (Ab1230), washed two

times with ice-cold lysis buffer, two times with lysis buffer containing 500Mm NaCl, and two times with phosphorylation buffer. The following phosphatase and protease inhibitors were present throughout the purification: 1mMNa3VO4, 1 mM NaF, 10 mM Na2MoO4,20mM b-glycerophosphate, 5 mM microcystin, 5 nM okadaic acid, 5 mg/ml aprotinin, 5 mg/ml leupeptin, and 5 mg/ml pepstatin, 1 mM PMSF, and 1 mM DTT. In vitro TAF1 phosphorylation assay was carried out using 120 ng of purified p53 as substrate with 350 uM ATP in 20 ul of phosphorylation buffer under conditions as described (Li et al., 2004). Thr55 phosphorylation of p53 was detected by anti-Thr55-Phos antibody and detected by autoradiography.

Seahorse Assays

OCR of cells was measured with the Seahorse Bioscience XF24 extracellular flux analyzer. Briefly, $5x10^4$ cells per well were seeded in 525 µL of XF assay medium. Cells were incubated in a CO2-free incubator for 1 h at 37°C to allow for temperature and pH equilibration prior to loading into the XF24 apparatus. XF assays consisted of a sequential mix (3 min), pause (3 min), and measurement (5 min) cycles, allowing for determination of OCR every 10 min.

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Chapter 3

High glucose effect on DNA damage-induced transcriptome profile in human bone osteosarcoma epithelial (U2OS) cells

3.1 Introduction

Our previous studies elucidated high glucose (HG) can inhibit p53 transcription factor activity and subsequent cellular stress response, including inhibition of cell growth or induction of cellular apoptosis (Chapter 2), and both of these cellular responses constitute hallmarks of cancer. Given that p53 is a major effector of DNA damage response (DDR) (Harper and Elledge, 2007), we were prompted to investigate whether HG can affect other DDR-dependent cellular processes. Studying the degree of HG inhibition on DDR might implicate HG as a tumor promoter in all steps of cancer progression, including self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, and metastasis in addition to promoting both insensitivity to anti-growth signals and evading apoptosis via p53 inhibition (Chapter 2; Hanahan and Weinberg, 2000).

Hyperglycemia, a state of high circulating blood glucose, occurs when the body is incapable of clearing glucose due to lack of insulin or the inability to use insulin productively. Hyperglycemia is most commonly associated with diabetes mellitus (DM), a group of metabolic disorders and a growing problem worldwide affecting slightly less than 10% of the population (Vigneri et al., 2009). Hyperglycemia can also occur as a result of other disorders such as obesity, pancreatitis, chronic stress, and cancer (Dobbs et al., 1975; Martyn et al., 2008; Fogar et al., 1998; Mechanick et al., 2006). Extensive epidemiology data suggest both hyperglycemia and diabetes are associated with certain cancer incidence and mortality, including cancer of the liver, pancreas, endometrium,

colon and rectum, breast, and bladder (Giovannucci et al., 2010). There is also accumulating research suggesting that high circulating glucose can promote carcinogenesis via the induction of cell proliferation and cell cycle progression (Okumura et al., 2002; Yamamoto et al., 1999). Recently, elevated blood glucose levels have been shown to lead to a non-enzymatic glycosylation of proteins that enhance DNA synthesis, the deliberation of free radicals, cytokines, and growth factors, all of which can promote tumorigenesis (Nishikawa et al., 2000; Suba et al., 2006). Those studies have elucidated some molecular associations between hyperglycemia and cancer.

It has previously been shown that endogenous or exogenous genomic insults activate DDR, which coordinates DNA repair mechanisms, cell cycle arrest, and cellular apoptosis (Zhou and Elledge, 2000). DDR is an integral part of genome stability and proper cellular survival, thus acting as a barrier for carcinogenesis (Harper et al., 2007; Bartkova et al., 2005). Interestingly, DDR induces gene expression changes to coordinate its cellular effects (Zhou and Elledge, 2000). It is well established that, upon DNA damage, healthy cells can either arrest at cell cycle checkpoint and repair damaged DNA or undergo cell apoptosis. However, cancerous cells continue to proliferate, due to loss of proper DDR. One striking observation is that proper DDR is vital to maintaining genomic integrity. Given that high circulating blood glucose (HG) may promote carcinogenesis, we aimed to investigate whether HG may promote cancer progression by inhibiting the DDR-regulated transcriptome change.

In this study, we employed a high-throughput RNA sequencing (RNA-seq) approach to analyze cellular transcriptome changes of UV-irradiated osteosarcoma cells (U2OS). We show UV-irradiation indeed affects canonical cancer-associated DDR pathways, such as p53 signaling, cell apoptosis, and cell cycle arrest. Moreover, our data also elucidates less studied DNA damage-related cancer pathways, including wnt, angiogenesis and integrin pathways. We also investigated the effect of HG on DDR-dependent transcriptome changes. Our results show that, while HG does not affect canonical DDR pathways, it affected these less-studied pathways, including integrin, angiogenesis and wnt signaling pathway. Further, analysis of the genes affected by HG using cBioPortal for Cancer Genomics reveals that some HG-affected genes are altered in cancer, suggesting that HG may promote cancer via regulation of gene expression. Importantly our analysis shows that several of the HG-affected genes are mutated in HG-associated cancers.

3.2 Results

Genome-Wide Analysis Reveals that HG Affects a Subset of DNA Damage Responsive Genes

To gain insight into how HG affects DDR at the transcriptome level, we integrated a high throughput RNA-seq experiment under the following three conditions: (A) normal growth (mock, M), (B) UV-irradiation (UV), and (C) UV-irradiation plus 25mM D-glucose treatment (UVHG). Our analysis revealed 7,288

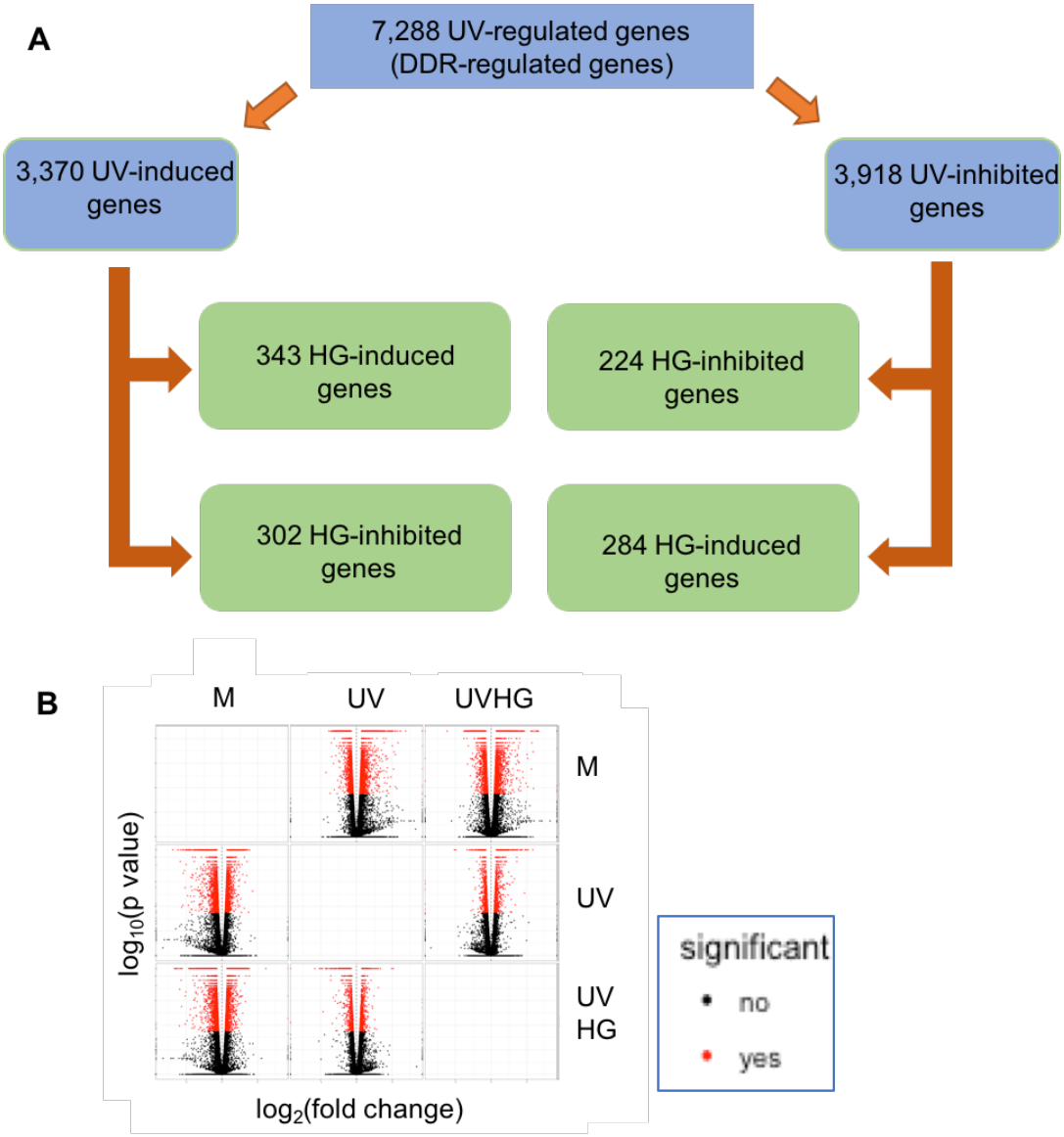

Figure 3.1. Genome-wide analysis reveals that high glucose affects a subset of DNA damage response genes. (A) Volcano plot from gene expression results. The x-axis represents fold change between each stated condition, and the y-axis represents statistical significance (p-value < 0.05). (B) Bioinformatics analysis pipeline used to reveal the DNA damage-dependent genes affected by HG.

transcripts differentially expressed (P<0.01, Cuffdiff differential expression analysis based on the beta-negative binomial distribution) between M and UV-irradiation. A total of 3,370 RNA transcripts were induced, and 3,919 were inhibited by UV-irradiation (Figure 3.1A). Out the of the UV-induced transcripts, HG inhibited the transcript level of 302 genes and further enhanced the transcript level of 343 genes. Also, out of the UV-inhibited transcripts, HG enhanced the transcript level of 284 genes and further inhibited the transcript level of 224 genes. In total, HG affected about 16% of the total UV-regulated transcriptome. We also analyzed our whole genome data using a volcano plot, and our results revealed that HG treatment induced statistically significant mRNA changes to the DNA damage affected genes (Figure 3.1B). Given that we were interested in studying DDR inhibition by HG, we continued our analysis using only the genes where HG hindered DDR regulation.

