MOLECULAR MECHANISMS OF ACQUIRED GEMCITABINE RESISTANCE IN PANCREATIC CANCER

Li Qin

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Pharmacology and Toxicology,
Indiana University

November 2014

Accepted by the Graduate Faculty, of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____
Jian-Ting Zhang, Ph.D., Chair

_____
Nickolay Brustovetsky, Ph.D.

Doctoral Committee

_____
Karen E. Pollok, Ph.D.

September 23, 2014

_____
Ahmad R. Safa, Ph.D.

_____
William J. Sullivan, Ph.D.

_____
Jingwu Xie, Ph.D.

ACKNOWLEDGEMENTS

My love for science was given to me by my sister, my parents and grandparents and it is to them that I owe my deepest debt of gratitude. They gave me encouragement of endless efforts for pursuing my goals, and the determination and confidence to reach them. Their many years of support have been a tremendous source of encouragement.

I would also like to express my deepest appreciation to the many fine individuals who make up this department, especially the members of my committee, Nickolay Brustovetsky, Karen E. Pollok, Ahmad R. Safa, William J. Sullivan, and Jingwu Xie. Together they have guided me through this precious journey, and helped me more than they can know. A very special thanks goes to Zi-zheng Dong and Jing Qi who have a way of making things go right and are always there patiently instructing me and guiding me through my projects, and my research would not be so smooth without their help. Finally, my gratitude also goes to the many fine scientists, present and passed, who have made the Zhang lab such a great place to be.

For my advisor I cannot begin to express the depth of my appreciation. JT Zhang has been more than my mentor; he has been my benefactor and patron saint. Whatever contribution I have made or will make to science I truly owe to his patient instruction and counsel. Last but not the least, thanks to the entire faculty, graduate students, and staff in Department of Pharmacology and Toxicology for their help and support throughout these years. I truly appreciate everyone encountered in my life in United States to make my adventure here colorful and unregretful.

Li Qin

MOLECULAR MECHANISMS OF ACQUIRED GEMCITABINE RESISTANCE IN PANCREATIC CANCER

Most pancreatic cancer patients receiving gemcitabine chemotherapy eventually develop resistance to gemcitabine. To improve survival and prognosis of pancreatic cancer patients, better understanding the mechanisms of gemcitabine resistance and discovery of new therapeutic targets are required. In this study, I investigated the molecular mechanisms of acquired gemcitabine resistance using a stepwise gemcitabine-selected pancreatic cancer cell line in comparison to the parental cell line. I found that 14-3-3σ is up-regulated in the drug resistant cell line due to demethylation in its first exon, and the up-regulation of 14-3-3σ gene expression, in turn, contributes to gemcitabine resistance. Intriguingly, I found that demethylation of the 14-3-3σ gene in gemcitabine resistant cells is reversibly regulated by DNMT1 and UHRF1. Furthermore, I found that 14-3-3σ over-expression causes gemcitabine resistance by inhibiting gemcitabine-induced apoptosis and caspase-8 activation possibly via binding to YAP1. The finding of demethylation of the 14-3-3σ gene in gemcitabine resistant cells led to a hypothesis that other genes may also be changed epigenetically following gemcitabine selection. By RRBS (Reduced Representation Bisulfite Sequencing) analysis, 845 genes were found to have altered methylation. One of these genes, PDGFD, was further

investigated and found to have reversible demethylation at its promoter region in the drug resistant cells and contribute to gemcitabine resistance possibly via autocrine activation of the STAT3 signaling pathway. Together, these findings not only provide evidence that 14-3-3σ and PDGFD over-expression contribute to acquired gemcitabine resistance and that reversible epigenetic changes may play an important role in acquired gemcitabine resistance, but also demonstrate that the molecular mechanisms of acquired gemcitabine resistance in pancreatic cancer cells are complex and multifaceted.

Jian-Ting Zhang, Ph.D., Chair

Table of Contents

List of Tables x

List of Figures xi

List of Abbreviations xiv

Introduction 1

A. Pancreatic ductal adenocarcinoma (PDAC) 1

B. Gemcitabine in pancreatic cancer treatment 2

C. Gemcitabine metabolism and known resistance mechanisms 3

D. 14-3-3 sigma and 14-3-3 family 8

E. Association of 14-3-3σ expression with drug resistance 10

F. YAP1, a potential binding partner of 14-3-3σ 13

G. 14-3-3σ gene methylation in cancers 15

H. Uhrf1/DNMT1 complex, an emerging regulator of gene methylation 18

I. Platelet-derived growth factor (PDGF) family 22

J. PDGFD over-expression in human cancers 24

K. Specific aims of the present work 25

Materials and Methods 29

A. Materials 29

B. Cell lines, cell cultures, and transfections 30

C. Cell lysate preparation, TCA protein precipitation and Western blot 31

D. Membrane preparation 32

E. Survival and apoptosis assay..... 33

F. Quantitative real-time RT-PCR..... 34

G. Immunofluorescence and confocal microscope imaging..... 35

H. Immunoprecipitation assay..... 36

I. Chromatin-immunoprecipitation (ChIP) Assay..... 36

J. Genomic DNA isolation, bisulfite modification, methylation-specific PCR and sequencing..... 37

K. Reduced representation bisulfite sequencing (RRBS) and data analysis..... 38

Results..... 40

Section I: Detailed mechanism of reversible epigenetic regulation of 14-3-3σ during gemcitabine selection

A. A gemcitabine-selected pancreatic cancer cell line is cross-resistant to Ara-C but not to other anticancer drugs..... 40

B. Ribonucleotide reductase and 14-3-3σ are up-regulated in G3K cells..... 43

C. 14-3-3σ over-expression contributes to acquired gemcitabine resistance.... 46

D. Differential methylation of 14-3-3σ gene in MiaPaca-2 and G3K cells..... 50

E. Demethylation of the 14-3-3σ gene is reversible..... 54

F. Uhrf1 and DNMT1 play important roles in regulating 14-3-3σ expression.... 57

G. Uhrf1 binds and helps recruit DNMT1 to methylated region of 14-3-3σ gene62

H. Gemcitabine treatment does not affect Uhrf1 and DNMT1 expression..... 65

Section II: Identification of PDGFD as a potential contributor to acquired gemcitabine resistance by Reduced Representation Bisulfite Sequencing (RRBS)

A. Identification of reversibly-methylated genes using Reduced Representation Bisulfite Sequencing (RRBS) 68

B. Reversible up-regulation of PDGFD 76

C. Validation of reversible methylation change of PDGFD gene 79

D. PDGFD up-regulation plays an important role in gemcitabine resistance 82

E. PDGFD over-expression contributes to gemcitabine resistance possibly by regulating STAT3 signaling pathway 86

F. Stat3 potentially contributes to gemcitabine resistance 89

Section III: Molecular mechanism of 14-3-3σ-mediated gemcitabine resistance in pancreatic cancer

A. YAP1 over-expression in G3K cells and its contribution to gemcitabine resistance 92

B. Gemcitabine resistance requires both 14-3-3σ and YAP1 98

C. 14-3-3σ and YAP1 form a complex 103

D. Both 14-3-3σ and YAP1 protect against gemcitabine-induced caspase-8 activation and apoptosis 105

Discussion 112

Summary and Conclusion 124

Future Plans 127

Appendices 132

Appendix 1. List of 230 genes the methylations of which are increased in G3K cells compared to MiaPaca-2 cells 132

Appendix 2. List of 615 genes the methylations of which are decreased in G3K cells compared to MiaPaca-2 cells 134

Appendix 3. List of 140 genes of which the methylations are increased in G3KRev cells compared to G3K cells 137

Appendix 4. List of 142 genes of which the methylations are decreased in G3KRev cells compared to G3K cells 138

Appendix 5. List of 309 genes of which the methylations are increased in G3KRev cells comparing with MiaPaca2 cells 139

Appendix 6. List of 645 genes of which the methylations are decreased in G3KRev cells comparing with MiaPaca2 cells 141

References 145

Curriculum Vitae

LIST OF TABLES

Table 1. Frequency of 14-3-3σ gene methylation in different types of cancers 17

Table 2. List of reversibly methylated genes 72

Table 3. Identification and characterization of candidate genes 75

LIST OF FIGURES

Figure 1. Gemcitabine metabolic schema and proposed pharmacological mechanisms of gemcitabine and its metabolites 6

Figure 2. A successive DNA transfer model for maintenance DNA methylation by Uhrf1 and DNMT1 21

Figure 3. PDGF proteins and PDGF-PDGFR interactions 23

Figure 4. Drug response profiles of MiaPaca-2 and its derivative G3K cells 42

Figure 5. Ribonucleotide reductase and 14-3-3σ are up-regulated in G3K cells 45

Figure 6. 14-3-3σ up-regulation contributes to gemcitabine and Ara-C resistance 48

Figure 7. Methylation status of 14-3-3σ gene in MiaPaca-2 and G3K cells 52

Figure 8. 14-3-3σ up-regulation in Adriamycin-selected MCF7/AdVp3000 cells is not due to gene demethylation 56

Figure 9. Role of Uhrf1 and DNMT1 in 14-3-3σ expression 60

Figure 10. Binding of Uhrf1 and DNMT1 to the CpG islands of the 14-3-3σ gene 64

Figure 11. Effect of gemcitabine treatment on Uhrf1 and DNMT1 expression 66

Figure 12. Schematic model of epigenetic regulation of 14-3-3σ gene following gemcitabine selection and drug removal 67

Figure 13. RRBS sequencing results comparison and characterization of genomic coverage 69

Figure 14. Flowchart of DMR filteration of genes 71

Figure 15. Identification of transcriptional changes in candidate genes 74

Figure 16. Identification of TRPC3 and PDGFD expression in MiaPaca-2 and G3K cells .. 77

Figure 17. Reversible transcription and expression of PDGFD 78

Figure 18. Reversible methylation change of PDGFD gene 80

Figure 19. PDGFD over-expression contributes to gemcitabine resistance in both G3K and Aspc-1 cells..... 84

Figure 20. PDGFD over-expression leads to increased gemcitabine resistance in MiaPaca-2 cells 85

Figure 21. PDGFD actively regulates STAT3 signaling pathway..... 88

Figure 22. Stat3 over-expression promotes gemcitabine resistance in MiaPaca-2 cells . 90

Figure 23. Schematic model of PDGFD-mediated gemcitabine resistance during drug selection..... 91

Figure 24. YAP1 is over-expressed in resistant G3K cells 94

Figure 25. Regulation of YAP1 expression by 14-3-3σ protein level 95

Figure 26. YAP1 over-expression contributes to gemcitabine resistance, but requires the presence of 14-3-3σ..... 96

Figure 27. Gemcitabine resistance requires both 14-3-3σ and YAP1..... 100

Figure 28. Decreased expression of both 14-3-3σ and YAP1 by RNA knockdown does not further reduce drug resistance..... 102

Figure 29. 14-3-3σ interacts and binds with YAP1 in vitro 104

Figure 30. Decreased expression of 14-3-3σ by RNA knockdown promotes parp-1 cleavage and apoptotic cell death 107

Figure 31. Decreased expression of either 14-3-3σ or YAP1 by RNA knockdown leads to increased parp-1 cleavage and caspase-8 activation 108

Figure 32. 14-3-3σ over-expression in MiaPaca-2 cells protects against parp-1 cleavage and caspase-8 activation..... 110

Figure 33. Schematic model of 14-3-3σ-mediated gemcitabine 111

List of Abbreviations

5-Aza-dC	5-aza-2'-deoxycytidine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CDA	Cytidine deaminase
CDC2	Cell division cycle protein 2
CDP	Cytidine diphosphate
ChIP	Chromatin immunoprecipitation
CHK1	Checkpoint kinase 1
CM	Conditioned medium
Co-IP	Co-immunoprecipitation
dCK	Deoxycytidine kinase
dCDP	Deoxycytidine diphosphate
dCTP	Deoxycytidine triphosphate
dCMPD	Deoxycytidylate deaminase
dFdC	2’,2’-difluorodeoxycytidine
dFdCDP	dFdC diphosphate
dFdCTP	dFdC triphosphate
dFdCMP	dFdC monophosphate
dFdU	2’,2’-difluorodeoxyuridine
dFdUMP	2’,2’-difluorodeoxyuridine monophosphate
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases

DMEM	Dulbecco's modification of Eagle's medium
DMR	Differentially methylated region
DMSO	Dimethyl sulfoxide
DRs	Death receptors
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDAC1	Histone Deacetylase
hENT1	Human equilibrative nucleoside transporters 1
LATS	Large tumour suppressor
MMP	Matrix metalloproteinase
MSP	Methylation-specific PCR
NDPK	Nucleoside diphosphate kinase
NMPK	Nucleoside monophosphate kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCNA	Proliferating-cell nuclear antigen
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PHD	Plant Homeo Domain

PI3K	Phosphatidylinositol 3 kinase
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RB1	Retinoblastoma protein 1
RING	Really Interesting New Gene
RNA	Ribonucleic acid
RRBS	Reduced Representation Bisulfite Sequencing
RRF	Relative resistance factor
RRM1/2	Ribonucleotide reductase M1/M2
SEM	Standard error of mean
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRA	Set and Ring Associated
STAT	Signal transducer and activator of transcription
TCA	Trichloroacetic acid
TE buffer	Tris-EDTA buffer
Tip60	Tat-Interactive Protein of 60 KDa
TRPC3	Transient receptor potential cation channel, subfamily C
TSGs	Tumor suppressor genes
TTD	Cryptic Tandem Tudor Domain
UBL	Ubiquitin-like domain
UHRF1	Ubiquitin-like, containing PHD and RING finger domains 1
VEGF	Vascular endothelial growth factor
YAP1	Yes-associated protein

Introduction

A. Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic cancer ranks the fourth most common cause of human death by cancer in the western world, with a 5-year survival rate less than 5% for all stages of the disease and a median survival of 6 months after diagnosis, thereby exhibiting the poorest prognosis of all solid tumors [1-3]. Pancreatic cancer has an annual mortality rate of approximately 95% with over 250,000 patients dying worldwide [4]. In 2014, an estimated 46,420 people will be diagnosed with pancreatic cancer in the United States, and approximately 39,590 people will die from the disease (please see http://www.cancer.org/cancer/pancreaticcancer/detailedguide/pancreatic-cancer-key-statistics). What makes it so lethal is its stealth, which means that it exhibits no clear early warning signs or symptoms and therefore often goes undetected until it is advanced and too late for resection. In the vast majority of cases, symptoms only develop after pancreatic cancer has already grown and begun to spread.

Pancreatic ductal adenocarcinoma, or PDAC, is by far the most common type of pancreatic malignancy. Although surgical resection remains the only curative intervention and offers the best patient outcome for this disease, surgical removal of the tumor is possible in only approximately 15% of the patients [5]. Therefore, the poor survival rate is mainly attributed to the late detection of PDAC and emergence of a largely drug-resistant phenotype over time.

B. Gemcitabine in pancreatic cancer treatment

Routine treatment options to improve prognosis in patients with pancreatic cancer are limited. Its array of treatments includes but is not limited to chemotherapy, radiotherapy, immunotherapy, hormonal therapy, medications, surgery, and nutritional therapy. At present, single-agent gemcitabine, which has been considered the standard of care since 1997, is recommended as first-line chemotherapy for patients with advanced pancreatic cancer and has been extensively studied in phase II and III trials.

Gemcitabine is a deoxycytidine analogue and was approved by FDA in 1997 as the first-line chemotherapeutic drug for patients with locally advanced or metastatic pancreatic adenocarcinoma [6, 7]. It functions by either directly and competitively incorporating into DNA or inhibiting ribonucleotide reductase M1 or M2 (RRM1/RRM2) to prevent DNA replication and, thereby, interrupting DNA synthesis and inhibiting cancer cell growth [8]. However, although gemcitabine is the standard and most commonly used drug for treatment of pancreatic cancer, almost all patients would eventually develop resistance to this therapeutic agent. Over the past decade, numerous trials have been conducted to improve the outcome in patients by combination therapies using gemcitabine as backbone. Currently, combinational treatments using gemcitabine and other therapeutics have shown some promise but no real significant improvements in overall survival rates. So far, gemcitabine with erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, is the only FDA-

approved combination treatment for PDAC. Unfortunately, this regimen only has a modest effect to prolong median overall survival of patients for less than 2 weeks [9] . Although gemcitabine monotherapy or in combination with other agents has become standard chemotherapy for the treatment of PDAC, gemcitabine imparts a progression-free survival interval ranging from 0.9 to 4.2 months only [10]. Therefore, the effect of gemcitabine on survival has been disappointing. Due to the characterization of PDAC by a high propensity for local invasion and distant metastasis as well as early relapses and largely drug-resistant phenotype, overcoming the gemcitabine drug-resistant phenotype has become a hot topic in this field. Understanding acquired gemcitabine resistance could lead to better improvements in the outcome for patients with pancreatic cancer. In order to do so, I strongly suggest that a better understanding of the molecular mechanisms by which gemcitabine resistance arises is likely to lead to novel therapeutic strategies for the successful treatment of patients diagnosed with pancreatic cancer.

C. Gemcitabine metabolism and known resistance mechanisms

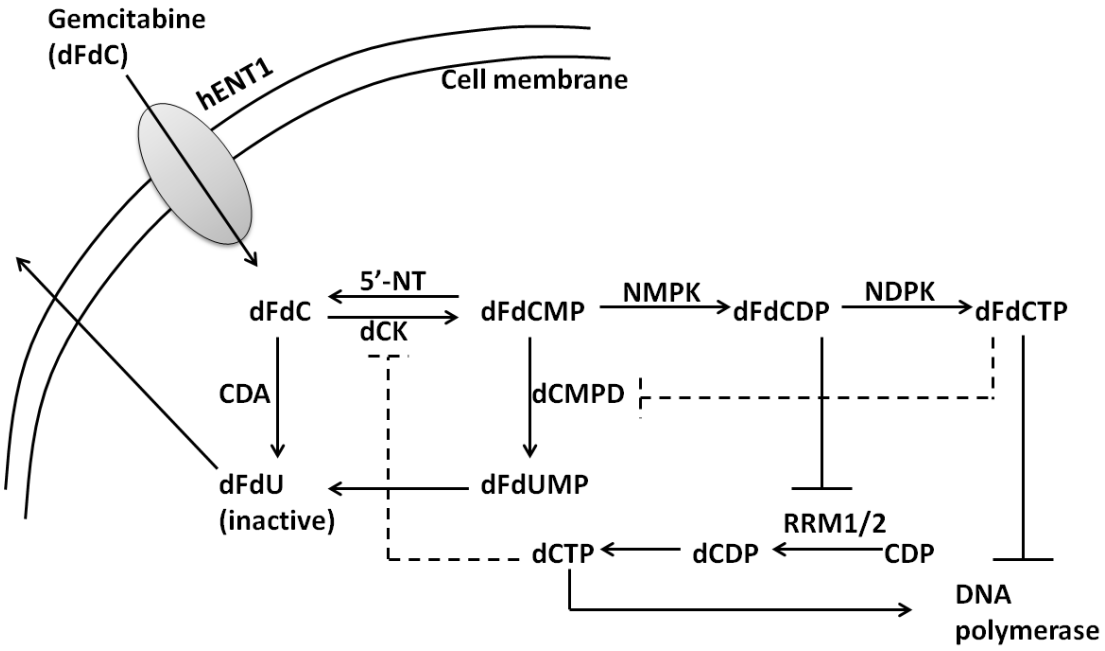
Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a prodrug that requires cellular uptake and intracellular phosphorylation into its active metabolites, gemcitabine diphosphate and triphosphate [8]. As shown in **Figure 1**, gemcitabine is transported into cells via human equilibrative nucleoside transporter-1 protein (hENT1), where it is phosphorylated by the rate-limiting enzyme deoxycytidine kinase (dCK) into gemcitabine monophosphate (dFdCMP), and is further phosphorylated into active

metabolites, the gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) by nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK) respectively [11, 12]. Gemcitabine triphosphate (dFdCTP) is incorporated into DNA, thereby competing with dCTP for incorporation. Once dFdCTP is incorporated, two phosphate molecules are split off and thus leave dFdCMP in the DNA chain, while it allows only one more deoxynucleoside triphosphate to be incorporated, after which DNA replication terminates. dFdCMP is resistant to be removed from the DNA strand by proofreading enzymes (i.e., polymerase ε), leading to impairment of their ability to repair the DNA strand, which is the mechanism also known as "masked-chain termination." Gemcitabine diphosphate (dFdCDP), on the other hand, inhibits ribonucleotide reductase M1 or M2 (RRM1/RRM2) that convert CDP to dCDP, leading to depletion of dCTP pools and facilitating incorporation of dFdCTP into DNA [13]. As depicted in **Figure 1**, gemcitabine has various self-potentiating mechanisms that contribute to the maintenance of dFdCDP and dFdCTP levels for prolonged periods of time. For example, since dCTP inhibits dCK, decreased dCTP pools from RRM1/RRM2 inhibition by dFdCDP can result in higher dCK activity and thus an increase in phosphorylation of dFdC to its active metabolites. In addition, dFdCTP can inhibit deoxycytidylate deaminase (dCMPD), an enzyme that deaminates dFdCMP to its inactive form dFdUMP, leading to a potentiation of its own formation.

It is known that high intracellular accumulation of dFdCTP and incorporation into DNA are associated with greater sensitivity to gemcitabine in preclinical tumor models

[14]. Moreover, clonogenic survival assays demonstrated that increased gemcitabine concentrations result in a decrease of cell viability, which suggested a prolonged periods of time for intracellular retention of active gemcitabine metabolites [15, 16]. The intracellular accumulation of active metabolites and cytotoxicity of gemcitabine are influenced by multiple factors, such as (a) the dosing schedule, (b) phosphorylation/activation by dCK, (c) cellular uptake via hENT1, (d) degradation/inactivation through CDA, and (e) genetic factors (e.g., single nucleotide polymorphisms in dCK and CDA) [17-20].

Figure 1. Gemcitabine metabolic schema and proposed pharmacological mechanisms of gemcitabine and its metabolites. Transportation of dFdC into the cell is mediated by hENT1, which is followed by intracellular phosphorylation by dCK to its monophosphate dFdCMP, and subsequently into its active dFdCDP and dFdCTP metabolites. dFdCTP is incorporated into DNA, thereby competing with dCTP for incorporation. dFdCDP inhibits RRM1/2, which prevents the conversion of CDP to dCDP and thus reduced synthesis of dCTP, leading to an elevation of intracellular dFdCTP/dCTP ratio and enhanced incorporation of dFdCTP into DNA.



Remarkable progress has been made during the last decade toward identifying and understanding the complicated signaling pathways that contribute to the initiation, progression of PDAC, and signaling pathways that contribute to intrinsic and acquired gemcitabine resistance in pancreatic cancers. To date, several molecular markers to predict gemcitabine sensitivity have been reported and investigated with or without relation to gemcitabine metabolism, including messenger RNA (mRNA) and microRNA, as well as genes related to gemcitabine metabolism and transport, such as deoxycytidine kinase, ribonucleotide reductase, and human equilibrative nucleoside transporter-1 [21-24]. However, the potential use of such markers in clinical settings remains limited due to the difficulties in evaluating their protein or mRNA levels in clinical samples. For example, accurate quantitative analyses of mRNA from clinical samples are often difficult as a result of degradation. Therefore, more reliable methods-based biomarkers are needed to predict responses to gemcitabine.

The most studied gemcitabine resistance mechanisms are the dysregulation of the enzymes participating in gemcitabine metabolism pathways, including down-regulation of transporter hENT1, down-regulation of rate-limiting enzyme dCK, and up-regulation of RRM1/RRM2 [25-31]. It suggests that the ratio of the expression level of these four genes (hENT1 X dCK)/ (RRM1 X RRM2) decreased progressively with development of acquired gemcitabine resistance and, thus, this ratio correlates with acquired gemcitabine resistance in pancreatic cancer cells, which may be a useful predictive marker for the efficacy of gemcitabine chemotherapy in pancreatic cancer

patients [30]. Other studies show changes in a variety of cellular signaling pathways such as Akt/mTOR signaling pathway and NF-kB signaling pathway, as well as malfunction of proteins involved in cell survival/apoptosis pathway [32-34]. Emerging evidence suggests molecular and phenotypic association between gemcitabine resistance and acquisition of epithelial-mesenchymal transition (EMT)-like phenotype of pancreatic cancer cells [35-39]. This process is also believed to be reminiscent of "cancer stem-like cells" characteristics in many cancer systems including pancreatic cancer [40-43]. EMT has been classified as a unique process by which epithelial cells undergo remarkable morphological changes characterized by transition from epithelial phenotype (cobblestone phenotype) to mesenchymal phenotype (elongated fibroblastic phenotype) with increased motility and invasion [44, 45]. Despite our improved understanding, it is crucial to continue efforts toward discovering biomarkers and unraveling the molecular mechanisms that support and drive this gemcitabine-resistant phenotype, which will ultimately provide means to improve treatment of this deadly disease.

D. 14-3-3 sigma and 14-3-3 family

Our lab has previously found that high expression of 14-3-3σ (sigma) associates with intrinsic gemcitabine resistance in human pancreatic cancer [46], but whether or not 14-3-3σ associates with acquired gemcitabine resistance is not known and needs to be investigated. It was previously found that 14-3-3σ protein level was increased

significantly in about 71% of human pancreatic cancer tissues compared with matched normal tissues, and that the 14-3-3σ protein level in pancreatic cancers correlated with lymph node metastasis and poor prognosis of the patients [46]. Importantly, the Kaplan-Meier survival curves demonstrated a trend of higher patients' survival with low 14-3-3σ expression compared to high 14-3-3σ expression ($p=0.06$). These evidences suggest that 14-3-3σ may be used as a potential biomarker and promising therapeutic target for treating pancreatic cancer.

14-3-3σ, also known as human mammary epithelial marker 1 or stratifin, is a member of highly conserved family called 14-3-3 proteins that are present in all eukaryotic organisms [47]. 14-3-3 proteins belong to a highly conserved multigene family of phosphoserine/phosphothreonine-binding molecules with consensus RSXpSXP-binding motif and play an essential role in multiple biological processes such as cell signaling, cell division, survival and cell death [48-51].

Among the seven human 14-3-3 family members (β, ε, θ/τ, ζ, σ, γ, η), 14-3-3σ is uniquely induced by p53 activation and has a positive feedback effect on p53 activity in response to DNA damage [52]. Therefore, 14-3-3σ might function as a potential tumor suppressor. Moreover, 14-3-3σ is a negative regulator of the cell cycle by initiating cell cycle checkpoint control after DNA damage, and is also required to prevent mitotic catastrophe after DNA damage [53-56]. 14-3-3σ was demonstrated to be induced by DNA damage such as γ-irradiation and Adriamycin treatment in a p53-dependent

manner [53]. Moreover, exogenously over-expressing 14-3-3σ caused a cellular phenotype remarkably similar to that observed following γ-irradiation, with an increase in cell size and failure to progress through G2/M cell cycle. These results strongly suggest that one of the molecular mechanisms underlying the G2/M cell cycle arrest following γ-irradiation is based on the activation of p53, which in turn transcriptionally activates 14-3-3σ [53]. Furthermore, 14-3-3σ appears to sequester CDC2-CyclinB1 complexes in the cytoplasm, causing G2/M cell cycle arrest, and 14-3-3σ knockout fails to arrest CDC2-Cyclin B1 complexes in cytoplasm, resulting in mitotic catastrophe [55]. In addition, inactivation of CDC25C, an activator of CDC2-Cyclin B1 complex, via phosphorylation at serine 216 by serine/threonine protein kinase CHK1, created a binding site for 14-3-3 proteins and resulted in cytoplasmic arrest of CDC25C [57, 58]. Therefore, 14-3-3 proteins act on both CDC25C and CDC2-Cyclin B1 complexes to ensure that mitosis does not occur in the presence of DNA damage [55, 59].

E. Association of 14-3-3σ expression with drug resistance

14-3-3σ was not only found to correlate with intrinsic gemcitabine resistance, it was also previously found to contribute to cisplatin resistance, Adriamycin resistance, and mitoxantrone resistance in several human cancer cells. It was found that the parental colon cancer HCT116 cells were six times more tolerance to cisplatin than the 14-3-3σ-KO HCT116 cells [60]. In human pancreatic cancer cell lines, it was found that in response to cisplatin treatment, 14-3-3σ-over-expressing PANC-1 cells exhibited

attenuated PARP cleavage and significantly decreased activation of caspase-3, compared with sham-transfected PANC-1 cells. Moreover, T3M4 pancreatic cancer cells with silenced 14-3-3σ exhibited an elevated PARP cleavage and caspase-3 activation following cisplatin treatment [61]. These findings suggest that 14-3-3σ over-expression contributes to intrinsic cisplatin resistance by inhibiting apoptosis. In addition, 14-3-3σ over-expression was also found to be associated with acquired cisplatin resistance in non-small cell lung cancer cells. It was found that 14-3-3σ mRNA expression levels were significantly increased in acquired cisplatin-resistant A549 and Calu1 cells compared with parental A549 and Calu1 cells that are cisplatin-sensitive, and that suppressing 14-3-3σ expression in cisplatin-resistant Calu-1 cells magnified cisplatin response [62].

Previous proteomic analysis identified 14-3-3σ as a contributor to acquired Adriamycin resistance in breast cancer cells [63]. By utilizing two-dimensional gel electrophoresis and mass spectrometry analysis, 14-3-3σ was one of the 17 proteins identified with differential expression levels between parental MCF7 cells and acquired Adriamycin-resistant cells MCF7/AdVp3000. Further studies by knocking down 14-3-3σ expression in these cells as well as ectopic over-expression of 14-3-3σ in parental MCF7 cells showed that the increased 14-3-3σ expression in resistant MCF7/AdVp3000 cells contributed to the drug-resistant phenotype [63].

Experiments were also conducted by ectopically over-expressing 14-3-3σ in HEK293 cells, and results showed that ectopic over-expression of 14-3-3σ in HEK293

cells resulted in increased resistance to mitoxantrone [63]. In addition, by perfoming two-dimensional gel electrophoresis, 14-3-3σ was also found to be over-expressed in the mitoxantrone-selected atypical multidrug-resistant cell line EPP85-181RNOV, suggesting its association with mitoxantrone resistance in human pancreatic adenocarcinoma [64].

In prostate cancers, it was found that the expression level of 14-3-3σ was much higher in androgen-independent prostate cancer cell lines DU145, PC3, and CWR22RV than that in the androgen-dependent cell line LNCaP, and that the androgen-independent cells are more resistant to mitoxantrone and Adriamycin than the androgen-dependent cells [65]. Moreover, depleting 14-3-3σ expression in androgen-independent DU145 and CWR22RV cells significantly sensitized these cells to mitoxantrone and Adriamycin treatment by abrogating G2/M cell cycle checkpoint and promoting apoptosis, whereas restoring 14-3-3σ expression in androgen-dependent LNCaP cells enhanced drug resistance [65]. This study indicates that advanced and hormone-refractory prostate cancers may have an increased level of 14-3-3σ, which in turn contributes to mitoxantrone and Adriamycin resistance in advanced and hormone-refractory prostate cancers. Thus, therapeutic intervention targeting 14-3-3σ may be useful for sensitizing hormone-refractory prostate cancers to chemotherapeutic drugs by both G2/M checkpoint abrogation and apoptosis enhancement.

Our lab has previously found that high expression of 14-3-3σ associates with intrinsic gemcitabine resistance in human pancreatic cancer, and that over-expression of 14-3-3σ caused resistance to γ-irradiation and anticancer drugs including Adriamycin, mitoxantrone, and gemcitabine in pancreatic cancer cell lines [46]. Therefore, 14-3-3σ may serve as a prognosis marker predicting survival of pancreatic cancer patients. Despite the evidence that high 14-3-3σ expression level contributes to intrinsic gemcitabine resistant in pancreatic cancer cell lines and prognosis in pancreatic cancer patients, it is unknown if and how 14-3-3σ contributes to acquired gemcitabine resistance.

F. YAP1, a potential binding partner of 14-3-3σ

As a chaperon protein, the potential contribution of 14-3-3σ to drug resistance may be by regulating its binding partners. One potential binding partner of 14-3-3σ is called YAP. YAP is a 65 kDa protein (sometimes termed YAP65 or YAP1) that was originally identified due to its interaction with the Src family tyrosine kinase Yes [66]. YAP is a transcriptional coactivator without a DNA-binding motif while maintaining a potent transactivation domain at its C terminus [67]. Thus it binds and activates several transcription factors including Runx [67] and four highly conserved TEAD/TEF transcription factors [68]. Structurally, it contains a proline-rich domain, a TEAD-binding domain, either one or two WW domains depending on alternative splicing [69], a SH3-binding motif, a transactivation domain (TAD), and a PDZ interaction motif. The WW

domain is the domain for protein-protein interaction, while the N terminus of YAP has a binding domain for the TEA domain (TEAD) family of DNA-binding proteins, which have been linked to the growth-promoting function of YAP [68]. These DNA-binding proteins include other possible transcriptional partners for YAP (ErbB, Runx2, and chromatin modeling proteins) as well as the negative YAP regulator LATS (large tumour suppressor) kinase [69-72]. Through its carboxyl terminus, YAP was reported to bind to the PDZ-containing protein EBP50, a submembranous scaffolding protein [73].

By transducing signals from cytoplasm to nucleus, YAP is important for transcriptional regulation. The important role of YAP was first discovered in *Drosophila,* where its homolog, Yorkie, was shown to promote tissue growth by increasing cell proliferation and inhibiting apoptosis [72]. Genetically, Yorkie is the ultimate effector of the evolutionarily conserved Hippo pathway [72]. The localization of YAP is controlled by phosphorylation. Five phosphorylation sites (S61, S109, S127, S164, S381) of YAP have been described and various kinases from Hippo-like pathways including LATS have been shown to be directly involved in the subcellular localization, transcriptional coactivator activity and biological functions of YAP [74-77]. Once phosphorylated at a key serine (S127), YAP is sequestered in the cytoplasm, where it can no longer function to promote target gene expression [78].

YAP was also found to bind to apoptosis-related proteins or transcription factors including p53 binding protein-2 (p53BP-2) [79], an important regulator of the apoptotic

activity of p53 [80]. YAP also interacts with the p53 family member p73, resulting in an enhancement of p73's transcriptional activity [81]. Since YAP is homologous to TAZ (45% identity), a transcriptional coactivator that is regulated by interaction with 14-3-3 [82], it may also potentially bind to 14-3-3 proteins. It was reported that YAP phosphorylation by Akt induces its interaction with 14-3-3 and suppresses its ability to promote p73-mediated transcription of pro-apoptotic genes in response to DNA damaging agents [83]. More importantly, the crystal structure of 14-3-3σ/YAP phosphopeptide with pSer127 complex has been resolved with 1.15 Å resolution [84]. However, whether or not YAP interacts with 14-3-3σ needs to be further investigated.

G. 14-3-3σ gene methylation in cancers

For a long time, cancer has been widely recognized as a complex disease characterized by both multiple genetic and epigenetic alterations [85, 86]. Epigenetic regulations like chromatin modifications are known to exert a significant impact on gene expression. Several chromatin-modifying enzymes have been identified and known to catalyze specific modifications including methylation, acetylation, phosphorylation and ubiquitination. DNA methylation is one of the most important epigenetic alterations and plays a critical functional role in various biological and physiological processes including development, differentiation and progression of various diseases such as tumorigenesis [87-90]. Hypermethylation of important genes including tumor suppressor genes has

been extensively studied and frequently described in many human cancers including pancreatic cancer [91-97].

14-3-3σ has been found to be frequently lost or decreased in various human tumors (**Table 1**) including breast cancer [98], esophageal squamous cell carcinoma [99], human oral squamous cell carcinoma [100], salivary gland adenoid cystic carcinoma [101], gastric carcinoma [102], urinary bladder carcinoma [103], and prostate cancer [104]. Its inactivation in these human cancers is broadly believed to be caused by promoter hypermethylation, which leads to block of DNA transcription and results in gene silencing [98, 101-104]. However, whether or not and how the methylation status of 14-3-3σ gene is regulated during development of acquired gemcitabine resistance is not known.

Table 1. Frequency of 14-3-3σ gene methylation in different types of cancers.

Type of cancer	Frequency	Sample size	References
Basal-cell carcinoma	68%	41 LMT	[105]
Benign prostate hyperplasia	100%	29 (MT)	[104]
Breast cancer	86%	50 (T)	[54]
Breast cancer	100%	32 (MT)	[54]
Breast ductal carcinoma *in situ*	83%	18 (MT)	[106]
Breast invasive ductal carcinoma	96%	25 (MT)	[106]
Gastric carcinoma	43%	60 (T)	[102]
Hepatocellular carcinoma	89%	19 (T)	[107]
Large-cell lung cancer	57%	7 (L)	[108]
Non-small-cell lung cancer	6%	17 (L)	[108]
Oral squamous-cell carcinoma	35%	92 (T)	[109]
Prostate carcinoma (Pca)	99%	121 (MT)	[104]
Prostate intraepithelial neoplasia (high-grade)	100%	39 (MT)	[104]
Small-cell lung cancer	69%	13 (L)	[108]
Small-cell lung cancer	33%	24 (ML)	[108]
Urinary bladder carcinoma (invasive)	57%	14 (MT)	[103]
Urinary bladder carcinoma (noninvasive)	21%	14 (MT)	[103]
Vulval pre-malignant neoplastic lesions VIN III	59%	22 (T)	[110]
Vulval squamous-cell carcinoma	55%	36 (T)	[110]

L, cell line; LMT, laser-microdissected tumour; MT, microdissected tumour; T, non-dissected tumour; VIN III, vulval intraepithelial neoplasia

H. Uhrf1/DNMT1 complex, an emerging regulator of gene methylation

It is well known that DNA methylation is established and maintained by three active DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b [111, 112]. Both DNMT3a and DNMT3b are regarded as de-novo DNA methyltransferases, whereas DNMT1 has a strong preference for hemi-methylated CpG substrates generated during DNA replication and is regarded as maintenance DNA methyltransferase [111-113]. Consistent with its central role in maintenance of DNA methylation, DNMT1 is associated with DNA replication forks in the S phase of cell cycle [114, 115], making it a good target for studying epigenetic alteration or inheritance during biological and pathological processes. Recent studies have focused on the central topic how DNMT1 is recruited to the DNA replication foci.

