CHARACTERIZATION OF P53-MEDIATED TRANSCRIPTION DEREGULATION BY THE HEPATITIS B VIRUS X PROTEIN

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Summary

Hepatitis B virus X protein (HBx) is strongly implicated in hepatitis B virus-associated hepatocellular carcinoma (HCC). One of the main mechanisms by which HBx contributes to neoplastic transformation is by functioning as a transcription co-factor that alters host cellular transcription regulation. HBx is known to interact with and modulate several key transcription factors that have been shown to be deregulated in various human cancers such as p53, E2F1 and CBP/p300, and the resulting gene expression aberrations are thought to upset the delicate balance of cellular homeostasis in favour of oncogenesis. However, the role of HBx in modulating cellular transcription factors and the underlying mechanisms(s) of this deregulation are poorly understood.

In this thesis, we have examined the role of HBx in modulating transcription of the master regulator p53. This is of great interest since altered p53-mediated transcription by HBx consequentially deregulates p53 target genes that are involved in many critical cellular processes including cell cycle arrest, DNA repair, apoptosis and senescence. Although several isolated studies have shown that HBx modulates p53 transcription primarily by altering its sequence-specific DNA-binding selectivity, the findings have thus far been controversial. We therefore first examined global potentially functional p53-DNA binding alterations by HBx by integrating (i) p53 chromatin immunoprecipitation-on-chip that identified differential p53-DNA binding patterns, with (ii) expression profiling that identified differentially expressed genes in a HBx-expressing cell culture system. We found that HBx altered p53 DNA-binding characteristics in several ways: HBx enhanced, alleviated as well as induced a novel shift in p53-DNA binding, and that a subset of these

alterations was associated with deregulated expression of the corresponding genes. Interestingly, we did not find any patterns between each type of p53-DNA binding alteration (enhancement, abolishment or shift) and the corresponding gene deregulation (up-regulation or down-regulation), alluding to a more complex mode of transcription deregulation than previously proposed.

To dissect this modulation of p53 sequence-specific DNA binding by the viral X protein, we further characterized a HBx-deregulated candidate from the global p53 ChIP-on-chip and expression profiling studies - p53-regulated apoptosis-inducing protein 1 (*p53AIP1*). We present strong evidence from detailed mutagenesis and promoter studies that HBx induced a novel shift in p53 binding from the promoter to intron 1 of the *p53AIP1* regulatory region and that this directly resulted in a deregulated, increased expression of *p53AIP1*. Importantly, we also found significantly higher *p53AIP1* expression in HCC patients with high HBx protein levels, highlighting the relevance of our *in vitro* findings in hepatocarcinogenesis. We further show that increased *p53AIP1* expression has biologically functional consequences – that of mediating HBx-induced apoptosis. Having demonstrated the potentially detrimental consequences of p53 transcription deregulation by the viral protein, we further investigated the mechanism(s) by which HBx modulated p53.

Using bioinformatics analysis complemented with experimental validation studies, we demonstrate that the shift in p53 binding at the *p53AIP1* regulatory region was linked to a mechanism by which HBx perturbs specific p53-associated co-regulatory modules. Essentially, HBx disrupted a transcriptionally repressive p53-YY1-GATA-1-HDAC1

complex at the *p53AIP1* promoter, but instead favoured recruitment of p53 with the co-activator Sp1 at the intron 1 region that further recruited the transcription co-activator PCAF in a transcriptionally stimulating complex. We show that by tipping the HDAC-HAT balance, HBx induced a specific p53 Lys320 'acetylation switch' that is in part responsible for altering p53 binding site selectivity and consequent *p53AIP1* deregulation. Consistent with our finding that particular transcription co-factors are favourably recruited with p53 in the presence of HBx, we found that distinct transcription factor motifs - including that of Sp1 - were selectively co-enriched in the vicinity of the p53 binding sites in the presence of HBx from the global p53 ChIP study. Moreover, in agreement with our finding that HBx-induced acetylated p53 Lys320 preferentially bound to the more structurally conserved intron 1 consensus site of *p53AIP1*, analysis of the consensus sites bound by p53 in the presence of HBx from the global p53 ChIP study revealed a similar preference for more conserved p53 response elements. These universal findings provide support for our proposed model of deregulated p53-mediated transcription as a global mechanism of p53-regulated transcription by the viral X protein

Collectively, we have demonstrated for the first time, a role for the viral X protein in upsetting the carefully orchestrated transcription regulation by the master regulator p53. Using *p53AIP1* as a model, we showed that HBx perturbs the dynamic interplay of transcription co-factors and co-regulators as well as specific p53 post-translational modifications that are critically needed for proper cellular homeostasis. The work in this thesis has thus provided invaluable insights to the transcription co-factor role of HBx in contributing to hepatocarcinogenesis and provides important directions for future efforts in the field of HBx research.

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List of Abbreviations

7-AAD	7-Amino-actinomycin D
ATM	ataxia telangiectasia mutated
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Cy	cyanine fluorescent dye
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGFP	enhanced green fluorescence protein
FBS	fetal bovine serum
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	Hepatocellular carcinoma
hr	hour
HRP	horse radish peroxidase
Ig	immunoglobulin
kDa	kilodalton
MAT	model-based analysis of tiling arrays
min	minute
mRNA	messenger RNA
NP-40	Nonidet P-40
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween 20
PCR	polymerase chain reaction
PE	phycoerythrin
PTM	post-translational modification
PVDF	polyvinylidene fluoride
PWM	position weight matrix
qPCR	quantitative real-time polymerase chain reaction
RE	response element
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.	standard error
sec	second
siRNA	small interfering RNA
TFBS	transcription factor binding site
Tris	tri-hydroxymethyl-aminomethane
UV	ultraviolet
WT	wild-type

List of Publications

C. Chan, Y. Wang, P.K.H. Chow, A.Y.F. Chung, L.L.P.J. Ooi, and C.G. LEE. A Novel Modulation of p53 Sequence-Specific DNA Binding by the Hepatitis B Virus X Protein. (In submission)

C Chan, C.G.L. Lee. (2010) Insights into host transcription modulation by the hepatitis B virus X protein. *Trends in Cancer Research*. 6: 55-68.

Abstracts Presented at Conferences

"Hepatitis B virus X protein alters p53 regulation of p53AIP1 gene". The EMBO Meeting 2011, Vienna, Austria, September 2011. Poster.

"Hepatitis B virus X protein modulates p53 transcriptional activity, deregulating p53AIP1 gene". Singhealth Duke-NUS Scientific Congress, Singapore, October 2010. Poster*.

* Winner of Best Poster Award (Basic Sciences), Student Category

Other Awards

Best Research in Progress Presentation, Duke-NUS, September 2009 – February 2010. "Elucidating p53-associated pathways that are deregulated by the hepatitis B virus X protein, HBx in hepatocellular carcinoma".

Chapter 1 Introduction

1.1 Hepatitis B virus associated hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer deaths with an estimated 500,000 HCC-related deaths annually (El-Serag and Rudolph, 2007; Parkin *et al.*, 2005). Its poor prognosis of less than a 3% 5-year survival rate is largely due to late symptom manifestation and unresponsiveness to treatment. At present, surgical resection and liver transplantation are still the most effective methods to treat the disease, but these options are only feasible in the minority of cases where the tumours are small and localized. Several risk factors are known to be associated with HCC. These include hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, aflatoxin b1 exposure and excessive alcohol intake. Of these risk factors, chronic HBV infection is most strongly associated with HCC, accounting for more than half of HCC cases worldwide and an estimated 80% in highly endemic areas such as Southeast Asia and sub-Saharan Africa. With approximately 350 million people chronically infected with HBV worldwide (Seeff and Hoofnagle, 2006), this presents a pressing global health problem that needs greater attention.

HBV is a small, partially double-stranded DNA virus belonging to the family of the Hepadnaviruses and has a tropism for hepatocytes. The 3.2 kb HBV genome contains four partially overlapping open reading frames (ORFs), *preS/S*, *preC/C*, *P* and *X*. (Figure 1.1A) The *preS/S* ORF encodes the preS1 (Large), preS2 (Middle) and S (small) viral surface proteins. The *preC/C* ORF encodes the core antigen (HBcAg) and the soluble antigen e

(HBeAg) while the P ORF encodes the viral polymerase. The smallest ORF is the X ORF that is regulated by HBV enhancer 1 and the X promoter and encodes the regulatory X protein (HBx). Of the four viral proteins, HBx has been strongly implicated in the carcinogenesis process of HBV-associated HCC. HBx is selectively over-expressed (Parkin *et al.*, 2005; Poussin *et al.*, 1999) and is the most frequently integrated viral factor in tumours of HCC patients (Paterlini et al 1995), highlighting its importance in establishing and maintaining viral infection.

1.2 Hepatitis B virus X protein

1.2.1 Structure of HBx

The X ORF is regulated by HBV enhancer 1 and the X promoter and encodes the small 17 kDa regulatory X protein (HBx). Characterization of the viral X protein has been largely hampered by the absence of a three-dimensional structure due to its insoluble nature and its weak sequence homology to known motifs and domains. Nevertheless, sequence analysis and deletion studies of HBx have provided some insights to its functional domains. Several conserved regions comprising amino acid (aa) residues 1-20, 58-84 and 120-140 were identified by analyzing the X protein sequences of mammalian hepadnavirus. Deletion studies of the viral X protein identified a negative regulatory domain at the amino-terminus (aa 1-20) (Misra *et al.*, 2004; Murakami *et al.*, 1994) where its cysteine residues have been implicated in folding (Rui *et al.*, 2005) or dimerization of the protein (Urban *et al.*, 1997) (Figure 1.1B). A trans-activation domain was identified at the carboxy-terminus (aa 48-150) that contained essential trans-activation elements of HBx (Kim et al., 1993; Kumar et al., 1996b; Renner et al., 1995; Takada and Koike, 1990a; Yoo et al., 2004). Also found within the trans-activation domain were two regions that exhibited significant homology to the

Figure 1.1

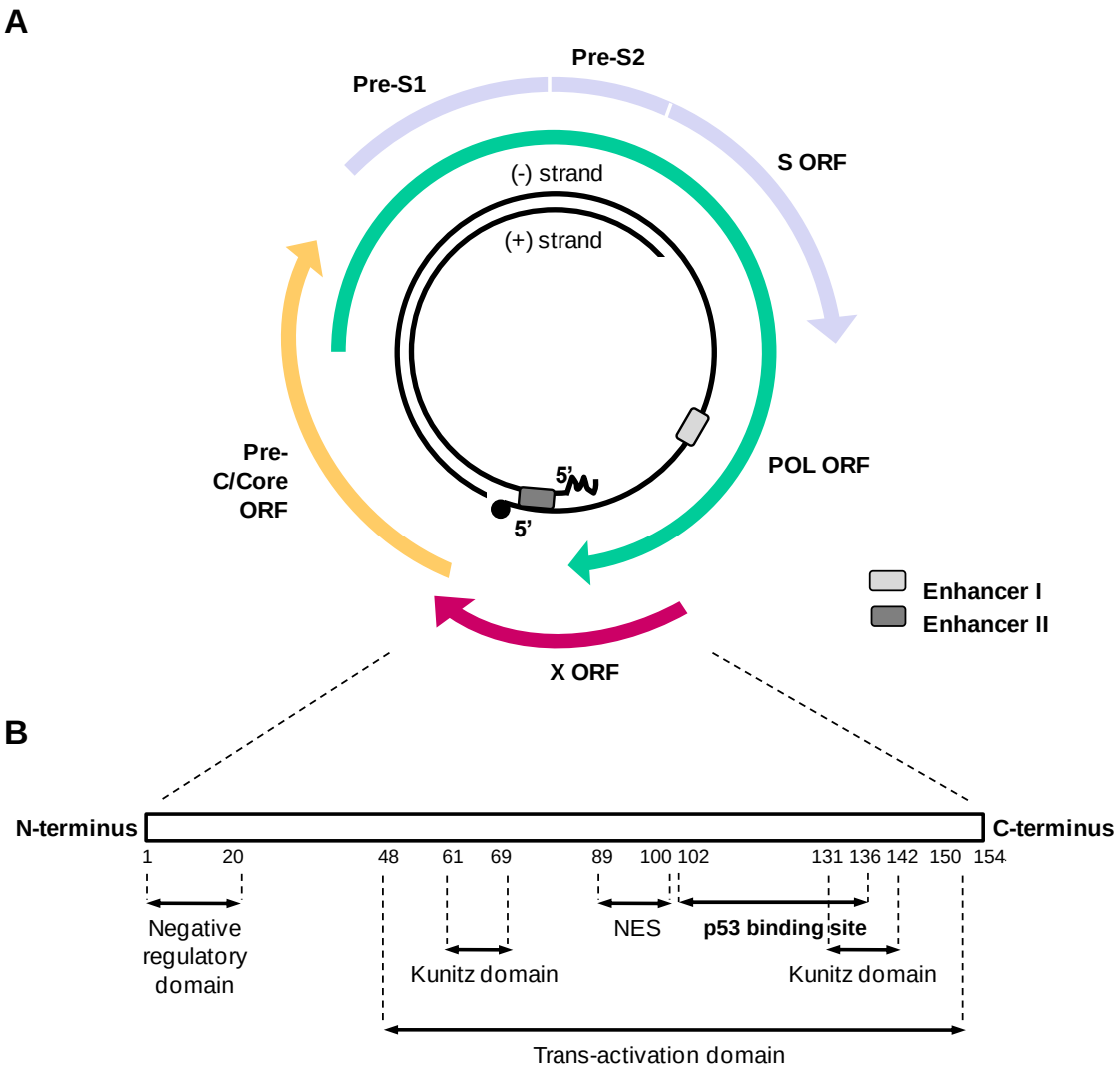


Figure 1.1. HBV genome and HBx protein. A. Structure of the HBV genome. The partially double-stranded 3.2kb viral DNA is depicted by two circular black lines. The black circle represents the viral polymerase attached to the 5' of the minus strand. The 4 arrows represent the 4 open reading frames (ORFs) and the rectangles represent the viral enhancers I and II. B. The HBx protein. The 154-amino acid HBx protein is depicted by the rectangle and the relevant amino acid positions are indicated by the numbers directly below. The regions containing the reported functional domains are demarcated by double-arrows. The nuclear export signal is abbreviated by NES. Adapted from Cheryl Chan and Caroline G. L. Lee. 2010, Insights into host transcription modulation by the hepatitis B virus X protein, *Trends in Cancer Research*, Vol. 6, 55-68.

Kunitz domain that is characteristic of Kunitz-type serine protease inhibitors, although no studies have since confirmed this proposed function of HBx (Takada and Koike, 1990b). Additionally, a leucine-rich nuclear export signal motif was mapped to residues 89-100 that has been reported to influence the subcellular distribution of HBx in a nuclear export receptor Crm1-dependent manner (Forgues *et al.*, 2001). Adjacent to the nuclear export signal is a p53 binding region (aa 102-136) that was identified by deletion studies to be essential for the interaction of HBx with the tumour suppressor p53 protein (Lin et al., 1997b). Spectroscopic assays showed that HBx appears to be an unstructured protein and proposed that HBx adopts a structured conformation following interaction with host proteins. The structural flexibility of HBx is thought to facilitate its interaction with a myriad of host proteins that accounts for its multifunctional nature (Rui *et al.*, 2005).

1.2.2 Function of HBx

HBx is a multifunctional viral regulator. It is conserved in mammalian hepadnaviruses and plays a key role in viral replication. This is exemplified by the woodchuck hepatitis virus (WHV) genome that is deficient in the X gene (WHx) where viral replication in the animal host was found to be either absent (Chen *et al.*, 1993), or compromised, akin to that of attenuated viruses (Zhang *et al.*, 2001). Conversely, introduction of HBx in *trans* restored production of viral components in a cell culture model (Nakatake *et al.*, 1993) and restored HBx-deficient HBV replication to wild-type levels in an acute hepatitis *in vivo* mouse model (Keasler *et al.*, 2007, 2009). These studies strongly support the important role of the viral X protein in HBV replication.

HBx is strongly implicated in neoplastic transformation. The multifunctional viral protein has been shown to induce neoplastic transformation in various cell culture systems (Seifer *et al.*, 1991; Shirakata *et al.*, 1989) and induces HCC in HBx transgenic mice studies either alone (Kim *et al.*, 1991; Koike *et al.*, 1994) or in combination with oncogenes such as c-Myc (Terradillos *et al.*, 1997), H-Ras (Kim *et al.*, 2001b) or upon exposure to the hepatocarcinogen diethylnitrosamine (Madden *et al.*, 2001; Slagle *et al.*, 1996; Zhu *et al.*, 2004). Moreover, treatment with short-interfering RNAs (siRNAs) that artificially reduced HBx expression levels reduced tumour development in nude mice (Chan and Ng, 2006). Whether HBx functions as a tumour initiator or a tumour promoter, the pivotal role of HBx in cellular transformation and malignancy is evident.

HBx was found largely localized to the cytoplasm in hepatocytes of human liver biopsies, while a fraction of hepatocytes contained nuclear HBx (Hoare *et al.*, 2001).Several *in vitro* studies have also detected HBx in association with the mitochondria (Clippinger and Bouchard, 2008; Takada et al., 1999). Li *et al.* identified 7 amino acid residues in the carboxy-terminus of HBx (aa 111-117) in which cysteine115 is the most crucial residue for mitochondrial targeting (Li et al., 2008). Separately, HBx has also been reported to localize to the mitochondria through its interaction with human voltage-dependent anion channel HVDAC3, altering mitochondrial transmembrane potential (Rahmani et al., 2000).

Generally, HBx deregulates host processes through direct interaction with host proteins in two ways (Doria *et al.*, 1995). On the one hand, cytoplasmic HBx trans-activates cellular signalling pathways including MAP kinase signalling (Lin et al., 1997c; Tarn et al., 2001), Jak1/STAT protein kinase C signalling, transforming growth factor beta (TGF-β)

signalling (Lee *et al.*, 2001) and Wnt/β-catenin signalling (Cha *et al.*, 2004). On the other hand, nuclear HBx functions as a transcription co-factor that interferes with the regulation of host genes. Unlike other DNA viral proteins such as the Epstein-Barr Virus Nuclear Antigen 1 (EBNA1) that deregulates cellular gene transcription by binding directly to regulatory elements of DNA, HBx does not bind DNA directly (Avantaggiati *et al.*, 1993; Siddiqui *et al.*, 1989). Instead, HBx is indirectly recruited to DNA through interaction with various sequence-specific transcription factors.

Importantly, HBx has been found to interact with various factors that regulate cellular transcription. HBx reportedly interacts with components of the basal transcription machinery such as RPB5, a common subunit of RNA polymerases (Cheong *et al.*, 1995), TFIIB (Lin *et al.*, 1997a), TATA-binding protein TBP (Qadri *et al.*, 1995) as well as ERCC3 and ERCC2 DNA helicase subunits of TFIIH (Qadri *et al.*, 1996) to stimulate transcription (Maguire *et al.*, 1991; Seto *et al.*, 1990; Unger and Shaul, 1990) (Table 1.1). In addition, HBx also associates with transcription factors and modulates their DNA-binding characteristics and/or transcription activity. Notably, HBx has been reported to associate with and modulate key transcription factors that are deregulated in various human cancers including E2F1 (Choi *et al.*, 2001; Sung *et al.*, 2009), p53 (Feitelson et al., 1993; Truant et al., 1995a; Wang et al., 1994a), YY1 (Sung *et al.*, 2009), CREB-binding protein/p300 (Cougot *et al.*, 2007) and the bZip family of transcription factors. A list of reported HBx-interacting transcription factors and the consequences on transcription regulation are summarized in Table 1.2. Several other transcription factors were also identified to mediate HBx-induced gene deregulation but have not been shown to directly associate with HBx (Table 1.3). With the exception of the Sp1 transcription factor that has been shown not to interact with HBx (Lee *et al.*, 1998b), the association of HBx with the other transcription factors AP-1, AP-2, NF-κB and Oct-1

Table 1.1 Summary of components of the basal transcription machinery that interact with HBx.

Transcription regulator	HBx interaction^		Interaction assay	References
	in vitro	*cellular*		
TFIIH (ERCC2 and ERCC3)	Y	N	Affinity chromatography using Mal-ERCC2 or GST-ERCC3 and ^{35}S methionine-labeled *in vitro* translated HBx and vice versa	(Qadri *et al.*, 1996)
RPB5	Y	Y	Far-Western blotting using GST-HBx or GST-RBP5; co-IP of transiently transfected HBx and RBP5; sedimentation and IP of endogenous RPB5 and detection of over-expressed HBx	(Cheong *et al.*, 1995)
TBP	Y	N	GST affinity chromatography using GST-HBx and ^{35}S methionine-labeled *in vitro* translated TBP; co-IP of HA-tagged TBP and GST-HBx	(Qadri *et al.*, 1995)
TFIIB	Y	Y	Far-Western blotting and GST pull-down	(Lin *et al.*, 1997a)

^Y and N denote Yes and No respectively; IP denotes immunoprecipitation. Reproduced from Cheryl Chan and Caroline G. L. Lee. 2010, Insights into host transcription modulation by the hepatitis B virus X protein, *Trends in Cancer Research*, Vol. 6, 55-68.

Table 1.2 Summary of transcription factors that interact with HBx, with resulting effect on gene transcription.

^

Transcription factor	HBx interaction		Interaction assay	Effect on gene transcription	References
	in vitro	*cellular*			
bZip family					
ATF-2	Y	N	I^{125}-labeled recombinant peptides in solution to detect direct protein-protein interaction with membrane-immobilized proteins	Enhances trans-activation of PEPCK	(Kong *et al.*, 2000)
CREB	Y	N		Increases CREB binding to CRE sites; enhances trans-activation in CREB-dependent assay system; trans-activates IL-8	(Maguire *et al.*, 1991; Mahe *et al.*, 1991; Williams and Andrisani, 1995)
ATF-2/CREB	Y	N		Increases ATF-2/CREB binding to HBV enhancer	(Maguire *et al.*, 1991)
ATF3	Y	Y	GST pull-down of GST-HBx and detection of ^{32}P-labeled bZip proteins; mammalian two-hybrid assay	Increases ATF3 binding to DNA in *in vitro* DNA binding assay, enhances trans-repression activity of ATF3 in reporter assay	(Barnabas *et al.*, 1997; Zhou *et al.*, 1994)
C/EBPβ	Y	Y			
gadd153/Chop10	Y	Y			
ICER IIγ	Y	Y			
AR	N	Y	co-IP of transiently-transfected AR and HA-tagged HBx	Increases AR DNA binding in presence of DHT ligand, enhances trans-activation of reporter gene	(Lee *et al.*, 1998a; Su and Schneider, 1996)
C/EBPα	Y	N	*In vitro* DNA binding assay of GST-C/EBPα and MBP-HBx	Enhances trans-activation of PPAR-γ, PEPCK	(Choi *et al.*, 1999; Kim *et al.*, 2007; Kong *et al.*, 2000)
E2F1	Y	N	Amylose pull-down assay using MBP-fused HBx and GST-E2F1	Trans-activates *Rb* promoter cooperatively with E2F; trans-activates SOAT2	(Choi *et al.*, 2001; Choi *et al.*, 2002; Sung *et al.*, 2009)

E4F1	Y	N	Yeast two-hybrid assay and GST pull-down of GST-p120E4F and His-tagged HBx	Reduces trans-activation of reporter gene	(Rui *et al.*, 2006)
HIF-1	Y	Y	GST pull-down assay; co-IP of FLAG-HBx and GFP-HIF-1α	Trans-activates MDR1 and carbonic anhydrase 9	(Amaro *et al.*, 1999; Holotnakova *et al.*, 2010; Moon *et al.*, 2004)
HNF1	Y	Y	IP of HA-tagged HBx and HNF1	Enhances trans-activation of reporter gene	(Weil *et al.*, 1999)
MAZ	Y	Y	GST pull-down assay; co-IP of endogenous MAZ and transiently-transfected tagged HBx/HBx-expressing stable cells	Enhances trans-repression of hTERT	(Kim *et al.*, 2001a)
NF-AT1	Y	Y	GST pull-down assay, co-IP of HA-tagged NF-AT1 and Flag-tagged HBx	Enhances trans-activation of reporter gene	(Carretero *et al.*, 2002)
p53	Y	N	GST pull-down assays with over-expressed and endogenous p53	Inhibits trans-activation of PTEN	(Feitelson et al., 1993; Truant et al., 1995a; Wang et al., 1994a)
SMAD4	Y	Y	GST pull-down assay, co-IP of HBx and endogenous SMAD4 in HBx-expressing stable cells	Trans-activates CYP17A1 and trans-represses IL17B	(Park *et al.*, 2006; Sung *et al.*, 2009)
SREBP1	N	Y	Co-IP of HBx and HA-SREBP1c, GST pull-down of GST-SREBP1c	Enhances trans-activation of Fas	(Kim *et al.*, 2007)
YY1	N	Y	Co-IP of transiently introduced HBx and endogenous YY1	Trans-represses AICDA and GRIN2D	(Sung *et al.*, 2009)
RXR	Y	Y	GST pull-down assay; mammalian yeast two-hybrid assay	Enhances trans-activation of PEPCK	(Kong *et al.*, 2000)

^Y and N denote Yes and No respectively. Reproduced from Cheryl Chan and Caroline G. L. Lee. 2010, Insights into host transcription modulation by the hepatitis B virus X protein, *Trends in Cancer Research*, Vol. 6, 55-68.

Table 1.3 Summary of transcription factors that have not been shown to interact with HBx, but are reported to mediate HBx-induced gene deregulation.

Transcription factor	HBx effect on gene transcription	Remarks	References
AP-1	Enhances trans-activation of c-jun	NA	(Seto *et al.*, 1990; Zhou *et al.*, 1994)
AP-2	Enhances trans-activation of TGF-α	NA	(Kim and Rho, 2002; Seto *et al.*, 1990)
NF-κB	Enhances trans-activation of HIV LTR, IL-8, IL-6, iNOS, cyclin D1	HBx activates NF-κB by acting on its cytoplasmic inhibitors, interacts with IκBα	(Amaro *et al.*, 1999; Gomez-Gonzalo *et al.*, 2001; Kim *et al.*, 2001a; Lee *et al.*, 1998a; Majano *et al.*, 2001; Park *et al.*, 2006; Su and Schneider, 1996; Twu *et al.*, 1989; Weil *et al.*, 1999)
Oct-1	Trans-activates/trans-represses human U6 depending on pre-initiation complex assembly	NA	(Antunovic *et al.*, 1993)
Sp1	Enhances trans-activation of IGF-II, reduces trans-activation of XPB, enhances trans-repression of XPD	Reported not to interact with HBx using protein affinity chromatography, but HBx augments DNA-binding activity of phosphorylated Sp1	(Jaitovich-Groisman *et al.*, 2001; Lee *et al.*, 1998b)

Reproduced from Cheryl Chan and Caroline G. L. Lee. 2010, Insights into host transcription modulation by the hepatitis B virus X protein, *Trends in Cancer Research*, Vol. 6, 55-68.

remains to be investigated. Nevertheless, the numerous reports of transcription factors that interact with and are deregulated by HBx highlight the important role of the viral X protein as a transcription co-factor in modulating cellular transcription, resulting in aberrant gene expression that may contribute to hepatocarcinogenesis.

1.3 HBx and p53

To understand the transcription co-factor role of HBx in hepatocarcinogenesis, this thesis is specifically focused on characterizing the modulation of the transcription factor p53 by HBx and its consequent effects on p53-regulated gene expression. P53 was chosen for the following reasons: firstly, the tumour suppressor protein is widely considered as a master regulator of critical cellular processes such as cell cycle arrest, DNA repair, apoptosis and senescence. Hence, deregulation of p53-mediated transcription by the viral X protein is of great biological significance as this would conceivably upset the maintenance of cellular genomic integrity and homeostasis. Although more than 60% of cancers have been reported to possess mutated or inactivated p53, p53 mutations in the early stages of HCC are infrequent (Feitelson *et al.*, 1993), thus highlighting the relevance of our study. Secondly, despite limited literature reports in this field, modulation of p53 by HBx is the most studied among the known HBx-interacting transcription factors. Thirdly, the availability of a relatively large body of knowledge on general p53 transcription regulation would serve to facilitate our study on the deregulation of p53 transcription by the viral X protein. On these biologically pertinent and pragmatic bases, the tumour suppressor p53 protein was selected for studying the role of the HBx transcription co-factor in contributing to hepatocarcinogenesis. To delineate the deregulation of p53 by HBx, it is first imperative to expound on the function and regulation of p53.

1.3.1 Transcription factor p53

p53 functions predominantly as a transcription factor that enables the cell to mount an appropriate adaptive response to various stress stimuli generally by either arresting the cell and repairing the damage, or eliminating the defective cell if the damage is irreparable. In unstressed cells, p53 is maintained at low levels by its negative regulator mouse double minute 2 (MDM2; HDM2 in humans), an E3 ubiquitin ligase that tags the protein for proteasomal degradation. Upon genotoxic stress such as DNA damage, oncogene activation and hypoxia, p53 is rapidly stabilized following MDM2 degradation and translocates to the nucleus. P53 is also phosphorylated and stabilized by protein kinases such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) and CHK2 that are activated in response to genotoxic stress (Figure 1.2). Depending on the stress signal and the stress reponse pathways that are activated, the p53 protein is further modified by a host of protein modifiers such as CREB-binding protein CBP/p300 and histone deacetylases (HDACs) that influence p53 binding site selectivity. Together with other sequence-specific DNA-binding transcription factors as well as transcription co-regulators, p53 transactivates or transrepresses discrete functional group(s) of target genes to direct an appropriate cellular response (Figure 1.2). Examples of p53 target genes that are involved in cell cycle arrest and DNA repair include *p21* (*cdkn1a*) and *Gadd45A*; p53 target genes that are involved in apoptosis regulation include *Bax, Fas, Puma, p53AIP1* and *Noxa*.

Figure 1.2

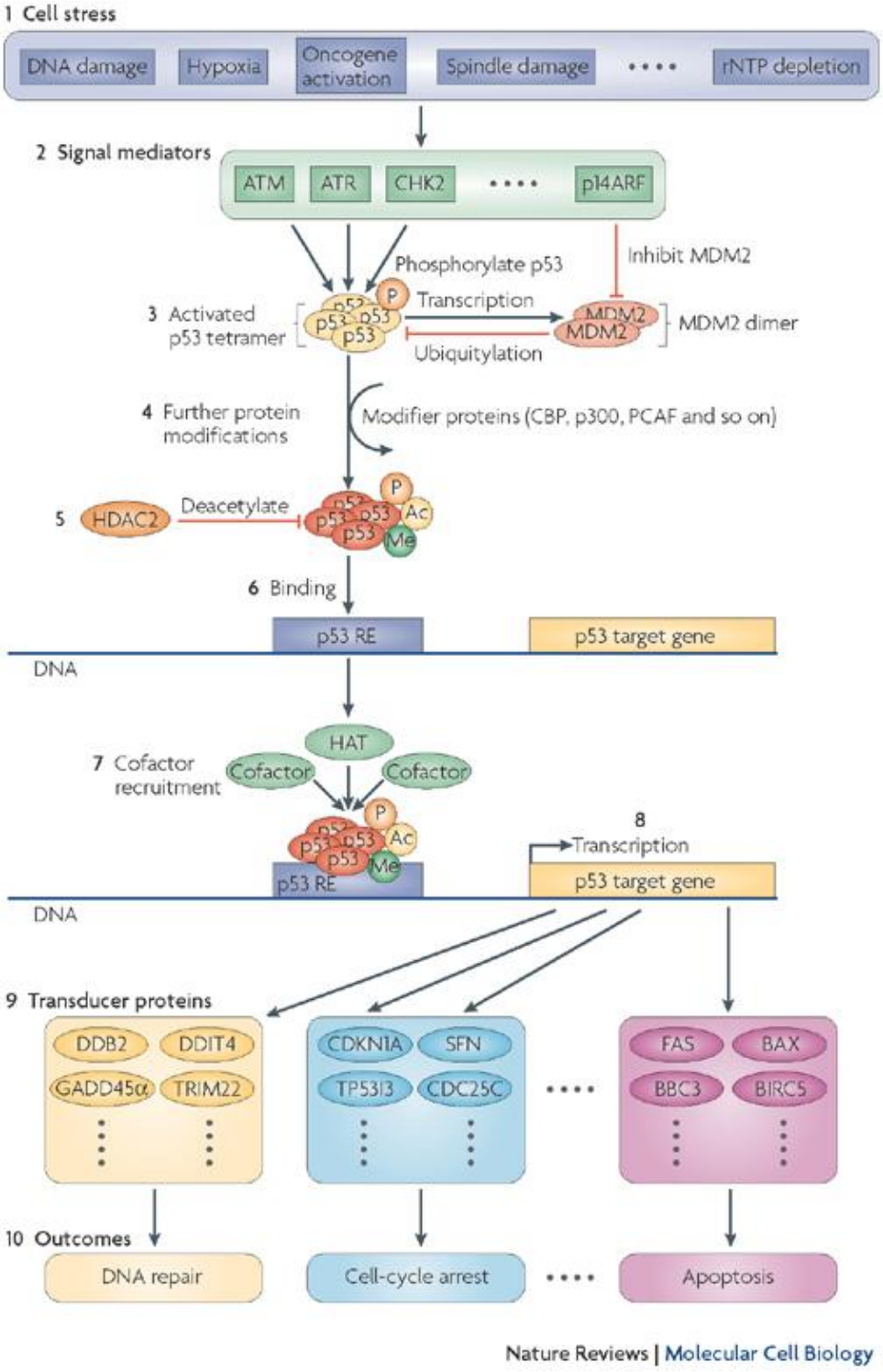


Figure 1.2. Illustration of p53-mediated response to cell stress. Various cell stress activate different signal mediators that mark p53 post-translationally. Post-translationally-modified p53 selectively binds its response element and recruits various transcription co-factors. Together, they effect transcription of genes that determine cell fate. Reprinted by permission from Macmillan Publishers Ltd. *Nat. Rev. Mol. Cell Biol.* (Riley *et al.*, 2008) © 2008.