The cBioPortal for Cancer Genomics Analysis Reveals Significant Alterations in HG-Affected Genes in All Cancers Investigated

We hypothesized that if HG is involved in carcinogenesis, it may function by targeting genes that are important in cancer. To test this hypothesis, we analyzed HG-affected DDR-regulated genes using cBioPortal for Cancer Genomics (cBioPortal) to examine their alteration in cancer. cBioPortal is a portal that provides visualization, analysis, and availability to download large-scale cancer genomics datasets (Gao et al., 2012). This tool allowed us to study

mutation, amplification, and deletion status of our affected genes across different types of cancer. Because hyperglycemia is tightly associated with cancer incidence and mortality in liver, pancreas, colon and rectum, breast, and bladder, we focused our analysis on those cancers (Giovannucci et al., 2010). Genomic alteration datasets for eleven breast cancers, nine pancreas cancers, seven bladder cancers, five liver cancers and five colorectal cancers were used in our studies. Endometrial cancer is another cancer showing HG-association. However, no data on this cancer is available in the cBioPortal data. We first analyzed the mutation status for the 302 HG-inhibited genes and found that within each type of cancer there was at least one dataset with alterations in more than 50% of their samples (Figure 3.2A). Alterations included missense mutations, deletion, amplification, and multiple alterations. Next, we analyzed the mutation status for the 284 HG-induced genes, and once again found that within each type of cancer there was at least one dataset with alterations in more than 50% of their samples (Figure 3.2B). Together, this data implies that some of the HG-affected genes have alteration changes in the cancers associated with hyperglycemia.

Functional Annotation of HG Affected DDR-Regulated Genes Reveals Cancer Pathways

Specific cancer-associated molecular pathways are responsible for the programming of cellular behaviors necessary for transformation, such as

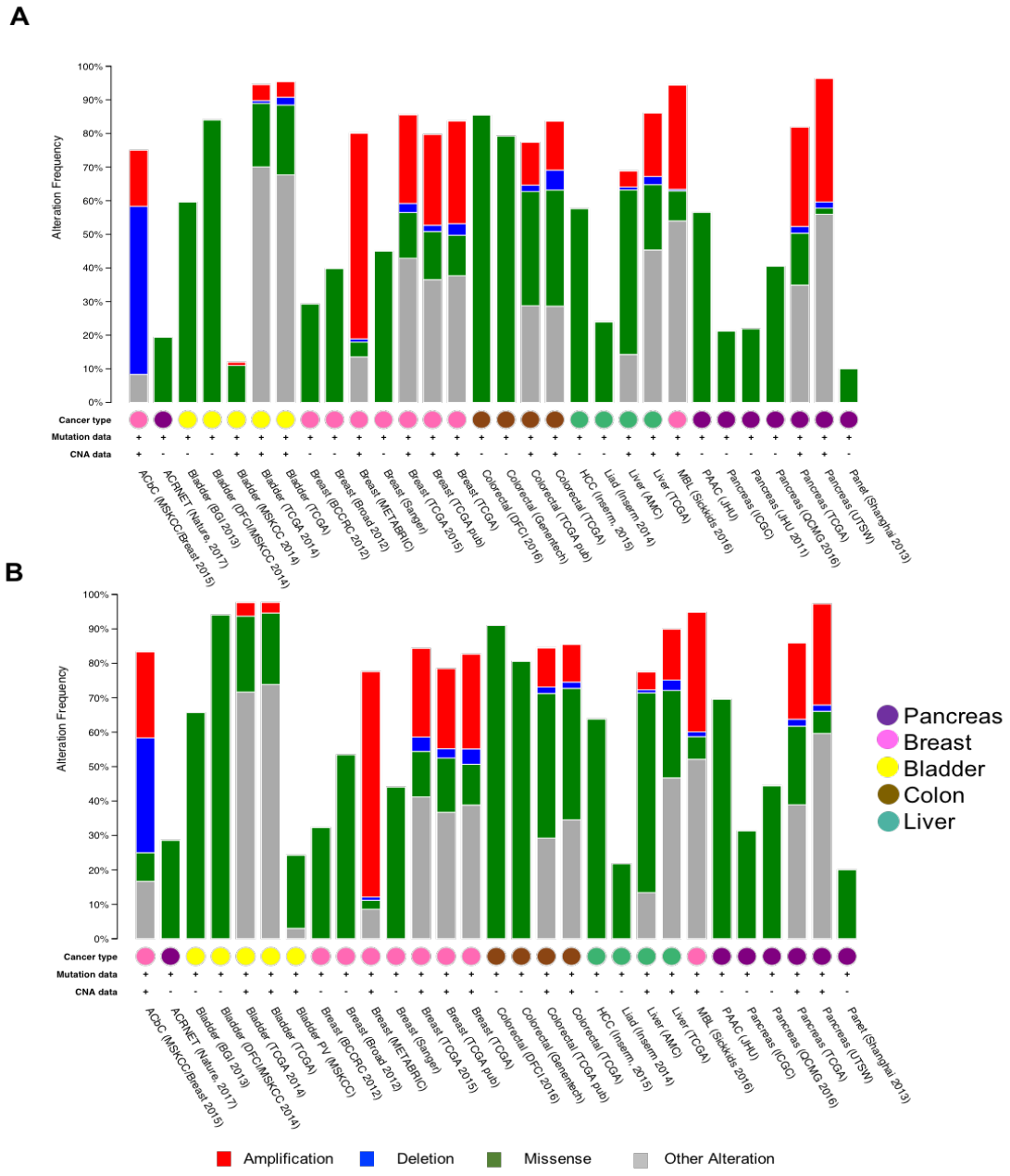

Figure 3.2. The cBioPortal Cancer Genomics analysis reveals significant alterations in HG-affected genes in all cancers investigated. cBioPortal Cancer Genomics tool was to study the mutational, amplification and deletion status of our genes across different types of cancer. Data includes eleven breast cancer, nine pancreas cancer, seven bladder cancer, five liver cancer and five colorectal cancer studies. We are only showing cancer studies with a minimum of 10% altered samples sorted alphabetically. (A) 284 genes up-regulated by DNA damage and repressed by HG were used for this analysis. (B) 302 genes down-regulated by DNA damage and rescued by HG were used for this analysis.

unlimited, self-sufficient growth and resistance to normal homeostatic regulatory mechanisms and these phenotypes are an outcome of genetic changes (Hanahan et al., 2000). To investigate which cancer-associated molecular pathways are affected by DDR, with the ultimate goal of elucidating which of those pathways HG inhibits, we used a gene functional annotation tool, Panther GeneOntology (Mi et al., 2016; Mi et al., 2009). The cancer-associated pathway analysis using either UV-induced genes (3370) or UV-inhibited genes (3918) identified canonical DDR pathways, such as apoptosis, cell cycle, inflammation and p53 response pathways (Table 3.1; 3.2). Surprisingly, our analysis also identified other cancer-associated pathways that are not typically related to DDR, including wnt, angiogenesis, cadherin, integrin signaling and several signal transduction pathways, suggesting their importance in DDR.

We next investigated which of those cancer-associated pathways were affected by HG. As shown in Table 3.3, our analysis revealed HG inhibits genes involved in wnt, G-protein, transforming growth factor beta (TGF-β), cadherin, angiogenesis, inflammation, and integrin signaling pathways. Of note, HG inhibited over 10% of the UV-induced genes that annotated to those pathways, except inflammation. Similarly, as shown in Table 3.4, our analysis revealed HG-induced genes involved in epidermal growth factor receptor (EGFR), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), inflammation, angiogenesis and integrin signaling pathways. Interestingly, HG up-regulated over 10% of the UV-inhibited genes that annotated to both angiogenesis and

Cancer Pathways	Genes
Hypoxia response via HIF activation (P00030)	4
DNA replication (P00017)	6
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	7
Interferon-gamma signaling pathway (P00035)	7
Interleukin signaling pathway (P00036)	7
p53 pathway feedback loops 2 (P04398)	7
FAS signaling pathway (P00020)	8
General transcription regulation (P00023)	9
p38 MAPK pathway (P05918)	9
T cell activation (P00053)	9
Cell cycle (P00013)	10
PI3 kinase pathway (P00048)	10
VEGF signaling pathway (P00056)	10
Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)	11
Transcription regulation by bZIP transcription factor (P00055)	11
Endothelin signaling pathway (P00019)	12
Ras Pathway (P04393)	12
Cadherin signaling pathway (P00012)	13
FGF signaling pathway (P00021)	14
PDGF signaling pathway (P00047)	14
EGF receptor signaling pathway (P00018)	15
TGF-beta signaling pathway (P00052)	15
Apoptosis signaling pathway (P00006)	17
Angiogenesis (P00005)	18
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	18
p53 pathway (P00059)	19
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	23
Wnt signaling pathway (P00057)	29
Integrin signaling pathway (P00034)	32
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	36

Table 3.1. Functional annotation of DNA damage induced genes reveals cancer pathways. Panther GeneOntology classification tool was used to uncover the cancer pathways regulated by DNA damage induced genes. We used 3370 genes for this analysis.