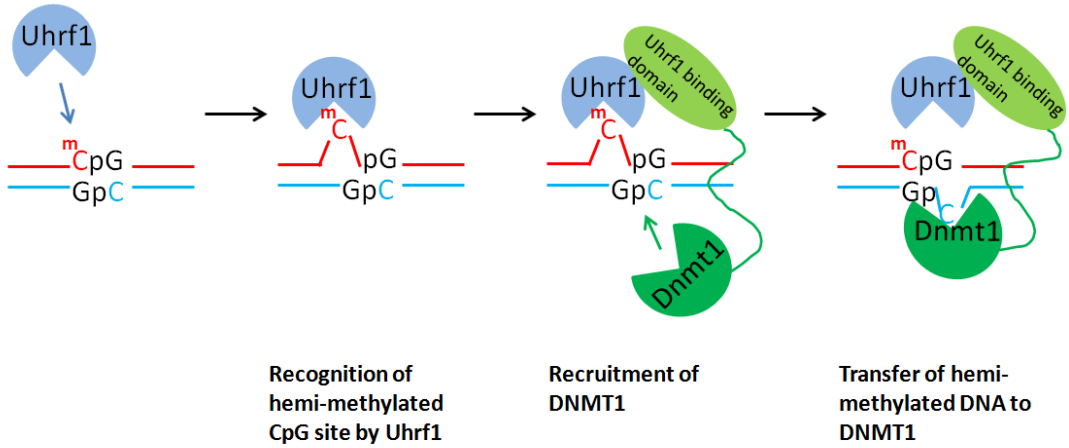
Recently, Uhrf1 (ubiquitin-like, containing PHD and RING finger domains 1) was identified as a DNMT1-interacting protein that recruits DNMT1 to replication forks to maintain DNA methylation and hence Uhrf1 is essential for epigenetic inheritance [116, 117]. Uhrf1 is a multi-domain protein associated with cell proliferation and epigenetic regulation, and is a putative oncogenic factor that is found over-expressed in numerous cancers [118, 119]. Structurally, it harbors an ubiquitin-like domain, a plant homeodomain (PHD), a Set and Ring Associated (SRA) domain and a RING domain. It is considered an efficient marker to differentially diagnose pancreatic adenocarcinoma, chronic pancreatitis and normal pancreas [120]. Moreover, Uhrf1 was also found to be

over-expressed in bladder cancer and the intensity of its over-expression appears to be related to the stage of the cancer, suggesting Uhrf1 as a novel molecular marker for diagnosis and prognosis of bladder cancer [121]. Furthermore, Uhrf1 could bind to histones and methyl-CpG dinucleotides with a preference for hemimethylated CpG sites via a unique SRA domain which is found only in Uhrf family [122, 123]. The consequence of Uhrf1 binding is recruitment of transcriptional repressors like DNMT1 (**Figure 2**) and histone deacetylase 1 (HDAC1) along with PCNA, resulting in methylation of the newly synthesized strands, which plays an important role in facilitating and maintaining DNA methylation in human genome [124-128]. Therefore, Uhrf1 is hypothetically involved in a macro-molecular protein complex called "Epigenetic Code Replication Machinery" that would be able to duplicate the epigenetic code by acting at the DNA replication fork and activating the right enzymatic activity at right moment [129, 130].

The ultimate outcome of Uhrf1 binding is repression of its target genes. By forming a complex with HDAC1, Uhrf1 was found to bind to methylated promoter regions of tumor suppressor genes such as p16 and p14 in cancer cells [131]. Moreover, Uhrf1 was also found to cooperate with G9a to enhance the transcriptional repression of p21 gene [132]. Furthermore, Uhrf1 was found to be over-expressed in colorectal cancer tissues to promote colorectal cancer growth and metastasis by repressing $p16^{ink4a}$ [133]. In addition, other tumor suppressor genes were also identified to be negatively regulated by Uhrf1, including RB1 and BRCA1 [127, 134]. However, whether

the putative tumor suppressor genes such as 14-3-3σ are epigenetically regulated by Uhrf1 needs to be investigated.

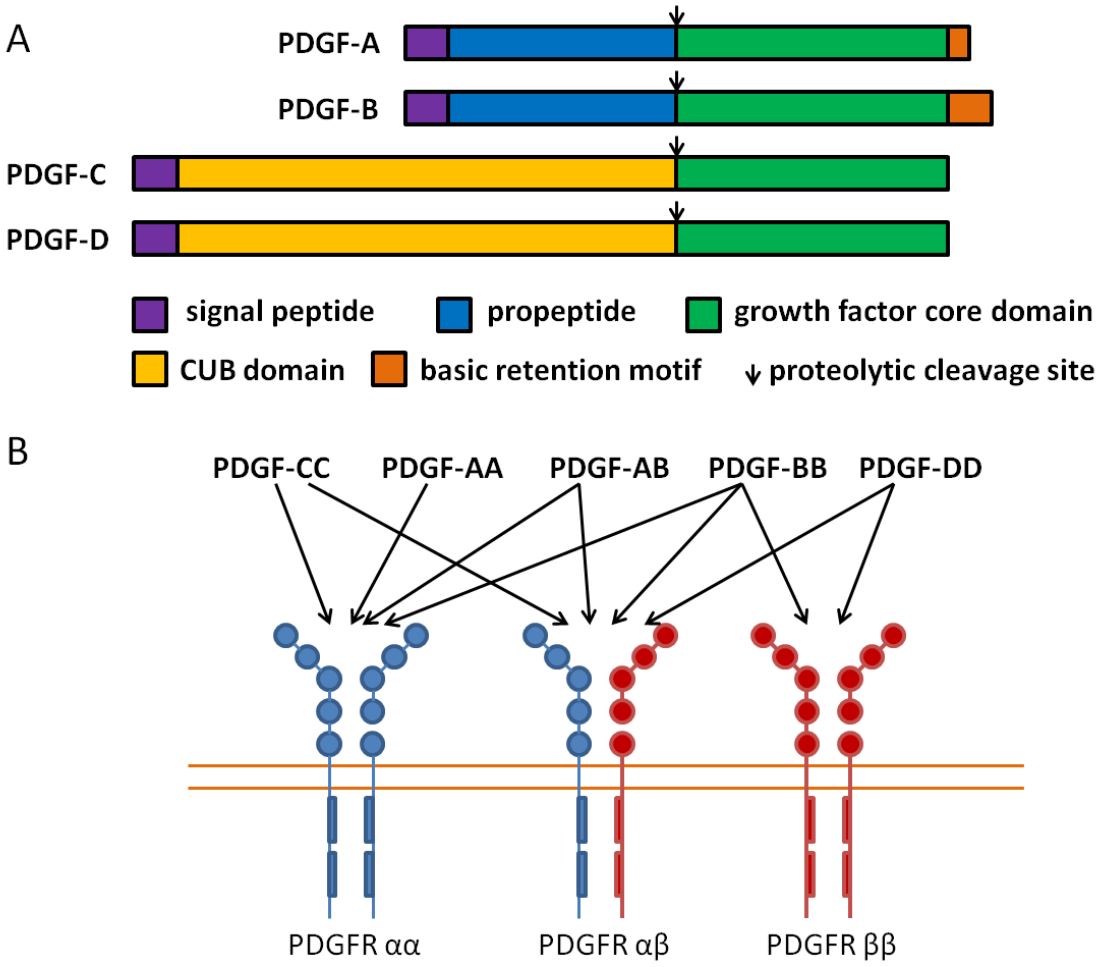
Figure 2. A successive DNA transfer model for maintenance DNA methylation by Uhrf1 and DNMT1. Schematic model showing cooperative action by Uhrf1 and DNMT1 for maintenance of DNA methylation. Uhrf1 recognizes and binds to hemi-methylated CpG sites of the genome, leading to the recruitment of DNMT1 to the site and transfer of hemi-methylated DNA to DNMT1, followed by subsequent methylation of newly synthesized strand by DNMT1 to maintain DNA methylation. Pre-existing and newly synthesized DNA strands are indicated by red and blue lines, respectively.



I. Platelet-derived growth factor (PDGF) family

Platelet-derived growth factor (PDGF) signaling pathway has been extensively studied and well characterized since PDGF was first described in 1970s as a serum factor that promoted the smooth muscle cell proliferation [135]. The PDGF family comprises of four different polypeptides encoded by different genes, which have been identified as PDGF-A, PDGF-B, PDGF-C and PDGF-D [136-138]. PDGFs need to be assembled into disulfide-bonded dimers via homodimerization or heterodimerization in order to play their functional role. To date, four homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD, and one heterodimer PDGF-AB have been described [139]. It is noteworthy that no heterodimers involving PDGF-C and PDGF-D chains have been identified. In addition, it is notable that PDGF-A and PDGF-B are secreted in their active forms, while PDGF-C and PDGF-D are secreted as inactive forms, requiring further activation for their function [136, 140, 141]. Structurally, as shown in **Figure 3A**, PDGF-A and PDGF-B mainly encode the growth factor domain and have short N-terminal extensions that undergo intracellular proteolytic processing for activation. However, both PDGF-C and PDGF-D encode a unique N-terminal CUB (for complement C1r/C1s, Uegf, Bmp1) domain, which is cleaved extracellularly followed by secretion for activation [142]. The domain structures of PDGF family members are provided in **Figure 3A**.

Figure 3. PDGF proteins and PDGF-PDGFR interactions. (A) Schematic drawing of the structure of four PDGF proteins (PDGF-A, B, C and D). (B) Representation of the PDGF-PDGFR interactions. The extracellular region of the PDGF receptor (PDGFR) consists of five immunoglobulin-like domains (shown as blue or red balls) while the intracellular part is the tyrosine kinase domain (shown as blue or red rectangles).



PDGFs exert their cellular effects by activating two structurally related receptor tyrosine kinases (PDGFR), PDGFR-α and PDGFR-β by phosphorylation [136, 137, 143, 144]. As shown in **Figure 3B**, the PDGF-AA activates PDGFR-α, whereas PDGF-BB activates PDGFR-α, PDGFR-α/β or PDGFR-β. PDGF-AB and PDGF-CC activate either PDGFR-α or PDGFR-α/β, while PDGF-DD specifically binds to and activates its cognate receptor PDGFR-β (homo or hetero- dimmers). Although all four PDGF ligands play their oncogenic roles through binding with two PDGFRs, they promote carcinogenesis through different targets. Specifically, the phosphorylation of PDGFR by PDGFD triggers a number of downstream signaling pathways including activation of phosphatidylinositol 3 kinase (PI3K), Akt, nuclear factor-κB (NF-κB), Notch, and extracellular signal-regulated kinase (ERK) [139, 145-147].

J. PDGFD over-expression in human cancers

PDGFD has generated considerable interest in recent years because of its up-regulation and involvement in the progression of several types of human cancers [147-153]. Although PDGFD was discovered over a decade ago, the functional role of PDGFD is just beginning to be understood. A growing body of literature strongly suggests that PDGFD may function as a key player in the development and progression of numerous human cancers by regulating the processes of cell proliferation, apoptosis, migration, invasion, angiogenesis, and metastasis [139, 153-156]. It has been reported that PDGFD signaling is frequently deregulated in human malignancies with up-regulated expression

of PDGFD in prostate, lung, renal, ovarian, brain, and pancreatic cancers [146, 147, 149-152]. Over-expression of PDGFD in breast cancer cells was found to promote tumor growth and lymph node metastasis through increased proliferation and decreased apoptosis via activation of MAPK and Akt signaling pathway [157]. In pancreatic cancer, PDGFD was reported to be strongly expressed in pancreatic adenocarcinomas, reactive cells of chronic pancreatitis, and in islets, but to a lesser degree in the normal ducts [147]. These findings make PDGFD a promising target for improvement in therapeutic treatment of pancreatic cancers. However, the association of PDGFD over-expression with resistance to therapeutic agents has not been extensively studied. PDGFD was only shown to be identified as one of the key genes that influenced semustine chemosensitivity in glioblastoma [158]. Also, in postate adenocarcinoma, increased PDGFD expression in PTEN knockout cells was shown to contribute to radio resistance observed in these cells [154]. However, whether or not and how PDGFD associates with gemcitabine resistance is unknown.

K. Specific aims of the present work

As discussed above, one of the major obstacles in successful treatment of pancreatic cancer is the development of drug resistance that makes the patients unresponsive to drug treatment and eventually leads to poor prognosis. Although gemcitabine is the first-line therapy used for treating pancreatic cancers, acquired gemcitabine resistance in a substantial number of patients appears to hinder its

effectiveness. Extensive studies have been carried out in the cancer research community to understand the mechanisms responsible for drug resistance in a hope to discover potential therapeutic targets for prognosis and therapeutic treatments of pancreatic cancer patients. However, progress in our understanding of acquired gemcitabane resistance has been very slow and more studies are needed.

To further investigate the mechanisms of gemcitabine resistance, a gemcitabine resistant pancreatic cancer cell line was generated by stepwise selection of a pancreatic cancer cell line MiaPaca-2 with increasing concentrations of gemcitabine. The resistant cell line was cloned and named G3K, and can survive and grow in the presence of 3000 nM of gemcitabine; this is ~6,500 (relative resistance factor or RRF) fold more resistant to gemcitabine than the parental MiaPaca-2 cells. In addition to the up-regulation of known gemcitabine resistant enzymes such as ribonucleotide reductase M1/M2 (RRM1 or RRM2), the expression of 14-3-3σ protein, but not other 14-3-3 family members, was dramatically increased in the resistant cells. Furthermore, our data indicate that 14-3-3σ up-regulation is widely recognized to be caused by gene demethylation of its first exon.

Therefore, **the first aim** of my present work is to investigate the detailed mechanism through which gemcitabine selection causes 14-3-3σ gene demethylation. By examining the DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) and the recruiter Uhrf1, I found that DNMT1 and Uhrf1, but not DNMT3a or DNMT3b, play a critical role in 14-3-3σ gene methylation, and knocking down expression of either one

leads to 14-3-3σ gene re-expression. Moreover, by ChIP assay, I found that Uhrf1 could recruit DNMT1, and both proteins bind to the methylated region of 14-3-3σ gene, demonstrating an important role of these two proteins for maintaining the methylation status of 14-3-3σ gene. Moreover, gemcitabine selection leads to the reduced expression of Uhrf1, which results in a decreased recruitment of DNMT1 to the 14-3-3σ gene and eventually leads to the 14-3-3σ gene demethylation. Because the altered expression of Uhrf1 and DNMT1 in G3K cells likely also influence the methylation status of other genes, which may also contribute to acquired gemcitabine resistance, **the second aim** was designed to profile global changes in gene methylation comparing MiaPaca-2 and G3K cells using Reduced Representation Bisulfite Sequencing (RRBS). Among 845 genes that have been found to have altered methylation, PDGFD was shown to play an important role in acquired gemcitabine resistance possibly by regulating STAT3. **The third aim** was designed to investigate the detailed mechanism of 14-3-3σ-mediated gemcitabine resistance in pancreatic cancer cells. My studies showed that 14-3-3σ over-expression protected cancer cells from gemcitabine-induced apoptosis likely by forming a complex with YAP1 and together inhibiting caspase-8 activation and gemcitabine-induced apoptosis.

The outcome of this study shall lead to a better understanding of epigenetic regulation of 14-3-3σ gene and molecular mechanisms of acquired gemcitabine resistance in pancreatic cancer, particularly the mechanism of PDGFD-mediated gemcitabine resistance. It may also help discover potential therapeutic targets and

develop better antineoplastic drugs and treatment regimens that maybe more effective for drug resistant pancreatic cancer patients. Moreover, this study strongly suggests that reversible epigenetic regulation may play an important role in development of acquired gemcitabine resistance in pancreatic cancer patients and targeting epigenetic regulation may provide a new direction for chemosensitization of gemcitabine resistant human pancreatic cancers.

MATERIALS AND METHODS

A. Materials

Metafectene Pro transfection reagent was obtained from Biontex. siRNAs targeting 14-3-3σ (sc-29590), YAP1 (sc-38637), Uhrf1 (sc-76805), DNMT1 (sc-35204), DNMT3a (sc-37757), DNMT3b (sc-37759) as well as PDGFD (sc-39709) and antibodies against 14-3-3θ (sc-732), 14-3-3ζ (sc-1019), DNMT1 (sc-135887), and DNMT3a (sc-20703) were purchased from Santa Cruz Biotechnology. Antibodies against GFP (ab290), YAP1 (ab52771), p-YAP1 (ab76252), TRPC3 (ab70603), and DNMT1 (ab13537) antibody for ChIP assay were from Abcam. Antibodies against 14-3-3σ (05-632), RRM1 (MABE567), ChIP Assay kit (17-295), and CpGenome Universal DNA Modification kit (17-295) were purchased from EMD Millipore. Antibodies against Uhrf1 (612264) and FASN (610963) were from BD Biosciences. Antibodies against histone H3 (9715), p-STAT3 (9145), STAT3 (9139), Caspase-8 (9746), and Parp-1 (9542) were from Cell Signaling. Antibodies against hENT1 (T0108), PDGFD (SAB1101911), and RRM2 were from Epitomics, Sigma, and generated in-house [159], respectively. Lipofectamine, pcDNA3.1(+) plasmid, and G418 were from Invitrogen. PDGFD cDNA was purchased from Thermo Scientific. RNeasy Mini kit and Qiagen Blood and Cell Culture DNA Kit were from Qiagen. The iScript^{TM} cDNA synthesis kit and the SYBR Green PCR master mix were from Bio-Rad and Applied Biosystems, respectively. Gemcitabine were purchased from Besse Medical whereas

Ara-C, 5-FU, Adriamycin, mitoxantrone, and nocodazole were form Sigma. All other chemicals were purchased from Sigma or Fisher Scientific.

B. Cell lines, cell cultures, and transfections

Human pancreatic cancer cell line MiaPaca-2 (ATCC) and its derivative lines G3K and G3KRev were cultured at 37℃, 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum and 2.5% horse serum. G3K cells were generated by stepwise selection of MiaPaca-2 using gradually increasing concentrations of gemcitabine starting at 4 nM. G3K cells were clonal and maintained in the presence of 3 μM gemcitabine. The G3KRev cell line was generated by culturing the drug-resistant G3K cells in the absence of gemcitabine for six months and partially lost its gemcitabine resistance phenotype. Human pancreatic cancer cell line Aspc-1 was a gift from Dr. Jingwu Xie (Indiana University) and was cultured in RPMI medium supplemented with 10% FBS. MCF7 and its derivative lines MCF7/AdVp3000, and MCF-7/AdVpG3K/REV were gifts from Dr. Susan E. Bates (National Cancer Institute) and cultured as previously described [63]. The cell lines were authenticated by analysis of tandem repeat sequences on September 17, 2013.

For transient knockdown or over-expression of target genes, cells were plated in a six-well plate at a density of 1.5-3×10^5 cells/well and cultured overnight in complete medium. About 60-120 pmol siRNAs of target genes or control scrambled siRNAs, or 1-2μg of over-expressing plasmid of target genes or vector control plasmid were diluted in

serum-free Opti-MEM medium and then transiently transfected into cells using Metafectene Pro transfection reagent as previously described [160].

For stable transfection, the cDNA of 14-3-3σ and PDGFD gene was engineered into pcDNA3.1(+) and transfected into MiaPaca-2 cells using Lipofectamine and Metafectene respectively. Stable clones were selected using 1 mg/ml G418 as previously described [63, 65]. The stable clones were maintained in complete medium supplemented with 200 μg/ml G418.

Similarly, the stable shRNA knockdown was generated as previously described [63, 65]. Briefly, G3K cells were transfected with pSilencer-σ (14-3-3σ shRNA cloned into pSilencer 3.1-H1neo vector) or scrambled shRNA construct [63, 65] using Lipofectamine followed by selection with 1 mg/ml G418 for 2 weeks. Individual clones were tested for 14-3-3σ knockdown and positive clones were propagated and maintained in complete DMEM medium.

C. Cell lysate preparation, TCA protein precipitation and Western blot

Cultured cells were harvested, washed with PBS, and lysed in TNN-SDS buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% SDS, and 2 mM phenyl-methylsulfonyl fluoride) for 30 minutes at 4°C with constant agitation. The cell lysates were then sonicated briefly and followed by centrifugation (14,000×*g* at 4°C) for 15 minutes to

remove insoluble materials. The protein concentrations of supernatants were measured by Bradford assay.

Cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane followed by a 2-hr incubation in blocking solution (PBS-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20) and a 2-hr incubation with primary antibodies. After extensive washing, immunoreactivity was detected with specific secondary antibodies conjugated to horseradish peroxidase. Signals were captured using ECL x-ray film.

For the detection of secreted proteins, 1 volume of TCA (100% w/v) was added to 4 volumes of protein samples collected from the conditioned medium, followed by incubation for 40 minutes at 4°C and centrifugation (14,000×*g* at 4°C) for 5 minutes to precipitation all proteins. After aspirating the supernatant, the protein samples were washed twice with pre-cold acetone, followed by centrifugation (14,000×*g* at 4°C) for 5 minutes. After removing the supernatant and air-dry, protein samples were solubilized by adding 2X loading buffer and ready for SDS-PAGE. When comparing two or more protein samples, the volume of 2X loading buffer was calculated based on cell numbers at the same time the conditioned medium was collected.

D. Membrane preparation

The membrane vesicles were prepared as described previously [161]. Briefly, the cells were washed with ice-cold PBS and resuspended in hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 2 mM PMSF) at 1×10^6 cells/ml followed by being homogenized 100 strokes with glass homogenizer and centrifugation at 4,000 × *g* for 10 min at 4°C. Crude membranes were obtained by centrifugation of the 4,000 × *g* supernatant at 100,000 × *g* for 1.5 hrs. The crude membrane pellet was then resuspended in STBS (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF), passed through a 26-gauge needle for 20 times, aliquoted, and stored at −80 °C.

E. Survival and apoptosis assay

Survival assay was performed as previously described using MTT colorimetric or colony formation assay [46, 162]. Briefly, cells were seeded in 96-well plate at 2000-3000 cells/well and cultured for 24 hrs followed by treatment with different dose of anticancer drugs and incubated continuously for 3 days followed by addition of MTT (5 mg/ml) to a final concentration of 0.5 mg/ml and incubation of the plates at 37°C for 4 hours. The OD_{570nm} and OD_{630nm} were measured using an automated plate reader and analyzed using GraphPad Prism software to generate fitted curve and IC_{50}. Relative resistance factor (RRF) is calculated using the following formula: $RRF=IC_{50(test)}/IC_{50(control)}$. For apoptosis assay, photometric enzyme immunoassay using a Cell Death Detection ELISA Plus kit (Roche Diagnostics, Indianapolis, IN) was performed for quantitative in

vitro determination of cytoplasmic histone-associated DNA fragments and apoptosis as previously described [163].

F. Quantitative real-time RT-PCR

Quantitative RT-PCR was performed as described previously [164, 165]. Briefly, total RNA was extracted using RNeasy Mini Kit followed by reverse-transcription using iScript^{TM} cDNA synthesis kit and quantitative PCR using the SYBR Green PCR master mix. The primer pairs used are: 5′-TAGGCGCTGTTCTTGCTCCAA-3′ (forward) and 5′-ACCAGTGGTTAGGTGCGCTCA-3′ (reverse) for 14-3-3σ; 5′-GGCAAGTTCTCCGAGGTCTCTG-3′ (forward) and 5′-TGGTACATGGCTTTTCGATAGGA-3′ (reverse) for DNMT3b, 5′-TCTGGCTTTCTTTGCAGCAA-3′ (forward) and 5′-CAGCGGGCTTCTGTAATCTGA-3′ (reverse) for RRM2; 5′-GCCTTTACCGTCACCCTTATC-3′ (forward) and 5′-AAAGGTACTACTTATGGGGGC-3′ (reverse) for PTPRG; 5′-GTGTCCCGCTCAGGTATAAAAG-3′ (forward) and 5′-GGGACCACATTCTCAAAGAGAC-3′ (reverse) for Adora2B; 5′-CCCCTTCCAACCAGAATGTA-3′ (forward) and 5′-TGCCAAGAGAAACTGCTGAA-3′ (reverse) for DUSP6; 5′-CCCCAAAAGTAGCGTAACCA-3′ (forward) and 5′-CCGGTACTCCTGCGTGTTA-3′ (reverse) for Olig1; 5′-TGAACACCCTGGGCTCTATC-3′ (forward) and 5′-GGCAGCTGGTCTCCACTTAG-3′ (reverse) for SLC35D3; 5′-CACCTCGGACTCTGTGTTCA-3′ (forward) and 5′-AAGGGCAACATGAGAGCTTG-3′ (reverse) for Cdc42EP3; 5′-CGTGGTCAGGTTGTTTGATGTG-3′ (forward) and 5′-ACTCGGTGTGAATGAAGAAAGTCC-3′ (reverse) for CDK6; 5′-ATTGCGATTTCGTGGTGTACAT-3′ (forward) and 5′-

CCATATTCACCAGTGCTGCTCTT-3′ (reverse) for SLC25A27; 5′-CCCAGGAATTACTCGGTCAA-3′ (forward) and 5′-ACAGCCACAATTTCCTCCAC-3′ (reverse) for PDGFD; 5′-GGCCGCACGACTATTTCT-3′ (forward) and 5′-AGCCCCTTGTAGGCATTG-3′ (reverse) for TRPC3; 5′-AAGGACTCATGACCACAGTCCAT-3′ (forward) and 5′-CCATCACGCCACAGTTTTC-3′ (reverse) for GAPDH.

G. Immunofluorescence and confocal microscope imaging

1-2 × 10^5 G3K cells were seeded on a glass coverslip in a six-well tissue culture plate. After the culture reaches confluence, the cells were washed 3 times with ice-cold PBS and fixed with acetone/methanol (1:1) at room temperature for 15 min and incubated with blocking solution (3% bovine serum albumin in PBS) for 1 h. The cells were then probed with primary antibody YAP1 (1:200) for 2 hrs followed by incubation with FITC-conjugated goat anti-rabbit IgG F(ab′)$_2$fragment (Sigma) (1:1000 dilution) for 30 min. After being washed 3 times with blocking solution, the cells were re-probed with another primary antibody 14-3-3σ (1:50) for 2 hrs followed by incubation with Alexa Fluor 647 dye (Life Technologies) for additional 30 min. Then, after being washed 3 times, the cell nucleus was counterstained with DAPI (25 μg/ml) for 20 min. The coverslips were then mounted on the slides before viewing with Olympus 2 confocal microscope. The laser excitation lines are as follows: 405 nM for DAPI, 488 nM for FITC, and 635 nM for Alexa Fluor 647. The image was then virtualized by Olympus Fluoview Ver.3.0 viewer (FV10-ASW 3.0 viewer).

H. Immunoprecipitation assay

Immunoprecipitation was performed as previously described [166]. Briefly, 1mg of cell lysates were first pre-cleaned by incubation with 1 µg of normal mouse IgG at 4 °C for 1 h, then mixed with 150 µL of protein G agarose beads (50% slurry) and incubated at 4 °C for 2 hrs followed by centrifugation at 500× *g* for 5 min. The cleared supernatants were split into two equal parts incubated with either normal mouse IgG (as a negative control) or incubated with primary antibodies (anti-Flag, anti-YAP1, anti-pYAP1, or anti-GFP) at 4 °C for 3 h, then each part was mixed with 50 µL of protein G agarose beads. After overnight incubation at 4 °C, the reaction was centrifuged to collect precipitates which were then washed five times with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) before being subjected to SDS-PAGE analysis for Western blot analysis.

I. Chromatin-immunoprecipitation (ChIP) Assay

The ChIP assay was performed using ChIP assay kit following manufacturer's instructions. Briefly, chromatin DNA was crosslinked by formaldehyde and sheared by sonication in 200 µl of SDS lysis buffer. After centrifugation, dilution, and pre-cleaning, the crosslinked protein-DNA complexes were precipitated by overnight incubation with the primary antibodies against histone H3, Uhrf1, DNMT1 or without any antibody as a negative control. The precipitated DNA was analyzed by PCR using primers 5'-CTGAACAGGCCGAACGGTATGAAGAC-3' and 5'-GAATCGATGATGCGCTTCTTGTCATC-3' (for

CpG island sequences of 14-3-3σ) and (5′-GCTCTTGGCTAGGTAACTGGACTCTTG-3′ and 5′-AGGGGCTTTCCTCATTCTGCCTGCTAC-3′ (for nonCpG island sequences of 14-3-3σ).

J. Genomic DNA isolation, bisulfite modification, methylation-specific PCR and sequencing

Genomic DNA was isolated from MiaPaca-2, G3K, and G3KRev cells using Qiagen Blood and Cell Culture DNA Kit and modified by sodium bisulfite using the CpGenome universal DNA modification kit according to the supplier's protocol, followed by Methylation-specific PCR as previously described [167]. Briefly, 10 μg bisulfite-modified genomic DNAs were subjected to PCR analysis using primers 5′-TGGTAGTTTTTATGAAAGGCGTC-3′ and 5′-CCTCTAACCGCCCACCACG-3′ for methylated sequence or primers 5′-ATGGTAGTTTTTATGAAAGGTGTT-3′ and 5′-CCCTCTAACCACCCACCACA-3′ for unmethylated sequence. The PCR products were then subjected to separation and analysis by agarose gel electrophoresis.

For sodium bisulfite sequencing, 10 μg bisulfite-modified genomic DNAs were first amplified using primers 5′-GAGAGAGTTAGTTTGATTTAGAAG-3′ and 5′-CTTACTAATATCCATAACCTCC-3′ (for 14-3-3σ gene) or primers 5′-TGAGTTTTTATAGGTTTAATTAGGAGGG-3′ and 5′-ACTCTCCCCAAACTTCCTACATACTA-3′ (for PDGFD gene) and subcloned into pGEM-T vectors (Promega). For sodium bisulfite sequencing of 14-3-3σ gene, six independent clones from MiaPaca-2 cells and 4 independent clones from G3K cells were isolated and subjected to DNA sequencing. For

sodium bisulfite sequencing of PDGFD gene, four independent clones from MiaPaca-2, G3K, and G3KRev cells were isolated and subjected to DNA sequencing.

K. Reduced representation bisulfite sequencing (RRBS) and data analysis

RRBS was applied on the DNA samples following the protocol described in reference [168]. Briefly, genomic DNA was isolated from MiaPaca-2, G3K, and G3KRev cells using Qiagen Blood and Cell Culture DNA Kit, respectively. For each cell line, three different DNA preparations were diluted to the same volume and same concentration, followed by being mixed together to reduce preparation bias and sent to BGI (Beijing Genomics Institute) for sequencing. Briefly, the DNA samples were treated with restriction enzyme MspI, and Illumina Paired-End protocol was used to construct the library following the digestion. 40-220bp fragments were selected and subjected to bisulfite treatment by ZYMO EZ DNA Methylation-Gold kit. All the bisulfite converted products were amplified by PCR and then followed by sequencing with IlluminaGAII.

The RRBS data was analysed in collaboration with Dr. Yunlong-Liu's lab. Briefly, the raw 49bp reads from sequencing were filtered before alignment, in which step the adapter sequences, contamination and low quality reads were removed. The cleaned reads were subjected to alignment to the genome using BGI SOAPaligner version2.01 [169]. Due to the strand specificity of DNA methylation, each bisulfite converted read pair were aligned twice: (1) the observed cytosines on the forward read of the pair were in silico converted to thymines and mapped to the genome converted the same way,

and (2) the observed guanines on the reverse read of the pair were in silico converted to adenosines and mapped to the genome converted the same way. To estimate methylation level for each region, only bases with quality >14 were considered to exclude sequencing errors. The level estimation is derived by dividing the number of converted cytosine bases in the region by the total number of bases covering CpG cytosines in the same region. Differentially methylated regions (DMR) were derived from windows with at least 5 CpG sites and a 2-fold change in methylation level, and also with Fisher's exact test p value <0.01, as described in [170].

Results

Section I: Detailed mechanism of reversible epigenetic regulation of 14-3-3σ during gemcitabine selection

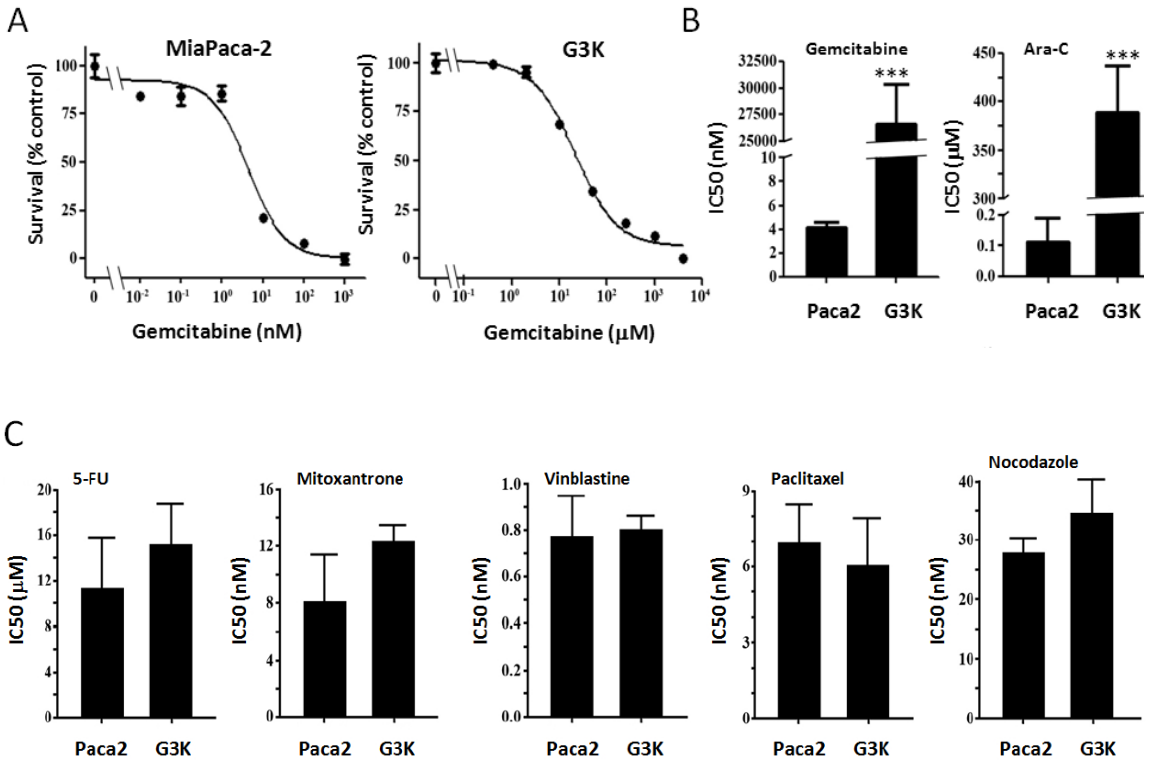
A. A gemcitabine-selected pancreatic cancer cell line is cross-resistant to Ara-C but not to other anticancer drugs.

To investigate acquired gemcitabine resistance, I subjected the pancreatic ductal adenocarcinoma cell line MiaPaca-2 to stepwise selection with escalating concentrations of gemcitabine starting at 4 nM. The final resistant cells were cloned and named G3K that was viable in the presence of 3000 nM of gemcitabine. Authentication using short tandem repeat sequence analysis confirms that G3K was derived from the parental MiaPaca-2 cells (data not shown). The G3K clone has an estimated IC_{50} of 26.6±3.8 μM while the parental MiaPaca-2 cells have an IC_{50} of 4.1±1.3 nM to gemcitabine (**Figure 4A-B**). Thus, G3K cells are ~6,500 (relative resistance factor or RRF) fold more resistant to gemcitabine than the parental MiaPaca-2 cells.

The G3K cells were next examined for cross-resistant to gemcitabine analogue, Ara-C, and other anticancer drugs using MTT assay. As expected, G3K cells are ~3,500 fold more resistant to Ara-C with an IC_{50} of 388.4±48.9 μM than the parental MiaPaca-2 cells with an IC_{50} of 112.8±74.4 nM (**Figure 4B**). However, G3K cells did not show any

significant resistance to vinblastine, paclitaxel, and nocodazole although G3K may be slightly more resistant to 5-FU and mitoxantrone than MiaPaca-2 cells (**Figure 4C**).

Figure 4. Drug response profiles of MiaPaca-2 and its derivative G3K cells. (A). Dose response of MiaPaca-2 and G3K cells to gemcitabine treatment. (B) and (C). IC_{50} of MiaPaca-2 and G3K cells to gemcitabine, Ara-C, 5-FU, mitoxantrone, vinblastine, paclitaxel, and nocodazole (n=4-8, **p<0.01, ***p<0.001).



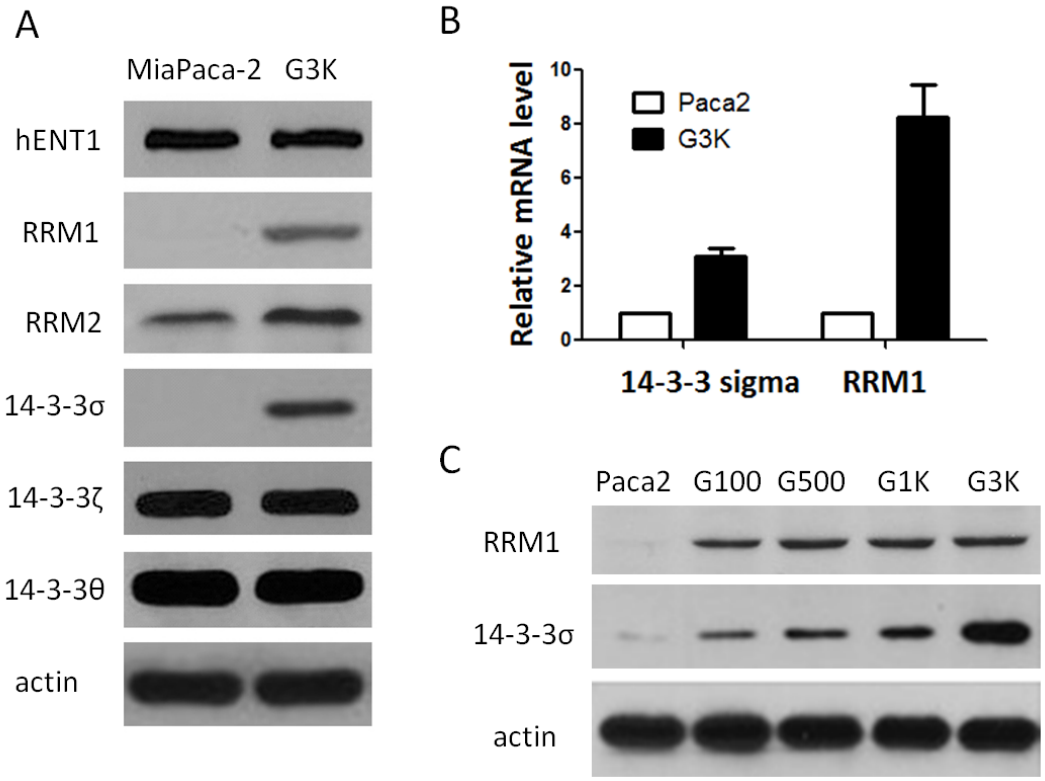
B. Ribonucleotide reductase and 14-3-3σ are up-regulated in G3K cells.

The finding that G3K cells are cross-resistant to Ara-C but lack of cross-resistance to multiple other anticancer drugs prompted us to investigate if any known mechanisms of gemcitabine resistance are up-regulated in G3K cells. These mechanisms include but are not limited to over-expression of hENT1, ribonucleotide reductase RRM1 and RRM2 [25, 30, 171]. The expression level of 14-3-3σ was also examined because of its association with intrinsic gemcitabine resistance [46]. As shown in **Figure 5A**, the protein level of hENT1 in G3K cells remains the same as in MiaPaca-2 cells, as determined by Western blot analysis. However, the expression of RRM1, RRM2, and 14-3-3σ are drastically up-regulated in G3K cells compared with the parental MiaPaca-2 cells. Interestingly, the other members of the 14-3-3 protein family, 14-3-3θ and 14-3-3ζ, did not increase in expression in the G3K cells.

To further validate the increased expression of RRM and 14-3-3σ, I next performed real time RT-PCR analysis. As shown in **Figure 5B**, the mRNA levels of both RRM1 and 14-3-3σ are significantly increased in G3K cells compared to MiaPaca-2 cells, up to ~3.1 fold and ~8.3 fold increase respectively. I also found that the expression of RRM1 and 14-3-3σ were up-regulated early during the selection process by testing the MiaPaca-2 cells with intermediate level of resistance generated during stepwise selections (**Figure 5C**). Although RRM1 remained in the same up-regulated level in all intermediate cells and the final G3K cells, 14-3-3σ appears to be further up-regulated in

G3K cells compared to the other preceding intermediate cells. This observation suggests that up-regulation of both RRM1 and 14-3-3σ may occur as an early event of acquired gemcitabine resistance.

