

The Role of FOXM1 and its Cofactors HMGA1 and HMGN1 in Breast Cancer Progression

Shang Yao

Department of Surgery and Cancer, Division of Cancer,
Imperial College London, Hammersmith Hospital Campus, London, United Kingdom

Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

June 2019

DECLARATION OF ORIGINALITY

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

Shang Yao (06.2019)

COPYRIGHT DECLARATION

'The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution- Non-Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.'

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Professor Eric W-F Lam, for his patient guidance and invaluable inspiration through each stage of this project. I would also like to thank my co-supervisor, Dr Ernesto Yague, for his kind advice and enthusiastic encouragement.

I would like to acknowledge my colleagues and collaborators. With a special mention to Dr Laura Bella, Dr Stefania Zona, Shi-qi Wang, Yannasittha Jiramongkol, Glow Alasiri, Lavender Fan, Jaysze Yong, Zimam Mahmud and George Leung. It was fantastic to have the opportunity to work with you all. You supported me greatly and were always willing to help me. To my friends, thank you for your thoughts, well-wishes and supports through this entire process. Thank you for being there whenever I needed it.

Lastly, I would like to express my deep gratitude to my beloved parents, Run-jie Gao and Chun-gui Yao, for their wise counsel and sympathetic ear. You have encouraged me in all of my pursuits and inspired me to follow my dreams. You are always there for me. I could not have done any of this without you. Thank you!

PUBLICATIONS

1. Characterization of FOXO Acetylation.

Yao S, Mahmud Z, Sachini N, Aimjongjun S, Saavedra-García P, Lam EW-F
Methods Mol Biol 1890:77-90 2019

2. The FOXO3-FOXm1 axis: A key cancer drug target and a modulator of cancer drug resistance

Yao S, Fan LY-N, Lam EW-F
SEMINARS IN CANCER BIOLOGY 50:77-89 (13 pages) 01 Jun 2018

3. FOXM1 modulates 5-FU resistance in colorectal cancer through regulating TYMS expression

Varghese V, Magnani L, Harada N, Mauri F, Szydlo R, Yao S, Lam E, Kenny L
Sci Rep 9(1):1505 06 Feb 2019

4. FOXM1 modulates 5-fluorouracil sensitivity in cholangiocarcinoma through thymidylate synthase (TYMS): implications of FOXM1-TYMS axis uncoupling in 5-FU resistance

Intuyod K, Saavedra-Garcia P, Zona S, Lai C-F, Jiramongkol Y, Vaeteewoottacharn K, Pairojkul C, Yao S, Yong J-S, Trakansuebkul S
CELL DEATH & DISEASE 9:15 pages Article number ARTN 1185 11 Dec 2018

5. SUMOylation modulates FOXK2-mediated paclitaxel sensitivity in breast cancer cells

de Moraes GN, Ji Z, Fan LY-N, Yao S, Zona S, Sharrocks AD, Lam EW-F
ONCOGENESIS 7:11 pages Article number ARTN 29 01 Mar 2018

6. p62/SQSTM1 interacts with vimentin to enhance breast cancer metastasis

Li S-S, Xu L-Z, Zhou W, Yao S, Wang C-L, Xia J-L, Wang H-F, Kamran M, Xue X-Y, Dong L
CARCINOGENESIS 38(11):1092-1103 (12 pages) 01 Nov 2017

7. Thienopyrimidinone Based Sirtuin-2 (SIRT2)-Selective Inhibitors Bind in the Ligand Induced Selectivity Pocket

Sundriyal S, Moniot S, Mahmud Z, Yao S, Di Fruscia P, Reynolds CR, Dexter DT, Sternberg MJE, Lam EW-F, Steegborn C
JOURNAL OF MEDICINAL CHEMISTRY 60(5):1928-1945 (18 pages) 09 Mar 2017

8. RNF168 cooperates with RNF8 to mediate FOXM1 ubiquitination and degradation in breast cancer epirubicin treatment

Kongsema M, Zona S, Karunarathna U, Cabrera E, Man EPS, Yao S, Shibakawa A, Khoo U-S, Medema RH, Freire R
ONCOGENESIS 5:16 pages Article number ARTN e252 01 Aug 2016

9. BRCA1 positively regulates FOXO3 expression by restricting FOXO3 gene methylation and epigenetic silencing through targeting EZH2 in breast cancer

Gong C, Yao S, Gomes AR, Man EPS, Lee HJ, Gong G, Chang S, Kim S-B, Fujino K, Kim S-W
ONCOGENESIS 5:15 pages Article number ARTN e214 01 Apr 2016

10. Forkhead box K2 modulates epirubicin and paclitaxel sensitivity through FOXO3a in breast cancer

de Moraes GN, Khongkow P, Gong C, Yao S, Gomes AR, Ji Z, Kandola N, Delbue D, Man EPS, Khoo US
ONCOGENESIS 4:12 pages Article number ARTN e167 01 Sep 2015

ABSTRACT

FOXM1 is a potent oncogenic transcription factor that plays essential roles in multiple hallmarks of cancer progression, thus having impacts on tumour initiation, invasion, angiogenesis, cancer stemness and drug sensitivity. Likewise, high mobility group proteins, HMGA1 and HMGN1 are also involved in tumorigenic and metastatic processes in cancer. The human protein-protein interactions prediction database has reported the potential interactions between these proteins, but no further experimental evidence to confirm and characterise this link. In my project, I demonstrated that FOXM1 binds to HMGA1 or HMGN1 as protein-protein complex, and such interactions affect the transcriptional regulation of *KIF20A* and *SQSTM1/p62*. Disruption of the complex or their protein expression levels significantly affects drug sensitivity, cell migration and invasion in breast cancer cells. Apart from the essential roles of HMGA1 and HMGN1 in DNA repair mechanisms, they influence these malignant processes may partially through regulating FOXM1 activities. Thus, the functions of these FOXM1's transcriptional targets, *KIF20A* and *p62* were further examined. The results showed the p62 is involved in modulating angiogenic and metastatic processes in breast cancer as silencing of p62 significantly attenuated tumour-induced angiogenesis and cell migration in zebrafish. Furthermore, KIF20A is a novel target which KIF20A inhibition increased drug sensitivity and reduced cell migration, invasion and mammosphere formation. In addition, the new KIF20A inhibitor, BKS0349 compound offers a viable therapeutic option for cancer treatment in future.

In summary, my results have established a potential mechanism underlying the interactions between FOXM1, HMGA1 and HMGN1 in breast cancer cells. Collectively, these findings strengthen our current understanding of the oncogenic protein FOXM1-

mediated cancer progression and provide new insights into cancer diagnosis and treatment.