Cancer Pathways	Genes
Cell cycle (P00013)	4
P53 pathway feedback loops 1 (P04392)	5
JAK/STAT signaling pathway (P00038)	8
FAS signaling pathway (P00020)	10
Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)	10
General transcription regulation (P00023)	12
DNA replication (P00017)	12
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	14
Hypoxia response via HIF activation (P00030)	15
p38 MAPK pathway (P05918)	18
Transcription regulation by bZIP transcription factor (P00055)	19
PI3 kinase pathway (P00048)	24
p53 pathway feedback loops 2 (P04398)	24
Endothelin signaling pathway (P00019)	26
Cadherin signaling pathway (P00012)	26
T cell activation (P00053)	27
Interleukin signaling pathway (P00036)	29
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	29
p53 pathway (P00059)	32
VEGF signaling pathway (P00056)	32
Ras Pathway (P04393)	32
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	33
TGF-beta signaling pathway (P00052)	34
Apoptosis signaling pathway (P00006)	44
FGF signaling pathway (P00021)	45
Integrin signaling pathway (P00034)	51
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	57
EGF receptor signaling pathway (P00018)	58
PDGF signaling pathway (P00047)	70
Angiogenesis (P00005)	71
Wnt signaling pathway (P00057)	79

Table 3.2. Functional annotation of DNA damage inhibited genes also reveals cancer pathways. Panther GeneOntology classification tool was used to uncover the cancer pathways regulated by DNA damage repressed genes. We used 3918 genes for this analysis.

Cancer Pathways	UV	UVHG	% inhibited
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	44	4	9%
Angiogenesis (P00005)	23	3	13%
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	27	4	15%
TGF-beta signaling pathway (P00052)	20	3	15%
Wnt signaling pathway (P00057)	39	7	18%
Integrin signaling pathway (P00034)	35	7	20%
Cadherin signaling pathway (P00012)	22	7	32%

Table 3.3. Functional annotation of DNA damage induced genes that are inhibited by HG. Panther GeneOntology classification tool was used to uncover the DNA damage-regulated cancer pathways inhibited by HG. We used 284 genes for this analysis.

Cancer Pathways	UV	UVHG	% rescued
EGF receptor signaling pathway (P00018)	58	3	5%
FGF signaling pathway (P00021)	45	3	7%
VEGF signaling pathway (P00056)	32	3	9%
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	57	4	7%
Angiogenesis (P00005)	71	7	10%
Integrin signaling pathway (P00034)	51	8	16%

Table 3.4. Functional annotation of DNA damage inhibited genes that are rescued by HG. Panther GeneOntology classification tool was used to uncover the DNA damage-regulated cancer pathways inhibited by HG. We used 302 genes for this analysis.

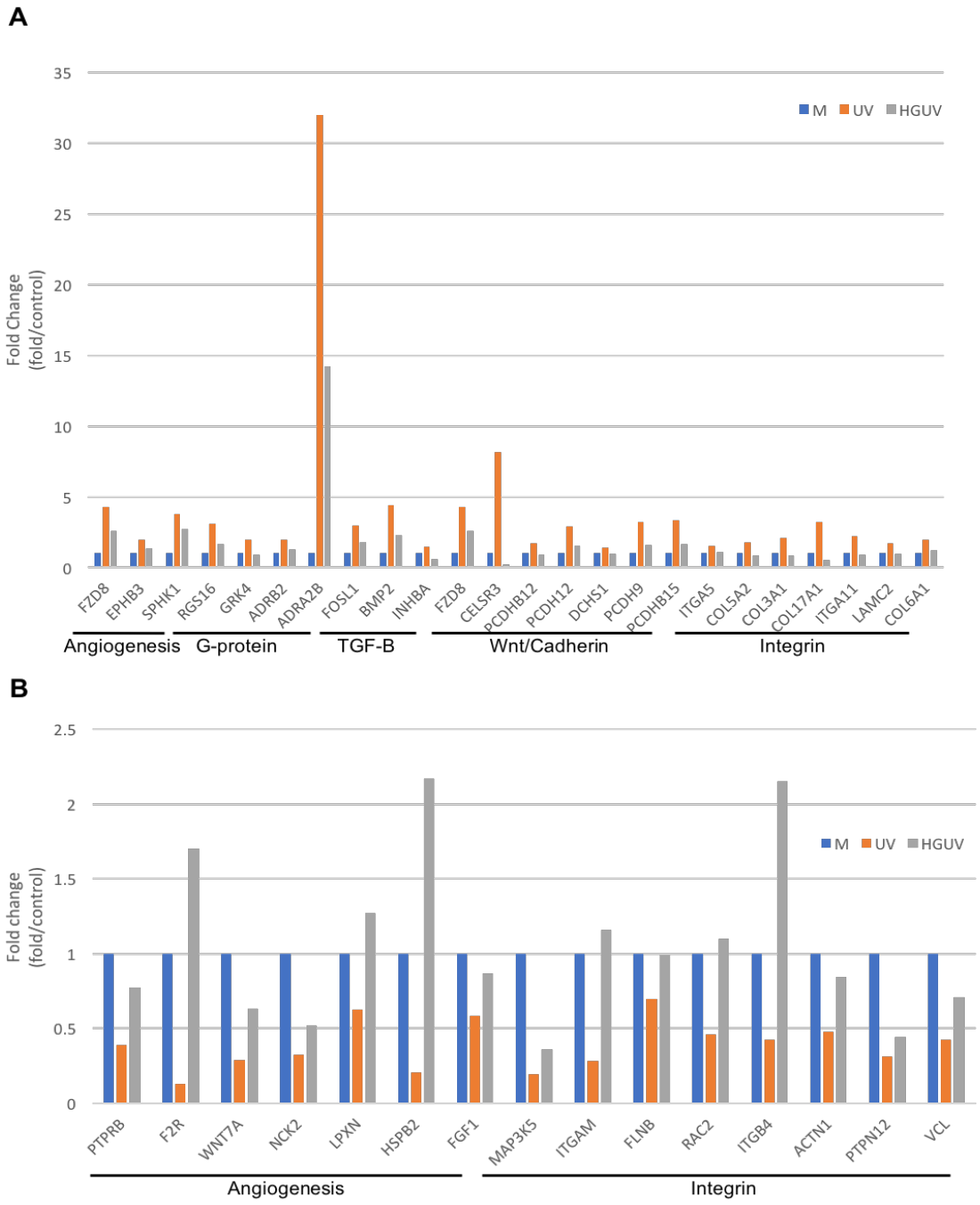

Figure 3.3. Expression of representative HG affected genes in cancer pathways. Relative FPKM expression for HG affected genes in cancer pathways. Mock (M) sample is presented in blue, DNA damage (UV) is shown in orange, and DNA damage with high glucose (UVHG) is presented in gray. Genes are grouped into the cancer pathways annotated by Panther GeneOntology tool. (A) DNA damage induced genes inhibited by HG. (B) DNA damage inhibited genes rescued by HG.

integrin signaling pathways, but no other pathway. The expression changes for the representative HG-affected genes involved in cancer pathways are summarized in Figure 3.3. Those analyzes suggest HG may affect cancer pathways via gene regulation.

The cBioPortal for Cancer Genomics Analysis of HG-Inhibited Cancer Pathways Suggests HG may Affect Integrin and Wnt/Cadherin Signaling in Cancer

Thus far, we found that HG can inhibit DDR pathways that are important in cancer. We speculated that perhaps the HG-affected genes from the cancer pathways we discovered are also the altered genes in the cancer genomic studies we found in Figure 3.2. To address this possibility, we individually investigated the genes in each HG-inhibited cancer pathway (Table 3.3) using cBioPortal on the cancers associated with HG as previously described. Of note, our analysis revealed deletions, and missense mutations in the genes annotated to integrin and wnt/cadherin pathways, but no other pathway (Figure 3.4). Both missense mutations and gene deletions can result in the down-regulation of gene expression, supporting that the HG-observed inhibition of those genes may promote cancer.

Interestingly, our analysis on the integrin pathway gene-set revealed high mutation rates for breast and colorectal cancer, and low mutation rates for bladder, liver and pancreatic cancer (Figure 3.4A). Primarily, we observed

deletions in over 40% of the samples from the adenoid cystic carcinoma (AdCC) breast cancer study, suggesting that inhibition of these genes by HG treatment may be important for this cancer. AdCC is a rare type of triple-negative breast cancer (TNBC) characterized by the presence of the *MYB – NFIB* fusion gene (Martelotto et al., 2015). Next, our studies also revealed mostly missense mutations in about 20% of the samples from two colorectal cancer studies (DFCI2016 and Genentech). DFCI2016 is a whole-exome sequencing study consisting of 619 colorectal cancers with clinicopathologic annotations (Gionnakis et al., 2016) and the Genentech colorectal cancer dataset is a comprehensive profiling study of 72 colon cancer samples (Seshagiri et al., 2012). Lastly, our analysis showed that some bladder, liver and pancreatic cancer studies had missense mutations in about 10-15% of their samples. These studies suggest that HG may play a role in regulating integrin signaling in AdCC breast and colorectal cancer and to a lesser extent in bladder, liver and pancreatic cancers.