Figure 5. Ribonucleotide reductase and 14-3-3σ are up-regulated in G3K cells. (A) Western blot analysis of hENT1, RRM1 and 2, 14-3-3σ, ζ, and θ expression in both MiaPaca-2 and G3K cells. (B) Real time RT-PCR analysis of RRM1 and 14-3-3σ mRNA in MiaPaca-2 and G3K cells (N=3, $p<0.001$). (C) Western blot analysis of RRM1 and 14-3-3σ in the intermediate gemcitabine-resistant cells G100, G500, and G1K. Beta-actin was used as a loading control for Western blot analyses and GAPDH was used as an internal control for PCR analyses.



C. 14-3-3σ over-expression contributes to acquired gemcitabine resistance.

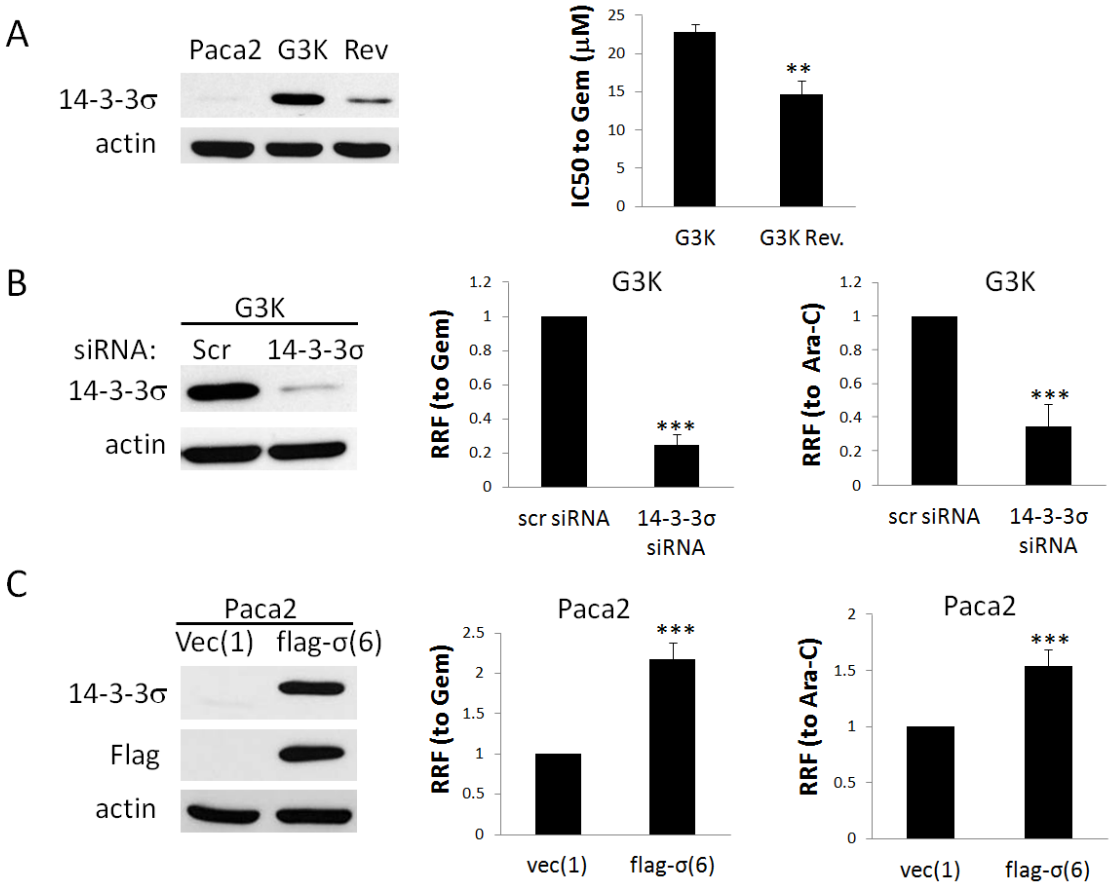
Because RRMs are well known contributors to acquired gemcitabine resistance [25, 30, 171], I chose to further investigate the potential contribution of 14-3-3σ to the acquired gemcitabine resistance in G3K cells. Although 14-3-3σ has been suggested to contribute to intrinsic gemcitabine resistance [46] and high 14-3-3σ levels correlate with resistance, there are no studies on its potential role in acquired gemcitabine resistance. For this purpose, I first knocked down the expression of 14-3-3σ in G3K cells using siRNA and tested if 14-3-3σ was involved in gemcitabine and Ara-C resistance in G3K cells. As shown in **Figure 6B**, 14-3-3σ was successfully knocked down in G3K cells by siRNA and the reduced 14-3-3σ expression was accompanied with ~80% reduction in gemcitabine resistance. Ara-C resistance was also reduced by ~65% by 14-3-3σ knockdown. Thus, 14-3-3σ over-expression in G3K cells may contribute to both gemcitabine and Ara-C resistance.

To further verify the role of 14-3-3σ in acquired gemcitabine resistance, I established a stable MiaPaca-2 cell line with over-expression of ectopic 14-3-3σ. **Figure 6C** shows the stable over-expression of ectopic 14-3-3σ in MiaPaca-2 cells and the significantly increased resistance of the stable cells to both gemcitabine and Ara-C, with ~2.2 fold and ~1.5 fold increase respectively, confirming that 14-3-3σ over-expression causes gemcitabine and Ara-C resistance.

To validate the role of 14-3-3σ in acquired gemcitabine resistance in G3K cells, I created a partially revertant cell line, G3KRev, by continuously culturing G3K cells in the absence of gemcitabine selection for 6 months and tested the level of gemcitabine resistance and 14-3-3σ expression. As shown in **Figure 6A**, G3KRev cells have a significantly lower IC_{50} to gemcitabine than the G3K cells (14.6±1.8 μM vs 22.7±1.1 μM). The expression level of 14-3-3σ in the G3KRev cells is also reduced compared with that of the G3K cells. Taken together, I conclude that the up-regulated 14-3-3σ likely contributes to the acquired gemcitabine resistance in G3K cells and that both the up-regulated 14-3-3σ expression and gemcitabine resistance are partially reversible in vitro.

Figure 6. 14-3-3σ up-regulation contributes to gemcitabine and Ara-C resistance. (A) Association of 14-3-3σ expression with gemcitabine resistance. Expression of 14-3-3σ and IC_{50} to gemcitabine in MiaPaca-2, G3K and the revertant G3KRev cells were determined using Western blot analysis and MTT assay, respectively. (N=4, **p<0.01). (B) 14-3-3σ knockdown reduces gemcitabine and Ara-C resistance in G3K cells. G3K cells were transiently transfected with 14-3-3σ siRNA (Si) or scrambled control siRNA (Scr) followed by Western blot analysis of 14-3-3σ expression and MTT analysis of cellular response to gemcitabine and Ara-C. RRF, relative resistance factor=$IC_{50(Si \text{ or } 14\text{-}3\text{-}3\sigma)}/IC_{50(Scr \text{ or } Vec)}$, (N=3-4, ***p<0.001). (C) 14-3-3σ over-expression in MiaPaca-2 cells causes gemcitabine and Ara-C resistance. Stable MiaPaca-2 cells with 14-3-3σ over-expression (flag-σ(6)) or transfected with vector control (Vec(1)) were established and subjected to Western blot analysis and MTT analysis of cellular response to gemcitabine and Ara-C. (N=3-4, ***p<0.001).

Figure 6 (cont).



D. Differential methylation of 14-3-3σ gene in MiaPaca-2 and G3K cells.

The 14-3-3σ gene was found frequently hypermethylated and thus its expression was suppressed in several cancer cells [54, 98, 101-104]. Thus, I hypothesize that the 14-3-3σ gene in the parental MiaPaca-2 cells may be hypermethylated and 14-3-3σ expression remains low. Following gemcitabine selection the methylation status of the 14-3-3σ gene may be altered, resulting in increased transcription and expression of 14-3-3σ. To test this hypothesis, I first treated the parental MiaPaca-2 cells with a well-known demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-dC, decitabine) that inhibits DNA methyltransferases, and determined 14-3-3σ expression level using Western blot. As shown in **Figure 7A**, 5-Aza-dC treatment increased 14-3-3σ expression in a dose-dependent manner. Thus, the 14-3-3σ gene is likely silenced in the parental MiaPaca-2 cells by methylation and reactivated in G3K cells.

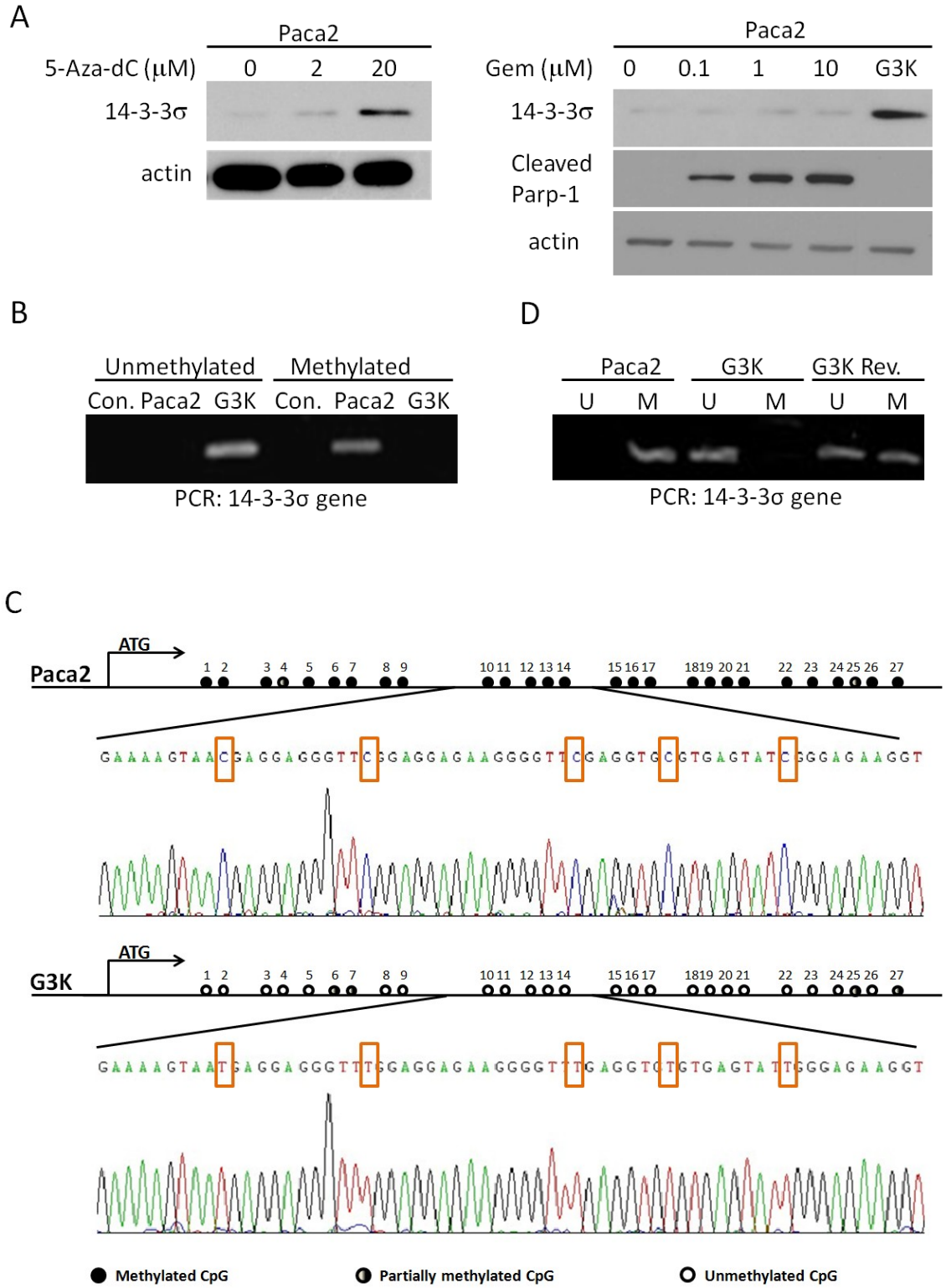
Although it has been reported that gemcitabine does not possess the pyrimindine ring modification at position 5, which is responsible for inhibition of DNA methyltransferases (DNMTs) and, thus, do not inhibit DNA methylation [172], a recent study showed that gemcitabine reactivated several epigenetically silenced genes possibly by inhibiting DNMT1 [173]. Thus, it is possible that treatment with gemcitabine during selection reactivated 14-3-3σ gene expression. To test this possibility, I treated the parental MiaPaca-2 cells with different concentrations of gemcitabine followed by Western blot analysis of 14-3-3σ expression. As shown in **Figure 7A**, unlike 5-Aza-dC,

gemcitabine treatment did not increase 14-3-3σ expression. Thus, reactivation of 14-3-3σ gene in G3K cells is unlikely due to direct gemcitabine-induced demethylation.

Next, I compared the methylation status of the 14-3-3σ gene in G3K and the parental MiaPaca-2 cells using methylation-specific PCR (MSP). As shown in **Figure 7B**, 14-3-3σ gene in the parental MiaPaca-2 cells was amplified only by primers for methylated sequences whereas in G3K cells it was amplified only by primers for unmethylated sequences. Thus, the 14-3-3σ gene in the parental MiaPaca-2 cells is heavily methylated whereas it is unmethylated in G3K cells. Analysis of the first exon with 27 CpG dinucleotides in the 14-3-3σ gene that are known to be methylated in cancer cells [54] using sodium bisulfite sequencing shows that 25 of these CpG dinucleotides in the parental MiaPaca-2 cells are fully methylated and the remaining 2 are partially methylated (**Figure 7C**). However, in G3K cells 23 of the 27 CpG dinucleotides are unmethylated and the remaining 4 are partially methylated. Clearly, the methylations of the 14-3-3σ gene in the parental MiaPaca-2 cells have been removed in G3K cells.

Figure 7. Methylation status of 14-3-3σ gene in MiaPaca-2 and G3K cells. (A) Effect of 5-Aza-dC or gemcitabine treatment on 14-3-3σ expression. MiaPaca-2 cells were treated with increasing concentrations of 5-Aza-dC or gemcitabine for 5 days or 72hrs respectively, followed by Western blot analysis of 14-3-3σ, cleaved Parp-1 and actin as a loading control. (B) Methylation-specific PCR (MSP) analysis of MiaPaCa-2 and G3K cells. Con., control MSP without genomic DNA input. (C) Sodium bisulfite sequencing analysis of MiaPaCa-2 and G3K cells. The 27 CpG islands in the first exon of 14-3-3σ gene is shown with solid circles for fully methylated, open circles for unmethylated, and half filled circles for partially methylated CpG dinucleotides. The sequence profile containing CpG dinucleotides #10-14 is shown for both MiaPaca-2 and G3K cells. (D) Methylation status of 14-3-3σ gene in the revertant G3KRev cells. MSP were used to determine the methylation status of 14-3-3σ gene in G3KRev cells as with MiaPaca-2 and G3K cells as controls. U, primers for unmethylated; M, primers for methylated.

Figure 7 (cont).



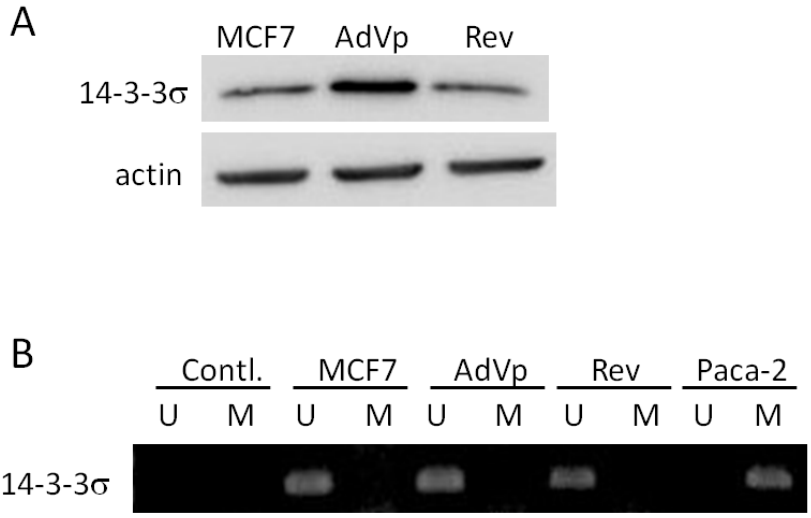
E. Demethylation of the 14-3-3σ gene is reversible.

As shown above, the increased expression of 14-3-3σ in G3K cells is partially reversed in the revertant G3KRev cells. The partial reversion in 14-3-3σ expression may be due to partial reversal of the methylation status of the 14-3-3σ gene. To test this possibility, I performed MSP of isolated genomic DNAs from the partially revertant G3KRev cells as described above. **Figure 7D** shows that the 14-3-3σ gene can be amplified by primers for both methylated and unmethylated sequences, indicating that the 14-3-3σ gene in G3KRev cells is likely partially reversed and that some cells have restored the methylation of their 14-3-3σ gene while others retains the demethylated 14-3-3σ gene. Thus, demethylation of the 14-3-3σ gene is likely partially reversible. The incomplete reversal of 14-3-3σ gene methylation following removal of gemcitabine suggests that the increased 14-3-3σ expression in G3K cells is unlikely due to direct gemcitabine-induced demethylation, consistent with the observation shown in **Figure 7A**.

Previously, up-regulation of 14-3-3σ expression was also observed in an Adriamycin-selected breast cancer cell line MCF7/AdVp3000 and it was decreased in the revertant MCF7/AdVp3000/Rev cells [63] (see also **Figure 8A**). However, the mechanism of 14-3-3σ regulation in these cell lines is not yet known. To determine if gene methylation is involved in regulating 14-3-3σ expression in these cells, I performed MSP of isolated genomic DNAs from the parental MCF7, Adriamycin-selected

MCF7/AdVp3000, and the revertant MCF7/AdVpG3K/Rev cells with MiaPaca-2 cells as a positive control. As shown in **Figure 8B**, while the 14-3-3σ gene in MiaPaca-2 cells can be amplified only using primers for methylated sequences, in all breast cancer cells it can be amplified only using primers for the unmethylated sequences. Thus, up-regulation of 14-3-3σ expression in MCF7/AdVp3000 cells and its reversal in MCF7/AdVp3000/Rev cells is unlikely due to changes in methylation status of the 14-3-3σ gene.

Figure 8. 14-3-3σ up-regulation in Adriamycin-selected MCF7/AdVp3000 cells is not due to gene demethylation. (A) Western blot analysis of 14-3-3σ expression in MCF7, MCF7/Advp3000 (AdVp), and MCF7/AdVp/Rev (Rev) cells. Actin was used as a loading control. (B) MSP analysis of 14-3-3σ gene in MCF7, MCF7/Advp3000 (AdVp), and MCF7/AdVp/Rev (Rev) cells with MiaPaCa-2 cells as a control. U, primers for unmethylated; M, primers for methylated. Contl., control MSP without genomic DNA input.



F. Uhrf1 and DNMT1 play important roles in regulating 14-3-3σ expression.

To determine what regulates the reversible methylation of 14-3-3σ gene in MiaPaca-2 and G3K cells, I first compared the expression level of enzymes important for DNA methylation among the parental MiaPaca-2, gemcitabine resistant G3K, and the partially revertant G3KRev cells using Western blot or real-time RT-PCR. As shown in **Figure 9A**, while DNMT3a and DNMT3b were increased in G3K and remained high in G3KRev cells, DNMT1 is increased in G3K but reduced back to the basal level in G3KRev cells. The expression pattern of these methyltransferases is peculiar and inconsistent with the expression pattern of 14-3-3σ in MiaPaca-2, G3K, and G3KRev cells.

Another protein of importance in gene methylation, Uhrf1, has been shown to play a major role in recruiting DNMTs [174] and loss of Uhrf1 results in 75% reduction in genomic methylation [175]. Thus, I tested the expression pattern of Uhrf1. As shown in **Figure 9A**, Uhrf1 protein is reduced in G3K cells and increased in the G3KRev cells. The profile of Uhrf1 expression in these cells is consistent with the partially reversible change in 14-3-3σ expression and gene methylation in these cells. The consequence of Uhrf1 binding is recruitment of DNMT1 and histone deacetylase 1, resulting in methylation of newly synthesized DNA strands [124, 126-128]. Thus, reduced Uhrf1 expression in G3K cells is likely responsible for reduced methylation of 14-3-3σ gene by reducing recruitment of DNMT1 protein to the methylated CpG islands despite the presence of high level of DNMT1. In MiaPaca-2 cells, however, the high level of Uhrf1

may efficiently recruit enough DNMT1 despite low DNMT1 level for efficient methylation of 14-3-3σ gene. If so, knocking down either Uhrf1 or DNMT1 in MiaPaca-2 cells would effectively reduce the level of the recruiter (Uhrf1) or the pool of DNMT1 to be recruited for methylation of 14-3-3σ gene and consequently increase 14-3-3σ expression. Furthermore, the slight increase of Uhrf1 expression in the G3KRev cells may be responsible for the increased methylation and reduced expression of 14-3-3σ gene. In addition, due to the fact that Uhrf1 has been shown to regulate p21 expression [132], I next tested the expression of p21 in MiaPaca-2, G3K, and G3KRev cells. As shown in **Figure 9A**, p21 protein level shows an expression profile in these cells similar to that of 14-3-3σ, consistent with the possible regulatory role of Uhrf1 in these cells.

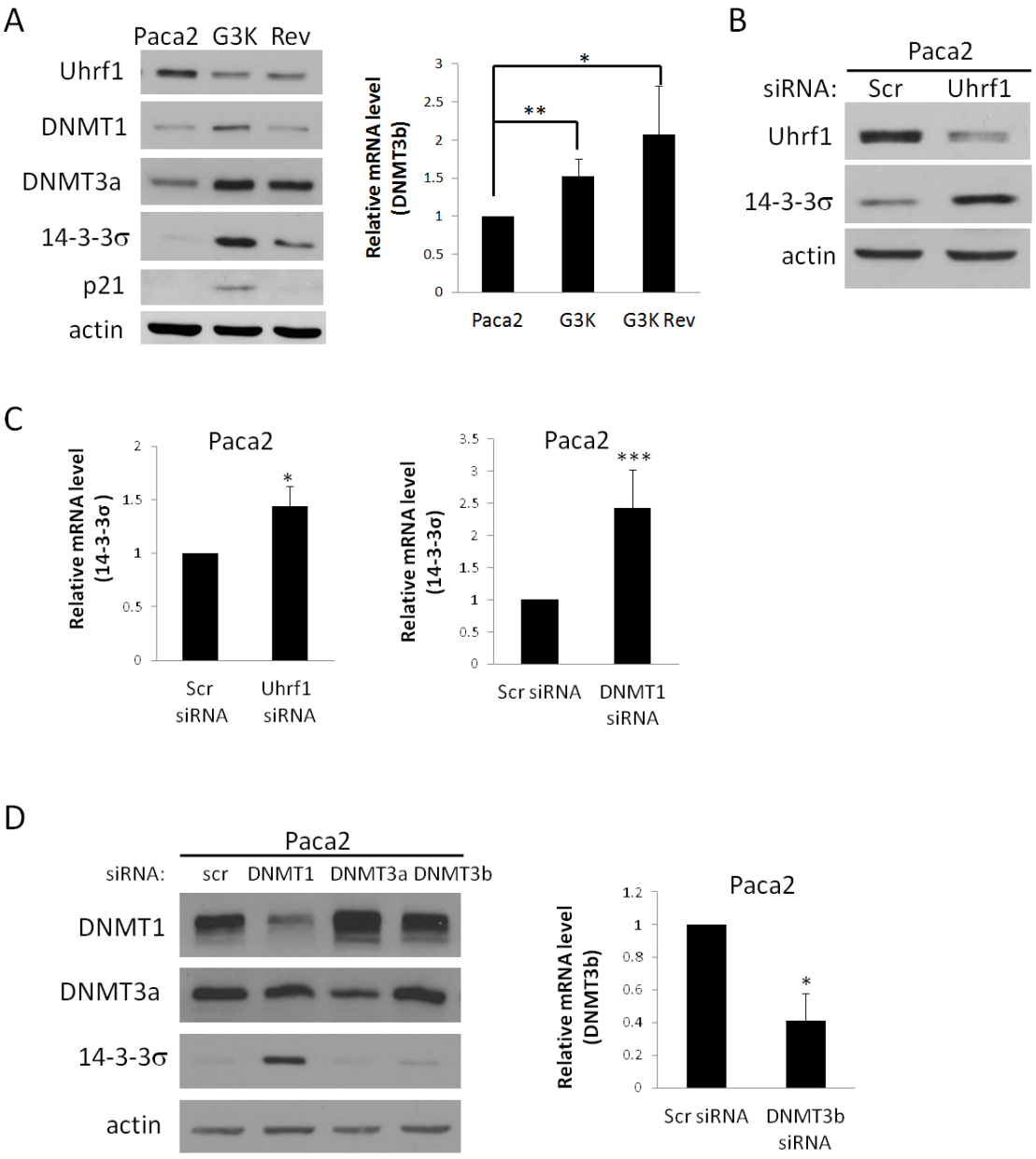
To determine if the reduced Uhrf1 expression is possibly responsible for increased expression of the 14-3-3σ gene in G3K cells, I knocked down Uhrf1 in the parental MiaPaca-2 cells using siRNA and tested its effect on 14-3-3σ expression. **Figure 9B** shows that 14-3-3σ protein level is dramatically increased by Uhrf1 knockdown. This observation is confirmed by real time RT-PCR analysis of 14-3-3σ mRNA, with ~1.4 fold and ~2.4 fold increase of 14-3-3σ mRNA by Uhrf1 and DNMT1 knockdown, respectively (**Figure 9C**).

To determine if DNMTs also contribute to 14-3-3σ expression regulation, I performed similar experiment by knocking down DNMT1, DNMT3a, and DNMT3b in MiaPaca-2 cells. **Figure 9D** shows successful knockdown of DNMT1, DNMT3a, and

DNMT3b as determined using Western blot or real-time RT-PCR. However, only DNMT1 knockdown significantly up-regulated the protein level of 14-3-3σ. DNMT3a and DNMT3b knockdown did not appear to affect 14-3-3σ expression. The effect of DNMT1 knockdown on 14-3-3σ expression was also confirmed by real time RT-PCR analysis (**Figure 9C**). Taken together, I conclude that likely both Uhrf1 and DNMT1 play important roles in regulating 14-3-3σ expression.

Figure 9. Role of Uhrf1 and DNMT1 in 14-3-3σ expression. (A) Western blot analysis of Uhrf1, DNMT1, DNMT3a, and p21 expression as well as real-time RT-PCR analysis of DNMT3b expression in MiaPaca-2, G3K, and G3KRev cells. (B-D) Effect of Uhrf1, DNMT1, DNMT3a and DNMT3b knockdown on 14-3-3σ expression. MiaPaca-2 cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting Uhrf1 (B), DNMT1, DNMT3a, and DNMT3b (D) followed by Western blot analysis of Uhrf1, DNMT1, DNMT3a, and 14-3-3σ protein levels (B, D) or real time RT-PCR analysis of DNMT3b and 14-3-3σ mRNA levels (C-D). (N=4-5, *$p<0.05$, ***$p<0.001$). Actin and GAPDH were used as a loading control for Western blot and internal control for PCR analyses, respectively.

Figure 9 (cont).



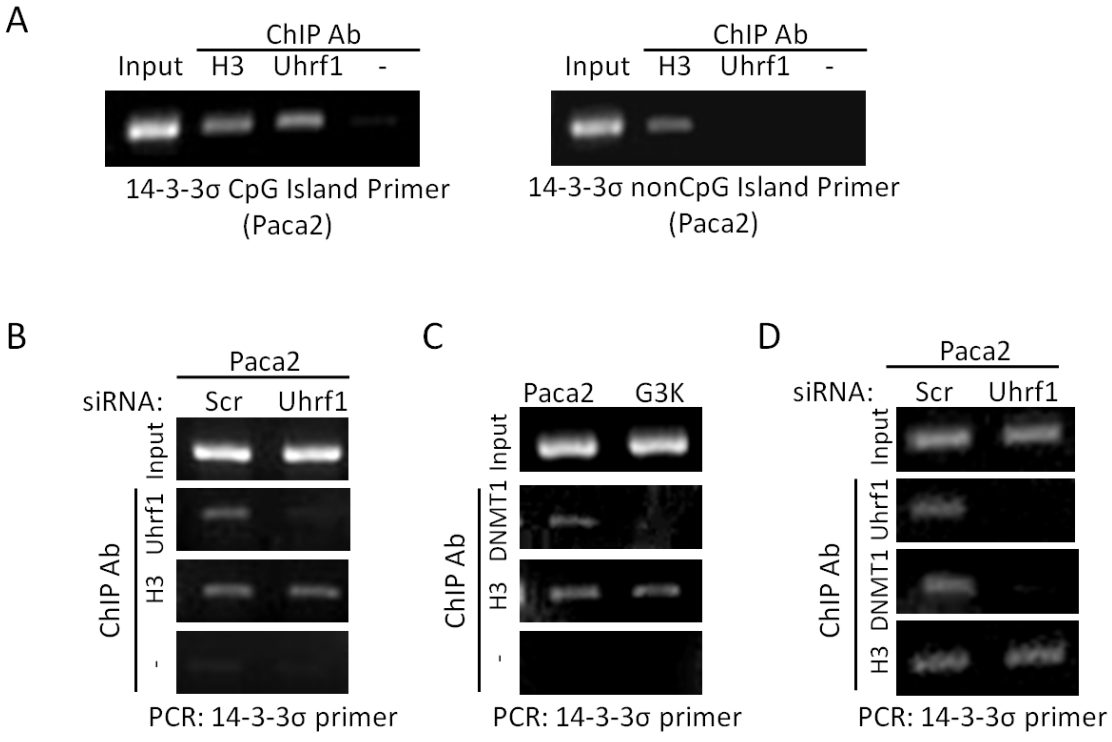
G. Uhrf1 binds and helps recruit DNMT1 to methylated region of 14-3-3σ gene.

To further determine the role of Uhrf1 and DNMT1 in regulating 14-3-3σ expression and gene methylation, I first determined if Uhrf1 binds to the methylated CpG islands in the first exon of 14-3-3σ gene in MiaPaca-2 cells using ChIP assay. As shown in **Figure 10A**, Uhrf1 was bound to the CpG islands but not to the CpG-free sequences in the promoter region of the 14-3-3σ gene in MiaPaca-2 cells. Knocking down Uhrf1 also reduced Uhrf1 binding to the CpG islands of 14-3-3σ gene (**Figure 10B**). Thus, Uhrf1 likely can bind to the methylated CpG island sequences of the 14-3-3σ gene.

As shown in **Figure 9A**, DNMT1 expression was found increased in G3K cells, which is peculiar since methylation of 14-3-3σ gene in G3K cells is reduced. However, because Uhrf1 binding to CpG island sequences helps recruit DNMT1 to maintain the methylation status of the DNA, it is possible that the reduced Uhrf1 expression in G3K cells reduces DNMT1 recruitment despite the higher level of DNMT1 in G3K cells. To test this possibility, I first compared the binding of DNMT1 to the CpG island sequences of the 14-3-3σ gene between MiaPaca-2 and G3K cells. As shown in **Figure 10C**, binding of DNMT1 to the CpG island sequences of 14-3-3σ gene is indeed much less in G3K than in MiaPaca-2 cells despite the higher expression level of DNMT1 in G3K cells. Importantly, Uhrf1 knockdown in MiaPaca-2 cells dramatically reduced DNMT1 binding to the CpG island sequences of the 14-3-3σ gene (**Figure 10D**), indicating that DNMT1 could not be effectively recruited to the CpG island sequences of the 14-3-3σ gene in the absence of

Uhrf1, and hence Uhrf1 is the recruiter of DNMT1. Thus, I conclude that Uhrf1 likely plays a major role in regulating 14-3-3σ expression by binding to its CpG-rich sequences and helps recruit DNMT1 to the site to reversibly methylate the 14-3-3σ gene during replication. The reduced Uhrf1 expression by gemcitabine selection in G3K cells likely decreased DNMT1 recruitment and methylation of 14-3-3σ gene while the slightly increase in Uhrf1 level in G3KRev cells is responsible for partial reversal of 14-3-3σ gene methylation.

Figure 10. Binding of Uhrf1 and DNMT1 to the CpG islands of the 14-3-3σ gene. (A) ChIP analysis of Uhrf1 binding to the CpG island sequences or nonCpG island sequences of 14-3-3σ gene. (B, D) Effect of Uhrf1 knockdown on Uhrf1 and DNMT1 binding to the CpG island sequences of 14-3-3σ gene in MiaPaca-2 cells. MiaPaca-2 cells were transiently transfected with scrambled control or Uhrf1 siRNAs followed by ChIP analysis of Uhrf1 and DNMT1 binding. (C) DNMT1 binding to the CpG island sequence of 14-3-3σ gene in G3K cells is less than that in MiaPaca-2 cells. ChIP with histone H3 antibody or without any primary antibody were used as positive and negative controls, respectively. Normal IgG was also used as negative control, which did not immunoprecipitate any DNA (data not shown).



H. Gemcitabine treatment does not affect Uhrf1 and DNMT1 expression.

Finally, to investigate if the altered expression of Uhrf1 and DNMT1 in G3K cells is potentially due to gemcitabine treatments during selection, I performed a Western blot analysis to detect both Uhrf1 and DNMT1 expression in MiaPaca-2 cells following treatments with gemcitabine at different concentrations for 72 hrs. As shown in **Figure 11**, gemcitabine treatments had no effect on Uhrf1 and DNMT1 expression. Therefore, the mechanism of regulation of Uhrf1 and DNMT1 expression in the drug resistant cells remains to be determined.

Overall, this section of my thesis demonstrated that 14-3-3σ over-expression contributed to acquired gemcitabine resistance and that the up-regulated 14-3-3σ expression was caused by lack of gene methylation regulation during gemcitabine selection. As summarized in **Figure 12**, Uhrf1 and DNMT1 bind to the methylated region of 14-3-3σ gene in parental MiaPaca-2 cells to maintain the methylation status, whereas during gemcitabine selection, the expression of Uhrf1 is dramatically decreased and thus insufficient to recruit DNMT1 to the site to methylate the DNA, resulting in 14-3-3σ gene demethylation and re-expression. After gemcitabine removal, however, the slight increase of Uhrf1 expression likely functions to recruit the methylation machinery to restore the methylation of 14-3-3σ gene.

Figure 11. Effect of gemcitabine treatment on Uhrf1 and DNMT1 expression. MiaPaca-2 were treated with/without increasing concentrations of gemcitabine, and G3K cells were used as control without treatment, followed by Western blot analysis of Uhrf1, DNMT1. Actin was used as a loading control.

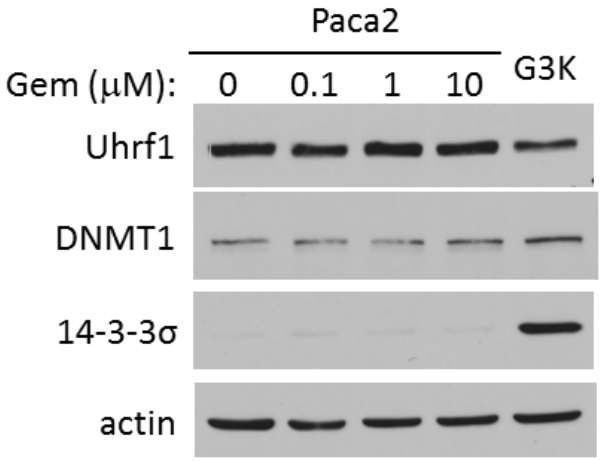
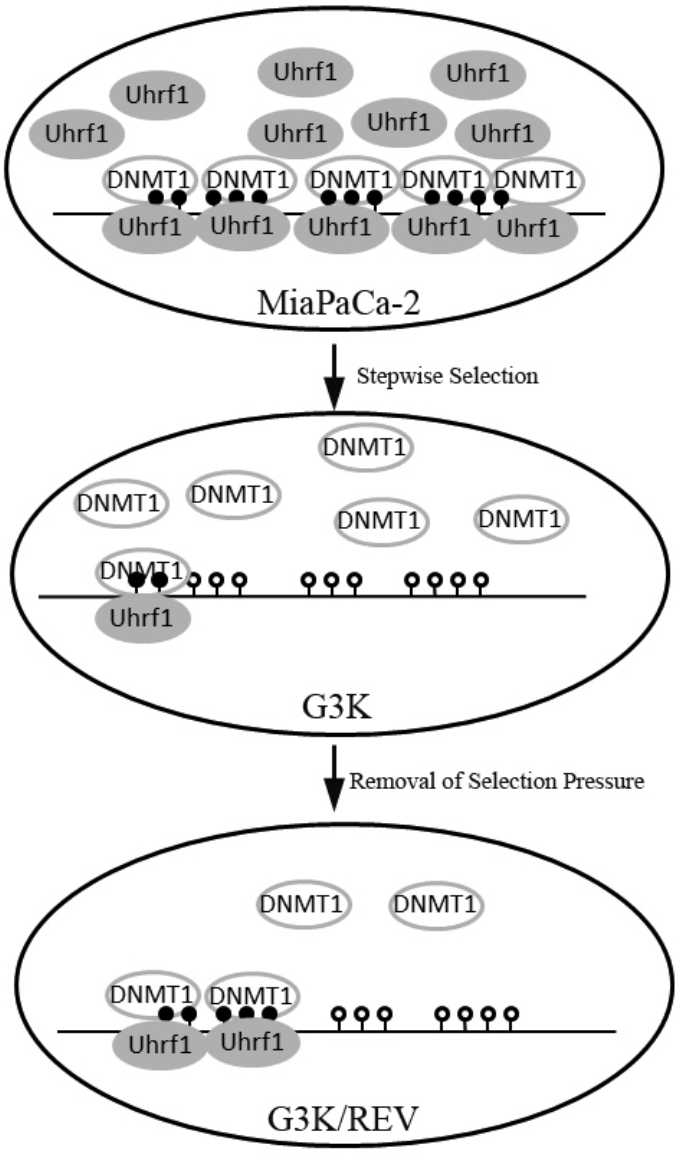


Figure 12. Schematic model of epigenetic regulation of 14-3-3σ gene following gemcitabine selection and drug removal. The relative expression levels of Uhrf1 and DNMT1, as well as their binding status to 14-3-3σ gene during gemcitabine selection or drug removal are shown in the model. Solid circles represent fully methylated CpG islands of 14-3-3σ gene, and open circles represent unmethylated CpGs.