Figure 1.3 illustrates the various structural domains of the p53 protein. This consists of two tandem amino-terminal transactivation domains TAD1 (aa 20-40) and TAD2 (aa 40-60) at the amino-terminus for transcription activation or repression of p53-responsive genes, an overlapping proline-rich domain for interaction with other proteins (aa 40-92), a core DNA-binding domain (aa 100-300), a linker region (aa 301-306), a tetramerization domain for oligomerization (aa 307-355) and a carboxy-terminal regulatory domain (aa 356-393). A nuclear export signal (NES) as well as a nuclear localization signal (NLS) have been found in the tetramerization domain and carboxy-terminal domain respectively.

p53 binds as a dimer of dimers (or a tetramer) to DNA in a sequence-specific manner. The p53 consensus motif is typically composed of two cognate half-sites that can be separated by a spacer sequence of 0-13 base pairs. Each half-site consists of a considerably degenerate decamer sequence 5'-RRRCWWGYYY-3' where R denotes a purine (adenine or guanine, A/G), Y denotes a pyrimidine (cytosine or thymine, C/T) and W denotes A/T. Based on X-ray crystallography studies, the core RCWWGY motif of the p53 response element was found to be in close contact with the amino acid residues of p53 DNA-binding domain and is thus regarded as the most important bases for p53-DNA binding (Tang et al., 2006a). Particularly important are the C and G nucleotides at positions 4 and 7 respectively of each half-site that are highly conserved. Since the p53 consensus sequence was defined, we now have a growing list of p53 target genes that contain one or more consensus sites in their promoter/intron/exon region.

Figure 1.3

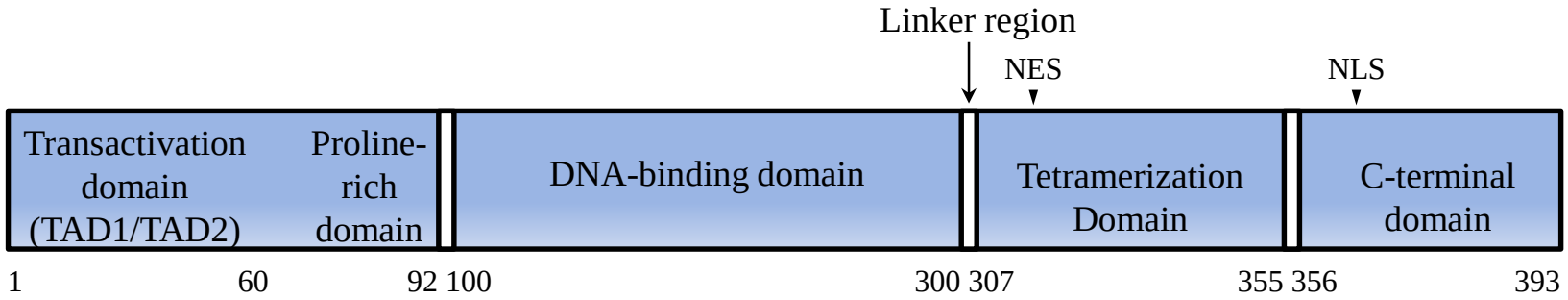


Figure 1.3 Structure of p53. The 393-amino acid p53 protein is depicted by the rectangle and the relevant amino acid positions are indicated by the numbers directly below. The regions containing the reported functional domains are indicated. The nuclear export signal is abbreviated by NES, nuclear localization signal is abbreviated by NLS.

1.3.2 P53 target gene selection

Binding of p53 to its response elements is spatially and temporally regulated. Also referred to as 'promoter selectivity', this trait is particularly evident in the response of p53 to various stress signals. Depending on the nature and extent of cellular stress, p53 induces distinct groups of p53-responsive genes by selectively associating with their promoters to elicit the desired response eg. cell cycle arrest or apoptosis. However, how p53 selects for some response elements and not others remains the subject of intense investigation. In the next section, the three major factors that can influence p53 promoter selectivity are described: (i) chromatin structure of the p53 response element, (ii) post-translational modification of the p53 protein, and (iii) p53 binding partners.

1.3.2.1 Chromatin structure of p53 response elements

In eukaryotes, DNA is packaged around histone proteins, forming nucleosomes which are the basic repeating units of chromatin. Thus, an obvious structural determinant that influences p53 promoter selectivity is the structure of the chromatin of the region encompassing the p53 response element of the target genes. It has been shown that some p53 response element-containing regulatory regions exist in a constitutively 'open' (nucleosome-free) conformation and hence accessible for p53 binding, while others exist in a 'closed' conformation, occluded by nucleosomes that render them inaccessible for p53 binding. Chromatin structure can be altered by ATP-dependent chromatin remodelers such as SWI/SNF, Polycomb and nucleosome remodelling and histone deacetylase (NuRD) complexes that alter nucleosome positioning along DNA, as well as factors that covalently modify histones such as histone acetyltransferases/deacetylases and methyltransferases/demethylases. These alter DNA accessibility to transcription regulators that consequently influences gene transcription. Notably, both chromatin remodelers and

histone modifiers have been implicated in oncogenesis and cancer progression (Lai and Wade, 2011). Of special mention is the NuRD complex of proteins. In addition to its ATP-dependent chromatin remodelling activity, the NuRD complex also possesses lysine deacetylase activity conferred by histone deacetylases HDAC1 and HDAC2 that has been shown to deacetylate lysine residues of the p53 protein, and inhibit p53-dependent transcriptional activation and its function in growth arrest and apoptosis (Kew, 2011).

1.3.2.2 p53 post-translational modifications

The p53 protein is extensively post-translationally modified (Figure 1.4). At least 21 different serine (Ser) and threonine (Thr) residues of p53 have been reported to be modified by phosphorylation. Majority of these residues are located at the amino-terminal trans-activation domain and the carboxy terminal regulatory domain and phosphorylation at these sites are thought to stabilize the p53 protein in response to stress. Moreover, p53 can be ubiquitinated at several lysine (Lys) residues residing mostly at the carboxy terminal regulatory domain. Mono-ubiquination at these sites has been shown to facilitate nuclear export of the p53 protein (Carter et al., 2007) while poly-ubiquitination is known to target p53 for proteasomal degradation (Li et al., 2003). Additionally, Lys residues at the tetramerization domain and carboxy-terminus regulatory domain of p53 can also be acetylated and have been shown to influence p53 sequence-specific DNA binding selectivity (Gu and Roeder, 1997; Liu *et al.*, 1999; Luo *et al.*, 2004; Sakaguchi *et al.*, 1998a). Lastly, p53 can also be altered by methylation, sumoylation (Chen and Chen, 2003; Gostissa et al., 1999; Rodriguez et al., 1999; Schmidt and Muller, 2002) and neddylation (Abida et al., 2007; Xirodimas et al., 2004), highlighting the myriad of post-translational modifications that regulate the p53 protein.

Figure 1.4

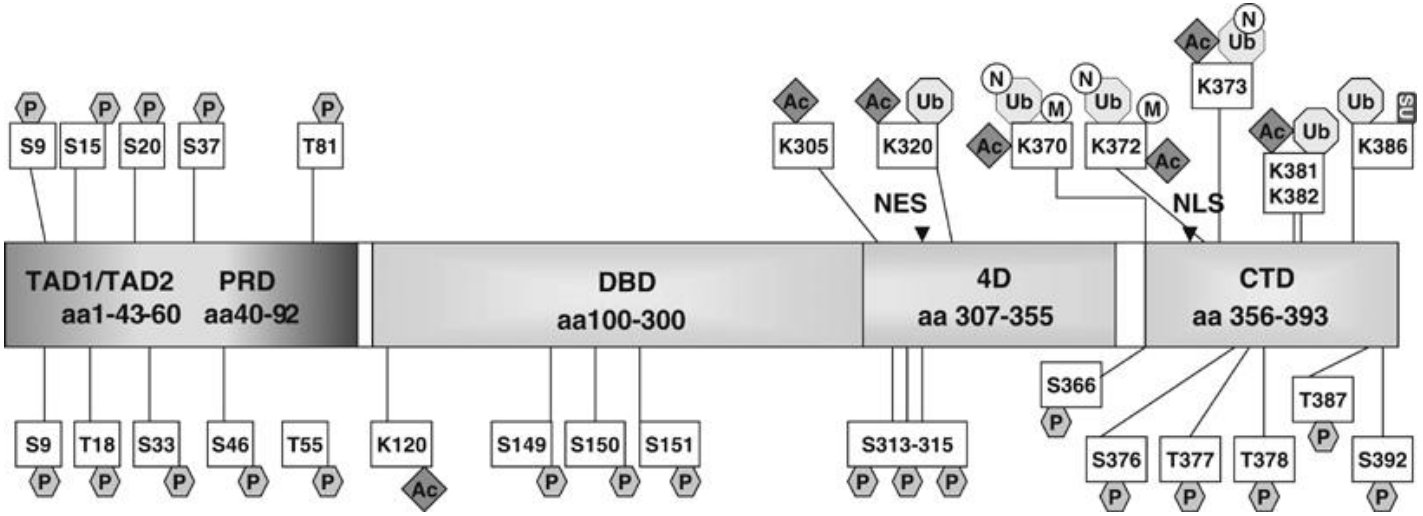


Figure 1.4. p53 post-translational modifications. Phosphorylation (P), acetylation (Ac), ubiquitination (Ub), sumoylation (SU), neddylation (N) and methylation (M) of p53 amino acid (aa) residues throughout the protein. Transactivation domain 1/2 (TAD1/TAD2), proline-rich domain (PRD), DNA-binding domain (DBD), tetramerization domain (4D) and carboxy-terminal domain (CTD) of p53. Reprinted by permission from Macmillan Publishers Ltd. *Cell Death Differ.* (Olsson *et al.*, 2007) © 2007.

Importantly, regulation of p53 by post-translational modifications has been shown to influence its target gene selection that directs a particular cellular outcome (Appella and Anderson, 2001; Jansson *et al.*, 2008). It has been reported that specific factors/pathways are activated in response to specific cell stresses that converge on the p53 in the form of a 'p53 post-translational code' to selectively activate (or repress) transcription of the appropriate functional group of genes to elicit the desired cellular response. For instance, ATM-dependent phosphorylation of p53 at Ser15 in response to γ-irradiation selectively transactivated genes that are involved in growth arrest and apoptosis (Banin *et al.*, 1998; Siliciano *et al.*, 1997).

Another example of p53 target gene selection by post-translational modifications is the phosphorylation of p53 Ser46 by various kinases such as protein kinase C delta (PKCdelta) (Chuikov *et al.*, 2004), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) (Oda *et al.*, 2000) and homeodomain-interacting protein kinase 2 (HIPK2) (Gu and Roeder, 1997) that are activated upon DNA damage. Phosphorylated Ser46 has been reported to specifically stimulate transcription of the pro-apoptotic gene *p53AIP1* (Oda *et al.*, 2000). The mechanism by which p53 phosphorylation modulates transcription of its target genes however, remains to be clarified. Acetylation of p53, on the other hand, has been shown to alter its sequence-specific DNA-binding property. It is thought that such modifications of p53 induce conformational changes in the protein that alter its affinity for response elements with particular DNA structural characteristics. For example, Knights *et al.* demonstrated that different p53 acetylation patterns show varying affinities for different types of p53 response elements that are associated with specific cellular outcomes (Knights *et al.*, 2006). The authors reported that acetylated-p53 Lys373 exhibited a preference for low-affinity binding sites that were found in several pro-apoptotic genes, thereby promoting cell death;

contrastingly, acetylated-p53 Lys320 exhibited a preference for high-affinity binding sites such as that of *p21*, thus promoting cell survival. These studies highlight the biological relevance of the various post-translational modifications in regulating p53-mediated transcription.

Clearly, the myriad of p53 post-translational modifications presents an intricate mechanism for fine-tuning p53 transcription regulation. Although the aforementioned examples illustrate simply the role of post-translational modifications in regulating p53 target gene selection, the p53 post-translational code is far more complicated. Firstly, each modifying factor, for example ATR, can phosphorylate multiple p53 Ser residues including Ser15 and 37. Secondly, various post-translational modifications can 'compete' for the same p53 residue. For instance, p53 Lys320 can be acetylated by p300/CBP-associated factor PCAF (Sakaguchi *et al.*, 1998a) or ubiquitinated by E3 ligase E4F1 (Le Cam *et al.*, 2006), while p53 Lys372 can be acetylated by CBP/p300 (Gu and Roeder, 1997), ubiquitinated by MDM2 (Nakamura *et al.*, 2000) or methylated by SET9 (Chuikov *et al.*, 2004). Thirdly, crosstalk also exists between the various post-translational modifications. Upon severe DNA damage, p53 is phosphorylated at Ser46 and this modification has been shown to induce acetylation at p53 Lys382, promoting apoptosis (Hofmann *et al.*, 2002; Puca *et al.*, 2009). Lastly, examination of various p53 post-translational modifications in knock-in animal models - albeit in limited cell types and on limited p53 target genes - have surprisingly revealed generally modest phenotypic changes, alluding to possible compensatory mechanisms *in vivo*. Nevertheless, a dynamic combination of p53 post-translational modifications can be induced in response to various stress stimuli that may influence p53 promoter selectivity to direct an appropriate cellular response.

1.3.2.3 p53 binding partners

Another factor that has been described to influence p53 promoter selectivity is its context-specific interaction with various protein partners such as members of the ASSP family, Brn3 family of POU domain transcription factors, Y-box binding protein YB1, NFkB, BRCA1 and Pin1. Interestingly, association of p53 with Brn-3b stimulated pro-apoptotic *bax* expression while association of p53 with Brn-3a repressed *bax* expression but augmented transactivation of *p21*, driving cell fate towards cell cycle arrest (Elmore et al., 1997; Lee et al., 2005a; Scheffner et al., 1990). Similarly, ASSP family members ASSP1 and ASSP2 were reported to selectively stimulate p53 binding to promoters of pro-apoptotic genes *bax* and *Pig3* but not to that of cell cycle arrest genes *cdkn1a* and *mdm2* while interaction of p53 with an anti-apoptotic ASSP family member iASSP inhibited transactivation of these pro-apoptotic genes (Scheffner *et al.*, 1993; Woo *et al.*, 2011). Notably, the interaction of p53 with selected binding partners can also be affected by post-translational modifications of the p53 protein. This is exemplified by the interaction between p53 and Pin1. Following genotoxic stress, the prolyl isomerase Pin1 specifically recognizes p53 phosphorylated at Ser46, displacing iASSP from p53 that drives cells towards apoptosis (Ryoo *et al.*, 2004; Zheng *et al.*, 2002). Hence, a complex interplay of structural and regulatory factors is likely to be involved in p53 target gene selectivity.

1.3.3 p53-mediated transcriptional repression

In addition to transcriptionally activating its target genes by binding and recruiting general transcription factors as well as histone modifiers to their promoter and/or enhancer regions, p53 can also transcriptionally repress its target genes. P53-mediated transcriptional repression however is less well defined.

Several mechanisms have been proposed to account for its direct transrepressive function. Firstly, binding of p53 to its response element can occlude overlapping or adjacent binding sites of other strong transcriptional activators, thus preventing their recruitment and abrogating their stimulatory effect of the target gene. This is evident from the mutually exclusive binding of p53 and hepatic nuclear factor HNF-3 at the repressor domain of alpha-fetoprotein (AFP) gene. HNF-3 recruitment stimulated AFP transcription while p53 sequence-specific binding conversely repressed AFP transcription (Liu *et al.*, 2000). Secondly, p53 can also repress transcription by recruiting and cooperating with transcription co-repressors such as the aforementioned histone deacetylases (Harrod *et al.*, 2003; Martinez-Balbas *et al.*, 2000). Another example is the interaction of p53 with YY1 that disrupts the association of p53 with the co-activator p300, abrogating transcription of the target gene (Tang et al., 2006b). In addition, some studies suggest that the DNA sequence of the p53 response element itself can determine whether p53 activates or represses gene transcription. Interestingly, a study that analyzed the spacer lengths of validated p53 response elements showed that sites associated with p53-mediated transcription repression (or repressor sites) contained motifs with longer spacers as compared to sites associated with transcription activation (or activator sites) (O'Connor *et al.*, 1995; Sykes *et al.*, 2006). Separately, another study reported that p53 transcriptional response was determined by the orientation of its quarter-sites (Brandner, 2010). However, these DNA sequence-centric theories are unable to predict transcription outcomes of p53-regulated genes that have a more complex regulatory landscape. For instance, some p53-responsive genes such as *p21* (Mertens *et al.*, 2002) and *PERP* (McPherson *et al.*, 2002; Sala *et al.*, 1996) harbour multiple functional p53 response elements in their regulatory region. Furthermore, not all p53-DNA binding events affect gene transcription. These studies thus underscore the complexity of p53-mediated transcription regulation that is currently still under investigation.

1.3.4 HBx modulation of p53

HBx interacts with p53 and alters its sequence-specific DNA binding that is associated with deregulated gene expression. Various studies to date however, report differing effects of HBx on p53-DNA binding. HBx was demonstrated to inhibit p53 sequence-specific DNA binding in *in vitro* DNA binding assays and reporter assays (Knights et al., 2006; Ogden et al., 2000b; Wang et al., 1994a). In contrast, a study by Truant *et al.* showed that HBx enhanced p53 oligomerization on DNA oligonucleotide (Truant et al., 1995a). Importantly however, the modulation of p53 sequence-specific DNA binding by HBx resulted in aberrant expression genes that have been implicated in heptocarcinogenesis. For example, HBx was reported to disrupt p53-mediated transcription of a tumour suppressor PTEN in cellular promoter assays, possibly by decreasing p53 binding to its response element at the PTEN promoter (Chung *et al.*, 2003). Although these few initial studies provided invaluable insights to the co-factor role of HBx in modulating p53 in hepatocarcinogenesis, little progress has been made since to meticulously characterize this modulation as well as to elucidate the mechanism of deregulation of the viral protein.

1.4 p53AIP1 and the role of HBx in apoptosis

1.4.1 p53AIP1

Our initial study identified the pro-apoptotic bona fide p53 target gene p53-regulated apoptosis-inducing protein *p53AIP1* to be deregulated by HBx in a p53-dependent manner. In this thesis, *p53AIP1* is used as a model to study the modulation of p53 by HBx and the mechanism(s) underlying this deregulation.

P53AIP1 was first discovered through direct cloning of p53 binding sequences from human genomic DNA by Oda *et al.* Its expression was demonstrated to be induced by p53 and a functional p53 response element that was identified in the intron1 region of the gene was implicated in its p53-mediated transactivation (Feitelson *et al.*, 1993). Significantly, the authors identified a unique DNA damage-inducible, site-specific phosphorylation of p53 at Ser46 that selectively stimulated *p53AIP1* transcription. They further demonstrated that Ser46-phosphorylated p53 selectively bound to the intron 1 p53 response element of *p53AIP1* but not to that of *p21*, implicating the p53 site-specific post-translational modification in p53 promoter selectivity and apoptosis induction. Since, various kinases such as protein kinase C delta (PKCdelta) (Chuikov *et al.*, 2004), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) (Oda *et al.*, 2000) and homeodomain-interacting protein kinase 2 (HIPK2) (Gu and Roeder, 1997) have been shown to phosphorylate p53 at Ser46 in response to DNA damage. The consequent increase in *p53AIP1* expression promoted apoptosis through the mitochondrial apoptotic pathway by disrupting the mitochondria membrane potential, resulting in cytochrome c release (Feitelson *et al.*, 1993; Sakaguchi *et al.*, 1998b).

1.4.2 The role of HBx-in p53-mediated apoptosis

P53 can initiate apoptosis, or programmed cell death, in response to a variety of stimuli including as viral infection and cell stress to eliminate damaged cells. Two apoptotic pathways have been described: the extrinsic pathway that responds to external stress signals through engaging cell surface ‘death receptors’, and the intrinsic pathway that alters responds to signals within the cell that alters mitochondrial membrane permeability through an interplay of pro- and anti-apoptotic regulators. The extrinsic and intrinsic pathways converge by activating caspases – a family of cysteine proteases that are key to the cell death

machinery in executing apoptosis and effecting cellular destruction. Evasion of apoptosis has long been considered one of the hallmarks of cancer. Recent reports however suggest that apoptosis can either promote or suppress tumourigenesis depending on the context and cell type examined.

Although there have been contradicting reports of the viral X protein in p53-mediated apoptosis, an increasing body of work points towards the pro-apoptotic role for HBx. Several initial studies described that HBx abrogates p53-mediated apoptosis (Knights *et al.*, 2006; Liu *et al.*, 1999; Sakaguchi *et al.*, 1998a). A subsequent study using stably-expressing HBx in various cell lines demonstrated that the effect of HBx on apoptosis induction might be cell type-dependent (Luo *et al.*, 2004). This study reported that an immortalized murine hepatocyte cell line AML12 stably expressing HBx showed a reduction in apoptosis, while a HBx stable human hepatoma cell line HepG2 exhibited enhanced apoptosis. Over the years, more and more reports have consistently shown that the viral protein is involved in apoptosis induction. The earliest reports advocated a pro-apoptotic role of the viral X protein both in cells stably-expressing HBx (Feitelson et al., 1993; Wang et al., 1994b) as well as in transiently-expressing HBx cells (Truant et al., 1995b). Wang *et al.* demonstrated that HBx sensitised hepatocytes to p53-mediated apoptosis through activating the p38 mitogen-activated protein kinase (MAPK) pathway that stabilized as well as enhanced transcription of p53, resulting in stimulated transcription of pro-apoptotic genes *Bax, Fas* and *Noxa* (Elmore et al., 1997; Lin et al., 1997b). We and others have independently demonstrated that HBx sensitised cells to programmed cell death after DNA damaging treatment (Chao et al., 2006; Ogden et al., 2000a; Yun et al., 2000). Recently, HBx was shown to induce apoptosis in tumour cell lines but not in non-tumorous cell lines (Lee et al., 1995). From these studies, it is

becoming apparent that HBx induces p53-mediated apoptosis but how this facilitates hepatocarcinogenesis is still being intensely researched.

1.5. Technological advancement

1.5.1 Chromatin immunoprecipitation and expression profiling

Traditional approaches used to characterize the modulation of p53-DNA binding by HBx as described above were analyzed mainly in an artificial context using oligonucleotide templates and purified proteins in *in vitro* DNA binding assays as well as mutagenesis studies and reporter assays. Significantly, the advent of chromatin immunopreciptation (ChIP) assay enabled protein-DNA interactions to be examined in a physiological context. This assay is now used widely to capture transcription factor-DNA interactions in its native environment, that is, in the context of the chromatin structure and cellular milieu. In ChIP, DNA-binding proteins are cross-linked to DNA and protein-DNA complexes are immunoprecipiated using an antibody specific for the protein of interest. Cross-links are reversed and the regions bound by the transcription factor (also known as ChIP-enriched DNA) can be recovered and quantified by quantitative real-time PCR. Moreover, recent advancement of technological platforms to detect ChIP-enriched DNA on a genome-wide scale such as using high density genome-wide tiling arrays (ChIP-on-chip) as well as massive parallel sequencing (ChIP-Seq) has afforded unprecedented capacity in mapping the binding sites of the transcription factor of interest. As the technology developed and became more cost-effective over the last few years, global maps of transcription factor binding sites have been elucidated. In addition, integration of genome-wide transcription factor-DNA interaction profiles with global gene expression profiles obtained using gene expression microarrays have facilitated the identification of direct gene targets of the transcription factor of interest (collectively known

as regulatory modules) (Abida et al., 2007; Feng et al., 2005; Lin et al., 1997b; Sung et al., 2009; Terui et al., 2003). Furthermore, bioinformatics analysis of the binding profile of a particular transcription factor can reveal other adjacent co-enriched transcription factors that co-operate with the transcription factor of interest (collectively termed co-regulatory modules). This strategy proved successful in identifying transcription factors that co-operate with SMAD in a context specific manner (SMAD co-regulatory modules) (Qin *et al.*, 2009) as well as co-regulatory modules of OCT4 and SRY (Nakatake *et al.*, 1993).

In addition to analysing the direct binding of a transcription factor of interest to DNA, ChIP has enabled the detection of proteins that bind DNA indirectly. As samples are cross-linked prior to ChIP, we can now capture huge protein complexes in association with DNA. This has been particularly useful in examining the transcription co-factor function of HBx. Previous work in our lab had demonstrated that ChIP using high-affinity HBx-specific antibodies successfully elucidated patterns of HBx indirect binding to DNA as well as HBx direct gene targets (Sung *et al.*, 2009). As HBx indirectly binds DNA through association with sequence-specific DNA-binding transcription factors, our lab had successfully used HBx-ChIP coupled with bioinformatics analysis to identify these HBx-interacting transcription factors such as E2F1, YY1 and SMAD4 that mediate the indirect binding of HBx to DNA. Furthermore, integration of global HBx-ChIP with expression profiling enabled the identification of target genes directly deregulated by HBx. Similarly, the binding profiles of transcription co-regulators such as histone deacetylases and acetyltransferases that bind DNA indirectly can also be examined using ChIP with the antibody specific for the factor of interest.

Thus, the advancement of technological platforms for genome-wide mapping of transcription factor binding sites together with the current widespread application of microarray profiling of global gene expression have brought with it an enormous capacity to uncover the complex regulatory circuits that govern cellular gene expression, and hence its deregulation.

1.5.2 Experimental tools in our lab

Research in the field of HBx has been largely hampered by the lack of high-affinity antibodies specific for the viral protein. Importantly, our lab has generated high affinity HBx-specific antibodies that have been shown to be amenable for use in various applications including immunoblotting, immunocytochemistry and immunoprecipitation (Chao *et al.*, 2006). Moreover, a recombinant HBx-expressing adenoviral system was also developed in our lab to efficiently introduce the viral X protein into HepG2 liver cells (Figure 1.5A) and that expresses HBx at physiologically relevant levels (Chao *et al.*, 2006) (Figures 1.5B and C).

Figure 1.5

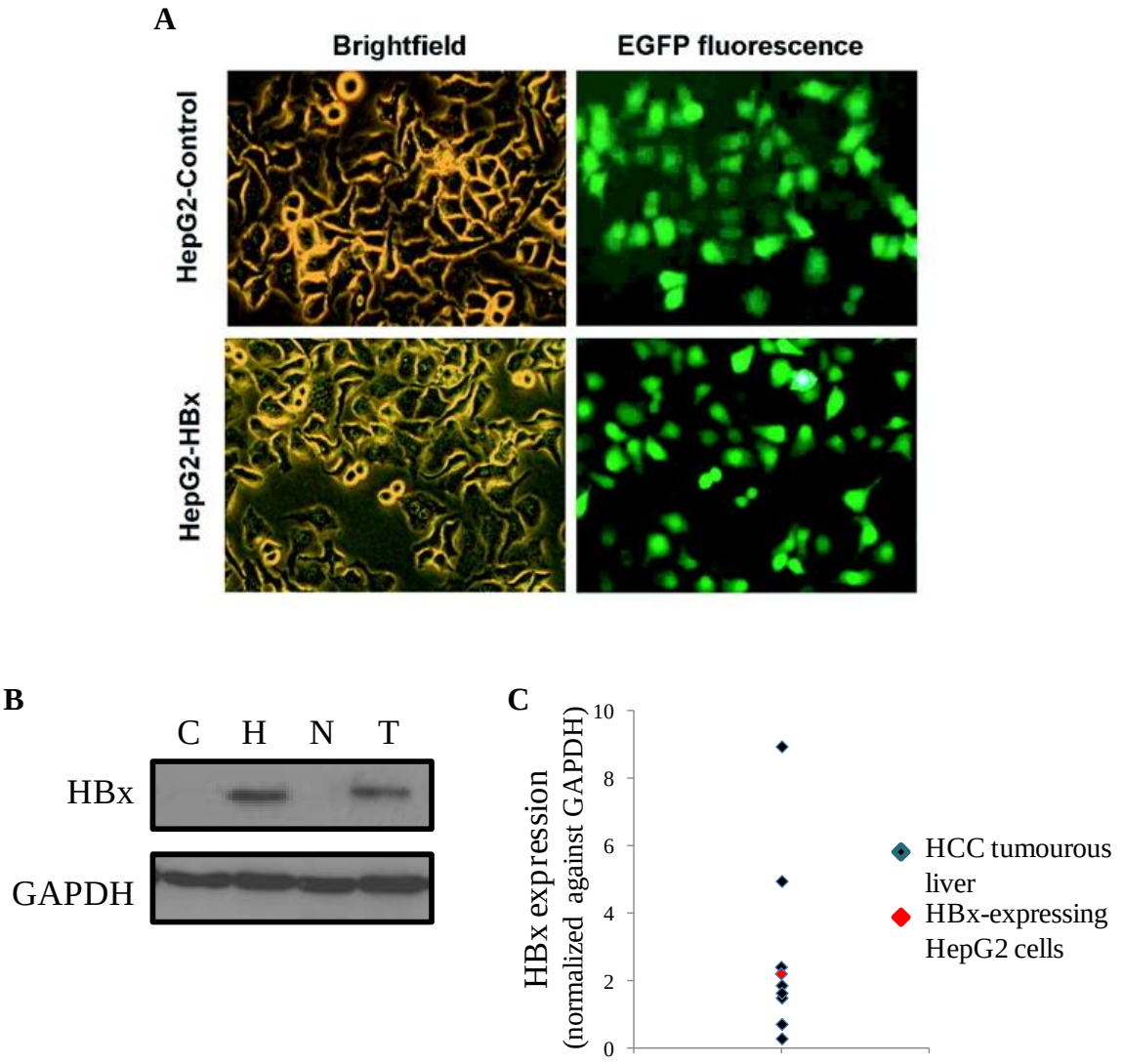


Figure 1.5. Experimental tools. A. Images of UV-treated HepG2 cells transduced with either control or HBx-expressing recombinant adenovirus. This research was originally published in The Journal of Biological Chemistry. Lee *et al.* The hepatitis B virus X protein sensitizes HepG2 cells to UV light-induced DNA damage. *J Biol. Chem*. 2005, 280(39): 33525-35. © the American Society for Biochemistry and Molecular Biology. B. Immunoblot of HepG2 cell lysates probed with antibodies specific for HBx and GAPDH. C and H denote control and HBx cell lysates respectively; N and T denote non-tumorous and HCC tumour lysates respectively. C. Range of HBx protein expression levels in HCC tumourous liver samples and in HBx-expressing HepG2 cells.

1.6 Objectives of this thesis

In this thesis, I sought to accomplish three main objectives to elucidate the role of HBx in hepatocarcinogenesis that are outlined below:

1. To identify patterns of modulated p53-DNA binding that are linked to p53 target gene deregulation by HBx. To this end, a cell culture HBx model was employed for p53 ChIP-on-chip and expression profiling (Figure 1.6). A known p53 target gene *p53AIP1* identified from this study that was deregulated by HBx with associated altered p53 binding at its regulatory region was selected for further characterization.

2. To characterize the p53-mediated deregulation of *p53AIP1* by HBx. Mutagenesis and promoter studies were used to systematically investigate the effect of HBx-induced altered p53-DNA binding on target gene expression. Furthermore, the functional and clinical importance of *p53AIP1* deregulation were also addressed.

3. To elucidate the mechanism by which HBx modulates p53 with respect to *p53AIP1* deregulation. Using a computational approach complemented by experimental validation, we define a novel mechanism of HBx in altering p53 sequence-specific DNA binding that involves an interplay of differential transcription co-regulator recruitment and p53 post-translational modification(s).