Likewise, our analysis of the wnt/cadherin pathway gene-set revealed high mutation rates for colorectal, bladder and pancreatic cancer, and low mutation rates for liver and breast cancer (Figure 3.4B). Firstly, our analysis revealed both missense mutations and deletions in over 20% of the samples for all colorectal cancer genomic datasets, The Cancer Genome Atlas (TCGA) bladder cancer dataset and the UT Southwestern (UTSW) pancreatic cancer dataset (Figure

3.4B). The TCGA bladder cancer dataset was compiled from a whole genome and RNA-seq study of 131 urothelial carcinomas (Weinstein et al., 2014), while the UTSW pancreatic dataset was collected from a whole exome sequencing of 109 micro-dissected pancreatic ductal adenocarcinoma (PDA) cases (Witkiewicz et al., 2015). Additionally, one liver, two pancreatic and two breast cancer datasets had both missense mutations and deletions in about 10-20% of their

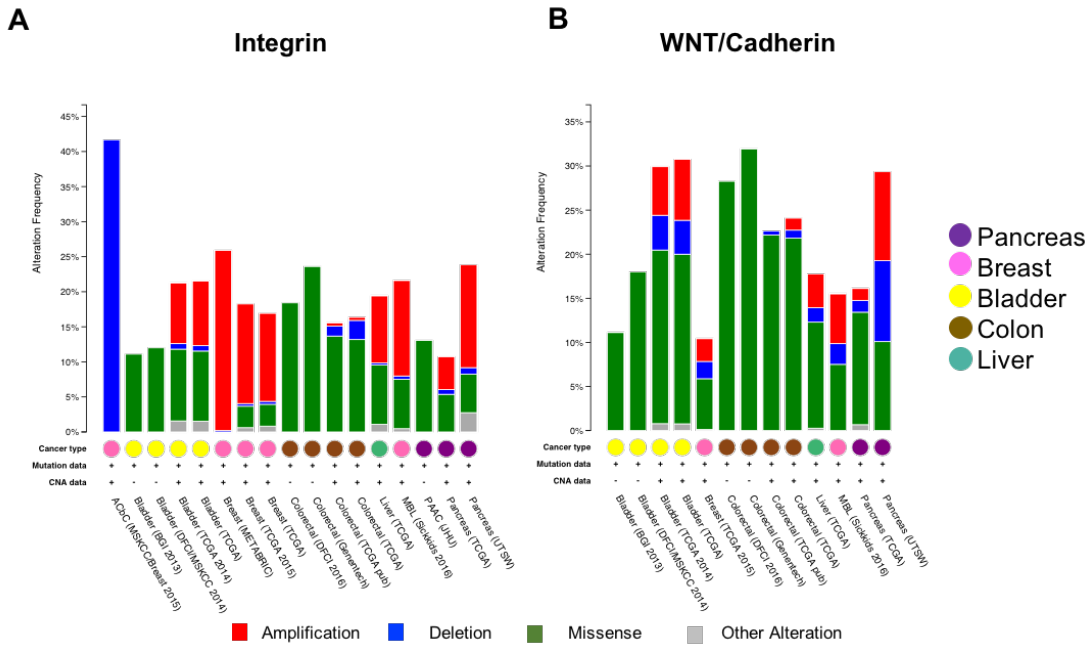

Figure 3.4. The cBioPortal Cancer Genomics analysis of HG-inhibited cancer pathways suggests HG may affect integrin and wnt/cadherin signaling in cancer. cBioPortal Cancer Genomics tool was to study the mutational, amplification and deletion status of our genes across different types of cancer. Data includes eleven breast cancer, nine pancreas cancer, seven bladder cancer, five liver cancer and five colorectal cancer studies. We are only showing cancer studies with a minimum of 10% altered samples sorted alphabetically. (A) Seven genes up-regulated by DNA damage and repressed by HG were used for the integrin analysis. (B) Seven genes up-regulated by DNA damage and repressed by HG were used for the wnt/cadherin analysis.

samples. Together, our analysis suggests HG may be involved in regulating wnt/cadherin signaling in colorectal, urothelial bladder and PDA cancer and to a lesser extent liver and breast cancer.

The cBioPortal for Cancer Genomics Analysis of HG-Enhanced Cancer Pathways Suggests HG may Affect Integrin and Angiogenesis Signaling in Cancer

Similarly, we individually investigated the genes in each HG-induced cancer pathway (Table 3.4) using cBioPortal on the cancers associated with HG as previously described. Of note, our analysis revealed gene amplifications and missense mutations in the genes annotated to integrin and angiogenesis pathways, but no other pathway (Figure 3.5). Both gene amplifications and missense mutations can result in the up-regulation of gene expression, supporting that the HG-observed induction of those genes may promote cancer.

Similar to our previous results, our analysis on the integrin pathway gene-set revealed high mutation rates for breast, bladder and pancreatic cancer, and low mutation rates for liver and colorectal cancer (Figure 3.5A). Primarily, our analysis revealed that breast (AdCC), bladder (TCGA) and pancreatic (UTSW) cancer datasets all had gene amplifications and missense mutations in about 25% of their samples. Interestingly, AdCC breast cancer had gene alterations in both HG-induced and inhibited gene-sets that were annotated to the integrin pathway, suggesting that integrin dysregulation may play an essential role in

AdCC breast cancer and that HG may be involved in this process. Moreover, most colorectal cancer datasets and one liver (TCGA) dataset had gene amplifications and missense mutations in 10-20% of their samples. Our studies suggest that HG may play a role in regulating integrin signaling in all cancers studied.

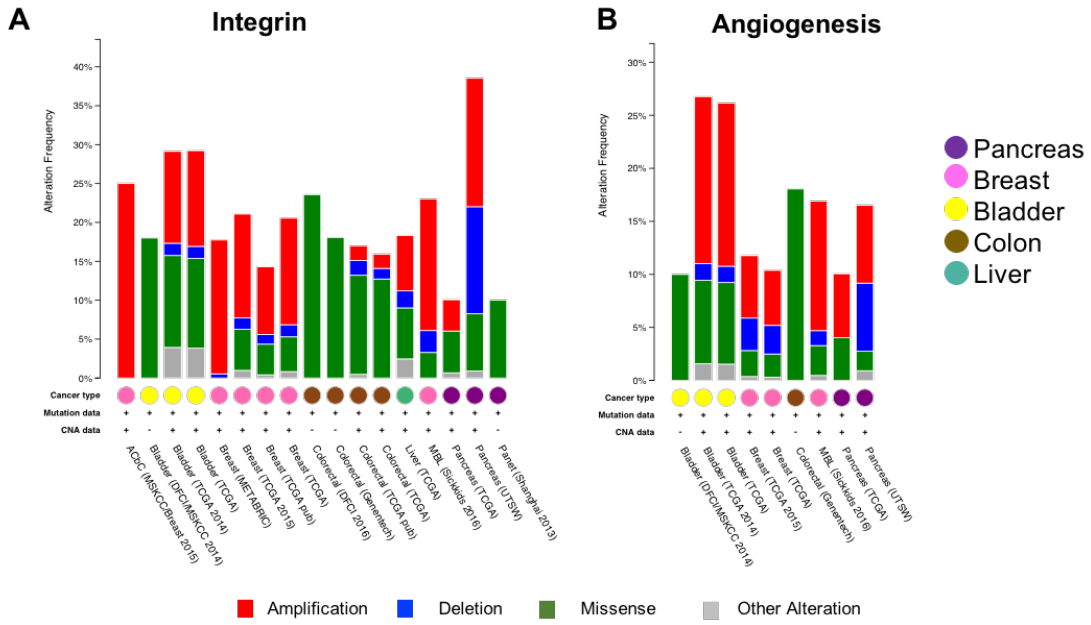

Figure 3.5. The cBioPortal Cancer Genomics analysis of HG-enhanced cancer pathways suggests HG may affect integrin and angiogenesis signaling in cancer. cBioPortal Cancer Genomics tool was to study the mutational, amplification and deletion status of our genes across different types of cancer. Data includes eleven breast cancer, nine pancreas cancer, seven bladder cancer, five liver cancer and five colorectal cancer studies. We are only showing cancer studies with a minimum of 10% altered samples sorted alphabetically. (A) Eight genes down-regulated by DNA damage and rescued by HG were used for the integrin analysis. (B) Seven genes down-regulated by DNA damage and rescued by HG were used for the angiogenesis analysis.

Our analysis of the angiogenesis pathway gene-set revealed high mutation rates for bladder cancer and low mutation rates for colorectal, breast and pancreatic cancer. Primarily, we found missense mutations and gene amplifications in 25% of the samples from the TCGA bladder cancer dataset (Figure 3.5B). Further, some colorectal (Genentech), breast (MBL), and pancreatic (UTSW) cancer datasets also had missense mutations and gene amplifications in 10-20% of their samples (Seshagiri et al., 2012; Witkiewicz et al., 2015). The breast MBL dataset is a whole-exome sequencing compilation of 216 tumor-normal (blood) pairs from metastatic breast cancer patients (Lefebvre et al., 2016). Alterations were not observed in liver cancer. Together, this data suggests HG may play a role in regulating angiogenesis in bladder cancer, and to a lesser extent colorectal, breast and pancreatic, but not liver cancer.

3.3 Discussion

In this study, we show that HG treatment affects the DDR-regulated transcriptome in U2OS cells. Our RNA-seq studies revealed that DDR regulates the transcript level of around 7,000 genes. Moreover, HG affected about 16% of those 7,000 genes. Although the percentage of change observed was small, many altered genes are associated with cancer pathways. To our surprise, part of the cancer-associated affected pathways included angiogenesis, integrin and wnt signaling pathways which are not typically associated with DDR.

Interestingly, those less associated DDR pathways including integrin, angiogenesis, and wnt pathways are inhibited by HG.