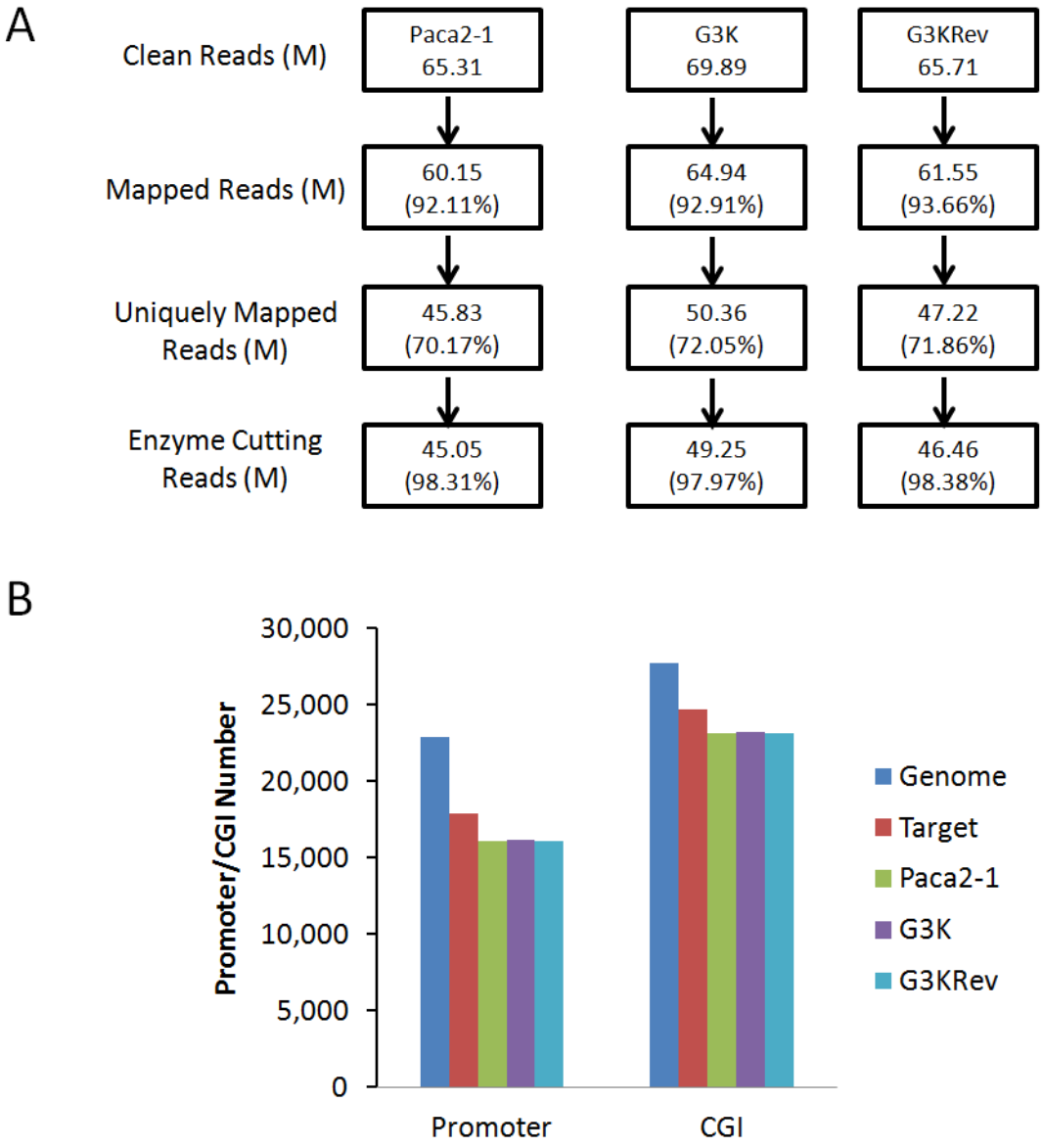
Section II: Identification of PDGFD as a potential contributor to acquired gemcitabine resistance by Reduced Representation Bisulfite Sequencing (RRBS)

A. Identification of reversibly-methylated genes using Reduced Representation Bisulfite Sequencing (RRBS).

The finding of demethylation of the 14-3-3σ gene in gemcitabine resistant cells led to a hypothesis that other genes may also have changed epigenetically following gemcitabine selection. To profile global changes in gene methylation in response to gemcitabine selection, genomic DNAs from MiaPaca-2, G3K, and G3KRev cells were isolated and subjected to RRBS sequencing, which yield genome-wide methylation profiles on a single nucleotide level, by BGI (Beijing Genomics Institute). The RRBS data were analysed in collaboration with Dr. Yunlong-Liu's lab.

As a result, the RRBS yields about 65~70 Million clean reads for each sample, with map rate of >92%, unique mapping rate of >70%, and >97% of unique reads with enzyme cutting site (**Figure 13A)**. Moreover, the RRBS targeted a reasonable proportion of genome of interest in all samples, with >70% of promoters and >83% of CG islands were covered respectively (**Figure 13B**). The individual CpG sites within CG islands and promoters were also examined and good depth coverage at 4X and 10X were achieved, and all samples showed consistent coverage (data not shown).

Figure 13. RRBS sequencing results comparison and characterization of genomic coverage. (A) RRBS sequencing results comparison. (B) Genomic coverage characterization of promoter and CG island (CGI) numbers.



According to RRBS outcome, 845 genes are differentially methylated between MiaPaca-2 and G3K cells, and 282 genes are differentially methylated between G3K and G3KRev cells. The list of these differentially methylated genes is shown in **Appendices**. Because the methylation changes in the promoter region most likely influence gene transcription and expression, I filtered these genes with differential methylation level in the promoter region and with a 2-fold change cutoff. As shown in **Figure 14**, 159 genes have increased methylation while 459 genes have decreased methylation in G3K cells compared with MiaPaca-2 cells. By comparing with G3K cells, G3KRev cells have 104 genes with increased and 88 genes with decreased methylation. Together, there are 65 genes that have reversible methylation changes from MiaPaca-2 to G3K and to G3KRev cells (**Table 2)**.

Figure 14. Flowchart of DMR filteration of genes. Genes with DMR p-value<0.01 were first filtered by DMR at the promoter regions, followed by further filter with 2-fold methylation level change.

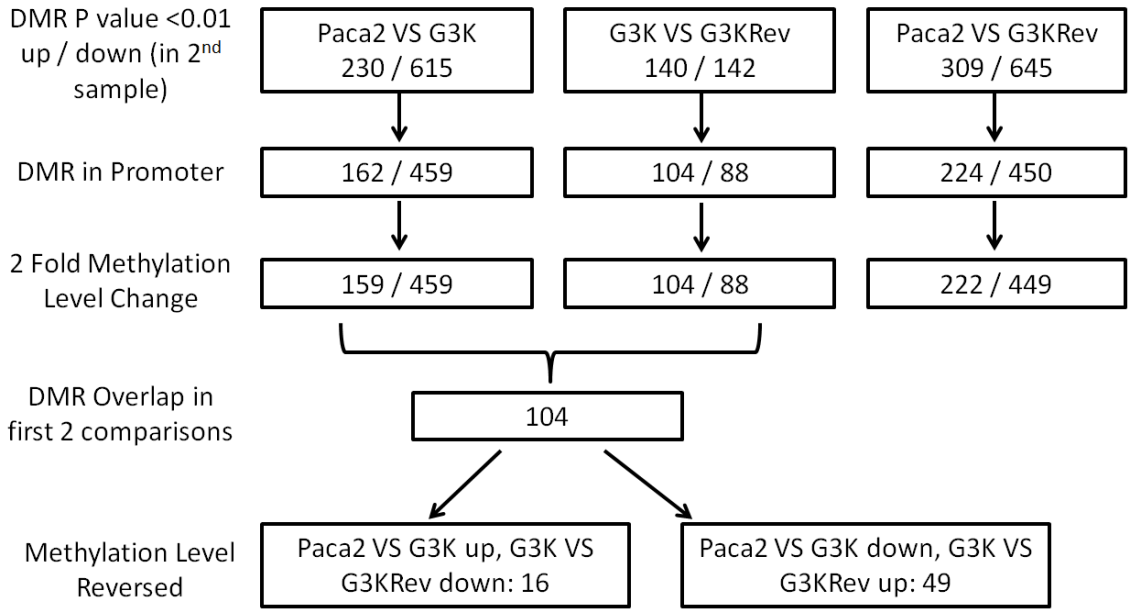


Table 2. List of reversibly methylated genes.

Paca2 vs G3K up, G3K vs G3KRev down	Paca2 vs G3K down, G3K vs G3KRev up
ADORA2B, **CDC42EP3**, CPNE7, FBXO27, JAG2, MIR1915, MRPS6, NPAS2, **OLIG1**, **PTPRG**, PWWP2B, SLC45A1, SNORD56B, SOX13, TNRC18, WNT9A	AFAP1L2, ANXA2P3, APBA1, CCNO, CDC42BPB, **CDK6**, CHRNA7, CRAMP1L, DDIT4L, DSE, **DUSP6**, EFNA5, ELOVL2-AS1, FAM133B, FRAS1, FUOM, GALNT7, GNS, GRM4, H2AFY2, JMJD8, KIAA1324L, KIF21B, LOC100506190, LONRF3, MCTP1, MDFIC, MIR205HG, MMP25, MTA1, NKX1-2, **PDGFD**, PRDM1, RAET1G, RASD2, RNASET2, SEMA5B, **SLC25A27**, SLC31A2, **SLC35D3**, SOWAHD, TMEM181, **TRPC3**, WDR1, WNT11, ZNF593, ZNF669, ZNF808, ZNF83

Among these 65 genes, 10 genes with the highest degree of methylation level change were selected for further analysis using real-time RT-PCR. **Figure 15** shows that the mRNA level of five genes (CDK6, SLC35D3, SLC25A27, PDGFD, and TRPC3) with decreased methylation increases in G3K cells compared with MiaPaca-2 cells. However, the other genes have different outcomes. While the mRNA level of DUSP6 with decreased methylation is reduced, the mRNAs of Olig1 and PTPRG with increased methylation are significantly increased and the mRNAs of Cdc42EP3 and Adora2B with increased methylation were not significantly affected in G3K cells (**Table 3**). Therefore, the methylation change of the genes in the promoter region may not be sufficient to predict change in the mRNA level of corresponding genes.

Figure 15. Identification of transcriptional changes in candidate genes. (A-B) Real time RT-PCR analysis of mRNA level of candidate genes including DUSP6, Olig1, Cdc42EP3, Adora2B, PTPRG, CDK6, SLC35D3, SLC25A27, PDGFD, and TRPC3 gene between MiaPaca-2 and G3K cells. GAPDH was used as internal control. (N=3-5, *p<0.05, **p<0.01, ***p<0.001). ↑: methylation is increased in G3K cells, ↓: methylation is decreased in G3K cells.

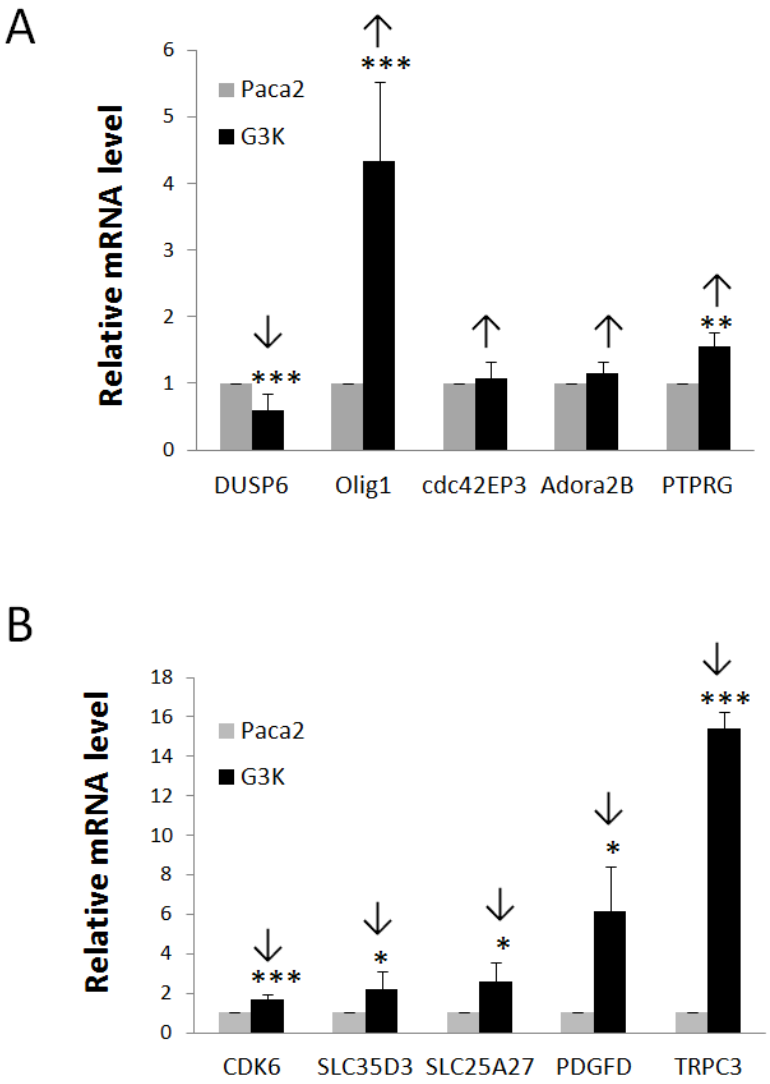


Table 3. Identification and characterization of candidate genes.

	DMR (G3K-MiaPaca-2)	mRNA level change (G3K/MiaPaca-2)	Significance
Negative correlation of methylation change with mRNA level change			
CDK6	-0.5	1.684548	p<0.001
SLC35D3	-0.57	2.171952	p<0.05
SLC25A27	-0.39	2.566402	p<0.05
PDGFD	-0.41	6.164361	p<0.05
TRPC3	-0.48	15.42079	p<0.001
Positive correlation of methylation change with mRNA level change			
PTPRG	0.21	1.551086	p<0.01
Olig1	0.34	4.334516	p<0.001
DUSP6	-0.41	0.596951	p<0.01
No correlation of methylation change with mRNA level change			
cdc42EP3	0.28	1.074495	NS
Adora2B	0.26	1.148135	NS

B. Reversible up-regulation of PDGFD.

Next, TRPC3 and PDGFD were selected for validation of their expression at protein level. Firstly, membrane fractions were prepared from both MiaPaca-2 and G3K cells for Western blot analysis of TRPC3. As shown in **Figure 16A**, TRPC3 protein level did not appear to change despite its mRNA level increased ~15 fold in G3K cells. Thus, TRPC3 mRNA level does not seem to correlate with its protein level. To determine PDGFD protein level, MiaPaca-2 and G3K cells were cultured in serum-free medium for 24 hrs, followed by collection of conditioned medium (CM) and TCA precipitation. The precipitated proteins were subjected to Western blot analysis. As shown in **Figure 16C**, PDGFD production was dramatically elevated in G3K cells compared with MiaPaca-2 cells.

To determine if increased PDGFD production in G3K cells was reversed in G3KRev cells, real-time RT-PCR analysis was performed. As shown in **Figure 17A**, the increased PDGFD mRNA level in G3K cells (~16 fold increase compared with MiaPaca-2 cells) was dramatically decreased in the G3KRev cells (only ~2.4 fold increase compared with MiaPaca-2 cells). Moreover, the Western blot analysis confirmed this finding (**Figure 17C**). Thus, the expression of PDGFD is reversibly up-regulated in G3K cells.

Figure 16. Identification of TRPC3 and PDGFD expression in MiaPaca-2 and G3K cells. (A) Western blot detection the expression level of TRPC3 protein in the cell membrane fraction. MRP3 was used as a loading control for membrane fraction. (B) The secreted proteins in MiaPaca-2 and G3K conditioned medium (CM) were precipitated by TCA precipitation method, followed by SDS-PAGD and coomassie blue staining. (C) The secretion of PDGFD is significantly increased in G3K cells. The secreted proteins in MiaPaca-2 and G3K conditioned medium (CM) were precipitated by TCA precipitation method, followed by Western blot detection of the expression of PDGFD.

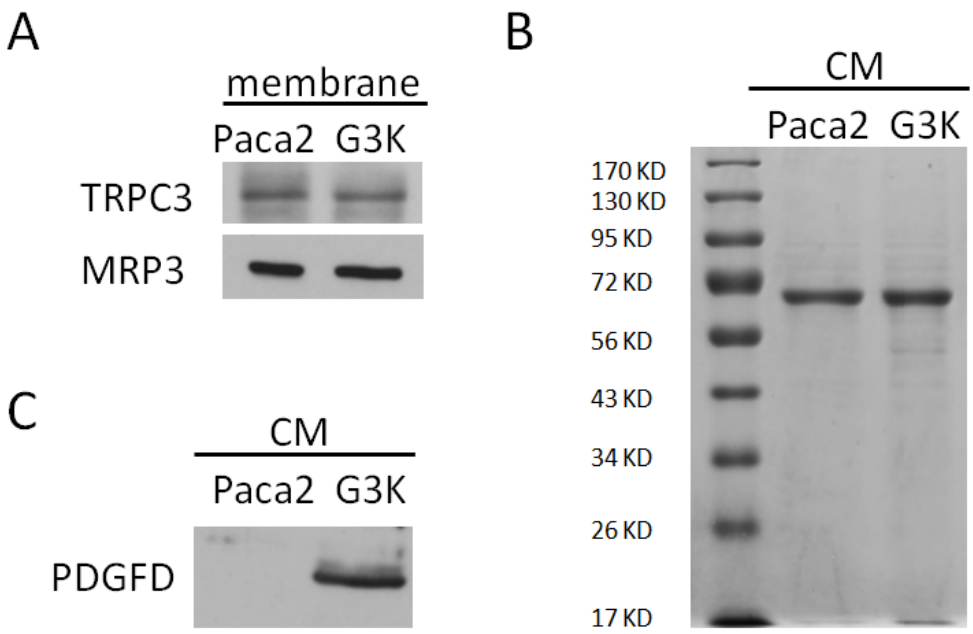
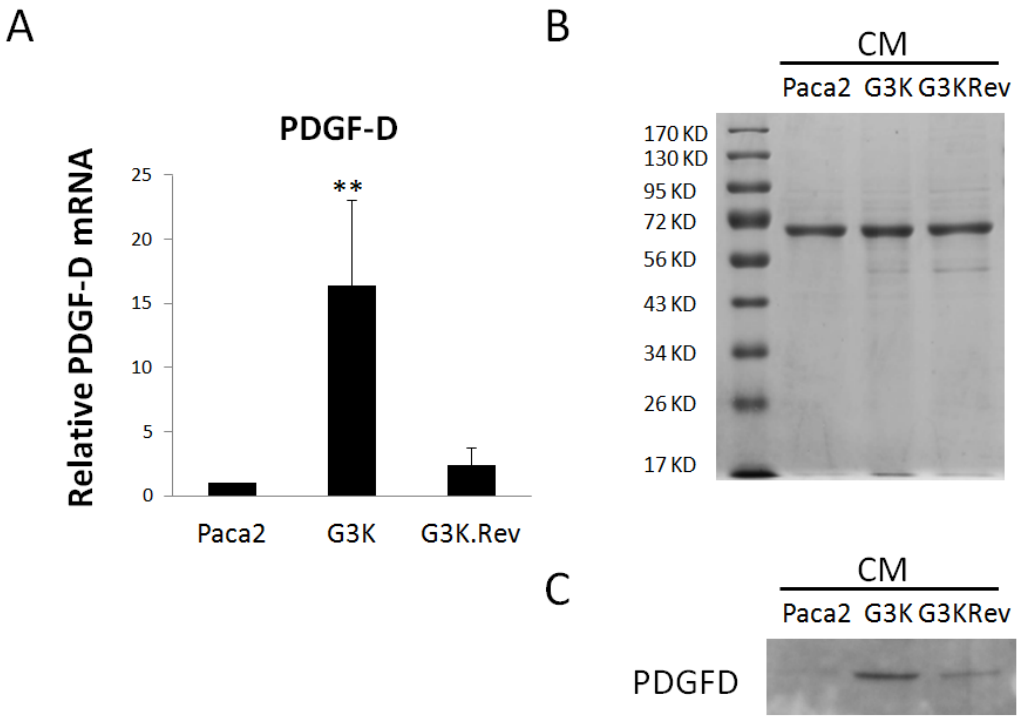


Figure 17. Reversible transcription and expression of PDGFD. (A) Real time RT-PCR analysis of PDGFD mRNA level in MiaPaca-2, G3K, and G3KRev cells. GAPDH was used as internal control. (N=4, **p<0.01). (B) The secreted proteins in MiaPaca-2, G3K, and G3KRev conditioned medium (CM) were precipitated by TCA precipitation method, followed by SDS-PAGD and coomassie blue staining. (C) PDGFD expression is reversibly increased in G3K cells. The secreted proteins in MiaPaca-2, G3K, and G3KRev conditioned medium (CM) were precipitated by TCA precipitation method, followed by Western blot detection of PDGFD expression.

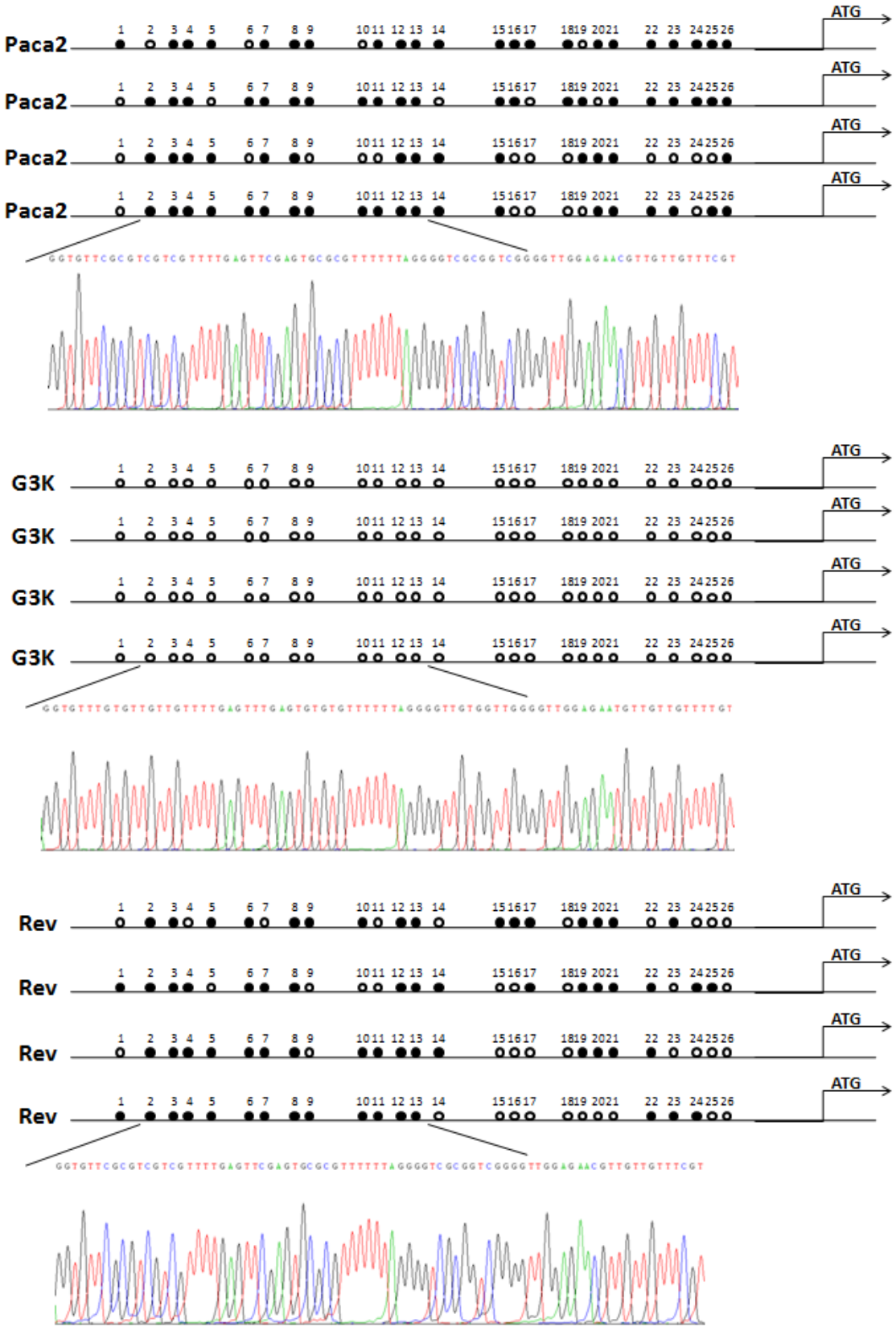


C. Validation of reversible methylation change of PDGFD gene.

To validate the RRBS data that suggest a reversible methylation change of PDGFD gene in G3K cells, I performed sodium-bisulfite gene sequencing analysis of the 26 CpG dinucleotides residing in the promoter region of PDGFD gene in all three cell lines. As shown in **Figure 18**, 14-22 out of 26 CpG dinucleotides were methylated in MiaPaca-2 cells. However, these CpG dinucleotides were all unmethylated in G3K cells. Interestingly, in G3KRev cells, 16-17 out of 26 CpG dinucleotides were again methylated. Thus, the methylation change of PDGFD promoter is reversible, and consistent with RRBS data.

Figure 18. Reversible methylation change of PDGFD gene. Sodium bisulfite sequencing analysis of PDGFD gene in MiaPaca-2, G3K, and G3KRev cells was conducted. Briefly, genomic DNA was treated with sodium bisulfite and the promoter region of PDGFD gene was amplified by PCR, followed by cloning into pGEM-T vector. 4 clones from each cell line were picked for sequencing. The 26 CpG islands in promoter region of PDGFD gene were shown with solid circles for fully methylated, open circles for unmethylated. The chromatogram represents sequence profile containing CpG dinucleotides #2-13 for all three cells.

Figure 18 (cont).



D. PDGFD up-regulation plays an important role in gemcitabine resistance.

It has been reported that PDGFD signaling is frequently deregulated in human malignancies with up-regulated expression in prostate, lung, renal, ovarian, brain, and pancreatic cancer [146, 147, 149-152]. However, its relationship with therapeutic drug resistance is limited to the discovery of its role in regulation of radiosensitivity in prostate adenocarcinoma, as well as its influence on sensitivity and resistance of glioblastoma to semustine [154, 158]. To determine if PDGFD potentially contributes to gemcitabine resistance in pancreatic cancer, I knocked down PDGFD in both G3K and Aspc-1 cells and performed the MTT assay to test its response to gemcitabine. **Figure 19A** and **Figure 19C** show knockdown of PDGFD in G3K and Aspc-1 cells at both protein and mRNA levels, respectively. As shown in **Figure 19B and Figure 19D**, the sufficient knockdown of PDGFD is accompanied with a dramatic reduction of gemcitabine resistance by 63.7%-76.5% in G3K and 46.4%-84.8% in Aspc-1 cells, respectively. Thus, PDGFD knockdown likely reduces gemcitabine resistance in both cells.

Next, to further test the influence of PDGFD over-expression on gemcitabine sensitivity, stable clones with PDGFD over-expression were generated from MiaPaca-2 cells. **Figure 20A-B** show increased PDGFD production in these clones compared with vector control clone by Western blot analysis of conditioned medium. Moreover, the increased production of PDGFD is accompanied with an increased resistance to gemcitabine by 4.8-4.9 fold (**Figure 20C**). Furthermore, MiaPaca-2 cells cultured in

conditioned medium from G3K cells are also more resistant to gemcitabine (by 2.4-fold increase averagely), than cells cultured with fresh medium (**Figure 20D**). Together, these results suggest that PDGFD plays an important role in gemcitabine resistance in human pancreatic cancers and that there may be bystander effect of resistant cells on sensitive cells.

Figure 19. PDGFD over-expression contributes to gemcitabine resistance in both G3K and Aspc-1 cells. (A, C) Western blot and PCR analysis of PDGFD knockdownin G3K and Aspc-1 cells. G3K (A) and Aspc-1 (C) cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting PDGFD followed by Western blot analysis of secreted PDGFD protein level, and RT-PCR analysis of the PDGFD mRNA level. GAPDH was used as an internal control for RT-PCR. (N=5-6, **p<0.01, ***p<0.001). (B, D) PDGFD knocking down in G3K and Aspc-1 cells reduces gemcitabine resistance. G3K (B) and Aspc-1 (D) cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting PDGFD followed by MTT assay for detection of the drug resistance. (N=3-4, **p<0.01, ***p<0.001).

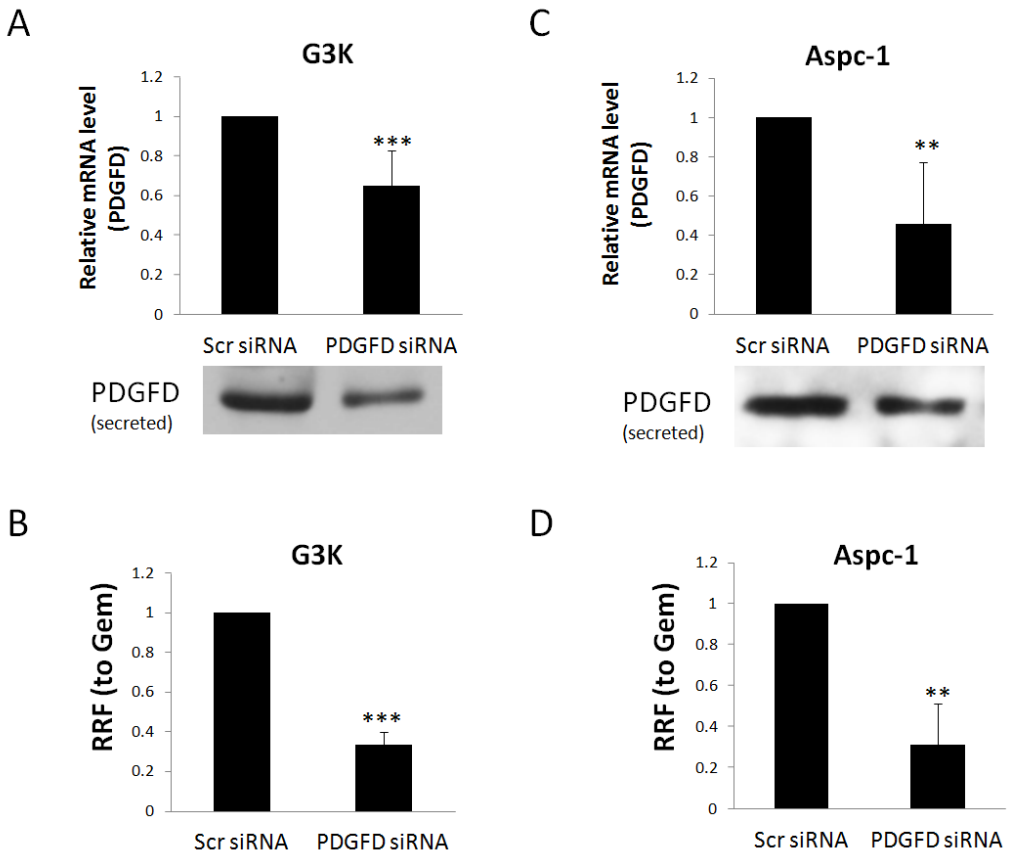
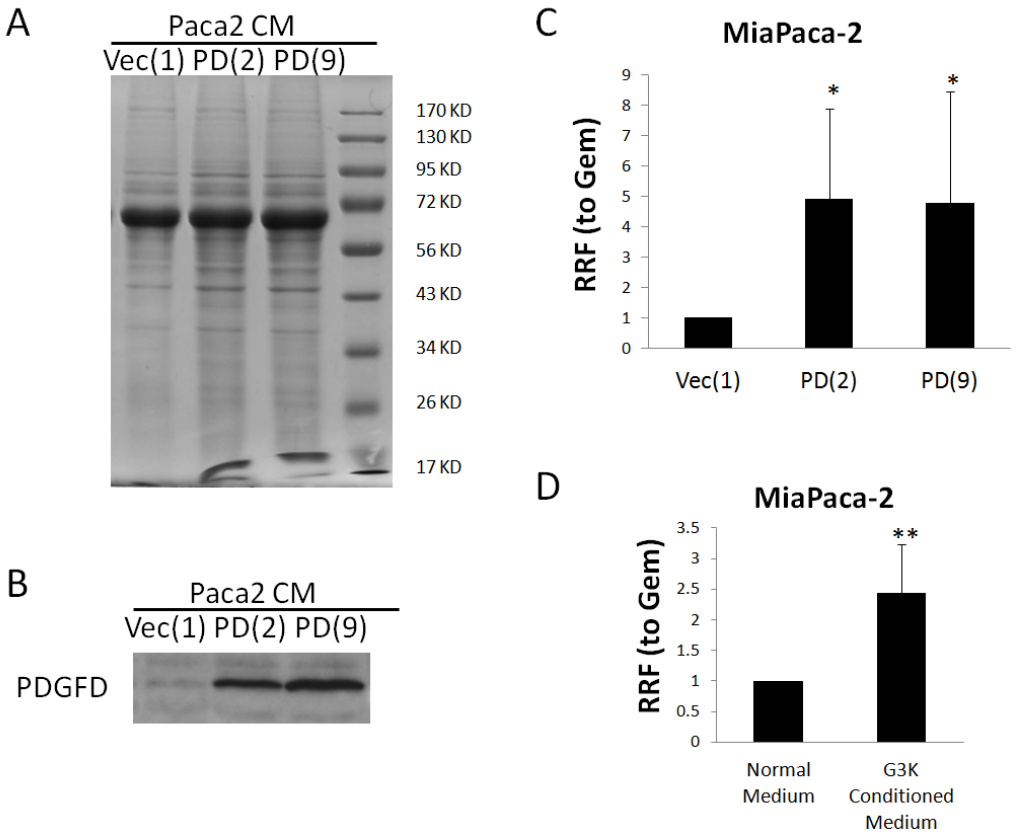


Figure 20. PDGFD over-expression leads to increased gemcitabine resistance in MiaPaca-2 cells. (A-B) MiaPaca-2 cells were stably transfected with vector control (vec(1)) or PDGFD plasmid (PD(2) and PD(9)) followed by SDS-PAGE analysis of total secreted proteins as a loading control (A) and Western blot analysis of PDGFD production (B). (C) PDGFD over-expression increases gemcitabine resistance. MiaPaca-2 cells were stably transfected with vector control (vec(1)) or PDGFD plasmid (PD(2) and PD(9)) followed by MTT assay for detection of gemcitabine resistance. (N=5, *p<0.05). (D) Bystander effect of resistant cells on sensitive cells. MiaPaca-2 cells were cultured in either fresh medium or G3K conditioned medium, followed by MTT assay for detection of gemcitabine resistance. (N=5, **p<0.01).



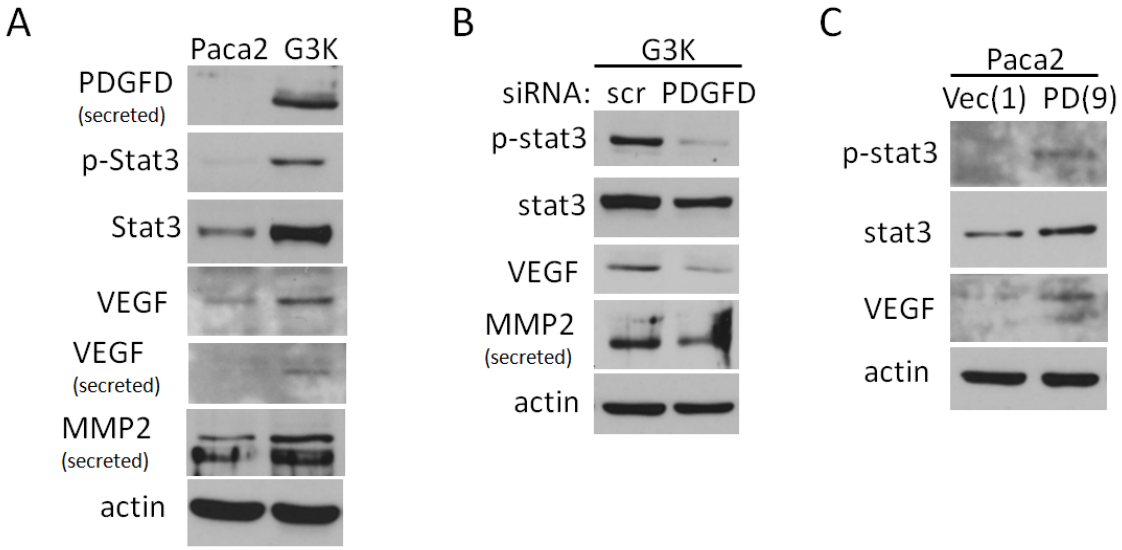
E. PDGFD over-expression contributes to gemcitabine resistance possibly by regulating STAT3 signaling pathway.

It has been reported that PDGFD activation triggers a number of downstream signaling pathways including activation of phosphatidylinositol 3 kinase (PI3K), Akt, nuclear factor-κB (NF-κB), Notch, and extracellular signal-regulated kinase (ERK) [139, 145-147]. It has also been reported that STAT3 is up-regulated in several drug-selected cancer cells including acquired resistance to erlotinib, vemurafenib, temozolomide, cisplatin, and paclitaxel [176-180]. Thus, I hypothesize that PDGFD over-expression may contribute to gemcitabine resistance by regulating STAT3 signaling pathway.

To test this hypothesis, I first compared the activation of STAT3 signaling pathway in MiaPaca-2 and G3K cells. As shown in **Figure 21A**, both the p-stat3 and total Stat3 as well as Stat3 downstream targets including VEGF and MMP2 were up-regulated in G3K cells. More importantly, knocking down PDGFD in G3K cells led to significant reduction of p-stat3/Stat3 and Stat3 downstream targets (**Figure 21B**). Similarly, knocking down PDGFD in Aspc-1 cells also reduced the level of p-stat3 and total Stat3 as well as Stat3 downstream targets (data not shown) while over-expression of PDGFD in parental MiaPaca-2 cells caused markedly increased level of p-stat3, total Stat3 and Stat3 downstream target VEGF (**Figure 21C**). Interestingly, the revertant G3KRev cells showed dramatically decreased expression of p-stat3 to the basal level compared with G3K cells although the total Stat3 level seems unchanged (**Figure 22C**). Taken together,

these findings strongly suggest that PDGFD actively regulates STAT3 signaling pathway, which may mediate PDGFD-induced gemcitabine resistance in pancreatic cancer cells.

Figure 21. PDGFD actively regulates STAT3 signaling pathway. (A) Western blot analysis of secreted PDGFD, p-stat3, stat3, VEGF, secreted VEGF, and secreted MMP2 expression in both MiaPaca-2 and G3K cells. (B) Effect of PDGFD knockdown on Stat3 signaling in G3K cells. G3K cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting PDGFD followed by Western blot analysis of p-stat3, stat3, VEGF, and MMP2 secretion. Actin was used as a loading control. (C) Effect of PDGFD over-expression on Stat3 signaling in MiaPaca-2 cells. MiaPaca-2 cells were stably transfected with vector control (vec(1)) or PDGFD plasmid (PD(9)) followed by Western blot analysis of p-stat3, stat3, and VEGF expression. Actin was used as a loading control.



F. Stat3 potentially contributes to gemcitabine resistance.

Finally, the potential role of Stat3 in gemcitabine resistance was tested by over-expressing wild type and constitutively activated mutant Stat3 (Stat3c) in parental MiaPaca-2 cells (**Figure 22A**). **Figure 22B** shows that over-expressing either the wild type or the constitutively activated Stat3 in parental MiaPaca-2 cells significantly increased gemcitabine resistance, by ~1.7 fold and ~2.3 fold increase respectively. Therefore, it is possible that PDGFD-induced up-regulation of Stat3 signaling may serve as a mediator for PDGFD-induced gemcitabine resistance.

Thus, I conclude that gemcitabine selection causes PDGFD gene demethylation and protein up-regulation, and its over-expression in turn contributes to gemcitabine resistance by activating STAT3 signaling pathway (**Figure 23**).

Figure 22. Stat3 over-expression promotes gemcitabine resistance in MiaPaca-2 cells. (A-B) Effect of PDGFD over-expression on gemcitabine resistance in MiaPaca-2 cells. MiaPaca-2 cells were stably transfected with vector control, stat3, or stat3c plasmid followed by Western blot analysis for detecting the expression of stat3 (A) or MTT assay for detecting the gemcitabine resistance. Actin was used as a loading control for Western blot. (N=3-4, **p<0.01). (C) Western blot detection of the expression of total Stat3 and p-stat3 in MiaPaca-2, G3K, and G3KRev cells. Actin was used as a loading control.