CONTENTS

DECLARATION OF ORIGINALITY	2
COPYRIGHT DECLARATION	2
ACKNOWLEDGEMENTS	3
PUBLICATIONS	4
ABSTRACT	5
LIST OF FIGURES.....	10
LIST OF TABLES	12
LIST OF APPENDIX	13
CHAPTER 1 INTRODUCTION	20
1.1 BREAST CANCER.....	20
1.1.1 <i>General overview</i>	20
1.1.2 <i>Metastatic breast cancer</i>	21
1.2 ZEBRAFISH MODELS FOR CANCER RESEARCH	23
1.3 FOX PROTEIN: FOXM1	27
1.3.1 <i>General overview</i>	27
1.3.2 <i>Regulation of FOXM1</i>	28
1.3.3 <i>Proteins interacting with FOXM1</i>	33
1.3.4 <i>FOXM1 in cancer progression</i>	35
1.3.5 <i>FOXM1 in chemotherapy sensitivity and resistance</i>	39
1.4 KINESIN: KIF20A.....	40
1.5 AUTOPHAGY ADAPTOR: P62.....	43
1.6 HISTONE MOBILE GROUP PROTEINS	49
1.6.1 <i>General overview</i>	49
1.6.2 <i>HMG proteins</i>	49
1.6.3 <i>HMGA protein: structure and function</i>	50
1.6.4 <i>Post-translational modifications of HMGA protein</i>	54
1.6.5 <i>Role of HMGA during development</i>	58
1.6.6 <i>HMGA proteins in cancer</i>	60
1.6.7 <i>HMGN protein: structure and function</i>	72
1.6.8 <i>Histone modifications and post-translational modifications of HMGNs</i>	76
1.6.9 <i>Role of HMGNs during development</i>	80
1.6.10 <i>HMGN proteins and cancer</i>	84
1.7 AIMS.....	90
CHAPTER 2 MATERIALS AND METHODS	91
2.1 CELL CULTURE	91
2.1.1 <i>Cell lines</i>	91
2.1.2 <i>Cell maintenance</i>	91
2.1.3 <i>Cell storage</i>	92
2.2 DRUG TREATMENT	92

2.3 PLASMID CONSTRUCTS	93
2.3.1 <i>Plasmids</i>	93
2.3.2 <i>Plasmid Miniprep and Maxiprep</i>	93
2.4 TRANSFECTION	93
2.4.1 <i>Gene Silencing with Small Interfering RNAs (siRNA)</i>	93
2.4.2 <i>FuGENE 6 transfection</i>	94
2.5 WESTERN BLOTTING	95
2.5.1 <i>Protein extraction and quantification</i>	95
2.5.2 <i>Subcellular Fractionation</i>	96
2.5.3 <i>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)</i>	96
2.5.4 <i>Immunoblotting</i>	97
2.5.5 <i>Antibodies</i>	98
2.6 REAL-TIME QUANTITATIVE PCR (RT-qPCR)	99
2.6.1 <i>RNA extraction and quantification</i>	99
2.6.2 <i>Complementary DNA (cDNA) synthesis</i>	99
2.6.3 <i>Primer design and optimization</i>	100
2.6.4 <i>Real time quantitative PCR (RT-qPCR)</i>	100
2.7 CO-IMMUNOPRECIPITATION	101
2.8 DUOLINK PROXIMITY LIGATION ASSAY (PLA)	102
2.9 CHROMATIN IMMUNOPRECIPITATION (CHIP)	103
2.9.1 <i>Cell harvest and lysis</i>	103
2.10 SULPHORHODAMINE B (SRB) ASSAY	106
2.11 CLONOGENIC ASSAY	106
2.12 TRANSWELL MIGRATION ASSAY	107
2.13 TRANSWELL INVASION ASSAY	108
2.14 MAMMOSPHERE FORMATION ASSAY	108
2.15 ZEBRAFISH	108
2.15.1 <i>Zebrafish husbandry</i>	108
2.15.2 <i>CM-Dil labelling</i>	109
2.15.3 <i>Microinjection</i>	109
2.16 STATISTICAL ANALYSIS	110
CHAPTER 3	111
3.1 INTRODUCTION	111
3.2 BASAL EXPRESSION LEVELS OF FOXM1, HMGA1 AND HMGN1	112
3.3 FOXM1 INTERACTS WITH HMGA1 AND HMGN1 AT PROTEIN LEVEL	114
3.4 FOXM1 BINDS TO <i>KIF20A</i> AND <i>P62</i> GENE PROMOTER REGIONS	118
3.5 HMGA1 ATTENUATES FOXM1 PROMOTER BINDING ABILITY WHEREAS HMGN1 PROMOTES FOXM1 BINDING	125
3.6 HMGA1 MODULATES THE EXPRESSION OF FOXM1 TARGET GENES- <i>KIF20A</i> AND <i>P62</i>	130
3.7 ALTERATION OF HMGN1 EXPRESSION AFFECTS <i>KIF20A</i> AND <i>P62</i> EXPRESSION ...	133
3.8 FOXM1 INTERACTS WITH HMGA1 AND HMGN1 IN RESPONSE TO DRUG TREATMENT	137
3.9 DISTINCT EXPRESSION PATTERNS OF FOXM1 AND HMG PROTEINS UPON DRUG TREATMENTS	149

3.10 SILENCING FOXM1, HMGA1 OR HMGN1 SENSITIZE BREAST CANCERS CELLS IN RESPONSE TO DRUG TREATMENT	154
3.11 SILENCING OF FOXM1, HMGA1 OR HMGN1 REDUCES CELL MIGRATION AND INVASION	160
3.12 DISCUSSION	165
CHAPTER 4.....	171
4.1 INTRODUCTION	171
4.2 SILENCING OF P62 SIGNIFICANTLY DIMINISHES TUMOUR-INDUCED ANGIOGENESIS IN ZEBRAFISH	173
4.3 KNOCKDOWN OF P62 ATTENUATES CELL MIGRATION IN ZEBRAFISH	176
4.4 DISCUSSION	179
CHAPTER 5.....	182
5.1 INTRODUCTION	182
5.2 THE CYTOTOXICITY EFFECT OF BKS0349 IN BREAST CANCER CELLS	183
5.3 THE CYTOTOXIC EFFECT OF BKS0349 FOLLOWING LONG-TERM EXPOSURE	186
5.4 BKS0349 SIGNIFICANTLY DIMINISHES CELL MIGRATION AND INVASION OF TAXR AND EPIR CELLS	188
5.5 INHIBITORY EFFECTS OF BKS0349 COMPOUND ON MAMMOSPHERE FORMATION ...	190
5.6 DISCUSSION	192
CHAPTER 6 FINAL DISCUSSION	195
REFERENCES.....	202
APPENDIX.....	225