The role of integrins in carcinogenesis is well established, as they are known to regulate diverse functions necessary for tumor growth i.e., migration, invasion, proliferation, disease progression and decreased survival (Ridley et al., 2003). Integrin adhesion receptors are heterodimeric molecules composed of α and β subunits that bind primarily to fibrous components of the extracellular matrix (ECM), such as collagen and laminin (White et al., 2007). It's believed, in both normal and transformed cells, that integrins are involved in cross-talk between integrin receptors and other growth-promoting molecules such as growth factor receptors (Moro et al., 1998; Miyamoto et al., 1996). In cancer, such cross-talk results in the concerted activation of downstream signaling pathways, such as those promoting cell cycle progression and oncogenic transformation (Guo et al., 2006; Gambaletta et al., 2000; Yoon et al., 2006). In normal tissues, this process provides a level of spatial control required for tissue integrity. For example, in epithelial and endothelial cells, integrin ligation regulates cell survival such that detachment from the ECM rapidly induces apoptosis (Meredith et al., 1993, Frish et al., 1994). When DDR is activated, cell death may result by detachment or inhibition of integrins or by overexpression of unligated integrins mediated by increased levels of p53 or Bax (Gilmore et al., 2000; Stromblad et al., 1996). Our studies elucidated β4 integrin subunit as a DDR inhibited integrin (2-fold) that is induced by HG (4-fold). Interestingly,

ablation of β4 integrin subunit or associated intracellular effectors such as integrin-linked protein kinase (ILK), focal adhesion kinase (FAK) or proto-oncogene tyrosine-protein kinase Src (c-Src) has been shown to impair tumorigenesis in various breast mouse models and skin carcinomas (Guo et al., 2006; Guy, 1994; McLean, 2001). Further, β4 integrin subunit polymorphism, rs743554, may enhance its ability to promote tumor cell growth, invasion and influence both tumor aggressiveness and survival in certain cancers (Brendle et al., 2017).

Correspondingly, α5 integrin subunit was also revealed by our analysis (up-regulated 1.5-fold by DDR and inhibited 1.5-fold by HG). α5 has been shown to be down-regulated or absent in pancreatic cancer (Linder et al., 2001; Weinel et al., 1992). More alarming, collagen 17 (COL17A1), another DDR repressed target that is induced by HG, has been shown to be lost in cancer cells, including invasive breast cancer (IBC) (Bergstraesser et al., 1995), and pancreatic ductal epithelium cancer (Laval et al., 2014). Recently, Yodsurang et al., elucidated COL17A1 as a target gene of tumor suppressor p53 involved in suppressing breast cancer cell migration and invasion, whereby a high level of COL17A1 expression led to a better prognosis of patients with IBC. Moreover, the specific role that integrins play in metastasis has been suggested from cell culture-based systems, and both in 2D and 3D reconstituted matrices in breast cancer models. It's believed integrins are involved at all stages of metastasis, including tumor cell

migration, invasion, and colonization of target tissues (White et al., 2017). Together, our data implicates HG in the regulation of integrins.

Wnt signaling pathway remains one of the best-defined pathways in carcinogenesis (Mah et al., 2017). Wnt signaling was one of the most affected pathways of DDR in both UV-induced and -repressed genes (Table 3.1 and 3.2). Although not intuitively, this was not surprising as wnt signaling is a target of p53 (Karimaian et al., 2017), a major effector of DDR. While DDR affected wnt signaling in both induced and repressed genes, HG only altered the wnt-annotated UV-induced gene subgroup by inhibiting the expression of 18% of those genes (Table 3.3). Surprisingly, the same subset of genes was also grouped into E-cadherin signaling pathway. As it turns out, all genes, except for FZD8, within this subset belongs to the cadherin superfamily of cell adhesion molecules. Furthermore, four of these genes (i.e., PCDHB12, PCDH12, PCDH9, PCDHB15) are protocadherins (Pcdhs), the largest group within the cadherin superfamily. Interestingly, recent research implicates the role of Pcdhs in the down-regulation of canonical wnt/β-catenin-dependent signaling pathways (Mah et al., 2017). Indeed, increasing evidence suggests that Pcdhs are potential tumor suppressor genes. Several studies have shown that Pcdhs expression is lost in cancer and re-expression can suppress tumor cell proliferation, inhibit cell migration, and induce apoptosis and autophagy in some cancer cell lines (Yu et al., 2008; Haruki et al., 2010; Hu et al., 2013; Imoto et al., 2006). Pcdh9, a Pcdh identified by our analysis, was up-regulated 3-fold by DDR and inhibited 2-fold by

HG. Pcdh9 has been reported to be silenced in glioblastomas and hepatocellular carcinoma (HCC) (Tayrac et al., 2009; Zhu et al., 2014). Studies by Zhu et al. found that over-expression of Pcdh9 in HCC-derived cell lines decreased migration and resulted in reduced phosphorylation of GSK3β. Further, loss of Pcdh9 in gastric cancer is associated with the differentiation of tumor cells, metastasis and predicts poor survival (Chen et al., 2015). Interestingly, Chocarro-Calvo et al. recently suggested a mechanism by which HG enhances signaling through the wnt/β-catenin pathway leading to increased β-catenin acetylation, its nuclear accumulation, and transcription activation of target genes. Perhaps HG has multiple effects within the cell that up-regulate the wnt pathway to promote cell growth.

To our knowledge angiogenesis has not been previously reported to be affected by DDR, and further research is necessary to establish whether UV-irradiation inhibits or promotes angiogenesis. Nonetheless, our analysis elucidated a group angiogenesis-annotated genes that are inhibited by DDR and rescued by HG (Figure 3.5B). Wnt7A was down-regulated 3.5-fold by DNA damage and induced 2-fold by HG. Wnt7A, a member of the wnt pathway, has been shown to transform cells efficiently and is overexpressed in ovarian carcinoma (Wong et al., 1994; Yoshioka et al., 2012). Wnt family members have been implicated in angiogenesis, notably in the developing retina, the placenta, and ovaries (Parmalee et al., 2013), but perhaps wnt family members angiogenic effect in cancer has yet to be elucidated.

Another gene discovered in our analysis, F2R, which was 8-fold down-regulated by DNA damage and 13-fold up-regulated by HG, has also been implicated in promoting carcinogenesis. F2R is a G protein-coupled receptor involved in the regulation of thrombotic response. F2R gene expression plays a central role in blood vessel recruitment in animal models and significantly enhances both angiogenesis and tumor growth in a VEGF-dependent manner (Yin et al., 2003). Our data suggest that UV-irradiation DDR may affect angiogenic pathways and perhaps this may present an important avenue in chemotherapy drugs that induce DNA damage to inhibit angiogenesis in cancer cells.

While the changes HG impaired on the DNA damage transcriptome were small, our analysis showed that HG changes the expression of genes involved in carcinogenesis. The HG effect on cancer has already gained interest in the research community, as studies correlate HG to increase in cell growth. The studies presented here suggest HG may be involved in all phases of cancer progression. It will be interesting to further elucidate the molecular mechanisms affected by HG to alter DDR-regulated gene expression. Of interest, TAF1 was found to bind to seventy percent of the HG-altered gene's promoters identified in this study, suggesting perhaps HG-enhanced TAF1 kinase activity also regulates the expression of these genes via the same mechanism identified in Chapter 2, i.e. via phosphorylation of transcription factors (TF) regulating these promoters.

Fully identifying those mechanisms or other TAF1 TF targets, in addition to p53, will help explain why diabetic individuals are more susceptible to certain cancers.

3.4 Methods

Library Generation and Illumina Sequencing

Total RNA was extracted using either TRIzol reagent (Sigma) according to manufacturer's protocol (Invitrogen.com), from two independent biological replicates. RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) according to the manufacturer's instructions. Poly(A)+ mRNA was purified from 4 μg of total RNA, and fragmented (~200 nt) before cDNA synthesis with random priming. The cDNA was converted into paired-end libraries using the Illumina TrueSeq RNA Preparation v2 Kit according to the manufacturer's protocol (www.Illumina.com). Each library was analyzed using Agilent Technologies 2100 Bioanalyzer to check size, purity, and concentration. Paired-end sequencing of the libraries was carried out with the Illumina HiSeq2000 using 100 cycles. Image deconvolution and quality values calculation were performed using the modules of the Illumina pipeline.

Bioinformatics Analysis

For the bioinformatics analysis, paired-end sequenced reads were aligned to the human reference genome (GRCh37/hg19) with Tophat (version 2.0.4) using the default parameters (http://tophat.cbcb.umd.edu). The data aligned by

Tophat was processed by Cufflinks (version 2.0.2) to assemble transcripts and to measure their relative abundances in FPKM units (fragments per kilobase of exon per million fragments mapped) (http://cufflinks.cbcb.umd.edu). Assembled transcripts from control and treated cells were compared with the RefSeq refFlat annotated transcriptome from the UCSC genome browser and tested for differential expression using the Cuffcompare and Cuffdiff utilities included in the Cufflinks package. Cuffdiff was run with FPKM upper-quartile normalization and a false discovery rate (FDR) threshold of 5%.

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Chapter 4

Conclusion

Several epidemiological data suggest individuals with diabetes are at higher risk for both cancer incidence and mortality. This association is more prominent in cancer of liver, pancreas, breast, endometrium and bladder (Giovannucci et al., 2010; Vigneri et al., 2009; Harding et al., 2015). As the prevalence of diabetes mellitus is substantially increasing worldwide, some associated cancers such as liver and pancreatic have also increased in both incidence and mortality (Siegel et al., 2016). While it is speculated that the associations are due to many factors, the work presented here suggests hyperglycemia, a hallmark of both type 1 and type 2 diabetes, could provide a link between diabetes and cancer (Johnson et al., 2012; Suh and Kim, 2011). Hyperglycemia is a state of excess glucose in the blood and it is the most widely studied metabolic change in diabetes (Vigneri, 2009; Suh and Kim, 2011). Recently, a significant part of cancer research has been dedicated to altered metabolism. Work on this field has shown that altered metabolism confers transformed cells the ability to overcome increased bioenergetic and biosynthetic requirements of cell proliferation and the means to survive the harsh tumor micro-environment (Jones and Thompson, 2009; Hanahan et al., 2000). Here we studied the role of high glucose (HG) as a tool used by cancer cells to confer growth advantage. We showed that HG elevates cellular ATP levels and induces the kinase activity of the TATA- binding protein (TBP) associated factor 1 (TAF1). Consequently, activated TAF1 kinase phosphorylates p53 at Thr55, and this phosphorylation dissociates p53 from its target promoters after DNA damage.

