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THE ONCOGENIC ROLE OF HISTONE CHAPERONE ASF1 PROTEINS IN SOLID TUMORS

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The Oncogenic Role of Histone Chaperone ASF1 Proteins in Solid Tumors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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‘ To my parents ’

致亲爱的爸爸妈妈

The absurd does not liberate; it binds.

—— Albert Camus <The Myth of Sisyphus>

ABSTRACT

Chromatin is the essential medium connecting regulatory signals such as transcription factors and signaling pathways to the alteration of gene activity and cellular phenotypes. Aberrant chromatin (epigenetic) environment plays an important role in carcinogenesis.

The fundamental unit of chromatin is the nucleosome which is composed of a histone core wrapped with 145-147 base pairs of DNA around. In the last decades, great efforts have been made to delineate the role of aberrant DNA methylation and chromatin/histone-remodeling factors in oncogenesis. However, recent evidence has merged that the dysregulation of histone chaperones also acts as a cancer-driver. Anti-silencing function 1 (ASF1) is the most conserved histone H3-H4 chaperone, regulating histone metabolism. ASF1 proteins include two paralogs ASF1A and ASF1B in mammals. ASF1A and ASF1B have been reported as oncogenes in human cancers. Data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases show that ASF1A and ASF1B are overexpressed in 20 and 24 different types of cancers, respectively. Thus, in this thesis, I explored the oncogenic role of histone chaperone ASF1 and underlying molecular mechanisms in several solid tumors.

In **Paper I**, the role for ASF1A in gastrointestinal cancer (GIC) was investigated. We discovered that ASF1A interacted with the oncogenic transcription factor β-catenin and promoted the transcription of β-catenin target genes (c-MYC, cyclin D1, ZEB1 and LGR5). The increased expression of these genes stimulated proliferation, stemness and migration/invasion of GIC cells. Over-expression and knockdown of ASF1A boosts and inhibits *in vivo* tumor growth and/or metastasis in mouse models, respectively. Higher levels of ASF1A expression predict significantly shorter patient survival in colorectal cancer (CRC). Further analyses of the Gene Expression Omnibus dataset validate higher ASF1A expression predicting a poor prognosis in CRC patients. Taken together, this study reveals the novel function of ASF1A as a transcription co-factor independent of its canonical role and the potential value of ASF1A for outcome prediction and targeted treatment in GIC.

In **Paper II**, we show that ASF1A overexpression is widespread in human malignancies and is required for the infinite proliferation of cancer cells. When ASF1A was knocked-down in wild-type (wt) p53 carrying cells derived from hepatocellular carcinoma (HCC) and prostate cancer (PCa), DNA damage response was activated and up-regulation of p53-$p21^{cip1}$ expression consequently occurred. These cells eventually underwent cellular senescence. Higher ASF1A expression and/or lower $p21^{cip1}$ expression predicts a poor outcome in HCC patients. Thus, ASF1A may be a therapeutic target and a prognostic factor in HCC and other cancers.

In **Paper III**, we evaluated whether ASF1B has diagnostic and prognostic values in adrenocortical carcinoma (ACC) and regulates invasion and metastasis. We first analyzed TCGA and GTEx data and found that the *ASF1B* gene was amplified in two thirds of ACC tumors and associated with its overexpression. ASF1B expression correlates with the ACC diagnostic criteria of the Weiss scoring system. Higher ASF1B expression and ASF1B copy number predict a poor outcome in the TCGA cohort of ACC patients. Knockdown of ASF1B in ACC cells impairs migration and invasion ability by inhibiting expression of the transcription factor FOXM1; whereas ASF1B over-expression exhibits opposing effects. These findings suggest that ASF1B may be a useful factor for ACC diagnostics and prognostication, and potentially a novel target for ACC therapy as well.

Collectively, the results presented in this thesis gain profound insights into the oncogenic role of ASF1 in several solid tumors and demonstrated novel activities of ASF1 proteins beyond their conserved histone chaperone function. These findings will inspire further exploration of both the clinical and biological roles of ASF1 in precision oncology.

LIST OF SCIENTIFIC PAPERS

I. Xiuming Liang, Xiaotian Yuan, Jingya Yu, **Yujiao Wu**, Kailin Li, Chao Sun, Shuyan Li, Li Shen, Feng Kong, Jihui Jia, Magnus Björkholm and Dawei Xu.
Histone chaperone ASF1A predicts poor outcomes for patients with gastrointestinal cancer and drives cancer progression by stimulating transcription of beta-Catenin target genes.
EBioMedicine. 2017, 21: 104-116.

II. **Yujiao Wu**, Xidan Li, Jingya Yu, Magnus Björkholm and Dawei Xu.
ASF1a inhibition induces p53-dependent growth arrest and senescence of cancer cells.
Cell Death Dis. 2019, 10(2): 76.

III. **Yujiao Wu**, Magnus Björkholm and Dawei Xu.
ASF1B over-expression is associated with aggressive adrenocortical carcinoma and poor patient outcomes.
Manuscript.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I. Yanxia Guo, Xiaotian Yuan, Kailin Li, Mingkai Dai, Lu Zhang, **Yujiao Wu**, Chao Sun, Yuan Chen, Guanghui Cheng, Cheng Liu, Klas Strååt, Feng Kong, Shengtian Zhao, Magnus Bjorkhölm and Dawei Xu.
GABPA is a master regulator of luminal identity and restrains aggressive diseases in bladder cancer.
Cell Death Differ. 2020, 27(6): 1862-1877.

II. **Yujiao Wu**, Li Shen, Xiuming Liang, Shuyan Li, Lin Ma, Lixin Zheng, Tongyu Li, Han Yu, Hillary Chan, Chunyan Chen, Jingya Yu and Jihui Jia
Helicobacter pylori-induced YAP1 nuclear translocation promotes gastric carcinogenesis by enhancing IL-1beta expression.
Cancer Med. 2019, 8(8): 3965-3980.

CONTENTS

LIST OF ABBREVIATIONS

3C	chromosome conformation capture
4C	chromosome conformation capture-on-chip
5C	chromosome conformation capture carbon copy
5mC	5-methylcytosine
ACA	adrenocortical adenoma
AcKs	acetylated lysines
ASF1	anti-silencing function 1
ATAC-seq	assay for transposase-accessible chromatin using
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
BET	the bromodomain and extra terminal domain
CAF-1	chromatin assembly factor 1
CC	colon cancer
ChIP	chromatin immunoprecipitation
CNV	copy number variation
CRC	colorectal cancer
Daxx	death domain-associated protein
DDR	DNA damage response
DFS	disease-free survival
DNase-seq	DNase I hypersensitive site sequencing
DNMT	DNA methyltransferase
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ESC	embryonic stem cell
FBS	fetal bovine serum
FGF	fibroblast growth factor
GC	gastric cancer
GIC	gastrointestinal cancer
GRN	gene regulatory network

GTEx	The Genotype-Tissue Expression Project
H3K56ac	H3K56 acetylation
HAT	histone acetyltransferases
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAC	histone deacetylase
HIRA	histone regulator A
HSV	herpes simplex virus
IF	immunofluorescence
IHC	immunohistochemistry
IP	immunoprecipitation
ITS	Insulin-Transferrin-Selenium
KMT	lysine methyltransferases
KO	knockout
MCM9	minichromosome maintenance complex 9
MeK	lysine methylation
mH2A	macroH2A
MNase-seq	micrococcal nuclease sequencing
Nap1	nucleosome assembly protein 1
NOMe-seq	nucleosome occupancy and methylome
NT	non-tumorous
OIS	oncogene-induced senescence
OS	overall survival
PCa	prostate cancer
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PTM	post-translational modification
qPCR	quantitative real-time PCR
RC	rectal cancer
RNA-seq	RNA sequencing
SAHF	senescence-associated heterochromatin foci

TAD	topologically associating domains
TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
TFs	transcription factors
TGF	tumor growth factor
UMSCs	umbilical-derived mesenchymal stem cells
VEGF	vascular endothelial growth factor
wt	wild-type
β-Gal	β-Galactosidase

1 INTRODUCTION

1.1 CELL FATE AND CANCER

It is always fascinating to think about how one fertilized egg develops to an organism composed of hundreds of different types of cells, all of which share one genome but express different sets of genes at different levels. Each cell identifies itself and restricts its role under the architecture of the whole life system, though in some pathological cases, cells lose their identities and drive their fate to another end, for example, becoming cancer cells (Figure 1).

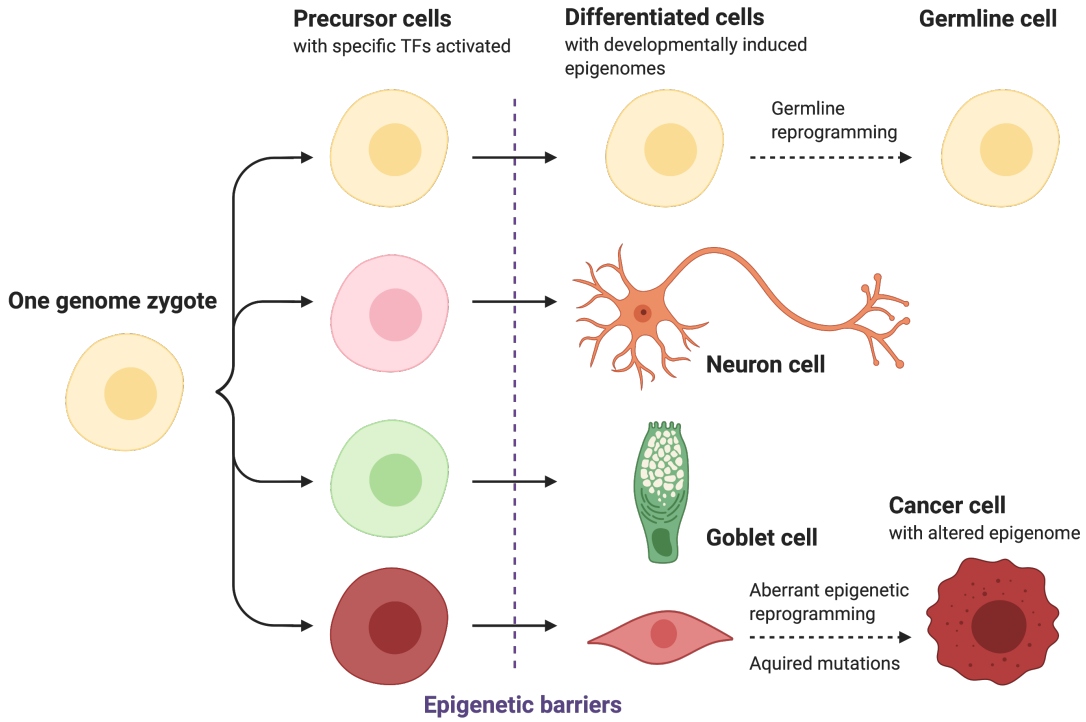

Figure 1. Cell identification in development and cancer. Life starts from a zygote with one genome. Stage- and cell type-specific transcription factors (TFs) initiate cell differentiation. Epigenomic factors may further influence the differentiation paths of the precursor cells. Under some disease conditions such as cancer, the differentiated cells may acquire oncogene mutations and undergo aberrant epigenetic re-programming and thus rewrite their fate.

1.1.1 Establishment and maintenance of cellular identity

Cells distinguish themselves from other cell types by expressing a unique spectrum of genes and generating a specific phenotype[1, 2]. This gene expression pattern and the phenotype must undergo two processes --- establishment and maintenance[1]. The establishment of cellular identity depends largely on how the pluripotential original cell coordinates hundreds of transcription factors to bind to specific DNA motifs to activate or repress the expression of cell lineage genes[3]. Genotype plays an important role in the establishment period and somehow decides the phenotype. In the maintenance period, a lot more non-DNA sequence specific chromatin cofactors are involved in to create and maintain certain chromatin states

through cell division --- delivering the precise hereditary information from the parental cells to the offspring cells, sometimes without the participation of the initial transcription factors[4]. Epigenetic regulation is inevitable to be mentioned in the maintenance phase --- passing down the alternative chromatin states without changing the DNA sequence.

1.1.2 Erasion of the barriers

Normally, cell fate is strictly and precisely restricted through the whole life cycle. A series of barriers are set up to limit the function and longevity of each cell type. However, once a neoplastic program happens to be switched on, the abnormal cells explore every means to break through these barriers and ultimately become malignant and immortalized. It is usually a long-time and multi-step process for the malignant transformation. The famous and classic theory that Hanahan and Weinberg brought up in 2000 and 2011 highlights eight hallmarks of cancer, which in turn are also the typical barriers protecting cell fate from tumorigenesis[5, 6]. Understanding the underlying molecular mechanisms of how cells overcome the obstacles and manipulate their destiny will be very crucial for the corresponding therapeutic intervention.

1.1.3 Genetic and epigenetic interplay

DNA sequence is undoubtedly an important determinant of cell fate. However, because eukaryotic genomic DNA is packaged into a highly ordered chromatin structure, how to approach and translate the DNA information correctly becomes another obstacle for eukaryotic cells on the road of self-identification[7]. Therefore, apart from the important information written in DNA, cellular identity is established and maintained with the aid of chromatin environment. The concept of epigenetics was then raised referring to “the study of molecules and mechanisms that perpetuate alternative gene activity states in the context of the same DNA sequence”[1].

Most human cancers display both genetic and epigenetic defects which inextricably interplay with each other[8-10]. Genetic factors such as sequence polymorphisms and mutations can affect epigenomic landscapes by altering chromatin accessibility or tempering histone and DNA modifying enzymes, chromatin remodelers and other chromatin factors[1]. For instance, pediatric tumors usually have a high possibility of harboring mutations in genes encoding chromatin modifying enzymes and have abnormality in DNA methylation[11, 12]. These types of mutations are also observed in adult tumors and are found to have a great influence on the epigenome[13]. For example, mutations in the genes *IDH1* and *IDH2* in gliomas and acute myeloid leukemia drive the pathological phenotype by inhibiting DNA demethylases and histone demethylases[14-16]. Epigenetic factors also influence the genome. For instance, chromatin states heavily affect the binding efficiency of transcription factors with DNA elements and thus regulate gene expression[17]. Epigenetic factors are also the intermediate between the environment and the intrinsic genome and can deliver environmental signals to the genome as well as affect genome integrity[1].

1.1.4 Theories on cell fate determination and how the rebellious cells develop to cancer

There is a metaphor called ‘epigenetic landscape’ proposed by the British developmental biologist Conrad H. Waddington[18]. The landscape is composed of a series of ridges and valleys and a ball is on the highest top where is its starting point to go down. This metaphor can be used to explain cell fate determination. The ball on the top is a progenitor cell. The end of each valley represents the final destiny the progenitor cell might drop to and the valleys stand for different developmental pathways the cells may follow (Figure 2).