Figure 1.6

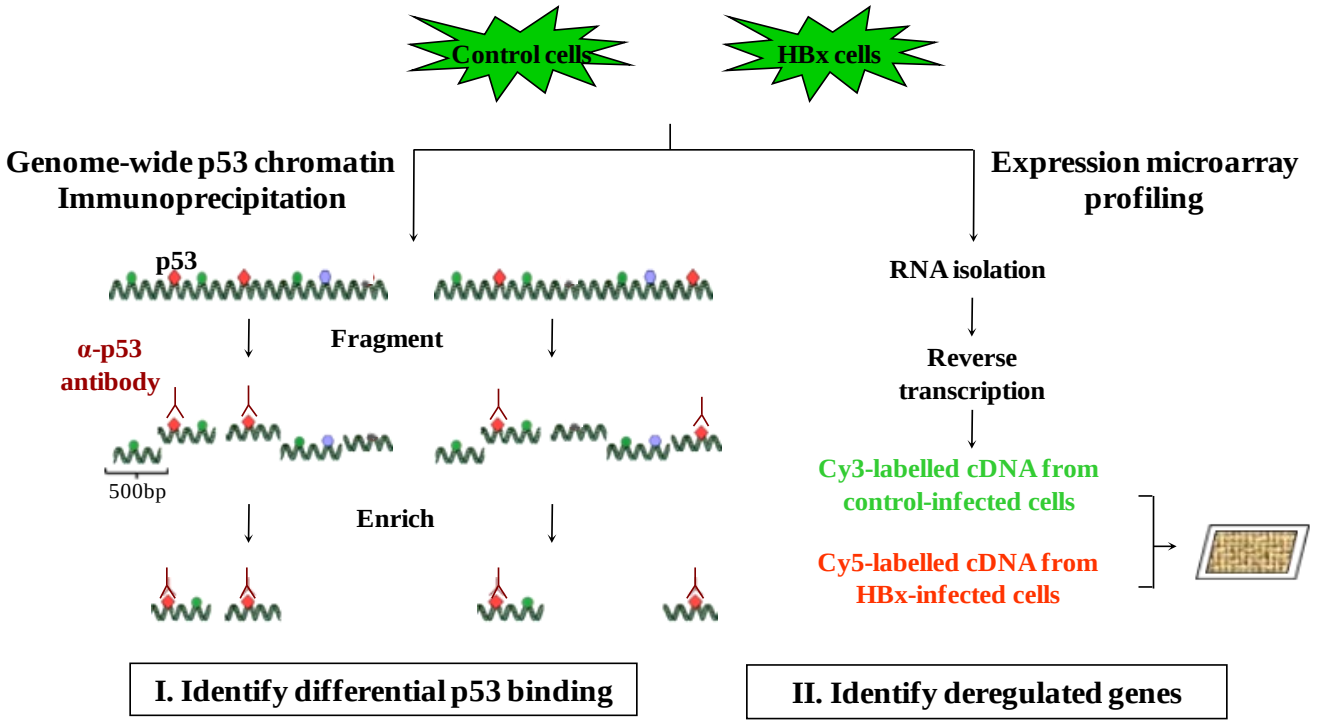


Figure 1.6. Illustration of our strategy to identify HBx-modulated p53-DNA binding with associated gene deregulation. Differential p53-DNA binding was identified by p53 chromatin immunoprecipitation using on control and HBx-expressing cells coupled with microarray or sequencing. HBx-deregulated genes were identified by expression array profiling.

1.7 Significance of this thesis

In delineating the oncogenic transcription co-factor function of the hepatitis B virus X protein, we present first evidence that HBx alters global p53 sequence-specific DNA binding properties that were linked to corresponding deregulated gene expression patterns. We observed that HBx enhanced, alleviated as well as induced a novel shift p53-DNA binding at the regulatory regions of genes and that these changes associated with alterations in their expression.

Using a validated candidate from the global study - a known p53-regulated gene *p53AIP1* as a model, I further characterized the role of HBx in modulating p53 sequence-specific binding and transcription regulation. I have demonstrated that HBx deregulates *p53AIP1* through a novel shift in p53-DNA binding at its regulatory region. Additionally, I have shown that *p53AIP1* expression is also aberrantly increased in tumours of HCC patients with high HBx expression, highlighting the relevance of our finding in hepatocarcinogenesis. Moreover, I have demonstrated the functional importance of increased *p53AIP1* expression in mediating HBx-induced apoptosis *in vitro*, emphasizing the potential of HBx-induced p53 transcription deregulation in disrupting cellular homeostasis.

Significantly, our findings further define a mechanism of action for the viral X protein in deregulating p53 transcription. I have shown by using *p53AIP1* as a model, that HBx perturbs the recruitment of distinct p53-associated transcription co-factors that result in the differential recruitment of co-regulators such as lysine deacetylases and acetyltransferases. This shift in turn feeds back into p53, altering the 'post-translational code' of the p53 protein that modulates its sequence-specific DNA-binding selectivity. The combination of HBx-

altered transcription co-factor/co-regulator recruitment as well as altered p53 post-translational modifications culminates in deregulated p53-mediated gene expression. This work presents a significant advancement in our understanding of the co-factor function of HBx in modulating the tumour suppressor protein p53, since the mechanism of action of the viral X protein had been largely unknown.

Table 1.4. Overview of thesis

Objective	Findings	Techniques
To identify patterns of modulated p53-DNA binding that are linked to p53 target gene deregulation by HBx	HBx modulates p53-DNA binding with associated gene deregulation	p53 ChIP-on-chip and expression profiling
To characterize the p53-mediated deregulation of *p53AIP1* by HBx	HBx induces a novel shift in p53-DNA binding at *p53AIP1*	ChIP-qPCR
	Altered p53-DNA binding by HBx deregulates *p53AIP1*	Mutagenesis studies Promoter assays RNA interference studies qRT-PCR
	p53AIP1 is increased in HCC patients with high HBx status	Immunoblotting qRT-PCR
	p53AIP1 mediates HBx-induced apoptosis	RNA interference studies Apoptosis assay (annexin V/7AAD staining and FACS analysis)
To elucidate the mechanism by which HBx modulates p53	HBx does not alter p53 Ser46 phosphorylation	Immunoblotting
	HBx alters transcription factor/co-regulator recruitment	Bioinformatics ChIP-qPCR Mutagenesis studies Promoter assays RNA interference studies
	HBx generally does not alter transcription factor/coregulator protein levels	Immunoblotting
	HBx does not alter chromatin state of p53AIP1 regulatory region	Acetylated H3/H4 ChIP-qPCR DNA methylation profiling
	HBx enhances PCAF-mediated p53 Lys320 acetylation that alters sequence-specific DNA binding	Immunoblotting ChIP-qPCR Mutagenesis studies RNA interference studies p53 ChIP-Seq

Chapter 2 Materials and Methods

Table 2.1 List of primary antibodies used in thesis.

Primary Antibody	Company	Catalogue Number	Working Dilution	Source
5-methyl-cytosine	Arraystar, Inc.			
Actin (I-19)	Santa Cruz	sc-1616	1:10,000	Goat polyclonal
EGFP	Roche	11814460001	1:20,000	Mouse monoclonal
GAPDH	Millipore	ABS16	1:20,000	Rabbit polyclonal
GATA-1(H-200)	Santa Cruz	sc-13053	1:5,000	Rabbit polyclonal
HBx	In house		1:10,000	Rabbit polyclonal
HDAC1 (H-51)	Santa Cruz	sc-7872	1:10,000	Rabbit polyclonal
p53 (DO-1)	Santa Cruz	sc-126	1:10,000	Mouse monoclonal
PCAF (E-8)	Santa Cruz	sc-13124	1:5,000	Mouse monoclonal
Acetyl-p53 (Lys 320)	Millipore	06-1283	1:5,000	Rabbit polyclonal
Acetyl-p53 (Lys 373)	Millipore	06-916	1:5,000	Rabbit polyclonal
Acetyl-p53 (Lys 380)	Millipore	04-1146	1:5,000	Rabbit monoclonal
Phospho-p53 (Ser 46)	Cell Signaling	2521	1:100	Rabbit polyclonal
Sp1 (1C6)	Santa Cruz	sc-420	1:1,000	Mouse monoclonal
YY1 (H-10)	Santa Cruz	sc-7341	1:1,000	Mouse monoclonal

Table 2.2 List of secondary antibodies used in thesis.

Secondary Antibody	Company	Working Dilution	Source
Goat anti-mouse IgG, HRP conjugated	Pierce	1:100,000	Goat polyclonal
Goat anti-rabbit IgG, HRP conjugated	Pierce	1:100,000	Goat polyclonal
Rabbit anti-goat IgG, HRP conjugated	Pierce	1:100,000	Rabbit polyclonal

Table 2.3 List of primers used in thesis.

Primer name	DNA sequence (5'-3')
ChIP-qPCR	
p53AIP1 promoter p53 RE	F TCAGGGTGAGATGTCTTATC R CACAGGCAGAATTGTCATTT
p53AIP1 intron 1 p53 RE	F CTCTTGCTAATGCCAGCCTG R GCATCAGGAAGTTCATCTCG
RT-qPCR	
Beta-actin	F AAAGACCTGTACGCCAACAC R GTCATACTCCTGCTTGCTGAT
p53AIP1	F CACCCAGTCACAGCAGCACA R CAGAGGAAGATCCCATCCAG
Promoter Cloning and Mutagenesis	
p53AIP1 wild-type promoter	F AGGAACGATGGAATCAGAGTCAC R GCAGCAGCAAGGCACCATCATG
p53AIP1 promoter p53 RE mutant	F TAGaATtTCTGAAAGTTGGCAAgtgGTAAAAAGGC R TTACcacTTGCCAACTTTCAGAaATtCTATTCCG
p53AIP1 intron 1 p53 RE mutant	F CTCTaTTacCCGGGtactTCGAGATGAAC R CATCTCGAagtaCCCGGgtAAtAGAGGAG
GATA-1 RE mutant	F GATGTCTTcTCCGGTTAACTGC R GCAGTTAACCGGAgAAGACATC
Sp1 RE mutant	F CCTCATCttGCCCCCTGCAC R GTGCAGGGGGCaaGATGAGG
YY1 RE mutant	F TACAATAAAAgacaGcCTAGGGAGAAATTACCCAGCAC R TTCTCCCTAGgCtgtcTTTTATTGTAGAGAATGGAAACCTG
p53 Cloning and Mutagenesis	
Wild-type p53	F AATTGGATCCATGGAGGAGCCGCAGTCAG R AATTGGATCCTCAGTCTGAGTCAGGCCCTT
S46A mutant	F TGCTGgCCCCGGACGATATTGAAC R CAATATCGTCCGGGGcCAGCATC
K320Q mutant	F CAGCCAAAGcAGAAACCACTGGATGG R TGGTTTCTgCTTTGGCTGGGGAGAGG
K320R mutant	F CAGCCAAAGAgGAAACCACTGGATGG R TGGTTTCcTCTTTGGCTGGGGAGAGG

Mutations introduced are denoted by lower case. RE denotes response element. F and R denote forward and reverse primers respectively.

2.1 Mammalian cell culture and assays

2.1.1 Mammalian cell culture

All cell lines used in this thesis were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human HCC cell lines HepG2 (p53 wild-type) and Hep3B (p53-deficient) as well as human embryonic kidney HEK293 cells (ATCC CRL-1573) were cultured in Dulbecco's Modified Eagle Medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Non-transformed immortalized human liver cells THLE-3 (ATCC CRL-11233) that have near diploid karyotypes and lack alpha-fetoprotein expression were maintained in bronchial epithelial basal medium media (Clonetics, San Diego, CA) without addition of Gentamycin/Amphotericin and Epinephrine and supplemented with 10 ng/ml EGF, 100 ng/ml Phosphoethanolamine and 10% FBS. Flasks and plates used for propagation of THLE-3 cells were coated with 0.03 mg/ml rat tail collagen type I. All cells were incubated in a humidified atmosphere at 37˚C, 5% CO_2.

2.1.2 Recombinant adenovirus transduction of cells

2.1.2.1 Construction of recombinant adenoviruses

An invaluable experimental tool used in this thesis is the control and HBx-expressing recombinant adenoviral system that facilitated efficient introduction the viral X protein into liver cells (Figure 2.1). As described previously (Murakami, 2001), the HBx gene was amplified from pEco63 plasmid, cloned into a pAdTrack-CMV shuttle vector and its integrity was verified by sequencing. The HBx-containing pAdTrack-CMV-HBx or control pAdTrack-CMV vectors were linearized using PmeI and subsequently co-transformed with enhanced green fluorescence protein (EGFP) gene-containing pAdEasy-1 plasmid into BJ5183 *E. coli* cells. Control and HBx-expressing recombinant adenoviral vectors were obtained by homologous recombination of the two vectors pAdEasy-1 and pAdTrack-CMV/pAdTrack-

CMV-HBx respectively, and successful recombination events were screened using *Eco*RV and *Pme*I restriction endonuclease analyses. Following enzymatic digestion by *Pac*I, the pAdControl and pAdHBx vectors (Figure 2.1A) were transfected into HEK293 cells that constitutively express the adenoviral E1 gene product for packaging into control and HBx-expressing recombinant adenoviruses respectively.

2.1.2.2 Propagation and titration of recombinant adenoviruses

Recombinant HBx and control adenoviruses were propagated and titrated using HEK293 cells. Essentially, cells were transduced with HBx and control viruses and harvested after 48 hr. Multiple rounds of freeze-thaw were performed to lyse cells. Supernatants containing the released viruses were stored in aliquots in -80˚C. To determine the titre of each batch of HBx and control viruses generated, 2.5 x 10^5 HEK293 cells were seeded in each well of a 24-well plate and transduced with serially diluted virus lysates (10^{-1} to 10^{-6}) performed in quadruplicates, 24 hr post-seeding. The titre of the viruses was determined by visually counting the number of cells with green fluorescence in each well and expressed as expression-forming units/per ml.

2.1.2.3 Determination of multiplicity of infection for transduction

Cells were transduced with a range of multiplicity of infection (MOI) of recombinant HBx and control viruses (MOI of 0, 2, 4, 6, 8 and 10). Dark field and phase contrast images were taken 24 hr post-transduction (Figure 2.1B) and cells were harvested and lysed for EGFP quantification by western blotting (Figure 2.1C). Appropriate MOIs for HBx and control adenovirus transduction for each cell line were determined using the following criteria: a) high transduction efficiency (>90%), b) minimal cytotoxicity and c) comparable

Figure 2.1

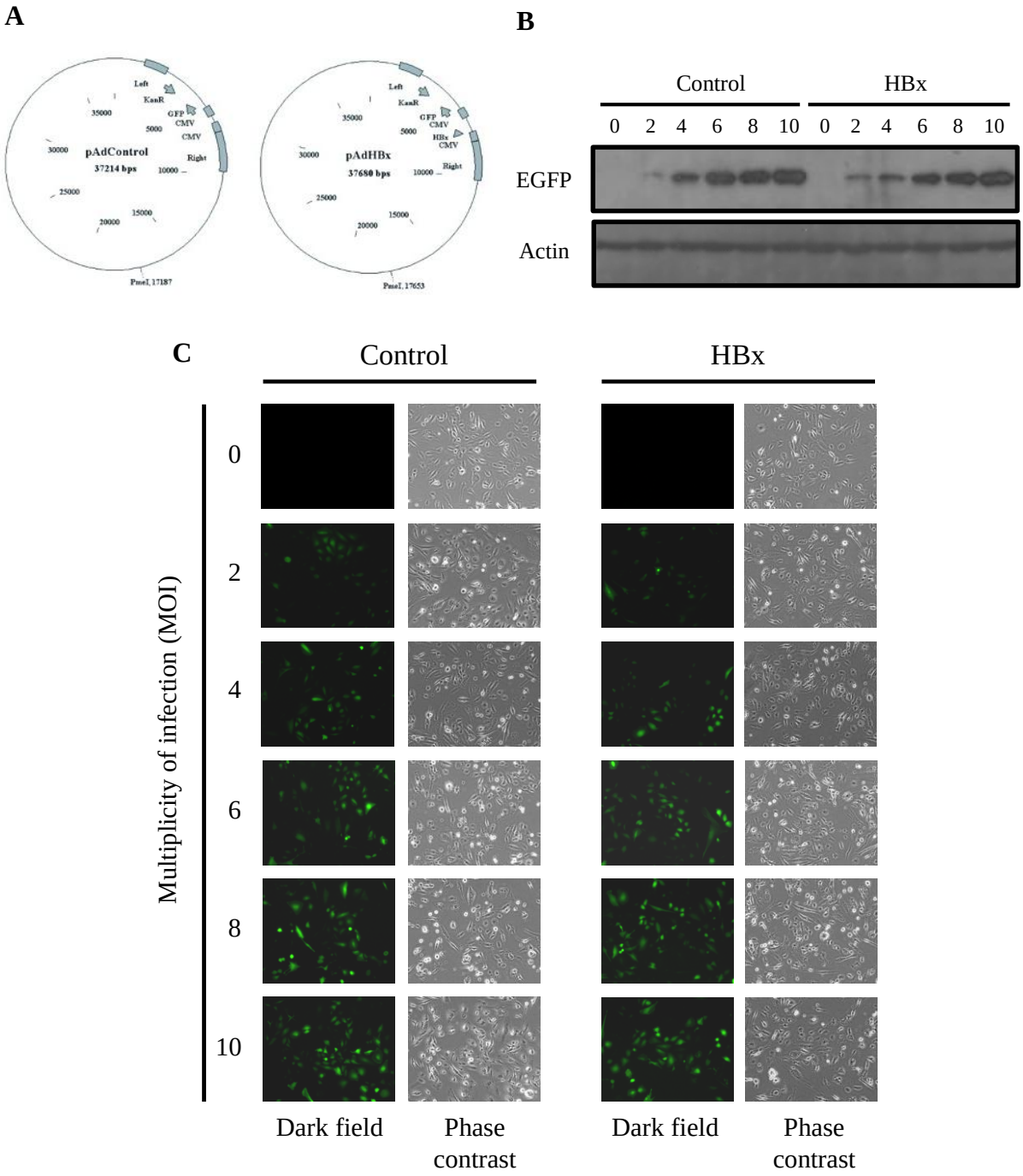


Figure 2.1. Control and HBx-expressing recombinant adenoviral system. A. Shown are schematic diagrams of the pAdControl and pAdHBx constructs used to generate the recombinant adenoviruses. This research was originally published in The Journal of Biological Chemistry. Lee *et al.* The hepatitis B virus X protein sensitizes HepG2 cells to UV light-induced DNA damage. *J Biol. Chem.* 2005, 280(39): 33525-35. © the American Society for Biochemistry and Molecular Biology. B and C. Control and HBx-transduced THLE-3 cells using a range of multiplicity of infection (MOI), from 0 to 10. B. Western blots of EGFP and actin loading control of the indicated protein lysates. C. Shown are dark field and phase contrast images of cells transduced with the indicated MOIs.

EGFP levels between cells transduced with HBx and control adenoviruses. Importantly, MOIs were chosen that reflected physiologically relevant HBx-expression levels (Figure1.5B). MOI of 6 and 10 for control and HBx-expressing recombinant adenovirus respectively were used for all subsequent experiments.

2.1.3 Ultraviolet treatment of HepG2 cells

As described previously, HepG2 cells were exposed to UVC (254 nm) irradiation for 30 sec with a germicidal lamp calibrated to deliver 8 J/m^2 48 hr post-transduction (Chao *et al.*, 2006). Cells were harvested 24 hr post-UV irradiation.

2.1.4 Transient transfection methods

2.1.4.1 Chemical transfection of siRNA and/or plasmid DNA in Hep3B and THLE-3 cells

0.5×10^6 cells were seeded in each well of a 6-well plate. 100 μM of short interfering RNAs (siRNAs) and/or 1-5 μg of plasmid DNA were chemically introduced to the cells 24 hr post-seeding using siPORT™ Amine Transfection Agent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Briefly, siPORT™ *Amine* Transfection Agent was diluted in OPTI-MEM I® (Invitrogen) reduced serum media in a 9 μl:100 μl ratio and incubated for 10 min at room temperature. Separately, siRNA and/or plasmid DNA was appropriately diluted in 100 μl OPTI-MEM I®. Diluted siPORT™ Amine Transfection Agent was subsequently mixed with the diluted siRNA and/or plasmid DNA by gentle pipetting up and down, and incubated for 30 min at room temperature. The nucleic acid/ siPORT™ *Amine* transfection complexes were then added onto the adherent cells that were pre-washed with PBS and replaced with fresh culture media. Cells were harvested 24 hr post-transfection unless otherwise indicated. SiRNAs specific for TP53 (s605), TP53AIP1 (241781) and

GATA-1 (s5595) as well as negative control scramble siRNA (AM4611) were purchased from Ambion. siRNAs specific for PCAF (sc-36198), Sp1 (sc-44221) and YY1 (sc-36863) were purchased from Santa Cruz Biotechnology, CA, USA.

2.1.4.2 Electroporation of siRNA and plasmid DNA into HepG2 cells

2 x 10^6 cells were used for electroporation experiments. Cells were trypsinized, washed once with PBS, resuspended in 200 μl OPTI-MEM I®. Appropriate amounts of siRNA and/or plasmid DNA was added into the cell suspension, gently mixed by tapping and subsequently transferred to a 4 mm BTX Electroporation Cuvette Plus™ (BTX® Harvard Apparatus, Inc., Holliston, MA, USA) and placed on ice. Cells were electroporated using a ECM 830 Square Wave Electroporator (BTX) using the following settings: Low voltage mode, 180 volts, 100 msec. Following electroporation, cuvettes were immediately placed back on ice. Cells were gently resuspended in fresh culture media and seeded in the respective wells of a 6-well plate. Cells were harvested 24 hr post-transfection unless otherwise stated.

2.1.5 Beta-galactosidase reporter assay

P53AIP1 promoter activity was assayed in p53-deficient Hep3B cells in which the effect of exogenously introduced p53 can be consistently tested. 5 μg of beta-galactosidase (β-gal) reporter construct (wild-type WT, mutants M1, M2 or M3), 1 μg *TP53*-expressing or control plasmid, and/or 100 μM *TP53*-specific or control siRNA was chemically introduced to Hep3B cells 24 hr post-seeding using siPORT™ Amine Transfection Agent (Ambion) according to the manufacturer's protocol. Cells were harvested 24 hr post-transfection. Transfected cells that were subsequently transduced with recombinant adenoviruses were harvested 24 hr post-transduction. Cells were washed with PBS, harvested and lysed using 200 μl per well of Complete Lysis-M EDTA-Free Lysis Buffer (Roche Applied Science,

Indianapolis, USA) supplemented with protease inhibitors (Roche), at 4°C for 10 min. Cell debris was removed by centrifugation at 13,000 rpm for 5 min and the supernatant was transferred to a clean eppendorf tube. 50 µl of each cell lysate sample (in triplicate) was dispensed into each well of a 96 well plate. Equal volume of assay buffer containing chlorophenol red-β-D-galactopyranoside (CPRG) as substrate was added to each well. The lysates were kinetically assayed for β-gal reporter activity by measuring at 30-sec intervals over 60 min at 570 nm using SpectraMax Plus^{384} microplate reader (Molecular Devices Corp, Sunnyvale, CA, USA). β-gal activity of each construct was normalized against protein concentration determined using BCA Protein Assay Kit (Pierce Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol, as well as the respective basal β-gal activity to take into account the small inherent differences in β-gal activity between promoter constructs in the absence of p53.

2.1.6 Apoptosis assay

2 x 10^6 HepG2 cells were electroporated with 100 µM *TP53AIP1* siRNA or negative control siRNA (Ambion) and HBx-expressing or control plasmid (generated previously in the lab). Cells were subjected to UV treatment 24 hr later. The apoptosis profiles of the cells were analyzed by PE Annexin V and 7AAD staining according to the manufacturer's protocol (BD Biosciences Pharmingen^{TM}, San Deigo, CA, USA), followed by flow cytometry using the BD FACSCalibur^{TM} (BD Biosciences) 24 hr post-treatment. Subsequent analysis of the cellular profiles was performed using FlowJo software with appropriate compensation (Tree Star, Inc., Ashland, OR, USA).

2.2. RNA/DNA methodology

2.2.1 RNA isolation and reverse transcription polymerase chain reaction

Total RNA was prepared from cells using RNeasy® Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions and quantified using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). For First-strand cDNA synthesis, a 12 µl reaction volume comprising 1 µg of RNA, 1 µl 50 µM oligo dT primers, 1 µl 10 mM dNTP and RNase/nuclease free water was first heated to 65°C for 15 min and put on ice for 5 min. The following was then added to each tube: 4 µl 5 X First-strand buffer, 2 µl 0.1 M DTT, 1 µl RNase/nuclease free water and 1 µl SuperScript® II Reverse Transcriptase (Invitrogen). The tubes were mixed and incubated at 25°C for 5 min followed by 42°C for 60 min and 70°C for 15 min.

2.2.2 Real-time polymerase chain reaction

Transcript abundance was determined by quantitative real-time PCR (qPCR) using ABI7500 Real Time PCR Detection System (Applied Biosystems™, Life Technologies™, Carlsbad, CA, USA). A 10 µl reaction volume was prepared comprising of 5 µl QuantiTect™ SYBR® Green Master PCR mix (Qiagen) and 0.25 µl each of forward and reverse primers (Table 2). The following qPCR reaction conditions were used: an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. All transcript abundance was normalized against β-actin housekeeping gene.

2.2.3 Mini- and maxi-preparation of plasmid DNA

Small scale plasmid DNA preparation was performed using QIAprep® Miniprep Kit (Qiagen) according to the manufacturer's instructions. Large scale plasmid DNA preparation

was performed using NucleoBond® Xtra Maxi EF kit (MACHEREY-NAGEL GmbH & Co., Dueren, Germany) according to the manufacturer's instructions.

2.2.4 Agarose gel electrophoresis

DNA was mixed with 6 X DNA loading dye (0.1% bromophenol blue, 40% sucrose, 240 mM Tris-HCL pH7.4, 60 mM EDTA-Na pH8.0) and separated on a 1% agarose gel prepared by dissolving agarose in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) with addition of 0.5 µg/ml ethidium bromide. The gel was run using 1 X TAE buffer, at 120 volts. DNA bands were visualized using a UV trans-illuminator.

2.2.5 DNA sequencing

40 ng of plasmid DNA was used for ABI BigDye® Terminator Cycle Sequencing (Applied Biosystems), with addition of the following in a 10 µl reaction volume: 2.5 µl BigDye Sequencing buffer, 0.5 µl 10 µM primer, 0.5 µl BigDye® Terminator v3.1 and deionised water. Cycle sequencing was carried out in a T3000 Thermocycler (Biometra GmbH, Goettingen, Germany) using the following conditions: 25 cycles of 96ºC for 10 sec, 50ºC for 10 sec, 60ºC for 4 min, then 4ºC on hold. The sequencing extension products were purified using ethanol precipitation. Briefly, 1 µl 250 mM EDTA, 1 µl 20 mg/ml glycogen and 50 µl 100% ethanol was added to each tube. Tubes were mixed well and centrifuged at 13,000 rpm for 30 min at 4ºC. DNA pellets were subsequently washed with 50 µl 70% ethanol, centrifuged at 13,000 rpm (10,000 x *g*) for 15 min at 4ºC and dried for 3 min at 94ºC. DNA pellets were resuspended in 10 µl highly deionised (Hi-Di) formamide (Applied Biosystems) and sequenced using ABI 3100 Genetic Analyzer (Applied Biosystems).

2.2.6 Generation of wild-type and mutant promoter constructs

To experimentally validate the predicted p53 response elements of the *p53AIP1* gene, a 3.8 kb fragment containing both the promoter and intron 1 p53 response elements was PCR-amplified from genomic DNA of non-tumorous human liver tissue using Expand High Fidelity PCR System (Roche Applied Science) and primers 5'-AGGAACGATGGAATCAGAGTCAC-3' (forward) and 5'-GCAGCAGCAAGGCACCATCATG-3' (reverse) in a total volume of 15 μl. PCR conditions used are as follows: initial denaturation at 94°C for 2 min, 10 cycles of 94°C for 15 sec, 55°C for 30 sec and 68°C for 4 min, followed by another 10 cycles of 94°C for 30 sec, 55°C for 30 sec and 68°C for 4 min with 5 sec cycle elongation of each successive cycle, and a final elongation at 72°C for 7 min. The amplified fragment was gel-purified and TA-cloned in front of a β-gal reporter gene (termed wild-type promoter construct). The promoter construct also contained the EGFP gene for visualization of transfection efficiency. In designing the mutant promoter and intron 1 p53 response elements, a bioinformatics approach using MatInspector (www.genomatix.de) transcription factor motif prediction tool was employed to identify mutations that abolish the response element of interest but that do not affect other proximal or overlapping transcription factor binding sites. The following mutant promoter constructs were generated: a) mutant promoter p53 response element (M1), b) mutant intron 1 p53 response element (M2), and c) double mutant containing both mutant promoter and intron 1 p53 response elements (M3). The mutant promoter constructs M1, M2 and M3 were generated by fusion PCR using primers containing the desired mutations: 5'-TAGaATtTCTGAAAGTTGGCAAgtgGTAAAAAGGC-3' (forward) and 5'-TTACcacTTGCCAACTTTCAGAaATtCTATTCCG-3' (reverse) for mutating the promoter p53 response element, and 5'-CTCTaTTaCCCGGGtactTCGAGATGAAC-3' (forward) and 5'-CATCTCGAagtaCCCGGGtAAtAGAGGAG-3' (reverse) for mutating the intron 1 p53

response element, where mutations are denoted by lower case letters. Fusion PCR conditions are as follows: an initial denaturation at 95°C for 15 min, followed by 20 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 3 min. The amplified fragments was gel-purified and similarly cloned upstream of the β-gal reporter gene. All constructs were sequenced to verify the integrity of the DNA sequences and the successful introduction of only the desired mutations.

2.3 Protein methodology

2.3.1 Protein isolation from cells and quantification

Cell pellets were lysed with 100 µl RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium Deoxycholate, 0.1% SDS) and incubated on ice for 10 min. Cells were sonicated using a Bioruptor® (Diagenode Denville, NJ, USA) for 10 min at high setting, 30 sec 'on' followed by 30 sec 'off'. Cell debris was removed by centrifugation at 13,000 rpm for 5 min at 4 ºC and cell lysates were transferred to new tubes. Protein concentrations were determined using BCA protein assay reagent (Pierce) according to the manufacturer's instructions.

2.3.2 Western blotting

20 µg of protein from each sample was mixed with 6 X loading buffer, boiled for 10 min and subjected to gel electrophoresis on a 12% SDS poly-acrylamide gel. Following transfer to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA), blots were blocked for 1 hr using Amersham ECL™ membrane blocking agent (GE Healthcare Biosciences, Uppsala, Sweden). The appropriate primary antibodies (Table 2.1) were then added to the blot and incubated for 1 hr: Blots were washed with PBST for 15 min and then incubated for

45 min with the appropriate horseradish peroxidise-conjugated secondary antibodies (Table 2.2). Following washing, signals were detected using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare). The membrane was then exposed on a Kodak® BioMax™ MR film (Kodak Inc., Rochester, NY).

2.3.3 Chromatin immunoprecipitation

Protein and DNA in cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was quenched with 125 mM glycine for 5 min. Cells were washed with PBS and aliquots of 2 x 10^6 cells were used for each chromatin immunoprecipitation (ChIP) assay, performed according to the manufacturer's instructions (Upstate Cell Signaling Solutions, Millipore, Billerica, MA, USA). Cells were lysed using SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH8.0) supplemented with protease inhibitor cocktail (Roche) and incubated for 10 min on ice. Chromatin was sheared to an average size of 300 bp using a Bioruptor® (Diagenode) at medium setting for 12 cycles and 23 cycles of 30 sec 'on' followed by 30 sec 'off' for HepG2/Hep3B and THLE-3 cells respectively. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C and cell lysate was diluted using ChIP dilution buffer (0.01% SDS, 1.1% Triton X- 100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl). Lysates were subsequently pre-cleared with 60 µl BSA-blocked protein G beads (Upstate) for 30 min at 4˚C on a rotating platform. A 100 µl portion of the cell lysate was saved as 'Input DNA'. Clarified lysates were immunoprecipitated with 1 µg of antibody specific for the protein of interest or 1 µg normal mouse/rabbit IgG antibody (non-specific) overnight at 4˚C on a rotating platform. Lysates were then incubated with 60 µl of BSA-blocked protein G beads for 3 hr at 4˚C on a rotating platform. Following gentle centrifugation at 1,000 rpm for 1 min to pellet the agarose, the supernatant containing unbound, non-specific DNA was carefully and completely removed.

The agarose was subjected to a series of washing using 1 ml of buffer for 10 min at 4˚C on a rotating platform each time in the following order: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 150 mM NaCl); high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 500 mM NaCl); and LiCl buffer (0.25 M LiCl, 1% NP-40, 1% SDS, 1 mM EDTA, 10 mM Tris-HCl pH8.0). The agarose was then washed twice with 1ml of TE buffer (20 mM Tris-HCl pH8.0, 1 mM EDTA pH8.0) for 10 min each at room temperature on a rotating platform. Protein-DNA complexes were eluted twice using 250 µl of elution buffer (1%SDS, 0.1 M NaHCO3) for 15 min each at room temperature on a rotating platform. The combined eluates were reverse cross-linked using 20 µl 5 M NaCl and incubated at 65˚C, overnight. Input DNA extracted from the total lysate that had not been immunoprecipitated was similarly reverse cross-linked. Protein was removed by addition of 10 µl 0.5 M EDTA, 20 µl 1 M Tris-HCl pH 6.5 and 2 µl 10 mg/ml Proteinase K and incubated at 45˚C for 1 hr. Input and ChIP DNA were subsequently recovered by phenol/chloroform extraction and ethanol precipitation. Briefly, an equal volume of phenol/chloroform was added to each sample and mixed thoroughly by vortex. Following centrifugation at 13,000 rpm for 10 min, the aqueous phase containing DNA was carefully aspirated and transferred to a new tube. To increase DNA yield during precipitation and to visualise the DNA pellet, 20 µg of the inert carrier glycogen was added in addition to 1 ml of 100% ethanol. Samples were incubated overnight at -20˚C. Following centrifugation at 13,000 rpm for 30 min at 4˚C, DNA pellets were washed with 1 ml of 70% ethanol and air-dried. All samples were resuspended in 100 µl deionised water.