Importantly, our results revealed that HG inhibits p53-dependent cellular responses such as cell growth and cell apoptosis. We further showed that HG treatment affects RNA levels in osteosarcoma cells (U2OS) after DNA damage. Interestingly, some of the HG regulated genes are involved in cancer pathways including integrin, angiogenesis, and wnt/cadherin pathways. More importantly, some of the HG-affected genes are also altered in diabetes-associated cancers. On the basis of those results, we propose that HG may promote cancer, at least in part, via regulation of DDR-mediated gene expression and more specifically via inhibition of p53-mediated cellular response.

Previous work on the hyperglycemia-cancer association suggested that HG promotes cancer cell proliferation via altering gene expression (Masur et al., 2011). Further, DDR, an integral part of genome stability and proper cellular survival, also mediates its cellular effect, at least in part, through gene expression changes and one of its major effectors is tumor suppressor p53. Interestingly, TAF1 has intrinsic kinase activity (Dikstein et al., 1996). Previously, our lab has shown that TAF1 can phosphorylate p53 at Thr55, leading to p53 inactivation (Li at al., 2004). Moreover, we showed TAF1 kinase activity is cellular ATP dependent (Wu et al., 2014). Given that glucose is the primary source for ATP production (Hsu and Sabatini, 2008) and high circulating glucose levels are commonly observed in patients with diabetes, our results prompted us to investigate the effect of HG treatment on p53 tumor suppressor function via TAF1 kinase activity. Our studies suggested a molecular model where HG

inhibits p53 cellular response via alteration of cellular ATP and TAF1 kinase activity. In particular, we showed that increase in glucose availability could inhibit p53-dependent cellular responses, such as anti-growth activity and induction of cellular apoptosis (Chapter 2). These studies indicate that a HG environment might be potentially dangerous when insults are made to the genomic material in the cell. The importance of these findings is further supported by the finding that transformed cells consume more glucose when it is readily available due to their altered metabolism (Hsu and Sabatini, 2008; Kim and Dang, 2006), suggesting that a diabetic-cancer patient will potentially suffer from a more aggressive cancer phenotype due to their high circulating blood glucose levels. Overall, our studies provide an explanation for the observed increase in both cancer incidence and mortality in diabetics.

We further extended our research on the overall effect of HG on DDR-mediated transcription changes. The studies presented in Chapter 3 elucidated HG-targeted RNA changes in a variety of genes involved in carcinogenesis, including genes shown to be mutated in HG-associated cancers. Importantly, some HG-affected genes are involved in commonly altered cancer pathways such as wnt, angiogenesis, and integrin pathways. All of the aforementioned pathways can promote transformation, further supporting the notion that HG promotes cancer. Analysis of HG-affected genes also suggests that HG may play a broader role in cancer in addition to its regulation of p53. To support this, our findings in Chapter 3 revealed that only a small subset of HG-affected genes are

p53 targets. In fact, only 8 out of the 62 genes with HG-affected p53 chromatin binding sites (Chapter 2) overlapped with the RNA-transcripts regulated by HG (Chapter 3). Further, overlapping of 343 literature-based p53 target genes (Fischer, 2017) with our HG-affected RNA transcripts, revealed that only 28 genes are regulated by HG. Of note, our HG genome-wide studies may have missed several HG-affected p53 target genes because they were done under DNA-damage conditions. Perhaps, investigating HG regulation on p53 genome-wide chromatin occupancy under normal and HG glucose conditions without DNA-damage might elucidate the overall effect of HG on p53. Completing such experiments would further advance our understanding of HG regulation on p53-mediated gene expression changes.

Additionally, while we firmly established the molecular mechanism by which HG inhibits p53 activity, the significance of this mechanism remains to be investigated in both *in vivo* animal studies and in humans. Thus far, animal studies have been limited since the Thr55 residue on p53 is not homologous in mice. One alternative would be to investigate p53 inhibition by HG and its biological outcome using xenograph models in both normal and diabetic animal models using a stable cell line carrying a p53 mutant, T55A, that can't be phosphorylated by TAF1. Analyzing Thr55 phosphorylation status in human cancer samples from non-diabetic and diabetic patients would also firmly establish the role of Thr55 phosphorylation in p53 de-activation as well as the overall role of p53 in HG-dependent cancer promotion. However, this approach

also has limitations because (a) epidemiology of diabetic status on cancer patients is not readily available and (b) p53 is very commonly mutated in human cancer.

Our results could lead to several exciting future studies. First, it is tempting to hypothesize, that perhaps HG-induced TAF1 kinase may have multiple phosphorylation targets. Feasibly, TAF1 is able to induce progression through the G1 phase (Wang and Tjian, 1994), through the phosphorylation of multiple targets. In this study, we have proposed that TAF1 serves as a nutrient sensor under normal homeostasis given that it can sensor changes in cellular ATP levels. We further suggest that perhaps, under excess nutrient conditions, TAF1 kinase activity may function in an oncogenic manner to inhibit p53 cellular response. Possibly other transcriptions factors (TF), such as those that regulate the genes affected by HG (Chapter 3) are also regulated via TAF1 kinase activity to promote cell growth. Interestingly, there are several genes elucidated in Chapter 3 that have both TAF1 binding and known oncogene/tumor suppressor TF promoter binding as shown via the ENCODE consortium (Gerstein et al., 2012; Wang et al., 2012; Wang et al., 2013). Some examples of these are β4 integrin subunit, α5 integrin subunit and PCDH9, all of which have known proto-oncogene TF promoter binding such as Myc (Cole et al., 1986), Fos (Verma and Graham, 1987) and c-Jun (Vogt et al, 1990), suggesting that perhaps TAF1 may also regulate proto-oncogenes.

Myc is a TF that regulates genes involved in almost every important cellular function (Miller et al., 2012). One interesting Myc-targeted cellular function is the regulation of cellular metabolism to induce more production of energy, proteins, lipids and nucleic acids necessary to support rapid cell division. Interestingly, overexpression of c-Myc is enough to confer its oncogenic effect and overexpression may occur as a result of post-translational modifications (PTMs), suggesting that potential regulation by TAF1 would be capable of activating Myc. Likewise, activator protein 1 (AP-1) is a dimeric-TF that comprises members of several protein families including Jun and Fos, among others (Eferl and Wagner, 2003). AP-1 has been implicated in both oncogenic functions and tumor suppressor functions. Nonetheless, AP-1 regulates genes involved in cell proliferation, differentiation, apoptosis, angiogenesis and tumor invasion. AP-1 has also been shown to be regulated via PTMs. However, the effect of PTMs on AP-1 in carcinogenesis is not completely understood. Overall, studies analyzing the activity of both TF's with or without TAF1 kinase inhibitor, apigenin, would indicate if these speculations are valid, and could widen our understanding of both TAF1 and cancer.

Second, in addition to its kinase activity, TAF1 also possesses several other enzymatic activities that are important in transcription regulation (Wassarman and Sauer, 2001; Dikstein et al., 1996; Mizzen et al., 1996; Pham and Sauer, 2000), one of which is histone acetyltransferase activity (HAT; Mizzen et al., 1996). HAT is another enzymatic activity that could be regulated via

nutrient availability (Lee et al., 2014). Interestingly, recent research by Lee et al. suggested that acetyl-CoA is also regulated by HG and that the ratio of acetyl-CoA:coenzyme A modulates global histone acetylation levels in cancer cells. This data implicated that increase in glucose might activate HATs to induce histone acetylation. It is tempting to speculate that HAT activity may be an additional enzymatic activity utilized by TAF1 in HG-associated cancer. Interestingly, a mutational analysis by Kandoth et al. not only elucidated TAF1 as a commonly mutated gene in cancer, but their data also shows that TAF1's HAT domain is one of the domains with most mutations (Kandoth et al., 2013). It would be interesting to investigate whether the mutations in the HAT domain are activating mutations that promote overall acetylation of histones and other targets by TAF1 in cancer. Elucidating if these assumptions are true, would establish TAF1 as a master player in both nutrient sensing and carcinogenesis.

Finally, while our work implicates glucose overload as one molecular link between diabetes and cancer, it's important to note that other nutrients may also be involved in cancer via the same mechanism utilized by HG, i.e., induction of cellular ATP via metabolic pathways, but independent of diabetic state. Cancer metabolism studies have shown that malignant cells can survive and grow in vascularity compromised environments by exploiting the full array of nutrients available extracellularly (Palm and Thompson, 2017). In particular, glutamine, the second most-consumed nutrient by proliferating cells, is a major source for bioenergy production (DeBerardinis et al., 2007; Yuneva et al., 2007). Work by

Fan et al. showed that glutamine could fuel cancer cells as the major source for ATP production even in oncogene-expressing or hypoxic cancer cells. Similarly, fatty acid carbons are also efficient bioenergetic substrates, and their oxidation generates two-fold more ATP per molecule than oxidation of glucose or glutamine (Palm and Thompson, 2017). Interestingly, fatty acid oxidation could provide sufficient ATP to rescue the viability of cells when glucose or glutamine becomes limiting (Bauer et al., 2005). Even under nutrient-replete conditions, some types of cancer use fatty acid oxidation to maintain oxidative phosphorylation (Camarda et al., 2016; Caro et al., 2012). It would be interesting to investigate if any of these nutrients can also induce cellular ATP production and activate TAF1 kinase activity.