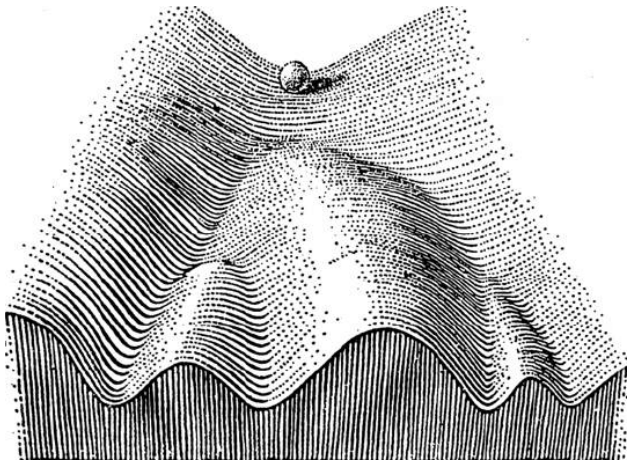

Figure 2. The Epigenetic Landscape. From the book <The strategy of the genes> written by Conrad H. Waddington and first published in 1957.

People have created multiple theories and models from the ‘Epigenetic landscape’, two of which are interesting. The first theory assumes that the organic system is an attractor network[19, 20]. It proposes that there are ‘attractors’ (potential well) among cell cohorts to restrict them in their destiny. The attractor is generated by the gene regulatory network (GRN) in the high-dimensional gene expression state space and can facilitate the cell to withstand the stochastic molecular fluctuations. The destabilization of their high-dimensional attractor state can cause the transition of cell fate. The ‘battle’ between an old attractor and a new one might be the cause the destabilization. There are relevant observations showing that the normal tissue microenvironment can shift the balance of the signal network to the precancerous state and rewrite the tumor fate in breast cancer, which creates a new angle for cancer treatment[21]. Oppositely, a tumor microenvironment can accelerate the tumorigenesis process and manipulate the normal cells to become malignant[6, 22].

The second theory assumes that the chromatin can adjust the heights of the ridges between the canals in the proposed epigenetic landscape by restricting the expression of lineage-specific genes. An overly permissive chromatin state can be created by either genetic or environmental factors. This state can result in epigenetic plasticity which makes the stochastic alteration of regulatory pathways possible and facilitates the selection of premalignant cells, and finally fuels the hallmarks of cancer[23]. Cancer hallmarks can be realized through the silencing of tumor suppressors, the activation of oncogenes by aberrant enhancers or cell fate transitions. From an epigenetic angle, epigenetic plasticity and

aberrations undoubtedly contribute to these processes. For example, disruption of chromatin insulators promotes proliferation by activating *PDGFRA* gene[24]. Promoter hypermethylation or EZH2 hyperactivity causes silencing of tumor suppressor $p16^{ink4a}$[25, 26]. DNMT3A or IDH mutations induce cell death resistance by altering DNA damage response[27, 28]. Mutations in genes coding histone H3.3 or its chaperones can drive replicative immortality[29, 30].

1.2 EPIGENETICS

1.2.1 Definition

Waddington first defined 'epigenetics' in 1942 as changes in phenotype without changes in genotype in the context of development[3, 31-33]. During the next three-quarters of a century, the understanding of this concept has evolved and now it is generally acceptable to refer to 'epigenetics' as the study of molecules and mechanisms that regulate gene activity state without involving changes in DNA sequence[1].

1.2.2 History of epigenetic research

The history of epigenetic research can be split into two periods: the fundamental age which can be traced back to the 19th century and the modern era which is from 1996 till now[34]. In the years between 1869 and 1928, the work completed by Miescher, Flemming, Kossel and Heitz included discovery of nucleic acids, chromatin and histone proteins, which was a foundation for the cytological distinction between euchromatin and heterochromatin[35]. In the following century, many epigenetic molecules and modifications were recognized or detected, including DNA methylation in the mid-1970s, the nucleosome (the chromatin subunit model) in 1974 and histone modifications in mid-1960s[36-38]. From the year 1996, along with the discovery of multiple histone and DNA modification enzymes and the boost of novel technologies such as genome-wide chromatin profiling, epigenetic research has entered a modern era[39]. More histone modifications and histone variants were recognized and the hypothesis of a histone code was raised[40-42]. Nucleosome remodeling and non-coding RNA were also brought into people's eyesight[34].

1.2.3 Major epigenetic information carriers

1.2.3.1 Histone modifications

Histone modifications are covalent post-translational modifications (PTMs) on histone proteins. There are a variety of forms of histone modifications including methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, etc.[34]. Different histone modifications convey different kinds of information. For example, histone lysine H3K9me3 is a repressive signal, while histone H3K4 methylation is associated with active promoters[34]. Histone modification information is edited by 'writers' and 'erasers' and accessed by 'readers'[40, 43]. The 'writers' and 'erasers' are generally enzymes which can add or remove post-translational modifications to or from histone proteins. Histone acetyltransferases (HATs) and histone lysine methyltransferases (KMTs) are typical histone 'writers'. The 'erasers' include for example histone deacetylase (HDAC) family enzymes and

histone demethylases. The histone modification 'readers' are molecules which can offer an accessible surface to interact with a modified histone residue and determine the modification and state specificity[44]. The 'readers' can also distinguish the flanking amino acids to achieve sequence specificity. There are a wide range of histone modification readers. For instance, the bromo domains and the tandem PHD domain can recognize acetylated lysines (AcKs)[45, 46]. The PHD, chromo, WD40, Tudor, double/tandem Tudor, MBT, Ankyrin Repeats, zf-CW and PWWP domains can recognize lysine methylation (MeK) modifications[44].

1.2.3.2 Canonical histones and histone variants

Chromatin is the physiological form of the eukaryotic genome and is composed of DNA and histone proteins. The canonical histone proteins include H1, H2A, H2B, H3, H4 and H5. In some contexts, the canonical histone proteins can be exchanged by histone variants. Histone variants have different sequences or structures from the canonical histones and compose a small portion of histone pool[47]. Usually they are tissue specific and are more prevalent in H2A, H3 and linker histone H1 family[48, 49]. For example, H2A variants consist of H2A.Z, H2A.X and macroH2A (mH2A). H3 variants include CENP-A and H3.3[47]. Histone variants play important roles in regulating chromatin segregation and gene expression. Some recently accumulated evidences have shown the role of histone variants in cancer progression. For instance, H2A.Z is an oncogenic histone variant and has increased expression in colorectal, breast, lung and bladder cancers[47, 50, 51]. H2A.X is involved in head and neck squamous cell carcinoma, non-Hodgkin lymphoma, gastrointestinal stromal tumor and breast cancer[52-55]. The histone variant mH2A suppresses melanoma progression by regulating CDK8[56].

1.2.3.3 DNA methylation

DNA methylation usually refers to the covalent addition of a methyl group on the 5th carbon of cytosine by DNA methyltransferases (DNMTs)[57]. DNA methylation has been well studied in the epigenetic regulation of gene expression, especially for 5-methylcytosine (5mC)[36]. The association between DNA methylation and gene expression was well established by 1980, where what was called CpG islands was discovered[58, 59]. CpG islands are prevalent present in human genome, especially at the transcription and promoter region[60]. In normal tissues, most of CpG islands have a low methylation level or are unmethylated, while are often found aberrantly methylated in tumor cells[57, 61].

1.2.3.4 Non-coding RNA

A non-coding RNA (ncRNA) usually refers to a functional RNA molecule which is not translated into a protein. There are several classes of ncRNAs including transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), and small RNAs such like microRNAs, siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs and the long ncRNAs (lncRNAs). Some ncRNAs are involved in post-translational regulation such as microRNAs, and some others are relevant to transcriptional regulation. The most representative example of non-coding RNA mediated regulation may be the X-chromosome inactivation[62]. Some nuclear lncRNAs are chromatin-associated and participate in the regulation of higher-order chromatin architecture[1].

1.2.4 Epigenetic inheritance versus plasticity

Many epigenetic characteristics can be transmitted across cell divisions and also through generations after they are established even when the original signals are absent[1]. Examples include heterochromatin inheritance in yeast, genomic imprinting in mammals and vernalization in plants[1]. It is much more difficult to achieve epigenetic inheritance than genomic inheritance because nucleosomes lack a DNA-template based duplication system. The inheritance of epigenetic marks has to depend on other mechanisms to survive DNA replication and mitosis. A series of chromatin factors such as heterochromatin complex, Polycomb complex, Trithorax complex, DNMT1-UHRF1 and EZH2 are involved in this process[63-66]. Besides, the inheritance of single nucleosome mark seems to be challenging and usually the establishment of chromatin hereditary domains requires several or hundreds of kilobases in size[67-70]. In summary, from DNA methylation, nucleosome modifications to 3D chromatin structures such as topologically associating domains (TADs), epigenetic inheritance includes multiple layers[71-74]. The multiple epigenetic machineries interact and cooperate to stabilize heritable states. Each layer adds an extent of stability, while at the same time diversifies the plasticity due to the reversible character of epigenetic modifications[75]. DNA sequence is very important and acts as the fundamental basis in some tough cases for the epigenetic inheritance system by offering binding sites for transcription factors or RNA-mediated mechanisms[76].

1.2.5 Novel approaches in epigenetic research

1.2.5.1 Chromatin accessibility and chromosome conformation measurement

Chromatin accessibility is crucial for transcription factors or transcription machineries to reach DNA information[77]. Based on polymerase chain reaction (PCR) technology[78], a number of quantitative methods have been designed and developed to measure site-specific chromatin accessibility using endonucleases and ligation-mediated PCR[79, 80], including DNase I hypersensitive site sequencing (DNase-seq)[81, 82], Assay for transposase-accessible chromatin using sequencing (ATAC-seq)[77, 83], micrococcal nuclease sequencing (MNase-seq)[84, 85] and Nucleosome occupancy and methylome sequencing (NOMe-seq)[77, 86]. The higher order of chromatin affects chromatin accessibility. A series of chromosome conformation capture (3C) based technologies have been developed to analyze the spatial organization of chromatin in cells, such as 3C, Chromosome conformation capture-on-chip (4C), Chromosome conformation capture carbon copy (5C), Hi-C, ChIP-loop and ChIA-PET[87-91].

1.2.5.2 Single-cell and lineage-tracing techniques

The ability to trace molecular changes through time and cell division is important for epigenetic inheritance study. Multi-labeling by introducing barcodes has solved the problem of limited labeling in dividing cells. Single-cell technology based quantitative measurements have also been matured. The combination of advanced lineage-tracing techniques and single-cell ‘omic’ technologies can help to better understand the gene expression timeline of different cell lineages[1, 92].

1.2.5.3 Genome editing and super-resolution microscopy technologies

The boost of genome editing methods such as CRISPR-Cas9 in the recent decade has made it possible and then convenient to edit the genome precisely[93]. This means that most of the epigenetic molecules and enzymes can be deleted or introduced in a more flexible way in multiple contexts for better functional study[94, 95]. Besides, the development of super-resolution microscopy technologies also contributes to epigenetic research for example in combination of single-cell Hi-C technology in chromosome conformation study[96].

1.3 HISTONE CHAPERONE ASF1

1.3.1 The nucleosome

The nucleosome is the fundamental unit of the chromatin[97]. One nucleosome unit is composed of a histone core wrapped with 145-147 base pairs of DNA[98]. The histone core consists of a histone (H3-H4)2 tetramer and two histone H2A-H2B dimers[98]. Since DNA is compacted around nucleosomes, nucleosomes have to be disassembled before DNA replication, transcription and repair. After these processes, nucleosome components are assembled to form new nucleosomes[98].

1.3.2 Histone chaperones

Histone chaperones are a diverse group of histone-binding proteins with distinct structural and functional properties. They help prevent or reverse incorrect interactions that occur when interactive surfaces are exposed to the environment before histones are assembled into chromatin[99, 100]. For instance, chromatin assembly factor 1 (CAF-1) is a H3.1-H4 chaperone. Nucleosome assembly protein 1 (Nap1) is a H3-H4 and H2A-H2B chaperone[99]. Although histone chaperones are broadly involved in chromatin assembly, they are not a permanent component of the final product --- the nucleosome[99]. Besides, histone chaperones also participate in multiple processes of histone metabolism, such as histone storage, transport, PTM and histone recycle[100].

Dysregulation of histone chaperones plays an important role in a variety of human diseases, especially in cancer[98, 101]. It was reported that mutations on histone chaperones ATRX and Daxx genes were detected in pancreatic neuroendocrine tumors[102]. Histone chaperone CHAF1A was observed to promote gastric cancer (GC) pathogenesis via upregulation of cMYC and CCND1 expression[103]. The histone chaperone FACT complex accelerates liver cancer progression by mediating oxidative stress response[104].

1.3.3 ASF1 --- a conserved H3-H4 histone chaperone

Anti-silencing function 1 (ASF1) is the most conserved histone H3-H4 chaperone, regulating histone metabolism in many biological processes, such as replication, transcription and DNA repair[105]. Among its conserved functions, on the one hand, ASF1 interacts with histone H3-H4 dimer and forms a heterotrimeric complex with H3 and H4[106]. On the other hand, ASF1 binds to two other histone H3-H4 co-chaperones: CAF-1 and histone regulator A (HIRA)[107]. CAF-1 is composed of three subunits named p48, p60 and p150. CAF-1 is

crucial for replication and DNA repair-dependent histone deposition, whereas HIRA has a more significant role in DNA repair-independent histone deposition[108].

1.3.4 ASF1 in nucleosome assembly and disassembly

1.3.4.1 ASF1 in replication-coupled nucleosome assembly

During replication, the chromatin needs to be reassembled after DNA is replicated. In this process, both newly synthesized and parental histones are deposited to the newly replicated and parental DNA[99]. The initial step of chromatin assembly is to load histones H3/H4 onto the DNA. Then the two H2A/H2B dimers are deposited, finally followed by the incorporation of linker histone H1[99]. The *de novo* incorporation of newly synthesized histone H3/H4 after replication is mediated by CAF-1[99]. CAF-1 binds to the replication-specific histone H3.1 instead of the replication-independent histone H3.3[109]. The task of ASF1 is to deliver the newly synthesized histone H3-H4 to CAF-1. Then ASF1 has to be removed from the interface with H3 because it is the same interface for H3 and H4 to form the H3/H4 heterotetramer[99]. The replication-coupled nucleosome assembly progress is illustrated in Figure3[98] and 5[99].