Detection of enriched ChIP DNA was determined by quantitative real-time PCR (qPCR) using QuantiTect^{TM} SYBR$^{®}$ Green Master PCR mix (Qiagen). Input DNA extracted from the total lysate that had not been immunoprecipitated but similarly reverse cross-linked

and recovered was used to normalize for differences in the starting amount of DNA in each sample. Primer sets that were designed to detect for the region of interest are listed in Table 2.3.

2.4 ChIP-on-chip and ChIP-Sequencing

2.4.1 Sample preparation and hybridization for ChIP-on-chip

ChIP-on-chip assays were performed on control and HBx UV-treated HepG2 cells with p53 DO-1 antibody (Santa Cruz Biotechnology) on NimbleGen 1.5kb promoter array according to the manufacturer's recommendations. P53 ChIP and input libraries were prepared as follows: the ends of the p53-immunoprecipitated ChIP DNA and input DNA were first polished using the End-It^{TM} DNA End-Repair Kit (EPICENTRE Biotechnologies, Madison, WI, USA) and subsequently ligated with pre-annealed oligonucleotide linkers (oligo 1, 5'-GCGGTGACCCGGGAGATCTGAATTC-3'; oligo 2, 5'-GAATTCAGATC-3'). The linker-ligated ChIP DNA and input DNA were then amplified using the following conditions: annealing at 55°C for 2 min using oligo 1 as primer; extension at 72°C for 2 min following addition of a mixture of *Taq* and *Pfu* DNA polymerase; denaturation by heating to 95°C for 2 min. This was followed by 22 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 5 min. ChIP DNA and input DNA were end-labeled with Cy5 and Cy3 respectively, and co-hybridized to a Nimblegen ChIP-chip promoter tiling array (Roche NimbleGen Systems, Inc., Madison, WI, USA). The array contained 382,884 probes with a probe length of 50 bp and median probe space of 100 bp tiled 1,200 bp upstream and 300 bp downstream of 24,134 human promoters, present in triplicate. Post-amplification steps of ChIP and input libraries such as DNA end-labeling, co-hybridization, and array scanning were performed by Roche NimbleGen Systems, Inc.

2.4.2 Bioinformatics analysis of ChIP-on-chip data

Paired raw data files of foreground signal intensities of p53 ChIP and input libraries provided by Roche NimbleGen Systems, Inc were analyzed using Partek® Genomics Suite™ 6.6 (Partek Incorporated, Missouri, USA). Control or HBx differentially enriched candidate p53 bound regions (termed peaks) were identified based on the following criteria: (i) regions that contained a minimum of five consecutive probes, (ii) MAT score on fold change greater than 3 (HBx-enriched candidate p53 bound regions) or less than -3 (control-enriched candidate p53 bound regions) and (iii) p value less than 0.01, and (iv) contained putative p53 response element(s) (binding score >60%) predicted using p53MH algorithm (Hoh *et al.*, 2002). Each differential candidate p53 bound region was subsequently annotated using Partek® Genomics Suite™ 6.6 (Partek Incorporated) with the nearest gene.

2.4.3 Expression microarray profiling

Agilent Whole Human Genome Oligo Microarray (G4112A) (Agilent Technologies California, USA) containing 44,000 60-mer oligonucleotide probes representing 41,000 unique human genes/transcripts, was employed for profiling of differential gene expression between control and HBx HepG2 UV and THLE-3 cells. RNA was isolated using MirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Subsequent cRNA generation, probe labeling and array hybridization were performed by Miltenyi Biotech, Cologne, Germany. Partek® Genomics Suite™ was used to analyze for differential gene expression profiles in control and HBx cells. HBx significantly deregulated genes were identified based on the following criteria: (i) fold change more than 1.5 (significantly up-regulated) or less than -1.5 (significantly down-regulated) and (ii) p value less than or equal to 0.05.

2.4.4 ChIP sample preparation for ChIP-Sequencing

P53 ChIP-DNA from 18 high quality biological repeats were pooled together and prepared as a single library for Solexa Sequencing (Illumina, Inc., California, USA) at the Genome Sequencing Facility, National Cancer Centre Singapore. This was performed for both control and HBx THLE-3 cells. The ChIP library was prepared using the Illumina ChIP-Seq Sample Preparation Kit according to the manufacturer's recommendations. Briefly, the ends of the p53 DO1 antibody-immunoprecipitated ChIP DNA were polished using the End-It^{TM} DNA end repair kit (EPICENTRE Biotechnologies). Adapters from the genomic adapter oligo mix were ligated to the DNA fragments following 'A' base addition to the 3′-ends. Adapter-modified DNA fragments were then amplified using Phusion DNA polymerase and Illumina Genomic PCR primers 1.1 and 1.2 using the following conditions: 98°C for 30 sec, followed by 15 cycles of 98°C for 10 sec, 65°C for 30 sec, 72°C for 30 sec and a final extension 72°C for 5 min. ChIP libraries were run on a 2% agarose gel and the 300 bp fraction of each library was gel extracted and purified. Samples were quantified using Agilent 2100 Bioanalyzer using Agilent DNA1000 kit (Agilent Technologies). Samples were then sequenced using Illumina Genome Analyzer II with a standard single read 36-cycle sequencing protocol and Illumina's sequencing reagents according to the manufacturer's instructions (Illumina, Inc.).

2.4.5 Mapping of ChIP-Seq reads, peak finding and motif search

Raw short sequence reads (36 bp) were mapped to human reference genome hg19 using Batman v2.0 software. A maximum of two mismatches between the read and mapping sites was allowed. Reads were restricted to mapping to only one unique location in the human genome. Control or HBx-enriched candidate p53 bound sites (or peaks) were identified using a peak calling software Control-based ChIP-Seq Analysis Tools (CCAT v3.0) (Tsai and

Chung, 2010) developed by the Genome Institute of Singapore using the default "transcription factor" setting. Subsequently, putative p53 response elements of the candidate peaks were identified using p53scan program (Benhenda et al., 2009) to scan +/-200 bp around peak summits. To estimate the FDR of the motif scanning program, 10,000 random positions in the reference genome were selected and the +/-200bp region scanned. Sites with a score less than the PWM score cut-off of 7% FDR were used.

2.5 Methylated DNA immunopreicpitation-chip and analysis

Methylated DNA immuoprecipitation coupled with microarray (MeDIP-chip) profiling was performed using a Human DNA Methylation 2.1M Deluxe Promoter Array. Genomic DNA was extracted from control and HBx THLE-3 cells using standard phenol-chloroform methods. Subsequent sample preparation and array hybridization were performed by Arraystar Inc (Rockville, MD, USA). Essentially, genomic DNA was fragmented by sonication, denatured and immunoprecipitated using an antibody that binds specifically to 5-methyl-cytosine (anti-5mC antibody). Purified immunoprecipitated methylated DNA was labelled with Cy5 and sonicated input DNA that was not subjected to immunoprecepitation was labelled with Cy3 and co-hybridized to the array. Partek® Genomics Suite™ 6.6 was used to analyze for differential methylated sites in control versus HBx samples. Control or HBx significantly enriched methylated sites were identified based on the following criteria: (i) regions that contained a minimum of five consecutive probes and (ii) p value of less than 0.01.

2.6 *p53AIP1* and HBx profiling of HCC patients

De-identified tumour and paired non-tumorous tissues from HCC patients were obtained from the NCCS/SingHealth Tissue Repository with prior approval from the

SingHealth CIRB (2007/437/B). *p53AIP1* gene expression profiles of 78 HCC patients were obtained by qPCR with a specific set of primers (Table 2.3). Transcript abundance of *p53AIP1* was normalized to that of β-actin housekeeping gene. HBx status was determined using immunoblot analysis with HBx-specific antibody previously generated in the lab.

2.7 Bioinformatics analysis of transcription factor motifs and gene functions

Two transcription factor motif prediction tools TRANSFAC® 7.0 (BIOBASE GmbH, Wolfenbuettel, Germany) and MatInspector release 8.0.1 (www.genomatix.de) were used to identify sequence-specific transcription factors that bind in close proximity (±300bp) to the promoter and intron 1 p53 response elements. Transcription factor binding sties that were predicted by both prediction tools were termed high confidence transcription factor binding sites. The GeneMANIA software application version 2.7.13 (www.genemania.org) that uses a large functional association data set to predict other potential members of a query protein complex was employed to identify potential transcription co-regulators that associate with each set of predicted sequence-specific transcription factors at the promoter and intron 1 regions but that may not bind DNA. Factors that were predicted to interact with more than half of the promoter/intron 1 predicted transcription factors were termed strongly-associated transcription co-regulators.

The putative functions of significantly deregulated genes with associated p53-DNA binding alterations identified from expression profiling and p53 ChIP-on-chip were predicted using the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7, http://david.abcc.ncifcrf.gov/).

2.8 Statistical analysis of experimental data

Data presented were obtained from at least three independent experiments, unless otherwise stated. Data were expressed as mean values of experimental triplicates ± standard error (SE). Student's two-sided unpaired *t* test was performed to analyze for statistical significance of differences between sample means. For transcript expression analysis in tumour and paired non-tumorous tissues of HCC patients, data were expressed as median values ± SE. Student's two-sided unpaired *t* test was performed to analyze for statistical significance of differences between sample medians.

Chapter 3 Results

3.1 HBx modulates p53-DNA binding

3.1.1 HBx abolishes, enhances and shifts p53-DNA binding

The viral transcription co-factor HBx disrupts host transcription regulation by interacting with and altering the activity of transcription factors. In this work, we are focused on the modulation of the central transcription regulator p53 by HBx. HBx has been demonstrated to interact with p53 in the nucleus, but the effect of HBx on p53 sequence-specific binding and transcription regulation is unclear.

To obtain a more comprehensive understanding of p53 modulation by HBx, we employed a global integrated approach to profile (i) the effect of HBx on p53-DNA binding by p53 chromatin immunoprecipitation coupled with microarray (p53 ChIP-on-chip), and (ii) the effect of HBx on gene expression by expression profiling of control and HBx HepG2 (UV-treated) cells (Figure 3.1A). Firstly, analysis of global p53 ChIP-on-chip revealed that HBx altered p53 sequence-specific DNA-binding in several ways. HBx abolished p53 binding to candidate p53 response elements of 98 genes (Figure 3.1B); HBx enhanced p53 binding to the candidate p53 response elements of 113 genes (Figure 3.1B); and strikingly, HBx also induced a novel shift in p53-DNA occupancy - simultaneously alleviating and enhancing p53 binding - to distinct candidate p53 response elements at the promoters of 21 genes (Figure 3.1B). An example of each HBx-altered p53-DNA binding pattern (abolishment, enhancement and shift) at the promoter of a representative gene is illustrated in Figures 3.1 C, D and E respectively.

Figure 3.1

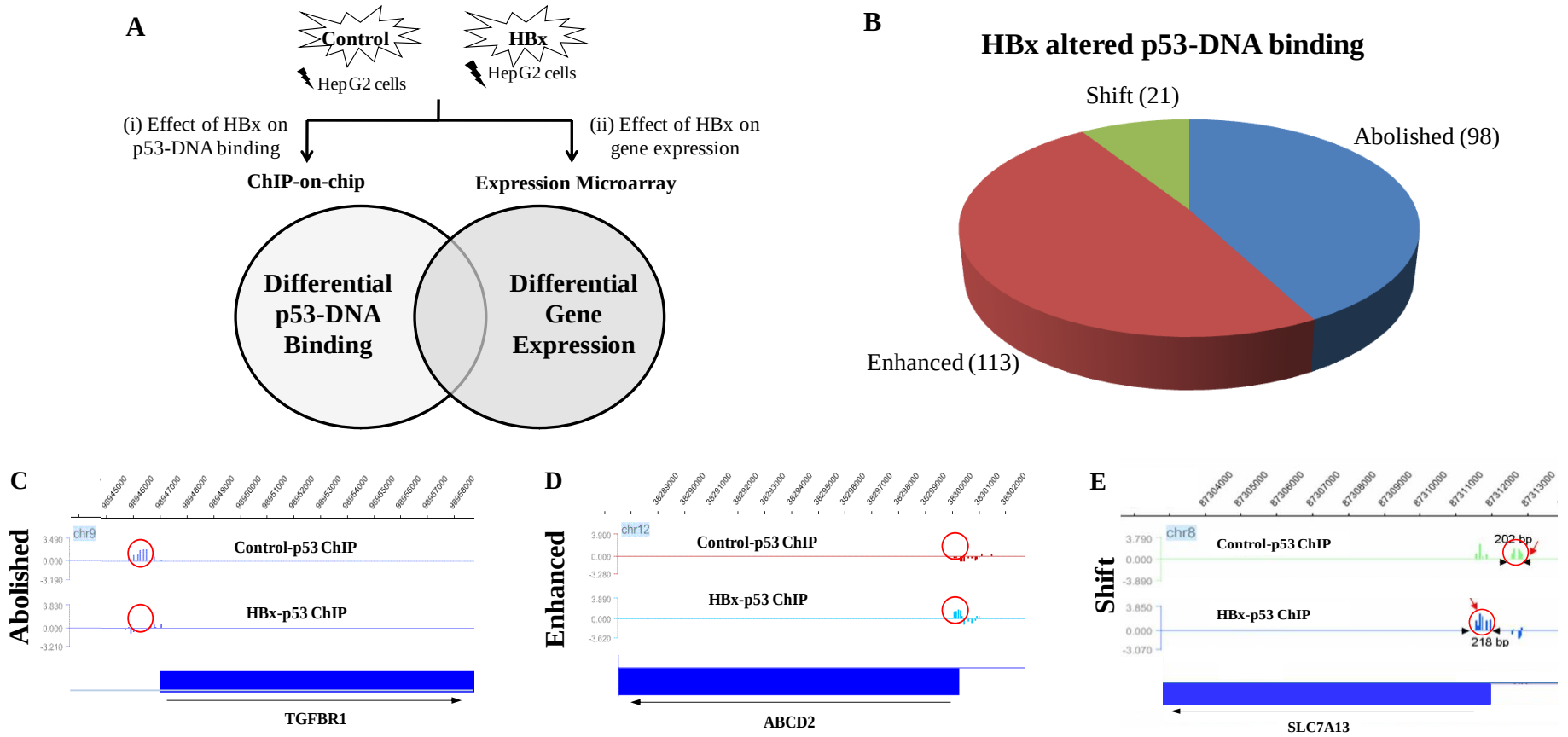


Figure 3.1 HBx alters p53-DNA binding. A Illustration of our strategy using a combination of ChIP-on-chip and expression profiling of UV-treated HepG2 cells transduced with recombinant HBx or control vectors. B. Patterns of p53-DNA binding alterations by HBx from p53 ChIP-on-chip analysis. Numbers within brackets refer to the number of genes with the respective p53-DNA binding alteration. C, D and E. Graphical representation of examples of abolished (C), enhanced (D) and shift (E) in p53-DNA binding by HBx. Figure was generated using SignalMap software (NimbleGen Systems).

This is the first report to show that HBx alters p53 sequence-specific DNA binding globally. Importantly, the findings suggest that HBx can alter p53-DNA binding in opposing ways (abolish as well as enhance p53-DNA binding), arguing for a more complex mechanism of p53 modulation by the viral protein than previously proposed.

3.1.2 HBx-altered p53-DNA binding is associated with gene deregulation

Secondly, to examine the biological significance of these observed p53-DNA binding alterations, microarray-based gene expression profiles were integrated with p53 ChIP-on-chip data (Figure 3.2A). As shown in Figure 3.2B, only a third of genes (32.8%) with HBx-altered p53-DNA binding at their promoters exhibited significantly deregulated expression (fold change >1.5 or <-1.5, $p \leq 0.05$). The majority of genes (67.2%) with HBx-altered p53-DNA binding at their promoters were not significantly deregulated by HBx (Figure 3.2B). This finding was not surprising as it is known that not all transcription factor-DNA binding events affect gene transcription (Wei et al, 2006). Nevertheless, of particular importance was the subset of HBx-altered p53-DNA binding that was associated with significantly deregulated corresponding gene expression. To examine the potential functions of these HBx-deregulated genes, the genes were queried using DAVID, a bioinformatics resource that predicts the most enriched Gene Ontology (GO) terms. Notably, the HBx-deregulated genes with altered p53-DNA binding were predicted to be involved in cell cycle, metabolism, protein localization, apoptosis, cellular response, DNA repair and transcription (Figure 3.2C). This suggests that modulation of p53 by HBx potentially disrupts the regulation of such key cellular processes that plausibly contributes to neoplastic transformation.

Figure 3.2

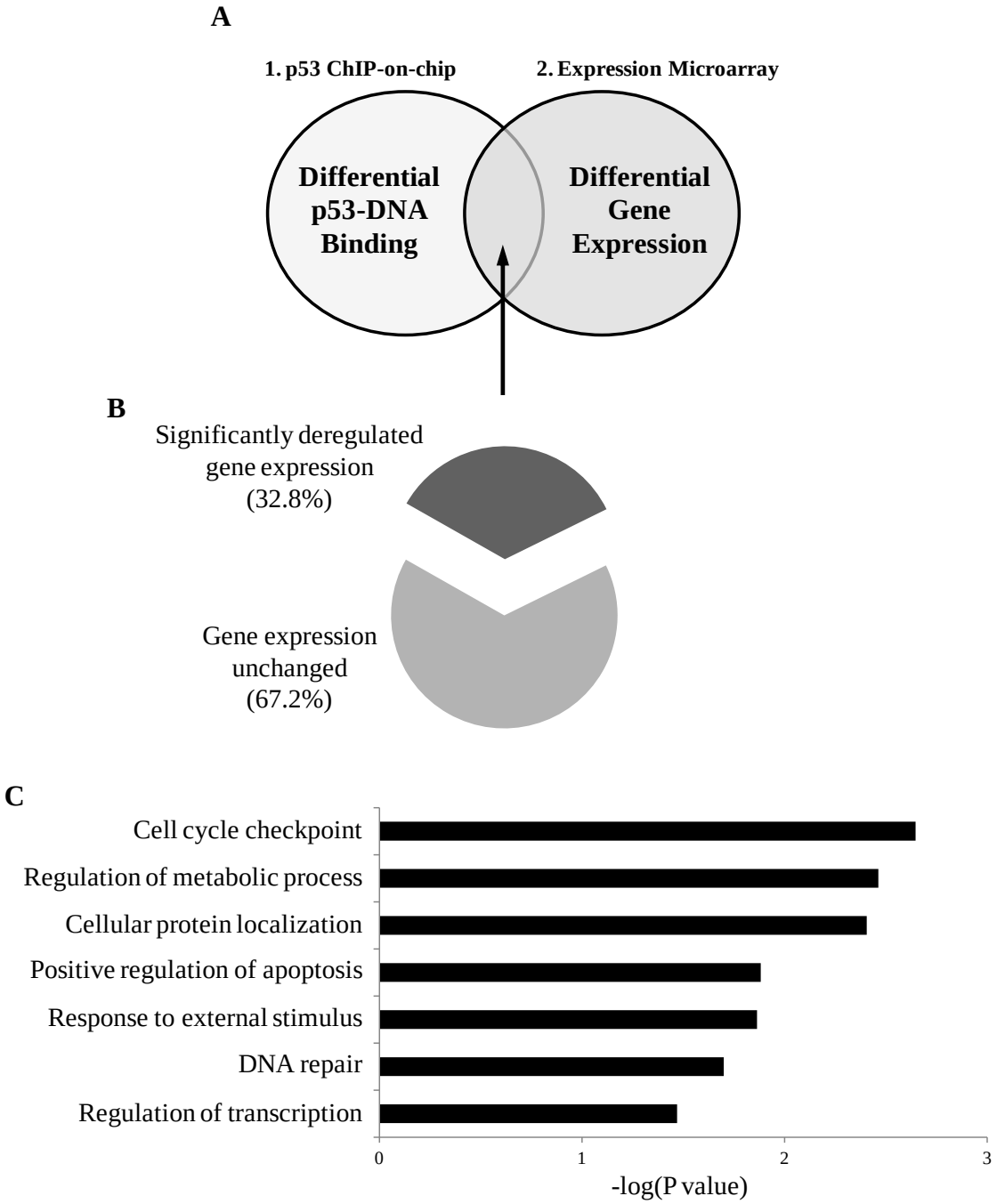


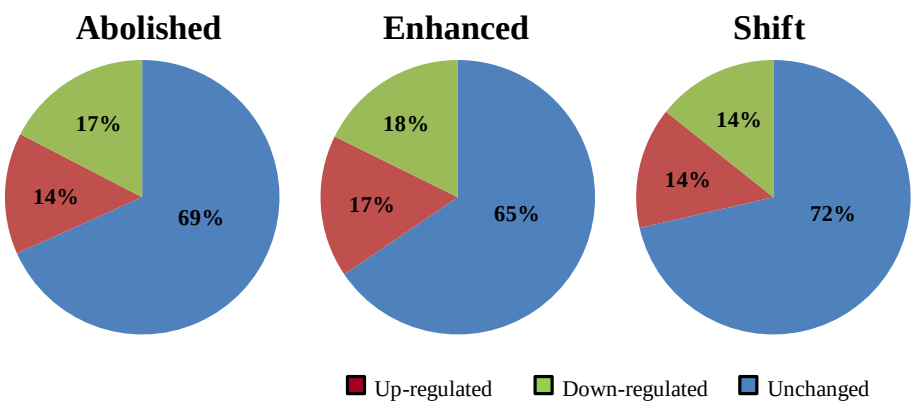
Figure 3.2 A subset of HBx-altered p53-DNA binding is associated with corresponding gene deregulation. A. Illustration of integrated p53 ChIP-on-chip and expression profiling to identify potentially functional HBx-altered p53-DNA binding (indicate by the arrow). B. Graphical representation HBx-altered p53-DNA binding associated with corresponding gene deregulation. Data is presented in percentages that are indicated in brackets. C. Gene Ontology (GO) term enrichment for significantly deregulated genes with associated p53-DNA binding alteration using DAVID, presented in order of decreasing significance –log(P value).

Next, we proceeded to examine the specific gene deregulation patterns that were linked to each type of p53-DNA binding alteration by HBx. Interestingly, each type of p53-DNA binding alteration (abolishment, enhancement or shift) did not correspond with any gene deregulation pattern (up- or down-regulation) (Figure 3.3A). Specifically, approximately equal numbers of genes were significantly up-regulated (indicated by red segment) or down-regulated (indicated by green segment) in each category of HBx-altered p53-DNA binding (Figure 3.3A). For example, 17% of genes with abolished p53-DNA binding at their promoter regions were significantly up-regulated, while a comparable proportion of genes (18%) were significantly down-regulated (Figure 3.3A). Using representative deregulated genes selected from each category of altered p53 binding, we experimentally validated the HBx-altered p53-DNA binding and gene deregulation patterns by ChIP-qPCR and qRT-PCR respectively (Figure 3.3B). Indeed, the abolishment, enhancement or shift in p53-DNA binding by HBx did not associate with any particular type of gene deregulation pattern. For example, HBx-enhanced p53 binding at the promoters of *FAS* and *ABCD2* were associated with increased gene expression, while HBx-enhanced p53 binding at the promoters of *AKT1S1* and *GDNF* were conversely associated with decreased gene expression (Figure 3.3B). Similarly, HBx-abolished p53 binding at the promoters of *p53AIP1* and *SPINK6* were linked to their up-regulation, while HBx-abolished p53 binding at the promoter of *DUX4* was conversely linked to its down-regulation. Taken together, the findings further support a complex mechanism of p53 modulation by the viral X protein.

While it is possible that the observed deregulated gene expression patterns could also be the result of the disruption of other modes of post-transcriptional regulation such as regulation by microRNAs and non-coding RNAs as well as the stability, intracellular

Figure 3.3

A



B

No	Gene Name	p53 binding (ChIP-on-chip)		Gene expression	
		Binding pattern	p53 binding (MAT score)	Fold Change (HBx/Control)	p value
1	**P53AIP1**	Abolished	**-15.17**	**1.66**	**0.05**
2	**SPINK6**		**-9.12**	**3.10**	**0.01**
3	**DUX4**		**-4.98**	**-1.50**	**0.00**
4	ERCC2		-14.42	1.01	0.97
5	C1D		-12.10	1.00	0.92
6	**FAS**	Enhanced	**7.20**	**2.11**	**0.00**
7	**ABCD2**		**6.30**	**1.62**	**0.04**
8	**AKT1S1**		**6.00**	**-2.51**	**0.00**
9	GDNF		4.00	-1.44	0.00
10	HNF4		10.60	1.18	0.91
11	TNP1		4.40	1.11	0.88
12	**SLC7A13**	Shift	**-13.80**	**1.61**	**0.04**
			4.20		
13	**UNKL**		**-4.30**	**-1.57**	**0.00**
			4.00		
14	KALRN		-9.80	-1.11	0.96
			5.10		

Figure 3.3. HBx-altered p53-DNA binding does not associate with any gene deregulation pattern. A. Graphical representation of HBx-altered p53-DNA binding patterns (abolished, enhanced, shift) with associated gene expression patterns (up-regulated, down-regulated and unchanged). Gene deregulation patterns associated with each p53-DNA binding alteration are presented in percentages. B. ChIP-qPCR and qRT-PCR validated p53-DNA binding alterations with corresponding gene deregulation by HBx. Shown are the gene name, ChIP-on-chip p53 binding pattern and MAT score, as well as corresponding gene expression fold change and p value. Significantly deregulated candidate genes (fold change >1.5 or <-1.5, p value ≤ 0.05) with altered p53 binding are indicated in bold.

localization and transport of mRNA by HBx, disruption of p53-mediated transcription primarily accounts for the gene deregulation patterns since HBx functions predominantly as a transcription co-factor in transcription regulation. Further, since mammalian gene transcription regulation is typically the result of an intricate interplay of transcription factors and co-regulators, these findings instead suggest that other transcription factors/co-regulators may co-operate with p53 to determine the resultant gene deregulation patterns.

Thus, to examine the role of HBx in modulating p53-DNA binding and transcription regulation in greater detail, *p53AIP1* – an experimentally validated HBx-deregulated gene with associated altered p53 binding at its promoter region identified from the global p53 ChIP-on-chip and expression profiling studies was selected for further characterization (Figure 3.3B). *P53AIP1* was selected for several reasons: it is a known p53-regulated gene, and its pro-apoptotic function is consistent with the reported role of HBx in apoptosis induction as well as with the predicted function of HBx-deregulated genes in positively regulating apoptosis (Figure 3.2C).

3.2 HBx induces a novel shift in p53 binding to the regulatory region of *p53AIP1*

3.2.1 HBx abolishes p53 recruitment to a novel *p53AIP1* promoter p53 response element

From the integrated global p53-DNA binding and expression profiling study of control and HBx HepG2 (UV-treated) cells, we identified a known p53-regulated gene *p53AIP1* that was deregulated by HBx with associated altered p53 binding at its promoter region. From the p53 ChIP-on-chip study, p53 bound to the promoter region of *p53AIP1* gene in control cells, and this binding was abolished in the presence of HBx. Figure 3.4A shows the novel p53 binding region indicated by a peak spanning 241 bp and comprising of signals

from five consecutive microarray probes. This candidate p53 binding region was further predicted to contain a p53 response element using the p53MH algorithm (Figure 3.4A). Located 861 bp upstream of the *p53AIP1* transcription start site, this previously unreported promoter p53 response element consisted of two half-sites separated by a 9 bp spacer and exhibited 65% similarity to the p53 consensus sequence as predicted by p53MH (Figure 3.4A).

To experimentally validate this observed HBx-induced differential binding of p53, ChIP using p53-specific antibody or normal IgG (non-specific control) was performed on control and HBx HepG2 (UV-treated) cells and ChIP DNA enrichment was determined by quantitative real-time PCR (qPCR) with primers specific for detection of the promoter region of interest (Table 2.3). Consistent with the p53 ChIP-on-chip results, significant enrichment of the promoter region was detected in control cells following p53 ChIP ($p<0.01$), but not in p53 ChIP of HBx cells and cells immunoprecipitated with the non-specific IgG antibody (Figure 3.4Bi). This confirmed that p53 bound to the promoter region of *p53AIP1* and this binding is abolished in the presence of HBx.

To examine if this HBx-induced differential p53 binding at the *p53AIP1* promoter was specific to the DNA-damaging treatment (UV) applied or the cell line used (transformed HepG2 liver cells), ChIP using p53-specific antibody or normal IgG was similarly performed on control and HBx-expressing non-transformed THLE-3 liver cells without DNA damaging treatment. Consistent with the observations in UV-treated HepG2 cells, p53 bound to the promoter region in control THLE-3 cells, and this binding was significantly reduced in the

Figure 3.4

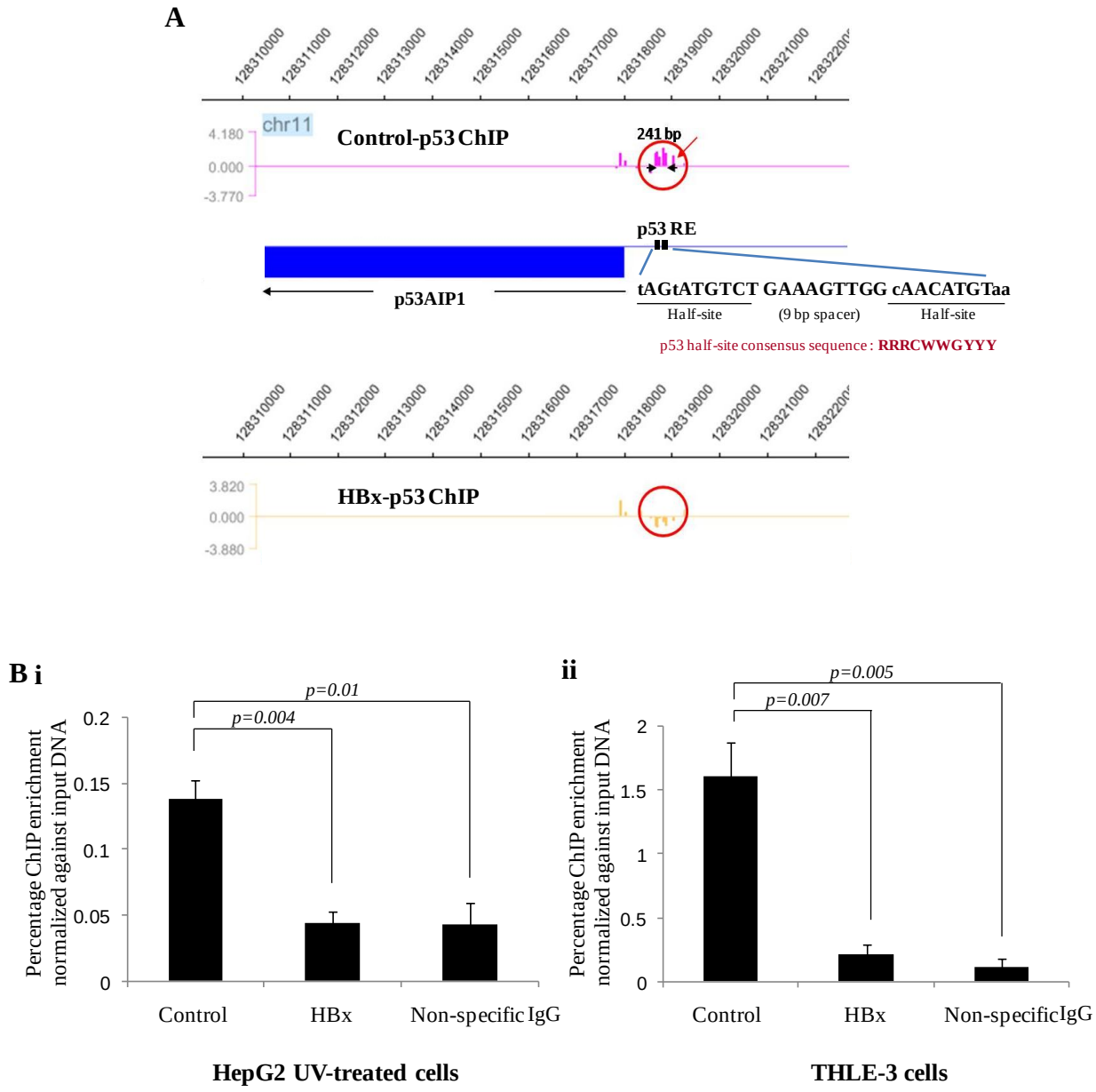


Figure 3.4. HBx abolishes p53 binding at a novel response element in *p53AIP1* promoter. A. A novel p53 RE in the promoter region of p53AIP1 gene is identified by ChIP-on-chip experiments. The DNA sequence of the novel p53 RE is 65% similar to the p53 consensus sequence. Nucleotides in capital letters represent identity of genomic sequence to the consensus; nucleotides in lower case letters represent disparity with the consensus. Figure was generated using SignalMap software (NimbleGen Systems). p53 binding at the novel p53 RE is abolished in the presence of HBx, indicated by the absence of a peak in HBx-p53 ChIP sample. B. Differential p53 binding at the novel p53 RE is successfully validated by ChIP-qPCR. p53 ChIP performed on control and HBx (i) HepG2 (UV-treated) and (ii) THLE-3 cells. All error bars show standard error of the mean (± SEM) of triplicate experiments.

presence of HBx ($p<0.01$) (Figure 3.4Bii). The data thus suggests that HBx abolishes p53 binding to the *p53AIP1* promoter response element regardless of treatment or cell line used.