In summary, this study elaborates on the importance of diet in cancer. Diet is a very important regiment in the treatment of diabetes, and there is substantial evidence supporting that dietary restrictions can lower insulin levels, insulin requirements, and improve glucose tolerance (Vernon, et al., 2003; Chandalia et al., 2000). Dietary regiments are not imposed as part of cancer treatment as the benefits of maintaining a proper diet in cancer patients are not entirely understood. Nonetheless, some studies suggest dietary restrictions can affect the risk of cancer incidence, recurrence, and mortality for some cancers (Kroenke et al., 2005; DPhill et al., 2002). At the molecular level, research like the one presented here and several others show that maintaining glucose homeostasis may be beneficial for cancer patients (Chocarro-Calvo et al., 2013;

Camarda et al., 2016; Masur et al., 2011; Lee et al., 2014). We propose that even minimal fluctuations in glucose levels are enough to promote growth via p53 inhibition. While more research is needed to fully elucidate the effects of altered nutrient consumption and aberrant metabolite concentration in cancer, perhaps it would be a good idea for implementation of dietary restrictions into overall cancer treatment.

4.1 References

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Appendix A

Supplemental Data for Chapter 2

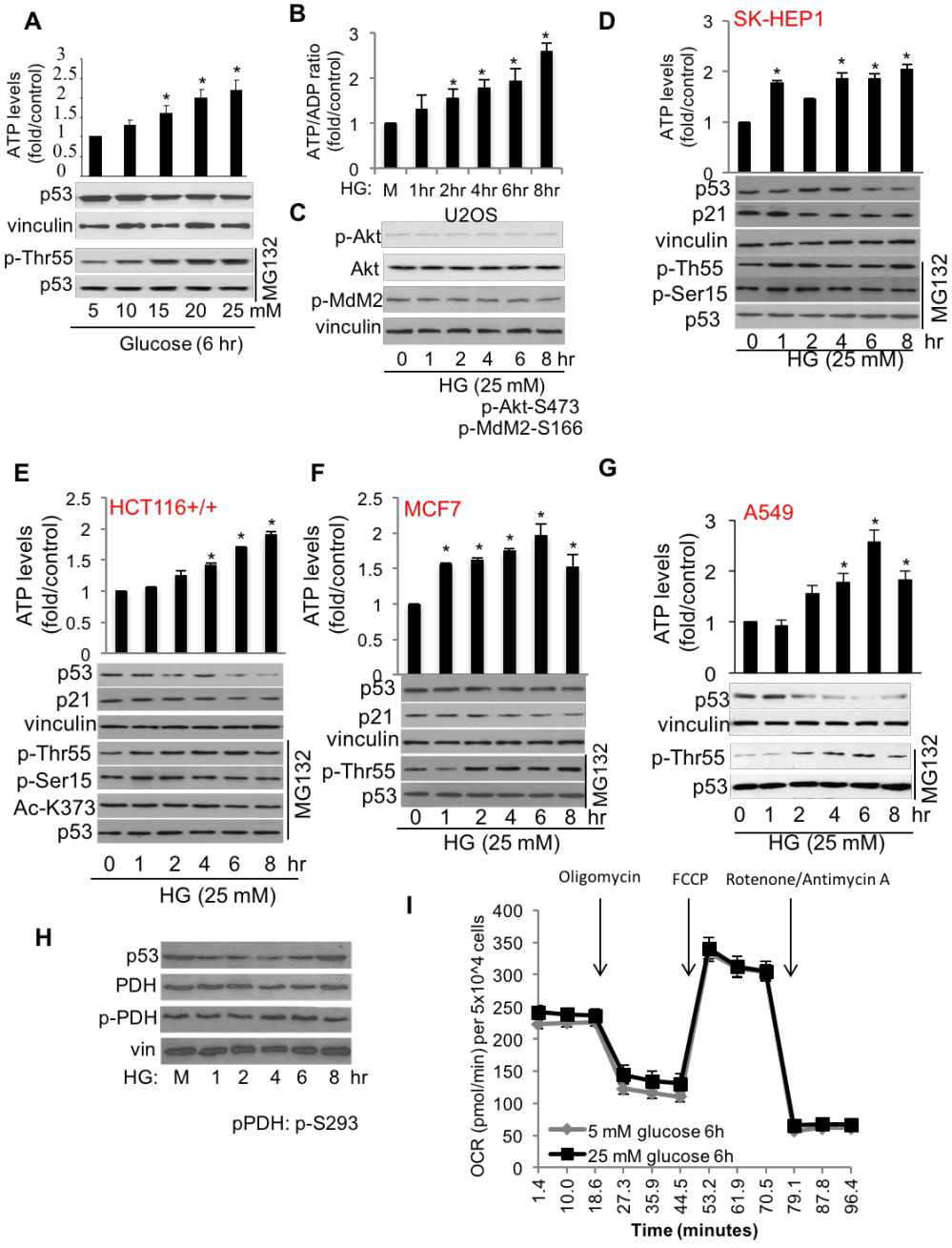

A
ATP levels (fold/control)
p53
vinculin
p-Thr55
p53
MG132
5 10 15 20 25 mM
Glucose (6 hr)
B
ATP/ADP ratio (fold/control)
HG: M 1hr 2hr 4hr 6hr 8hr
C
U2OS
p-Akt
Akt
p-MdM2
vinculin
0 1 2 4 6 8 hr
HG (25 mM)
p-Akt-S473
p-MdM2-S166
D
SK-HEP1
ATP levels (fold/control)
p53
p21
vinculin
p-Th55
p-Ser15
p53
MG132
0 1 2 4 6 8 hr
HG (25 mM)
E
HCT116+/+
ATP levels (fold/control)
p53
p21
vinculin
p-Thr55
p-Ser15
Ac-K373
p53
MG132
0 1 2 4 6 8 hr
HG (25 mM)
F
MCF7
ATP levels (fold/control)
p53
p21
vinculin
p-Thr55
p53
MG132
0 1 2 4 6 8 hr
HG (25 mM)
G
A549
ATP levels (fold/control)
p53
vinculin
p-Thr55
p53
MG132
0 1 2 4 6 8 hr
HG (25 mM)
H
p53
PDH
p-PDH
vin
HG: M 1 2 4 6 8 hr
pPDH: p-S293
I
Oligomycin
FCCP
Rotenone/Antimycin A
OCR (pmol/min) per 5x10^4 cells
5 mM glucose 6h
25 mM glucose 6h
1.4 10.0 18.6 27.3 35.9 44.5 53.2 61.9 70.5 79.1 87.8 96.4
Time (minutes)

Figure S2.1 Supplemental Material for Figure 2.1. (A, D-G) The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (A) U2OS cells treated with different concentrations of D-glucose treatment as indicated. Cell lysates underwent SDS-PAGE followed by Western blotting with various primary antibodies as shown and visualized by the enzyme-linked chemiluminescence system. The blot is a representative of 3 blots obtained from 3 separate experiments. To normalize the p53 protein level, cells were treated with MG132 before harvest. The level of Thr55-p was detected by immunoprecipitation with Thr-55 phospho-specific antibody (202 IP) and immunoblotting with anti-p53 antibody. (B) For measuring total ATP+ADP, ADP was first converted into ATP by mixing pyruvate kinase cell lysate. ADP levels were then calculated by subtracting ATP from the total ATP+ADP. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments. (C-H) Cell lysates from (C) U2OS, (D) SK-HEP1, (E) HCT116 p53 +/+, (F) MCF7, (G) A549, (H) U2OS treated with 25 mM D-glucose treatment for the time indicated. Cell lysates underwent SDS-PAGE followed by Western blotting with various primary antibodies as indicated and visualized by the enzyme-linked chemiluminescence system. The blot is a representative of 3 blots obtained from 3 separate experiments. To normalize the p53 protein level, cells were treated with MG132 before harvest. The level of Thr55-p was detected by immunoprecipitation with Thr-55 phospho-specific antibody (202 IP) and immunoblotting with anti-p53 antibody. (I) OCR measured in U2OS cells in 5mM or 25mM glucose treated cells for 6h using the XF-24 Seahorse system. Data presented are mean ± SD of five independent experiments.