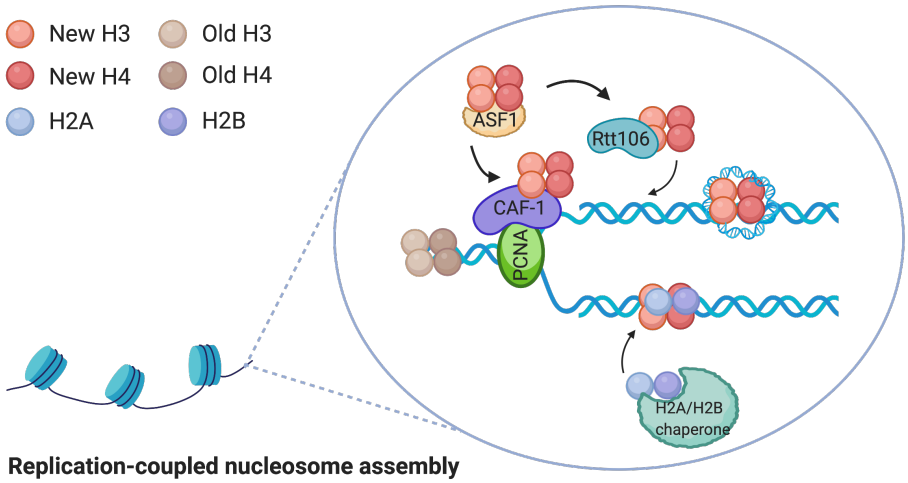

Figure 3. Replication-coupled nucleosome assembly. In replication-coupled nucleosome assembly, ASF1 passes the H3-H4 dimer to downstream chaperones CAF-1 and Rtt106. In human cells, CAF-1 and Rtt106 assemble (H3-H4)2 tetramers and deposit them to newly synthesized DNA. The interaction between CAF-1 and proliferating cell nuclear antigen (PCNA) facilitates the deposition process. (Illustration graph is modified based on Burgess et al., Nat Struct Mol Biol, 2013. 20(1): p. 14-22.)

1.3.4.2 ASF1 in replication-independent nucleosome assembly

In the transcription process, nucleosomes also pose as barriers for the transcriptional machinery and thus need to go through disassembly and reassembly as well. HIRA and death domain-associated protein (Daxx) are mediators of replication-independent nucleosome assembly[98]. They facilitate histone H3.3-H4 deposition. The ASF1A-H3.3-H4 complex is transferred to HIRA at genic regions. Daxx assists deposition of H3.3-H4 at telomere

regions[98]. The replication-independent nucleosome assembly process is shown in Figure 4[98].

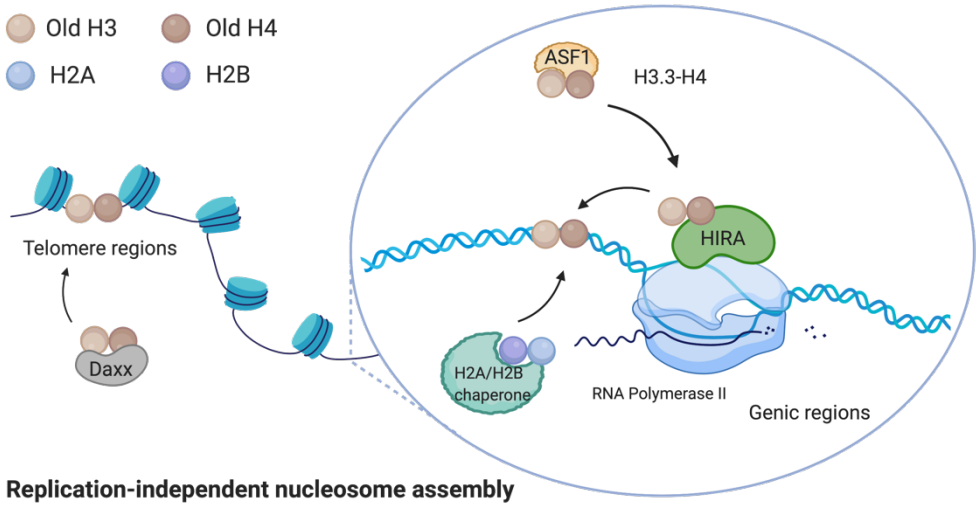

Figure 4. Replication-independent nucleosome assembly. In human cells, nucleosomes pose as barriers for the transcriptional machinery and thus undergoes disassembly and reassembly during transcription. This process is known as replication-independent nucleosome assembly. ASF1 delivers histone H3.3-H4 to downstream chaperone HIRA at genic regions, possibly through interactions with RNA polymerase II and DNA. In telomere regions, H3.3-H4 is deposited by Daxx. The mechanisms of Daxx-mediated histone deposition are still unclear. (Illustration graph is modified based on Burgess et al., Nat Struct Mol Biol, 2013. 20(1): p. 14-22.)

1.3.4.3 ASF1 in nucleosome disassembly

Nucleosomes need to be disassembled before replication and transcription. It is reported that around 300bp naked DNA lies ahead of the replication fork while approximately 250bp is behind the replication fork[110, 111]. The H2A/H2B histones are removed first, followed by the more stable histone H3/H4. ASF1 facilitates the removal of H3/H4 from DNA. The histones in the ASF1-H3/H4-MCM complex have a similar posttranslational modification pattern as the parental chromatin, which suggests the role of ASF1 in nucleosome disassembly[112]. The nucleosome disassembly process is presented in Figure 5[99].

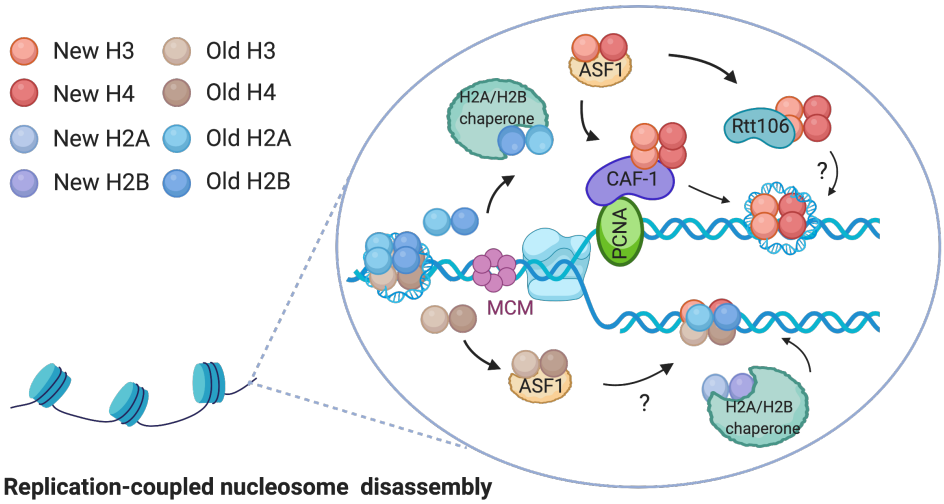

Figure 5. Nucleosome disassembly and assembly at replication fork. The nucleosomes ahead of the replication fork need to be disassembled before the replication machinery arrives. MCM2-7 helicase complex is needed for unwinding the DNA duplex. PCNA keeps the processivity of DNA replication. In human cells, histone chaperone ASF1 is responsible for removing histone H3-H4 from double-strand DNA. ASF1 binds to the MCM complex through the parental histones H3 and H4. The parental histones in this complex provide the information that the histones bound to ASF1 have been removed from the DNA ahead. Similar mechanisms are observed in H2A/H2B chaperones such as histone chaperone FACT. (Illustration graph is modified based on Ransom et al., Cell, 2010. 140(2): p. 183-95.)

1.3.4.4 ASF1 in DNA repair

There are several lines of evidence showing ASF1 participating in DNA repair. For example, ASF1A-facilitated histone H3K56 acetylation (H3K56ac) is necessary for nucleosome reassembly after DNA damage to promote DNA repair[113]. ASF1 assists checkpoint recovery after DNA damage repair[114]. ASF1A and ATM can also modulate UV-induced cell-cycle checkpoint recovery[115]. Moreover, ASF1A has a direct role in DNA double-strand break repair[116]. We have observed that ASF1A deletion triggers DNA damage response by activating the p53 signaling pathway[105].

1.3.5 Evolution of the *ASF1* gene

ASF1 was first identified in yeast as a single protein[117]. On the road of evolution, the *ASF1* gene duplicated itself at the origin of jawed vertebrates[107]. Afterwards, *ASF1* passed down in most vertebrates as two paralogs which were then named *ASF1A* and *ASF1B* in mammals[107]. After the duplication incident, *ASF1A* gene relocated into an intron of the *minichromosome maintenance complex component 9 (MCM9)* gene at the ancestor of tetrapods, leaving *ASF1B* gene at the original spot[107]. The new genomic environment at *MCM9* provided *ASF1A* gene a different GC content and replication timing, which might fuel the two paralogs towards different evolutionary directions[107].

1.3.6 Functional divergence of ASF1A and ASF1B

1.3.6.1 Structure-based interaction specificities

ASF1 gene has three structural regions: core, N- and C-terminus. The main core region is highly conserved while the N- and C- terminal regions have varied. The conserved core binding domain provides basis for the interaction with histone H3-H4 and cochaperones. The N-terminus of ASF1A keeps almost all the characters of their ancestor, whereas the ASF1B N-terminus largely evolves away. It is reported that the divergence of ASF1B N-terminal residuals decreases the affinity for HIRA, which is in charge of replication-independent histone deposition together with ASF1[108]. Instead of interacting with HIRA, ASF1B has higher affinity for CAF-1 p60, which plays an important role in replication-dependent histone deposition. The C-terminus domain of ASF1 is a phosphorylation substrate in both human and mouse[118, 119]. The ASF1A C-terminus remained quite stable since the ancestor of amniotes while ASF1B C-terminus kept evolving at similar rates all the time[107]. The positive selection on ASF1A C-terminus at amniotes might be an indicator of acquisition of novel functions of this gene in this lineage[107]. In human and mouse, many sites on ASF1A C-terminus mutated to Ser and Thr residues, whereas ASF1B C-terminus lost some Ser

residues and gained many Gly ad Pro residues[107]. ASF1A presents more phosphorylation sites on the C-terminus due to the presence of multiple Ser and Thr residues.

1.3.6.2 Biological function divergence of ASF1A and ASF1B

Although ASF1A and ASF1B preserved most of the ancestral characters, they acquired some novel and distinct functions. ASF1A played an important role in histone H3K56 acetylation[120, 121]. The interaction between ASF1A and histone H3-H4 is essential for histone H3K56 acetylation[121]. ASF1A is also involved in maintenance of pluripotency and cellular reprogramming[122]. ASF1B functions more in proliferation regulation. For example, ASF1B can promote human β-cell proliferation by recruiting histone H3.3[123].

1.4 ASF1 IN CANCER

Among numerous epigenetic factors, histone chaperones distinguish themselves in regulating all aspects of histone metabolism, which is the fundamental process of most chromatin-based epigenetic activities[122]. ASF1A and ASF1B have been reported as oncogenes in many types of cancer and predict a poor outcome in patients with higher expression, including gastrointestinal, liver, prostate and breast cancer[105, 124, 125]. Data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression Project (GTEx) databases show that ASF1A and ASF1B are overexpressed in 20 and 24 different types of cancers, respectively, suggesting their widespread dysregulation in cancer development.

Many studies have suggested ASF1s as crucial factors in carcinogenesis. We showed that ASF1A promoted gastrointestinal cancer pathogenesis by targeting β-catenin downstream genes. More recently, ASF1A has been found as an immunotherapeutic target in kras-mutant lung adenocarcinoma[126]. In addition, ASF1A is required for the constitutive expression of telomerase reverse transcriptase (TERT), which is essential for the immortal phenotype of cancer cells[127]. ASF1B was reported to accelerate breast cancer cell growth and to predict poor clinical outcomes [124, 125]. In clear cell renal cell carcinoma, ASF1B promoted cellular proliferation and migration [128]. Another study reported that prostate cancer (PCa) cells depleted of ASF1B underwent apoptosis resulting from the PI3K/Akt pathway inhibition [129].

1.5 ASF1 AND CELLULAR SENESCENCE

1.5.1 Cellular senescence

Cellular senescence is described as a process where cells quit from cell cycle and undergo distinctive phenotypic alterations, including morphology, chromatin, transcriptome and secretome changes[130-133]. Other than functioning in embryonic development, host immunity, wound healing, tissue repair and organismal aging, cellular senescence is also important in tumor suppression[134]. It is reported that cellular senescence can repress a variety of cancers, such like breast cancer, hepatocellular carcinoma, gastric, prostate, colorectal, and lung cancer[134]. Typically, normal human cells undergo senescence rather

than transformation upon oncogene activation, which is so-called oncogene-induced senescence (OIS). In certain cases, cellular senescence is regarded more significant than other forms of cell death for tumor suppression in mammals, since evidence has shown that subtle perturbations in senescence regulatory network influence cancer susceptibility dramatically in mice while defects in apoptosis do not[134]. Thus, cellular senescence induction serves as a novel strategy for cancer treatment.

1.5.2 The role of ASF1 in cellular senescence

ASF1A has been shown to participate in the formation of Senescence-Associated Heterochromatin Foci (SAHF)[135, 136]. SAHF is the condensed transcriptionally silent heterochromatin region which is observed in senescent cell nuclei under certain circumstances[135]. In other cases, however, cellular senescence and SAHF formation are not always coupled. For example, SAHF is preferentially induced in OIS rather than in replicative cellular senescence[137]. Besides, SAHF formation depends on cell types[138]. We have reported that ASF1A knockdown in liver and prostate cancer cell lines carrying wild-type (wt) p53 induces growth arrest and senescence via activation of the DNA damage response (DDR)/p53/$p21^{cip}$ pathway[105].

1.6 SOLID TUMORS STUDIED IN THIS THESIS

1.6.1 Gastrointestinal cancer (GIC)

1.6.1.1 Epidemiology

GIC is one of the most common malignancies and the leading cause of cancer-related death worldwide. In 2015, there were approximately 1,157,000 new cases of GIC and 798,500 related deaths in China[139]. In USA, although GC incidence overall is low, colorectal cancer (CRC) incidence is high. In 2015, there were 132,700 new cases and 49,700 deaths caused by CRC in USA[140].

1.6.1.2 Etiology

GIC can be triggered by genetic and environmental factors. Genomic instability and mutations have been reported in GIC[141]. GIC is more prevalent to be seen in developing countries like countries in east Asia. This phenomenon indicates that hygienic standards, eating habits and microbiota colonization may be associated with GIC development. Due to the daily exposure to the stimuli from food and microbiota, inflammation often occurs in the digestive tract. The function of inflammation is to resist infection, but persistent activation of inflammatory responses creates an enduring inflammatory microenvironment. This special microenvironment promotes tumor-favoring inflammation and finally leads to the unresolved inflammation which is regarded as a cancer hallmark[6, 142, 143].

1.6.1.3 Wnt/β-Catenin pathway in GIC

The Wnt/β-Catenin pathway is a highly conserved pathway involved in development from nematodes to humans[144]. The central character of Wnt/β-Catenin pathway is β-Catenin

protein which is encoded by *CTNNB1*. β-Catenin can either function at the cellular adherent junctions by interacting with E-cadherin or present in the nucleus as a transcriptional factor[144]. When lacking upstream activating signals, β-Catenin is rapidly phosphorylated by a 'destruction complex' which is composed of AXIN, APC, GSK3, CK1, followed by proteasomal degradation[145]. Thus, β-Catenin has a very low cytosolic concentration in normal circumstance. In certain developmental situations, the Wnt ligand binds to the FZD receptor and LRP coreceptor, and recruits DVL and Axin to the cell membrane[144]. In this way the 'destruction complex' is disrupted and β-Catenin is released from phosphorylation and degradation. β-Catenin is then translocated into the cell nucleus and mainly interacts with the T-cell factor/lymphoid enhancer factor TCF/LEF transcription factors and initiates transcription of downstream genes[144].