LIST OF FIGURES

Figure 1. 1 Schematic diagram of FOXM1 and transcription factors (both activator and repressor) in <i>FOXM1</i> promoter region.....	33
Figure 1. 2 Functional roles of FOXM1 in cancer progression.....	38
Figure 1. 3 (A)Three types of autophagy.....	48
Figure 1. 4 Multiple functions of HMGA protein.	54
Figure 1. 5 Flow-chart of research design.	90
Figure 3. 1 The basal expression levels of FOXM1, HMGA1 and HMGN1 in MCF-7, TaxR and EpiR cell lines.....	113
Figure 3. 2 FOXM1 interacts with HMGA1 and HMGN1.....	117
Figure 3. 3.1 FOXM1 binding sites on human <i>KIF20A</i> and <i>p62</i>	122
Figure 3. 3.2 FOXM1 directly binds to the <i>HMGA1</i> , <i>HMGN1</i> , <i>KIF20A</i> and <i>p62</i> at promoter regions	123
Figure 3. 3.3 Regulations of FOXM1 on <i>KIF20A</i> and <i>p62</i>	124
Figure 3. 4.1 HMGA1 restricts FOXM1 binding ability in MCF-7 cells, whereas HMGN1 is required for FOXM1 binding.....	127
Figure 3. 4.2 Binding of HMGA1 and HMGN1 at <i>KIF20A</i> and <i>p62</i> promoter regions	128
Figure 3. 5 Altercations of HMGA1 affect <i>KIF20A</i> and <i>p62</i> expression.	132
Figure 3. 6 HMGN1 overexpression and knockdown significantly affect <i>KIF20</i> and <i>p62</i> expression in MCF-7 cells.	135
Figure 3. 7.1 Protein-protein interactions of FOXM1 and HMGA1 in breast cancer MCF-7, MCF-7 TaxR and MCF-7 EpiR cell lines	143
Figure 3. 7.2 <i>In situ</i> PLA for protein-protein interactions between FOXM1 and HMGN1	148
Figure 3. 8 The expression levels of FOXM1 and relevant proteins in response to drug treatments at different time points.	153

Figure 3. 9.1 Knockdown of FOXM1, HMGA1 and HMGN1 reduces colony formation.	157
Figure 3. 9.2 Knockdown of FOXM1, HMGA1 and HMGN1 significantly reduces cell colony formation and sensitizes cellular response to epirubicin treatment	159
Figure 3. 10 Silencing of FOXM1, HMGA1 or HMGN1 significantly reduces breast cancer cell migration and invasion.	163
Figure 3. 11 The proposed mechanism of interactions between FOXM1, HMGA1 and HMGN1 in breast cancer MCF-7 cells.....	169
Figure 4. 1 Silencing of p62 in MCF-7 cells results in decreased tumour induced angiogenesis.....	174
Figure 4. 2 Depletion of p62 reduces cell migration in Tra/Nac zebrafish.....	177
Figure 4. 3 Multifunctional nature of p62 impacts on tumorigenesis and metastasis.	180
Figure 5. 1 The BKS0349 compound significantly reduces cell viability of TaxR and EpiR cells compared to MCF-7.....	184
Figure 5. 2 BKS0349 acts more efficiently on TaxR and EpiR cells over a longer term.....	187
Figure 5. 3 BKS0349 compound attenuates cell migration and invasion.....	189
Figure 5. 4 Inhibitory effect of BKS0349 on mammosphere formation in MCF-7, TaxR and EpiR cells.....	191

LIST OF TABLES

Table 1. 1 Advantages and disadvantages of zebrafish as a model for cancer research	27
Table 1. 2 Summary of differences between HMGA and HMGN proteins	89
Table 2. 1 Composition of resolving and stacking gel.....	97
Table 2. 2 List of antibodies used for Western blotting.	98
Table 2. 3 List of human RT-qPCR primers used in this study	100
Table 2. 4 List of primer sequences used for quantitative real-time PCR following ChIP.....	105
Table S. 1 Copyright permissions summary	225

LIST OF APPENDIXES

Table S. 1 Copyright permissions summary.	225
Figure S. 1 Copyright permission for Figure 1. 3 A	226
Figure S. 2 Copyright permission for Figure 1. 4.	227
Figure S. 3 Regulations of FOXM1 on KIF20A and p62.	228
Figure S. 4 FOXM1, HMGA1 and HMGN1 interactions in response to paclitaxel treatment.	229
Figure S. 5 The interactions between FOXM1, HMGA1 and HMGN1 in response to epirubicin treatment.	230
Figure S. 6 Subcellular localization of HMGA1 and HMGN1 upon drug treatment.. ..	231

ABBREVIATIONS

5-FU: 5-fluorouracil
ADOX: Adenosine Dialdehyde
Akt: Ak strain transforming, a.k.a Protein Kinase B (PKB)
AML: Acute Myeloid Leukaemia
AP1: Activator Protein 1
APC/C: Anaphase-promoting Complex/ Cyclosome
APS: Ammonium Persulphate
ATF2: Cyclic AMP-dependent Transcription Factor
ATG: Autophagy-related Gene
ATM: Ataxia Telangiectasia -mutated Kinase
ATP: Adenosine Triphosphate
AURKA: Aurora Kinase A
Bax: Bcl2-associated X Protein
Bcl2: B Cell Leukaemia/lymphoma 2
BMP: Bone Morphogenic Protein
BSA: Bovine Serum Albumin
CBP: CREB-binding Protein
CDC25A: Cell Division Cycle 25A
Cdk1: Cyclin-dependent Kinase 1
CDK1: Cyclin-dependent Kinase 1
Cdkn1a: Cyclin-dependent Kinase Inhibitor 1A
cDNA: Complementary DNA
Chk2: Checkpoint Kinase 2
CK2: Casein Kinase II
CMA: Chaperone-mediated Autophagy
COX2: Cyclooxygenase 2
CRC: Colorectal Cancer
CREB: cAMP Responsive Element-binding Protein
CSA: Cockayne Syndrome Protein A
CSC: Cancer Stem Cells
CTCF: CCCTC-binding Factor
DAPI: 4,6-diamidino-2-phenyl Indole

DBD: DNA Binding Domain
DES: Diethylstilbestrol
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Di-methyl Sulfoxide
dpc: Days-post-coitum
dpf: Days Post-Fertilization
DSB: Double Strand Break
DSBs: Double-strand Breaks
E2F: E2 Transcription Factor
ECM: Extracellular Matrix
EDTA: Ethylenediaminetetraacetic Acid
EGFR: Epidermal Growth Factor Receptor
EpiR: Epirubicin Resistant
ER: Estrogen Receptor
ERE: Estrogen-response Element
ERK1: Extracellular Signal-regulated Kinase 1
ER α : Estrogen Receptor α
ER β : Estrogen Receptor β
FBXW7: F-box/WD Repeat-Containing Protein 7
FCS: Foetal Calf Serum
FGF: Fibroblast Growth Factor
FKH: Forkhead
Fox: Forkhead Box
FOXM1: Forkhead Box M1
FRA1: Fos Related Antigen-1
FRA2: Fos Related Antigen-2
GEN: Genistein
GG-NER: Global Genome Nucleotide Excision Repair
Gli1: Glioma-associated Oncogene Homolog 1
GM-CSF: Granulocyte-macrophage Colony-stimulating Factor
GSCs: Glioma Stem-like Cells
GSK3: Glycogen Synthase Kinase 3
HATs: Histone Acetyltransferases
HCC: Hepatocellular Carcinoma