3.2.2 HBx enhances p53 binding to a previously reported *p53AIP1* intron 1 p53 response element

A functional p53 response element located in intron 1 of *p53AIP1* was previously reported by Oda *et al.* to be necessary for its p53-mediated regulation (Figure 3.5A). As the intron 1 p53 response element is located approximately 2 kb downstream of the *p53AIP1* transcription start site, it was not examined by the p53 ChIP-on-chip screen that probed only a 1.5 kb promoter region of each gene. A comparison of the intron 1 and promoter p53 response elements showed that the intron 1 response element exhibited a higher degree of similarity to the p53 consensus sequence (80% similarity as predicted by p53MH), as compared to the promoter response element (65% similarity) (Figure 3.5B). Another feature that distinguished the intron 1 from the promoter p53 response element is the absence of a spacer in the intron 1 response element where the two p53 half-sites occur in tandem (Figure 3.5B). As differences in the base and spacer composition of p53 response elements are thought to influence p53 binding and subsequent transcription regulation, we therefore examined the pattern of p53 binding at this intron 1 p53 response element and if HBx also affected this binding.

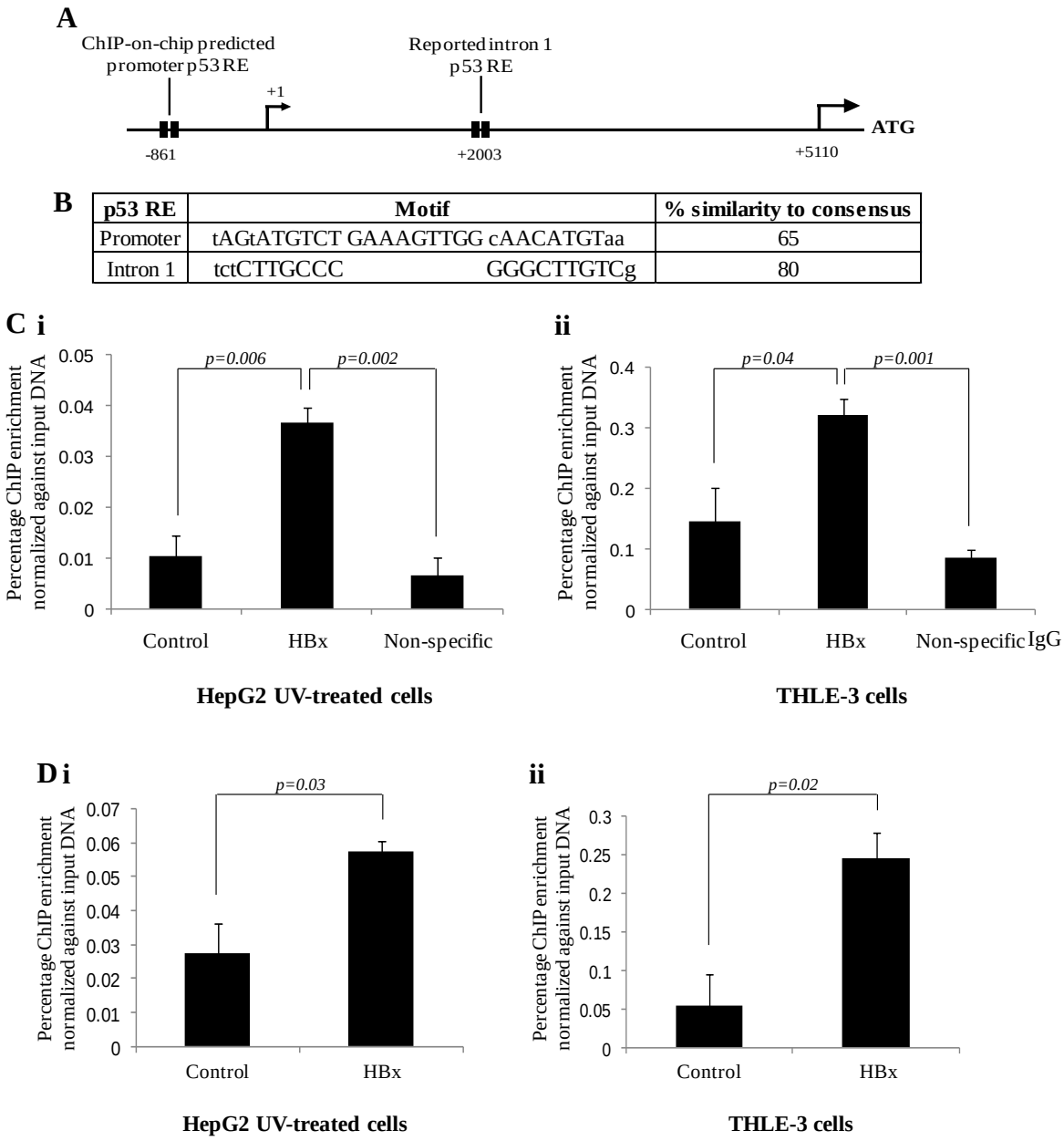
To this end, ChIP using p53-specific antibody or normal IgG was performed on control and HBx-expressing HepG2 (UV-treated) cells as well as THLE-3 cells and ChIP DNA enrichment was determined using qPCR with primers specific for detection of the intron 1 region (Table 2.3). Intriguingly, binding of p53 to the intron 1 response element was

significantly enhanced in the presence of HBx in both cell lines tested ($p<0.05$) and regardless of UV treatment applied (Figure 3.5Ci and ii). Further, since HBx is known to bind DNA indirectly via transcription factors such as p53, we asked if HBx was also recruited together with p53 to the intron 1 region of *p53AIP1*. ChIP using a HBx-specific antibody generated in our lab was performed on control and HBx-expressing HepG2 (UV-treated) and THLE-3 cells, and ChIP DNA enrichment was similarly determined using qPCR with primers specific for detection of the intron 1 region. Analysis of HBx ChIP-qPCR revealed significant enrichment of the intron 1 region in the presence of HBx ($p<0.05$), indicating that a HBx-p53 transcription complex may be recruited to the intron 1 RE (Figure 3.5Di and ii). Taken together, our findings suggest that HBx might directly induce a novel shift in p53 recruitment from the promoter to the intron 1 response element of *p53AIP1* gene.

3.2.3 The novel *p53AIP1* promoter p53 response element identified is essential for p53-mediated transcription

To determine if the previously unreported *p53AIP1* promoter p53 response element identified in our p53 ChIP-on-chip study is a bona fide p53 response element, a DNA fragment harbouring the promoter p53 response element was cloned upstream of a beta-galactosidase (β-gal) reporter gene and assayed for reporter activity in p53-deficient Hep3B cells. Hep3B cells were used as the effect of exogenously introduced p53 could be consistently and easily tested. Approximately 80% transfection efficiency in Hep3B cells was consistently achieved in all our reporter assays as shown by the proportion of green fluorescence cells detected following chemical transfection of reporter constructs harbouring the enhanced green fluorescence protein (EGFP) gene (Figure 3.6A). In the absence of p53, almost no reporter activity was detected (Figure 3.6B). However in the presence of p53, reporter activity increased 6-fold compared to that sans p53 ($p<0.01$) (Figure 3.6B).

Figure 3.5



p53 RE	Motif	% similarity to consensus
Promoter	tAGtATGTCT GAAAGTTGG cAACATGTaa	65
Intron 1	tctCTTGCCC GGGCTTGTCg	80

Figure 3.5. HBx enhances p53 binding to the known intron 1 response element of *p53AIP1*. A. Illustration of p53 RE positions relative to p53AIP1 gene. Each p53 RE is depicted as two black boxes, each representing one half-site of the p53 consensus sequence. The transcription start site (+1) and translation start site (ATG) of *p53AIP1* gene are also depicted. B. Comparison of promoter and intron 1 p53 response elements. Shown are the motifs and similarity to the consensus sequence. C. p53 binding at intron 1 p53 RE is enhanced in the presence of HBx. p53 ChIP-qPCR was performed on control and HBx (i) HepG2 (UV-treated) and (ii) THLE-3 cells. ChIP using normal IgG was performed as a non-specific control. D. HBx indirectly binds to intron 1 p53 RE of *p53AIP1*. HBx ChIP-qPCR was performed on control and HBx (i) HepG2 (UV-treated) and (ii) THLE-3 cells using antibody specific for HBx previously generated in the lab.

Moreover, depletion of p53 using short-interfering RNA (siRNA) specific for p53 negated the increase in reporter activity ($p<0.01$), indicating that the *p53AIP1* regulatory region tested that contained the promoter p53 response element is responsive to p53 (Figure 3.6B).

To confirm that the novel *p53AIP1* promoter p53 response element is indeed functional and essential for transcription regulation, core nucleotides of the p53 response element that are crucial for p53-DNA binding were mutated, inserted upstream of the β-gal reporter gene and assayed for reporter activity. In addition, reporter constructs harbouring mutated intron 1 p53 response element (M2) as well as a combination of mutated promoter and intron 1 p53 response elements (double mutant M3) were generated and similarly assayed. All mutations introduced were confirmed not to obliterate or create other overlapping transcription factor binding sites (TFBS) by comparing TFBS profiles of wild-type and mutant promoters using the transcription factor binding motif prediction tool MatInspector. Consistent with previous reports that the intron 1 p53 response element of *p53AIP1* is functional and essential for transcription, only marginal reporter activity was observed when the intron 1 p53 response element was mutated (M2 construct) as compared to that of the construct harbouring wild-type p53 response elements ($p<0.05$) (Figure 3.6C). Importantly, a complete loss in reporter activity was observed by mutating the promoter p53 response element (M1 construct) ($p<0.01$) (Figure 3.6C), demonstrating that the promoter p53 response element is indeed a bona fide p53 response element and essential for transcription stimulation. The construct harbouring both mutated promoter and intron 1 p53 response elements (M3) unexpectedly exhibited higher reporter activity than the individual mutants, but this activity was still markedly lower than that of the wild-type promoter (Figure 3.6C). We speculate that this may be due to the action of other transcription regulators that may bind to the examined promoter region, though their role in transcription activation may

Figure 3.6

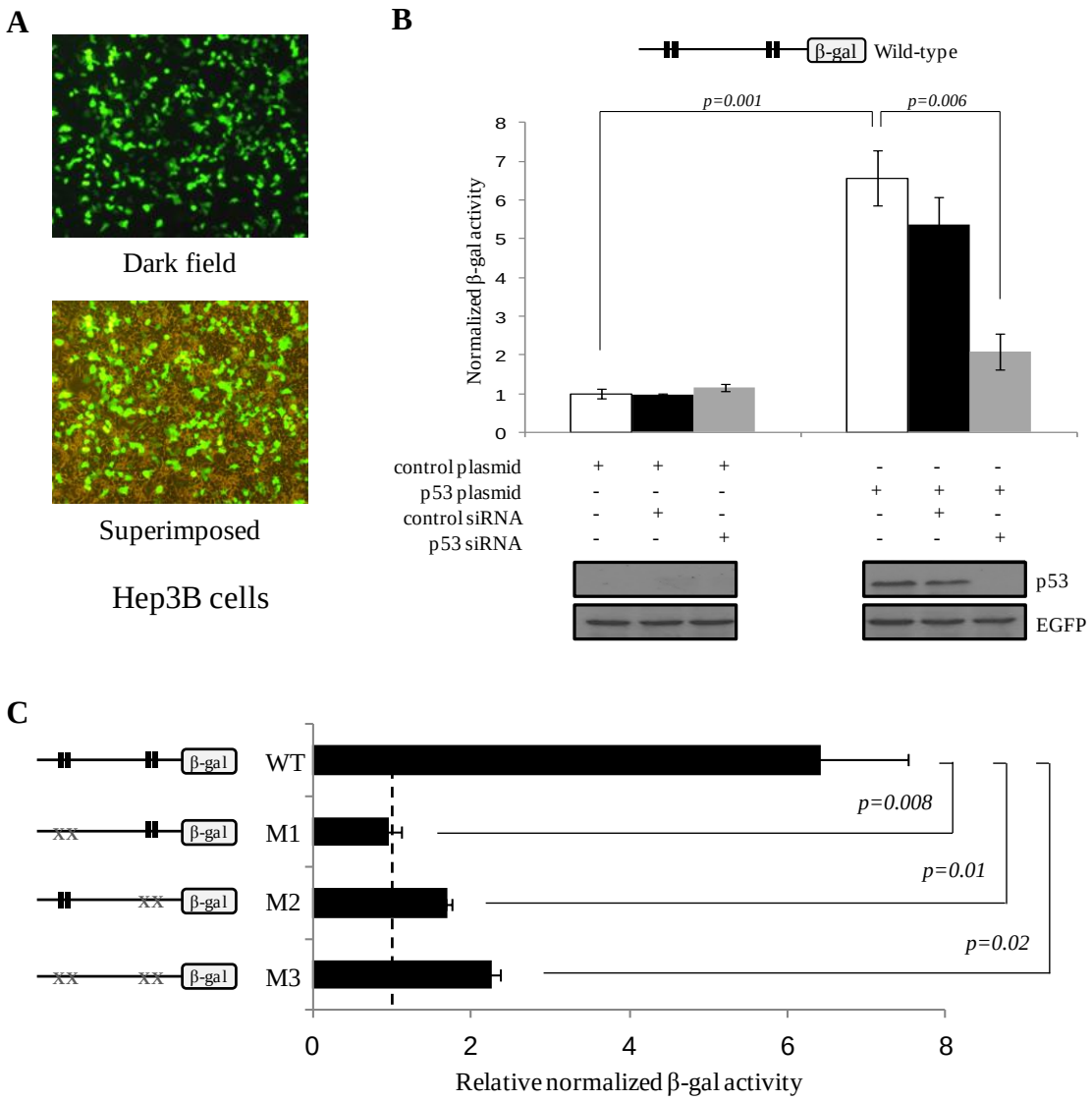


Figure 3.6. Both promoter and intron 1 p53 response elements are functional and necessary for *p53AIP1* regulation. A. High transfection efficiency (approximately 80%) is consistently achieved in Hep3B cells. Shown are the dark field and the corresponding superimposed image of a representative transfection experiment using the EGFP-bearing promoter construct in Hep3B cells. B. p53 stimulates *p53AIP1* promoter activity. p53-deficient Hep3B cells were co-transfected with wild-type promoter construct and indicated plasmids and/or siRNA, and assayed for β-gal activity. p53 and EGFP expression levels are examined by western blotting. C. Both p53 REs are functional and necessary for *p53AIP1* regulation. Hep3B cells were co-transfected with wild-type or mutant promoter constructs, and p53 or control plasmid. Basal β-gal activity is denoted by the vertical black dashed line. All error bars show ± SEM of triplicate experiments.

be of a lesser extent as compared to the central role of p53. Collectively, the data provides strong evidence that the novel promoter p53 response element of *p53AIP1* identified from the p53 ChIP-on-chip study is functional and essential for p53-mediated transcription.

3.3 HBx modulation of p53-DNA binding deregulates *p53AIP1* expression

3.3.1 HBx increases *p53AIP1* expression

Having successfully validated that HBx induces a novel shift in p53-DNA binding at the regulatory region of *p53AIP1*, we next investigated if this HBx-altered p53-DNA binding affects *p53AIP1* expression. As shown in Figure 3.2B, integration of p53 ChIP-on-chip and expression profiling of control and HBx UV-treated HepG2 cells revealed that the HBx-induced differential p53 binding at the *p53AIP1* regulatory region was associated with a 1.6-fold increase in *p53AIP1* expression. To experimentally validate the expression array data, *p53AIP1* expression was measured in control and HBx HepG2 (UV-treated) and THLE-3 cells using qRT-PCR. Consistent with the expression array results, *p53AIP1* expression increased 2-fold ($p<0.05$) and 1.6-fold ($p<0.01$) in HBx HepG2 (UV-treated) and HBx THLE-3 cells respectively, compared to the respective control cells (Figure 3.7Ai and ii).

To determine if this increase in *p53AIP1* expression by HBx is mediated by p53, siRNA specific for p53 or negative control siRNA was introduced into both HepG2 (UV-treated) and THLE-3 cells and cells were subsequently transduced with control or HBx vectors. As shown in Figures 3.7Bi and ii, transient depletion of p53 using p53-specific siRNA abrogated the increase in *p53AIP1* expression by HBx in both of the cell lines tested. These findings strongly suggest that HBx increases *p53AIP1* expression through p53.

Figure 3.7

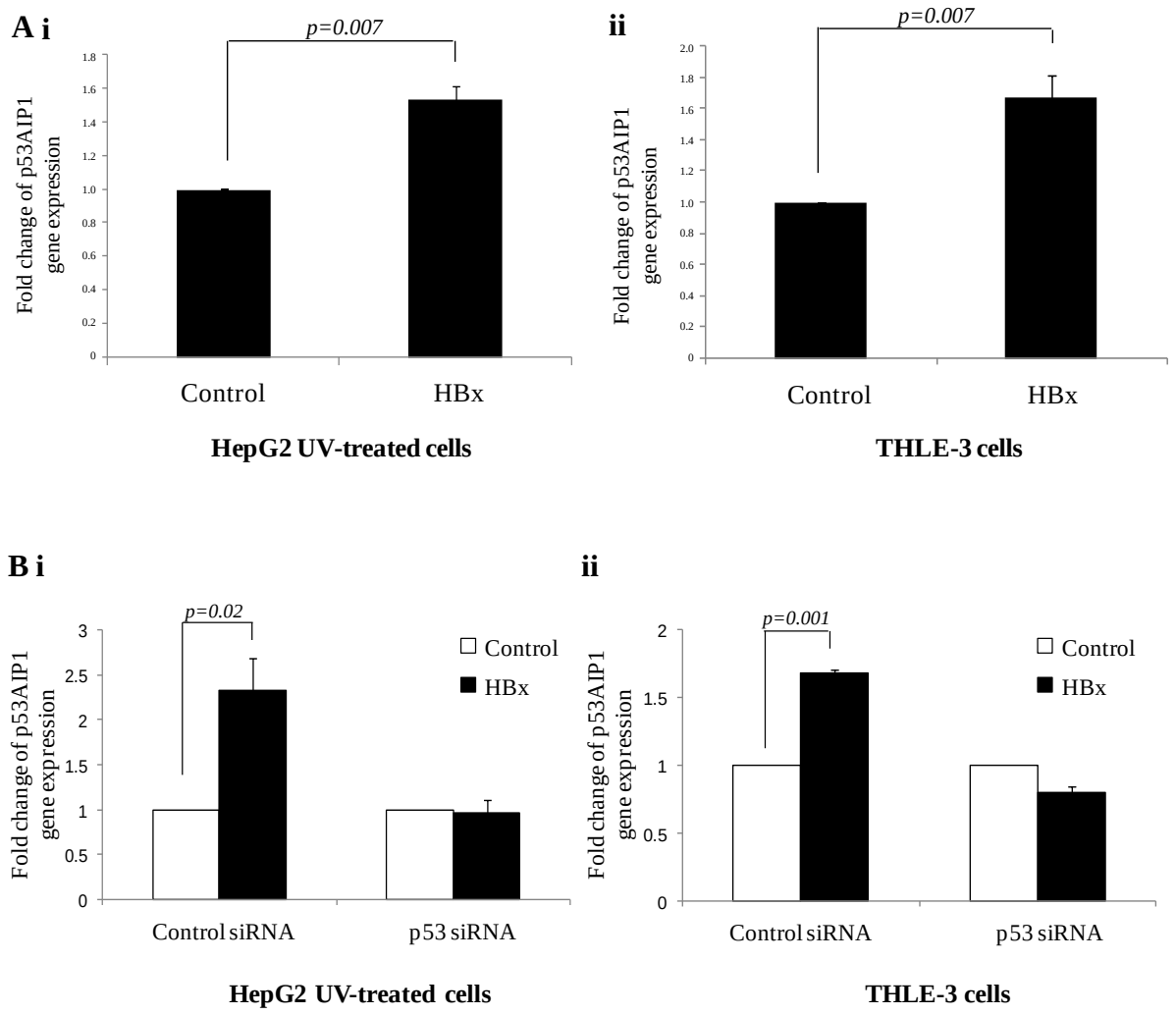


Figure 3.7. HBx up-regulates *p53AIP1* in a p53-dependent manner. A. p53AIP1 expression increases in the presence of HBx. (i) HepG2 (UV-treated) and (ii) THLE-3 cells were transduced with recombinant HBx and control vectors. *p53AIP1* expression levels were measured by qRT-PCR and normalized against beta-actin. B. (i) HepG2 (UV-treated) and (ii) THLE-3 cells were transfected with p53-specific or control siRNA, and transduced with HBx or control vectors 24 h post-transfection. *p53AIP1* expression levels were measured by qRT-PCR and normalized against beta-actin. All error bars show ± SEM of triplicate experiments.

3.3.2 A shift in p53-DNA binding is essential for HBx-induced increase in adjacent gene expression

Our data thus far suggests that HBx induces a novel shift in p53 binding from the promoter to intron 1 response element of *p53AIP1* gene, and that p53 mediates HBx-induced increase in *p53AIP1* expression. To determine if the shift in p53-DNA binding is essential for the increased gene expression by HBx, reporter activities of the wild-type (WT), promoter (M1) and intron 1 (M2) p53 response element single mutants as well as the double mutant (M3) were assayed for β-gal reporter activity in the presence or absence of HBx. Consistent with the findings that HBx increases *p53AIP1* expression, HBx increased reporter activity of the WT construct that harboured intact p53 response elements by approximately 1.4-fold ($p<0.01$) (Figure 3.8). Next, we used the construct bearing the mutated promoter p53 response element (M1) to recapitulate the shift in p53 binding to the intron 1 p53 response element and examined the effect of HBx on its reporter activity. Importantly, HBx increased M1 reporter activity by approximately 1.8-fold ($p<0.01$) (Figure 3.8). This increase in M1 promoter activity by HBx was significantly greater than that of the WT construct ($p<0.05$). In contrast, reporter activities of constructs containing the mutated intron 1 p53 response element (M2 and double mutant M3) were not significantly enhanced in the presence of HBx (Figure 3.8), demonstrating that the shift in p53 binding from the promoter to intron 1 response element of *p53AIP1* is essential for stimulation of adjacent gene expression by HBx. These findings also hint that HBx might relieve p53-associated repression at the promoter region and enhance p53-associated transcription stimulation at the intron 1 region of *p53AIP1*.

3.3.3 Increased *p53AIP1* expression in tumours of HCC patients with high HBx expression

To evaluate the clinical relevance of the *in vitro* findings that HBx increases *p53AIP1* expression, *p53AIP1* gene expression and HBx protein expression profiles were analyzed in 78 de-identified tumour (T) and paired non-tumorous (NT) tissues of HCC patients obtained from the NCCS/SingHealth Tissue Repository. *p53AIP1* expression was determined using qRT-PCR analysis and normalized to the respective *β-actin* expression. HBx protein expression was determined using western blotting with a HBx-specific antibody generated in our lab followed by quantification using densitometry analysis. HBx expression of each sample was normalized to the respective β-actin expression. Patients were subsequently classified into two groups according to their HBx status. Thirty-two HCC patients were classified in 'high HBx status' group where HBx expression is more than 2-fold T versus NT while 46 HCC patients were classified in 'low HBx status' group where HBx expression is less than 2-fold T versus NT. Strikingly, a comparison of the ratio of *p53AIP1* expression (T/NT) and HBx status of the 78 HCC patients showed significantly higher ($p<0.05$) *p53AIP1* expression in HCC patients with high HBx status (median 1.46) in contrast to that of HCC patients with low HBx status (median -1.36) (Figure 3.9). This finding lends clinical relevance to our *in vitro* observations thus far that HBx increases *p53AIP1* expression.

Figure 3.8

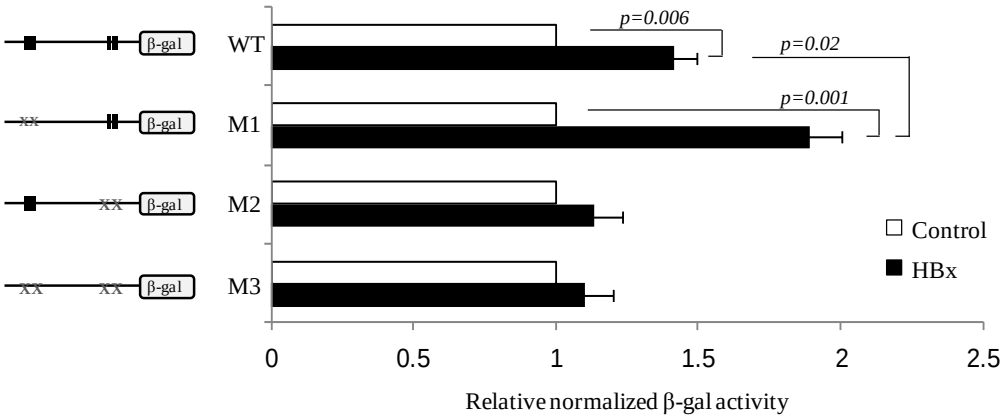


Figure 3.8. HBx stimulates target gene expression in promoter assay. Hep3B cells were co-transfected with wild-type or mutant promoter constructs, and p53 or control plasmid. Cells were transduced with HBx or control adenovirus 24 h post-transfection. All error bars show ± SEM of triplicate experiments.

Figure 3.9

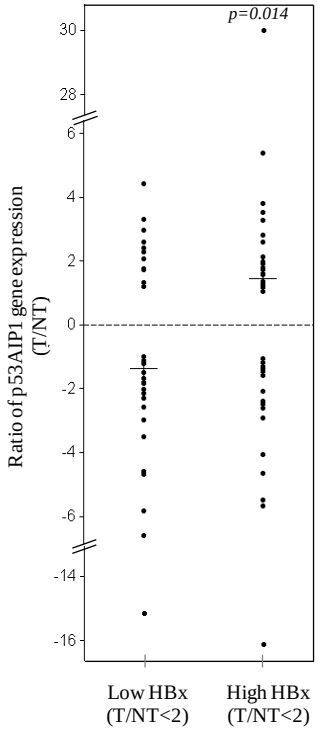


Figure 3.9. *p53AIP1* gene expression is significantly higher in HCC patients with high HBx protein expression. Gene expression profiles and HBx protein status of tumour (T) and adjacent non-tumorous (NT) samples of 78 HCC patients were obtained and analyzed for association. The median ratio of *p53AIP1* gene expression (T/NT) in 32 patients with low HBx protein expression (T/NT<2) and 46 patients with high HBx protein expression (T/NT>2) is -1.36 and 1.46 respectively (p=0.014).

3.3.4 Increased *p53AIP1* expression mediates HBx-induced apoptosis

Having demonstrated that HBx induces *p53AIP1* expression, we next examined the functional relevance of this deregulation. Our lab had previously reported that HBx sensitizes UV-treated HepG2 cells to apoptosis (Lee et al., 2005b). Therefore, we hypothesized that the apoptosis-inducing protein p53AIP1 may be a potential mediator of HBx-induced apoptosis. To this end, HepG2 cells were treated with control or HBx vectors as well as control or *p53AIP1*-specific siRNA, treated with UV irradiation and analyzed for their apoptosis profiles by detection of phosphatidylserine externalization through staining with Annexin V and membrane integrity via the exclusion of 7AAD. A 70% reduction in *p53AIP1* expression was consistently achieved using *p53AIP1*-specific siRNA (Figure 3.10A). In agreement with our lab's previous study, the population of apoptotic cells increased to approximately 20% in the presence of HBx compared to 12% in control cells ($p<0.01$) (Figures 3.10B and C). This increase was abrogated by transiently depleting *p53AIP1* using *p53AIP1*-specific siRNA ($p<0.01$) (Figure 3.10B, C), suggesting a role for p53AIP1 in HBx-induced apoptosis, possibly via a p53-dependent mechanism.

3.4. HBx alters co-regulator recruitment and specific p53 post-translational modification

3.4.1 HBx does not alter p53 phosphorylation at serine 46

p53 has been reported to be regulated by a myriad of site-specific post-translational modifications including phosphorylation, acetylation, ubiquitination and sumoylation. It is proposed that these post-translational modifications can change the conformation of the p53 protein, thereby altering its DNA-binding affinity or sequence-specific selectivity. Importantly, the site-specific phosphorylation at p53 serine 46 (Ser46) was previously

Figure 3.10

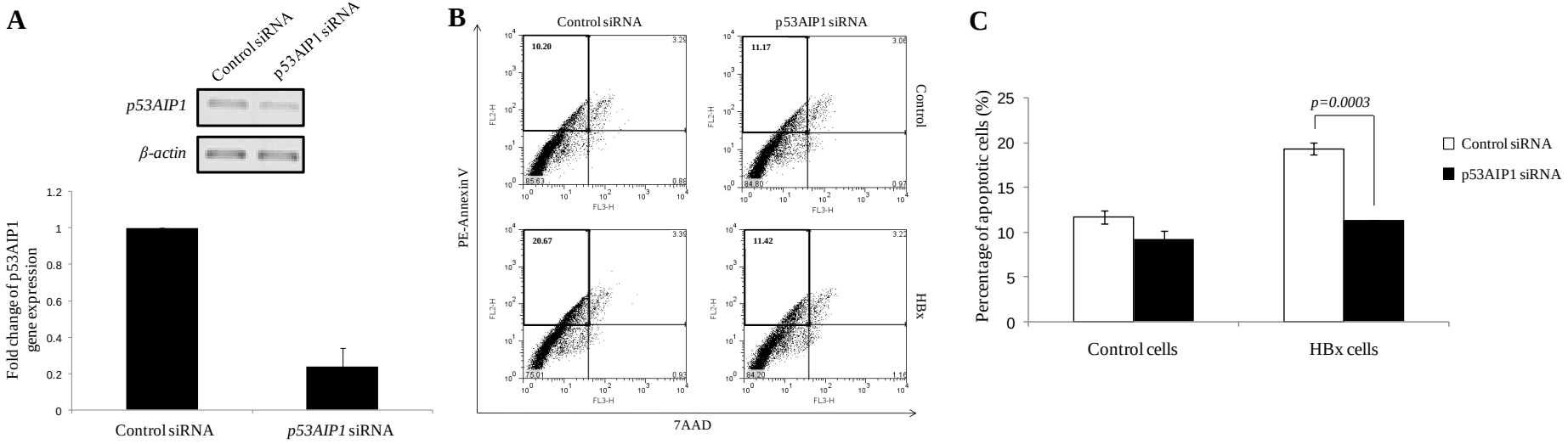


Figure 3.10. *p53AIP1* depletion abrogates HBx-induced apoptosis. A. Transient knock-down of *p53AIP1* using RNA interference. *p53AIP1*-specific and control siRNA was introduced into HepG2 cells using electroporation and cells were harvested 48 h post-transfection. A reduction in *p53AIP1* mRNA levels was achieved in cells with *p53AIP1*-specific siRNA compared to that with control siRNA as measured by RT-qPCR. Shown are *p53AIP1* and *beta-actin* expression levels analyzed by agarose gel electrophoresis. B, C. Increased apoptosis of HBx cells is negated by *p53AIP1* knockdown. HepG2 cells were co-transfected with HBx or control vectors and *p53AIP1*-specific or control siRNA. Apoptosis profiles of cells were determined using PE Annexin V and 7AAD staining, followed by flow cytometry detection and analysis. B. Shown are the staining profiles of a representative set of experiments. The apoptotic cell population is indicated in the upper left quadrant (Annexin V positive, 7AAD negative). C. Shown are the percentages of apoptotic cells described in B from 3 independent experiments. All error bars show ± SEM of triplicate experiments.

reported by Oda *et al.* to correlate with *p53AIP1* expression and apoptosis induction. We therefore hypothesized that HBx might enhance phosphorylation of p53 Ser46, altering its DNA-binding affinity and resulting in increased *p53AIP1* expression.

To test this hypothesis, phosphorylation levels of p53 Ser46 were examined in control and HBx HepG2 (UV-treated) and THLE-3 cells over a 72 hr time period, using an antibody specific for phosphorylated p53 Ser46. As shown in Figure 3.11, similar phosphorylation levels of p53 Ser46 were observed in control and HBx cells at each time point, and remained unchanged over the course of 72 hr. These observations were consistent regardless of treatment by UV irradiation or cell line used (Figure 3.11). Since p53 Ser46 phosphorylation is unaffected by HBx, this site-specific p53 post-translational modification is thus unlikely to be involved in HBx-induced *p53AIP1* deregulation.

3.4.2 HBx perturbs unique p53-associated transcription co-regulators

3.4.2.1 DNA-bound p53 co-regulators

Mammalian gene regulation typically involves the interplay of multiple sequence-specific transcription factors and/or co-regulators that bind to transcription factors (collectively termed co-regulatory module). As our findings also thus far hint at the possible involvement of other p53-associated transcription factors/co-regulators that might influence trans-activation or trans-repression of *p53AIP1* expression, we hypothesized that HBx may modulate p53 binding/transcription regulation depending on the specific combination of the surrounding associated transcription factors/co-regulators at the promoter and intron 1 region of *p53AIP1*.