The Wnt/β-Catenin pathway plays an important role in both intestinal development and CRC. It is reported that 90% of CRC tumors have mutations in key components of the Wnt/β-Catenin pathway, most of which appear in *APC* or *CTNNB1*[146]. Especially, activation of the Wnt/β-Catenin pathway is highly associated with tumor metastasis in GIC. One important mechanism underlying the Wnt/β-Catenin pathway-mediated cancer metastasis is the epithelial to mesenchymal transition (EMT).

1.6.2 Hepatocellular carcinoma (HCC)

1.6.2.1 Epidemiology

HCC is the most common liver cancer with high lethality, ranked as the sixth most common cancer and the third leading cause of cancer death in 2012[147]. Chronic liver disease has been indicated as an important trigger in HCC development[147]. HCC has been reported as a leading cause of death in cirrhosis patients[147]. Approximately 80% of HCC cases occur in sub-Saharan Africa and eastern Asia, which suggests a higher morbidity in less developed countries[147]. There is a higher incidence in men than in women[148].

1.6.2.2 Etiology

HCC is usually caused from chronic liver diseases, such as chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection and cirrhosis[149]. In sub-Saharan Africa and eastern Asia, the main risk factors are chronic hepatitis B and aflatoxin B1 exposure, whereas in USA, Europe, and Japan, the main risk factor is hepatitis C and alcohol abuse[147].

1.6.2.3 Genome instability and DDR in HCC

HCC is thus highly associated with HBV and HCV infection[150-152]. HCV, an RNA virus, is not able to integrate into the host genome, while HBV is a DNA virus which is frequently inserted into the host genome and contributes to the carcinogenesis[153]. The integration of HBV into the liver cell genome is a risk factor to cause genome instability and DNA damage[150]. Interestingly, HBV has a locational preference to be integrated in regions crucial to genome stability such as CpG islands, chromosomal fragile fractions and regions adjacent to telomere in tumors [150]. The prevalent presence of DNA damage and chromosomal instability is an important feature of HCC[154].

DDR refers to a series of reactions when cells encounter DNA damage usually caused by endogenous insults like ROS and replication errors or exogenous stimuli such as radiation and chemicals[155]. DNA damage can be divided into mainly two types: single-strand DNA breaks and double-strand DNA breaks. After a certain type of DNA damage happens, the cell cycle checkpoints usually at G1/S and G2/M boundaries are activated. The activation of checkpoints pauses the cell cycle progression and facilitates DDR. DNA damage sensation and checkpoint activation are controlled by two kinases which are also at the central position of the DDR: ataxia telangiectasia and Rad3-related (ATR) which reacts to single-strand DNA breaks or replication stress, and ataxia telangiectasia mutated (ATM) which responds to the more severe double-strand DNA breaks or disruptions in higher order chromatin[156]. ATR and ATM organize the downstream DDR cascades and result in either DNA damage repair or cell death/senescence. If the lesions on DNA are not so serious and are repairable, after the DNA repair process, the recovered cell will be released into the cell-cycle[156]. However, if there are sustained DNA damage signals, which indicates that the injured cell can be sacrificed, cell death or senescence program will be initiated. For example, ATR and ATM can activate p53 pathway and induce apoptosis or senescence[156].

1.6.2.4 p53 in the DDR

p53 is a tumor suppressor and the most frequently mutated gene in cancer[157-159]. p53 plays an important role in the DDR and thus is called the 'guardian of the genome'. Following DNA damage, p53 regulates cell cycle arrest and is crucial for the cell fate[157]. The fate of the cells with DNA damage goes to survival (DNA repair), death (apoptosis) or senescence. p53 regulates a variety of biological functions including DDR, cell cycle arrest, apoptosis, senescence and metabolism[157].

1.6.3 Prostate Cancer (PCa)

1.6.3.1 Epidemiology

PCa is the most prevalent malignancy in men. The incidence increases with age, quite often to be diagnosed among men aged > 79 years[160]. It is more common in the western countries than in the east, indicating lifestyle and environment as risk factors.

1.6.3.2 Etiology

Both genetic and environmental factors affect PCa development. A family history of PCa is a crucial risk factor. It has been reported that men who have first-degree relatives with PCa have twice the risk for PCa compared to men in the general population. If the first-degree relatives with PCa are diagnosed below the age of 60 years, this increased familial risk is more than four times higher than that for men in the general population[161]. Smoking and alcohol consumption also increase the risk of PCa[160].

1.6.4 Adrenocortical Carcinoma (ACC)

1.6.4.1 Epidemiology

ACC is a rare endocrine cancer carrying a poor prognosis[162]. Different from the benign adrenocortical adenoma (ACA) which is prevalent in 3%-10% of the world's population,

ACC only occurs in 0.7 - 2 persons per million[163, 164]. However, ACC is much more aggressive and malignant than ACA[165]. Patients with stage III/IV ACC only have a median survival of less than 1 year and the survival rate of patients with stage IV ACC is merely 6% - 13% at 5 years[164, 166]. Distinguishing ACC from ACA at presentation is very important for the further management.

1.6.4.2 Etiology

ACC is likely to be genetically predisposed and seems to be comparably more prevalent in children[163, 167, 168]. Germline TP53 mutations account for the relatively high incidence in childhood[169]. Generally, the molecular pathology of ACC includes abnormal clonality and DNA content, epigenetic changes, microRNA dysregulation and gene mutations. There are several forms of abnormal clonality and DNA content like aneuploidy, chromosomal aberrations such as chromosomal gains in 4q, 4p16, 5p15,9p34, 12q13,12q24 and 19p[170, 171]. Epigenetic changes include DNA methylation on 11p15 which contains important genes IGF2 and H19[163]. MicroRNA dysregulation and gene mutations include genetic alterations in TP53, MEN1, IGF2 and CDKN2A[163]. Abnormal cellular signaling pathways also play an important role in ACC development, such as the IGF pathway, WNT pathway and vascular endothelial growth factor (VEGF) pathways[163].

1.6.4.3 The Weiss scoring system

The Weiss scoring system is the most accepted pathological assessment standard for ACC in clinical practice[163, 165, 172]. The Weiss scoring system assesses 9 criteria which reflect the architectural, nuclear and invasion status of ACC tumors[165, 173-175] (Figure 6). Each item examined is ranked 0 if it is absent and ranked 1 if it is present in the tumor. The total Weiss score is a sum-up of the 9 sub-scores and is used to evaluate the aggressiveness of the tumor including prediction of prognosis[174, 175].

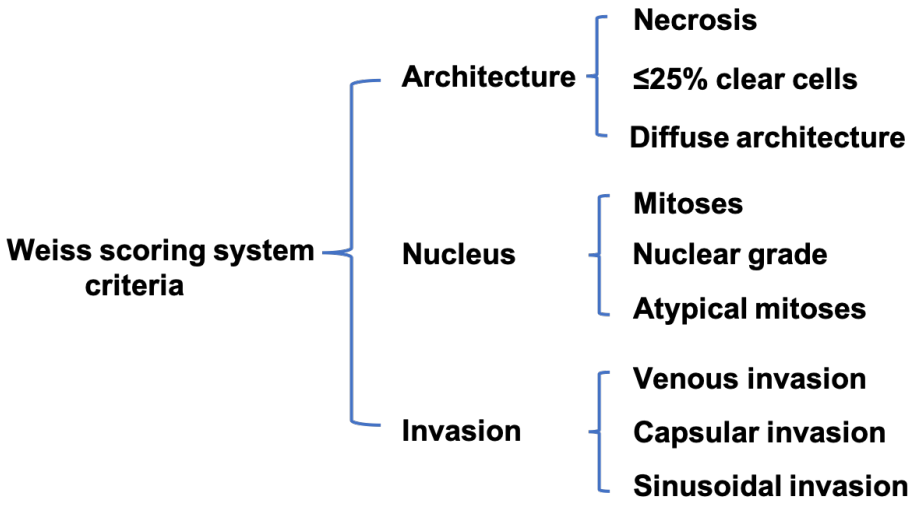

Figure 6. Weiss scoring system criteria for adrenocortical carcinoma.

2 AMIS OF THE STUDY

The overall aim of this thesis was to increase the knowledge of the oncogenic role of histone chaperone proteins ASF1A and ASF1B in solid tumors and the underlying molecular mechanisms, especially of their novel functions other than the canonical role in assisting nucleosome assembly and disassembly, as well as their diagnostic and prognostic values.

The specific aims of each study are:

Paper I

To explore the role of ASF1A in GIC and to determine the physical interaction between ASF1A and the oncogenic transcription factor β-Catenin and the impact on proliferation, stemness and migration/invasion on GIC cells. To evaluate the prognostic value of ASF1A in GIC patients.

Paper II

To define the effect of ASF1A on inducing p53 dependent cell cycle arrest and cellular senescence and the associated mechanisms in HCC and PCa. To assess the potential prognostic value of ASF1A in HCC patients.

Paper III

To evaluate the diagnostic and prognostic values of ASF1B in ACC and to explore the impact and mechanisms of ASF1B on metastasis and invasive capacity of ACC cells.

3 METHODS

3.1 PATIENT SAMPLES (PAPERS I AND II)

The 286 GIC patient samples in Paper I were collected at Qilu Hospital and Second Hospital of Shandong University, China. The 51 HCC patient samples and 29 non-tumorous adjacent liver tissues in Paper II were recruited from Qilu Hospital and Second Hospital of Shandong University, China. Surgical samples were collected from operations and were stored at -80 freezer or in TRIzol™ Reagent or paraffin-embedded immediately. The studies were approved by the ethics committee of Second Hospital of Shandong University.

3.2 CELL LINES AND CELL CULTURE (PAPERS I - III)

GC cell lines AGS, BGC-823 and HGC-27, CRC cell lines HCT116, HCT116-Cas9 and HCT116 p53-/- sublines, SW480, Caco2 and HT29, HCC cell lines HepG2, Huh.7 and Hep3B, PCa cell line LNCaP, breast cancer cell line MCF-7, normal human umbilical-derived mesenchymal stem cells (UMSCs), adrenocortical carcinoma cell line NCI-H295R and HEK-293T were used in Paper I – III. BGC-823, HGC-27, SW480, Caco2, HT29, HepG2, Hep3B, LNCaP and MCF-7 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100ug/ml streptomycin and 4mM L-glutamine. AGS cells were cultured in F12 medium with 10% FBS, antibiotics and L-glutamine. HCT-116 cells and the variants were cultured in Dulbecco's modified Eagle's medium supplied with 10% FBS and antibiotics. HEK-293T and Huh.7 cells were cultured in DMEM medium with 10% FBS, antibiotics and L-glutamine. UMSCs were cultured in F12 medium with 10% FBS, bFGF (10 ng/ml), antibiotics and L-glutamine. NCI-H295R cells were cultured in DMEM/F12 medium with 2.5% Nuserum and 1% Insulin-Transferrin-Selenium (ITS). All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

3.3 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (QPCR) (PAPERS I - III)

Total RNA was extracted from patient samples or cell lines using TRIzol™ Reagent liquid according to manufacturer's instructions. RNA was then reversely transcribed into cDNA. qPCR was performed using SYBR green PCR Master Mix. Relative mRNA expression was presented in arbitrary units based on the CT values and normalized with β -2m expression.

3.4 siRNA AND PLASMID TRANSFECTION (PAPERS I - II)

ASF1A, β-Catenin, ZEB1, p53 and negative control siRNAs were commercially available and were reconstituted to the concentration of 20 nM as working solution after purchase. Cells were transfected with siRNA by using Lipofectamine 2000 or transfected with plasmids by using Lipofectamine 3000 in antibiotic-free Opti-MEM medium according to the manufacturer's instruction.

3.5 PROMOTER ACTIVITY ASSAY (PAPERS I - II)

Wt E-cadherin promoter, E-box-mutated (E-box1 and 3) E-cadherin promoter and $p21^{cip1}$/WAF1 promoter plasmids were obtained from Addgene (Middlesex, UK). The pGL3 Basic luciferase reporter vector was purchased from Promega (Madison, WI, USA). Firefly

and Renilla luminescence was measured by using the Luciferase Assay (Promega). The Renilla luminescence activity was set for normalization.

3.6 WESTERN BLOT (PAPERS I - III)

Total protein was extracted from cell lines or patient samples by using RIPA lysis buffer [Cell Signaling Technology (CST)]. Samples were then loaded in Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA, USA) and transferred to 0.2 um PVDF membranes (Bio-Rad). The membranes were blocked with 5% non-fat milk for 2 hrs at room temperature and then incubated with primary antibodies at 4 overnight with gentle shaking. The second antibodies were added the next day and the target proteins on the membranes were detected. β-Actin was used for normalization.

3.7 SOUTHERN BLOT (PAPER II)

Genomic DNA was extracted from cells using QIAamp DNA Blood Mini Kit (Cat no 51104, Qiagen, Hilden, Germany). Southern blot protocol was from Telo *TAGGG* Telomere Length Assay (Merck, Cat no. 12209136001).

3.8 IMMUNOFLUORESCENCE (IF) STAINING (PAPERS I - II)

Cells under different treatments were cultured on coverslips. Cells were fixed in 4% formaldehyde, washed with PBS and blocked in blocking buffer (Dako, Santa Clara, CA, USA) for 1 h. Primary antibodies were then added and incubated at 4 °C overnight. Triton-100 (Sigma-Aldrich; St. Louis, MO, USA) was used to increase the permeability of cellular and nuclear membranes. The nuclei were stained with 4-6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories Inc., Burlingame, CA, USA).

3.9 COLONY-FORMATION ASSAY (PAPERS I - II)

GIC, HCC and PCa cells under different treatments were seeded into 6-well plates at proper concentrations and incubated for 12 – 14 days in a humidified atmosphere containing 5% CO2 at 37 °C. The colonies were fixed with methanol and stained with Giemsa buffer. Colony numbers (colonies with > 50 cells) were then counted.

3.10 FLOW CYTOMETRY AND CELL CYCLE ANALYSIS (PAPERS I - II)

Flow cytometry was used for LGR5 staining in Paper I and cell cycle analysis in Paper I and Paper II. For LGR5 staining, cells were harvested, washed with PBS. After the blocking treatment, cells were incubated with the LGR5 primary antibody for 1 h at room temperature. The second antibody was then added for 1h incubation. Cells were then analyzed. For cell cycle analysis, cells were fixed in 70% ethanol at 4 °C overnight and stained with a solution containing RNase (0.5 μg) (Sigma - Aldrich) and propidium iodide (50 μg/ml) (Sigma - Aldrich). Cell cycle was analyzed using flow cytometry and further data analysis was performed by using ModFit (BD Biosciences, Franklin Lakes, NJ, USA) and Kaluza software (Beckman Coulter, Indianapolis, IN, USA).