HDAC1: Histone Deacetylase 1
 HER2: Human Epidermal Growth Factor 2
 HFG: Hepatocyte Growth Factor
 HG-PSG: Human High-grade Papillary Serous Carcinoma
 HIF: Hypoxia Inducible Factor
 HIPK2: Homeodomain-interacting Protein Kinase 2
 HIV-1: Human Immunodeficiency Virus Type 1
 HMG: High Mobility Group
 HP1: Heterochromatin Protein 1
 hpf: Hours Post-Fertilization
 HPV18: Human Papillomavirus Type 18
 HR: Hormone Receptor
 HSC: Haematopoietic Stem Cell
 Hsc70: Heat Shock Cognate 70
 HSF1: Heat Shock Transcription Factor 1
 IE: Immediate-early
 IFN- β : Interferon- β
 IgE: Immunoglobulin E
Igf2bp2: Insulin-like Growth Factor 2 mRNA Binding Protein 2
 IL-2R α : Interleukin-2 Receptor α
 INCENP: Inner Centromere Protein
 lncRNAs: Long Noncoding RNAs
 iNOS: Inducible Nitric Oxide
 IP: Immunoprecipitation
 IR: Insulin Receptor
 IRFs: Interferon Regulatory Factors
 JNK1: c-Jun N-terminal Kinases
 Keap1: Kelch-like ECH-associated Protein 1
 KIFs: Kinesins
 KIR: Keap1-interacting Region
 Lamp2A: Lysosomal Associated Membrane Protein 2
 LC3: Microtubule-associated Protein 1A/1B-light Chain 3
 LIR: LC3- interacting Region
 LXR α : Liver X Receptor α

Mad2: Mitotic Arrest Deficient 2
MARs: Matrix Attachment Regions
MBC: Metastatic Breast Cancer
MBT: Mid-blastula Transition
MDM2: Mouse Double Minute 2 Homologue
MEFs: Embryonic Fibroblasts
MEKK3: Mitogen-activated Protein Kinase Kinase Kinase 3
MELK: Maternal Embryonic Leucine-zipper Kinase
MET: Mesenchymal-Epithelial Transition
miRNAs: microRNAs
MMPs: Matrix Metalloproteinases
mRNA: Messenger RNA
mTORC1: Mammalian Target Of Rapamycin Complex 1
NBD: Nucleosomal Binding Domain
NBR1: Neighbor of BRCA1 Gene 1 Protein
NEM: N-Ethylmaleimide
NER: Nucleotide Excision Repair
NES: Nuclear Export Signal
NF- κ B: Nuclear Factor kappa-light-chain-enhancer Of Activated B Cells
NLS: Nuclear Localization Signal
NPCs: Neural Precursor Cells
NPM: Nucleophosmin
NRD: N-terminal Repressor Domain
NRF2: Nuclear Factor Erythroid 2-related Factor 2
p53: Transformation Related Protein 53
PAI: Plasminogen Activator Inhibitor 1
PB1: Phox-BEM1 Domain
PBS: Phosphate Buffered Saline
PDX1: Pancreatic And Duodenal Homeobox 1
PEST1: Pectinesterase 1
PgR: Progesterone Receptor
PI3K: Phosphoinositide 3 Kinase
PIC: Preintegration Complex
Pin1: Peptidyl-prolyl cis-trans Isomerase NIMA-interacting 1

PITX2: Paired Like Homeodomain 2
PKC: Protein Kinase C
PLA: Duolink Proximity Ligation Assay
PLK1: Polo-like Kinase 1
PMSF: Phenylmethanesulfonyl Fluoride
pRB: Retinoblastoma Protein
PRD: Proline Rich Domain
PRMT6: Protein Arginine Methyltransferase 6
PTMs: Post-translational Modifications
PTU: Phenylthiourea
PVT1: Plasmacytoma Variant Translocation 1
RB: Retinoblastoma
RD: Regulatory Domain
RHOA: Ras Homolog Family Member A
RIP: Receptor-interacting serine/threonine-protein Kinase 1
SAHF: Senescence-associated Heterochromatic Foci
SARs: Scaffold Attachment Regions
SDS-PAGE: Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis
SETD3: SET Domain Containing 3
siRNAs: Small-interfering RNAs
SQSTM1: Sequestosome 1
SRF: Serum-response Factor
SSBs: Single-strand Breaks
STAT3: Signal Transducer and Activator of Transcription 3
TAD: Transactivation Domain
TaxR: Paclitaxel Resistant
TB: TRAF6-binding Domain
TBP: TATA-box Binding Protein
TCA: Trichloroacetic Acid
TCR: Transcription-coupled Repair
TEMED: Tetramethylethylenediamine
TFF1: Trefoil Factor 1
TGF- β : Transforming Growth Factor- β
TGF β : Transforming Growth Factor β

TNBC: Human Triple-negative Breast Cancer
Twist1: Twist-related Protein 1
UBA: Ubiquitin-associated Domain
Ubc9: SUMO-conjugating Enzyme 9
UK: United Kingdom
uPA: Urokinase Plasminogen Activator
UTR: Untranslated Region
UV: Ultraviolet Light
VEGF: Vascular Endothelial Growth Factor
VPR: Viral Protein R
XRCC1: X-Ray Repair Cross Complementing 1

CHAPTER 1 INTRODUCTION

1.1 Breast cancer

1.1.1 General overview

Breast cancer is the most prevalent cancer in women worldwide and the leading cause of cancer death among women [1]. Over 55,000 women are diagnosed with breast cancer each year in the UK, which is about one in eight of women developing breast cancer in the duration of their lives. Globally, there were approximately 2.1 million diagnoses of breast cancer in 2018, contributing to about 11.6% of the total cancer incidence [2]. The causes of breast cancer are not fully understood. However, many risk factors have been shown to affect the likelihood of developing breast cancer, which includes age, family history and inherited genes, breastfeeding, hormone replacement therapy, obese, alcohol consumption, X-ray and radiation [3].

In general, cancer cells harbour disturbances in their proliferation pathways by acquiring functional capabilities involved in self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, and tissue invasion [4]. Toxic agents are generated from external environmental factors or internal metabolic processes, which adversely disturb cellular signalling pathways. A permanent disturbance may be introduced by damaging and mutating the DNA that encodes for certain genes and proteins involved in the signalling pathway. Despite the environmental and genetic factors, more recently, the tumour microenvironment and breast cancer stem cells (CSC) have also been identified as contributors to breast cancer tumorigenesis.

Breast cancer is categorized into 3 major subtypes depend on the presence of molecular markers for estrogen receptor (ER), progesterone receptor (PR) and human

epidermal growth factor 2 (HER2), in which hormone receptor-positive and HER2-negative (HR+/HER2-) accounts for approximately 60%-70%, HER2+ (15%-20%), and triple-negative (15%) of all breast cancer cases [5]. Moreover, breast cancer can be divided into four stages, 1 to 4, which are characterised by their severity. The current treatments include surgery, radiotherapy, chemotherapy, hormone therapy and targeted therapy, single or combinational treatment strategies is administrated depend on tumour subtype, cancer stage and patient preference.