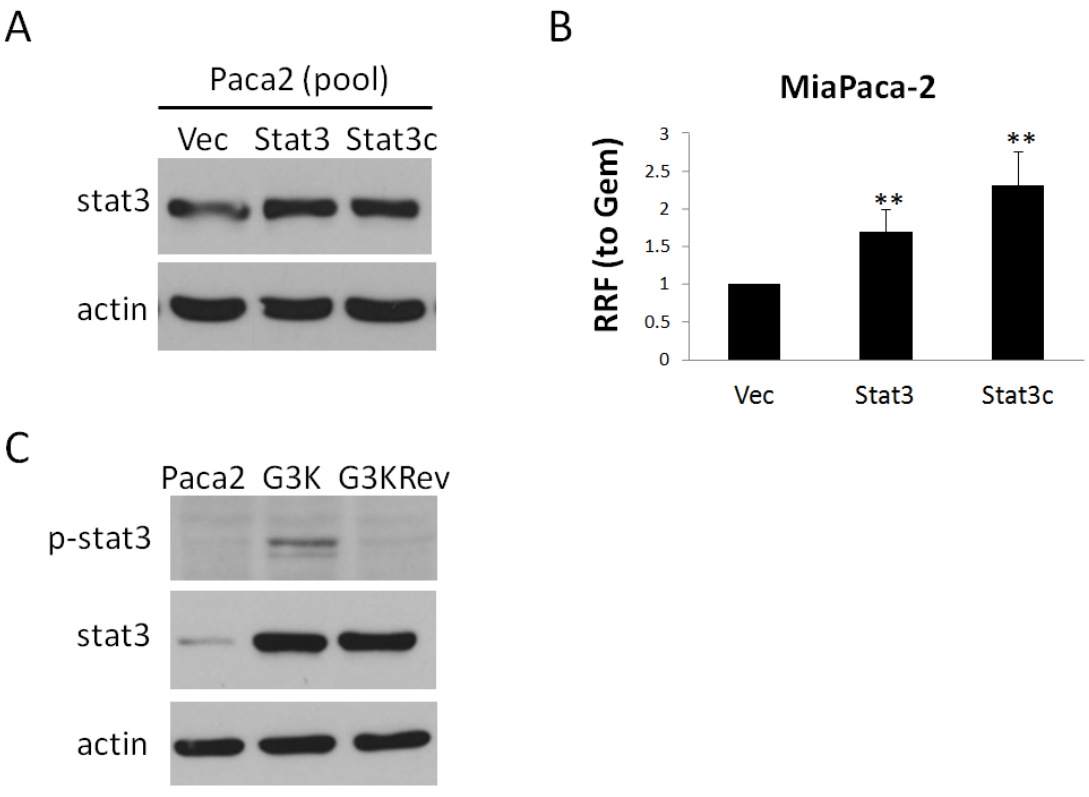
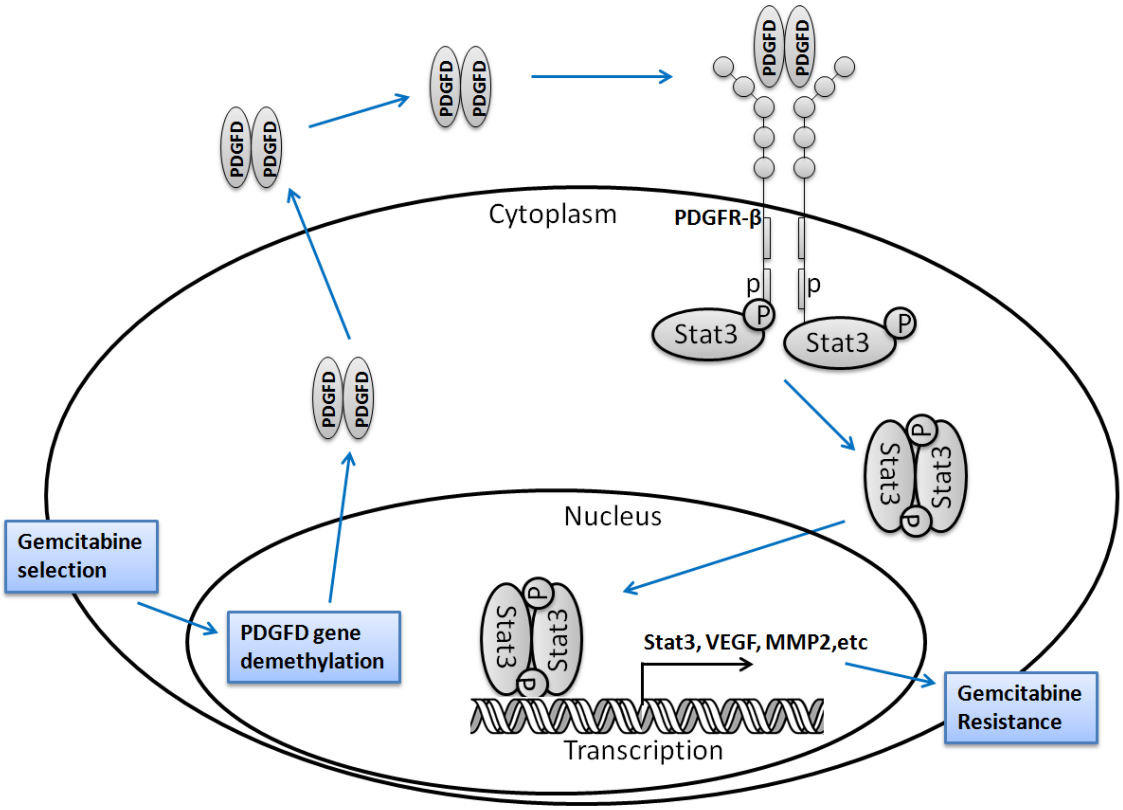


Figure 23. Schematic model of PDGFD-mediated gemcitabine resistance during drug selection. Gemcitabine selection leads to PDGFD gene demethylation and protein up-regulation, which binds to its cognate receptor and activates Stat3 signaling. Activation of Stat3 signaling pathway causes transcription of its downstream target genes and contributes to gemcitabine resistance.



Section III: Molecular mechanism of 14-3-3σ-mediated gemcitabine resistance in pancreatic cancer

The previous studies showed that 14-3-3σ over-expression in G3K cells contribute to gemcitabine resistance. However, the underlying mechanism through which 14-3-3σ contributes to acquired gemcitabine resistance remains unknown. Here, based on the escalating interest in recent years on one potential binding partner of 14-3-3σ, YAP1, I tested the hypothesis that 14-3-3σ contributes to acquired gemcitabine resistance by binding to YAP1.

A. YAP1 over-expression in G3K cells and its contribution to gemcitabine resistance.

To determine the potential role of YAP1 in 14-3-3σ-mediated gemcitabine resistance, I first tested the level of total YAP1 and p-YAP1 in both MiaPaca-2 and G3K cells. As shown in **Figure 24**, the level of both YAP1 and p-YAP1 as well as the mRNA level of YAP1 (~4.7 fold increase) were found highly elevated in G3K compared with MiaPaca-2 cells, suggesting that YAP1 may be transcriptionally up-regulated in G3K cells. Interestingly, 14-3-3σ knockdown in G3K cells also reduced total YAP1, p-YAP1, and YAP1 mRNA levels (~50% reduction) (**Figure 25A-B**). Moreover, over-expression of 14-3-3σ in MiaPaca-2 cells resulted in an increase of YAP1, p-YAP1, and YAP1 mRNA levels (~2.5 fold increase) (**Figure 25C-D**), indicating that YAP1 may be transcriptionally regulated by 14-3-3σ. However, knocking down of YAP1 in G3K cells did not affect the

protein level of 14-3-3σ (**Figure 26A**). Thus, 14-3-3σ may regulate the transcription and expression of YAP1, but not vice versa.

To determine whether the increased expression of YAP1 in G3K cells contributes to gemcitabine resistance, I first knocked down YAP1 in G3K cells by using specific siRNA followed by examining the difference in gemcitabine resistance using MTT assay. As shown in **Figure 26A-B**, knocking down YAP1 dramatically reduced the drug resistance in G3K cells, by up to 80% reduction. However, over-expression of YAP1 in the parental MiaPaca-2 cells did not significantly influence the gemcitabine resistance (**Figure 26C-D**). The reason for the discrepancy between these two experiments could be that YAP1 over-expression in MiaPaca2 cells may need 14-3-3σ for gemcitabine resistance. To test this possibility, I over-expressed YAP1 in MiaPaca-2 cells with stable flag-14-3-3σ-over-expression and tested the effect on gemcitabine resistance. As shown in **Figure 26E-F**, over-expression of YAP1 in these cells further increase gemcitabine resistance by ~2.4 fold. Thus, it is likely that YAP1 requires 14-3-3σ to contribute to gemcitabine resistance.

Figure 24. YAP1 is over-expressed in resistant G3K cells. (A) Western blot analysis of YAP1, p-YAP1 and 14-3-3σ expression in both MiaPaca-2 and G3K cells. Actin was used as a loading control. (B) Real time RT-PCR analysis of YAP1 mRNA level in MiaPaca-2 and G3K cells (N=3, **p<0.01).

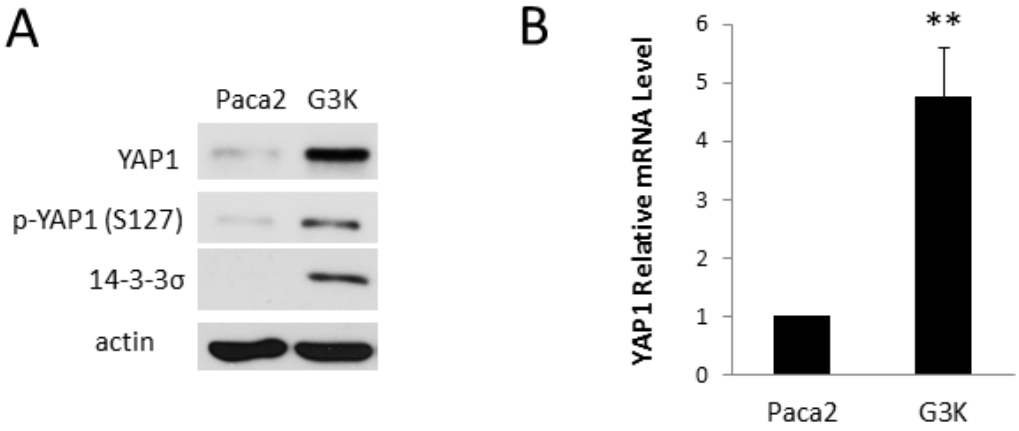


Figure 25. Regulation of YAP1 expression by 14-3-3σ protein level. (A) Effect of 14-3-3σ knockdown on YAP1/p-YAP1 expression. G3K cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting 14-3-3σ followed by Western blot analysis of YAP1, p-YAP1 and 14-3-3σ protein level. Actin was used as a loading control. (B) Real time RT-PCR analysis of YAP1 mRNA level in G3K cells stably transfected with scrambled control shRNA (scr sh3') or shRNA targeting 14-3-3σ (14-3-3 sh(11)). GAPDH was used as internal control. (N=3, **p<0.01). (C-D). Effect of 14-3-3σ over-expression on YAP1 expression. MiaPaca-2 cells were stably transfected with vector control (vec(1)) or pcDNA3.1(+)-flag-14-3-3σ plasmid (flag-σ(6)) followed by Western blot analysis of YAP1, p-YAP1, and 14-3-3σ protein levels (C) or real time RT-PCR analysis of YAP1 mRNA level (D). (N=3, **p<0.01). Actin and GAPDH were used as a loading control for Western blot and internal control for PCR analysis, respectively.

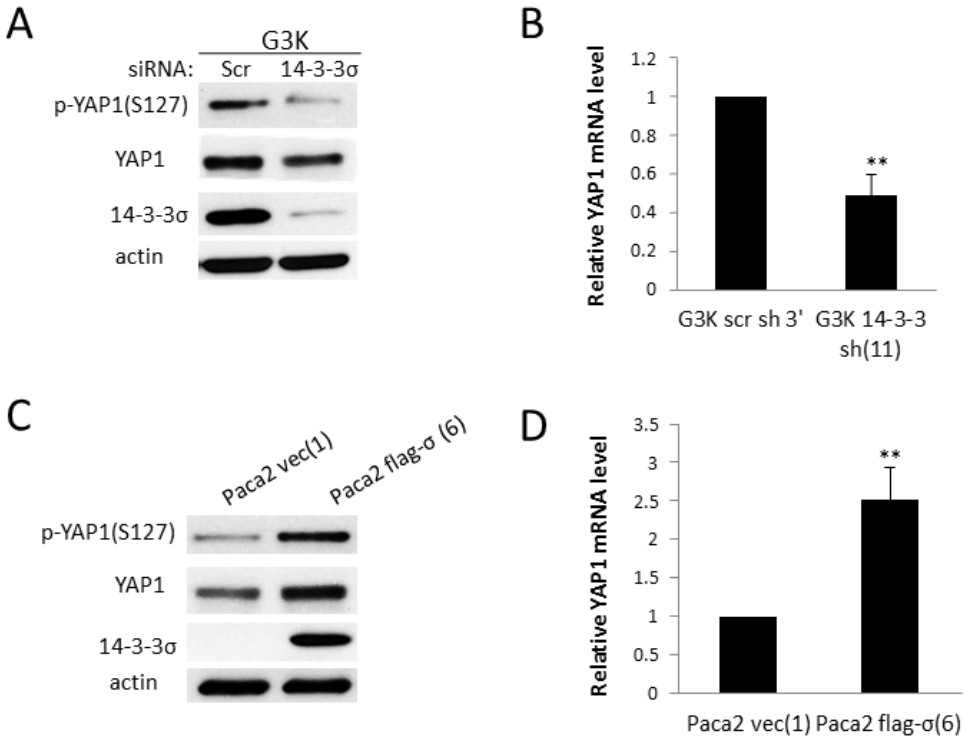
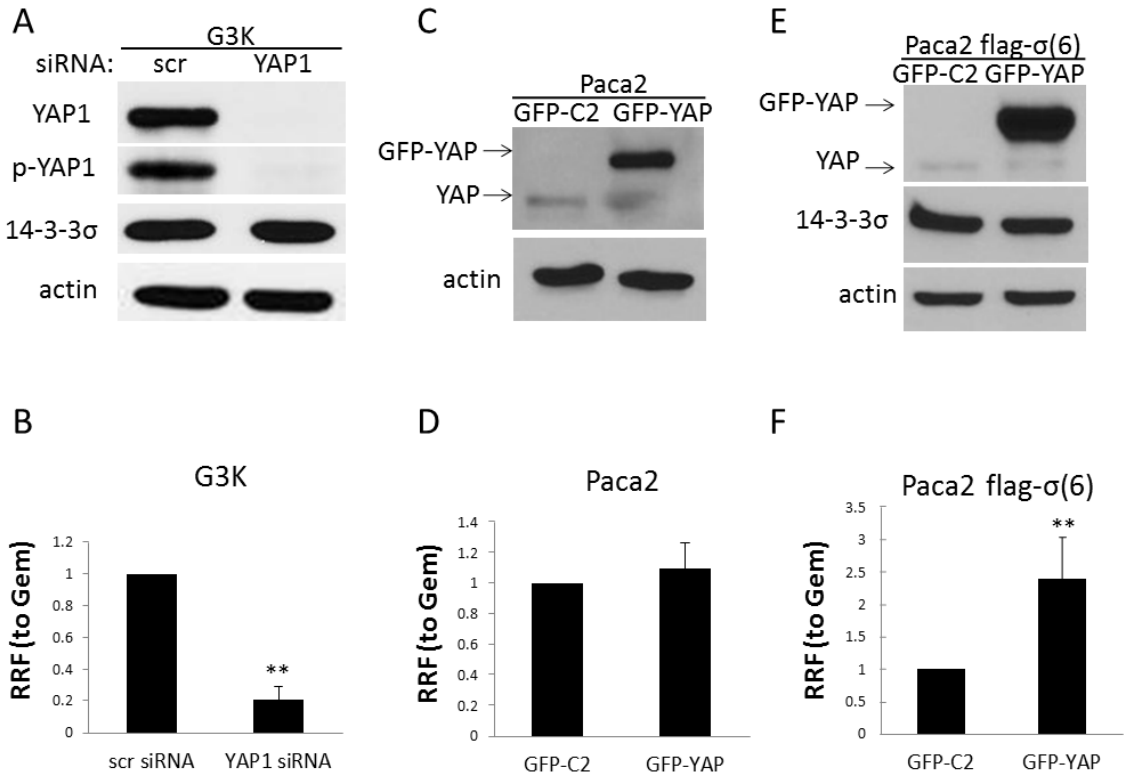


Figure 26. YAP1 over-expression contributes to gemcitabine resistance, but requires the presence of 14-3-3σ. (A-B) Effect of YAP1 knockdown on gemcitabine resistance in G3K cells. G3K cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting YAP1 followed by Western blot analysis of YAP1 and 14-3-3σ protein level (A) or MTT assay for detection of drug resistance(B). (N=4, **p<0.01). Actin was used as a loading control for Western blot. (C-D) Effect of YAP1 over-expression on gemcitabine resistance in MiaPaca-2 cells. MiaPaca-2 cells were transfected with vector control (GFP-C2) or GFP-YAP plasmid followed by Western blot analysis of YAP1 protein level (C) or MTT assay for detection of drug resistance (D). (N=4, not significant). Actin was used as a loading control for Western blot. (E-F) Effect of YAP1 over-expression on gemcitabine resistance in 14-3-3σ-over-expressing MiaPaca-2 cells. 14-3-3σ-over-expressing MiaPaca-2 cells (MiaPaca-2 flag-σ (6)) were transfected with vector control (GFP-C2) or GFP-YAP plasmid followed by Western blot analysis of YAP1 protein level (E) or MTT assay for detection of drug resistance (F). (N=3, **p<0.01). Actin was used as a loading control for Western blot.

Figure 26 (cont).



B. Gemcitabine resistance requires both 14-3-3σ and YAP1.

To better understand the mechanism of 14-3-3σ and YAP1 in contributing to acquired gemcitabine resistance, either 14-3-3σ or YAP1 was up-regulated but down-regulated the other, followed by MTT assay to determine gemcitabine response. As shown in **Figure 27A-B**, over-expressing 14-3-3σ markedly increased YAP1 protein level and gemcitabine resistance in MiaPaca-2 cells. However, knocking down YAP1 in 14-3-3σ-over-expressing MiaPaca-2 cells diminished the 14-3-3σ-induced gemcitabine resistance. Similarly, up-regulation of YAP1 in 14-3-3σ-over-expressing MiaPaca-2 cells significantly increased gemcitabine resistance, whereas knocking down 14-3-3σ in these cells abolished gemcitabine resistance despite that the ectopic YAP1 expression maintains at high level (**Figure 27C-D**). Together, these findings suggest that both 14-3-3σ and YAP1 are required for gemcitabine resistance.

To better address this observation, I performed the double knockdown experiment. Either 14-3-3σ or YAP1, or both proteins were knocked down in G3K (**Figure 28A-B**) or Aspc-1 (**Figure 28C-D**) cells to test the changes in gemcitabine resistance. Not surprisingly, knocking down either 14-3-3σ or YAP1 alone dramatically reduces gemcitabine resistance in both cell lines, by ~61% and ~69% reduction in G3K cells respectively. However, knocking down both 14-3-3σ and YAP1 simultaneously did not further reduce drug resistance, by ~72% reduction in G3K cells. Thus, it is possible that 14-3-3σ and YAP1 cooperates with each other to contribute to gemcitabine resistance,

and hence it is likely that the mechanism of 14-3-3σ-mediated gemcitabine resisance is the same as YAP1-mediated gemcitabine resistance.

Figure 27. Gemcitabine resistance requires both 14-3-3σ and YAP1. (A-B) YAP1 knockdown counteracts increased gemcitabine resistance caused by 14-3-3σ over-expression. MiaPaca-2 vec(1) and MiaPaca-2 flag-σ(6) cells were transiently transfected with scrambled control siRNA (Scr) or siRNAs targeting YAP1 followed by Western blot analysis of YAP1 and 14-3-3σ (A) or MTT assay for detection of drug resistance (B). (N=3, **p<0.01, ***p<0.001). Actin was used as a loading control for Western blot. (C-D) 14-3-3σ knockdown counteracts with increased gemcitabine resistance caused by YAP1 over-expression. MiaPaca-2 flag-σ(6) cells were transiently co-transfected with (1) GFP-C2 vector and scrambled control siRNA (GFP-C2/Scr si), or (2) GFP-YAP over-expression plamid and scrambled control siRNA (GFP-YAP/scr si), or (3) or GFP-YAP over-expression plamid and siRNA targeting 14-3-3σ (GFP-YAP/14-3-3σ si), followed by Western blot analysis of YAP1 and 14-3-3σ (C) or MTT assay for detection of drug resistance (D). (N=3, **p<0.01, ***p<0.001). Actin was used as a loading control for Western blot.

Figure 27 (cont).

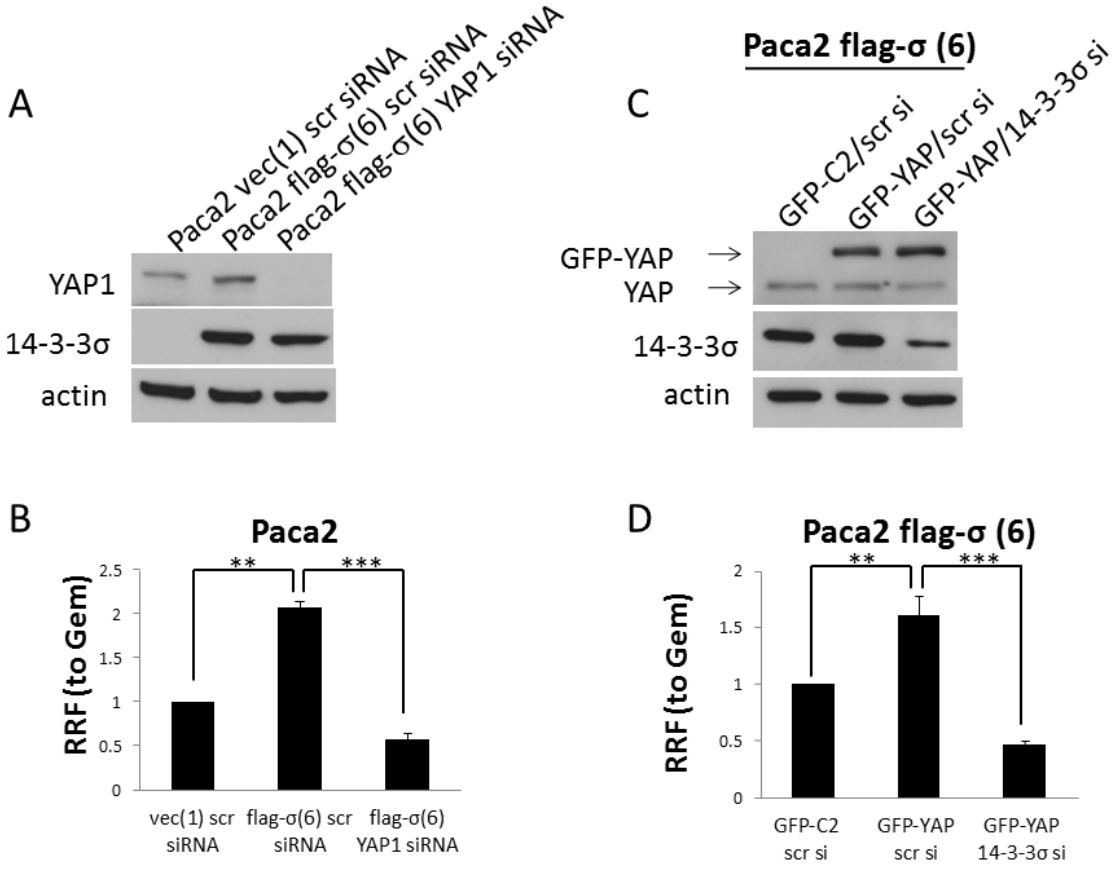
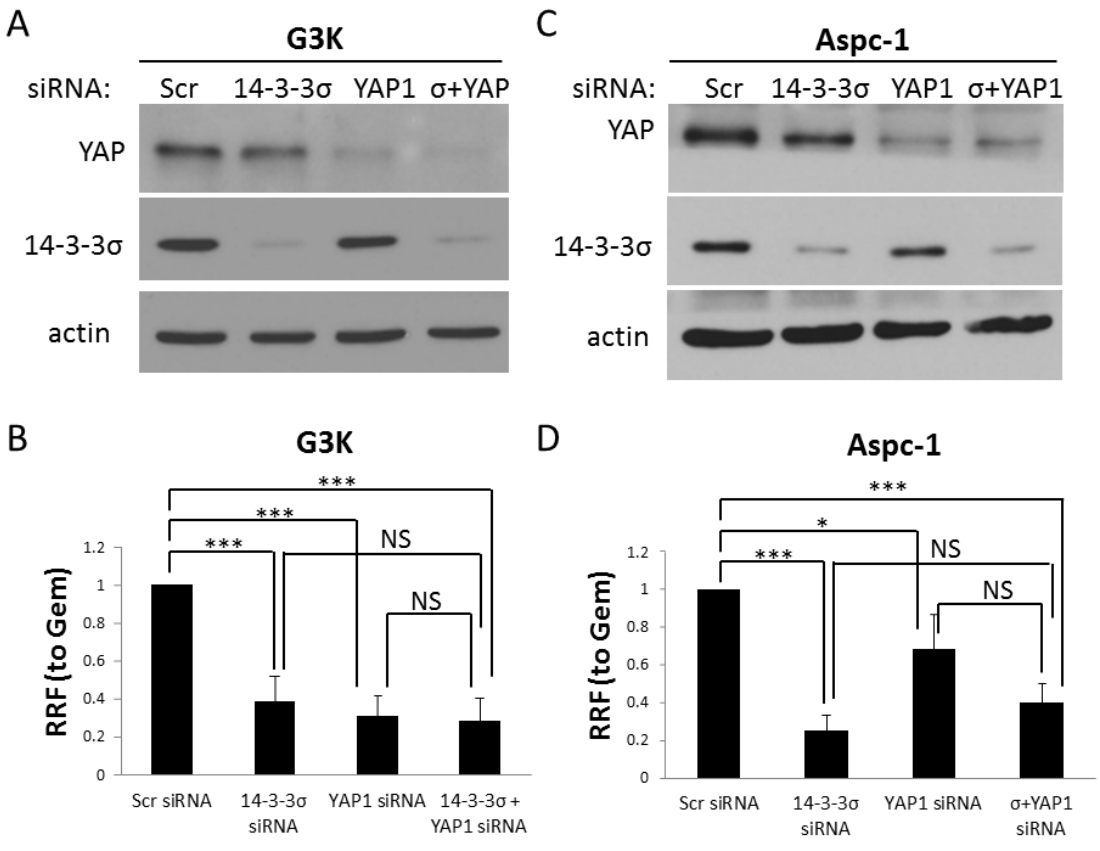


Figure 28. Decreased expression of both 14-3-3σ and YAP1 by RNA knockdown does not further reduce drug resistance. G3K cells (A-B) or Aspc-1 cells (C-D) were transiently transfected with either scrambled (Scr) siRNA, 14-3-3σ siRNA, and YAP1 siRNA alone, or co-transfected with both 14-3-3σ and YAP1 siRNA simultaneously, followed by Western blot analysis of YAP1 and 14-3-3σ (A, C) or MTT assay for detection of drug resistance (B, D). (N=3-5, *p<0.05, **p<0.01, ***p<0.001, NS: not significant). Actin was used as a loading control for Western blot.

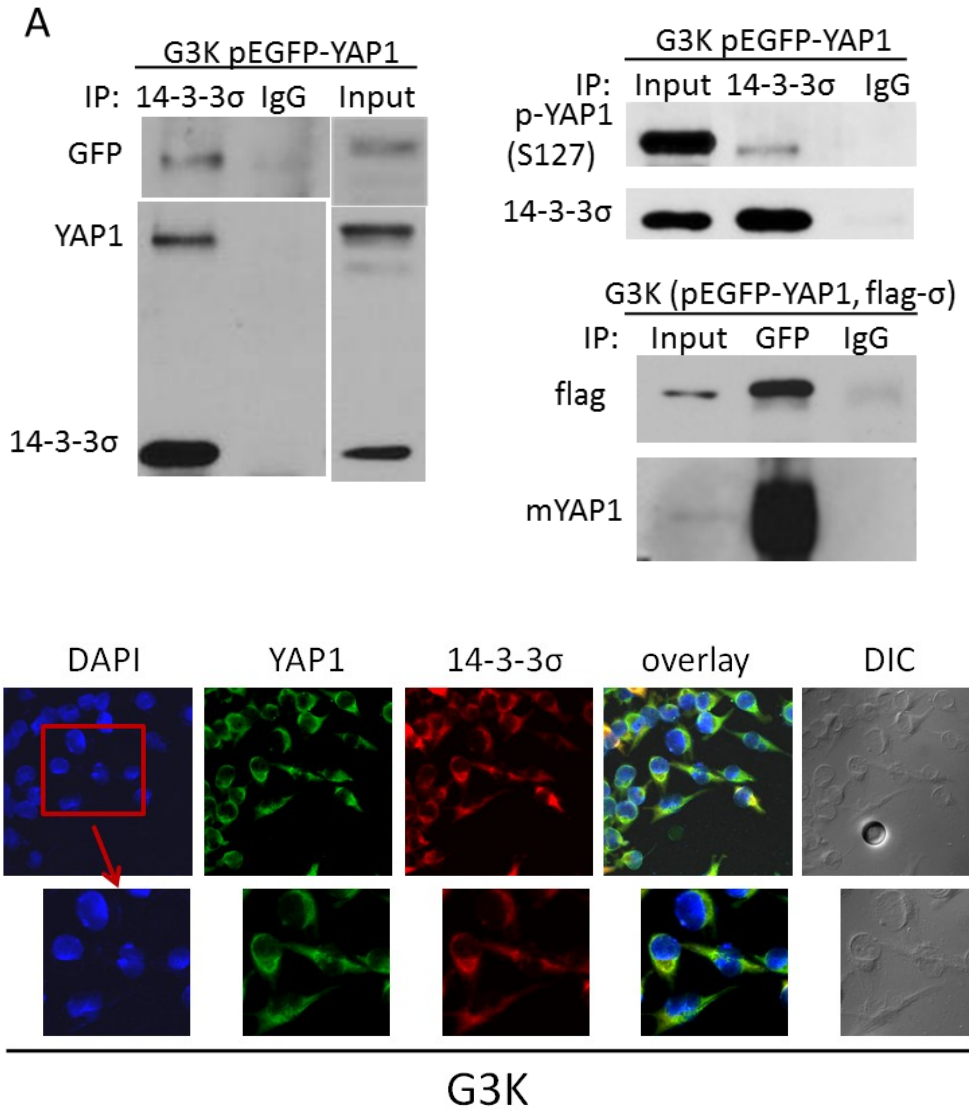


C. 14-3-3σ and YAP1 form a complex.

The finding of indispensable roles of both 14-3-3σ and YAP1 in gemcitabine resistance suggests that 14-3-3σ may form a complex with YAP1. To test this possibility, co-immunoprecipitation assay was performed in G3K cells transiently transected with GFP-YAP1 and flag-14-3-3σ. As shown in **Figure 29A**, YAP1 and p-YAP1 proteins could be co-immunoprecipitated by 14-3-3σ antibody and flag-14-3-3σ protein could be co-immunoprecipitated by GFP antibody. Thus, 14-3-3σ may bind with YAP1/p-YAP1 to form a complex in G3K cells.

Next, co-localization analysis of these two proteins was performed using immunofluorescence staining. As shown in **Figure 29B**, both 14-3-3σ and YAP1 appear to localize to the cytoplasm of G3K cells and the overlay image indicates their co-localization, which supports the possibility that these two proteins reside and form a complex in the cytoplasm.

Figure 29. 14-3-3σ interacts and binds with YAP1 in vitro. (A) 14-3-3σ co-immunoprecipitates with YAP1, and vise versa. G3K cells were transiently transfected with pEGFP-YAP1 plasmid or co-transfected with both pEGFP-YAP1 and pcDNA3.1-flag-σ plasmid, followed by immunoprecipitation with either 14-3-3σ or GFP antibody, and Western blot detection the existence of YAP1, p-YAP1 or 14-3-3σ in the complex. (B) YAP1 co-localizes with 14-3-3σ in vitro. The localization of both YAP1 and 14-3-3σ in the G3K cells was visualized by confocal microscopy.



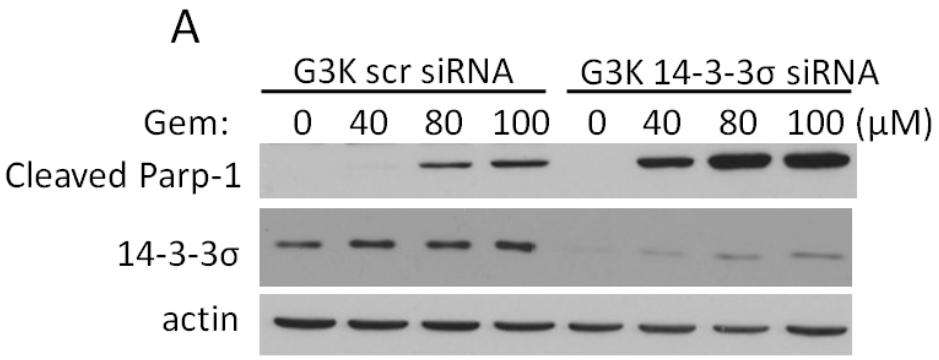
D. Both 14-3-3σ and YAP1 protect against gemcitabine-induced caspase-8 activation and apoptosis.

To further investigate the mechanism of 14-3-3σ and YAP1-induced gemcitabine resistance, I tested their effect on gemcitabine-induced apoptosis. 14-3-3σ was knocked down in G3K cells to test its influence on gemcitabine-induced apoptosis. As shown in **Figure 30A**, knocking down 14-3-3σ in G3K cells led to dose-dependent increase in gemcitabine-induced Parp-1 cleavage, suggesting its potential protective role in apoptosis. To confirm this result, a stable knockdown clone was established by transfecting the G3K cells with 14-3-3σ shRNA followed by apoptotic assay. Treatment the G3K cells with scrambled shRNA with 40 μM or 80 μM of gemcitabine for 24 hrs induced apoptosis by ~1.9 fold and ~4.8 fold increase compared with no treatment control respectively, whereas in 14-3-3σ knockdown cells it induced more apoptosis, by ~4.8 fold and ~9.5 fold increase (**Figure 30B**). The apoptotic assay showed that 14-3-3σ knockdown cells appeared to undergo more apoptosis following gemcitabine treatment, confirming the protective role of 14-3-3σ against gemcitabine-induced apoptosis. Moreover, gemcitabine-induced Parp-1 cleavage was found to be markedly increased in 14-3-3σ knockdown cells compared with vector shRNA transfected cells (**Figure 31A**). Similarly, knocking down YAP1 in G3K cells also led to dose-dependent increase in gemcitabine-induced Parp-1 cleavage, suggesting that YAP1 over-expression may also protect G3K cells from gemcitabine-induced apoptosis (**Figure 31B**). Together, these

experiments strongly suggest that both 14-3-3σ and YAP1 up-regulation contribute to gemcitabine resistance by inhibiting Parp-1 cleavage and gemcitabine-induced apoptosis.

To further investigate the specific apoptotic pathway that 14-3-3σ and YAP1 participated in, the Western blot analysis was conducted to detect the caspase-8 and caspase-9 activation under gemcitabine treatment in G3K cells. As shown in **Figure 31A**, cleaved and active caspase-8 was found to be dramatically elevated in 14-3-3σ knockdown G3K cells compared with control cells upon gemcitabine treatment. Similarly, knocking down YAP1 in G3K cells also led to an increased activation of caspase-8 following gemcitabine treatment (**Figure 31B**). However, caspase-9 activation was not affected (data not shown). Thus, likely 14-3-3σ and YAP1 function in inhibiting gemcitabine-induced apoptosis by attenuating caspase-8 activation.

Figure 30. Decreased expression of 14-3-3σ by RNA knockdown promotes parp-1 cleavage and apoptotic cell death. (A) G3K cells were transiently transfected with either scrambled (Scr) siRNA or siRNA targeting 14-3-3σ, followed by Western blot analysis of cleaved parp-1 and 14-3-3σ. Actin was used as a loading control. (B) 14-3-3σ knocking down leads to compromised apoptosis. G3K cells stably transfected with either scrambled shRNA (scr sh3') or 14-3-3σ shRNA (14-3-3σ sh(11)) were treated with/without various dose of gemcitabine, followed by apoptotic assay to measure the enrichment of nucleosomes released in the cytoplasm. (N=5, *p<0.05, **p<0.001).



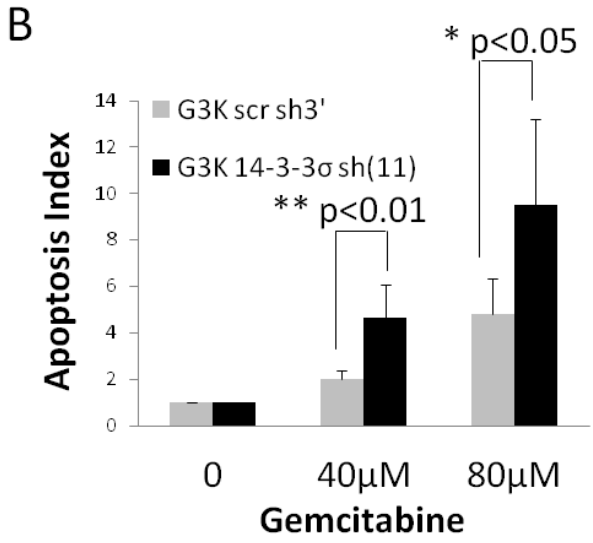
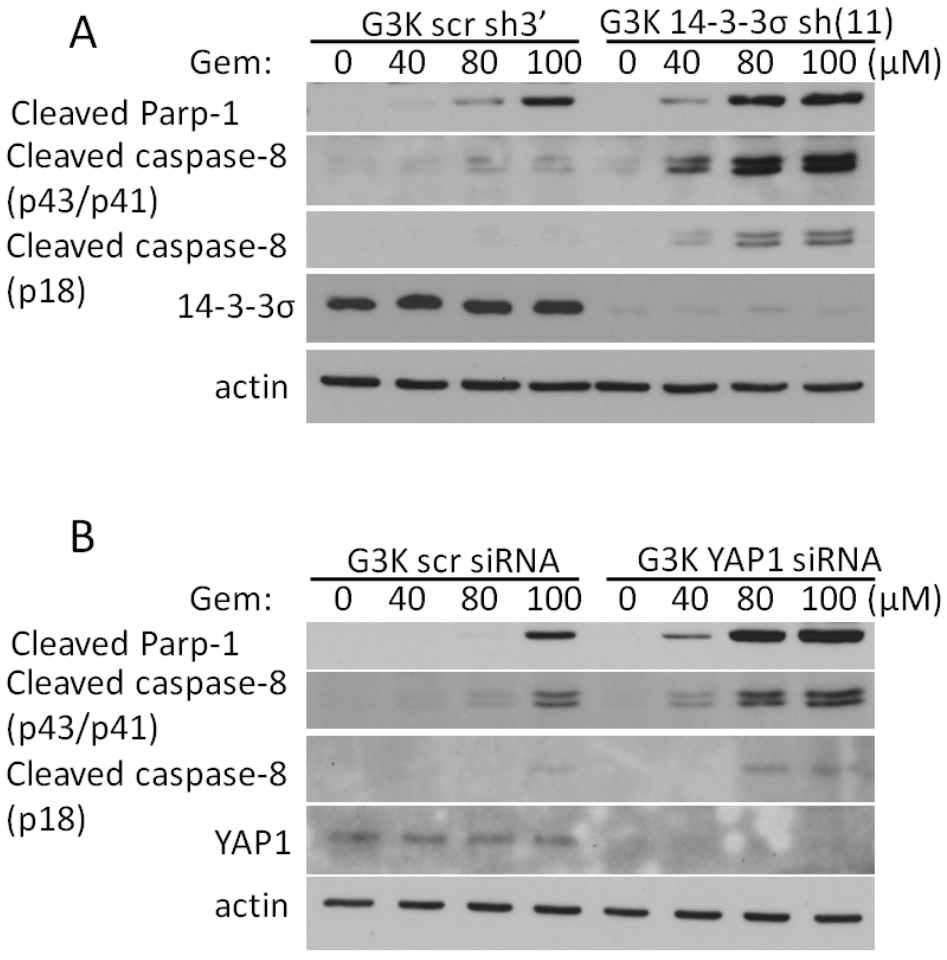


Figure 31. Decreased expression of either 14-3-3σ or YAP1 by RNA knockdown leads to increased parp-1 cleavage and caspase-8 activation. (A) G3K cells stably transfected with either scrambled shRNA (scr sh3') or 14-3-3σ shRNA (14-3-3σ sh(11)) were treated with or without various dose of gemcitabine, followed by Western blot analysis of cleaved parp-1, cleaved caspase-8 and 14-3-3σ. Actin was used as a loading control. (B) G3K cells were transiently transfected with either scrambled (Scr) siRNA or siRNA targeting YAP1, followed by Western blot analysis of cleaved parp-1, caspase-8 activation and YAP1. Actin was used as a loading control.