3.11 β-GALACTOSIDASE (β-GAL) STAINING (PAPER II)

β-Gal staining was used for the detection of cellular senescence in HCC and PCa cell lines in Paper II. Cells under different treatments were cultured for 8-10 days, rinse with PBS and fixed with 4% formaldehyde and incubated with freshly prepared β-Gal staining solution (Cellular Senescence Assay KAA002; Merck) at 37 °C overnight without CO2.

3.12 MONO-SPHEROID FORMATION ASSAY (PAPER I)

Cells were seeded and cultured in ultra-low-attachment 96-well plates in RPMI-1640/10 mM HEPES serum-free medium. Two types of growth factors were needed during the culture: 10 ng/ml bFGF and 20 ng/ml EGF. The spheroid colonies formed in 15 days and were counted by using a light microscopy.

3.13 MIGRATION AND INVASION ASSAY (PAPERS I AND III)

Cells were seeded and cultured in serum-free medium in the upper chamber. For the migration assay, the lower chamber contained RPMI-1640 medium with 20% FBS for HGC-27, HCT116, SW480 and HT29 cells in Paper I and DMEM/F12 medium with 5% Nuserum and 1% ITS for NCI-H295R cells in Paper III. Migrated cells were stained with crystal violet solution at proper time points and then photographed and quantified. For the invasion assay, 50 μl Matrigel (Corning life sciences, Flintshire, UK) was injected into the bottom of the upper chamber and incubated for 1 h before cells were seeded. The following procedures were the same as in the migration assay.

3.14 IMMUNOPRECIPITATION (IP) (PAPER I)

IP protocol was based on the instruction of Pierce™ Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific) which was used in the experiment. 50 μg protein extracts were incubated with IP antibodies at 4 °C overnight. The Protein A/G Magnetic Beads were then covalently crosslinked with the antibody using disuccinmidyl suberate. The antibody-crosslinked beads were incubated with the interested protein-containing cell lysate of interest. Washing buffer was used to remove the non-specific unbound materials. Elution buffer was used for the clearance of bound antigen from the antibody-crosslinked beads. Neutralization buffer was used to prevent the precipitation of the isolated antigen. The precipitated product was loaded in Mini-PROTEAN TGX Gels for Western blot examination.

3.15 CHROMATIN IMMUNOPRECIPITATION (CHIP) (PAPER I)

The ChIP protocol was based on the instruction of SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology), which was used in the experiment. Cells under different treatments were crosslinked with formaldehyde. Micrococcal Nuclease was used for chromatin digestion and the products were analyzed by running agarose gels. DNA concentration was determined by Nanodrop 2000 (Thermo Fisher Scientific). The primary antibodies and positive control (histone H3) antibody were incubated with the digested products at 4 °C overnight with rotation. The DNA-antigen-antibody complex was precipitated with Protein G Magnetic beads. The chromatin was then eluted from Antibody/Protein G Magnetic Beads, followed by the reversal of cross-links. The DNA products were purified and amplified using PCR.

3.16 IMMUNOHISTOCHEMISTRY (IHC) (PAPER I)

Paraffin embedded slides were deparaffinized and rehydrated. Citric acid buffer was used for antigen-retrieval and H2O2 was used for the deactivation of endogenous peroxidase. Ten percent of goat serum was used as blocking buffer. Slides were incubated with primary antibodies at 4 °C overnight. Second antibodies were added the second day and incubated for 45 mins at room temperature. DAB staining was then carried out for the detection of the specific signals.

3.17 ANIMAL EXPERIMENTS (PAPER I)

The animal experiments in Paper I included subcutaneous tumor formation and metastasis of CRC cells in a murine xenograft model. For the subcutaneous tumor formation experiment, HT29 and HCT116 cells under different treatments were injected into six weeks old male nude mice subcutaneously (2×10^6 cells/mouse, 5 mice/group). Tumor size was measured weekly for 6 weeks and mice were then sacrificed. Tumors were separated and collected for further experiments and analysis.

For the metastasis of CRC cells in the murine xenograft model, HT29 cells under different treatments were injected into six weeks old NOD-SCID mice via the tail vein (3×10^6 cells/mouse, 6 mice/group). Mice were sacrificed after 7 weeks. The lungs were collected. Half of each lung was embedded with paraffin and the other half was immediately frozen at -80 °C freezer for further experiments and analysis.

3.18 LENTIVIRUS INFECTED STABLE CELL LINES (PAPERS I AND III)

A plasmid cocktail including 15 μg viral vector, 10.5 μg sPAX2 vector and 6 μg pMD2.G plasmid was transfected into HEK293T cells which were cultured in a 75 cm^2 flask at 90% confluency by using Lipofectamine 3000. Supernatants were collected at 48 hrs and 72 hrs after the transfection and was filtered through 0.45 μm filters. PEG-it ™ Virus Precipitation Solution (System Biosciences, Palo Alto, CA, USA) was used for supernatant concentration (1 volume PEG-it™ Virus Precipitation Solution in 4 volumes viral supernatant). The mixture was stored at 4 °C overnight and centrifuged for virus pellets. The pellets were reconstituted with DMEM medium and HEPES buffer. The lentivirus was added to cells for infection at modified concentrations. Polybrene Infection/Transfection Reagent (Sigma-Aldrich, St Louis, MO, USA) was used to improve the infection efficiency. Successfully infected cells were selected by using puromycin (Thermo Fisher Scientific) at different concentrations (HCT116 cells: 5 μg/ml; NCI-H295R cells: 10 μg/ml).

3.19 CRISPR-CAS9 KNOCKOUT (KO) CELL LINES (PAPER II)

CRISPR plasmids were transfected into cells using Lipofectamine TM 3000 Transfection Reagent (Thermo Fisher Scientific) according to the product instructions. Twenty-four – 48 hrs after the transfection, when GFP fluorescence could be observed, GFP positive cells were sorted out and cultured. When the cell population grew to 90% confluence, the cells were digested by using 0.25% Trypsin (Thermo Fisher Scientific) and single cells were sorted into 96-well plates and expanded. Western Blot was applied to validate the KO efficiency.

3.20 RNA SEQUENCING (RNA-SEQ)

Total RNA was extracted form NC, siASF1A, siASF1B, siASF1A+siASF1B groups by using miRNeasy Mini Kit (Qiagen). RNA-seq was carried out on Illumina platform. Data were analyzed through R.

3.21 DATA ANALYSIS FROM ONLINE DATABASES (PAPERS I - III)

In Paper I, ASF1A mRNA expression values were related to prognosis of CRC patient survival and were downloaded from GEO datasets (http://www.prognoscan.org/) (GSE17536 and GSE17537). The data for 32 colorectal adenoma and 32 normal mucosa samples were obtained from GEO (GSE8671) and analyzed via GEO2R. Data for progressive colorectal diseases, including adenoma (17 samples), carcinoma (17 samples) and carcinoma with metastasis (11 samples) were downloaded from GEO (GSE77953). The data of 211 primary CRC samples for gene expression correlation analyses were from GEO (GSE75315).

In Paper II, data of ASF1A mRNA expression in HCC, PCa, GC and BC and their normal tissue counterparts were obtained from TCGA (https://cancergenome.nih.gov) and GTEx (https://gtexportal.org/home/) databases. Data of ASF1A and $p21^{cip1}$ expression from TCGA used above were downloaded via cBioPortal (http://www.cbioportal.org) (January, 2018). ASF1A and $p21^{cip1}$ survival analysis using TCGA data was achieved by GEPIA (http://gepia.cancer-pku.cn).

In Paper III, TCGA data of ACC samples were downloaded via UCSC-Xena (http://xena.ucsc.edu, March, 2020) and cBioPortal (https://www.cbioportal.org, March, 2020).

3.22 STATISTICAL ANALYSES (PAPERS I - III)

Experimental quantitative data in Paper I, II and III were obtained from biological replicates and shown as means ± standard deviation (mean $\pm$ SD) if not otherwise indicated. Student's *t* test (two-tailed) was applied to examine the differences between experiment groups. Disease-free survival (DFS) and overall survival (OS) were visualized by Kaplan–Meier plots. The log-rank test was used to compare survival distributions between groups.

4 RESULTS

4.1 PAPER I: ASF1A IN GIC PROGRESSION

4.1.1 ASF1A overexpression in GIC cell lines/primary tumors

As there were not many reports on the expression of ASF1A in human cancers, especially it was vacant in GIC, we first examined ASF1A expression in several GIC cell lines including GC derived cell lines AGS, BGC-823 and HCG-27 and CRC-derived cell lines HCT116, SW480, HT29 and Caco-2 (Figure 7A). The results revealed that ASF1A had the most abundant expression in the most aggressive or poorly differentiated GC cell line HGC-27 and CRC cell line HCT-116. We then evaluated ASF1A expression in primary GIC tumors and their adjacent non-tumorous (NT) counterparts from 286 GIC patients (106 GC and 180 CRC patients) by performing IHC staining and found that ASF1A had a significant higher expression in GIC tumors compared with the NT tissues (GC vs NT, CC vs NT and rectal cancer (RC) vs NT) (Figure 7B and C). Interestingly, ASF1A expression increased orderly in normal, metaplasia and cancer tissues in gastric sections. These findings collectively indicated an important role of ASF1A in GIC progression and evolution.

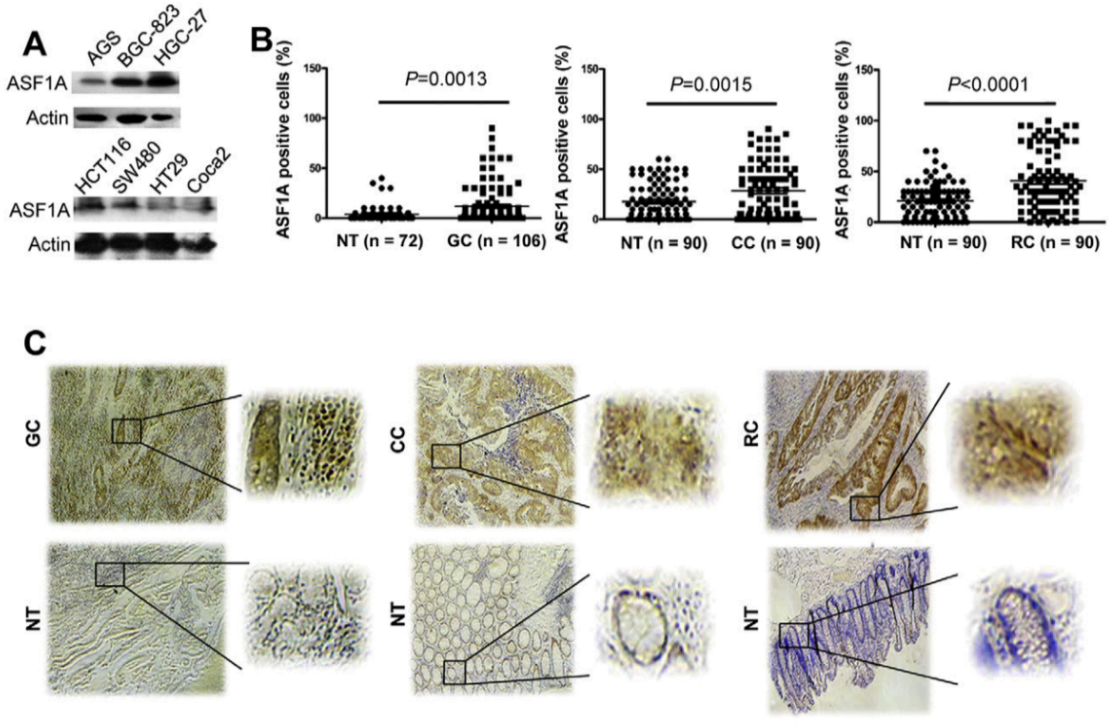

Figure 7. ASF1A overexpression is observed in GIC cell lines/primary tumors and is associated with the evolution of GIC. (A) ASF1A protein expression in GIC cell lines. (B) Quantification of 7C showing the percentage of ASF1A positive cells in tumors and their non-tumorous (NT) counterparts. (C) immunohistochemistry (IHC) staining of ASF1A in primary GIC tumors and their NT counterparts.

4.1.2 The interaction between ASF1A and β-Catenin

To investigate ASF1A's function in GIC cells, we used CRC cell line HT29 which had very low basal ASF1A expression to generate ASF1A stable overexpression sublines (HT29-

ASF1A) with the aid of lentivirus system. HT29-ASF1A cells had higher ASF1A expression on protein level and accumulated histone H3K56 acetylation. Cell cycle analysis showed significant declines in G1 phase and increases in S phase in HT29-ASF1A cells compared with control cells. Colony formation assays exhibited a higher colony formation ability of HT29-ASF1A cells than control cells. ASF1A overexpression was also associated with stemness and migration/invasion ability. We examined the CRC stem cell marker Lgr5 using flow cytometry and found a higher Lgr5 expression in HT29-ASF1A cells than in control cells. An increase in sphere formation ability was observed in ASF1A overexpressing cells, which also indicated the enhanced stemness of HT29-ASF1A cells. Increased migration ability was shown in ASF1A overexpressing HT29 cells by using a Transwell assay. Knockdown of ASF1A using siRNAs in ASF1A high expressing GIC cell lines HGC-27, HCT116 and SW480 led to significantly impaired migration and invasion ability in all the three cell lines. Cellular morphology examination showed less elongated or spindle-like shapes in ASF1A-depleted cells, which was a feature more prevalent in epithelial cells than in mesenchymal cells. These functional studies together revealed the effect of ASF1A in facilitating proliferation, stemness and migration/invasion ability in GIC cells.

Because the Wnt/β-Catenin pathway alteration was an important cause of the GIC pathogenesis, we examined the potential connection between ASF1A and β-Catenin. First, we found a physical interaction between ASF1A and β-Catenin proteins using IP (Figure 8A). We then introduced a TCF/LEF assay to evaluate the impact of the observed interaction on β-Catenin's function. The TCF/LEF assay was designed to measure the transcription activity of β-Catenin. We validated the efficiency of the TCF/LEF assay by transfecting β-Catenin expression plasmids into HCT116 cells and found an elevated reporter activity in these cells. Synchronous transfection of β-Catenin and ASF1A expression plasmids showed enhanced reporter activity in a synergistic manner. Thus, ASF1A boosted the transcriptional activating function of β-Catenin as a consequence of the interaction.

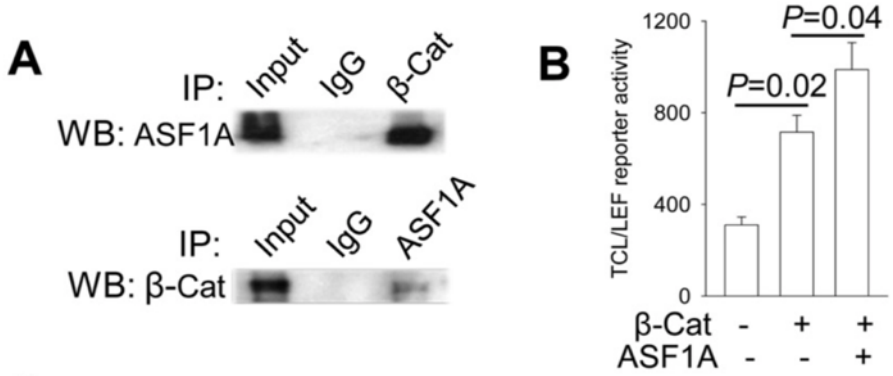

Figure 8. ASF1A physically interacts with β-Catenin and activates the β-Catenin target promoters. (A) Immunoprecipitation (IP) showed a physical interaction between ASF1A and β-Catenin proteins. (B) TCL/LEF reporter activity is regulated by ASF1A. In HCT116 cells, β-Catenin overexpression can increase TCL/LEF reporter activity. Overexpression of both β-Catenin and ASF1A amplifies this effect.