1.1.2 Metastatic breast cancer

The most devastating aspect of cancer is the emergence of metastatic tumour developed in organs distant from the primary tumour; thus most cancer mortality is due to metastases. Metastatic cancers develop through a series of stages entail primary tumour expansion, epithelial-mesenchymal transition (EMT), local invasion, intravasation, extravasation and establishment of tumour cells in the secondary sites [6, 7]. It is generally accepted that accumulated genetic mutations in the cell could trigger severe alterations in gene expression, tissue differentiation and tissue growth, thus eventually initiating progressive primary tumour growth [7, 8]. In the early stage, benign tumours are localised with no chance of spreading, since limited oxygen and nutrients supplied via diffusion from the nearest capillary. Once primary tumour grows beyond specific size (1mm^3), it secretes of angiogenic activators and stimulates neovascularisation [9]. Increased tumour-associated vascular and lymphatic density not only provide nutrients for progressive tumour growth but also provide an escape route for tumour dissemination [10]. Cancer cells invade by breaching basement membrane barriers via secreting proteases and changing their cell-to-cell adhesion properties, in a transformation process named EMT [11]. The mesenchymal cells that

lost the attachment with neighbouring cells penetrate the blood or lymphatic system and invade normal tissues elsewhere in the body. During this process, cells must evolve to ignore the default death response and host immune response. After the mesenchymal cells invade a secondary tissue, they need to regain the epithelial cell-to-cell junction for colonisation in a process called 'mesenchymal-epithelial transition' (MET) [11]. Subsequent enhanced blood supply and enriched supporting connective tissue framework are crucial in the development of cancer cells at secondary sites [12]. Besides, EMT and MET have been discussed most frequently among others in the metastatic cascade, in which cancer cells invade collectively as epithelial sheets or detached clusters, followed by incomplete or complete EMT have also been observed during metastatic progression [13, 14].

Likewise, breast cancer starts as a local disease but can spread to distant organs, namely metastatic breast cancer (MBC). MBC development requires the metastatic cell dissociate from the primary tumour, invade surrounding tissues and penetrate local vasculature, survive within the circulation, and be immobilised in the capillaries at the secondary sites where it can extravasate to enter, survive and adapt to a new microenvironment [15, 16]. MBC is also known as advanced or stage IV breast cancer, which accounts for the majority of deaths from breast cancer. In fact, 10%-15% of breast cancer patients develop metastatic cancer within three years after the initial diagnosis [17]. MBC has also been observed in patients with >10 years of initial detection of the primary tumour [18]. Therefore, the risk of developing MBC is throughout the entire lifetime of breast cancer patients. Moreover, like many other tumours, breast cancer cells preferentially spread into certain tissues or organs, including the bone (occurrence rate at 47%-60%), lung (16%-34%), liver (19%-20%), and brain (10%-16%) [19]. Thus, detection of breast cancer metastasis at the early

stage is important for management and prediction of breast cancer progression. The American Society of Clinical Oncology has identified 13 categories of breast tumour markers of clinical, which include estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2), carcinoembryonic antigen (CEA), serum tumour markers CA 15-3 and CA 27.29, urokinase plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI), and certain multi-parameter gene expression assays [20]. Furthermore, decades of research into the cancer progression have demonstrated a strikingly complex set of molecular mechanisms, but the detailed mechanisms are far from being understood.

1.2 Zebrafish models for cancer research

Cell culture is a powerful tool for experimental studies, yet animal models remain indispensable for investigating the fundamental mechanisms of malignancies and for discovering enhanced treatments. Moreover, in the view of oncology drug discovery, preclinical animal studies are essential for toxicological risk assessments and for supporting human drug approvals [21]. The mouse is the most common animal model for preclinical studies in which its genome is 99% similar to the human [22]. However, the mouse model also has significant disadvantages including challenging to assess the early stage of metastasis, long duration for the whole process of metastasis, real-time imaging of tumour lesions in deep tissues, impossible without autopsy, immunodeficiency mice may still have a residual anti-tumour competence, and requiring high costs throughout the experiment [22, 23]. In contrast, zebrafish (*Danio rerio*) model provides several advantages, such as high genetic homology to humans, transparent embryos which allow real-time imaging without termination, the utilisation of large numbers of animals in cell transplantation assays, and its husbandry costs are

about 20 times less than mice [22, 24]. The usage of zebrafish has been applied in several areas of cancer research, including the establishment of cancer models by carcinogenic agents, genetic alterations, xenotransplantation; evaluation of tumour metastasis and angiogenesis, and also the screening of anti-tumour drugs.

Zebrafish share genetical and physiological similarities with humans, including the brain, digestive tract, musculature, vasculature, and the innate immune system [25-30]. *Howe. et al* has generated a high-quality sequence assembly of the zebrafish genome, showing that approximately 70% of protein-coding human genes are related to genes found in the zebrafish, and 84% of genes known to be associated with human disease have zebrafish counterpart [31]. These advantages make zebrafish a desirable tool for studying the development and progression of human diseases. Moreover, zebrafish larvae are transparent through to 7 days post-fertilization (dpf), and this phase can be extended to 9-14 dpf by treating with PTU (a tyrosinase inhibitor prevents melanin synthesis) [32]. The transparent embryo and larvae provide non-invasive imaging and live tracking, which significantly aids the observation of the entire process, particularly in the studies of angiogenesis, metastasis and anti-neoplastic drug screens. Besides zebrafish have high fecundity, with a single pair of adult zebrafish can produce over 100 embryos per week. The embryo size is 0.7 mm diameter at fertilisation, optically transparent, and develop rapidly of which the major organs are fully developed by 24 hours post-fertilization (hpf) and ready at 3 dpf for specific experiments [33]. Furthermore, like humans, zebrafish also have innate and adaptive immune systems of which the adaptive immune system in zebrafish is morphologically and functionally mature only after 4-6 weeks post-fertilization [34, 35]. This time duration allows most transplanted cancer cells to survive and metastasise in the body of zebrafish which is significant for cancer research.

Given these advantages of zebrafish, it has become a superior animal model in cancer research, particularly with regard to establishing tumour models, and investigating angiogenesis, metastasis and drug screening. There are several methods for establishing a cancer model in zebrafish, including carcinogenic treatment, transgenic modification, and transplantation of mammalian tumour cells. Among these, xenotransplantation represents its great strengths through pre-staining the transplanted tumour cells to enable them to be distinguished from original zebrafish cells. Thus, the complete process of tumour development, angiogenesis and metastasis can be observed and captured. Since the first human xenotransplantation was carried out in zebrafish in 2005, a variety of human tumours have been transplanted into zebrafish for cancer studies, which including hepatocellular carcinoma, melanoma, glioma, ovarian carcinoma, retinoblastoma, leukaemia, lung cancer, pancreatic cancer, prostate cancer and breast cancer [36-45].

Moreover, angiogenesis is considered an essential process in tumour growth and subsequent metastasis. It is difficult to detect the vascularisation in mammalian models without biopsy, and often only assessable in the late stage of the tumour. In contrast, the zebrafish provides an ideal model for tumour angiogenesis research, which not only because the transparent zebrafish is suitable for visualisation but also because the tumour microenvironment is strikingly similar to humans [46]. Furthermore, a pigmentation mutant *casper* strain with a completely transparent body has allowed the non-invasive imaging of the vasculature of the whole body [32]. Another transgenic zebrafish line, Tg (*fli:GFP*), has enhanced green fluorescent protein in all blood vessels, which allows observation of precise vessel formation under confocal microscopy [47]. In red fluorescent tumour tissues, the green fluorescent vascularisation is highlighted which allowing precise observation of the angiogenesis

process. Additionally, the transparent Tg (*fli:GFP*) transgenic zebrafish model is also ideal for observing tumour cell migration and angiogenesis throughout the transparent body.