Figure 3.11

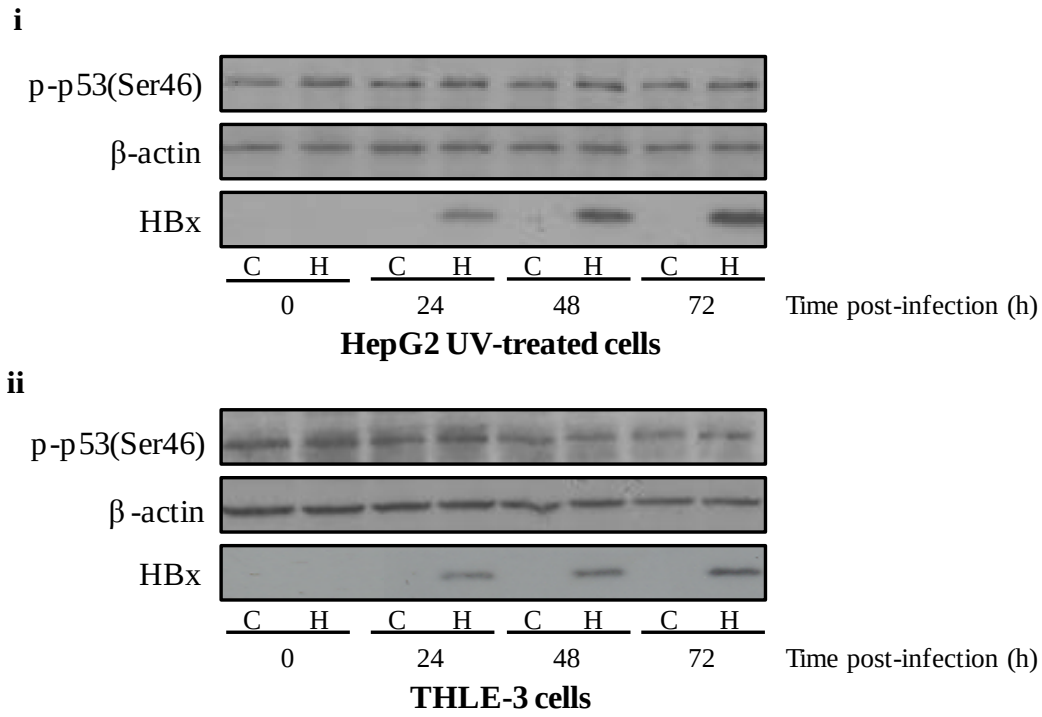


Figure 3.11. HBx does not induce phosphorylation of p53 at Serine 46. Phosphorylation levels of p53 serine 46 residue (Ser46) are unchanged in HBx cells. HepG2 (UV-treated) and THLE-3 cells were transduced with recombinant HBx (H) or control (C) vectors and harvested at various time points. Immunoblot detection of p53 Ser46 phosphorylation levels in (i) HepG2 (UV-treated) and (ii) THLE-3 cells was performed using an antibody specific for the phosphorylated residue.

To identify potential proximal transcription factors at the promoter and intron 1 regions, a computational approach was employed to predict for transcription factor motifs in close proximity (±300 bp) to the p53 response element in both regions (Figure 3.12A). Five high confidence transcription factor motifs were predicted by both TRANSFAC and MatInspector transcription factor binding site prediction tools to reside adjacent to the promoter p53 response element (Figure 3.12Bi). These are heat shock transcription factor 1 (HSF1), globin transcription factor 1 (GATA-1), Yin Yang 1 (YY1), myeloid zinc finger 1 (MZF1) and transcription factor CP2 (Figure 3.12B). At the intron 1 region, four high confidence transcription factor motifs were predicted, namely transcription factor PU.1/SPI1, specificity protein 1 (Sp1), ets variant gene 4 (ETV4/PEA3) and aryl hydrocarbon receptor (AHR) (Figure 3.12Bii).

To further select for strong p53-interacting candidates, these predicted high confidence transcription factors were then searched in the literature for known interactions with p53. Notably, only transcription repressors YY1 and GATA-1 located -102 bp and -175 bp respectively relative to the promoter p53 response element, and transcription activator Sp1 located -145 bp relative to the intron 1 p53 response element were shortlisted as strong p53-interacting candidates (Figure 3.12B and 3.13A). To assess if these strong p53-interacting candidates were recruited to their respective response elements and if HBx affected their binding, ChIP was performed on control and HBx THLE-3 cells using antibodies specific for YY1, GATA-1 and Sp1. Similar to the pattern of p53 binding at the promoter response element, YY1 and GATA-1 bound to their respective predicted binding sites in control cells but this binding was abolished in the presence of HBx ($p<0.05$ and $p<0.01$ respectively) (Figure 3.13B and C). There was no significant ChIP enrichment in both control and HBx

Figure 3.12

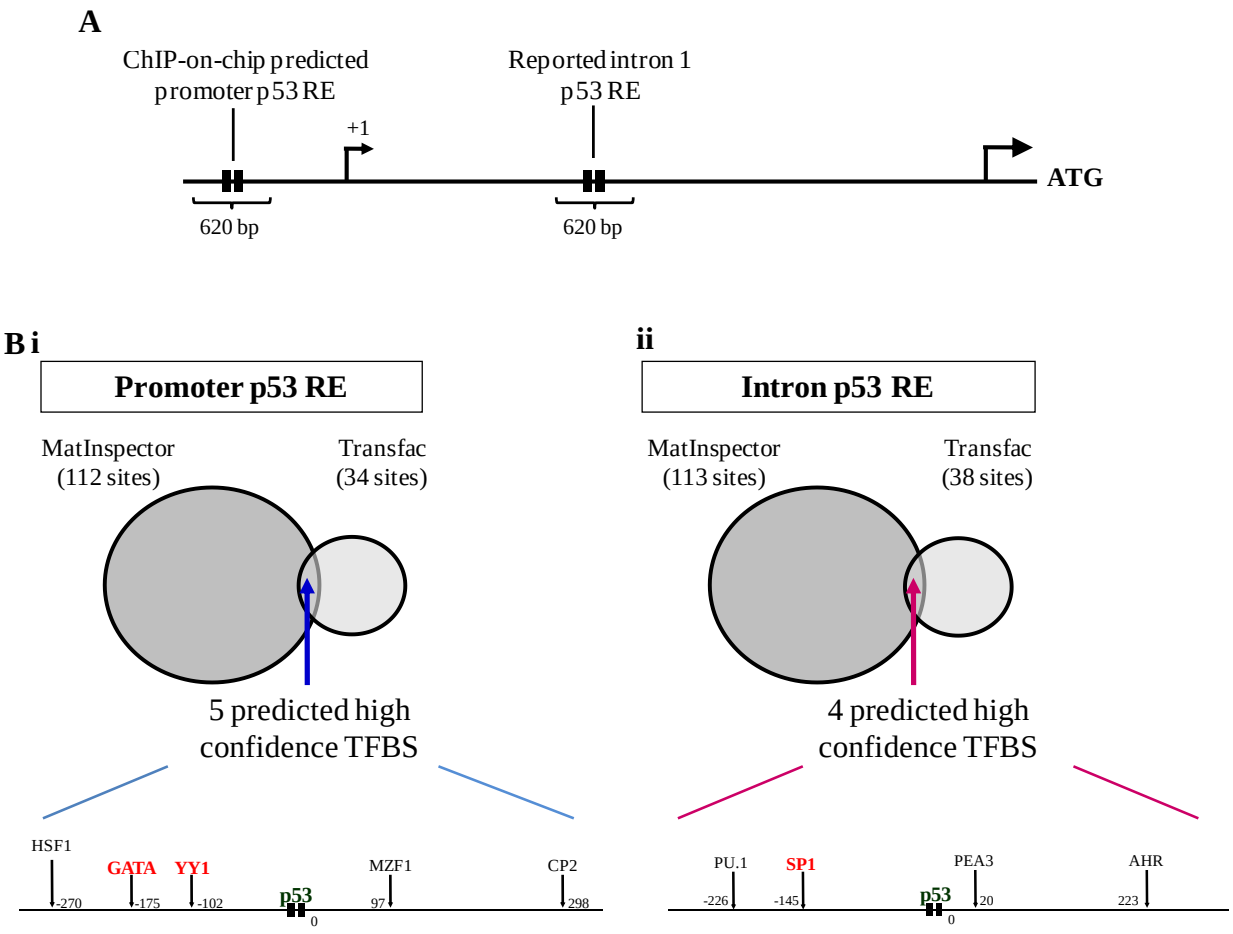


Figure 3.12. High confidence transcription factor binding motifs adjacent to promoter and intron 1 p53 response elements predicted by both TRANSFAC and MatInspector. A. DNA sequence ±300 bp of each p53 response element was queried for transcription factor binding motifs using MatInspector and TRANSFAC prediction tools. B. Shown are the predicted high confidence transcription factors and their respective motif positions relative to the (i) promoter and (ii) intron 1 p53 response element that were predicted by both algorithms. Strong p53-interacting candidates are highlighted in red.

Figure 3.13

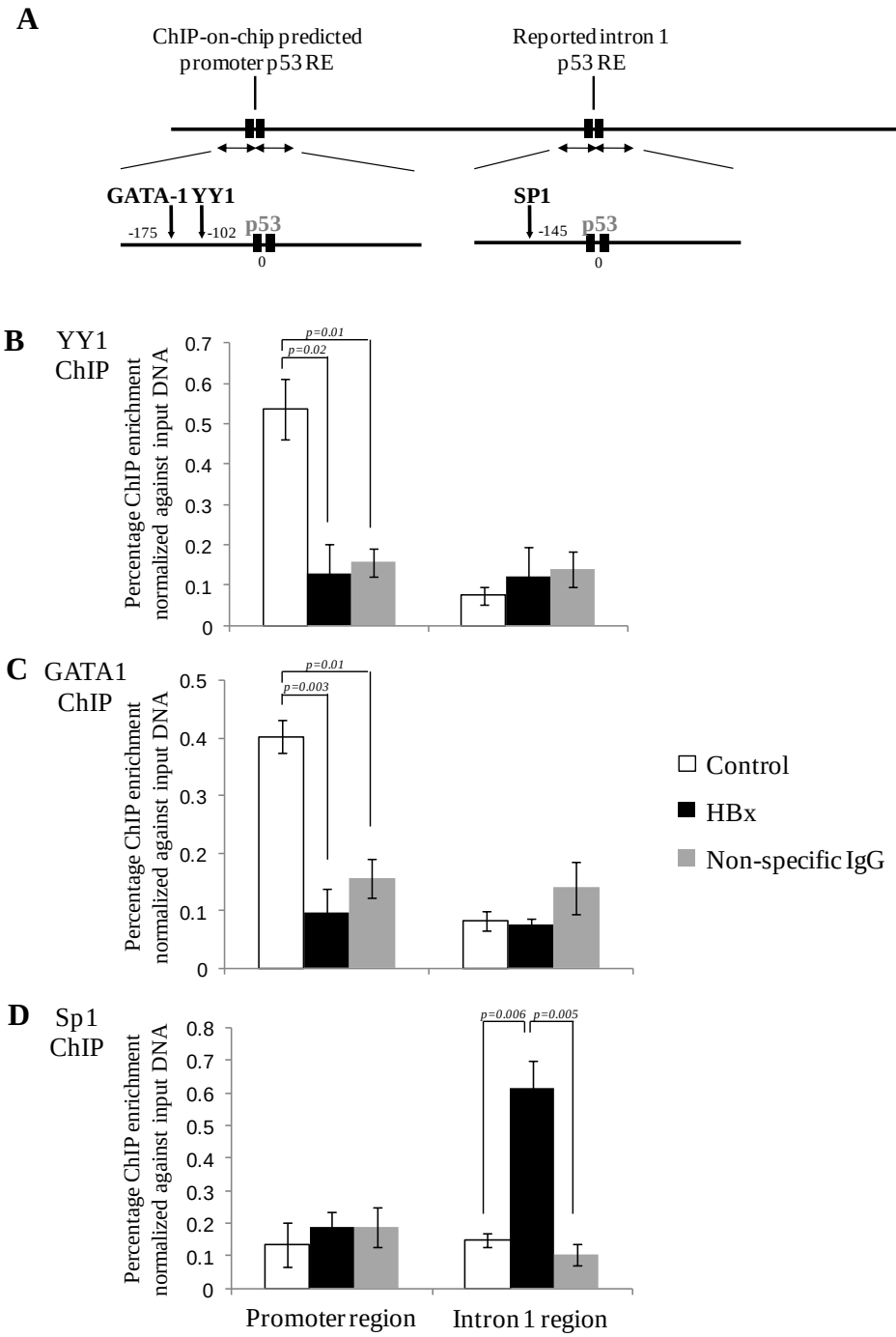


Figure 3.13. HBx perturbs recruitment of distinct transcription co-regulators. A. MatInspector- and TRANSFAC-predicted high confidence transcription factor binding motifs (TFBS) adjacent to the p53 REs (±300 bp) that interact with p53. Shown are their positions relative to the respective p53 RE. B, C and D. Differential binding of transcription factors YY1, GATA-1 and Sp1 in the presence of HBx were validated using ChIP-qPCR on control and HBx THLE-3 cells using specific antibodies for YY1 (B), GATA-1 (C) and Sp1 (D). ChIP using normal IgG was performed as a non-specific control. All error bars show ± SEM of triplicate experiments.

cells at the intron 1 region where no YY1 or GATA-1 motifs were predicted (Figure 3.13B and C). On the other hand, Sp1 binding to its predicted response element was enhanced in the presence of HBx, analogous to that of p53 at the intron 1 RE ($p<0.01$) (Figure 3.13D). There was no significant ChIP enrichment in both control and HBx cells at the promoter region where no Sp1 motif was predicted (Figure 3.13D). These findings suggest that HBx disrupts the recruitment of a transcriptionally repressive p53-YY1-GATA1 complex and favours the recruitment of a transcriptionally activating p53-Sp1 complex to the regulatory region of *p53AIP1*.

3.4.2.2 p53-associated transcription factors modulate gene transcription

To examine if the recruitment of the p53-associated transcription factors YY1, GATA-1 and Sp1 modulate *p53AIP1* transcription, each transcription factor of interest was depleted using the respective specific siRNA and its effect on *p53AIP1* expression was determined. In addition, a complementary two-pronged approach using RNA interference and mutagenesis studies in the aforementioned reporter assay system was also employed. Essentially, the transcription factor of interest was either depleted using siRNA or prevented to bind to their respective binding site in the *p53AIP1* regulatory region by mutating the core nucleotides of the response element, and assayed for β-gal reporter activity in Hep3B cells.

First, by chemically transfecting siRNA specific for transcription repressors YY1 and GATA-1 into cells, efficient knockdown in their respective expression was achieved (Figure 3.14Ai and ii). qRT-PCR analysis of *p53AIP1* expression levels in THLE-3 cells revealed that depletion of either YY1 or GATA-1 resulted in a significant increase in *p53AIP1* expression as compared to that treated with the negative control siRNA ($p<0.01$) (Figure 3.14B). Moreover, analysis of the reporter activity of wild-type *p53AIP1* promoter construct

in YY1- and GATA-1- depleted Hep3B cells showed a moderate increase in reporter activity in the presence of p53 ($p<0.05$) (Figure 3.14C). To test if this increase in gene transcription is indeed a result of decreased transcription factor recruitment, and not due to unanticipated off-target effects of the siRNAs used, we employed the second approach that specifically examines the effect of abolishing transcription factor recruitment to the binding site of interest by mutating the response element. Significantly, abolishment of YY1 or GATA-1 binding to their respective response elements by mutagenesis studies showed enhanced reporter activity of the *p53AIP1* promoter ($p<0.05$ and $p<0.01$ respectively) (Figure 3.14D), confirming that the transcription repressors YY1 and GATA-1 negatively modulate *p53AIP1* expression.

To examine if Sp1 recruitment to *p53AIP1* intron 1 region influences *p53AIP1* expression in the presence of HBx, Sp1-specific RNA interference and mutagenesis studies were similarly performed in the presence or absence of HBx. Following efficient knockdown of Sp1 in THLE-3 cells (Figure 3.15A), qRT-PCR analysis of *p53AIP1* expression revealed that Sp1 depletion negated the increase in *p53AIP1* expression by HBx (Figure 3.15B). In addition, transient depletion of Sp1 using Sp1-specific siRNA moderately blunted the increase in reporter activity in HBx cells, albeit not as pronounced as that observed in Sp1-depleted THLE-3 cells (Figure 3.15C). Consistently, obliteration of Sp1 binding to the *p53AIP1* intron 1 region by mutating its response element also moderately blunted the HBx-induced increase in reporter activity (Figure 3.15D). We speculate that over-expression of p53 by exogenously introducing p53 into Hep3B cells in the reporter assays might have some compensatory effects that may mask the effect of Sp1 depletion on reporter activity. This might account for the modest effect in reporter activity observed in Sp1-depleted HBx cells

Figure 3.14

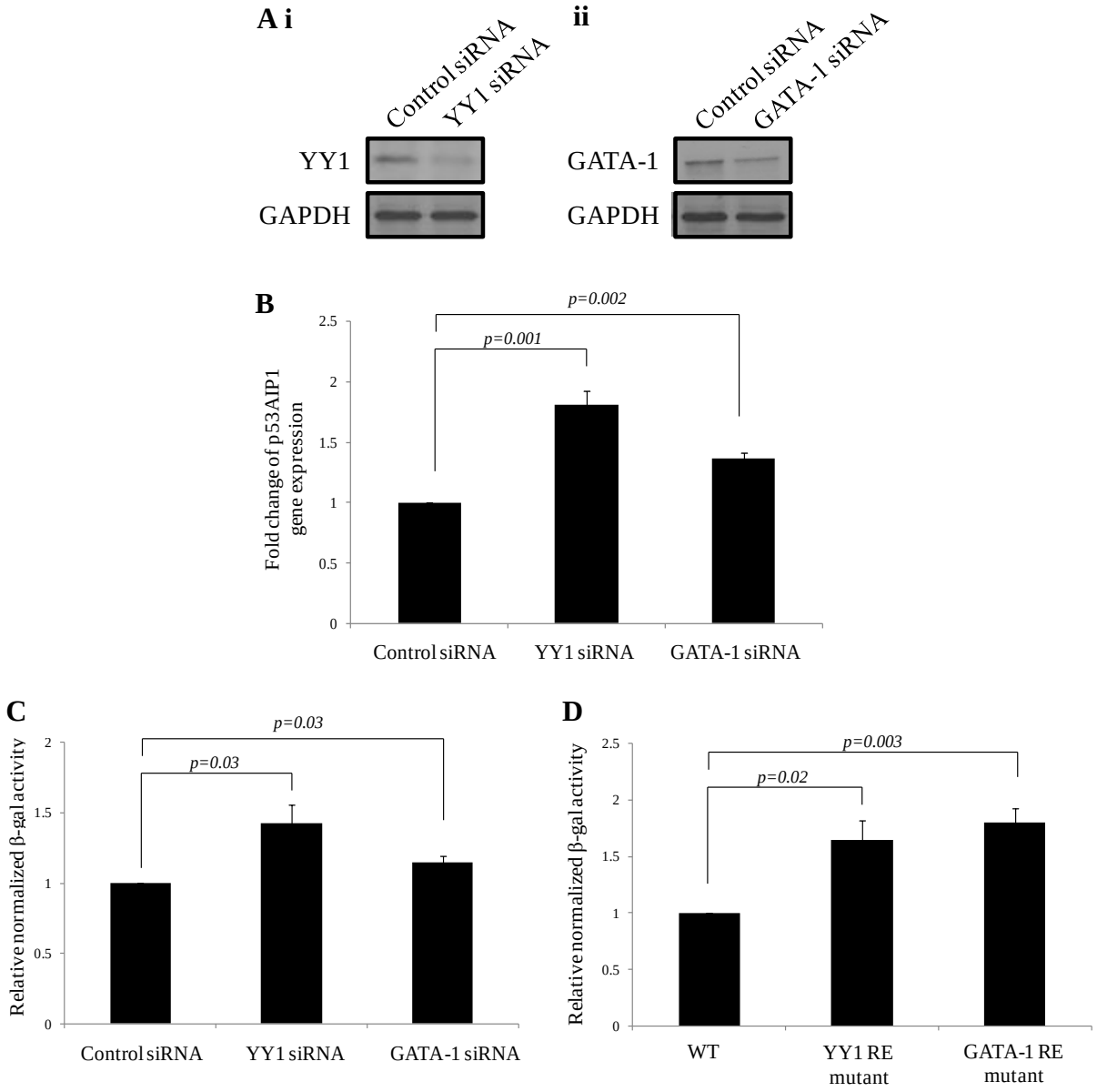


Figure 3.14. YY1 and GATA-1 negatively modulate *p53AIP1* expression. A. Efficient knockdown of (i) YY1 and (ii) GATA-1 is achieved using the respective specific siRNAs. Shown are western blots of YY1, GATA-1 and loading control GAPDH of THLE-3 cells transfected with the indicated siRNAs. B and C. Knockdown of YY1 and GATA-1 using specific siRNAs increases gene expression. B. THLE-3 cells were transfected with YY1, GATA-1 or control scrambled siRNA. *P53AIP1* expression was measured using qRT-PCR. C. Hep3B cells were co-transfected with wild-type *p53AIP1* promoter construct, p53 or control plasmid and YY1, GATA-1 or control scrambled siRNA, and assayed for β-gal activity. D. Mutation of YY1 and GATA-1 binding sites increases gene expression. Hep3B cells were co-transfected with wild-type, YY1 or GATA-1 RE mutant *p53AIP1* promoter construct and p53 or control plasmid, and assayed for β-gal activity. D. All error bars show ± SEM of triplicate experiments.

Figure 3.15

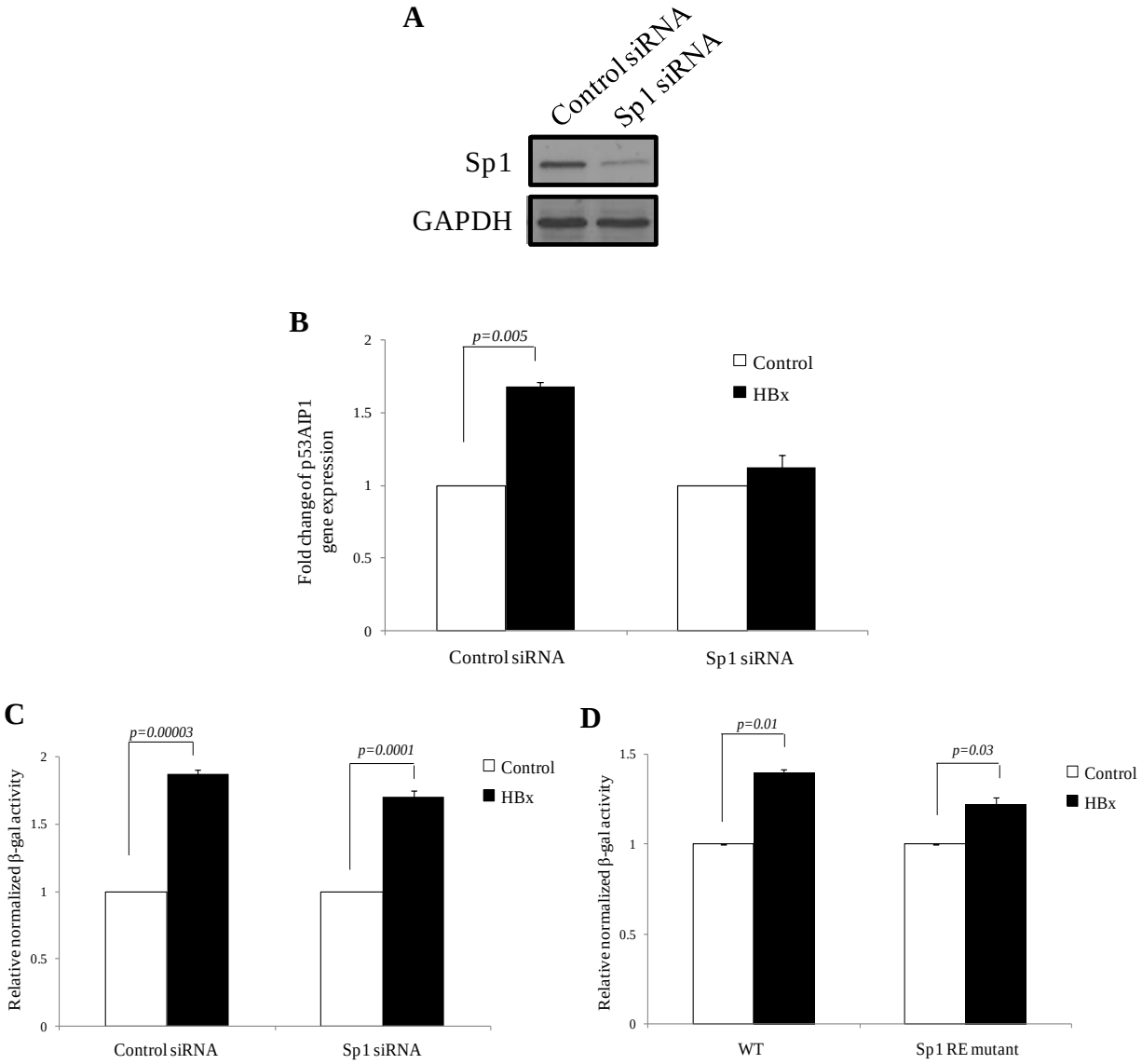


Figure 3.15. Sp1 positively modulates HBx-induced increase in *p53AIP1* expression. A. Efficient knockdown of Sp1 is achieved using the Sp1-specific siRNA. Shown are western blots of Sp1 and loading control GAPDH of THLE-3 cells transfected with the indicated siRNAs. B and C. Knockdown of Sp1 using specific siRNAs blunts HBx-induced increase in gene expression. B. THLE-3 cells were transfected with Sp1 or control scrambled siRNA. *P53AIP1* expression was measured using qRT-PCR. C. Hep3B cells were co-transfected with wild-type *p53AIP1* promoter construct, p53 or control plasmid and Sp1 or control siRNA. Cells were transduced with control or HBx recombinant adenovirus 24 h post-transfection and assayed for β-gal activity. D. Mutation of Sp1 binding site blunts HBx-induced increase in gene expression. Hep3B cells were co-transfected with wild-type or Sp1 RE mutant *p53AIP1* promoter construct and p53 or control plasmid. Cells were transduced with control or HBx recombinant adenovirus 24 h post-transfection and assayed for β-gal activity. All error bars show ± SEM of triplicate experiments.

versus control cells, as compared to that in THLE-3 cells. Nevertheless, our findings suggest that Sp1 positively modulates *p53AIP1* expression in the presence of HBx. Taken together, these findings suggest that the p53-associated transcription co-regulators recruited to the *p53AIP1* regulatory region - YY1 and GATA-1 in the absence of HBx, and Sp1 in the presence of HBx – function to modulate *p53AIP1* gene transcription.

3.4.2.3 Non-DNA-bound p53 co-regulators

Next, to identify other potential members of the promoter and intron 1 regulatory complexes that do not exhibit sequence-specific DNA binding (termed transcription co-regulators), a computational approach that assessed associated networks of protein factors was employed. Given that each member of a regulatory complex associates with at least one other member of the complex (not necessarily p53), all the predicted high confidence transcription factors in each region were queried using GeneMANIA association network prediction tool.

Strikingly, the most strongly-associated factor predicted at the promoter region was histone deacetylase 1 (HDAC1) that associated with three of the five high confidence transcription factors namely YY1, GATA-1 and HSF1, in addition to p53 (Figure 3.16A). To examine whether HDAC1 is recruited to *p53AIP1* promoter region and if this is affected by HBx, ChIP was performed on control and HBx THLE-3 cells using an antibody specific for HDAC1. The pattern of HDAC1 recruitment strikingly resembled that of p53, YY1 and GATA-1 at the *p53AIP1* promoter region – HDAC1 was recruited to the promoter region in control cells, and this recruitment was markedly reduced in the presence of HBx ($p<0.05$)

Figure 3.16

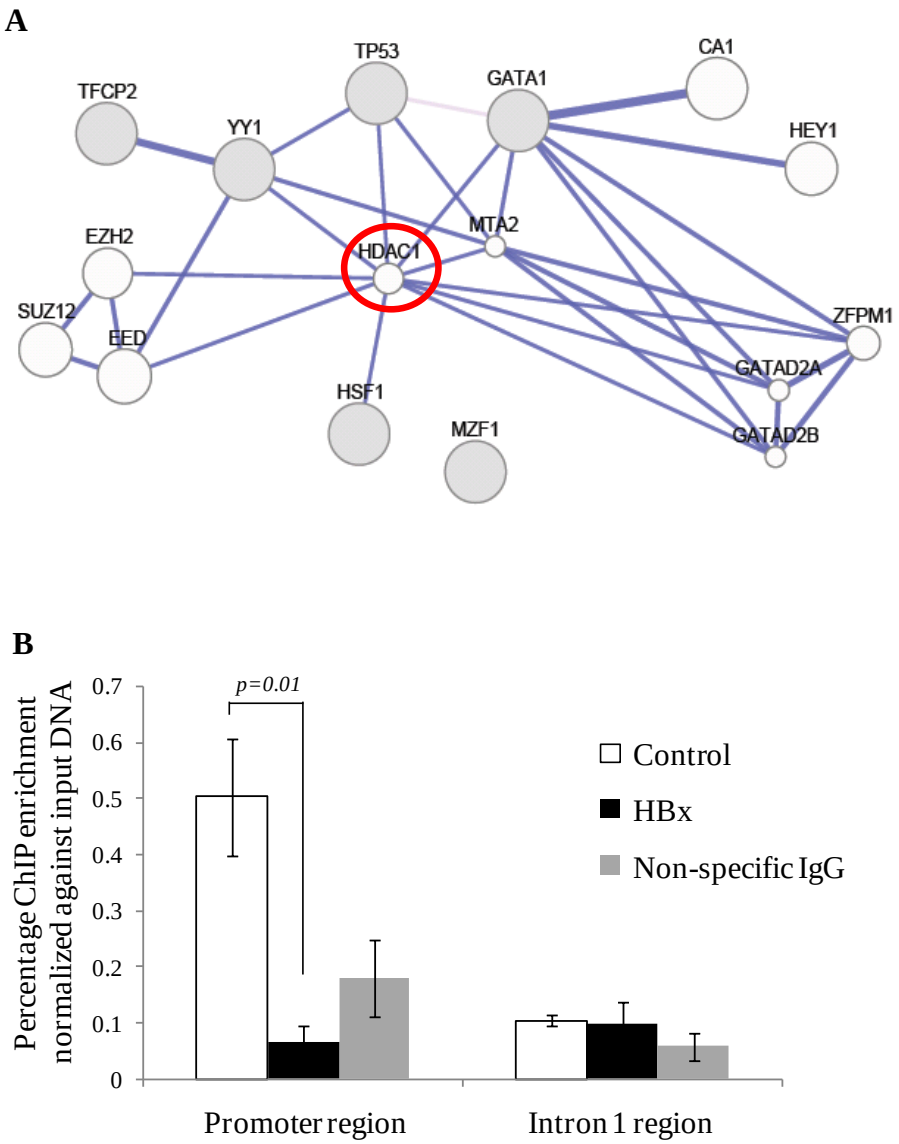


Figure 3.16. HDAC1 recruitment is perturbed by HBx. A. HDAC1 is predicted to associate with p53, YY1 and GATA1. Associated co-regulators adjacent to the promoter p53 RE was predicted using GeneMANIA. TRANSFAC and MatInspector predicted transcription factors are represented by solid grey circles, other associated factors predicted by GeneMANIA are represented by white circles. Solid lines linking two circles indicate reported association between the two factors. B. Recruitment of HDAC1 to the promoter region of p53AIP1 is abolished by HBx. HDAC1 ChIP-qPCR was performed on control and HBx THLE-3 cells. ChIP using normal IgG was performed as a non-specific control. All error bars show ± SEM of triplicate experiments.

(Figure 3.16B). No significant ChIP enrichment at the intron 1 region was detected in both control and HBx cells. This suggests that a huge transcriptionally repressive complex of transcription factors and co-regulator(s) is recruited to the *p53AIP1* promoter region in the absence of HBx that negatively regulates *p53AIP1* expression. No factors were predicted to be strongly-associated with the high confidence transcription factors at the intron 1 region. Taken together, our results indicate that HBx abolishes the binding of a p53-containing repressive transcription complex at the promoter region and favours the recruitment of a transcriptionally activating p53-Sp1 complex at the intron 1 region of *p53AIP1*, plausibly resulting in increased *p53AIP1* gene expression.

3.4.3 Expression of transcription co-regulators is generally unaffected by HBx

Since our findings thus far demonstrate that p53-associated transcription factors and co-regulator(s) are differentially recruited to the *p53AIP1* regulatory region in the presence of HBx, we asked if this was due to alterations in their expression levels in the presence of the viral X protein. We postulated that enhanced degradation or stabilization of the transcription factors and co-regulator(s) would affect their availability and thus recruitment to the respective response elements.

To test this hypothesis, protein expression levels of transcription repressors YY1, GATA-1 and HDAC1 as well as transcription activator Sp1 were examined in control and HBx THLE-3 cells using immunoblotting with the respective specific antibodies. As shown in Figure 3.17, YY1, GATA-1 and HDAC1 protein levels were found to be comparable in the presence or absence of HBx, demonstrating that HBx does not alter their expression. Only moderately higher Sp1 expression was detected in HBx cells compared to control cells.