Combined with the functional study, we investigated whether the ASF1A/ β-Catenin interaction could explain the mechanisms behind the effects of ASF1A on proliferation, stemness and migration/invasion. We focused on several important β-Catenin target genes including *LGR5*, *CCND1*, *c-MYC* and *ZEB1*, and determined CCND1, c-MYC and ZEB1 expression at mRNA and protein levels in control and HT29-ASF1A cells. The results showed accumulated expression of the target genes in ASF1A overexpressing cells. Oppositely, depletion of ASF1A with siRNAs in HCT116 cells displayed decreased expression of CCND1, c-MYC and ZEB. By analyzing data of 210 patients in the GEO database, positive correlations could be drawn between ASF1A and CCND1, c-MYC or ZEB1, respectively. To further validate the activity of ASF1A on β-Catenin targeted promoters, ChIP assay was performed in HCT116 cells which had abundant endogenous expression of both ASF1A and β-Catenin. The results showed the enrichment of both ASF1A and β-Catenin on the promoter regions of CCND1, c-MYC and ZEB1. These findings demonstrated the amplifying function of ASF1A on the β-Catenin transcription activity, thereby promoting the malignant phenotypes of GIC.

Because losing the epithelial marker E-Cadherin is a key process for increasing cell mobility and ZEB1 impairs E-Cadherin expression, we hypothesized that the ASF1A/ β-Catenin/ZEB1 axis might promote invasion by inhibiting E-Cadherin expression. To verify this hypothesis, first, we knocked down ASF1A in HCT116 cells and observed accumulation of E-Cadherin and diminished ZEB1 expression at both mRNA level and protein levels. In contrast, overexpression of ASF1A in HT29 cells induced ZEB1 expression and decreased E-Cadherin expression. Knockdown of β-Catenin in ASF1A overexpressing HT29 cells abolished ASF1A's effect on ZEB1 and E-Cadherin. Inhibition of ZEB1 also led to the recovery of E-Cadherin. Since it is well documented that ZEB1 regulates E-Cadherin by binding to the E-boxes in the promoter region of E-Cadherin, we evaluated the E-Cadherin promoter activity in ASF1A-depleted HCT116 cells. We found that E-Cadherin promoter activity was upregulated in ASF1A-inhibited HCT116 cells while mutation on E-boxes could attenuate the effect.

4.1.3 ASF1A effects on GIC in mouse xenograft models

To examine the effects of ASF1A on proliferation in GIC *in vivo*, we injected control HT29/HT29-ASF1A cells, and control HCT116/HCT116-ASF1AshRNA cells subcutaneously into 6 week old nude mice, respectively. Mice were sacrificed after 6 weeks. Tumor volume was recorded during this period weekly. The HT29-ASF1A tumors grew much faster and were bigger than the control ones, while the HCT116-ASF1A sh tumors grow slower and were smaller than their control counterparts. IHC staining showed stronger ASF1A, E-cadherin and PCNA signals in HT29-ASF1A tumor slides than in the control sections, whereas weaker signals were observed in HCT116-ASF1A-shRNA tumor sections than in control ones.

For the *in vivo* metastasis experiments, we injected control HT29 and HT29-ASF1A cells into 6 weeks old NOD-SCID mice via the tail vein. Mice were killed after 7 weeks. Metastatic lung tumors were quantified. The results showed that the control HT29 cells led to a total of 19 metastatic nodules in the lungs while the HT29-ASF1A cells caused a total of 93

metastatic lung nodules. Besides, the tumor nodules in HT29-ASF1A group were generally larger than those in the HT29 control group. IHC staining revealed more intense ASF1A and PCNA signals while reduced E-Cadherin expression in tumors with ASF1A overexpression. Taking together, ASF1A promoted *in vivo* tumor growth and metastasis, which was in accordance with the results obtained from the *in vitro* experiments.

4.1.4 The prognostic value of ASF1A overexpression in CRC patients

The prognostic value of ASF1A in CRC patients was examined by using Kaplan-Meier analysis. A cut-off of 35% based on the proportion of ASF1A positive tumor cells in CRC IHC staining was set to define low (<35%) and high (>35%) ASF1A expressing tumors/patients. The results displayed that higher ASF1A expression predicted shorter OS sometimes in both colon cancer (CC) and rectal cancer (RC) patients. Further validation of ASF1A overexpression in CRC patients and its prognostic potential was carried out by GEO data analyses. The results showed that ASF1A was over-expressed in adenomas compared with normal intestinal mucosa, and in a small cohort of patients (n = 17), ASF1A was observed to have a gradual accumulation in adenomas, CRC and CRC with liver metastasis. Kaplan-Meier analysis further consolidated the association between higher ASF1A expression and shorter patient OS in two CRC patient cohorts (Cohort 1: n = 49; Cohort 2: n = 177). These results confirm the prognostic value of ASF1A in CRC patients.

The work model for this study is shown in Figure 9.

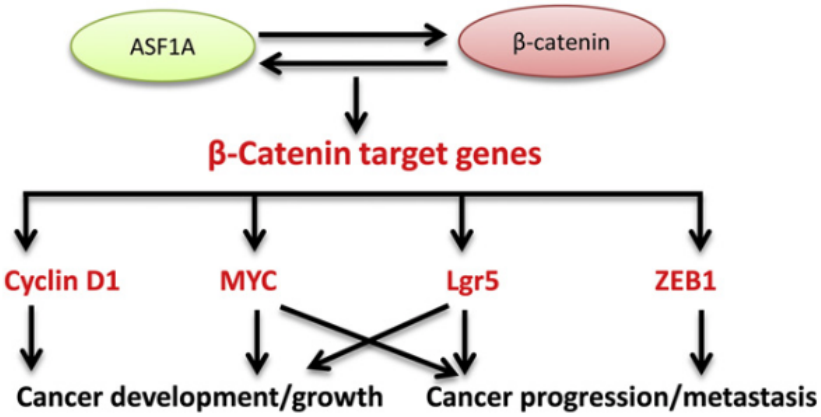

Figure 9. Work model for ASF1A mediated oncogenesis in CRC. ASF1A physically interacts with β-Catenin and they synergistically promote β-Catenin downstream genes *CCND1*, *MYC*, *LGR5* and *ZEB1* expression. Overactivation of these genes leads to GIC development and progression.

4.2 PAPER II: ASF1A INHIBITION-INDUCED SENESCENCE IN WT P53 CARRYING CANCER CELLS

4.2.1 ASF1A overexpression in multiple solid tumors with the prognostic value

We first found that ASF1A was overexpressed in primary HCC tumors compared to their non-tumorous counterparts at both mRNA and protein levels. This finding was supported by the data from TCGA and GTEx databases. Apart from liver cancer, ASF1A expression was also increased in PCa, GC and BC based on TCGA and GTEx data. In addition, higher ASF1A mRNA expression predicted a worse OS and DFS in TCGA HCC patients, which supported ASF1A's potential as a predictor of outcome.

4.2.2 ASF1A knockdown-induced senescence in wt p53 carrying HCC and PCa cells

To investigate the functions of ASF1A, we knocked down ASF1A using siRNAs in the HCC cell line HepG2 and PCa cell line LNCaP. Proliferation was the first parameter variable we assessed. We discovered that the ASF1A-depleted cells had a lower proliferation rate than the control cells. Colony formation assay showed decreased colonies in ASF1A knockdown cells than control ones in both HepG2 and LNCaP cells, indicating a weaker clonogenic ability. Cell cycle analysis revealed an accumulation in G0/G1 phase and a reduction in G2/M phase in both cell lines, which exhibited a cell cycle arrest phenotype (Figure 10A). Morphologically, the ASF1A depleted cells were generally bigger and flatter than the control cells, which was an indicator of cellular senescence. We then used β-Gal staining to validate the senescence phenotype in HepG2 and LNCaP cells. The results showed higher β-Gal-positive (blue) staining in ASF1A-depleted HepG2 and LNCaP cells, which verified our hypothesis that ASF1A knockdown induced cellular senescence (Figure 10B and C). Notably, both HepG2 and LNCaP were wt p53-carrying cell lines. Because cellular senescence is in general triggered via the activation of p53-$p21^{cip1}$ and/or p16 pathways, we questioned whether the ASF1A inhibition-mediated cellular senescence also happened in cells with defective p53. To this end, we introduced p53 mutant HCC cell line Huh.7 and p53 null HCC cell line Hep3B, and knocked down ASF1A in these cell lines. We did not observe any significant cellular senescence phenotype in ASF1A-depleted Huh.7 and Hep3B cells, as seen in HepG2 and LNCaP cells. These findings demonstrated the effect of ASF1A inhibition on cellular senescence in wt p53-carrying cell lines HepG2 and LNCaP.

4.2.3 ASF1A inhibition-induced senescence and the DDR/p53/$p21^{cip1}$ pathway

To further elucidate the mechanism underlying the p53-dependent growth arrest and senescence, we first performed a screen to check the mRNA expression of several cellular senescence-related genes: $p16^{ink4a}$, $p21^{cip1}$, $p27^{kip1}$ and TERT in control and ASF1A knockdown HepG2 and LNCaP cells. The result showed that only $p21^{cip1}$ was upregulated in ASF1A-inhibited cells. It is well documented that $p21^{cip}$ is transcriptionally regulated by p53, which was accordant with the findings above showing that the cellular senescence phenotype occurred in wt p53 cell lines HepG2 and LNCaP rather than p53 defective cell lines Huh.7 and Hep3B. We then knocked down ASF1A using siRNAs in HepG2, LNCaP, AGS and

MCF-7 cell lines, all of which carried wt p53, and examined p53 and $p21^{cip1}$ expression. Western blot and qPCR results revealed both p53 and $p21^{cip1}$ to be upregulated in all the four cell lines upon ASF1 knockdown. We then introduced p53 binding site-containing $p21^{cip1}$ promoter plasmid. We found elevated $p21^{cip1}$ promoter activity in ASF1A knockdown groups compared with control groups in both HepG2 and LNCaP cells. Interestingly, when we knocked down both ASF1A and p53 using siRNAs, the $p21^{cip1}$ upregulation caused by ASF1A inhibition was attenuated, indicating the role of p53 in ASF1A depletion-mediated $p21^{cip1}$ overexpression. To further validate the p53-$p21^{cip1}$ mechanism, we knocked down ASF1A in Huh.7 and Hep3B cells and did not observed any p53 or $p21^{cip1}$ accumulation. Luciferase assay also did not show higher $p21^{cip1}$ promoter activity in ASF1A-inhibited cells. We knocked down ASF1A in wt p53-baring HCT116 cells and observed upregulation of both p53 and $p21^{cip}$. Then we introduced p53 KO HCT116 sublines constructed by using CRISPR-Cas9 system. We found that inhibition of ASF1A in p53 KO HCT116 cells was not able to cause $p21^{cip1}$ increase any longer.

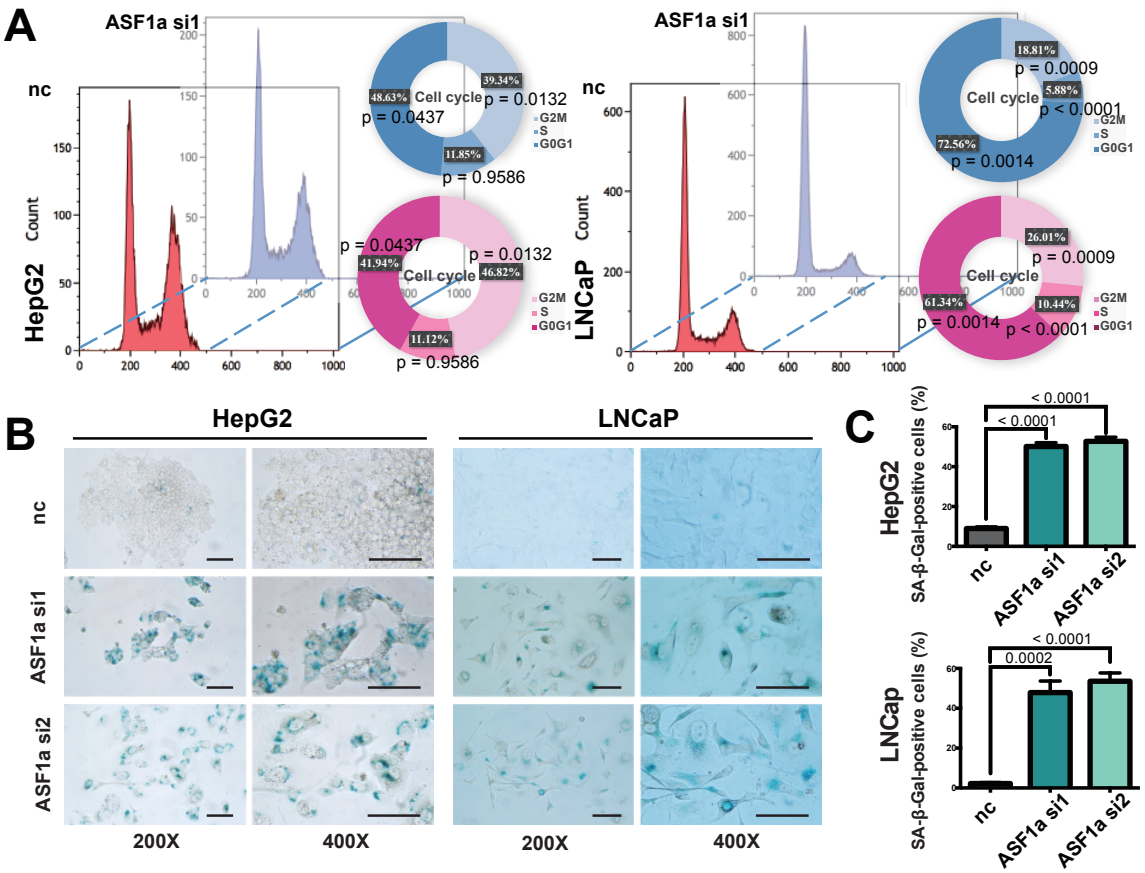

Figure 10. Knockdown of ASF1A induces growth arrest and cellular senescence in wt p53 carrying HCC and PCa cells. (A) Cell cycle analysis reveals that ASF1A knockdown induces a G0/G1 phase arrest and a reduction in G2/M phase in HepG2 and LNCaP cells. (B) β-Gal staining shows that ASF1A inhibition leads to cellular senescence. (C) Quantification of 10B. The percentages of β-Gal staining-positive HepG2 and LNCaP cells in different experimental groups are presented in bar charts.