However, as an underdeveloped model, there are several weaknesses of zebrafish, limiting its application in cancer research. Zebrafish have many duplicate genes that complicate genetic manipulation in establishing cancer models. Forward genetical manipulation cannot completely knockout both copies of the target genes. In this respect, the advanced technology, CRISPR, can overcome this difficulty as it can remove multiple genes. Besides, it is challenging to use water-insoluble drugs to zebrafish because the solvents may be toxic before the drugs dissolve. Moreover, the embryos need to be raised in the water at 28°C, which may not be optimum for the metabolism of mammalian tumour cells that maintaining homeostasis at 37°C. The previous study has revealed that maintaining embryos with transplanted tumour cells in 34 °C does not affect the viability of the embryos or the growth of tumour cells [48]. Therefore, raising the incubator temperature to 34 °C may offer a practical compromise.

In conclusion, zebrafish is a valuable tool for cancer studies for deepening our knowledge of tumour development and metastasis, as well as for discovering new and more effective diagnosis and treatments for cancer in the near future.

Advantages
• Genetic similarity to humans (70% homology with human genome) • High reproductive rate/ fast development • External fertilisation and embryogenesis • Non-invasive observation and manipulation due to transparency (larvae and <i>casper</i> mutants) • High throughput genetic/ drug screening • Direct administration of compounds to the medium of embryos • Cost/ time efficiency • Fewer ethical impediments
Disadvantages
• Some duplicated genes • Not as many well-characterised inbred strains as mice • More distantly related to human genes than mice

Table 1. 1 Advantages and disadvantages of zebrafish as a model for cancer research

1.3 Fox protein: FOXM1

1.3.1 General overview

Forkhead Box M1 (FOXM1) is a transcription factor of the forkhead box (Fox) superfamily which is defined by a conserved 100 amino acid long winged-helix DNA binding domain across all Fox family members and across species [49]. Since the first *Fox* gene was discovered in *Drosophila melanogaster*, it has attracted wide attention and abundant studies have emerged [50]. Until 2000, Fox proteins lacked a unified naming convention, based on the phylogenetic analysis of the forkhead domain, Fox proteins were divided into 19 subfamilies from FoxA to FoxP [51]. The human *FOXM1* gene locates on chromosome 12p13.3 and it consists of 10 exons, of which exons Va and VIIa can be alternatively spliced, giving rise to four common isoforms: FOXM1a, FOXM1b, FOXM1c and FOXM1d [52, 53]. FOXM1 protein is composed of three functional regions, an N-terminal repressor domain (NRD), a forkhead/winged-helix domain (FKH) and a C-terminal transactivation domain (TAD). The transactivation

activity of TAD can be suppressed by direct interaction with the NRD [54]. The FKH DNA binding domain binds promoter regions with a preference for tandem repeats of a consensus 5'-A(C/T) AAA(C/T) AA-3' sequence [55]. Moreover, FOXM1 seems to regulate a broad spectrum of essential biological functions, including DNA damage repair, cell cycle progression, proliferation, differentiation, renewal, migration, angiogenesis and cell survival. The FOXM1 protein is dominantly involved in embryonic development, adult tissue homeostasis, cancer initiation and progression [56]. Indeed, increased expression of FOXM1 is observed in a variety of human cancers, including hepatoma, angiosarcoma, melanoma, ovarian cancer, prostate cancer, lung cancer, gastric cancer, colorectal cancer and breast cancer [52]. Inhibition of FOXM1 in cancers cells results in reduced cell proliferation, EMT, migration, angiogenesis, drug resistance and poor prognosis in most solid tumours [57-61]. Therefore, FOXM1 has been considered as a potential diagnostic biomarker as well as a vital therapeutic target for the treatment of cancer.

1.3.2 Regulation of FOXM1

The core promoter region of *FOXM1* contains several classic regulatory elements, such as E-boxes, and other cis-acting elements. Several transcription factors involving cAMP responsive element-binding protein (CREB), glioma-associated oncogene homolog 1 (Gli1), CCCTC-binding factor (CTCF), signal transducer and activator of transcription 3 (STAT3) and E2F interact directly by binding to FOXM1 promoter region and thereby, upregulate FOXM1 expression [62-67]. In hepatitis B virus-associated hepatocellular carcinoma (HCC), FOXM1 is induced by CREB binding to its promoter region upon hepatitis B virus x protein, which in turn, facilitates hepatoma cell invasion and metastasis [66]. The DNA-binding factor, CTCF, interacts with the CTCF-binding

sites located in the promoter region of *FOXM1*, which may promote the migration and invasion properties of HCC cells [63]. In human colorectal cancer (CRC), Gli1 directly bound to *FOXM1* promoter at the BS4 (GCCCACCCA) site and contributes to colorectal cancer cell proliferation [62]. In the chronic myeloid leukaemia K562 cell line, STAT3 regulates *FOXM1* and is essential for the cell proliferation, cell cycle checkpoints and viability [65]. Other transcription factors also regulate *FOXM1* expression in different ways, for example, twist-related protein 1 (Twist1) recruits the coactivator p300 to form a complex on *FOXM1* promoter region and subsequently modulates its mRNA transcription [68]. Another transcription factor, HIF-1 α , is generally unstable under normoxic conditions. However, under hypoxic circumstances, it directly binds to the promoter for *FOXM1* and activates its expression. Consequently, the upregulation of *FOXM1* increases hepatoma cell proliferation and promotes resistance to apoptosis [69, 70]. Moreover, in response to heat shock stress, HSF1 was released from Hsp90 and translocated from cytoplasm to nucleus where it binds to the *FOXM1* promoter and induced *FOXM1* transcription [71]. In glioma cell lines, HSF1 regulates the *FOXM1* expression under both heat shock stress and normal conditions [71].

The *FOXM1* promoter region also contains other cis-suppression elements. For example, liver X receptor α (LXR α) and p53 can bind to the *FOXM1* promoter and repress its expression [72-74]. Furthermore, some responsive elements including the estrogen-response element (ERE) and the E-box, within the *FOXM1* promoter region show dual roles. In ER α -positive breast cancer cells, ER α binds to the ERE and induces *FOXM1*, whereas ER β binds to the same element and suppresses *FOXM1* expression [75, 76]. Likewise, the activator c-Myc and the repressor Mxi1-SR α bind to the E-box within the *FOXM1* promoter region and display opposite regulatory roles [77,

78]. More interestingly, there is a positive auto-regulation of FOXM1, which FOXM1 protein can potentially bind to the *FOXM1* promoter region and enhance its expression. Besides, it has been shown that nuclear AURKA can be recruited by FOXM1 as a cofactor to transactivate FOXM1 target genes, while AURKA can also bind to the *FOXM1* promoter through forkhead response elements, probably via FOXM1 and induce FOXM1 expression [79].