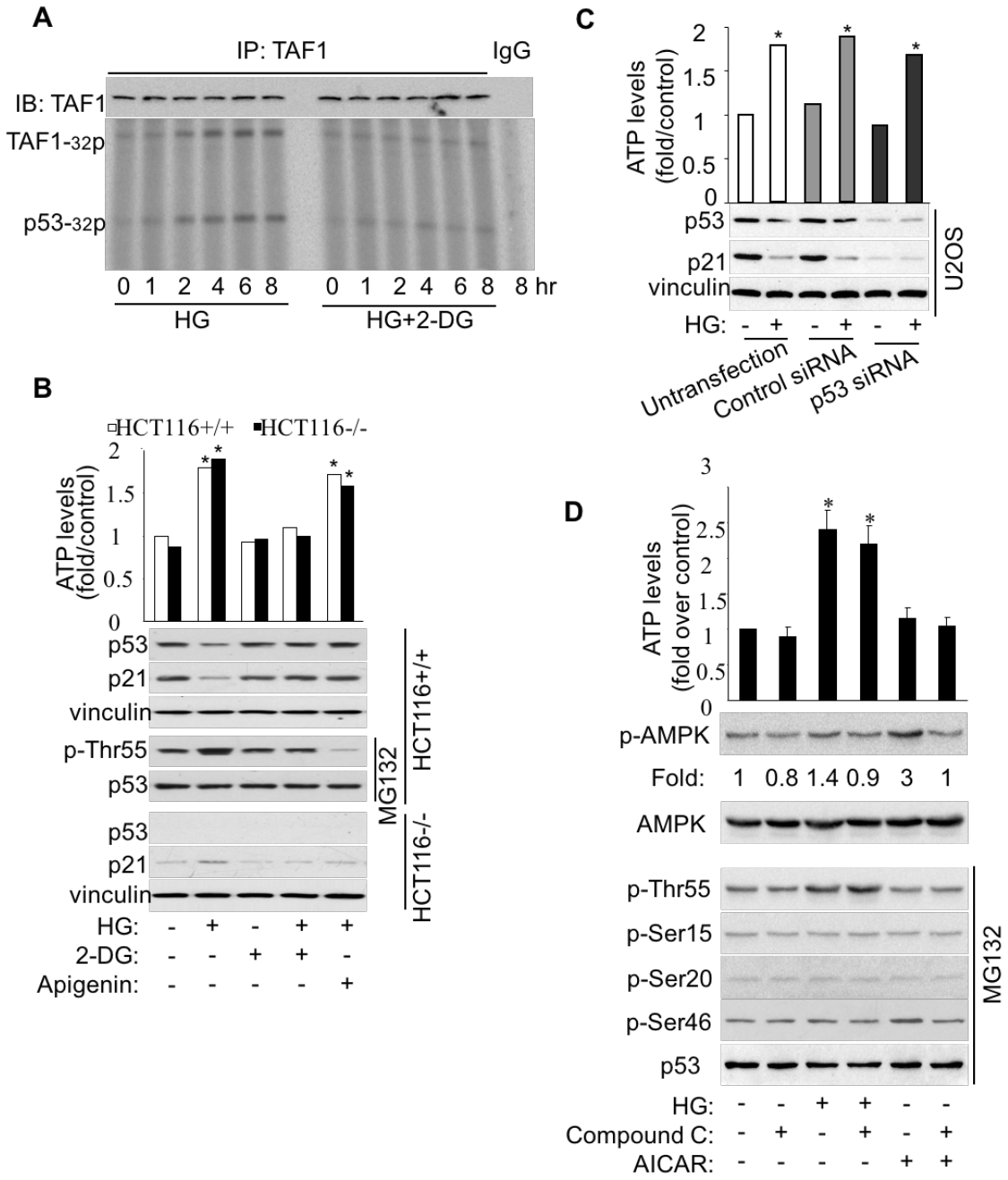

A
IP: TAF1
IgG
IB: TAF1
TAF1-32p
p53-32p
0 1 2 4 6 8 0 1 2 4 6 8 8 hr
HG
HG+2-DG
B
HCT116+/+
HCT116-/-
ATP levels (fold/control)
p53
p21
vinculin
p-Thr55
p53
MG132
HCT116+/+
HCT116-/-
HG:
2-DG:
Apigenin:
C
ATP levels (fold/control)
p53
p21
vinculin
U2OS
HG:
Untransfection
Control siRNA
p53 siRNA
D
ATP levels (fold over control)
p-AMPK
Fold: 1 0.8 1.4 0.9 3 1
AMPK
p-Thr55
p-Ser15
p-Ser20
p-Ser46
p53
MG132
HG:
Compound C:
AICAR:

Figure S2.2. Supplemental Material for Figure 2.2. (A) U2OS cells were treated with 25mM D-glucose for the times indicated, in the presence or absence of 2-DG, and TAF1 was immunoprecipitated using Ab1230 antibody. In vitro TAF1 kinase assay was performed using immunoprecipitated TAF1 along with baculovirus-expressed and purified p53. TAF1 and p53 phosphorylation were detected by either autoradiography or immunoblotting as indicated. (B-C) Protein levels of indicated proteins were detected by Western blotting using primary antibodies. The blot is a representative of 3 blots obtained from 3 separate experiments. To normalize the p53 protein level, cells were treated with MG132 before harvest. The level of Thr-55-phosphorylation was detected by immunoprecipitation with Thr-55 phospho-specific antibody (202 IP) and immunoblotting with anti-p53 antibody. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (B) Confluent HCT116+/+ and HCT116-/- cells were incubated with HG for 6 hours in the presence or absence of 2-DG (20 mM) or apigenin (40 μM). (C) U2OS cells were transfected with p53–specific siRNA or control siRNA for 42 h and then treated with HG for 6 h. (D) U2OS cells were treated with or without 25mM D-glucose for 6h, 2 mM AICAR for .5 h, or 10uM Compound C for 6h.

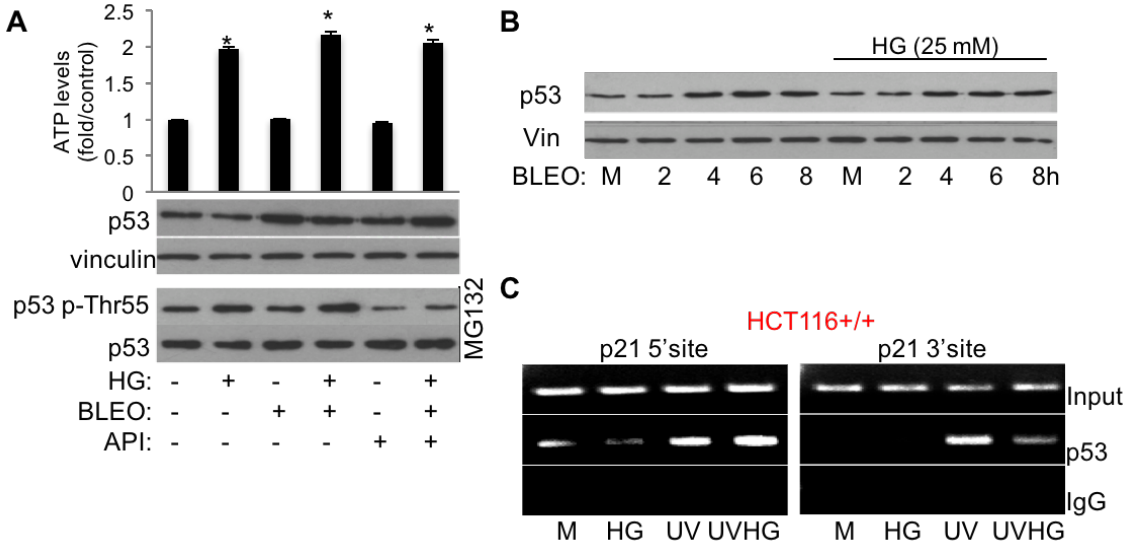
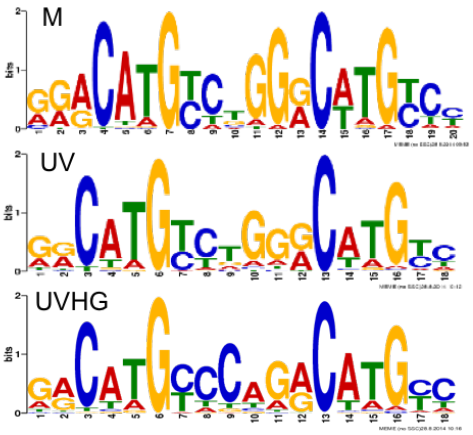


Figure S2.3. Supplemental material for Figure 2.4. (A-B) U2OS cells were treated with (A) high glucose (25 mM), bleomycin, or apigenin as indicated or (B) Bleomycin (Bleo) with or without 25mM D-glucose treatment for the times indicated. Cell lysates underwent SDS-PAGE followed by Western blotting with various primary antibodies as indicated, and visualized by the enzyme-linked chemiluminescence system. To normalize the p53 protein level, cells were treated with MG132 before harvest. The level of Thr55-p was detected by immunoprecipitation with Thr-55 phospho-specific antibody (202 IP) and immunoblotting with anti-p53 antibody. The intracellular ATP levels were determined using a luciferin/luciferase method according to manufacturer's protocol. Bars represent mean fold increases (±SD) in treated groups over basal values from 3 independent experiments (*$p < 0.05$). (C) HCT116 *p53*+/+ cells treated with 25mM D-glucose with or without bleomycin. Protein extracts were prepared and subjected to ChIP assays using the indicated antibodies. PCR was performed to test for enrichment of the indicated p21 promoter regions. PCR products were resolved by agarose-gel electrophoresis and visualized by ethidium bromide staining.

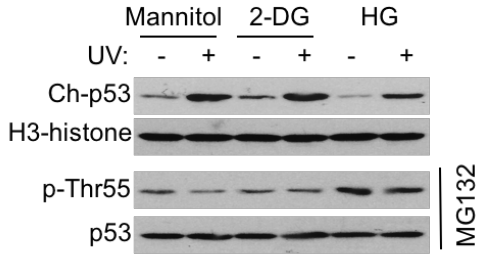
A

ChIP-Seq Sample	UV	UV HG
Numbers of p53-bound Peaks	1722	1073
Enriched peaks with UV DNA damage	700	
(+/-)10kb TSS (Peaks)	228	
(+/-)10kb TSS of Ensembl Genes	224	
(+/-)10kb TSS of RefSeq Genes	144	
Enriched peaks (UV) Inhibited by HG		286
(+/-)10kb TSS (Peaks)		95
(+/-)10kb TSS of Ensembl Genes		93
(+/-)10kb TSS of RefSeq Genes		62

B



C



D

DAVID Annotation Cluster	ES p<0.05
Response to DNA Damage Stimulus	2.75
Regulation of Cell Cycle	2.63
Regulation of Apoptosis	2.45
Cellular Response to Stress	2.3
Response to UV	2.23
Metal Ion Binding	1.46
Macromolecule Catabolic Process	1.43

Figure S2.4. Supplemental material for Figure 2.5. (A) A summary of p53 ChIP-Seq results. (B) p53-binding motif found enriched at mock-treated cells (M) (top), UV-irradiated (UV) (middle), and UV-irradiated in the presence of 25mM D-glucose treatment (bottom). (C) U2OS cells were untreated (M) or subjected to UV irradiation (20 J/m2) in the presence or absence of mannitol, 2-DG or HG. The treated U2OS cells were fractionated into chromatin-bound and unbound fractions and analyzed for p53 and histone H3 protein levels by immunoblotting of chromatin-bound fraction. The level of Thr-55-phosphorylation was detected by immunoprecipitation with Thr-55 phospho-specific antibody (202 IP) and immunoblotting with anti-p53 antibody. To normalize the p53 protein level, cells were treated with MG132 before harvest. (D) DAVID functional annotation clustering of genes where p53 DNA damage induced binding is inhibited by HG. Enriched clusters of genes with enrichment score above 1.4 (p-value < 0.05).