Because p53 accumulation in general is caused by DDR, we questioned whether it was DDR induced by ASF1A depletion that triggered p53 activation. So we stained two DDR specific markers γ H2AX and 53BP1 using IF staining. Our findings showed DDR foci in ASF1A knockdown HepG2 and LNCaP cells. These results collectively demonstrate the role of DDR/p53/p21^{cip1} pathway in ASF1A inhibition-mediated cellular senescence.

The graphic illustration of this study is shown in Figure 11.

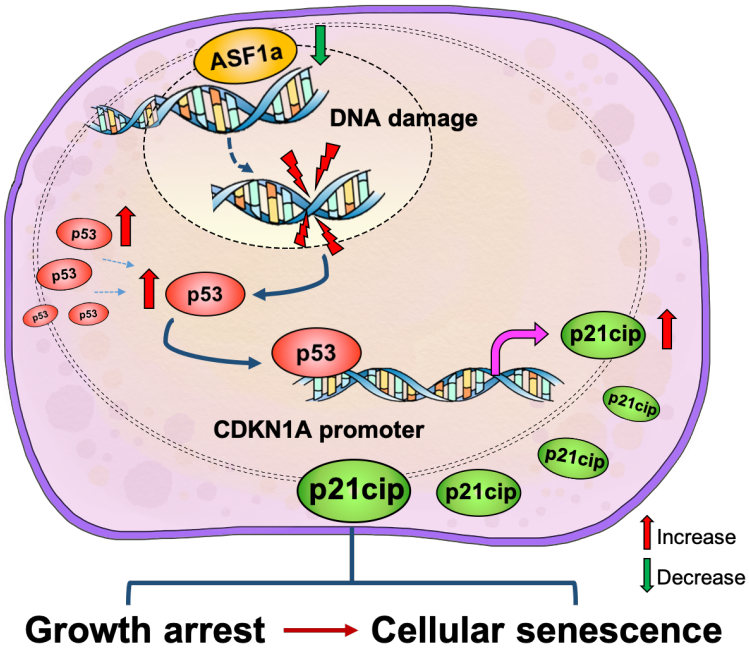

Figure 11. Graphic illustration of the tentative mechanism of the ASF1A inhibition induced cellular senescence. ASF1A inhibition causes growth arrest and cellular senescence in wt p53 carrying cancer cells through the DDR/p53/p21^{cip1} pathway.

4.3 PAPER III: THE DIAGNOSTIC AND PROGNOSTIC VALUES OF ASF1B AND ITS ASSOCIATION WITH METASTASIS IN ACC

4.3.1 A higher copy number of the *ASF1B* gene and ASF1B mRNA overexpression in the TCGA cohort of ACC patients

There are a lot of chromosomal aberrations presented in ACC. The TCGA cohort of ACC analysis revealed that chromosome 19p13.12, where the *ASF1B* gene is resident, had amplification. Analysis of ASF1B copy number variation (CNV) showed gain/amplification of *ASF1B* gene in 63.16% patients of the TCGA ACC cohort. ASF1B was overexpressed in TCGA ACC tumors on the mRNA level. By comparing ASF1B mRNA expression in *ASF1B* gene diploid tumors and *ASF1B* gene gain/amplification tumors, we found that ASF1B had higher expression in *ASF1B* gene gain/amplification tumors than did the diploid ones, which indicated that increased *ASF1B* gene copy numbers caused higher ASF1B expression. In summary, more than 60% of ACC tumors had ASF1B gains/amplification and this CNV resulted in increased ASF1B expression.

4.3.2 The association between ASF1B alterations and Weiss scoring system criteria

Weiss scoring system is the universally applied clinical diagnostic standard for ACC in the clinic. It examines 9 criteria which pinpoint the architectural, nuclear and invasion features of ACC tumors. By analyzing the TCGA ACC tumors, we found that ASF1B was associated with 7 criteria of the Weiss scoring system. Higher ASF1B expression was associated with necrosis, higher mitoses count, higher mitotic rate, higher nuclear grade, atypical mitoses and higher invasion ability including capsule invasion, sinusoid invasion and venous invasion. We could also see that tumors with a more abundant ASF1B expression had a higher Weiss score, which indicated a more malignant status.

4.3.3 The correlation between ASF1B expression and ACC tumor stage & patient survival

Tumor stage is an important prognostic indicator. Patients with stage III/IV ACC tumors frequently have metastatic disease and have a poor outcome. We found that ASF1B had higher expression in stage III/IV TCGA ACC tumors than in stage I/II tumors (Figure 12 A and B). Patients were divided into high and low ASF1B expression groups based on a cut off 0.5. Survival analysis showed that ACC patients with higher ASF1B expression had a poorer OS and DFS, confirming the prognostic value of ASF1B in ACC (Figure 12C and D).

4.3.4 The effect of ASF1B on ACC cell migration and invasion via FOXM1

Because ASF1B was highly expressed in stage III/IV tumors which carrying a higher metastatic potential, we evaluated the effect of ASF1B on migration and invasion in the ACC cell line NCI-H295R. ASF1B knockdown and overexpression stable sublines were made with the aid of a lentivirus system. Results from the migration and invasion assay showed that ASF1B inhibition impaired both the migration and invasion capacity while overexpression of ASF1B enhanced both the migration and invasion ability of NCI-H295R cells.

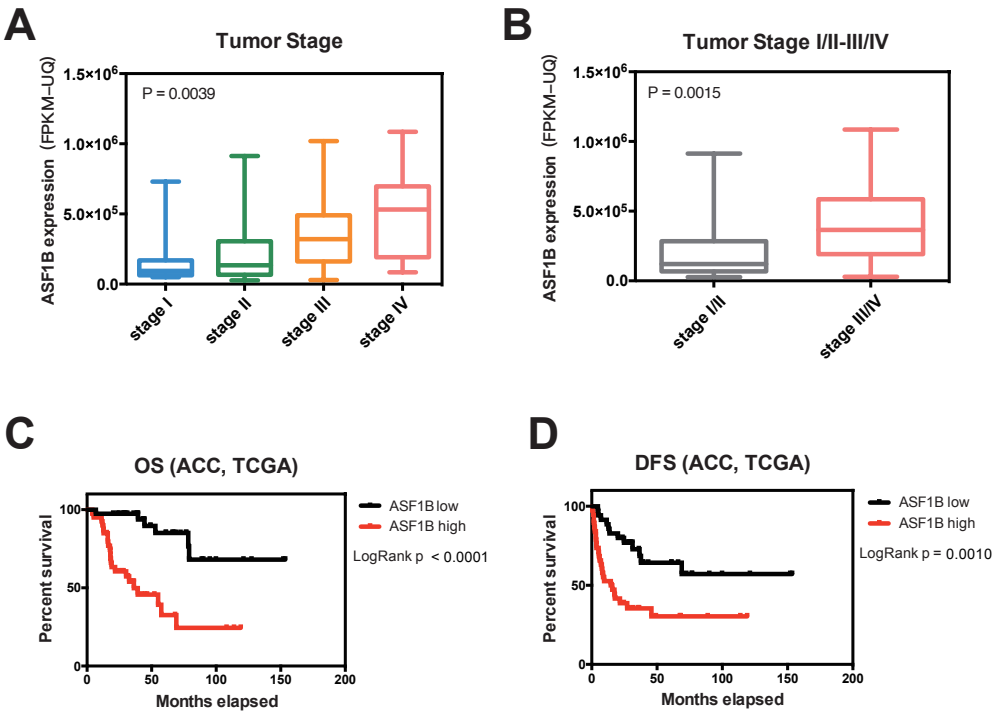

Figure 12. ASF1B expression correlates with ACC tumor stage and patient survival. (A) ASF1B mRNA expression in stage I/II/III/IV TCGA ACC tumors. (B) ASF1B has a higher expression in stage III and IV ACC tumors than in stage I and II ACC tumors. (C) Patients with higher ASF1B expression have a shorter overall survival (OS). (D) Patients with higher ASF1B expression have a shorter disease-free survival (DFS).

We then worked on the mechanisms behind the migration/invasion-promoting effect of ASF1B. By Analyzing the TCGA ACC transcriptome data, we found that FOXM1, which played an important role in regulating migration and invasion, was among the top genes that correlated with ASF1B expression. Western blot results showed diminished FOXM1 expression in ASF1B knockdown NCI-H295R sublines as compared to control cells. Oppositely, ASF1B overexpressing NCI-H295R cells led to an elevated FOXM1 expression. These findings suggest that ASF1B boosted the migration/invasion ability of ACC cells through activating FOXM1 expression.

5 DISCUSSION

5.1 ASF1 IN CANCER: THE CANONICAL ROLE AND BEYOND

Both ASF1A and ASF1B are highly expressed in a variety of cancers based on the TCGA dataset analysis, suggesting their role in carcinogenesis. Both of the two paralogs are deeply involved in regulating rapidly dividing cancer cells and the actively transcribed cancer genome. As introduced above, ASF1A and ASF1B share the conserved duty of being a histone H3-H4 chaperone for histone transport[125]. Meanwhile, they also have divergent and novel functions in cancer pathogenesis and other biological processes. Due to the priority that ASF1A and ASF1B physically interact with histone H3/H4, they are ones of the most ideal candidates to deliver epigenetic information during DNA and chromatin metabolism. For example, Gao and colleagues reported that the ASF1A-histone interaction is required for transcription regulation by modifying histone H3K56 instead of the role of ASF1A in nucleosome assembly[176]. Thus, exploring the novel role of ASF1 and other histone chaperones in DNA and chromatin dynamics will provide new momentum to cancer epigenetic research.

ASF1A or ASF1B deletion results in a distinct cell fate. In a study published in 2010, Corpet and colleagues knocked down ASF1A, ASF1B and ASF1A+ASF1B in human U-2-OS cells and performed transcriptome analysis[125]. The results from GO analysis revealed distinct transcriptional signatures in the three experimental groups. The most affected cellular process in the ASF1A inhibition group was Cytokinesis, while ASF1B deletion highly influenced the DNA replication and G1/S transition of mitotic cells. There is an overlap between the genes altered by ASF1A or ASF1B knockdown. They also discovered that ASF1B knockdown induced abnormal cell nucleus, macronucleus and DNA bridges which were typical phenotypes of genome instability in the breast cancer cell line Hs578T. However, these phenomena were not found in ASF1A-depleted Hs578T cells. Besides, they noticed a potential compensation effect between ASF1A and ASF1B by observing a slight upregulation of ASF1A in ASF1B-inhibited U-2-OS cells. But they did not find any ASF1B increase in ASF1A knockdown cells. We also performed similar experiments in the PCa cell line LNCaP. We knocked down ASF1A, ASF1B and ASF1A+ASF1B respectively in LNCaP by using siRNAs and did RNA-seq. Our pathway analysis suggested that apart from some common functions ASF1A and ASF1B shared, they behaved differently in a variety of pathways, which was similar to the GO analysis in Armelle's paper (Figure 12A). In a still unpublished study, we made ASF1B CRISPR-Cas9 KO sublines in the lung cancer cell lines A549 and NCI-H1299. Interestingly, we observed the similar nuclear abnormalities in ASF1B KO cells as seen in the study described above, which confirmed that ASF1B deletion heavily affected genome stability. However, results from our ASF1B CRISPR-Cas9 KO experiments in lung cancer cell lines A549 and NCI-H1299 showed that ASF1A expression did not increase significantly in ASF1B KO cells (Figure 12B). Furthermore, in the ASF1B overexpressing lung cancer cell lines A549, NCI-H1299, NCI-H1975 and ACC cell line NCI-H295R, only little changes in ASF1A protein expression were detected either (Figure 12B). In the ASF1A or ASF1B knockdown LNCaP cells, an around 20% ASF1A mRNA increase was observed in ASF1B-inhibited cells while no significant ASF1B fluctuation was seen in

ASF1A-depleted cells. These observations suggested little or very low compensatory effect between the two paralogs under these different cellular contexts.

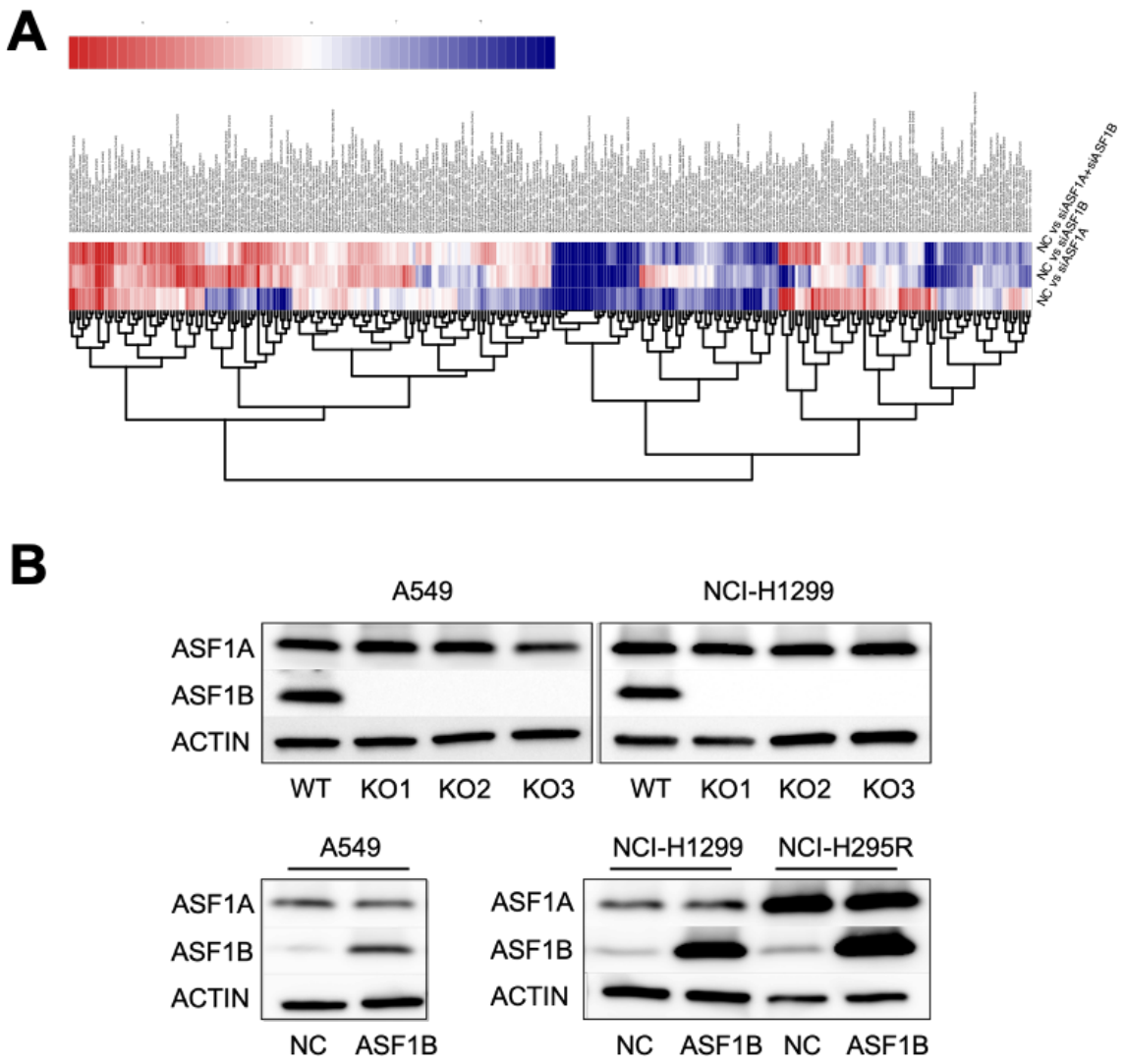

Figure 13. ASF1B and ASF1B have distinct functions other than their shared duty. (A) Pathway analysis of ASF1A and ASF1B-depleted LNCaP cells. (B) ASF1A and ASF1B protein expression in wild type and ASF1B knockout A549 and NCI-H1299 cells (the upper panel). ASF1A and ASF1B protein expression in control and ASF1B overexpressing A549, NCI-H1299 and NCI-H295R cells (the lower panel).