Figure 3.17

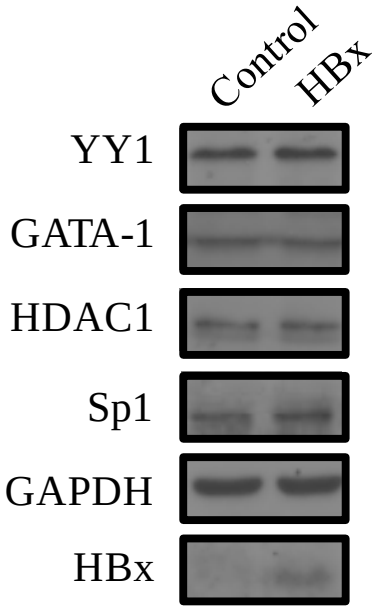


Figure 3.17. Expression of transcription co-regulators in the presence of HBx. Western blot analysis of YY1, GATA-1 and HDAC1 expression levels using specific antibodies in control (C) and HBx (H) THLE-3 cells. GAPDH was used as a loading control.

It is possible that increased Sp1 expression may contribute to the enhanced recruitment of the transcription activator to its response element observed in our study. However, since the protein expression levels of the majority of the transcription factors and co-regulator(s) involved are largely unchanged by HBx, this is unlikely to represent the main mode of action of the viral X protein. We therefore proceeded to explore other possible mechanisms of action of the viral protein in modulating the recruitment of p53-associated transcription factors/co-regulators.

3.4.4 Chromatin structure of *p53AIP1* regulatory region is not affected by HBx

We have shown in this study that HDAC1 is recruited together with transcription co-repressors to the promoter region of *p53AIP1* and that this recruitment is abolished in the presence of HBx. HDAC1 has been reported to deacetylate lysine (Lys) residues at the N-terminal tails of histones that is associated with a condensed chromatin state favouring transcription repression (Koike, 2009). To investigate if HDAC1 deacetylates histones at the promoter region of *p53AIP1* following recruitment to this region in the absence of HBx, ChIP assay was performed using antibodies specific for acetylated H3 and acetylated H4 on control and HBx THLE-3 cells. qPCR analysis of ChIP-enriched *p53AIP1* promoter region revealed no significant difference in H3/H4 acetylation in the presence or absence of HBx (Figure 3.18). Since the histone acetylation patterns at the *p53AIP1* promoter are unaffected by HBx, this suggests that HBx is unlikely to alter chromatin organization at the *p53AIP1* regulatory region.

Figure 3.18

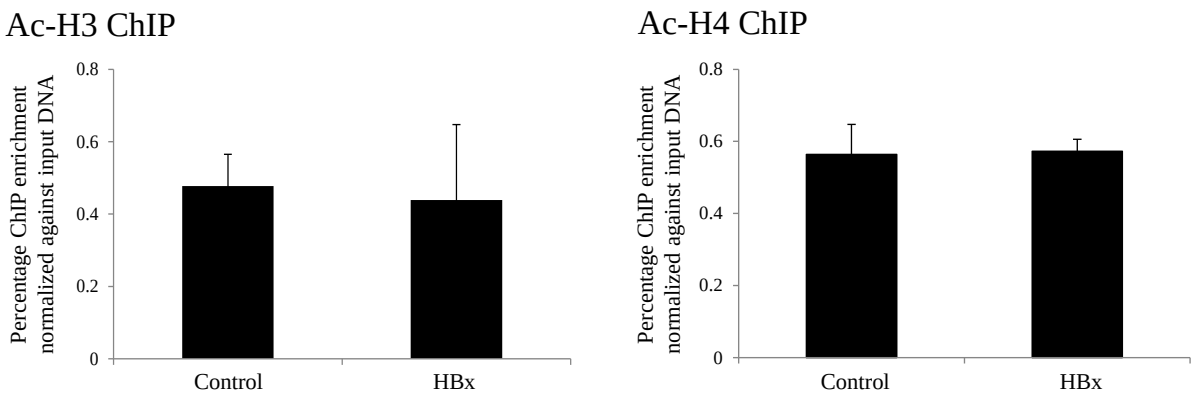


Figure 3.18. Acetylation of histones H3 and H4 at *p53AIP1* promoter region is not altered by HBx. ChIP using antibodies specific for acetylated H3 and acetylated H4 was performed on control and HBx THLE-3 cells. ChIP enrichment was measured using qPCR with primers specific for *p53AIP1* promoter region.

To further examine if the chromatin state of *p53AIP1* regulatory region is indeed not altered by HBx, DNA methylation profiles that is associated with a repressed chromatin state were analyzed using methylated DNA immunoprecipitation coupled with microarray (MeDIP-chip) of control and HBx THLE-3 cells. Notably, analysis of the DNA methylation profiles of the *p53AIP1* promoter and intron 1 regions of control and HBx THLE-3 cells did not show any significantly differentially methylated sites at both regions (Figure 3.19). Collectively, these findings suggest that the differential recruitment of p53-associated transcriptional complexes at the *p53AIP1* promoter and intron 1 regions are not a result of changes in chromatin states of these regions.

3.4.5 HBx enhances p53 site-specific acetylation at lysine 320 that is important for HBx-deregulated *p53AIP1* expression

In addition to deacetylating histones, HDAC1 also functions to deacetylate non-histone proteins such as p53 (Kew, 2011). Luo *et al.* showed that deacetylation of p53 by HDAC1 repressed p53-dependent transcription activation. We thus investigated if the promoter-recruited HDAC1 deacetylates p53 and if the relief in HDAC1 recruitment by HBx restores p53 acetylation. To this end, site-specific acetylation of p53 Lys residues was examined in control and HBx THLE-3 cells. Of the four p53 Lys residues known to be deacetylated by HDAC1 at positions 120 (Mellert *et al.*, 2011), 320, 373 and 382 (Ito *et al.*, 2002), the latter three residues for which site-specific antibodies were commercially available were tested. Strikingly, acetylation of p53 Lys320 was markedly enhanced in the presence of HBx, while that of residues Lys373 and Lys382 remained unchanged (Figure 3.20A). Since p53 Lys320 is differentially acetylated in control and HBx cells, and as acetylation of p53 can alter its

Figure 3.19

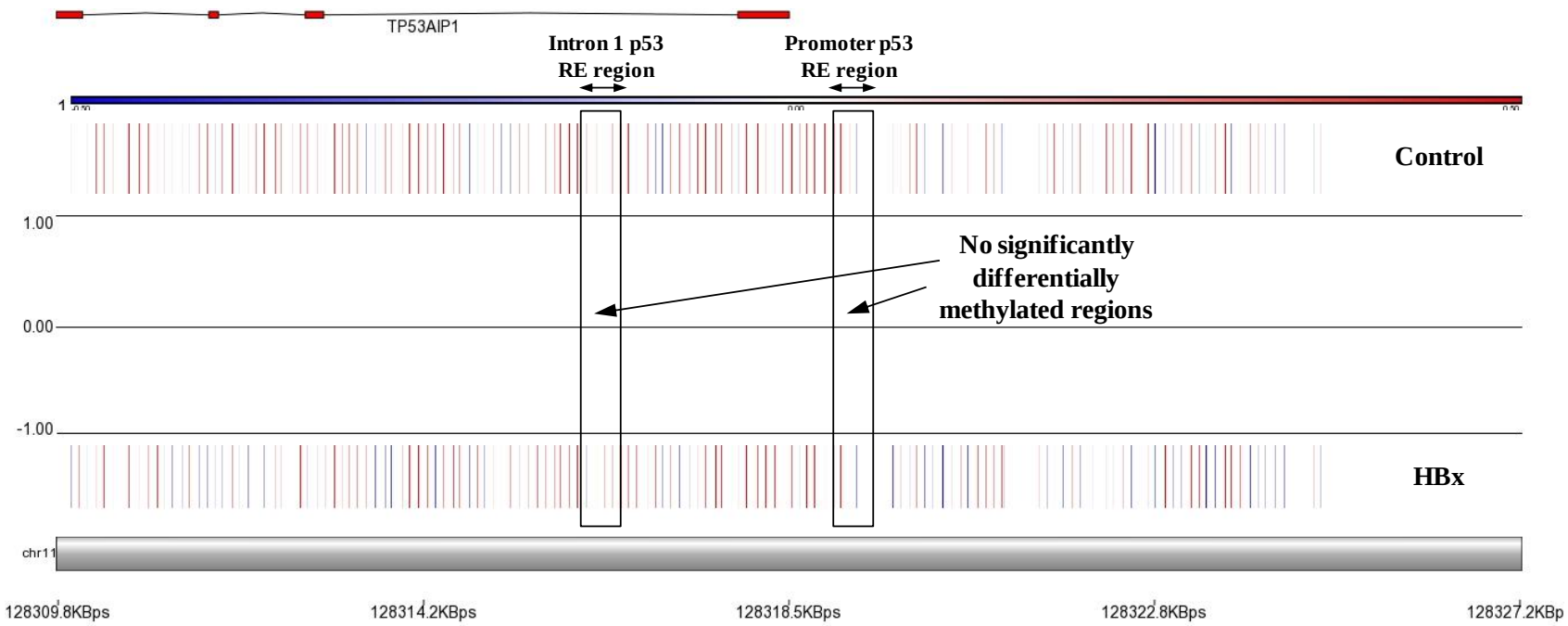


Figure 3.19. DNA methylation profiles of *p53AIP1* promoter and intron 1 regions. MeDIP analysis of p53 response element-containing promoter and intron 1 regions of *p53AIP1* in control and HBx THLE-3 cells. Uppermost panel shows the relative position of the *p53AIP1* transcript; bottom panel indicates the chromosomal position on chromosome 11. Each vertical line in control or HBx panel represents a probe; red represents a methylation signal, blue represents a demthylation signal.

site-specific DNA binding, we hypothesized that HBx might modulate p53-DNA binding through altering p53 Lys320 acetylation.

To examine the sequence-specific DNA binding pattern of acetylated p53 Lys320, ChIP was performed on control and HBx THLE-3 cells using an antibody that is specific for acetylated p53 Lys320. Intriguingly, binding of acetylated p53 Lys320 to the more conserved intron 1 p53 response element of *p53AIP1* was enhanced in the presence of HBx ($p<0.05$) (Figure 3.20B). No significant binding of p53 Lys320 to the less conserved promoter p53 response element was detected (Figure 3.20B). Next, to determine if this site-specific acetylation of p53 at Lys320 was essential for p53-mediated deregulation of *p53AIP1* by HBx, wild-type p53, p53 K320Q acetyl-mimic or non-acetylatable p53 Lys320 (K320R) mutant as well as recombinant HBx or control vectors were introduced into p53-deficient Hep3B cells, and examined for *p53AIP1* expression. Significantly, HBx increased *p53AIP1* expression only in cells with wild-type p53 or the constitutively acetylated K320Q mutant (Figure 3.20C). In contrast, comparable *p53AIP1* expression levels were detected in control and HBx cells with the non-acetylated K320R mutant (Figure 3.20C), suggesting that acetylation of p53 Lys320 is necessary for *p53AIP1* deregulation by HBx. Taken together, these findings strongly implicate p53 Lys320 site-specific acetylation in the modulation of p53 sequence-specific DNA binding by HBx.

Figure 3.20

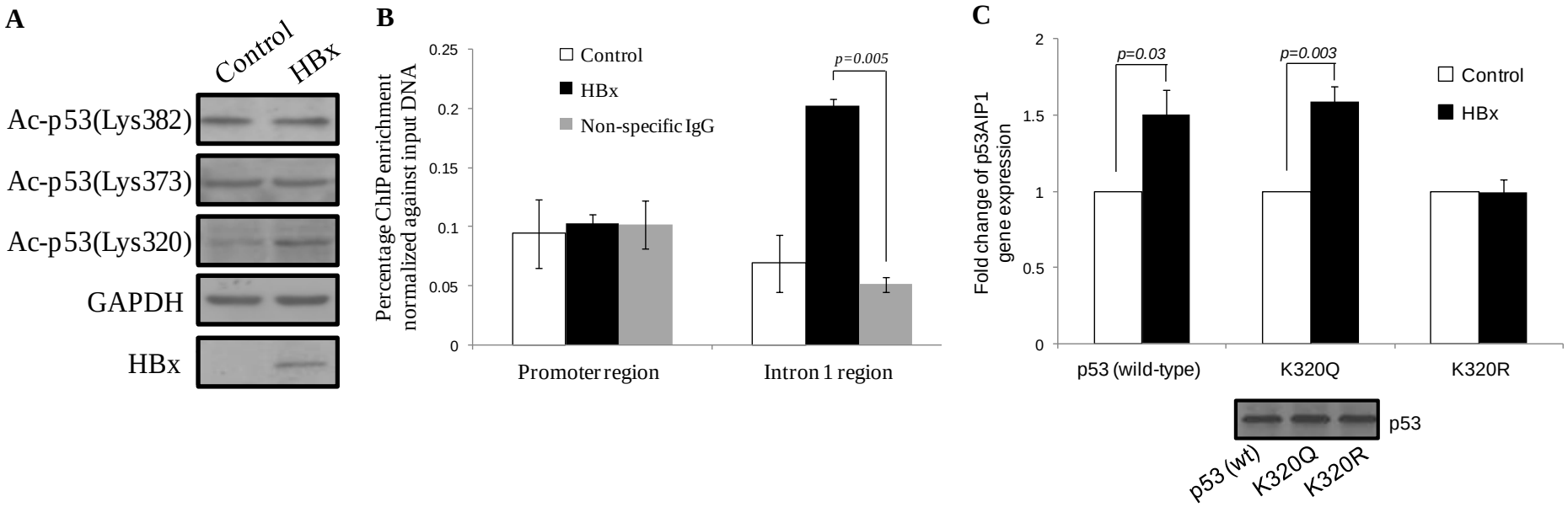


Figure 3.20. Enhanced p53 Lys320 acetylation by HBx. A. Western blot analysis of p53 Lys 320, 373 and 382 acetylation levels in control and HBx THLE-3 cells was performed using site-specific antibodies. B. Acetylated-p53 Lys320 is recruited to *p53AIP1* intron 1 RE in the presence of HBx. ChIP-qPCR was performed on control and HBx THLE-3 cells using Ac-p53 Lys320-specific antibody. ChIP using normal IgG was performed as a non-specific control. C. p53 Lys320 acetylation is essential for HBx-induced *p53AIP1* expression. Wild-type, constitutively acetylated (K320Q) or non-acetylated (K320R) p53 Lys320 was transfected into Hep3B cells and transduced with HBx or control adenovirus 24 h post-transfection. *p53AIP1* expression levels were measured by qRT-PCR and p53 protein levels were determined by western blotting.

3.4.6 HBx-enhanced p53 Lys320 acetylation is mediated by PCAF

To investigate if HBx enhances p53 Lys320 acetylation by relieving HDAC1, acetylation levels of p53 Lys320 were examined following depletion of HDAC1 using specific siRNA. As shown in Figure 3.21A, acetylated p53 Lys320 levels were comparable between control and HDAC1-knockdown cells, indicating that the relief of lysine deacetylase activity by HDAC1 depletion is not sufficient for spontaneous acetylation of p53 Lys320 in the absence of HBx. Since lysine acetylation is the result of the balance of deacetylase (HDAC) and acetyltransferase (HAT) activities, the findings here suggest that lysine acetyltransferase(s) may be required to promote p53 Lys320 acetylation in the presence of HBx.

Interestingly, as only site-specific acetylation of p53 at Lys320 was enhanced in the presence of HBx, and since the lysine acetyltransferase p300/CBP-associated factor PCAF is known to selectively acetylate p53 Lys320 (Liu *et al.*, 1999; Sakaguchi *et al.*, 1998a), we hypothesized that PCAF may mediate HBx-induced p53 Lys320 acetylation and subsequent *p53AIP1* deregulation. To this end, we first examined if PCAF is recruited to the regulatory region of *p53AIP1*. ChIP was performed on control and HBx THLE-3 cells using a PCAF-specific antibody. Similar to the recruitment pattern of acetylated p53 Lys320, recruitment of PCAF to the intron 1 region was enhanced in the presence of HBx ($p<0.01$) (Figure 3.21B). No significant enrichment of the promoter region was detected in control and HBx cells. Next, we investigated the possible role of PCAF in mediating the observed HBx-induced increase in p53 Lys320 acetylation and *p53AIP1* gene expression. To this end, THLE-3 cells were treated with PCAF-specific or control siRNA and control or HBx vectors, and examined for p53 Lys320 acetylation and *p53AIP1* gene expression using immunoblot and RT-qPCR analysis respectively. As shown in Figure 3.21C, efficient knockdown of PCAF expression

was achieved using PCAF-specific siRNA as compared to that of control siRNA. Significantly, PCAF depletion using PCAF-specific siRNA abrogated the HBx-induced increase in *p53AIP1* gene expression (Figure 3.21C). Concomitantly, a reduction in PCAF expression blunted acetylation of p53 Lys320 in HBx cells (Figure 3.21C). Taken together, the data strongly suggest that PCAF may play a key role in mediating HBx-induced increase in p53 Lys320 acetylation and consequent *p53AIP1* gene deregulation.

3.5 Genome-wide p53 chromatin immunoprecipitation-sequencing study reveals unique p53-DNA binding characteristics in the presence of HBx

3.5.1 p53 motif selectivity is altered by HBx

From the detailed characterization of p53-mediated *p53AIP1* deregulation by HBx, our findings so far suggest that HBx modulates p53-DNA binding at least in part by altering specific post-translational modification(s). This plausibly results in a conformational change in the tumour suppressor protein that now preferentially binds to a more conserved consensus sequence. Further, in co-operation with adjacent transcription factors and co-regulators, p53 mediates HBx-induced gene deregulation.

To investigate if this observation represents a general mechanism of p53 modulation by the viral X protein, genome-wide p53 chromatin immunoprecipitation coupled with sequencing (ChIP-Seq) was performed on THLE-3 cells transduced with recombinant HBx or control adenoviral vectors (Figure 3.22A). ChIP-Seq was employed to overcome the main limitations of our initial ChIP-on-chip study that only examined a 1.5 kb promoter region of genes and was restricted by probe design and coverage. This was particularly important so as

Figure 3.21

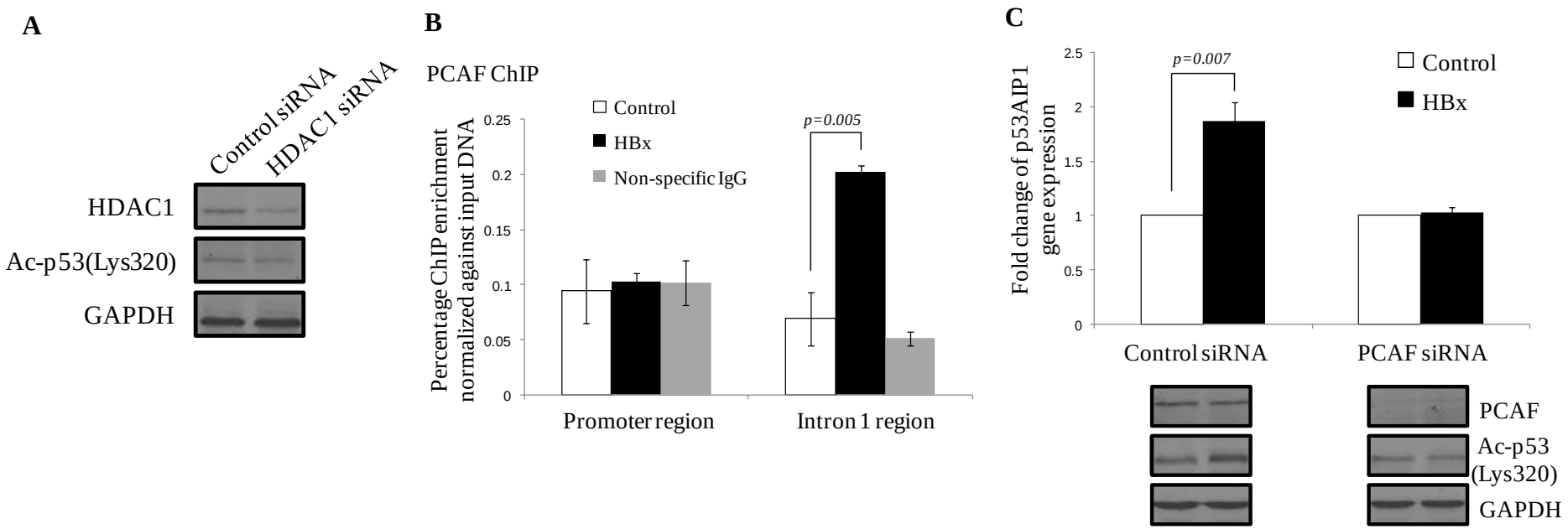


Figure 3.21. PCAF mediates HBx-induced p53Lys320 acetylation. A. Depletion of HDAC1 does not affect acetylation of p53 Lys320. THLE-3 cells were transfected with HDAC1-specific or control siRNA and examined for Ac-p53(Lys320) expression. Shown are the immunoblots of HDAC1, Ac-p53 Lys320 and GAPDH (loading control). B. PCAF is recruited to p53AIP1 intron 1 RE in the presence of HBx. ChIP-qPCR was performed on control and HBx THLE-3 cells using PCAF-specific antibody. ChIP using normal IgG was performed as a non-specific control. C. Depletion of PCAF blunts p53 Lys320 acetylation and *p53AIP1* upregulation by HBx. THLE-3 cells were transfected with PCAF-specific or control siRNA, and transduced with HBx or control vectors 24 h post-transfection. Ac-p53(Lys320) levels were determined using western blot analysis while *p53AIP1* expression levels were measured by qRT-PCR. All error bars show ± SEM of triplicate experiments.

to obtain a truly genome-wide HBx-modulated p53 binding profile to investigate in - an unbiased manner - the general mechanism of p53 modulation by HBx.To identify candidate p53 binding sites (termed peaks) in control and HBx samples, the peak finder Control-based ChIP-Seq Analysis Tools (CCAT) (Tsai and Chung, 2010) was employed and candidate peaks were then queried for the presence of the p53 motif using p53scan. As shown in Figure 3.22B, 426 unique candidate p53 binding sites were identified in control cells while 343 unique candidate p53 binding sites were identified in HBx cells. To experimentally validate these candidate p53 binding sites identified in the control and HBx p53-ChIP samples, 10 candidate p53 binding sites each from the list of control and HBx were randomly selected and validated using p53 ChIP-qPCR performed on control and HBx THLE-3 cells (Appendix). Majority (approximately 80%) of these control and HBx candidate p53 binding sites were successfully validated by p53 ChIP-qPCR (Appendix A and B), demonstrating that the p53 ChIP-Seq data is reliable.

We therefore proceeded to examine the nature of the p53 motif(s) in control and HBx samples. To this end, the position weight matrices (PWM) of control and HBx p53 motifs were obtained. A comparison of PWMs of control and HBx p53 motifs showed that p53 motifs in the control sample exhibited greater degeneracy in the core nucleotides C, A, T and G at positions 4 ,5, 6 and 7 respectively than that in the HBx sample (Figure 3.22C). In contrast, the p53 motif in the HBx sample was highly conserved particularly at the core nucleotides C, A, T and G at positions 4 to 7 (first half-site) and 14 to 17 (second half-site) (Figure 3.22C). This difference in p53 motif degeneracy observed in control and HBx samples is especially important since the core nucleotides CATG are critical for interaction with the p53 protein as determined by X-ray crystallography studies (Tang et al., 2006a). Another difference observed between control and HBx p53 motifs is that the highly

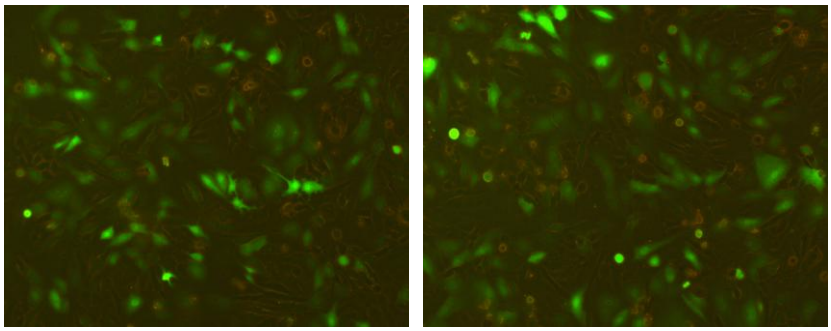
conserved p53 motif in the HBx sample consists of two half-sites in tandem, devoid of a spacer sequence while that of the control sample consists only of one half-site, indicating possible tolerance of variable spacer lengths (Figure 3.22C). This is also particularly significant as spacer sequences and lengths between the two half-sites have been reported to affect the binding affinity of the p53 protein, possibly contributing to the observed modulation of p53-DNA binding by HBx (O'Connor *et al.*, 1995; Sykes *et al.*, 2006). Taken together, the global p53 binding patterns observed in control and HBx THLE-3 cells showed a preference for a more conserved p53 motif in the presence of HBx. Notably, this is consistent with our finding that p53 preferentially bound to a more conserved response element at intron 1 of *p53AIP1* in the presence of HBx, indicating that the HBx-induced change in p53 binding site selectivity at least in part by p53 Lys320 acetylation may represent a global phenomenon.

3.5.2 Distinct transcription factors are co-associated with p53 in the presence of HBx

Since our findings on the deregulation of p53-mediated regulation of *p53AIP1* by HBx advocate that unique p53-associated transcription factors are favoured in the presence of HBx, we therefore investigated if distinct surrounding sequence-specific transcription factors are enriched at the candidate p53 binding sites from the genome-wide p53 ChIP-Seq study. This analysis was feasible with the use of the p53 ChIP-Seq data as it captured more p53 binding sites as compared to that of the p53 ChIP-on-chip study that had identified too few p53 binding sites for analysis by the co-motif scanning program CENTDIST (Anzola, 2004). Together with the superior resolution of ChIP-Seq, our p53 ChIP-Seq study was key to examining the possible global mechanism of HBx in modulating p53 sequence-specific DNA binding.

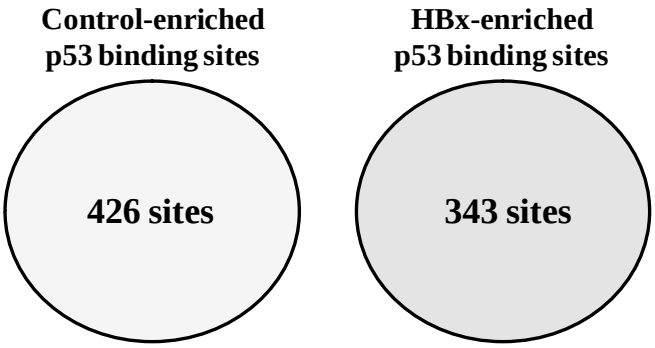
Figure 3.22

A



Control THLE-3 cells HBx THLE-3 cells

B



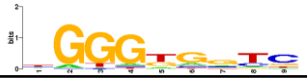
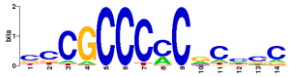
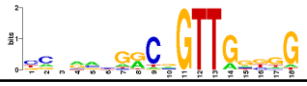
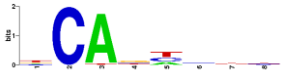
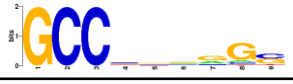
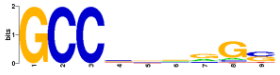
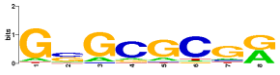
C

Sample	Enriched p53 motif
Control	
HBx	

Figure 3.22. Genome-wide p53 ChIP-Seq study reveals differential p53 motif selectivity in the presence of HBx. A. A representative set of control- and HBx-transduced THLE-3 cells. Approximately 90% transduction efficiency of recombinant HBx and control adenovirus (bearing enhanced green fluorescence gene) was consistently achieved in THLE-3 cells. B. Graphical representation of the number of candidate p53 binding sites identified in control and HBx cells using CCAT peak finder tool. C. Different p53 motifs are enriched in control and HBx cells. Shown are the position weight matrix logos of the p53 motifs identified in control- and HBx-enriched candidate p53 binding sites.

To this end, control and HBx candidate p53 binding sites were first queried for proximal co-enriched transcription factor motifs using the co-motif scanning program CENTDIST. Differentially co-enriched transcription factor motifs in HBx versus control candidate p53 binding regions were then predicted using MotifDiff (unpublished). Table 3.1 shows the transcription factors that are predicted to be significantly co-associated with p53 in the presence of HBx (but not in control cells). Notably, motifs of transcription factors that are known to interact with both HBx and p53 were predicted by CENTDIST/MotifDiff to be co-associated with p53 at candidate p53 binding sites in the presence of HBx, indicating that our analysis and findings are relevant *in vitro*. An example is the transcription factor E2F1 that was the third most co-enriched factor (Table 3.1). Additionally, motifs of known p53-interacting transcription factors such as Sp1 and AP2 were also predicted by CENTDIST/MotifDiff to be enriched (Table 3.2). Significantly, the transcription regulator Sp1 that we have shown in this work to be preferentially associated with p53 at the *p53AIP1* regulatory region in the presence of HBx was the second most highly co-associated transcription factor in the presence of HBx (Table3.1). Taken together, these data support our findings that HBx favours distinct transcription co-regulator-p53 associations (or co-regulatory modules) including Sp1 globally.

Table 3.1 List of transcription factors that significantly co-associate with p53 in HBx sample.

Rank	Family	Motif	Logo	HBx Score	Control Score
1	EBOX	V$LMO2COM_01		1.55	0.64
2	SP1	V$GC_01		1.51	0.65
3	E2F	V$E2F1_Q6_01		1.51	0.66
4	GLI	V$ZIC3_01		1.50	0.66
5	EGR	V$KROX_Q6		1.45	0.68
6	MYB	V$CMYB_01		1.45	0.68
7	CAP	V$CAP_01		1.42	0.70
8	AP2	V$AP2ALPHA_01		1.39	0.71
9	jaspar Helix Loop Helix	V$jaspar_TFAP2A		1.39	0.71
10	ZF5	V$ZF5_01		1.38	0.72

Differentially co-enriched transcription factor motifs in candidate p53 binding sites in HBx THLE-3 cells were predicted using MotifDiff. Shown are the rank, transcription family and motif, PWM logo of the motif and scores obtained in HBx and control samples of the top 10 co-enriched motifs. A score greater than 1.3 indicates co-enrichment.

Chapter 4 Discussion

4.1 Basis of this thesis

The hepatitis B virus X protein, HBx is strongly implicated in hepatitis B virus associated hepatocellular carcinoma. A major way by which HBx promotes hepatocarcinogenesis is through its function as a transcription co-factor. It is widely accepted that HBx does not possess sequence-specific DNA binding properties, but instead perturbs cellular gene expression program by interacting with and modulating a variety of host transcription regulators. As transcription regulators are a vital component of the cellular apparatus that carefully orchestrates the gene expression program to maintain cellular homeostasis, disrupting the integrity of transcription regulation therefore has potentially detrimental consequences in tumourigenesis and tumour progression (Woo *et al.,* 2011). It is thus pertinent to understand the transcription co-factor role of the viral X protein in contributing to hepatocarcinogenesis.

To study the transcription co-factor function of HBx, we chose to examine its modulatory effect on a known HBx-interacting transcription factor p53. P53 was carefully selected for the following reasons: firstly, the tumour suppressor protein p53 is regarded as a master regulator of transcription that controls key cellular processes such as cell cycle, DNA repair, apoptosis and senescence and is thus particularly biologically relevant. Although more than 60% of cancers have been reported to possess mutated or inactivated p53, p53 mutations in the early stages of HCC are infrequent (Feitelson *et al.,* 1993). Secondly, albeit limited literature reports in this field, modulation of p53 by HBx is the most studied among the known HBx-interacting transcription factors. Moreover, the availability of a relatively large body of knowledge on general p53 transcription regulation would serve to facilitate our study

on the deregulation of p53 transcription by the viral X protein. For these biologically pertinent and practical reasons, the tumour suppressor protein p53 was selected for studying the transcription co-factor function of HBx in hepatocarcinogenesis.

4.2 Importance of altered p53-mediated regulation by HBx

The literature to date has provided some clues on how HBx modulates p53. Firstly, we and several independent groups have established that HBx interacts with p53 and co-localize in the nucleus. Secondly, unlike the human papillomavirus E6 oncoprotein and the Epstein-Barr virus nuclear antigen 3C that disrupt p53 function by enhancing p53 ubiquitination and proteasomal degradation (Scheffner *et al.*, 1993; Scheffner *et al.*, 1990), we and others have shown that HBx does not affect p53 expression levels (Chung et al., 2003; Lee et al., 2005a). Consistently, we have also demonstrated in this work that p53 expression is comparable in the presence or absence of HBx, and that a large fraction of p53 is localized to the nucleus. How then does HBx modulate p53? The first clues to this question came from a few early *in vitro* binding assays that showed changes in p53-DNA binding in the presence of HBx, although the changes reported were seemingly contradictory: HBx inhibited p53-DNA binding in some studies (Chung *et al.*, 2003; Elmore *et al.*, 1997), but potentiated p53-DNA binding in another study (Truant et al., 1995a). This discrepancy in findings was thought to be partly attributed to the different *in vitro* systems in which the effect of HBx was examined. Nevertheless, subsequent work by Chung *et al.* significantly highlighted the potential biological consequences of such a modulation: abrogation of p53-DNA binding at the promoter of the tumour suppressor PTEN by HBx was linked to its deregulated expression (Chung *et al.*, 2003). However, the lack of progress made in this area

thereafter continued to present a huge gap in knowledge of the transcription co-factor role and the mechanism of action of the viral X protein.