More recently, some microRNAs (miRNAs) have been identified in associated with FOXM1 expression. MicroRNAs represent a class of small (18 to 28 nucleotides), non-coding RNA molecules, which can regulate protein expression at the post-transcriptional level in diverse species. They act to guide mRNA degradation or suppress translation by binding to complementary sequences in the 3' untranslated region (3' UTR) of targeted mRNAs [80]. To date, dozens of miRNAs have been shown bound to 3'UTR of FOXM1 mRNA to affect the proliferation, migration, invasion, senescence, apoptosis, EMT and drug sensitivity of cancer. MiRNA-214, a tumour suppressor, acts by binding to 3'UTR of FOXM1 mRNA and inhibits FOXM1-mediated cancer metastasis and induces sensitivity to cisplatin in cervical cancer [81]. Likewise, miRNA-149 can inhibit EMT in non-small cell lung cancer cells through the same mechanism [82]. Another major family of the noncoding transcripts is the long noncoding RNAs (lncRNAs), which is defined as transcripts longer than 200 nucleotides that are not translated into protein. Moreover, lncRNA H19 has been shown to upregulate FOXM1 expression and promote proliferation and invasion of gallbladder cancer, through competitively binding to miRNA-342-3p and counteracting its inhibition on FOXM1 [83, 84]. In the same way, another lncRNA, colon cancer-associated transcript 2 (CCAT2) interacts with and inhibits miRNA-34a and thus

promoting FOXM1 expression in HCC. To summarize, multiple transcription factors, cofactors and noncoding transcripts can modulate FOXM1 expression in the cell.

Apart from these, FOXM1 protein can be modified through post-translational modifications (PTMs) that include phosphorylation, ubiquitination, SUMOylation, acetylation and methylation. Significantly, phosphorylation of FOXM1 is involved in the FOXM1-mediated cell cycle progression, in which the FOXM1 phosphorylation is constantly changing through cell cycle progression [85]. In the G1/S phase, FOXM1 mRNA reaches its peak while FOXM1 shows low transcriptional activity due to cytoplasmic localisation and autorepression by the NRD. In the late G1 phase, cyclin D-CDK4/6 phosphorylates multiple sites of FOXM1 (T⁶²⁰, T⁶²⁷ and S⁶⁷²) which triggers the G1 to S transition [86]. In the late S and G2/M phases, the Raf/MEK/MAPK pathway stimulates the phosphorylation of FOXM1 at S³³¹ and S⁷⁰⁴, which in turn induces FOXM1 nuclear translocation and promotes FOXM1 transcription [87]. During G2, cyclin A/E-CDK2 complex phosphorylates FOXM1 at T⁶⁰⁰, T⁶¹¹ and S⁶³⁸, which relieves repression of TAD by NRD [88, 89]. Moreover, phosphorylation of FOXM1 by cyclin B-CDK1 (S²⁵¹) and PLK1 (S⁷¹⁵, S⁷²⁴) results in the activation of FOXM1 transcription activity, while Chk2 phosphorylates FOXM1 at S³⁶¹ leads to enhanced FOXM1 activation and protein stabilization, as well as inducing the transcription of XRCC1 and BRCA2, which are required for DNA damage repair system [90-92]. Furthermore, FOXM1 phosphorylation is also connected to other PTMs such as ubiquitination and SUMOylation. It has been shown that GSK3 phosphorylates FOXM1 at S⁴⁷⁴, which subsequently induces ubiquitin-mediated degradation by FBXW7 [93]. PLK1-mediated phosphorylation of FOXM1 abrogates the inhibitory effect of FOXM1 by SUMOylation, thereby promoting its nuclear translocation and preventing protein degradation in the cytoplasm [94].

Ubiquitination is an enzymatic PTM in which ubiquitin protein is attached to target proteins and ultimately led to protein degradation. In the late M and early G1 phases, Cdh1 interacts with FOXM1 and then recruits APC/C E3 ubiquitin ligases to degrade FOXM1, which inhibits exit from mitosis [95, 96]. This E3 ligase system is fine-tuning of FOXM1 activity throughout the cell cycle. Moreover, SUMO isoforms are also involved in regulating FOXM1 activity and stability. For example, SUMO-2 promotes FOXM1 transcriptional activity by blocking the dimerisation and relieving the autorepression of FOXM1 [97]. Furthermore, FOXM1 can be regulated by acetyltransferases and methyltransferases. For instance, p300/CBP acetylates FOXM1 at K⁶³, K⁴⁰⁰, K⁴²², K⁶⁰³ and K⁶¹⁴, which promoting FOXM1 binding affinity, protein stability and phosphorylation sensitivity [98]. The histone H3 methyltransferase, SETD3 methylates FOXM1 to regulate VEGF expression under normoxic and hypoxic conditions. VEGF is a crucial factor involved in the formation of new blood vessels (angiogenesis) and is critical for tumour growth [99]. Taken together, the expression of FOXM1 can be modulated by multiple PTMs such as phosphorylation, ubiquitination, SUMOylation, acetylation and methylation.

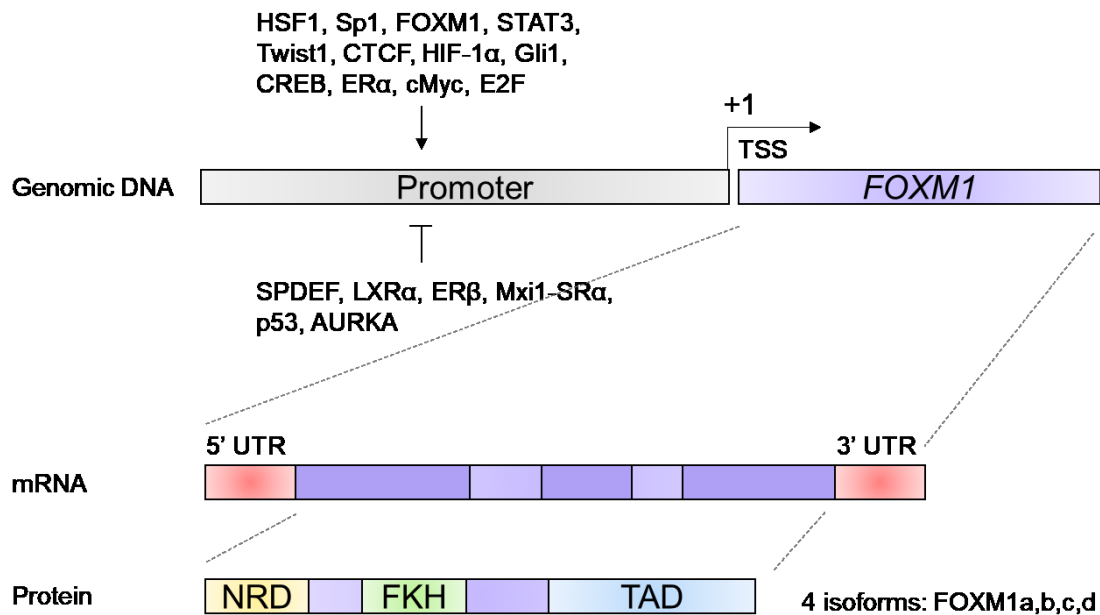


Figure 1. 1 Schematic diagram of FOXM1 and transcription factors (both activator and repressor) in *FOXM1* promoter region. NRD: N-terminal repressor domain, FKH: Forkhead winged helix DNA binding domain, TAD: Transactivation domain.