In summary, based on our findings and those from us and others, we conclude that single inhibition of the ASF1A or ASF1B paralog in cancer cells may cause distinct phenotypes and transcriptional signatures leading to different cell fate which mainly include proliferation inhibition, cell cycle arrest, apoptosis or senescence under multiple and complex cellular environments. ASF1A and ASF1B should be redundant in their overlapped duty in cancer cells so that there is little or weak compensation observed between them. Meanwhile, they might be quite independent from each other in their distinct roles. Further studies elucidating the different functions of ASF1A and ASF1B in cancer development and the underlying mechanisms will be necessary for precision oncology application.

The divergence in ASF1A and ASF1B functions can be explained on a structural basis. As described in the introduction, ASF1A and ASF1B follow distinct evolutionary paths since some time point[107]. The structures of the two paralogs vary mainly in the C-terminus while they have a conserved core region and N-terminus. The structural difference may account for the functional divergence of ASF1A and ASF1B. Both ASF1A and ASF1B interact with CAF-1, though ASF1B has a much higher affinity than ASF1A[107]. However, only ASF1A binds to HIRA, which is involved in replication-independent nucleosome assembly and disassembly. This preference for the interaction with difference downstream chaperone partners is consistent with the major role of ASF1A in transcription regulation or DNA repair whereas ASF1B is involved in proliferation. In Paper I and Paper II, we reported that ASF1A acted as a transcriptional co-factor promoting β-catenin downstream gene expression and induce cellular senescence by triggering DDR/p53/$p21^{cip}$ pathway. In a study published in 2014, Gonzalez-Muñoz and colleagues reported that ASF1A activated the expression of core pluripotency genes by acetylating histone H3K56[122]. Zhang and colleagues found that that ASF1 facilitated H3K56 acetylation by stabilizing the very C-terminal β strand of histone H4 and unwinding of the K56 located histone H3 α_N, further unveiling the structural fundament of ASF1 function[177]. These findings may provide some clues on why and how ASF1A and ASF1B exert their specific functions under specific contexts and inspire future research on the more detailed mechanisms of how ASF1 and other histone chaperones behave in chromatin dynamics during replication, transcription and DNA repair.

5.2 POSSIBLE MECHANISMS OF HOW ASF1 PROTEINS PARTICIPATE IN GENE EXPRESSION REGULATION

Based on the limited publications on ASF1 proteins in transcription regulation and our research, we conclude that ASF1 proteins function in regulating gene expression in the following ways:

ASF1 proteins interact with transcriptional factors/co-factors. In Paper I, we showed that ASF1A physically interacted with transcriptional co-factor β-Catenin and promoted the downstream gene expression in GIC. Gao and colleagues found that ASF1A was recruited to some bivalent promoters in part by associating with transcription factors such as Nanog and Oct4 and trigger lineage specific gene expression in mouse embryonic stem cell (ESC) differentiation[176]. Another paper reported that ASF1B interacted with transcriptional coactivator HCF-1 and facilitated the herpes simplex virus (HSV) DNA replication and early gene expression[178]. Thus, ASF1 proteins may have the ability to selectively combine with specific transcriptional co-factors or transcription factors to increase the promoter activity and induce downstream gene expression.

ASF1 proteins interact with histone H3/H4 and recruit enzymes for histone modification. ASF1A has been shown to induce core pluripotency genes by facilitating histone H3K56 acetylation[122]. In Paper I, we also detected decreased H3K56 expression in ASF1A inhibition GIC cells which showed impaired ability of proliferation, stemness and invasion. Hereby, ASF1 may serve as a linker protein to connect the transcription factors/cofactors, histone H3/H4 and histone modification enzymes, facilitating these factors to form a complex to finally mark the histones at the certain promoter region with specific histone modifications such as H3K56 acetylation. It was reported that histone H3K56

acetylation increased chromatin accessibility by driving chromatin toward the disassembled state during transcription activation[121]. Besides, ASF1 also promotes the histone disassembly process in these regions. Thus, ASF1 proteins may affect gene expression through promoting the communication between DNA elements and the chromatin environment in the regulatory regions to create a more opened chromatin state for transcription machineries. In addition, different from the downstream histone chaperone CAF-1 and HIRA that also interact with the replication and transcription machineries, ASF1 proteins may have more freedom and less complexity in the regulating processes.

There are still many questions to be answered. Which types of transfection factors/co-factors do ASF1 proteins prefer to bind? Which kinds of genes are regulated by ASF1 proteins? Is there any "reader" function of ASF1 proteins? Are there any other histone metabolisms in which ASF1 proteins are involved? How about the roles of other histone chaperones? Some studies have provided a few of clues. For instance, Clément and colleagues observed that H3K36me3 and H3K9me3 histone marks decreased in volume or density in ASF1-depleted cells by using super-resolution microscopy[179]. More research is required to further elucidate these unresolved questions.

5.3 ASF1 IN CELLULAR SENESCENCE

The role of ASF1 in cellular senescence has been studied and discussed in many publications, most of which mentioned the SAHF. SAHF refers to the phenomenon that the euchromatin regions containing proliferation-promoting genes are compacted into transcriptionally silent heterochromatin under cellular senescence state[135, 180-182]. Both ASF1A and HIRA are required to form SAHF[135, 136]. In Paper II, we discovered that ASF1A inhibition induced cellular senescence and did not find SAHF formation in ASF1A knockdown cells, which was in contrast with the previous findings of the presence of SAHF triggered by senescence. It is reasonable that cellular senescence and SAHF formation are not always coupled. First, whether SAHF can form is dependent on cell types[138]. For instance, SAHF is present in the senescent human embryonic fibroblast cell lines IMR90 and WI38 but is not observed in the primary human foreskin fibroblast cell line BJ[180]. Besides, SAHF formation favors to be fueled by the $p16^{ink4}$ pathway which is usually activated by OIS rather than the DDR/p53/$p21^{cip1}$ pathway induced by replicative cellular senescence[137]. In addition, TERT expression and telomerase activation is important for cancer cells to escape cellular senescence and become immortalized[183]. ASF1A inhibition was shown to decrease TERT expression, which impairs cancer cells' ability to overcome senescence. In Paper II, however, we did not observe significant changes in TERT expression and telomere length in ASF1A-inhibited HepG2 and LNCaP cells. Thus, the cellular senescence induced by ASF1A inhibition should not be caused by reduced TERT/telomerase or shortened telomere length.

Cellular senescence is considered more important than cell death for tumor repression under certain circumstances. For example, the susceptibility of cancer in mice is heavily affected by senescence regulatory network rather than deficiency in apoptosis[134]. Thereby, targeting cellular senescence associated genes may be an efficient strategy for cancer treatment. Further evaluation of the ASF1A-depletion induced senescence may provide a fundamental basis for clinical intervention design and novel drug discovery.

5.4 ASF1 IN DNA DAMAGE AND GENOME INSTABILITY

In Paper II, we found that ASF1A inhibition triggered DDR and activated sustained p53/p21^{cip1} pathway, finally inducing cellular senescence. ASF1A's role in DNA repair has been well documented: ASF1A-mediated acetylation of histone H3K56 is necessary for DNA repair by participating in nucleosome reassembly in DNA damage repair process[113]. ASF1 promotes checkpoint recovery after DDR[114]. The involvement of ASF1A and ATM is important for UV-induced cell-cycle checkpoint recovery[115]. To be noted, ASF1A directly takes part in DNA double-strand break repair, which is a non-canonical role of ASF1A as a histone chaperone[116]. It is also reported that ASF1A is required for 53BP1 accumulation at the DNA damage region and in DNA damage situation, less 53BP1 foci are observed in ASF1A-inhibited U2OS cells compared with the control cells[116]. Oppositely, in Paper II, more 53BP1 foci were detected in ASF1A knockdown HepG2 and LNCaP cells than in control cells. This result may be accounted by the different ASF1A-associated DNA repair mechanisms under different contexts such as different cell lines with distinct ASF1A expression or genetic backgrounds. Collectively, based on the documented role of ASF1A in DNA repair, it is conceivable that ASF1A is a necessary factor involved in a range of cases of DNA repair processes. ASF1A inhibition may result in an impaired DNA damage repair outcome and lead to a sustained unresolved DNA damage state, which persistently activates p53/p21^{cip1} pathway and eventually arrests cell cycle and drives cell fate to cellular senescence.

DNA damage is one form of genome instability, an important hallmark of cancer. Interestingly, in addition to the role of ASF1A in DNA repair, ASF1B may also contribute to protection of genome stability. As described above, ASF1B depletion caused nuclear abnormalities in several cancer cell lines[125]. There are also clues from our studies that ASF1B may be associated with chromosomal instability and aneuploidy. Besides, the expression pattern of lamin A, a nuclear periphery marker, was aberrant in ASF1A-inhibited cells where the abnormal nuclei were observed[125]. These findings collectively point out ASF1's role in genome stability which should be an indispensable direction for ASF1 research.

5.5 ASF1 AND CANCER METASTASIS

Cancer metastasis is the movement and colonization of tumor cells from their original sites to distant organs, becoming the major cause of cancer-related deaths[184]. The outcome of patients with or without metastasis differs substantially. Metastasis is considered a multi-step processes. During cancer development, tumor cells gain mutations and aberrant epigenetic features gradually and finally achieve the ability to invade and migrate to other tissues or organs. There are basically three types of metastasis genes based on expression signatures[185]: 1) metastasis initiation genes for the escape of cancer cells from the primary tumor such as genes favoring EMT. 2) metastasis progression genes for the survival of cancer cells in circulation system like EREG, MMP1 and LOX. 3) metastasis virulence genes for the final colonization in new organs including PTHRP, IL-11, IL-6 and TNF α)[186, 187]. Prevention and treatment in the earlier phase of carcinogenesis are important for the outcome.

EMT is a very conserved biological process in evolution[188]. It also occurs in cancer development. EMT facilitates the transformation of cancer cells' features from epithelial to

mesenchymal. Thus, cancer cells gain the ability for long-term migration and obtain some new features such as movement and stemness. Several major pathways are involved in EMT[188, 189]: Wnt/β-catenin, Notch, tumor growth factor (TGF), fibroblast growth factor (FGF) and epidermal growth factor receptor (EGFR) pathways. In GIC, the Wnt/β-catenin pathway plays an important role in malignant transformation. So in Paper I, we explored the interaction between ASF1A and Wnt/β-catenin in GIC. ASF1 proteins are likely to be highly involved in the metastasis process. In Paper III, we found that ASF1B expression was highly associated with ACC invasion and metastasis, which was a good biomarker for diagnosis since the survival of ACC patients was significantly longer in metastasis-free cases. Further studies are required to better elucidate the mechanisms of ASF1-mediated tumor metastasis.

5.6 ASF1 AND EPIGENETIC THERAPY

Cancer progression is a result of the interplay between genome and epigenome. The normal cells overcome genetic and epigenetic barriers and evolve to cancer cells[8]. In some even worse situations, cancer cells exposed to chemotherapeutic agents obtain new aggressive features and become resistant to further treatment which accounts for the major cause of cancer relapse[8]. A part of these newly-gained alterations during the rapid resistance period are epigenetic alterations. Different from the genetic alterations such as gene mutations which are difficult to change, all epigenetic modifications discovered by now are reversible[34]. Thus, targeting the epigenetic alterations and integrating epigenetic therapies into the traditional therapies should provide a new angle for cancer treatment. In addition, epigenomic targets are usually enzymes or small covalent modifications which are more ideal candidates for drug design than genomic targets[8].

Research on epigenetic drugs has made a lot of progress by now, including drugs targeting DNA methylation and histone modifications, some of which have been approved by the FDA. Examples are such as the DNA methylation inhibitors azacitidine, decitabine, and histone deacetylation inhibitors belinostat and panobinostat[8]. Notably, the bromodomain and extra terminal domain (BET) inhibitor JQ1 has been shown to be very efficient in specifically inducing growth inhibition and/or apoptosis in cancer cells[190]. JQ1 has also been proved to have the ability to inhibit histone H3K56 acetylation[191]. As mentioned above, ASF1A participates in and promotes H3K56 acetylation[122]. It will be interesting to investigate whether the inhibitory effect of H3K56 acetylation by JQ1 is mediated through interaction with ASF1A.

6 SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, the oncogenic role of the histone chaperones ASF1A and ASF1B was studied. Both ASF1A and ASF1B are overexpressed in the vast majority of human solid tumors. In GIC, ASF1A stimulates proliferation, stemness and migration/invasion of tumor cells, leading to poor patient outcomes. Mechanistically, ASF1A interacts with β-catenin and promotes the transcription of β-catenin target genes including c-MYC, cyclin D1, ZEB1 and LGR5. When ASF1A expression is inhibited, the DNA damage response is induced through which wt p53-bearing cancer cells undergo cellular senescence. Senescence similarly occurs in HCC, breast and PCa-derived cells with a wt p53 upon ASF1A depletion. For ACC, the *ASF1B* gene is amplified in two-thirds of tumors, which contributes to ASF1B overexpression and poor patient outcomes. ASF1B overexpression is highly correlated with the ACC diagnostic criteria of the Weiss scoring system. Our results further show that ASF1B up-regulates expression of the oncogene FoxM1, thereby promoting migration and invasion of ACC cells. Collectively, the results presented in this thesis reveal a key role of ASF1A and ASF1B in cancer cell proliferation, stemness and metastasis, suggesting that they may be novel epigenetic targets for cancer therapy. These findings, together with the potential values of ASF1A and ASF1B in cancer diagnostics and prognostication, should be of both biological and clinical importance. Further translational investigations are expected to pave the path towards the application of ASF1A and ASF1B in precision oncology.

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The only way to deal with an unfree world is to become so absolutely free that your very existence is an act of rebellion.

—— Albert Camus <The Rebel>