Finally, in order to further confirm its protective role in gemcitabine-induced apoptosis, 14-3-3σ was over-expressed in parental MiaPaca-2 cells followed by Western blot detection of caspase-8 activation. As shown in **Figure 32A**, following treatment with various doses of gemcitabine, 14-3-3σ over-expression led to much less Parp-1 cleavage and caspase-8 activation, with Parp-1 cleavage and caspase-8 activation saw at 20 nM of gemcitabine treatment compared to 10 nM in vector control cells, confirming that 14-3-3σ up-regulation protects MiaPaca-2 cells from gemcitabine-induced caspase-8 activation and apoptosis. Furthermore, 14-3-3σ over-expression in MiaPaca-2 cells also caused a delay of caspase-8 activation at different time points under 100 nM of gemcitabine treatment, with caspase-8 activation at 48 hrs compared to 24 hrs in vector control cells (**Figure 32B**), suggesting that the protective role of 14-3-3σ in gemcitabine-induced apoptosis and caspase-8 activation is not only dose-dependent, but also time-dependent.

Taken together, 14-3-3σ appears to contribute to gemcitabine resistance by up-regulating YAP1 protein level and then forming a complex with YAP1 and p-YAP1, then both 14-3-3σ and YAP1 in the complex may protect against gemcitabine-induced apoptosis via attenuating caspase-8 activation (**Figure 33**).

Figure 32. 14-3-3σ over-expression in MiaPaca-2 cells protects against parp-1 cleavage and caspase-8 activation. (A) MiaPaca-2 cells stably transfected with vector control or pcDNA3.1-flag-σ plasmid were treated with/without various dose of gemcitabine, followed by Western blot detection the expression level of cleaved parp-1, cleaved caspase-8, and 14-3-3σ. Actin was used as a loading control. (B) MiaPaca-2 cells stably transfected with vector control or pcDNA3.1-flag-σ plasmid were treated with 100nM of gemcitabine, followed by Western blot detection the expression level of cleaved caspase-8 and 14-3-3σ at different time points. Actin was used as a loading control.

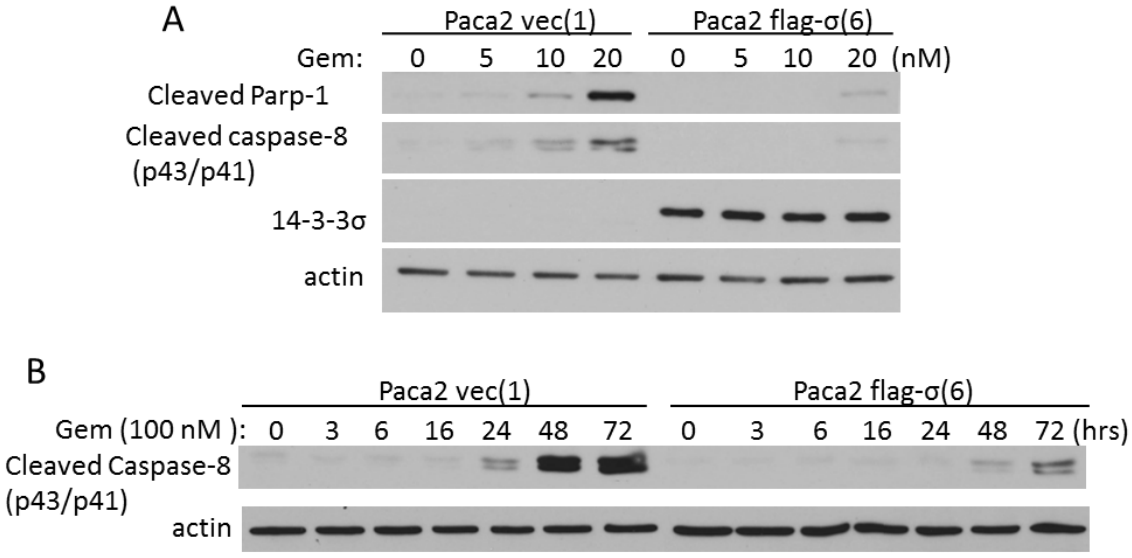
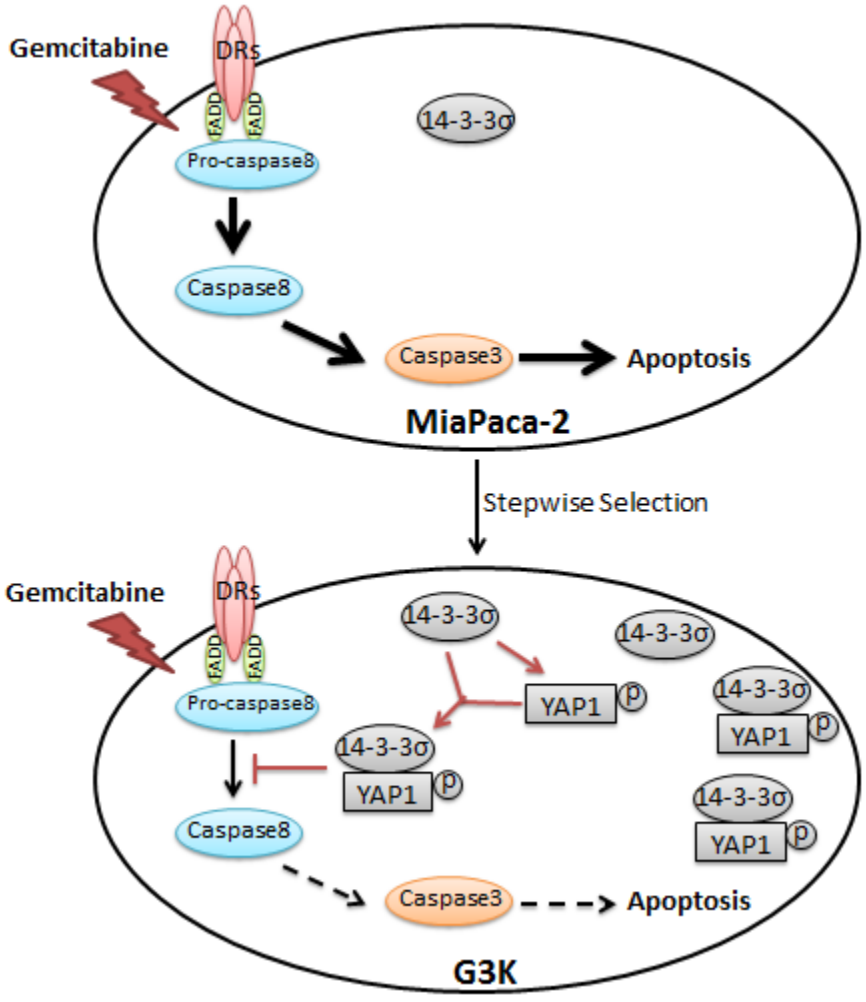


Figure 33. Schematic model of 14-3-3σ-mediated gemcitabine. 14-3-3σ up-regulation in resistant G3K cells mediated gemcitabine resistance by up-regulating YAP1 expression, binding with YAP1/p-YAP1 as a complex, and together inhibiting gemcitabine-induced caspase-8 activation.



Discussion

Stepwise selections with anticancer drugs have been used as a standard method to create model cell lines for laboratory studies and identify novel mechanisms of acquired resistance. G3K cells selected with gemcitabine in this study are clonal and cross resistant to Ara-C. Considering the similarity in structure, mechanism of action, and metabolism between gemcitabine and Ara-C, it was not surprising to find the cross-resistance of G3K cells to Ara-C. The observation of increased expression of RRM is also as expected as these proteins have previously been shown to be up-regulated and contribute to gemcitabine resistance in other gemcitabine-selected cells [181, 182]. However, the findings of the up-regulated expression of 14-3-3σ via reversible epigenetic regulation during gemcitabine selection and its role in acquired gemcitabine resistance are unexpected and novel.

14-3-3σ, a homo-dimeric protein that functions as a chaperone, binds to >100 phospho-serine/phospho-threonine proteins and plays an important role in cell survival [183]. Up-regulated expression of 14-3-3σ has been found in PDAC and appears to associate with poor prognosis of PDAC by causing resistance to gemcitabine [46, 184]. However, it remains to be determined if 14-3-3σ plays an important role in clinically acquired gemcitabine resistance in PDAC. The possible role of 14-3-3σ in resistance to other anticancer drugs such as Adriamycin and cisplatin has also been reported previously [60, 61, 63, 65]. Similar to the finding of this study that 14-3-3σ can be

selected and responsible for acquired gemcitabine resistance, it can also be selected and responsible for acquired Adriamycin resistance [63].

Although some of the other members of the 14-3-3 protein family such as 14-3-3ζ and 14-3-3θ/τ have been reported to also contribute to [185, 186] and associate with [187] drug resistance, respectively, I did not find up-regulation in expression of 14-3-3ζ and 14-3-3θ/τ in the gemcitabine resistant G3K cells. This observation suggests that 14-3-3ζ and 14-3-3θ/τ may not participate in the acquired gemcitabine resistance in PDAC.

This study is the first time to find that 14-3-3σ-mediated gemcitabine resistance may be possibly through binding with YAP1 and protecting against gemcitabine-induced caspase-8 activation and apoptosis. There are two distinct apoptotic pathways, the mitochondria pathway with subsequent activation of caspase-9, or the death receptor pathway via activation of caspase-8. In this study, I found that 14-3-3σ exerts its protective effect mainly by binding to YAP1 and inhibiting gemcitabine-induced apoptosis and caspase-8 activation, suggesting that the apoptotic pathway induced by gemcitabine is likely the cell death receptor pathway although it remains unclear how 14-3-3σ/YAP1 complex protects against caspase-8 activation. This study is consistent with previous report that gemcitabine induces caspase-8 and caspase-3 activation in H292 cells and enhances cell sensitivity to Fas-mediated cytotoxic activity [188], which supported our evidence that gemcitabine induces apoptosis by activating caspase-8. Moreover, it is also reported that gemcitabine induces apoptosis in non-small cell lung

cancer (NSCLC) cells by increasing functionally active Fas (CD95, APO-1) expression, as well as up-regulation of Fas ligand (FasL) which triggers cell apoptosis via an autocrine/paracrine loop [188, 189], demonstrating the important role of death receptor pathway (extrinsic pathway) in contributing to gemcitabine-induced apoptosis. Therefore, it will be interesting to investigate whether or not 14-3-3σ and YAP1-mediated inhibition of gemcitabine-induced caspase-8 activation by down-regulating or inactivating Fas or FasL. Fas stimulation triggers apoptosis via the so-called type I extrinsic signaling pathway. Central to this pathway is the direct caspase-8-mediated cleavage and activation of caspase-3 as compared to the type II pathway which first requires caspase-8-mediated Bid cleavage to trigger mitochondrial cytochrome c release for caspase-3 activation. However, in this study, caspase-9 activation under gemcitabine treatment seems not be interfered by 14-3-3σ and YAP1 expression, suggesting type I extrinsic signaling pathway as the main pathway. In contrast to the well established role of caspase-3 as an effector caspase, caspase-3 is also delineated to induce feedback activation of the apical caspases including caspase-2, caspase-8, caspase-9 and caspase-10A in doxorubicin and TNFα-induced apoptosis [190]. Moreover, the positive feedback loop between active caspases-3 and caspase-8 is also reported in lymphocytes, hepatocytes and Hela cells [191]. It has shown that fully processed, active p17 caspase-3 feeds back on caspase-8 by cleaving its partially processed p43 form into the fully processed p18 species [191], suggesting a possibility that 14-3-3σ protects against gemcitabine-induced caspase-8 activation by suppressing caspase-3 activation

first. Therefore, it will be intriguing to investigate if 14-3-3σ-mediated inhibition of gemcitabine-induced caspase-8 activation is dependent on caspase-3 activation or not.

Although it is unknown if other mechanisms of 14-3-3σ-induced gemcitabine resistance exist, the fact that it binds to various proteins important for different cellular processes suggest that other protein partners may also mediate 14-3-3σ-induced gemcitabine resistance. Somatic knockout of 14-3-3σ in colon cancer cells has been shown to cause drug-induced mitotic catastrophe by reducing cellular ability to arrest in G2/M phase [55]. In addition, increased 14-3-3σ expression in breast cancer cells was found to make cancer cells more resistant to drug-induced apoptosis [63], possibly due to 14-3-3σ binding and arresting cyclin B1 and CDC2 [55, 65] and pro-apoptotic proteins such as Bax and Bad [192, 193] in cytoplasm. 14-3-3σ over-expression was also found to contribute to cisplatin resistance in gastric cancer cells through Erk and p38 activation [194], suggesting a possible role of Erk and p38 in mediating 14-3-3σ-induced gemcitabine resistance. Nevertheless, it remains to be determined if 14-3-3σ-induced gemcitabine resistance is also via cytoplasmic retention of cyclin B1 and CDC2, interference of Erk and p38 signaling pathway, and blockade of mitotic catastrophe. In addition, based on similar expression profile of RRM1, RRM2, and 14-3-3σ, and the regulatory role of 14-3-3σ on YAP1 expression, it is intriguing to determine whether or not 14-3-3σ potentially regulates the expression of RRM1 and RRM2. The possible role of 14-3-3σ in regulating gemcitabine metabolism pathway is not studied and remains to be investigated. I am currently working toward this direction.

The regulation of 14-3-3σ expression occurs in multiple steps and epigenetic regulation by methylation has been observed in cell line models and clinical samples although the mechanism of regulation by methylation was unknown [195, 196]. However, the increased 14-3-3σ expression following drug selection in different cell lines appears to be regulated by different mechanisms. Epigenetic regulation likely occurs only in the parental cells where the 14-3-3σ gene is epigenetically silenced in MiaPaca-2 cells due to hypermethylation of the promoter. Up-regulation of 14-3-3σ expression in Adriamycin-selected and resistant MCF7 cells is likely mediated by other mechanisms. In response to DNA damage, 14-3-3σ is induced in a p53-dependent manner, and in turn it positively regulates p53 and suppresses tumor cell growth, indicating a positive feedback between 14-3-3σ and p53 [197]. Similar to our finding that 14-3-3σ over-expression in Adriamycin-selected and resistant MCF7 cells is not mediated by epigenetic regulation, 14-3-3σ mRNA level change in 5-fluorouracil (5-FU)-selected and resistant MCF7 cells was due to p53 protein expression but not methylation level change [198]. Like p53, 14-3-3σ was also found to be transactivated by p73 and, in turn, stabilize p73 and enhance the p73-mediated transcriptional activity as well as its pro-apoptotic function [199]. Our preliminary study has found that p73 expression was increased in G3K cells, whereas in parental MiaPaca-2 cells p53 gene is mutated, thus it is likely that in G3K cells 14-3-3σ is transactivated by p73. Furthermore, except for being regulated by gene methylation and transcription, 14-3-3σ protein was also found to be regulated by phosphorylation and proteolytic inactivation [200, 201]. It was found that TGFβ1 induced phosphorylation of 14-3-3σ at Ser69 and Ser74, which

regulates MCF7 tumor progenitor population and acts as a feed-forward mechanism in TGFβ/Smad3-dependent transcription [201]. In addition, the estrogen-responsive E3 ubiquitin ligase Efp was identified to specifically targets 14-3-3σ for degradation in breast cancers [200]. Therefore, both epigenetic silencing by gene methylation, p53 inactivation, and proteasome-dependent proteolysis leads to loss of 14-3-3σ.

In this study, it is the first time to discover that Uhrf1 and DNMT1 bind to methylated region and regulate and maintain the hypermethylation status of 14-3-3σ gene. The finding that the 14-3-3σ gene could be reversibly methylated by DNMT1 and Uhrf1 during gemcitabine selection is consistent with the observation of reduced Uhrf1 expression in G3K cells, which recruits DNMT1. The finding that 14-3-3σ expression is progressively up-regulated during gemcitabine selection suggests that the gene demethylation may also be progressive. Although the decreased methylation of the 14-3-3σ gene in the drug resistant G3K cells is inconsistent with the increased expression of DNMT1, but it is consistent with the reduced level of Uhrf1.

Uhrf1, a multi-domain protein associated with cellular proliferation and epigenetic regulation, binds to histones and methyl-CpG dinucleotides with a preference for hemi-methylated CpG sites. The consequence of Uhrf1 binding was recruitment of DNMT1 and histone deacetylase 1, resulting in methylation of nascent DNA strands [124, 126-128]. Thus, reduced Uhrf1 expression in G3K cells is likely responsible for reduced methylation of 14-3-3σ gene by reducing recruitment of DNMT1 to the methylated CpG

islands despite the presence of high level of DNMT1. In MiaPaca-2 cells, however, the high level of Uhrf1 may efficiently recruit enough DNMT1 despite low DNMT1 level for sufficient methylation of 14-3-3σ gene. Therefore, knocking down either Uhrf1 or DNMT1 in MiaPaca-2 cells would effectively reduce the level of the recruiter (Uhrf1) or the pool of DNMT1 to be recruited for methylation of 14-3-3σ gene and consequently increase 14-3-3σ expression. Furthermore, the slight increase of Uhrf1 expression in the G3KRev cells may be responsible for the increased methylation and reduced expression of 14-3-3σ gene. These findings also suggest that other genes that are under epigenetic regulation, specifically DNA methylation, may also change in their expression similar as 14-3-3σ. In addition, I found that p21, a known Uhrf1 downstream target gene, has a similar expression profile as 14-3-3σ in the parental MiaPaca-2, gemcitabine resistant G3K and revertant G3KRev cells. What other genes have similar expression profile in these cells and whether these genes such as p21 also contribute to the acquired gemcitabine resistance are very intriguing, and have been partly answered by RRBS analysis of global gene methylations.

There are several methods available for global DNA methylation evaluation. Comparing with MeDIP, MethylCap, the bisulfite-based method RRBS is slightly more accurate than the other two enrichment-based methods in identifying Differential Methylated Regions (DMRs) [202]. Due to single nucleotide resolution, RRBS has the advantage of offering a direct measure of methylation levels without requiring statistical correction of CpG bias as in MeDIP and MethylCap. However, the method design of

RRBS only focuses on CpG-rich regions (promoters and CG islands) and thus has a much lower genomic coverage (RRBS reads are clustered in approximately 1% of the genome) than MeDIP and MethylCap, the latter two methods are theoretically capable of covering all methylation regions across the whole genome. Despite the great difference in genomic coverage, the identified DMRs are similar among these three methods due to depth requirement for reliably detecting DMRs that are statistically significant.

14-3-3σ gene, which was confirmed to be differentially methylated, nevertheless was not listed among the total 65 candidate genes. The reason is that the RRBS read coverage for 14-3-3σ gene is only 11.71% and among 27 CpG dinucleotides analyzed using traditional bisulfite sequencing, only 4 were covered in the RRBS sequencing regions. It is, however, noteworthy that RRBS revealed significant difference in differential methylation of 14-3-3σ gene between MiaPaca-2 and G3K cells with a P-value of 9.23e-15. Moreover, studies on 14-3-3σ gene methylation are focused on its first exon, and whether or not 14-3-3σ gene is methylated at its promoter or other regions is unknown. Based on our RRBS study that significantly differential methylation of 14-3-3σ gene appears only in the region of first exon, CpG islands of 14-3-3σ gene at promoter and other regions may not be frequently methylated.

It is widely recognized that gene methylation at the promoter may affect gene transcription and expression. However, among the 10 candidate genes selected by DMRs from RRBS analysis, two had no change in mRNA levels as determined using real

time RT-PCR. Thus gene methylation change in the promoter region may not necessarily lead to mRNA level change. In general, gene silencing has been thought to be either due to direct inhibition of transcription factor binding by DNA methylation or mediated by methyl-binding domain (MBD) proteins that recruit chromatin-modifying complexes to methylated DNA to bring about further changes in chromatin structure: prototypically those associated with nucleosomal compaction and transcriptional silencing [203, 204]. The linkage between gene promoter methylation and heritable transcriptional suppression is well recognized, however, it seems that CG Island (CGI) methylation is not the initiating event in gene silencing, but acts to lock in the silent state. For example, during X-chromosome inactivation, X-linked CGIs do not become methylated until after gene silencing and the acquisition of several silencing chromatin modifications, such as H3K27me3 [205, 206]. Moreover, the majority of CGIs that gain methylation during differentiation are already silent in embryonic stem cells, providing further evidence that gene silencing precedes DNA methylation [207]. Therefore, the causative relationship between gemcitabine selection and differential gene methylation is unclear. In addition, using regionally methylated plasmids, it has been reported that parameters such as position, length, or density of methylated cytosines are crucial for the efficiency of gene repression [208-210]. Furthermore, other studies also suggested that transcriptional inhibition relies on methylation at specifically critical CpG sites [211-213]. Thus, it is likely that DMRs of some genes in our RRBS analysis are not the key CpG sites and hence the gene transcription is not affected. Noticeably, it was also reported that densely methylated elements (DMEs) of the genome are disproportionately enriched for

exons, and that DNA methylation in the region of the first exon is much more tightly linked to transcriptional silencing than methylation in the upstream promoter region [214], indicating a more predictive role of first exon rather than promoter region in gene silencing. Moreover, it was demonstrated that methylation of promoter sequences does not have a greater repressive effect than the modification of flanking, nonregulatory DNA sequences, and that the presence of a transcription factor can compete for the establishment of an inactive promoter conformation, thus reducing the silencing effect [215]. Additionally, the existence of factors indifferent to DNA methylation status and the demonstration that this modification is often capable of repressing transcription only after chromatin has been assembled suggest that other factors such as chromatin structure may impede DNA methylation-mediated gene silencing [210, 216, 217]. Thereby, gene transcription and repression is a complicated process and the methylation of CpG islands located in gene promoters is not always equated with transcriptional inactivity, other factors such as methylation density, critical CpG methylation sites, competing transcription factors as well as chromatin status and other player participating in gene transcription and repression complex should also be considered.

PDGFD, as the latest addition to PDGF family, has generated considerable interest in recent years because of its up-regulation and involvement in the progression of many types of human cancers [147-153]. The growing body of literature strongly suggests that PDGFD may function as a key player in the development and progression

of human cancers by regulating the processes of cell proliferation, apoptosis, migration, invasion, angiogenesis, and metastasis. It has been reported that PDGFD signaling is frequently deregulated in human malignancies and PDGFD expression was up-regulated in many human cancers including prostate, lung, renal, ovarian, brain, and pancreatic cancer [146, 147, 149-152]. Pancreatic cancer, like many other tumors, has been shown to over-express PDGFD. However, the association of PDGFD with drug resistance, especially gemcitabine resistance in pancreatic cancers, is unknown. This study not only identified PDGFD as an important contributor of gemcitabine resistance for the first time, but also newly investigated its methylation status in the promoter region. Thus it is very possible that the up-regulated expression of PDGFD found in many other cancers was also accredited to gene demethylation, and if so, targeting PDGFD gene methylation and over-expression may be a useful therapeutic approach to overcome different human tumors.

It is known that PDGFD upregulation triggers a number of downstream signaling pathways including activation of phosphatidylinositol 3 kinase (PI3K), Akt, nuclear factor-κB (NF-κB), Notch, and extracellular signal-regulated kinase (ERK) [139, 145-147]. Here, it is the first time to show a direct association of PDGFD expression and activation of Stat3 signaling pathway. However, other signaling pathways that might regulate PDGFD-mediated gemcitabine resistance are not excluded. Thus it is interesting to investigate other downstream signaling pathways of PDGFD such as NF-κB and ERK to complete the study of molecular mechanisms of PDGFD-induced gemcitabine resistance.

It is noteworthy that in addition to PDGFD, the methylations of PDGFB and PDGFC genes were also found significantly reduced in G3K cells compared with MiaPaca-2 cells, based on the RRBS analysis. Therefore, it will be of interest to determine if the methylation change of both PDGFB and PDGFC genes also leads to increased gene transcription and protein expression, and whether these two genes also play important roles in acquired gemcitabine resistance.

The overall outcome of this study suggests the importance of epigenetic alterations, specifically methylation of chromatin DNA, which may play an important role in gemcitabine resistance. It also revealed a critical role of 14-3-3σ and PDGFD in acquired gemcitabine resistance. Finally, targeting 14-3-3σ, PDGFD, and perhaps DNA methylation may be considered for therapeutic development in combinational treatment to overcome gemcitabine resistance.

Summary and Conclusion

The experimental results of this dissertation can be summarized as follows:

1. Acquired gemcitabine resistant cell line G3K is cross-resistant to Ara-C, but not to other anticancer therapeutic drugs been tested.

2. In addition to increased RRM1/2, 14-3-3σ expression and transcription level are dramatic elevated in gemcitabine-resistant cells.

3. Down-regulation of 14-3-3σ expression in gemcitabine-resistant cell line G3K decreases drug resistance level to both gemcitabine and Ara-C.

4. Ectopic over-expression of 14-3-3σ in the parental MiaPaca-2 cells increases resistance to both gemcitabine and Ara-C.

5. 14-3-3σ gene in the parental MiaPaca-2 cells is hyper-methylated, whereas in gemcitabine-resistant G3K cells it is hypo-methylated.

6. Demethylation of 14-3-3σ gene during gemcitabine selection is partially reversible followed by drug retrieval.

7. Although 14-3-3σ expression is up-regulated in Adriamycin-resistant breast cancer cell line MCF7/Advp3000, the methylation status of 14-3-3σ gene remains unchanged during Adriamycin selection.

8. The expression level of DNMT1 in gemcitabine-resistant G3K cells is markedly increased, while down-regulation of DNMT1 in parental MiaPaca-2 cells restores 14-3-3σ gene expression.

9. The expression level of Uhrf1 in gemcitabine-resistant G3K cells is dramatically decreased, and down-regulation of Uhrf1 in parental MiaPaca-2 cells leads to 14-3-3σ gene re-expression.

10. Both Uhrf1 and DNMT1 bind to methylated region of 14-3-3σ gene in the parental MiaPaca-2 cells, together function to repress 14-3-3σ gene expression.

11. DNMT1 protein is recruited by Uhrf1 for binding to 14-3-3σ gene, and down-regulation of Uhrf1 in MiaPaca-2 cells affect DNMT1 binding.

12. Through RRBS, 845 genes are found differentially methylated comparing MiaPaca-2 and G3K cells, and 282 genes found differentially methylated comparing G3K and G3KRev cells.

13. PDGFD gene is reversibly demethylated during gemcitabine selection, and the reversible demethylation leads to corresponding increase/decrease of gene transcription and protein expression.

14. Down-regulation of PDGFD in gemcitabine resistant G3K cells and Aspc-1 cells compromises gemcitabine resistance.

15. Ectopic over-expression of PDGFD in parental MiaPaca-2 cells escalates cell resistance to gemcitabine.

16. The expression level of STAT3, p-stat3, and STAT3 downstream targets MMP2 and VEGF is increased in gemcitabine resistant G3K cells compared with MiaPaca-2 cells.

17. Down-regulation of PDGFD in gemcitabine resistant G3K cells impairs STAT3 signaling pathway, whereas ectopic over-expression of PDGFD in parental MiaPaca-2 cells activates STAT3 signaling pathway.

18. Ectopic over-expression of STAT3 in parental MiaPaca-2 cells increases drug resistance to gemcitabine.

19. The expression level of YAP1/p-YAP1 is increased in gemcitabine-resistant G3K cells.

20. Both the protein and mRNA levels of YAP1 are regulated by 14-3-3σ expression.

21. Down-regulation of YAP1 in gemcitabine-resistant G3K cells leads to sensitization to gemcitabine treatment.

22. Ectopic over-expression of YAP1 in MiaPaca-2 cells dramatically increases gemcitabine resistance, whereas it requires existence of 14-3-3σ.

23. Down-regulation of 14-3-3σ or YAP1 reduces gemcitabine resistance caused by the up-regulation of YAP1 or 14-3-3σ.

24. Down-regulation of either 14-3-3σ or YAP1 in gemcitabine resistant G3K cells and Aspc-1 cells reduces gemcitabine resistance, while down-regulation of both proteins do not further reduce drug resistance.

25. 14-3-3σ and YAP1/p-YAP1 binds to each other in gemcitabine resistant G3K cells, and co-localizes at the cytoplasm.

26. Down-regulation of either 14-3-3σ or YAP1 in gemcitabine resistant G3K cells leads to increased PARP-1 cleavage and caspase-8 activation under gemcitabine treatment.

27. Over-expression of 14-3-3σ inhibits and delays parental MiaPaca-2 cells from gemcitabine-induced PARP-1 cleavage and caspase-8 activation.

Future Plans

The key findings of my current study showed: 1) the contribution of 14-3-3σ over-expression in acquired gemcitabine resistance and its reversibly epigenetic regulation by Uhrf1 and DNMT1 during gemcitabine selection; 2) identification of PDGFD as a differentially methylated gene during gemcitabine selection and as a critical contributor to gemcitabine resistance; 3) 14-3-3σ mediated gemcitabine resistance at least partially by binding with YAP1 protein and together protecting against caspase-8 activation and apoptosis induced by gemcitabine. Future directions that may extend the current are:

1. Based on similar expression and methylation profile of 14-3-3σ and PDGFD, it is possible that PDGFD gene is epigenetically regulated by the same machinery as 14-3-3σ. Therefore, it is very interesting to investigate whether or not Uhrf1 and DNMT1 also binds to the methylated region of PDGFD gene and function to repress its expression. Another gene of interest is p21 the protein expression profile of which is found to be same as 14-3-3σ, yet other studies has demonstrated that Uhrf1 binds to p21 promoter to repress its gene expression. Thereby, it will be interesting to investigate the methylation status of p21 gene during gemcitabine selection and whether or not the increased expression of p21 contributes to acquired gemcitabine resistance. In addition, other proteins such as HDAC1 and methyl-CpG binding proteins like MeCP2 should also

be tested to better understand dynamic changes of "Epigenetic Code Replication Machinery" of 14-3-3σ and PDGFD gene during gemcitabine selection.

2. RRBS data shows a global methylation profile change during gemcitabine selection, yet our top candidate gene PDGFD was only screened among 10 genes transcriptionally tested. Therefore, it is highly possible that there are other genes with methylation status change also play important roles in acquired gemcitabine resistance. Hence continuous discovery of other genes participating in gemcitabine resistance is critical and helps to better understand the complex process of gemcitabine resistance, as well as helps improvement of clinically therapeutic treatment for pancreatic cancer patients in the future.

3. 14-3-3σ and YAP1 over-expression are shown to inhibit gemcitabine-induced caspase-8 activation, which belongs to death receptor pathway. Therefore, whether or not 14-3-3σ and YAP1 mediate through extracellular signals and what is the detail mechanism of inhibiting caspase-8 activation are intriguing and need to be further investigated. Moreover, solid evidence is lacked to prove the importance of caspase-8 inhibition in 14-3-3σ-mediated gemcitabine resistance, and thus the usage of caspase-8 inhibitor or siRNA would help demonstrate this hypothesis. Furthermore, since caspase-3 feeds back on caspase-8 for its activation, it is very interesting to dissect the process of which 14-3-3σ and YAP1 inhibit caspase-8 activation and whether or not inhibition of caspase-3 activation precedes inhibition of capsase-8 activation. Therefore, utilizing a

caspase-3 inhibitor or siRNA would be necessary to help understand the process. If inhibition of caspase-3 precedes inhibition of caspase-8 activation, then future research should focus on the molecular mechanism by which 14-3-3σ and YAP1 inhibit gemcitabine-induced caspase-3 activation.

4. This study showed that 14-3-3σ up-regulation contributes to acquired gemcitabine resistance by up-regulating YAP1 and together inhibit gemcitabine-induced apoptosis. However, since 14-3-3σ is a chaperon protein and binds >100 protein ligands, YAP1 binding followed by inhibition of gemcitabine-induced apoptosis may not the sole mechanism by which 14-3-3σ contributes to gemcitabine resistance. Thereby, other possibilities including but not limited to cytoplasmic retention of CDC2-Cyclin B1 complexes, prevention of mitotic catastrophe, and interference of gemcitabine metabolic pathway should also be investigated. In addition, this study also showed that PDGFD over-expression contributes to acquired gemcitabine resistance possibly by activating Stat3 signaling pathway, yet other signaling pathways downstream of PDGFD activation are not investigated. Therefore, future studies should also focus on other possible signaling pathways like NF-κB and ERK pathway that might mediate PDGFD-induced acquired gemcitabine resistance.

5. This study suggests a bystander effect of resistant cells on sensitive cells as shown in **Figure 20D**, indicating that co-culturing MiaPaca-2 cells with G3K cells likely makes MiaPaca-2 cells more resistant to gemcitabine treatment compared to MiaPaca-2

cells being surrounded by its own. Hence, it will be very interesting to test this hypothesis. In order to do so, stable clones of MiaPaca-2 cells with GFP vector plasmid (MiaPaca-2-GFP cells) should first be established, followed by performing a colony formation assay comparing colonies with green fluorescence under gemcitabine treatment in both experimental group of MiaPaca-2-GFP cells co-cultured with G3K cells and control group of MiaPaca-2-GFP cells co-cultured with MiaPaca-2 cells. The long-term goal of this direction is to inject MiaPaca-2-GFP cells with either G3K cells or MiaPaca-2 cells into mice xenograft model to compare the growth of MiaPaca-2-GFP cells in each group and their responses to gemcitabine treatment.

6. Overall, this research identified 14-3-3σ and PDGFD overexpression by epigenetic regulation and their contribution to acquired gemcitabine resistance, and long-term goals should move forward toward this direction. I have previously established stable clones of 14-3-3σ over-expression and PDGFD over-expression in MiaPaca-2 cells, and stable knockdown clones of 14-3-3σ in G3K cells, and thus stable knockdown clones of PDGFD should also be generated. Then these four pairs of stably over-expression or knockdown cells will be injected into nude mice xenograft, followed by gemcitabine treatment and comparison of parameters including tumor size, tumor volume, mouse survival and death, response to gemcitabine treatment, tumor growth and apoptosis, and tumor angiogenesis and metastasis. These in vivo experiments will help verify the critical role of 14-3-3σ and PDGFD in gemcitabine resistance. Because both 14-3-3σ and PDGFD were identified to be up-regulated in tissue specimens of

pancreatic cancer patients [46, 147], if these in vivo experiments show any significance of survival benefit, it will be very intriguing and important to test this effect in pancreatic cancer patients. First of all, inhibitors of both 14-3-3σ and PDGFD need to be developed. Then pancreatic cancer patients, especially those with advanced and resistant pancreatic cancers, will receive combinational therapies of gemcitabine with 14-3-3σ inhibitor or gemcitabine with PDGFD inhibitor within a safe dose range. This study may finally lead to a discovery and improvement of a successful treatment regimen for pancreatic cancer patients.