To address this, we employed a global integrative approach to obtain an overall view of HBx-altered p53 DNA-binding patterns that are associated with corresponding aberrant gene expression. Using a HBx-expressing cell culture system, we employed two powerful tools: (i) chromatin immunoprecipitation coupled with microarray (ChIP-on-chip) to identify global differential p53-DNA binding patterns in the context of the chromatin and cellular milieu and, (ii) microarray expression profiling to identify genes deregulated by HBx. This is the first report that shows the global effect of HBx on p53 sequence-specific DNA binding. We found that HBx can potentiate, relieve as well as shift p53 DNA-binding and that these alterations were associated with deregulated corresponding gene expression. This finding suggests that HBx can indeed alter biologically functional p53-DNA binding characteristics in opposing manners, suggesting that the seemingly contradicting reports of HBx on p53-DNA binding were likely to have been real observations and were not mere artefacts of different *in vitro* systems. A model previously proposed purported that HBx repressed gene transcription through a repressive domain that contacts the basal transcription machinery (Truant et al., 1995a). However, the findings in this thesis suggest that the deregulation of p53-mediated transcription is far more complex than that. This is evidenced by the observation that HBx-altered p53-DNA binding was linked to transcriptional activation of some genes and transcriptional repression of other genes. The complexity of transcription deregulation by HBx is highlighted by the finding that the various HBx-induced p53-DNA binding alterations (enhancement, abolishment or a shift) did not associate with any specific type of gene deregulation pattern (stimulation or inhibition). This suggests that a more intricate, multifaceted interplay of virus-host interactions is at work. Instead, our findings

suggest that the viral X protein may deregulate p53-mediated transcription through a more complex mechanism that possibly involves other transcription factors and/or co-regulators.

To gain a better understanding of the modulation of p53 sequence-specific DNA binding property by HBx, we characterized a HBx-deregulated p53-regulated candidate gene that was identified from our global study - *p53AIP1*. *P53AIP1* was deemed as an attractive candidate for further characterization studies for several reasons. First, as its name suggests, *p53AIP1* is a known p53-regulated gene. Secondly, its function in promoting apoptosis – the most ancestral function of p53 - has been characterized and the observed HBx-deregulated increased *p53AIP1* expression from microarray expressing profiling is consistent with the apoptosis-inducing role of HBx.

Our findings here provide strong evidence that HBx disrupts p53-mediated transcription regulation by modulating p53 recruitment to its regulatory elements. Using *p53AIP1* as a model, we have successfully showed that HBx induced a novel shift in p53 recruitment from the promoter to intron 1 region of *p53AIP1* and that this directly resulted in a deregulated increase in expression. This finding is significant in several ways: this is the first evidence demonstrating that HBx can alter p53 selectivity for distinct binding sites within the regulatory region of a particular gene. Importantly, this change in p53 selectivity induced by the viral X protein has serious biological consequences – that of aberrant gene expression. This is potentially detrimental to the cell since p53 regulates important cellular processes by the co-ordinated transcription regulation of critical genes. Modulation of p53-mediated transcription by the viral X protein would thus conceivably upset the delicate balance of cellular homeostasis. This is particularly pertinent in an environment of chronic

inflammation and constant liver injury and regeneration such as that in hepatitis and liver cirrhosis. HBx-altered p53 function might impair the cell's physiological response to these stresses and predispose hepatocytes to neoplastic transformation.

Indeed, the findings in this thesis allude to the adverse biological consequences of deregulated p53 regulation by the viral X protein. Specifically, we found that the enhanced expression of *p53AIP1* in the presence of HBx plays a key role in tipping the balance in favour of cellular apoptosis. This is consistent with the increasing amount of evidence that points to the pro-apoptotic function of the viral X protein. In this work, we have also found that pro-apoptotic *p53AIP1* expression is significantly elevated in HCC patients with high expression of the hepatitis B virus X protein. At first glance, the pro-apoptotic function of HBx appears to be incompatible with its role in neoplastic transformation and hepatocarcinogenesis, a hallmark of which is the cell's resistance to apoptosis. How then do we reconcile the apoptotic-promoting effect of the viral X protein with its role in hepatocarcinogenesis? A possible explanation may lie in the way normal tissue homeostasis is maintained by a delicate balance between cell growth and apoptosis. In the event of cell stress or injury such as during viral infection, apoptotic cells release mitogenic factors that stimulate proliferation of neighbouring cells – a process termed 'apoptosis-induced compensatory proliferation' (Ryoo *et al.*, 2004). Accordingly, induction of apoptosis by the viral X protein may consequently enhance compensatory proliferation and promote regeneration of hepatocytes. Such a cellular environment may exert a selective pressure for premalignant hepatocytes that are resistant to apoptosis, a precursor to hepatocarcinogenesis.

4.3 A novel mechanism of the HBx transcription co-factor

In dissecting the molecular mechanism by which the viral X protein altered p53 sequence-specific DNA binding characteristics and consequence p53-regulated transcription, our findings strongly suggest that HBx alters the recruitment of distinct transcription factors and co-regulators(s) to the regulatory region of *p53AIP1* and that these transcription complexes consequently modulate *p53AIP1* expression. This was founded on the knowledge that mammalian transcription regulation generally involves the co-ordinated action of multiple transcription factors and chromatin modifiers. Furthermore, a variety of transcription factors have been reported to mediate the indirect binding of the viral X protein to DNA, suggestive of possible multi-protein transcription complexes that are involved.

Using the power of bioinformatics predictions and chromatin immunoprecipitation assays to identify transcription co-regulators that are directly as well as indirectly bound to the *p53AIP1* regulatory region, our findings strongly suggest that p53 functions together with other transcription co-regulators in a multi-protein complex at the *p53AIP1* regulatory region. Our findings suggest that specific combinations of p53 and proximal sequence-specific transcription factors together recruit distinct non-DNA-bound transcription regulators such as HATs and HDACs. In addition to modulating gene transcription, we found that the HATs and HDACs in turn modify specific p53 acetylation patterns, altering p53 selectivity for its binding sites. Intriguingly, we found that the viral X protein alters p53 transcription regulation by perturbing the recruitment of unique p53-transcription co-factor combinations and thus the non-DNA-bound transcription regulators that are recruited to the regulatory regions of genes. Using *p53AIP1* as a model, we showed that HBx disrupted a transcriptionally repressive p53-YY1-GATA-1-HDAC1 complex at the *p53AIP1* promoter that served to keep *p53AIP1* expression under tight control. Instead, HBx favoured

recruitment of p53 with the co-activator Sp1 at the intron 1 region that further recruited the transcription co-activator PCAF in a transcriptionally stimulating complex.

To further delineate the mechanism by which HBx perturbs the recruitment of the distinct transcription complexes, we explored several hypotheses. Firstly, we hypothesized that the observed modulated patterns of p53-DNA binding might be a consequence of altered transcription factor/co-regulator protein levels and thus their availability for recruitment in HBx-expressing cells. However, our findings suggest that the protein expression levels of the transcription factors/co-regulators examined were generally unaffected by the viral X protein, effectively ruling out this possibility.

Secondly, we hypothesized that the modulated p53-DNA binding patterns may be the result of HBx-induced global changes in chromatin structure and accessibility. Chromatin structure can be altered by various modifications such as DNA methylation and covalent modifications of histone tails. Here, we show that comparable DNA methylation profiles of the *p53AIP1* regulatory region were detected in the presence or absence of HBx. Moreover, we demonstrate that HDAC1-regulated histone acetylation patterns at the *p53AIP1* promoter region were also unaffected by HBx, suggesting that the differential p53-DNA binding patterns observed in the presence of HBx are unlikely a result of a change in gross chromatin structure but through other mechanisms.

Next, since the HBx-altered recruitment of transcription co-regulators HDAC1 and PCAF regulate acetylation of non-histone proteins such as p53, and as post-translational modification of p53 is associated with altered sequence-specific DNA binding, we

hypothesized that HBx might alter p53 binding site selection by affecting its acetylation pattern via perturbing the HDAC1-PCAF balance. Indeed, our findings suggest that tipping of the HDAC-HAT balance by HBx activated a PCAF-specific p53 Lys320 'acetylation switch' that is in part responsible for conferring p53 binding site selectivity for more conserved p53 response elements. Based on these findings, we postulate that under normal conditions, HDAC1-deacetylated p53 Lys320 preferably binds to the less conserved p53 response element, locking the transcriptionally repressive p53-YY1-GATA-1-HDAC1 complex at the *p53AIP1* promoter, thus preventing aberrant transcription of the pro-apoptotic gene (Figure 4.1). This mode of transcription inhibition is conceivably advantageous to the cell as it safeguards the cell against undergoing aberrant apoptosis under normal conditions, and is 'primed' to activate *p53AIP1* transcription in response to genotoxic stress through the rapid disassembly and re-assembly of desired transcription regulators on an 'open' chromatin. In fact, upon cellular stress by the expression of the viral X protein, HBx instead favours recruitment of a p53-Sp1 transcription co-activator combination that engages PCAF, which steers p53 to a more conserved response element at the *p53AIP1* intron 1 region by activating a p53 Lys320 'acetylation switch'. We postulate that enhanced acetylation of p53 Lys320 induces a conformational change in the p53 protein that now preferably binds more conserved p53 response elements. Consequently, this HBx-induced shift in transcription regulator recruitment from a repressor to an activator complex aberrantly stimulates *p53AIP1* expression that directs the cell towards apoptosis.

Figure 4.1

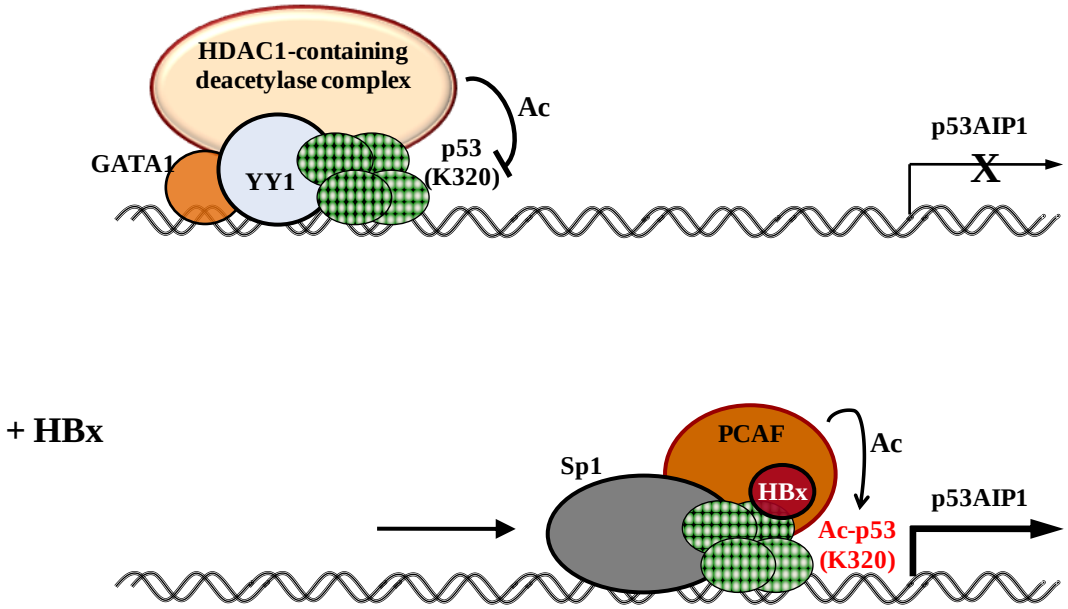


Figure 4.1 Model of p53-mediated p53AIP1 deregulation by HBx. In unstressed cells, a repressive p53-YY1-GATA-1-HDAC1 transcription complex occupies the promoter of p53AIP1, keeping gene transcription under tight control. Upon HBV infection and the selective over-expression of HBx, HBx enhances PCAF-mediated acetylation of p53Lys320 and induces a conformational change in p53 that favours binding to a stronger consensus sequence in intron 1. Together with the transcription activator Sp1, the HBx-PCAF-p53-Sp1 complex is recruited to the intron 1 region of p53AIP1, resulting in aberrant stimulation of its gene expression.

Several lines of evidence further indicate that our proposed model of deregulated p53-mediated transcription may represent a global mechanism of the viral X protein. Firstly, distinct transcription factor motifs - including that of Sp1 - were found to be selectively co-enriched in the vicinity of the p53 binding sites in the presence of HBx from the global p53 ChIP study. In addition, many of these transcription co-factors motifs were also significantly enriched in HBx direct target genes from a global HBx ChIP study that was previously conducted in our lab (Sung *et al.*, 2009). Many of these transcription co-factors have also been reported to interact and co-operate with p53 in regulating cell growth and/or apoptosis such as E2F1 (O'Connor *et al.*, 1995), GLI (Brandner, 2010), c-myb (Sala *et al.*, 1996), and AP2 (McPherson *et al.*, 2002; Mertens *et al.*, 2002). Collectively, this suggests that specific p53-transcription co-regulator combinations are globally favoured by the viral X protein. Secondly, p53 universally bound to more conserved response elements in the presence of HBx. This was revealed by a comparison of the structural characteristics of the p53 DNA consensus sequence of p53 binding sites in the presence and absence of HBx from the global p53 ChIP study. We postulate that specific post-translational modifications of the p53 protein such as enhanced p53 Lys320 acetylation as a result of altered transcription regulator recruitment may be responsible for the altered preference for more conserved p53 response elements. Taken together, these findings from global ChIP studies strongly suggest that the mechanism of p53-mediated deregulation the viral X protein illustrated by the *p53AIP1* model may represent a global mechanism by which HBx deregulates cellular transcription.

The finding that HBx perturbs acetylation of p53 only at specific Lys residue(s) is particularly fascinating. HBx enhanced PCAF-mediated acetylation of p53 Lys320 but did not affect p300/CBP-associated acetylation of p53 Lys373 and Lys382. Additionally, our findings suggest that selective engagement of the acetyltransferase PCAF is critical to the

differential acetylation patterns observed since removal of p53 deacetylation by HDAC1 did not lead to a spontaneous increase in p53 Lys320 acetylation levels. Taken together, these findings allude to the specific hijacking of the PCAF-mediated acetylation system by the viral X protein. Interestingly, several other viral proteins such as the adenovirus E1B-55kDa and human immunodeficiency virus type-1 Tat proteins have been reported to deregulate p53 transcription by interfering with the physical interaction of PCAF and p53, thus inhibiting PCAF-mediated p53 Lys320 acetylation (Harrod *et al.*, 2003; Liu *et al.*, 2000). It will be of importance to investigate the mechanism by which HBx conversely enhances PCAF-mediated p53 Lys320 acetylation. Additionally, it will be particularly interesting to investigate if deregulation of PCAF by HBx represents a mode of general transcription deregulation by HBx. Analysis of other transcription factors that are acetylated by PCAF such as E2F1 (Martinez-Balbas *et al.*, 2000) – a transcription factor that is co-enriched with p53 in the presence of HBx as well as enriched at HBx direct target genes – will provide clues to this question. If so, PCAF could present an attractive drug target that is more specific as compared to the broad range HDAC inhibitors that are used in cancer treatment.

Additionally, it is possible that HBx may disrupt other factors in addition to PCAF that modify the p53 protein post-translationally, thus contributing to an altered 'post-translational code'. First, to obtain a more complete understanding of the effect of HBx on p53 acetylation, it may be worthwhile to examine the potential effect of HBx on another family of acetyltransferases – hMOF and TIPP60 of the MYST family that was recently shown to acetylate p53 at Lys120 (Sykes et al., 2006; Tang et al., 2006b). In addition, it is possible that HBx may also interfere with other p53 site-specific post-translational modifications such as phosphorylation (other than Ser46), ubiquitination, sumoylation, neddylation and methylation through the interaction with specific p53 modifying enzymes.

We predict that crosstalk between the various p53 site-specific modifications induced by HBx may determine modulation of p53 sequence-specific binding characteristics and consequent p53 transcription deregulation.

Going forward, in addition to investigating the role of wild-type HBx on p53 regulation/site specific binding, it will be pertinent to examine the role of clinically relevant mutant forms of the viral protein on p53 as well. Of particular mention are HBx mutants that have been frequently found to be deleted at the 3'-end in HCC tumours (Iavarone et al., 2003; Ma et al., 2008; Poussin et al., 1999; Tu et al., 2001; Wei et al., 1995). Several studies have reported that carboxy-terminal truncated HBx retain their transactivational activities (Balsano et al., 1991; Kumar et al., 1996a). Notably, Kumar *et al.* demonstrated that a HBx truncated mutant (residues 58-140) – that contained intact p53 binding domain – maintained its transactivating function (Kumar et al., 1996a). On the contrary, other studies that examined larger portions of 3'-end truncated HBx mutants identified from HCC tumours report a lost in their transctivational activities, their inhibitory effect on cell proliferation and transformation as well as their pro-apoptotic effect (Ma et al., 2008; Tu et al., 2001; Wang et al., 2004; Zhang et al., 2008). Hence, it will also be important to dissect the role of these clinically relevant HBx mutants on the regulation of p53 as well as on p53-independent pathways that may facilitate hepatocarcinogenesis.

4.4 Significance of our work on the field of p53 research

Investigation of p53 transcription deregulation by the hepatitis B viral X protein not only provides new insight on virus-host interactions, but also advances our understanding of general p53-mediated transcription regulation.

Departing from the initial simplistic one transcription factor-one gene model of transcription regulation, it is now widely accepted that p53 transcription regulation typically involves the intricate interplay of other sequence-specific transcription factors and co-regulators that modulate p53 target gene expression. Additionally, there is growing support for the importance of p53 post-translational modifications in regulating p53 transcription activity. Consistently, our work in this thesis provides evidence here that indeed the transcription factor p53 does not function in isolation, but rather as 'p53 cassettes' – a collective term used to describe the combination of p53 modifications and associated transcription factors and/or regulators. Additionally, from our study of p53 transcription deregulation by the hepatitis B virus X protein, we show that in response to stress, distinct p53 cassettes are recruited to specific regulatory regions of selected target genes that co-ordinate their expression to elicit an appropriate cellular response. So how are different p53 cassettes specifically recruited to selected response elements? Characterization of p53-mediated *p53AIP1* deregulation by the viral X protein presented in this thesis provides some clues to the regulation of p53-DNA binding selectivity.

Importantly, we found that a specific reversible p53 Lys320 acetylation mark determined by the opposing action of HDAC and HAT recruited by distinct p53-transcription co-factor combinations confers selectivity for p53 response elements with particular DNA structural characteristic. Indeed, there has been growing advocacy for the idea of such an acetylation 'sensor system'. In this theory, distinct HATs/HDACs are activated/recruited in response to specific genotoxic stresses and mediate differential acetylation of distinct p53 Lys residues. The p53 'acetylation code' thus acts as a sensor by which p53 selectively engages classes of p53 target genes that directs the appropriate cell fate. It is speculated that attaching

acetyl moieties to the ε-amino group of p53 Lys side chains changes the conformation of the p53 protein in such a way that it binds response elements that have specific DNA structural characteristics. In support of this, we have found that acetylated p53 Lys320 preferentially binds p53 response elements that exhibit greater sequence similarity to the consensus sequence. The exact physical mechanism however, remains to be clarified and would undoubtedly be facilitated by X-ray crystallography studies of various p53 acetylation mutants in complex with DNA. It is also important to note that this theory assumes that each class of p53 target genes (eg. cell cycle) is regulated by p53 response elements of similar DNA structural characteristics, and that this differs from those in other classes of target genes (eg. apoptosis). However, attempts at defining a hard and fast rule for classifying p53 response elements according to their specific classes of target genes based solely on their DNA structural characteristics have shown that it is not so clear cut. Moreover, our findings here highlight an additional level of complexity in p53 target gene selectivity. We have demonstrated that p53 can differentially bind to distinct response elements that regulate a single gene. As an increasing number of p53-regulated genes are found to contain more than one (or a cluster of) functional p53 response elements at their regulatory region, we believe that it may be more meaningful to further dissect p53 target site specificity instead in the context of each p53-regulated gene.

Various studies have reported conflicting roles of site-specific p53 Lys320 acetylation in influencing cellular outcome. On the one hand, acetylation of p53 Lys320 has been associated with promoting cell survival. Knights *et al.* demonstrated that acetylation of p53 Lys320 repressed several pro-apoptotic genes while positively regulating some genes involved in cell survival in lung carcinoma cells (Knights *et al.*, 2006). Similarly, using a

murine model, Chao *et al.* showed that non-acetylatable p53 Lys320 mutants resulted in stimulation of pro-apoptotic genes and was associated with apoptosis induction in particular cell types such as thymocytes, epithelial cells of the small intestine and retinal cells (Chao *et al.*, 2006). On the other hand, Terui *et al.* showed that acetylated p53 Lys320 lead to an up-regulation of pro-apoptotic genes *Pig* and *Noxa* in gastric carcinoma cells (Terui *et al.*, 2003). Consistent with the proposed pro-apoptotic function of acetylation p53 Lys320 by Terui *et al.*, we found that acetylated p53 Lys320 is important for stimulating *p53AIP1* expression. A likely explanation for the discrepancy in findings could lie in the different cell types used in each study and generalization of p53 regulation mechanisms would be perilous. The specific repertoire of proteins and regulatory networks at work in each cell type may exert different regulatory effects on the p53 protein that might not have been examined in these studies. There is a possibility that recruitment of unique cell type-specific p53 cassettes may mark p53 with unique post-translational codes whose crosstalk and/or compensatory functions are still being investigated. Additionally, assessing the effect of these post-translational codes in various cell types in an *in vivo* model will provide a more complete understanding of the physiological relevance of p53 modifications. Unfortunately, Chao *et al.* did not examine the effect of non-acetylatable p53 Lys320 mutants in liver cells in the murine model used that could have provided useful information for our study. Collectively, these studies caution against drawing general conclusions about particular p53 modifications without carefully take into account the unique landscape of each cell type used. Moreover, this highlights the complexity of p53 transcription regulation but may also present exciting possibilities for targeted cell-type specific therapy.

Our findings strongly suggest that the main function of p53 site-specific acetylation of Lys320 is in conferring sequence-specific binding properties and not in affecting p53 stability. It is suggested that since the same p53 Lys residue can be modified by acetylation and ubiquitination, exclusion of ubiquitination by acetylation of the residue prevents degradation of p53. However, there are no reports to suggest that p53 is ubiquitinated at Lys320 and the only other modification reported at this residue, neddylation, was shown not to affect its stability but to inhibit p53 activity (Abida *et al.*, 2007). In support for the role of acetylation in altering p53 sequence-specific DNA-binding/activity, knock-in studies of carboxy-terminal lysines of the mouse p53 gene showed no effect on p53 stability but functioned by fine-tuning p53 activity (Feng *et al.*, 2005).

From our study of how the viral X protein hijacks the p53 transcription machinery in this thesis, our findings allude to the great complexity of several inter-linked regulatory mechanisms that govern p53-mediated transcription. The importance of regulation by post-translational modifications of the p53 protein described above is important in so far as in influencing p53 binding site selectivity, but the intricacy of p53-mediated gene expression is highlighted by the observation that only a subset of all p53-DNA binding events actually affects gene transcription. In concordance with this, we show that p53-regulated gene transcription is likely the result of the convergence of multiple layers of regulation including, but not limited to (i) recruitment of specific combinations of transcription co-factors and co-regulators, (ii) recruitment of chromatin remodelling factors and, (iii) combinations of p53 post-translational modifications. Other equally important factors though not examined in this thesis such as p53 binding factors, other p53 family members such as p63 and p73 that exhibit overlapping functions with p53 as well as the emerging role of p53-responsive

microRNAs may also be involved in modulating p53 transcription and ultimately determining cellular outcome. These various mechanisms reflect the intricacy of fine-tuning the master regulator p53 response to various cellular stresses and also present multiple safeguards against breaches by exogenous agents such as viral proteins.

4.5 Conclusion and future perspectives

Our work presented in this thesis has provided new insights to how the hepatitis B virus X protein deregulates p53 transcription regulation and offers clues as to how the virus has evolved to hijack the p53 transcription machinery and alter cellular outcome.

We have shown that p53-regulated gene transcription is carefully orchestrated by (but not limited to) a dynamic interplay of transcription co-factors and co-regulators as well as specific p53 post-translational modifications, and that the viral X protein functions by upsetting this system. Using *p53AIP1* gene regulation as a model, our findings suggest that HBx does this by hijacking the p53 acetylation sensor system through differentially favouring the recruitment of unique HDAC- or HAT-containing p53 cassettes. The shift in the HDAC-HAT balance alters the specific acetylation code of the p53 protein, modulating p53 sequence-specific DNA-binding selectivity that can result in aberrant corresponding gene expression with functional consequences and clinical relevance in hepatocarcinogenesis. Based on our findings, we advocate that future efforts be focused on elucidating the specific ‘p53 cassettes’ – the combination of transcription co-factors and co-regulators as well as p53 post-translational modifications – that are perturbed by HBx., This effort needs to amalgamate a host of information by harnessing the power of chromatin immunoprecipitation coupled with massive parallel sequencing: genome-wide HBx and HBx-altered p53 binding

profiles, differential recruitment patterns of p53 transcription co-factors and co-regulators, HBx-altered histone modification profiles as well as combinations of HBx-altered p53 post-translational modifications. This must be further integrated with HBx-deregulated gene expression profiles to elucidate functional p53 cassettes that are responsible for transcription deregulation. Dissecting the intricate interaction of all these factors presents a mammoth task, but is one that will undeniably provide a more coherent model of how the viral protein deregulates transcription of the cell's master regulator.

In the greater scheme of things, we envisage that HBx may similarly deregulate other transcription factors that may function either independently or in combination with p53. Co-ordinated efforts in dissecting the patterns of altered co-regulatory modules by the viral X protein will undoubtedly advance our understanding of how such a small viral protein perturbs cellular transcription regulation. In addition, future work will also need to analyze the mechanism of HBx modulation of these co-regulatory modules in the context of chromosomal looping. We would need to examine if HBx perturbs the interaction of proximal and distant transcription factors, co-regulators, chromatin modifiers and remodelers that were brought together into so called 'transcription factories' by chromosomal looping.

Lastly, these studies should ideally be performed in normal liver cells as well as in tumours obtained at various stages of the disease to obtain a more comprehensive understanding of the co-factor role of the viral X protein in the process of hepatocarcinogenesis.

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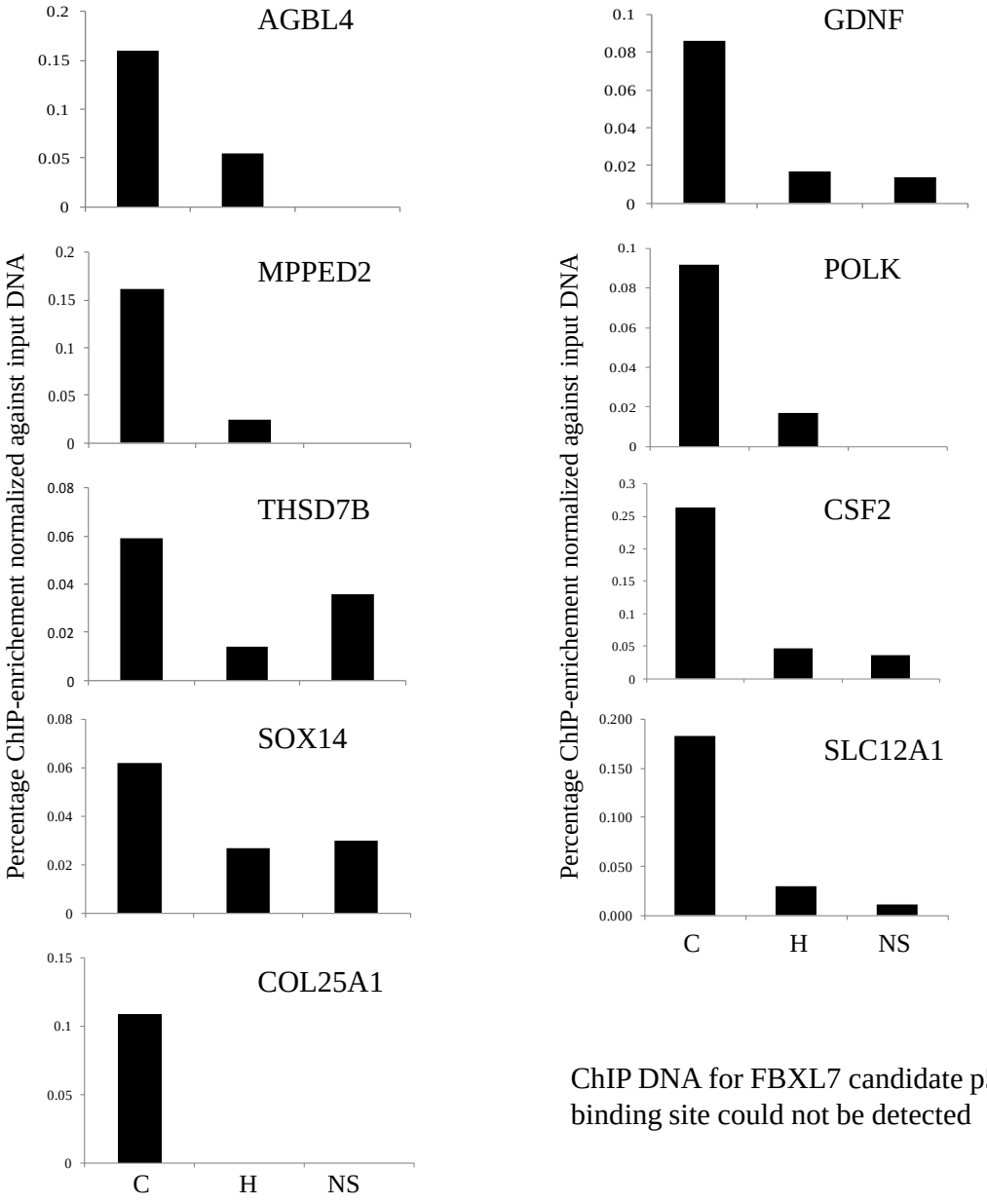
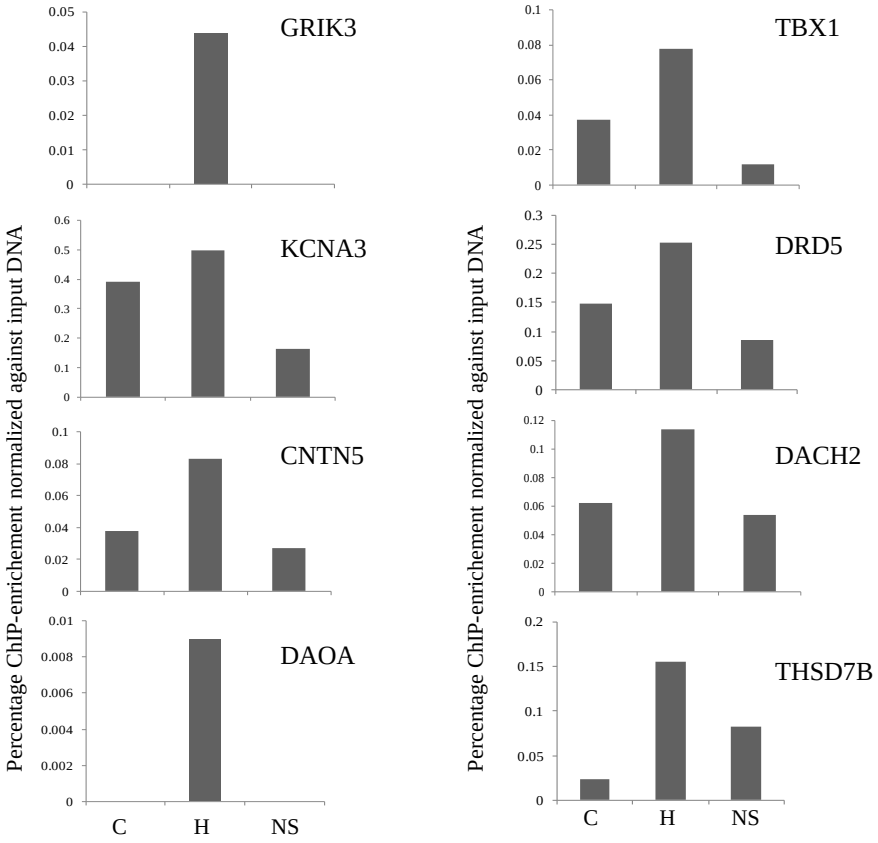
Appendices

Appendix A. ChIP-qPCR validation of 10 randomly selected Control-enriched candidate p53 binding sites



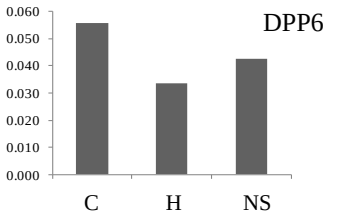
ChIP DNA for FBXL7 candidate p53 binding site could not be detected

Appendix B. ChIP-qPCR validation of 10 randomly selected HBx-enriched candidate p53 binding sites



Validated

Not validated



ChIP DNA for APOBEC3A candidate p53 binding site could not be detected

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