Appendices

Appendix 1. List of 230 genes the methylations of which are increased in G3K cells compared to MiaPaca-2 cells:

ABHD14A, ABHD14A-ACY1, ACSS1, ADAM23, ADCY3, ADM, ADORA2A, ADORA2B, ADRA2B, AGXT2L1, AKNA, ANGPTL6, ANKRD20A2, AP3B1, ARHGAP22, ARHGDIA, ARHGEF6, ARHGEF7, ART5, ASAH2B, ATG5, B4GALT6, BCKDK, BCL11B, BDH1, BMP6, BMPR1B, BTBD11, C16orf59, C17orf96, C17orf97, C1orf229, C22orf34, C4orf48, C7orf25, CABP1, CACNG2, CAMK2D, CD8A, CDC42EP1, CDC42EP3, CDH15, CDH4, CDK5R1, CDKN2AIP, CERKL, CHID1, CHRND, CHSY1, COBLL1, CPLX2, CPNE7, CRHR1, CTAGE1, CYP1B1, DAB2IP, DACH1, DACT2, DFNB59, DUSP4, EBF3, ECHDC2, EFNA3, EPHA4, EPHA8, ERVK13-1, ESRP2, FAM167A, FAM176C, FAM219A, FASN, FBXO27, FBXO44, FGFRL1, FLJ45983, FLJ46257, FOS, FOXF2, FYCO1, GADD45B, GALNTL4, GBX2, GJB2, GLIS3, GPC2, GPR160, GPSM1, HBQ1, HCN2, HMHA1, HRASLS, HTT-AS1, IAH1, IGF2-AS, IQSEC2, ITPKA, JAG2, JDP2, KBTBD11, KCNQ2, KCNQ4, KCNS2, KCTD15, KDELC1, KIF1A, KLF11, KLF8, KLHDC7B, KREMEN2, LARGE, LCP1, LEMD3, LINC00159, LINC00293, LINC00461, LINC00473, LLGL2, LMF1, LOC100128946, LOC100131825, LOC285577, LOC286083, LOC643923, LOC728613, LOC728716, LRRC32, LRRFIP1, LYNX1, MAFK, MAP2K3, MAPRE2, MAST3, MDFI, MESDC1, MGMT, MIR1915, MIR3201, MIR3621, MIR4724, MIR598, MMP17, MORN3, MRPS6, MST1P2, MYH11, MYO1B, NCR3LG1, NFATC1, NFIB, NGEF, NINL, NOD2, NPAS2, NRIP3, OLIG1, OR1F1, OSMR, OVOL2, PALLD, PARD3B,

PCGF5, PCSK6, PDLIM1, PEX6, PODXL2, POU3F1, PPM1E, PPOX, PRAF2, PRELID1, PTGFRN, PTK6, PTMA, PTPN21, PTPRG, PWWP2B, RASSF2, RHBDD1, RHOB, RIMBP2, RNF144B, RPS6KL1, RRAGC, RSPO1, SALL1, SAMD4A, SIM2, SLC16A14, SLC3A2, SLC45A1, SLC7A5P2, SLCO5A1, SMARCA2, SNORD56B, SNORD68, SNTG2, SOWAHC, SOX13, ST6GAL1, STK3, SUPT7L, SYCE3, TBR1, TFCP2L1, TMEM17, TMEM179, TMEM181, TMEM200B, TNFRSF6B, TNRC18, TPO, TRABD2B, TSC22D4, USH1G, VEGFC, VPS37D, VWCE, WNT3, WNT9A, XIRP1, ZBTB47, ZDHHC2, ZNF213, ZNF232, ZNF396, ZNF442, ZNF512, ZNF717, ZNF763, ZYX

Appendix 2. List of 615 genes the methylations of which are decreased in G3K cells compared to MiaPaca-2 cells:

AATK-AS1, ABCG1, ABHD8, ABLIM2, ABR, ABRACL, ACTB, ADAM19, ADAMTS3, ADAMTSL4, ADM, ADRA2A, ADRB1, ADRB2, ADSSL1, AFAP1L2, AFF3, AGFG2, AGPAT3, AKR1B1, ALKBH7, ALS2CR8, AMIGO1, AMOTL1, ANK1, ANKRD18DP, ANKRD6, ANXA2P3, APBA1, APBB2, AQP7P1, ARAP3, ARC, ARHGAP5, ARNTL, ARNTL2, ASTE1, ATP8B2, ATRNL1, BAD, BANK1, BARX2, BATF3, BBX, BCL7A, BCOR, BCR, BDH1, BIK, BIN1, BLM, BMP6, BRSK2, C10orf10, C10orf129, C11orf70, C14orf132, C14orf180, C15orf59, C18orf25, C1orf204, C22orf34, C2orf82, C5orf55, C6orf141, C9orf106, C9orf96, CABLES2, CACNA1D, CACNA1I, CACNG4, CAMK2B, CAMK2G, CAV2, CBR3-AS1, CBS, CBX8, CCDC109B, CCDC149, CCDC174, CCDC71L, CCNO, CDC42BPB, CDK6, CDYL, CEBPA, CELSR1, CERS4, CHAC1, CHD7, CHRM4, CHRNA7, CHST1, CHST15, CIB2, CIDEA, CKAP4, CLDN11, CLDN4, CLK3, CLPTM1L, CMTM3, COL11A2, CPE, CPEB2, CPNE5, CRADD, CRAMP1L, CREG2, CT62, CTAG1A, CTAG1B, CTIF, CTSO, CXCL1, CXXC5, CYP2W1, DACT2, DBNDD1, DBP, DCAF5, DCTD, DDIT4L, DENND3, DGAT1, DGKZ, DIXDC1, DLL1, DLX4, DNAJB1, DNAJB2, DNMT3B, DOC2A, DPF1, DPYSL5, DRD3, DSE, DUS3L, DUSP15, DUSP6, EBF3, ECE1, EFNA5, EGLN3, EHBP1L1, ELANE, ELFN1, ELOVL2-AS1, ELOVL7, ENO3, ENOX1, EPS8, ERF, ERICH1, ESYT2, ETHE1, ETS2, ETV2, EXD3, EXPH5, F3, FAM102A, FAM105A, FAM133B, FAM155A, FAM160B1, FAM196A, FAM198B, FAM211A, FAM213A, FAT4, FBXL16, FBXO6, FGF18, FGF19, FGF8, FHOD1, FLJ30403, FLJ42102, FMNL1, FMNL3, FOXF1, FRAS1, FTMT, FUCA1, FUOM, GAB1, GADD45B, GADD45G, GALNT7, GALNTL4,

GAS1, GGN, GLCCI1, GMNC, GNG7, GNS, GORASP1, GPR123, GPR132, GPR146, GPS1, GPX4, GRHL1, GRIA2, GRM4, GSN, GSTO2, H2AFY2, H3F3AP4, HDAC5, HERPUD2, HEY1, HIPK3, HIVEP2, HLA-B, HLA-C, HLA-L, HLX, HMP19, HNRNPD, HOXA3, HOXB2, HOXB9, HS3ST3B1, HSD11B2, HTR6, HUNK, IDH2, IFITM3, IGF2BP2, IGSF3, IKBKE, IL15RA, IL17RA, IL20RB, INPP4B, INS, INSIG1, INSIG2, INTU, IRF2BPL, IRF5, IRS1, ITGA9, JARID2, JMJD8, KANK3, KANK4, KC6, KCNC1, KCNH3, KCNJ12, KCNK18, KCNK9, KIAA1024, KIAA1199, KIAA1324L, KIF1A, KIF21B, KIF6, KIT, KLF13, KLHL36, KLHL4, KLK6, KNDC1, KRBA1, KRT19P2, KSR2, L3MBTL2, LACC1, LEF1-AS1, LGI3, LHB, LIMCH1, LINC00112, LINC00290, LINC00347, LINC00461, LINC00667, LITAF, LMO1, LOC100128511, LOC100271722, LOC100288974, LOC100506190, LOC158572, LOC255512, LOC283663, LOC283922, LOC284751, LOC389895, LOC643355, LOC645752, LOC648987, LOC728875, LONRF3, LPAL2, LPHN2, LPPR3, LRIG1, LRRC43, LRRC6, LRRCC1, LRRK1, LSP1P3, LTBP3, LYPD3, MAML3, MAMSTR, MAN1A1, MAN1C1, MAPK4, MARK1, MB21D1, MBOAT2, MC4R, MCOLN3, MCTP1, MDFIC, MED12L, MEGF9, MEIS2, MERTK, METTL10, MFAP3L, MGAT5B, MGC21881, MGC45800, MICALL1, MIDN, MIR101-1, MIR130B, MIR153-1, MIR205HG, MIR3182, MIR4456, MIR4479, MIR4634, MIR4787, MIR548I2, MIR573, MIR589, MLL3, MLL4, MLLT6, MMP25, MMP28, MN1, MNX1, MSI1, MTA1, MUC6, MYO6, NAAA, NAGPA-AS1, NAT8L, NCKAP5L, NDRG4, NDUFV2, NEIL1, NEK11, NINJ2, NIPAL1, NIPAL4, NKX1-2, NMU, NOTUM, NQO2, NR4A1, NR4A2, NRARP, NRN1, NSG1, NTNG2, NTSR1, NUDT16, OGDHL, OR2L1P, OSBPL7, OSTF1, OTOF, PABPC1L, PABPC5, PALM, PANX2, PAOX, PAQR5, PAQR8, PARD6G, PARP11, PARP8, PARP9, PAX2, PCDHGC3, PDGFB, PDGFC, PDGFD, PDK3, PDPR, PDZD2, PF4, PGAP2, PGF, PHLDA2, PHLDA3, PIK3IP1,

PIK3R1, PITPNM2, PKNOX2, PLEKHA5, PLEKHG1, PLXNA2, PMAIP1, PPCS, PPFIBP2, PPM1E, PPM1H, PPM1K, PPP1R14C, PPP4R4, PRDM1, PRDM5, PRDM6, PRKCDBP, PRKCZ, PSD2, PSMD1, PTBP1, PTCH2, PTPRK, PXDN, QPCT, RAB12, RAB26, RAB3B, RAET1G, RANBP17, RAPGEF4, RASAL2-AS1, RASD2, RASSF9, RCCD1, RHPN1-AS1, RIMBP3B, RIPK4, RNASET2, RNF126, RNF152, RNF207, RNLS, RNPEPL1, ROBO2, ROCK2, RPA4, RTN1, RXRA, SAPCD2, SARM1, SBNO2, SCAND2, SCD5, SCOC, SCRN1, SDC3, SDK1, SDK2, SEL1L3, SEMA4F, SEMA5B, SEPHS2, SEPT11, SEPT4, SEPT5, SEPT8, SEPT9, SERPINE2, SETMAR, SFRP1, SH3BGRL2, SHH, SHOX2, SKIDA1, SLC16A7, SLC16A9, SLC22A20, SLC25A27, SLC27A1, SLC2A11, SLC31A2, SLC35D3, SLC37A2, SLC44A3, SLC4A8, SLC6A8, SLITRK5, SMCR8, SMTNL2, SNORA47, SNORD82, SNX24, SNX33, SOCS1, SOCS3, SOWAHD, SOX4, SOX6, SOX7, SPAG1, SPATA31D1, SPATA6, SPATA6L, SPECC1, SPHK1, SQSTM1, ST3GAL1, ST3GAL5, STK11, STK32C, STOX1, STOX2, SULF2, SUV420H1, SYNE4, SYT12, SYT7, TACC3, TALDO1, TBC1D14, TBC1D9, TBL1XR1, TBX2, TBXAS1, TCF7, TCTE1, TDRD7, TEAD3, TENM3, TFAP2A, TFAP4, THBS2, THEMIS2, TIGD2, TMEFF1, TMEM150C, TMEM179B, TMEM181, TMEM38A, TMEM56-RWDD3, TMX4, TNFSF9, TNK2, TNNI3, TNS3, TP53TG1, TPBG, TPM1, TPM2, TPRA1, TRIL, TRPC3, TSKU, TSPAN14, TSPAN15, TSPAN18, TSPAN5, TTC23L, TTC9, TXN2, TXNRD1, UBE2E2, UHRF1, ULK1, UPP1, UST, VAX2, VEGFC, VPS13D, VTI1B, WDR1, WIPF3, WNT11, WNT2B, WNT4, WNT8B, WSCD2, WWC2-AS2, ZC3HAV1L, ZFAND2A, ZFAND4, ZFHX3, ZFP36, ZFYVE28, ZNF136, ZNF14, ZNF223, ZNF253, ZNF32, ZNF362, ZNF385C, ZNF419, ZNF44, ZNF440, ZNF469, ZNF516, ZNF517, ZNF555, ZNF593, ZNF669, ZNF700, ZNF771, ZNF808, ZNF83, ZNF833P

Appendix 3. List of 140 genes of which the methylations are increased in G3KRev cells compared to G3K cells:

AFAP1L2, ANXA2P3, APBA1, APOBEC3A, B3GNT5, C10orf11, C14orf37, C1orf21, C22orf26, CAMK2D, CCNO, CDC42BPB, CDCA7L, CDK6, CHRNA7, CHURC1-FNTB, CNN3, CRAMP1L, CTAGE1, CYP11A1, DDIT4L, DDX60, DGCR6, DSE, DUSP6, EFNA5, EGR4, ELOVL2-AS1, EPHA7, FAM133B, FAR2, FLJ30838, FRAS1, FRMD6, FUOM, GALNT6, GALNT7, GALNTL1, GDPD5, GFPT2, GLIS3, GNS, GPR160, GREB1L, GRM4, GULP1, H2AFY2, HIC2, HIVEP2, HLA-A, HLX, HNRNPD, HRASLS, IL15, INSIG1, IRAK1BP1, JMJD8, KCNQ4, KIAA1324L, KIAA1609, KIF21A, KIF21B, KLF15, KLHL13, LINC00290, LOC100287042, LOC100506190, LOC285577, LOC648987, LONRF3, LRR1, MCTP1, MDFIC, MIR205HG, MIR4473, MIR4479, MIR573, MLPH, MMP25, MTA1, NFIA, NKX1-2, NOBOX, PARP4, PCDH9, PDGFD, PHF10, PLXNA1, PPM1H, PRDM1, PRKAG2, PRR5L, PTGES, RAB39B, RAD21L1, RAET1G, RASD2, RASSF1, RGS2, RNASET2, ROR2, RSPO4, RUNDC3A, SAMD13, SAMD5, SEMA5B, SERTAD1, SFXN3, SLC16A7, SLC19A2, SLC25A27, SLC31A2, SLC35D3, SLC35G2, SLC7A5P1, SOWAHD, SPATA31D1, STK17A, TMA16, TMEM181, TMEM200B, TRABD2A, TRIL, TRPC3, VDR, VENTXP7, VGLL3, WDR1, WNT11, WNT6, ZNF160, ZNF470, ZNF593, ZNF611, ZNF630, ZNF669, ZNF704, ZNF789, ZNF808, ZNF83

Appendix 4. List of 142 genes of which the methylations are decreased in G3KRev cells compared to G3K cells:

ABCB6, ACAP3, ACOT11, ADAP1, ADORA2B, AHNAK2, AKT3, AMOTL2, ARAF, ARHGAP29, ARHGEF6, ARX, ASAP1-IT1, BCL7A, BCOR, C15orf59, C9orf106, CACNG6, CBFA2T3, CBX8, CDC42EP3, CELSR1, COL18A1, COL18A1-AS1, CPNE7, CSTB, CUX1, CXXC5, DGKE, DOCK3, DPYSL2, EDN2, EIF4E3, ELFN1, EPS8L1, ERVK13-1, EXD3, FAM131B, FAM174A, FAM84B, FBXO27, FGFRL1, FOXQ1, GADD45B, GAS1, GBX2, GFOD1, GPM6B, GPSM1, GRIN1, HCN2, IKBKE, IMPA2, INS, IRX3, JAG2, KCNH3, KLF4, KLHL29, LHX4, LHX9, LINC00523, LINC00674, LMX1B, LOC284751, LOC339874, LOC643355, LRRK1, MAGED4, MAGIX, MB21D1, MFI2, MIR124-3, MIR1915, MIR4456, MIR4516, MIR4686, MMEL1, MN1, MPG, MRPS6, MUC17, MXRA7, NCOR2, NEDD4L, NFIA, NPAS2, NR2F6, NT5DC2, NUDT11, NUP210, NXN, OAZ1, OLIG1, OR1F1, PAX2, PDGFB, PFKFB2, PIP5K1C, PLEC, PNPLA7, PPARG, PRDM16, PRR5, PTPRG, PWWP2B, RALGPS2, RBMS1, RIPK4, RNU6ATAC, SATB1, SCARNA3, SERPINE2, SESTD1, SH3GL1, SHOX2, SIM2, SLC25A17, SLC25A39, SLC45A1, SLC9A3R2, SMAD3, SNCG, SNORD56B, SNORD68, SOCS1, SOX13, SSTR5, TENC1, TET3, TJP3, TNRC18, TPRN, TTYH1, TXN2, WNT9A, WSCD2, WWC3, XIRP1, ZNF213, ZNF555, ZNF761

Appendix 5. List of 309 genes of which the methylations are increased in G3KRev cells comparing with MiaPaca2 cells:

ABHD14A, ABHD14A-ACY1, ACSS1, ADM, ADORA2A, AGXT2L1, AKNA, ANGPTL6, ANKH, ANKRD20A2, APOBEC3A, ARHGAP22, ARHGDIA, ARHGEF16, ARHGEF7, ARL6IP4, ARRDC4, ARSD, ART5, ASAH2B, ATF3, ATG5, B3GNT5, B4GALT6, BAHCC1, BBX, BCKDK, BCL11B, BDH1, BMP6, BMPR1B, BSX, BTBD11, C14orf132, C17orf96, C17orf97, C1orf21, C1orf229, C20orf112, C22orf34, C4orf48, C7orf25, CA12, CABP1, CALN1, CAMK2D, CCND2, CD8A, CDC42EP1, CDCA7L, CDH15, CDH4, CDS1, CERKL, CHID1, CHRND, CHSY1, CNNM1, COBLL1, COL5A1, CPLX2, CPOX, CRHR1, CTAGE1, CTBP2, CYP1B1, DACH1, DDIT4L, DFNB59, DHPS, DLGAP1-AS1, DLGAP2, DSE, DUSP4, EBF3, ECHDC2, EGR1, EGR4, ELFN1, EPHA4, EPHA8, FAHD1, FAM176C, FAM219A, FBXO27, FBXO44, FGFRL1, FLJ44511, FLJ45983, FLJ46257, FOS, FOXA1, FOXF2, FOXP1, FZD1, FZD10, GADD45B, GALNTL4, GBX2, GFPT2, GJB2, GLIS3, GPC1, GPC2, GPR160, GPSM1, GRIN3B, GULP1, H19, HBQ1, HDAC9, HLA-A, HLA-E, HMHA1, HRASLS, IAH1, IFT140, IGF2-AS, IQSEC2, IRX2, ITGB8, ITPKA, JDP2, JHDM1D, KBTBD11, KCNQ2, KCNQ4, KCNS2, KCTD15, KDELC1, KIAA0284, KIAA0513, KIF1A, KLF11, KLF15, KLF6, KLHDC7B, KREMEN2, LARGE, LCP1, LINC00461, LINC00473, LINC00628, LLGL2, LMF1, LOC100128946, LOC100131825, LOC100287042, LOC285577, LOC286083, LOC339807, LOC643923, LOC648987, LOC728613, LOC728716, LPAR3, LRRC32, LRRFIP1, LUZP2, LYNX1, MAFK, MAPRE2, MAST3, MBLAC1, MESDC1, MGMT, MIR3201, MIR3621, MIR3914-2, MIR4655, MIR4724, MIR581, MIR598, MKI67, MLPH, MMP17, MORN3, MST1P2, MYH11, MYO1B, NCR3LG1, NDST3, NFASC, NFIA,

NFIB, NGEF, NINL, NKX6-1, NOD2, NRIP3, NRL, NUDT16L1, OR1F1, OSMR, OVOL2, P4HA2, PALLD, PARD3B, PCDH9, PCGF5, PCOLCE-AS1, PDE4D, PDLIM1, PEX6, PGM2L1, PGPEP1L, PODXL2, POU3F1, PPM1E, PPM1H, PRAF2, PRELID1, PRR5L, PTGFRN, PTK6, PTMA, PTPN21, PWWP2B, RAB39B, RAD21L1, RASGEF1A, RASSF1, RASSF2, RBM33, RHBDD1, RHOB, RIMBP2, RNF130, ROR2, RPP40, RPS6KL1, RPUSD1, RRAGC, RSPO1, RUNDC3A, S1PR1, SALL1, SAMD13, SAMD4A, SARM1, SATB2-AS1, SCAMP1, SERTAD1, SFXN3, SFXN4, SLAIN1, SLC10A3, SLC16A14, SLC35G2, SLC3A2, SLC7A5P1, SLC7A5P2, SLCO5A1, SMARCA2, SNTG2, SOWAHC, ST6GAL1, STK17A, STK3, SUPT7L, SVIL, SYCE3, SYNPO, SYT17, TBR1, TFCP2L1, TGFA, TGFB1I1, TMEM171, TMEM179, TMEM181, TMEM200B, TMEM238, TNRC6C, TPBG, TPO, TRABD2A, TRABD2B, TSC22D3, TSC22D4, TUB, TUBA4A, UBB, USH1G, USP25, VDR, VEGFC, VGLL3, VLDLR, VPS37D, VWCE, WNT2B, WNT3, WNT6, ZDHHC2, ZNF160, ZNF20, ZNF396, ZNF423, ZNF442, ZNF470, ZNF512, ZNF528, ZNF611, ZNF669, ZNF717, ZNF789, ZNF808, ZNRF1

Appendix 6. List of 645 genes of which the methylations are decreased in G3KRev cells comparing with MiaPaca2 cells:

AATK-AS1, ABCB6, ABHD8, ABLIM2, ACAP3, ACOT11, ACTB, ADAM19, ADAMTS3, ADAMTSL4, ADAP1, ADM, ADRB1, ADRB2, ADSSL1, AFF3, AGFG2, AKT3, ALG10, AMOTL1, AMOTL2, ANK1, ANKRD18DP, ANKRD29, APBB2, AQP7P1, ARAP3, ARC, ARHGAP23, ARHGAP29, ARHGAP5, ARHGEF6, ARNTL, ARTN, ARX, ASTE1, ATP8B2, ATRNL1, B4GALNT4, BAHCC1, BARX2, BATF3, BCL2L14, BCL7A, BCOR, BCR, BIK, BIN1, BLM, BMP6, BRSK2, BTNL9, C10orf10, C10orf129, C11orf70, C12orf57, C14orf132, C15orf59, C18orf25, C1orf115, C1orf170, C1orf204, C22orf34, C2CD4C, C3orf79, C6orf141, C9orf106, CABLES2, CACHD1, CACNA1D, CACNA1I, CACNG4, CACNG6, CAMK2B, CAMK2G, CAV2, CBFA2T3, CBLN2, CBR3-AS1, CBX4, CBX8, CCDC109B, CCDC149, CCDC174, CCDC71L, CCNO, CD34, CDC42EP1, CDH8, CDK6, CEBPA, CELSR1, CERS4, CHAC1, CHD7, CHRM4, CHST1, CIB2, CIDEA, CKAP4, CLDN4, CLK3, CMTM3, COL11A2, COL18A1, COL18A1-AS1, CPE, CPEB2, CPNE5, CPNE9, CRADD, CREG2, CRISPLD2, CSNK1E, CSTB, CT62, CTAG1A, CTAG1B, CTIF, CXCL1, CXXC5, CYB5R3, CYP1A1, CYP24A1, CYP2W1, DACT2, DBNDD1, DBP, DCAF5, DCTD, DDHD1, DEFA5, DGKZ, DHRS3, DIXDC1, DLK2, DLL1, DLX4, DNAJB1, DNAJB2, DNAJC6, DNMT3B, DOC2A, DOCK3, DPYSL2, DPYSL5, DSE, DUS3L, DUSP1, DUSP15, EBF3, ECE1, EDN2, EFNA5, EGLN3, EHBP1L1, EIF4E3, ELANE, ELFN1, ELOVL6, ENTHD2, EPHB3, EPS8L1, ERICH1, ESYT2, ETHE1, ETV2, EXD3, FAM105A, FAM184A, FAM196A, FAM198B, FAM20C, FAM211A, FAM213A, FAM27A, FAM65A, FAT4, FBXL16, FBXO6, FGF8, FGFRL1, FLJ42102, FMNL1, FMNL3, FOXF1, FOXQ1, FRG2,

FTMT, FUCA1, FUOM, GAB1, GAD1, GADD45B, GADD45G, GALNT11, GAS1, GBX1, GBX2, GFRA2, GGN, GINS2, GLCCI1, GLIS3, GMNC, GNG7, GORASP1, GPM6B, GPR132, GPR137B, GPR146, GPS1, GRIA2, GRM4, GSTO2, GUK1, H1F0, H3F3AP4, HDAC11, HDAC5, HERPUD2, HEY1, HHEX, HIPK3, HIVEP2, HLA-L, HLX, HNRNPD, HOXA3, HOXB1, HOXB2, HOXB9, HRC, HS3ST3B1, HSD11B2, HTR6, HUNK, IFITM3, IGF2BP2, IGSF3, IGSF8, IKBKE, IL15RA, IL17RA, IL20RB, IMPA2, INF2, INPP4B, INS, INSIG2, INTU, IRF2BPL, IRF5, IRS1, IRX3, ITGA9, JARID2, JMJD8, JUNB, KANK3, KCNC3, KCND3, KCNG3, KCNH3, KCNK18, KCNK9, KIAA0895L, KIAA1024, KIAA1324L, KIF1A, KIF6, KLC2, KLF13, KLF16, KLF4, KLF9, KLHL29, KLHL36, KLK6, KRT19P2, KSR2, L3MBTL2, LEF1-AS1, LGI3, LHB, LHX2, LHX4, LHX9, LIMCH1, LINC00112, LINC00290, LINC00461, LINC00518, LINC00523, LINC00667, LINC00674, LITAF, LMO1, LMO2, LMX1B, LOC100128511, LOC100271722, LOC100288974, LOC100506190, LOC255512, LOC283663, LOC283856, LOC283922, LOC284751, LOC339874, LOC400958, LOC643355, LOC645752, LOC648987, LPAL2, LPIN1, LPPR3, LRIG1, LRRC43, LRRCC1, LRRK1, LSP1P3, LTBP3, LYPD3, MAGED4, MAGIX, MAML3, MAMSTR, MAN1A1, MAN1C1, MAP1B, MAPK4, MARK1, MB21D1, MBOAT2, MCM5, MCOLN3, MDFIC, MECOM, MED12L, MEIS2, METTL10, MFI2, MFNG, MGAT4A, MGAT5B, MGC45800, MICALL1, MIDN, MIR101-1, MIR124-3, MIR130B, MIR153-1, MIR183, MIR3180-4, MIR339, MIR3615, MIR4456, MIR4634, MIR4664, MIR4686, MIR4787, MIR5189, MIR548I2, MIR573, MIR589, MLL3, MLLT6, MMEL1, MMP25, MN1, MNX1, MRPS10, MSI1, MUC17, MUC6, MYZAP, N4BP2L1, NAAA, NBL1, NCOA1, NCOR2, NDRG4, NDUFV2, NEDD4L, NEIL1, NFIA, NINJ2, NIPAL1, NIPAL4, NKX1-2, NOTUM, NPAS2, NPPC, NQO2, NR2F6, NR4A2, NRARP, NRN1, NSUN5, NTNG2, NTSR1, NUDT11, NUDT16,

NUP210, OAZ1, OR1F1, OSBPL7, OTOF, PABPC1L, PAOX, PAQR5, PAQR8, PARD6G, PARP9, PAX2, PCDHGC3, PDGFA, PDGFB, PDGFC, PDPR, PDZD2, PF4, PFKFB2, PHKA2-AS1, PIK3IP1, PIP5K1C, PITPNM2, PLEC, PLEKHA5, PLEKHG1, PLXNA2, PLXNA3, PMAIP1, PNMA2, PNPLA7, PPAPDC3, PPARG, PPCS, PPFIBP2, PPM1H, PPM1K, PPP1R14C, PPP4R4, PRDM1, PRDM16, PRDM6, PRKCQ, PRKCZ, PRR5, PRRX2, PRTG, PSD2, PSMD1, PTBP1, PTCH2, PTDSS2, PTGS1, PTPRK, PXDN, RAB26, RABAC1, RALGPS2, RANBP17, RAPGEF3, RAPGEF4, RASAL2-AS1, RASGEF1A, RASSF9, RBM20, RBMS1, RBPMS, RCAN2, RCCD1, RHPN1-AS1, RIMBP3B, RIPK4, RNF126, RNF152, RNF207, RNLS, RNPEPL1, RNU6ATAC, ROBO2, RSPH1, SAMD14, SBNO2, SCAND2, SCARNA3, SCD5, SCN8A, SCRN1, SDK1, SDK2, SEL1L3, SEMA4F, SEPHS2, SEPT11, SEPT5, SEPT8, SEPT9, SERPINE2, SFRP1, SH3BGRL2, SH3KBP1, SHOX2, SIM2, SKIDA1, SLC16A9, SLC22A20, SLC25A17, SLC25A27, SLC27A1, SLC29A4, SLC2A11, SLC37A2, SLC38A1, SLC44A3, SLC45A1, SLC4A8, SLC52A3, SLC6A8, SLC9A3R2, SMAD3, SMCR8, SMTNL2, SNCG, SNORA47, SNORD56B, SNORD82, SOCS3, SOX4, SOX6, SOX7, SOX8, SOX9, SPAG1, SPATA6, SPATA6L, SPC24, SPECC1, SPHK1, SQSTM1, SRCIN1, ST3GAL1, STK11, STOX2, STX17, SULF2, SUV420H1, SYNE4, SYNM, SYT12, SYT7, TACC1, TACC3, TALDO1, TAOK3, TBC1D14, TBC1D9, TBL1XR1, TBX2, TCF7, TDRD7, TEAD3, TENC1, TENM3, TFAP2A, THEMIS2, TIGD2, TJP3, TMC6, TMEM107, TMEM150C, TMEM179B, TMEM38A, TMEM56-RWDD3, TMTC2, TMX4, TNFSF9, TNNI3, TNRC18, TNS3, TP53TG1, TPBG, TPM1, TPM2, TPRN, TRIM62, TRPC3, TSKU, TSPAN14, TSPAN18, TSPAN5, TTC23L, TTC9, TTYH1, TXN2, TXNRD1, UBAC1, UBE2E2, UHRF1, ULK1, UNC5A, USP49, UST, VAMP3, VEGFC, VPS13D, VTI1B, VWA1, WDR81, WDR83, WIPF3, WNK2, WNT11, WNT2B, WNT4, WNT9A, WSCD2, WWC2-AS2, WWC3, ZBTB7A,

ZC3HAV1L, ZFAND2A, ZFAND4, ZFP36, ZNF136, ZNF14, ZNF223, ZNF304, ZNF362, ZNF385C, ZNF419, ZNF44, ZNF440, ZNF469, ZNF516, ZNF517, ZNF555, ZNF700, ZNF761, ZNF771, ZNF777, ZNF833P

References

1. Siegel R, Naishadham D, Jemal A: **Cancer statistics, 2013**. *CA: a cancer journal for clinicians* 2013, **63**(1):11-30.
2. Alexakis N, Halloran C, Raraty M, Ghaneh P, Sutton R, Neoptolemos JP: **Current standards of surgery for pancreatic cancer**. *The British journal of surgery* 2004, **91**(11):1410-1427.
3. Michaud DS: **Epidemiology of pancreatic cancer**. *Minerva chirurgica* 2004, **59**(2):99-111.
4. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: **Global cancer statistics**. *CA: a cancer journal for clinicians* 2011, **61**(2):69-90.
5. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M: **Pancreatic cancer**. *Lancet* 2011, **378**(9791):607-620.
6. el-Kamar FG, Grossbard ML, Kozuch PS: **Metastatic pancreatic cancer: emerging strategies in chemotherapy and palliative care**. *The oncologist* 2003, **8**(1):18-34.
7. Rosemurgy AS, Serafini FM: **New directions in systemic therapy of pancreatic cancer**. *Cancer control : journal of the Moffitt Cancer Center* 2000, **7**(5):437-451.
8. Mini E, Nobili S, Caciagli B, Landini I, Mazzei T: **Cellular pharmacology of gemcitabine**. *Ann Oncol* 2006, **17 Suppl 5**:v7-12.
9. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA *et al*: **Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group**. *J Clin Oncol* 2007, **25**(15):1960-1966.
10. Stathopoulos GP, Androulakis N, Souglakos J, Stathopoulos J, Georgoulias V: **Present treatment and future expectations in advanced pancreatic cancer**. *Anticancer Res* 2008, **28**(2B):1303-1308.
11. Garcia-Manteiga J, Molina-Arcas M, Casado FJ, Mazo A, Pastor-Anglada M: **Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2',2'-difluorodeoxycytidine- induced cytotoxicity**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2003, **9**(13):5000-5008.
12. Veltkamp SA, Beijnen JH, Schellens JH: **Prolonged versus standard gemcitabine infusion: translation of molecular pharmacology to new treatment strategy**. *The oncologist* 2008, **13**(3):261-276.
13. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W: **Cellular elimination of 2',2'-difluorodeoxycytidine 5'-triphosphate: a mechanism of self-potentiation**. *Cancer research* 1992, **52**(3):533-539.
14. Ruiz van Haperen VW, Veerman G, Boven E, Noordhuis P, Vermorken JB, Peters GJ: **Schedule dependence of sensitivity to 2',2'-difluorodeoxycytidine (Gemcitabine) in relation to accumulation and retention of its triphosphate in**

solid tumour cell lines and solid tumours. *Biochem Pharmacol* 1994, **48**(7):1327-1339.

15. Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC, Grindey GB: **Evaluation of the antitumor activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine)**. *Cancer research* 1990, **50**(14):4417-4422.
16. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W: **Action of 2',2'-difluorodeoxycytidine on DNA synthesis**. *Cancer research* 1991, **51**(22):6110-6117.
17. Galmarini CM, Mackey JR, Dumontet C: **Nucleoside analogues: mechanisms of drug resistance and reversal strategies**. *Leukemia* 2001, **15**(6):875-890.
18. Fukunaga AK, Marsh S, Murry DJ, Hurley TD, McLeod HL: **Identification and analysis of single-nucleotide polymorphisms in the gemcitabine pharmacologic pathway**. *Pharmacogenomics J* 2004, **4**(5):307-314.
19. Maring JG, Groen HJ, Wachters FM, Uges DR, de Vries EG: **Genetic factors influencing pyrimidine-antagonist chemotherapy**. *Pharmacogenomics J* 2005, **5**(4):226-243.
20. Joerger M, Bosch TM, Doodeman VD, Beijnen JH, Smits PH, Schellens JH: **Novel deoxycytidine kinase gene polymorphisms: a population screening study in Caucasian healthy volunteers**. *Eur J Clin Pharmacol* 2006, **62**(8):681-684.
21. Ohuchida K, Mizumoto K, Kayashima T, Fujita H, Moriyama T, Ohtsuka T, Ueda J, Nagai E, Hashizume M, Tanaka M: **MicroRNA expression as a predictive marker for gemcitabine response after surgical resection of pancreatic cancer**. *Ann Surg Oncol* 2011, **18**(8):2381-2387.
22. Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, Wang Z, Philip PA, Sarkar FH: **Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF**. *Cancer research* 2010, **70**(9):3606-3617.
23. Mahon PC, Baril P, Bhakta V, Chelala C, Caulee K, Harada T, Lemoine NR: **S100A4 contributes to the suppression of BNIP3 expression, chemoresistance, and inhibition of apoptosis in pancreatic cancer**. *Cancer research* 2007, **67**(14):6786-6795.
24. Ueno H, Kiyosawa K, Kaniwa N: **Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy?** *British journal of cancer* 2007, **97**(2):145-151.
25. Ohhashi S, Ohuchida K, Mizumoto K, Fujita H, Egami T, Yu J, Toma H, Sadatomi S, Nagai E, Tanaka M: **Down-regulation of deoxycytidine kinase enhances acquired resistance to gemcitabine in pancreatic cancer**. *Anticancer Res* 2008, **28**(4B):2205-2212.
26. Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, Cass C, Lai R, Mackey JR: **The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2004, **10**(20):6956-6961.

27. Wang H, Word BR, Lyn-Cook BD: **Enhanced efficacy of gemcitabine by indole-3-carbinol in pancreatic cell lines: the role of human equilibrative nucleoside transporter 1**. *Anticancer Res* 2011, **31**(10):3171-3180.
28. Santini D, Vincenzi B, Fratto ME, Perrone G, Lai R, Catalano V, Cass C, Ruffini PA, Spoto C, Muretto P *et al*: **Prognostic role of human equilibrative transporter 1 (hENT1) in patients with resected gastric cancer**. *J Cell Physiol* 2010, **223**(2):384-388.
29. Mori R, Ishikawa T, Ichikawa Y, Taniguchi K, Matsuyama R, Ueda M, Fujii Y, Endo I, Togo S, Danenberg PV *et al*: **Human equilibrative nucleoside transporter 1 is associated with the chemosensitivity of gemcitabine in human pancreatic adenocarcinoma and biliary tract carcinoma cells**. *Oncol Rep* 2007, **17**(5):1201-1205.
30. Nakano Y, Tanno S, Koizumi K, Nishikawa T, Nakamura K, Minoguchi M, Izawa T, Mizukami Y, Okumura T, Kohgo Y: **Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells**. *British journal of cancer* 2007, **96**(3):457-463.
31. Ohmine K, Kawaguchi K, Ohtsuki S, Motoi F, Egawa S, Unno M, Terasaki T: **Attenuation of phosphorylation by deoxycytidine kinase is key to acquired gemcitabine resistance in a pancreatic cancer cell line: targeted proteomic and metabolomic analyses in PK9 cells**. *Pharm Res* 2012, **29**(7):2006-2016.
32. Kagawa S, Takano S, Yoshitomi H, Kimura F, Satoh M, Shimizu H, Yoshidome H, Ohtsuka M, Kato A, Furukawa K *et al*: **Akt/mTOR signaling pathway is crucial for gemcitabine resistance induced by Annexin II in pancreatic cancer cells**. *J Surg Res* 2012, **178**(2):758-767.
33. Wang YW, Wang SJ, Zhou YN, Pan SH, Sun B: **Escin augments the efficacy of gemcitabine through down-regulation of nuclear factor-kappaB and nuclear factor-kappaB-regulated gene products in pancreatic cancer both in vitro and in vivo**. *J Cancer Res Clin Oncol* 2012, **138**(5):785-797.
34. Wang SJ, Gao Y, Chen H, Kong R, Jiang HC, Pan SH, Xue DB, Bai XW, Sun B: **Dihydroartemisinin inactivates NF-kappaB and potentiates the anti-tumor effect of gemcitabine on pancreatic cancer both in vitro and in vivo**. *Cancer letters* 2010, **293**(1):99-108.
35. Wang R, Cheng L, Xia J, Wang Z, Wu Q, Wang Z: **Gemcitabine Resistance is Associated with Epithelial-Mesenchymal Transition and Induction of HIF-1 in Pancreatic Cancer Cells**. *Curr Cancer Drug Targets* 2014.
36. Wang Z, Li Y, Kong D, Banerjee S, Ahmad A, Azmi AS, Ali S, Abbruzzese JL, Gallick GE, Sarkar FH: **Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway**. *Cancer research* 2009, **69**(6):2400-2407.
37. Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, Gallick GE, Logsdon CD, McConkey DJ, Choi W: **Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer**. *Cancer research* 2009, **69**(14):5820-5828.

38. Shah AN, Summy JM, Zhang J, Park SI, Parikh NU, Gallick GE: **Development and characterization of gemcitabine-resistant pancreatic tumor cells**. *Ann Surg Oncol* 2007, **14**(12):3629-3637.
39. Li Y, VandenBoom TG, 2nd, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH: **Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells**. *Cancer research* 2009, **69**(16):6704-6712.
40. Du Z, Qin R, Wei C, Wang M, Shi C, Tian R, Peng C: **Pancreatic cancer cells resistant to chemoradiotherapy rich in "stem-cell-like" tumor cells**. *Dig Dis Sci* 2011, **56**(3):741-750.
41. Croker AK, Allan AL: **Cancer stem cells: implications for the progression and treatment of metastatic disease**. *J Cell Mol Med* 2008, **12**(2):374-390.
42. Ponti D, Zaffaroni N, Capelli C, Daidone MG: **Breast cancer stem cells: an overview**. *Eur J Cancer* 2006, **42**(9):1219-1224.
43. Soltysova A, Altanerova V, Altaner C: **Cancer stem cells**. *Neoplasma* 2005, **52**(6):435-440.
44. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW: **Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression**. *J Cell Physiol* 2007, **213**(2):374-383.
45. Min C, Eddy SF, Sherr DH, Sonenshein GE: **NF-kappaB and epithelial to mesenchymal transition of cancer**. *J Cell Biochem* 2008, **104**(3):733-744.
46. Li Z, Dong Z, Myer D, Yip-Schneider M, Liu J, Cui P, Schmidt CM, Zhang JT: **Role of 14-3-3sigma in poor prognosis and in radiation and drug resistance of human pancreatic cancers**. *BMC Cancer* 2010, **10**:598.
47. Li Z, Liu JY, Zhang JT: **14-3-3sigma, the double-edged sword of human cancers**. *American journal of translational research* 2009, **1**(4):326-340.
48. Hermeking H: **The 14-3-3 cancer connection**. *Nature reviews Cancer* 2003, **3**(12):931-943.
49. Muslin AJ, Tanner JW, Allen PM, Shaw AS: **Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine**. *Cell* 1996, **84**(6):889-897.
50. Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ, Cantley LC: **The structural basis for 14-3-3:phosphopeptide binding specificity**. *Cell* 1997, **91**(7):961-971.
51. van Hemert MJ, Steensma HY, van Heusden GP: **14-3-3 proteins: key regulators of cell division, signalling and apoptosis**. *BioEssays : news and reviews in molecular, cellular and developmental biology* 2001, **23**(10):936-946.
52. Lee MH, Lozano G: **Regulation of the p53-MDM2 pathway by 14-3-3 sigma and other proteins**. *Seminars in cancer biology* 2006, **16**(3):225-234.
53. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B: **14-3-3 sigma is a p53-regulated inhibitor of G2/M progression**. *Molecular cell* 1997, **1**(1):3-11.
54. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR *et al*: **High frequency of hypermethylation**

at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(11):6049-6054.

55. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B: **14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage**. *Nature* 1999, **401**(6753):616-620.
56. Chu K, Teele N, Dewey MW, Albright N, Dewey WC: **Computerized video time lapse study of cell cycle delay and arrest, mitotic catastrophe, apoptosis and clonogenic survival in irradiated 14-3-3sigma and CDKN1A (p21) knockout cell lines**. *Radiat Res* 2004, **162**(3):270-286.
57. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, Elledge SJ: **Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25**. *Science* 1997, **277**(5331):1497-1501.
58. Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H: **Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216**. *Science* 1997, **277**(5331):1501-1505.
59. Lopez-Girona A, Furnari B, Mondesert O, Russell P: **Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein**. *Nature* 1999, **397**(6715):172-175.
60. Han Z, Dimas K, Tian X, Wang Y, Hemmi H, Yamada K, Kato N, Pantazis P, Ramanujam RJ, Anant S *et al*: **14-3-3sigma-dependent resistance to cisplatin**. *Anticancer Res* 2009, **29**(6):2009-2014.
61. Neupane D, Korc M: **14-3-3sigma Modulates pancreatic cancer cell survival and invasiveness**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2008, **14**(23):7614-7623.
62. Cetintas VB, Tetik A, Cok G, Kucukaslan AS, Kosova B, Gunduz C, Veral A, Eroglu Z: **Role of 14-3-3sigma in resistance to cisplatin in non-small cell lung cancer cells**. *Cell Biol Int* 2013, **37**(1):78-86.
63. Liu Y, Liu H, Han B, Zhang JT: **Identification of 14-3-3sigma as a contributor to drug resistance in human breast cancer cells using functional proteomic analysis**. *Cancer research* 2006, **66**(6):3248-3255.
64. Sinha P, Hutter G, Kottgen E, Dietel M, Schadendorf D, Lage H: **Increased expression of epidermal fatty acid binding protein, cofilin, and 14-3-3-sigma (stratifin) detected by two-dimensional gel electrophoresis, mass spectrometry and microsequencing of drug-resistant human adenocarcinoma of the pancreas**. *Electrophoresis* 1999, **20**(14):2952-2960.
65. Han B, Xie H, Chen Q, Zhang JT: **Sensitizing hormone-refractory prostate cancer cells to drug treatment by targeting 14-3-3sigma**. *Molecular cancer therapeutics* 2006, **5**(4):903-912.
66. Sudol M: **Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product**. *Oncogene* 1994, **9**(8):2145-2152.

67. Yagi R, Chen LF, Shigesada K, Murakami Y, Ito Y: **A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator**. *EMBO J* 1999, **18**(9):2551-2562.
68. Vassilev A, Kaneko KJ, Shu H, Zhao Y, DePamphilis ML: **TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm**. *Genes Dev* 2001, **15**(10):1229-1241.
69. Sudol M, Bork P, Einbond A, Kastury K, Druck T, Negrini M, Huebner K, Lehman D: **Characterization of the mammalian YAP (Yes-associated protein) gene and its role in defining a novel protein module, the WW domain**. *The Journal of biological chemistry* 1995, **270**(24):14733-14741.
70. Linn H, Ermekova KS, Rentschler S, Sparks AB, Kay BK, Sudol M: **Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP**. *Biol Chem* 1997, **378**(6):531-537.
71. Chen HI, Sudol M: **The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules**. *Proceedings of the National Academy of Sciences of the United States of America* 1995, **92**(17):7819-7823.
72. Huang J, Wu S, Barrera J, Matthews K, Pan D: **The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP**. *Cell* 2005, **122**(3):421-434.
73. Mohler PJ, Kreda SM, Boucher RC, Sudol M, Stutts MJ, Milgram SL: **Yes-associated protein 65 localizes p62(c-Yes) to the apical compartment of airway epithelia by association with EBP50**. *The Journal of cell biology* 1999, **147**(4):879-890.
74. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L *et al*: **Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control**. *Genes Dev* 2007, **21**(21):2747-2761.
75. Hao Y, Chun A, Cheung K, Rashidi B, Yang X: **Tumor suppressor LATS1 is a negative regulator of oncogene YAP**. *The Journal of biological chemistry* 2008, **283**(9):5496-5509.
76. Oka T, Mazack V, Sudol M: **Mst2 and Lats kinases regulate apoptotic function of Yes kinase-associated protein (YAP)**. *The Journal of biological chemistry* 2008, **283**(41):27534-27546.
77. Zhang L, Ren F, Zhang Q, Chen Y, Wang B, Jiang J: **The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control**. *Dev Cell* 2008, **14**(3):377-387.
78. Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D: **Elucidation of a universal size-control mechanism in Drosophila and mammals**. *Cell* 2007, **130**(6):1120-1133.
79. Espanel X, Sudol M: **Yes-associated protein and p53-binding protein-2 interact through their WW and SH3 domains**. *The Journal of biological chemistry* 2001, **276**(17):14514-14523.

80. Samuels-Lev Y, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, Campargue I, Naumovski L, Crook T, Lu X: **ASPP proteins specifically stimulate the apoptotic function of p53**. *Molecular cell* 2001, **8**(4):781-794.
81. Strano S, Munarriz E, Rossi M, Castagnoli L, Shaul Y, Sacchi A, Oren M, Sudol M, Cesareni G, Blandino G: **Physical interaction with Yes-associated protein enhances p73 transcriptional activity**. *The Journal of biological chemistry* 2001, **276**(18):15164-15173.
82. Kanai F, Marignani PA, Sarbassova D, Yagi R, Hall RA, Donowitz M, Hisaminato A, Fujiwara T, Ito Y, Cantley LC *et al*: **TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins**. *EMBO J* 2000, **19**(24):6778-6791.
83. Basu S, Totty NF, Irwin MS, Sudol M, Downward J: **Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis**. *Molecular cell* 2003, **11**(1):11-23.
84. Schumacher B, Skwarczynska M, Rose R, Ottmann C: **Structure of a 14-3-3sigma-YAP phosphopeptide complex at 1.15 A resolution**. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2010, **66**(Pt 9):978-984.
85. Feinberg AP, Ohlsson R, Henikoff S: **The epigenetic progenitor origin of human cancer**. *Nature reviews Genetics* 2006, **7**(1):21-33.
86. Jones PA, Baylin SB: **The fundamental role of epigenetic events in cancer**. *Nature reviews Genetics* 2002, **3**(6):415-428.
87. Sarkar S, Goldgar S, Byler S, Rosenthal S, Heerboth S: **Demethylation and re-expression of epigenetically silenced tumor suppressor genes: sensitization of cancer cells by combination therapy**. *Epigenomics* 2013, **5**(1):87-94.
88. Fukushige S, Horii A: **DNA methylation in cancer: a gene silencing mechanism and the clinical potential of its biomarkers**. *The Tohoku journal of experimental medicine* 2013, **229**(3):173-185.
89. Jacinto FV, Esteller M: **Mutator pathways unleashed by epigenetic silencing in human cancer**. *Mutagenesis* 2007, **22**(4):247-253.
90. Esteller M: **Epigenetic gene silencing in cancer: the DNA hypermethylome**. *Human molecular genetics* 2007, **16 Spec No 1**:R50-59.
91. Dammann R, Schagdarsurengin U, Seidel C, Strunnikova M, Rastetter M, Baier K, Pfeifer GP: **The tumor suppressor RASSF1A in human carcinogenesis: an update**. *Histology and histopathology* 2005, **20**(2):645-663.
92. Fukushima N, Sato N, Ueki T, Rosty C, Walter KM, Wilentz RE, Yeo CJ, Hruban RH, Goggins M: **Aberrant methylation of preproenkephalin and p16 genes in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma**. *The American journal of pathology* 2002, **160**(5):1573-1581.
93. Fukushima N, Sato N, Sahin F, Su GH, Hruban RH, Goggins M: **Aberrant methylation of suppressor of cytokine signalling-1 (SOCS-1) gene in pancreatic ductal neoplasms**. *British journal of cancer* 2003, **89**(2):338-343.
94. Miyamoto K, Asada K, Fukutomi T, Okochi E, Yagi Y, Hasegawa T, Asahara T, Sugimura T, Ushijima T: **Methylation-associated silencing of heparan sulfate D-**

glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. *Oncogene* 2003, **22**(2):274-280.

95. Waraya M, Yamashita K, Katoh H, Ooki A, Kawamata H, Nishimiya H, Nakamura K, Ema A, Watanabe M: **Cancer specific promoter CpG Islands hypermethylation of HOP homeobox (HOPX) gene and its potential tumor suppressive role in pancreatic carcinogenesis**. *BMC cancer* 2012, **12**:397.
96. Zhao G, Qin Q, Zhang J, Liu Y, Deng S, Liu L, Wang B, Tian K, Wang C: **Hypermethylation of HIC1 Promoter and Aberrant Expression of HIC1/SIRT1 Might Contribute to the Carcinogenesis of Pancreatic Cancer**. *Annals of surgical oncology* 2012.
97. Zhao L, Cui Q, Lu Z, Chen J: **Aberrant methylation of RASSF2A in human pancreatic ductal adenocarcinoma and its relation to clinicopathologic features**. *Pancreas* 2012, **41**(2):206-211.
98. Zurita M, Lara PC, del Moral R, Torres B, Linares-Fernandez JL, Arrabal SR, Martinez-Galan J, Oliver FJ, Ruiz de Almodovar JM: **Hypermethylated 14-3-3-sigma and ESR1 gene promoters in serum as candidate biomarkers for the diagnosis and treatment efficacy of breast cancer metastasis**. *BMC Cancer* 2010, **10**:217.
99. Okumura H, Kita Y, Yokomakura N, Uchikado Y, Setoyama T, Sakurai H, Omoto I, Matsumoto M, Owaki T, Ishigami S *et al*: **Nuclear expression of 14-3-3 sigma is related to prognosis in patients with esophageal squamous cell carcinoma**. *Anticancer Res* 2010, **30**(12):5175-5179.
100. Hayashi E, Kuramitsu Y, Fujimoto M, Zhang X, Tanaka T, Uchida K, Fukuda T, Furumoto H, Ueyama Y, Nakamura K: **Proteomic profiling of differential display analysis for human oral squamous cell carcinoma: 14-3-3 sigma Protein is upregulated in human oral squamous cell carcinoma and dependent on the differentiation level**. *Proteomics Clin Appl* 2009, **3**(11):1338-1347.
101. Uchida D, Begum NM, Almofti A, Kawamata H, Yoshida H, Sato M: **Frequent downregulation of 14-3-3 sigma protein and hypermethylation of 14-3-3 sigma gene in salivary gland adenoid cystic carcinoma**. *British journal of cancer* 2004, **91**(6):1131-1138.
102. Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K: **Inactivation of the 14-3-3 sigma gene is associated with 5' CpG island hypermethylation in human cancers**. *Cancer research* 2000, **60**(16):4353-4357.
103. Kunze E, Wendt M, Schlott T: **Promoter hypermethylation of the 14-3-3 sigma, SYK and CAGE-1 genes is related to the various phenotypes of urinary bladder carcinomas and associated with progression of transitional cell carcinomas**. *Int J Mol Med* 2006, **18**(4):547-557.
104. Henrique R, Jeronimo C, Hoque MO, Carvalho AL, Oliveira J, Teixeira MR, Lopes C, Sidransky D: **Frequent 14-3-3 sigma promoter methylation in benign and malignant prostate lesions**. *DNA Cell Biol* 2005, **24**(4):264-269.
105. Lodygin D, Yazdi AS, Sander CA, Herzinger T, Hermeking H: **Analysis of 14-3-3sigma expression in hyperproliferative skin diseases reveals selective loss**

associated with CpG-methylation in basal cell carcinoma. *Oncogene* 2003, **22**(35):5519-5524.

106. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S: **Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer**. *Oncogene* 2001, **20**(26):3348-3353.
107. Iwata N, Yamamoto H, Sasaki S, Itoh F, Suzuki H, Kikuchi T, Kaneto H, Iku S, Ozeki I, Karino Y *et al*: **Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma**. *Oncogene* 2000, **19**(46):5298-5302.
108. Osada H, Tatematsu Y, Yatabe Y, Nakagawa T, Konishi H, Harano T, Tezel E, Takada M, Takahashi T: **Frequent and histological type-specific inactivation of 14-3-3sigma in human lung cancers**. *Oncogene* 2002, **21**(15):2418-2424.
109. Gasco M, Bell AK, Heath V, Sullivan A, Smith P, Hiller L, Yulug I, Numico G, Merlano M, Farrell PJ *et al*: **Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity**. *Cancer research* 2002, **62**(7):2072-2076.
110. Gasco M, Sullivan A, Repellin C, Brooks L, Farrell PJ, Tidy JA, Dunne B, Gusterson B, Evans DJ, Crook T: **Coincident inactivation of 14-3-3sigma and p16INK4a is an early event in vulval squamous neoplasia**. *Oncogene* 2002, **21**(12):1876-1881.
111. Goll MG, Bestor TH: **Eukaryotic cytosine methyltransferases**. *Annual review of biochemistry* 2005, **74**:481-514.
112. Cheng X, Blumenthal RM: **Mammalian DNA methyltransferases: a structural perspective**. *Structure* 2008, **16**(3):341-350.
113. Hermann A, Goyal R, Jeltsch A: **The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites**. *The Journal of biological chemistry* 2004, **279**(46):48350-48359.
114. Leonhardt H, Page AW, Weier HU, Bestor TH: **A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei**. *Cell* 1992, **71**(5):865-873.
115. Liu Y, Oakeley EJ, Sun L, Jost JP: **Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci**. *Nucleic acids research* 1998, **26**(4):1038-1045.
116. Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE: **UHRF1 plays a role in maintaining DNA methylation in mammalian cells**. *Science* 2007, **317**(5845):1760-1764.
117. Sharif J, Muto M, Takebayashi S, Suetake I, Iwamatsu A, Endo TA, Shinga J, Mizutani-Koseki Y, Toyoda T, Okamura K *et al*: **The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA**. *Nature* 2007, **450**(7171):908-912.
118. Bronner C, Achour M, Arima Y, Chataigneau T, Saya H, Schini-Kerth VB: **The UHRF family: oncogenes that are drugable targets for cancer therapy in the near future?** *Pharmacol Ther* 2007, **115**(3):419-434.

119. Unoki M, Brunet J, Mousli M: **Drug discovery targeting epigenetic codes: the great potential of UHRF1, which links DNA methylation and histone modifications, as a drug target in cancers and toxoplasmosis**. *Biochem Pharmacol* 2009, **78**(10):1279-1288.
120. Crnogorac-Jurcevic T, Gangeswaran R, Bhakta V, Capurso G, Lattimore S, Akada M, Sunamura M, Prime W, Campbell F, Brentnall TA *et al*: **Proteomic analysis of chronic pancreatitis and pancreatic adenocarcinoma**. *Gastroenterology* 2005, **129**(5):1454-1463.
121. Unoki M, Kelly JD, Neal DE, Ponder BA, Nakamura Y, Hamamoto R: **UHRF1 is a novel molecular marker for diagnosis and the prognosis of bladder cancer**. *British journal of cancer* 2009, **101**(1):98-105.
122. Berkyurek AC, Suetake I, Arita K, Takeshita K, Nakagawa A, Shirakawa M, Tajima S: **The DNA methyltransferase Dnmt1 directly interacts with the SET and RING finger-associated (SRA) domain of the multifunctional protein Uhrf1 to facilitate accession of the catalytic center to hemi-methylated DNA**. *The Journal of biological chemistry* 2014, **289**(1):379-386.
123. Liu X, Gao Q, Li P, Zhao Q, Zhang J, Li J, Koseki H, Wong J: **UHRF1 targets DNMT1 for DNA methylation through cooperative binding of hemi-methylated DNA and methylated H3K9**. *Nat Commun* 2013, **4**:1563.
124. Hervouet E, Lalier L, Debien E, Cheray M, Geairon A, Rogniaux H, Loussouarn D, Martin SA, Vallette FM, Cartron PF: **Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells**. *PLoS One* 2010, **5**(6):e11333.
125. Cartron PF, Blanquart C, Hervouet E, Gregoire M, Vallette FM: **HDAC1-mSin3a-NCOR1, Dnmt3b-HDAC1-Egr1 and Dnmt1-PCNA-UHRF1-G9a regulate the NY-ESO1 gene expression**. *Molecular oncology* 2012.
126. Felle M, Joppien S, Nemeth A, Diermeier S, Thalhammer V, Dobner T, Kremmer E, Kappler R, Langst G: **The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1**. *Nucleic Acids Res* 2011, **39**(19):8355-8365.
127. Achour M, Jacq X, Ronde P, Alhosin M, Charlot C, Chataigneau T, Jeanblanc M, Macaluso M, Giordano A, Hughes AD *et al*: **The interaction of the SRA domain of ICBP90 with a novel domain of DNMT1 is involved in the regulation of VEGF gene expression**. *Oncogene* 2008, **27**(15):2187-2197.
128. Cartron PF, Blanquart C, Hervouet E, Gregoire M, Vallette FM: **HDAC1-mSin3a-NCOR1, Dnmt3b-HDAC1-Egr1 and Dnmt1-PCNA-UHRF1-G9a regulate the NY-ESO1 gene expression**. *Molecular oncology* 2013, **7**(3):452-463.
129. Alhosin M, Sharif T, Mousli M, Etienne-Selloum N, Fuhrmann G, Schini-Kerth VB, Bronner C: **Down-regulation of UHRF1, associated with re-expression of tumor suppressor genes, is a common feature of natural compounds exhibiting anti-cancer properties**. *J Exp Clin Cancer Res* 2011, **30**:41.
130. Bronner C, Chataigneau T, Schini-Kerth VB, Landry Y: **The "Epigenetic Code Replication Machinery", ECREM: a promising drugable target of the epigenetic cell memory**. *Curr Med Chem* 2007, **14**(25):2629-2641.

131. Unoki M, Nishidate T, Nakamura Y: **ICBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain**. *Oncogene* 2004, **23**(46):7601-7610.
132. Kim JK, Esteve PO, Jacobsen SE, Pradhan S: **UHRF1 binds G9a and participates in p21 transcriptional regulation in mammalian cells**. *Nucleic Acids Res* 2009, **37**(2):493-505.
133. Wang F, Yang YZ, Shi CZ, Zhang P, Moyer MP, Zhang HZ, Zou Y, Qin HL: **UHRF1 promotes cell growth and metastasis through repression of p16(ink(4)a) in colorectal cancer**. *Ann Surg Oncol* 2012, **19**(8):2753-2762.
134. Jin W, Chen L, Chen Y, Xu SG, Di GH, Yin WJ, Wu J, Shao ZM: **UHRF1 is associated with epigenetic silencing of BRCA1 in sporadic breast cancer**. *Breast Cancer Res Treat* 2010, **123**(2):359-373.
135. Andrae J, Gallini R, Betsholtz C: **Role of platelet-derived growth factors in physiology and medicine**. *Genes Dev* 2008, **22**(10):1276-1312.
136. LaRochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, Sullivan C, Boldog FL, Yang M, Vernet C *et al*: **PDGF-D, a new protease-activated growth factor**. *Nature cell biology* 2001, **3**(5):517-521.
137. Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U: **PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor**. *Nature cell biology* 2001, **3**(5):512-516.
138. Li X, Eriksson U: **Novel PDGF family members: PDGF-C and PDGF-D**. *Cytokine & growth factor reviews* 2003, **14**(2):91-98.
139. Wang Z, Kong D, Li Y, Sarkar FH: **PDGF-D signaling: a novel target in cancer therapy**. *Curr Drug Targets* 2009, **10**(1):38-41.
140. Ostman A, Thyberg J, Westermark B, Heldin CH: **PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly**. *The Journal of cell biology* 1992, **118**(3):509-519.
141. Siegfried G, Khatib AM, Benjannet S, Chretien M, Seidah NG: **The proteolytic processing of pro-platelet-derived growth factor-A at RRKR(86) by members of the proprotein convertase family is functionally correlated to platelet-derived growth factor-A-induced functions and tumorigenicity**. *Cancer research* 2003, **63**(7):1458-1463.
142. Fredriksson L, Li H, Eriksson U: **The PDGF family: four gene products form five dimeric isoforms**. *Cytokine & growth factor reviews* 2004, **15**(4):197-204.
143. Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H *et al*: **PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor**. *Nature cell biology* 2000, **2**(5):302-309.
144. Gilbertson DG, Duff ME, West JW, Kelly JD, Sheppard PO, Hofstrand PD, Gao Z, Shoemaker K, Bukowski TR, Moore M *et al*: **Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF alpha and beta receptor**. *The Journal of biological chemistry* 2001, **276**(29):27406-27414.
145. Kong D, Wang Z, Sarkar SH, Li Y, Banerjee S, Saliganan A, Kim HR, Cher ML, Sarkar FH: **Platelet-derived growth factor-D overexpression contributes to epithelial-**

mesenchymal transition of PC3 prostate cancer cells. *Stem Cells* 2008, **26**(6):1425-1435.

146. Kong D, Banerjee S, Huang W, Li Y, Wang Z, Kim HR, Sarkar FH: **Mammalian target of rapamycin repression by 3,3'-diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells**. *Cancer research* 2008, **68**(6):1927-1934.
147. Wang Z, Kong D, Banerjee S, Li Y, Adsay NV, Abbruzzese J, Sarkar FH: **Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling**. *Cancer research* 2007, **67**(23):11377-11385.
148. LaRochelle WJ, Jeffers M, Corvalan JR, Jia XC, Feng X, Vanegas S, Vickroy JD, Yang XD, Chen F, Gazit G *et al*: **Platelet-derived growth factor D: tumorigenicity in mice and dysregulated expression in human cancer**. *Cancer research* 2002, **62**(9):2468-2473.
149. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA: **Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors**. *Cancer research* 2002, **62**(13):3729-3735.
150. Ustach CV, Taube ME, Hurst NJ, Jr., Bhagat S, Bonfil RD, Cher ML, Schuger L, Kim HR: **A potential oncogenic activity of platelet-derived growth factor d in prostate cancer progression**. *Cancer research* 2004, **64**(5):1722-1729.
151. Xu L, Tong R, Cochran DM, Jain RK: **Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model**. *Cancer research* 2005, **65**(13):5711-5719.
152. Ustach CV, Kim HR: **Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells**. *Molecular and cellular biology* 2005, **25**(14):6279-6288.
153. Zhao L, Zhang C, Liao G, Long J: **RNAi-mediated inhibition of PDGF-D leads to decreased cell growth, invasion and angiogenesis in the SGC-7901 gastric cancer xenograft model**. *Cancer Biol Ther* 2010, **9**(1):42-48.
154. Christensen M, Najy AJ, Snyder M, Movilla LS, Kim HR: **A critical role of the PTEN/PDGF signaling network for the regulation of radiosensitivity in adenocarcinoma of the prostate**. *Int J Radiat Oncol Biol Phys* 2014, **88**(1):151-158.
155. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, Sarkar FH: **Emerging roles of PDGF-D signaling pathway in tumor development and progression**. *Biochim Biophys Acta* 2010, **1806**(1):122-130.
156. Li H, Fredriksson L, Li X, Eriksson U: **PDGF-D is a potent transforming and angiogenic growth factor**. *Oncogene* 2003, **22**(10):1501-1510.
157. Liu J, Liao S, Huang Y, Samuel R, Shi T, Naxerova K, Huang P, Kamoun W, Jain RK, Fukumura D *et al*: **PDGF-D improves drug delivery and efficacy via vascular normalization, but promotes lymphatic metastasis by activating CXCR4 in**

breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2011, **17**(11):3638-3648.

158. Zhao Z, Liu Y, He H, Chen X, Chen J, Lu YC: **Candidate genes influencing sensitivity and resistance of human glioblastoma to Semustine**. *Brain Res Bull* 2011, **86**(3-4):189-194.
159. Dong Z, Liu Y, Zhang JT: **Regulation of ribonucleotide reductase M2 expression by the upstream AUGs**. *Nucleic Acids Res* 2005, **33**(8):2715-2725.
160. Liu H, Liu Y, Zhang JT: **A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction**. *Mol Cancer Ther* 2008, **7**(2):263-270.
161. Yang Y, Chen Q, Zhang JT: **Structural and functional consequences of mutating cysteine residues in the amino terminus of human multidrug resistance-associated protein 1**. *The Journal of biological chemistry* 2002, **277**(46):44268-44277.
162. Yang Y, Liu Y, Dong Z, Xu J, Peng H, Liu Z, Zhang JT: **Regulation of function by dimerization through the amino-terminal membrane-spanning domain of human ABCC1/MRP1**. *J Biol Chem* 2007, **282**(12):8821-8830.
163. Huang W, Dong Z, Wang F, Peng H, Liu JY, Zhang JT: **A small molecule compound targeting STAT3 DNA-binding domain inhibits cancer cell proliferation, migration, and invasion**. *ACS Chem Biol* 2014, **9**(5):1188-1196.
164. Liu Z, Dong Z, Yang Z, Chen Q, Pan Y, Yang Y, Cui P, Zhang X, Zhang JT: **Role of eIF3a (eIF3 p170) in intestinal cell differentiation and its association with early development**. *Differentiation* 2007, **75**(7):652-661.
165. Dong Z, Liu Z, Cui P, Pincheira R, Yang Y, Liu J, Zhang JT: **Role of eIF3a in regulating cell cycle progression**. *Exp Cell Res* 2009, **315**(11):1889-1894.
166. Yang Y, Li Z, Mo W, Ambadipudi R, Arnold RJ, Hrncirova P, Novotny MV, Georges E, Zhang JT: **Human ABCC1 interacts and colocalizes with ATP synthase alpha, revealed by interactive proteomics analysis**. *J Proteome Res* 2012, **11**(2):1364-1372.
167. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: **Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands**. *Proceedings of the National Academy of Sciences of the United States of America* 1996, **93**(18):9821-9826.
168. Wang L, Sun J, Wu H, Liu S, Wang J, Wu B, Huang S, Li N, Wang J, Zhang X: **Systematic assessment of reduced representation bisulfite sequencing to human blood samples: A promising method for large-sample-scale epigenomic studies**. *Journal of biotechnology* 2012, **157**(1):1-6.
169. Li R, Yu C, Li Y, Lam TW, Yiu SM, Kristiansen K, Wang J: **SOAP2: an improved ultrafast tool for short read alignment**. *Bioinformatics* 2009, **25**(15):1966-1967.
170. Li Y, Zhu J, Tian G, Li N, Li Q, Ye M, Zheng H, Yu J, Wu H, Sun J *et al*: **The DNA methylome of human peripheral blood mononuclear cells**. *PLoS biology* 2010, **8**(11):e1000533.
171. Nakahira S, Nakamori S, Tsujie M, Takahashi Y, Okami J, Yoshioka S, Yamasaki M, Marubashi S, Takemasa I, Miyamoto A *et al*: **Involvement of ribonucleotide**

reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. *International journal of cancer Journal international du cancer* 2007, **120**(6):1355-1363.

172. Goffin J, Eisenhauer E: **DNA methyltransferase inhibitors-state of the art**. *Ann Oncol* 2002, **13**(11):1699-1716.
173. Gray SG, Baird AM, O'Kelly F, Nikolaidis G, Almgren M, Meunier A, Dockry E, Hollywood D, Ekstrom TJ, Perry AS *et al*: **Gemcitabine reactivates epigenetically silenced genes and functions as a DNA methyltransferase inhibitor**. *Int J Mol Med* 2012, **30**(6):1505-1511.
174. Bronner C, Fuhrmann G, Chedin FL, Macaluso M, Dhe-Paganon S: **UHRF1 Links the Histone code and DNA Methylation to ensure Faithful Epigenetic Memory Inheritance**. *Genetics & epigenetics* 2010, **2009**(2):29-36.
175. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE: **Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning**. *Nature* 2008, **452**(7184):215-219.
176. Li R, Hu Z, Sun SY, Chen ZG, Owonikoko TK, Sica GL, Ramalingam SS, Curran WJ, Khuri FR, Deng X: **Niclosamide overcomes acquired resistance to erlotinib through suppression of STAT3 in non-small cell lung cancer**. *Molecular cancer therapeutics* 2013, **12**(10):2200-2212.
177. Liu F, Cao J, Wu J, Sullivan K, Shen J, Ryu B, Xu Z, Wei W, Cui R: **Stat3-targeted therapies overcome the acquired resistance to vemurafenib in melanomas**. *The Journal of investigative dermatology* 2013, **133**(8):2041-2049.
178. Huang S, Chen M, Shen Y, Shen W, Guo H, Gao Q, Zou X: **Inhibition of activated Stat3 reverses drug resistance to chemotherapeutic agents in gastric cancer cells**. *Cancer letters* 2012, **315**(2):198-205.
179. Duan Z, Foster R, Bell DA, Mahoney J, Wolak K, Vaidya A, Hampel C, Lee H, Seiden MV: **Signal transducers and activators of transcription 3 pathway activation in drug-resistant ovarian cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2006, **12**(17):5055-5063.
180. Lee ES, Ko KK, Joe YA, Kang SG, Hong YK: **Inhibition of STAT3 reverses drug resistance acquired in temozolomide-resistant human glioma cells**. *Oncology letters* 2011, **2**(1):115-121.
181. Goan YG, Zhou B, Hu E, Mi S, Yen Y: **Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line**. *Cancer Res* 1999, **59**(17):4204-4207.
182. Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA: **An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines**. *Cancer Res* 2004, **64**(11):3761-3766.
183. Li Z, Liu J-Y, Zhang J-T: **14-3-3σ, the double-edged sword of human cancers**. *American Journal of Translational Research* 2009, **1**(4):326-340.
184. Hustinx SR, Fukushima N, Zahurak ML, Riall TS, Maitra A, Brosens L, Cameron JL, Yeo CJ, Offerhaus GJ, Hruban RH *et al*: **Expression and Prognostic Significance of**

14-3-3sigma and ERM Family Protein Expression in Periampullary Neoplasms. *Cancer Biol Ther* 2005, **4**(5):596-601.

185. Maxwell SA, Li Z, Jaye D, Ballard S, Ferrell J, Fu H: **14-3-3zeta mediates resistance of diffuse large B cell lymphoma to an anthracycline-based chemotherapeutic regimen**. *J Biol Chem* 2009, **284**(33):22379-22389.
186. Neal CL, Yao J, Yang W, Zhou X, Nguyen NT, Lu J, Danes CG, Guo H, Lan KH, Ensor J *et al*: **14-3-3zeta overexpression defines high risk for breast cancer recurrence and promotes cancer cell survival**. *Cancer Res* 2009, **69**(8):3425-3432.
187. Hodgkinson VC, D EL, Agarwal V, Garimella V, Russell C, Long ED, Fox JN, McManus PL, Mahapatra TK, Kneeshaw PJ *et al*: **Proteomic identification of predictive biomarkers of resistance to neoadjuvant chemotherapy in luminal breast cancer: a possible role for 14-3-3 theta/tau and tBID?** *Journal of proteomics* 2012, **75**(4):1276-1283.
188. Siena L, Pace E, Ferraro M, Di Sano C, Melis M, Profita M, Spatafora M, Gjomarkaj M: **Gemcitabine sensitizes lung cancer cells to Fas/FasL system-mediated killing**. *Immunology* 2014, **141**(2):242-255.
189. Pace E, Melis M, Siena L, Bucchieri F, Vignola AM, Profita M, Gjomarkaj M, Bonsignore G: **Effects of gemcitabine on cell proliferation and apoptosis in non-small-cell lung cancer (NSCLC) cell lines**. *Cancer chemotherapy and pharmacology* 2000, **46**(6):467-476.
190. Yang S, Thor AD, Edgerton S, Yang X: **Caspase-3 mediated feedback activation of apical caspases in doxorubicin and TNF-alpha induced apoptosis**. *Apoptosis : an international journal on programmed cell death* 2006, **11**(11):1987-1997.
191. Ferreira KS, Kreutz C, Macnelly S, Neubert K, Haber A, Bogyo M, Timmer J, Borner C: **Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes**. *Apoptosis : an international journal on programmed cell death* 2012, **17**(5):503-515.
192. Samuel T, Weber HO, Rauch P, Verdoodt B, Eppel JT, McShea A, Hermeking H, Funk JO: **The G2/M regulator 14-3-3sigma prevents apoptosis through sequestration of Bax**. *J Biol Chem* 2001, **276**(48):45201-45206.
193. Subramanian RR, Masters SC, Zhang H, Fu H: **Functional conservation of 14-3-3 isoforms in inhibiting bad-induced apoptosis**. *Exp Cell Res* 2001, **271**(1):142-151.
194. Kim IK, Park SM, Cho HJ, Baek KE, Nam IK, Park SH, Ryu KJ, Ryu J, Choi J, Hong SC *et al*: **14-3-3sigma attenuates RhoGDI2-induced cisplatin resistance through activation of Erk and p38 in gastric cancer cells**. *Oncotarget* 2013, **4**(11):2045-2056.
195. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR *et al*: **High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer**. *PNAS* 2000, **97**(11):6049-6054.
196. Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K: **Inactivation of the 14-3-3 {{sigma}} Gene Is Associated with 5' CpG Island Hypermethylation in Human Cancers**. *Cancer Res* 2000, **60**(16):4353-4357.

197. Yang HY, Wen YY, Chen CH, Lozano G, Lee MH: **14-3-3 sigma positively regulates p53 and suppresses tumor growth**. *Molecular and cellular biology* 2003, **23**(20):7096-7107.
198. Zheng G, Xiong Y, Yi S, Zhang W, Peng B, Zhang Q, He Z: **14-3-3sigma regulation by p53 mediates a chemotherapy response to 5-fluorouracil in MCF-7 breast cancer cells via Akt inactivation**. *FEBS letters* 2012, **586**(2):163-168.
199. Sang M, Li Y, Ozaki T, Ono S, Ando K, Yamamoto H, Koda T, Geng C, Nakagawara A: **p73-dependent induction of 14-3-3sigma increases the chemo-sensitivity of drug-resistant human breast cancers**. *Biochemical and biophysical research communications* 2006, **347**(1):327-333.
200. Horie-Inoue K, Inoue S: **Epigenetic and proteolytic inactivation of 14-3-3sigma in breast and prostate cancers**. *Seminars in cancer biology* 2006, **16**(3):235-239.
201. Zakharchenko O, Cojoc M, Dubrovska A, Souchelnytskyi S: **A role of TGFss1 dependent 14-3-3sigma phosphorylation at Ser69 and Ser74 in the regulation of gene transcription, stemness and radioresistance**. *PloS one* 2013, **8**(5):e65163.
202. Bock C, Tomazou EM, Brinkman AB, Muller F, Simmer F, Gu H, Jager N, Gnirke A, Stunnenberg HG, Meissner A: **Quantitative comparison of genome-wide DNA methylation mapping technologies**. *Nature biotechnology* 2010, **28**(10):1106-1114.
203. Bogdanovic O, Veenstra GJ: **DNA methylation and methyl-CpG binding proteins: developmental requirements and function**. *Chromosoma* 2009, **118**(5):549-565.
204. Klose RJ, Bird AP: **Genomic DNA methylation: the mark and its mediators**. *Trends in biochemical sciences* 2006, **31**(2):89-97.
205. Payer B, Lee JT: **X chromosome dosage compensation: how mammals keep the balance**. *Annual review of genetics* 2008, **42**:733-772.
206. Okamoto I, Heard E: **Lessons from comparative analysis of X-chromosome inactivation in mammals**. *Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology* 2009, **17**(5):659-669.
207. Mohn F, Weber M, Rebhan M, Roloff TC, Richter J, Stadler MB, Bibel M, Schubeler D: **Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors**. *Molecular cell* 2008, **30**(6):755-766.
208. Hsieh CL: **Dependence of transcriptional repression on CpG methylation density**. *Molecular and cellular biology* 1994, **14**(8):5487-5494.
209. Hsieh CL: **Stability of patch methylation and its impact in regions of transcriptional initiation and elongation**. *Molecular and cellular biology* 1997, **17**(10):5897-5904.
210. Kass SU, Goddard JP, Adams RL: **Inactive chromatin spreads from a focus of methylation**. *Molecular and cellular biology* 1993, **13**(12):7372-7379.
211. Chen C, Yang MC, Yang TP: **Evidence that silencing of the HPRT promoter by DNA methylation is mediated by critical CpG sites**. *The Journal of biological chemistry* 2001, **276**(1):320-328.

212. Graessmann A, Sandberg G, Guhl E, Graessmann M: **Methylation of single sites within the herpes simplex virus tk coding region and the simian virus 40 T-antigen intron causes gene inactivation**. *Molecular and cellular biology* 1994, **14**(3):2004-2010.
213. Yisraeli J, Frank D, Razin A, Cedar H: **Effect of in vitro DNA methylation on beta-globin gene expression**. *Proceedings of the National Academy of Sciences of the United States of America* 1988, **85**(13):4638-4642.
214. Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND, Scandura JM: **DNA methylation of the first exon is tightly linked to transcriptional silencing**. *PloS one* 2011, **6**(1):e14524.
215. Curradi M, Izzo A, Badaracco G, Landsberger N: **Molecular mechanisms of gene silencing mediated by DNA methylation**. *Molecular and cellular biology* 2002, **22**(9):3157-3173.
216. Buschhausen G, Wittig B, Graessmann M, Graessmann A: **Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene**. *Proceedings of the National Academy of Sciences of the United States of America* 1987, **84**(5):1177-1181.
217. Kass SU, Landsberger N, Wolffe AP: **DNA methylation directs a time-dependent repression of transcription initiation**. *Current biology : CB* 1997, **7**(3):157-165.

Curriculum Vitae

Li Qin

EDUCATION

2009-2014	Indiana University, Indianapolis, United States	Ph.D. Pharmacology
2005-2009	Nankai University, Tianjin, China	B.S. Biological Science

ACADEMIC EXPERIENCES

2009-2014 Graduate student

Indiana University, Indianapolis, United States with Dr. Jian-Ting Zhang
Thesis title: Molecular mechanisms of acquired gemcitabine resistance in pancreatic cancer

2005-2009 Undergraduate Research

Nankai University, Tianjin, China with Dr. Zhi-Nan Yin

ACADEMIC ACTIVITIES

5/2014	The 9th Annual Meeting of the Great Lakes Drug Metabolism & Disposition Group, Indianapolis, IN, United States
3/2014	The 6th Yao Yuan Biotech-Pharma Symposium, Chicago, IL, United States
7/2013	Molecular Therapeutics of Cancer Meeting, Boulder, CO, United States
3/2012	2012 American Association for Cancer Research Annual Meeting, Chicago, IL, United States

AWARDS

1/2014	Paradise Travel Award, Indiana University
2009-2014	Indiana University Scholarship
7/2007	Top-grade National Encouragement Scholarship, Nankai University
7/2006	The Scholarship for Excellent Undergraduate of Nankai University

PUBLICATIONS

- **Qin L**, Dong ZZ, Zhang JT. Reversible epigenetic regulation of 14-3-3σ expression in acquired gemcitabine resistance by Uhrf1 and DNA methyltransferase 1. *Molecular Pharmacology.* 2014, 86(5):561-9.
- **Qin L**, Dong ZZ, Zhang JT. Emerging role of 14-3-3σ/YAP1 complex in acquired gemcitabine resistance in pancreatic cancer. (in preparation)
- **Qin L**, Hao YY, Dong ZZ, Liu YL, Zhang JT. Identification of PDGFD as a contributor of gemcitabine resistance by Reduced Representation Bisulfite Sequencing. (in preparation)
- Wu X, **Qin L**, Fako V, Zhang JT. Molecular mechanisms of fatty acid synthase (FASN)-mediated resistance to anti-cancer treatments. *Advances in Biological Regulation*. 2014, 54:214-21
- Li Z, Peng H, **Qin L**, Qi J, Zuo X, Liu JY, Zhang JT. Determinants of 14-3-3σ protein dimerization and function in drug and radiation resistance. *The Journal of Biological Chemistry*. 2013, 288(44):31447-57.