1.3.3 Proteins interacting with FOXM1

The oncogenic potential of FOXM1 has been postulated to be exerted predominantly through its capacity to transactivate target genes that are implicated in cancer development directly. However, emerging studies have also shown that FOXM1 may cooperate with other proteins through direct protein interactions to promote cancer progression.

Like another proliferation-related transcription factor E2F, FOXM1 is repressed by the Retinoblastoma protein (pRB) through direct binding to the central domain of FOXM1 and thereby indirectly represses the *trans*activation domain (TAD). The repression of FOXM1 by pRB can be relieved by cyclin D1/Cdk4 activity. The cyclin D1/Cdk4 complex phosphorylates pRB and interrupts FOXM1-pRB interaction, and thereby

releases the TAD of FOXM1 from NRD [100]. This process is highly important for the regulation of FOXM1 transcriptional activity during the G1/S transition. Moreover, another tumour suppressor, p19^{ARF} can directly bind the C-terminal TAD of FOXM1 and inactivate its transcriptional activity [101].

Several other proteins have found to increase FOXM1 activity by promoting its stabilisation, nuclear location, and inhibiting its ubiquitination/degradation. The nucleophosmin (NPM) is a phosphoprotein that is ubiquitously expressed in mammalian cells and shuttled between the nucleus and the cytoplasm. It has been found that the interaction between FOXM1 and NPM is necessary for sustaining the level and localisation of FOXM1 in cancer cell lines [102]. In acute myeloid leukaemia (AML), data shows that FOXM1 colocalises with NPM, and the strong nuclear interactions may confer chemoresistance on leukaemia cells [103]. It also has been shown that NF- κ B (p65) interacts with FOXM1/ β -catenin and transcriptionally induces FOXM1 expression in chronic myelogenous leukaemia (CML), suggesting that the physical interaction between FOXM1 and p65 promotes oncogenic activity in CML cells [104]. Likewise, in glioma cells, the FOXM1- β -catenin interaction enhances β -catenin expression and therefore induces its tumorigenicity [105]. Moreover, the STAT3-FOXM1 interaction may contribute to the activation of FOXM1 expression and resistance to radiation in glioma stem cells [106]. In glioma stem-like cells (GSCs), maternal embryonic leucine-zipper kinase (MELK) increases the activity of FOXM1 by directly binding to its N-terminus and regulating its phosphorylation [107]. Interestingly, activation of FOXM1 is also dependent on the binding and phosphorylation by PLK1 during mitotic progression. Besides, cell division cycle 25A (CDC25A) interacts with the C-terminus of FOXM1 and thus promotes CDK1-dependent phosphorylation of FOXM1 [108]. This phosphorylation is required to release the inhibitory function of the

NRD during the G2 phase. Moreover, Pin1 has been found to bind to FOXM1 and increase FOXM1 activity in melanoma cells [109]. The Pin1-FOXM1 interaction through MEK-dependent regulation during the cell cycle perturbs the growth of metastatic melanoma [109]. Furthermore, it has been shown that FOXM1 directly interacts with SMAD3 and regulates the nuclear retention of the SMAD3/SMAD4 complex, thus inducing the TGF β signalling and the development of metastasis in breast cancer [110]. In addition, the lncRNA-PVT1 has been found to bind to FOXM1 protein and to inhibit its degradation in gastric cells [111]. Therefore, lncRNA-PVT1 is determined as a stabilizing cofactor for FOXM1, and this interaction is essential for tumour growth and metastasis in gastric cancer.

Taken all the evidence together, the oncogenic transcription factor FOXM1 is overexpressed in the majority of cancers. Typically, it induces cancer development by promoting the transcriptional activation of target genes involved in all hallmarks of cancer. Moreover, multiple proteins and RNAs directly interact with FOXM1 to either promote or inhibit their activity. FOXM1 may also act as a cofactor for contributing to the induction of other oncogenic pathways. For instance, FOXM1 stabilises β -catenin or SMAD3 to activate the Wnt and TGF β signalling pathways, respectively.

1.3.4 FOXM1 in cancer progression

FOXM1 regulates a variety of biological processes in mammalian cells through regulating the transcription of target genes directly and also cooperate with other cofactors essential for cell cycle progression, cell proliferation and survival, cell differentiation, DNA damage repair, angiogenesis, cell metastasis and chemotherapeutic drug response. Genome-wide gene expression profiling of cancers

has identified *FOXM1* as one of the most commonly overexpressed genes in solid human tumours [112-114]. Such aberrant expression is observed in various cancers of the brain, liver, breast, lung, prostate, cervix, ovary, colon and nervous system [115-125]. Consistently, *FOXM1* deficiency constrains tumour initiation, growth and progression [126-129].

As discussed earlier (section 1.3.2), *FOXM1* is a key regulator in cell cycle progression. Under normal conditions, its expression is restricted to proliferating cells and is therefore absent in quiescent or terminally differentiated cells. Upon the cell re-entry the cell cycle process, the expression level of *FOXM1* is highly regulated for G1/S and G2/M transition, and M phase progression. Aberrant regulation of *FOXM1* disrupts the proliferative homeostasis to the cell and make them more susceptible to oncogenic transformation. Moreover, recent studies suggest that *FOXM1* also contributes to cell transformation by overriding cellular senescence. Cellular senescence is a tumour-suppressive mechanism that arrests the proliferation of potential tumour cells. In gastric cancer, *FOXM1* depletion triggers p53 and p61-independent senescence of cancer cells [130]. The embryonic fibroblasts (MEFs) derived from *FOXM1*-deficient mice also display a premature cellular senescence phenotype as evidenced by high expression of the senescence-associated β -galactosidase, p19^{ARF} and p16^{INK4A} proteins [131]. In contrast, increased *FOXM1* expression in MEFs exerts an antagonistic effect against oxidative stress-induced premature senescence which suggesting an active role for *FOXM1* in protecting against cellular senescence [132]. Taken all these observations together, *FOXM1* has been shown to be involved in restricting the senescence programme of human malignant cells. Furthermore, *FOXM1* has been observed in promoting cancer initiation through stimulating stem

cell-like properties, including self-renewal, immortalization and sustained proliferative ability.

Besides its initial role in tumorigenesis, FOXM1 is also involved in multiple steps of cancer progression by promoting proliferative and survival signals, tumour cell migration, invasion and angiogenesis. Firstly, abundant studies have shown that the expression of FOXM1 is crucial to tumour cell proliferation and growth. Consistent with this, the depletion of FOXM1 by siRNAs in various cancer cells results in a reduction in cell proliferation and colony formation on soft agar. Indeed, FOXM1 knockdown has been shown to downregulate the cell cycle proteins, cyclin A2, cyclin B1 and Cdc25 phosphatases, and to upregulate the expression of the cell cycle inhibitor p21^{Waf1/Cip1} and p27^{Kip1} [117, 121, 133]. Moreover, FOXM1 plays important roles in embryonic and organ development as well as tissue regeneration. During embryogenesis, FOXM1 is highly expressed in tissues of epithelial and mesenchymal origins, including cardiomyocytes, hepatocytes, neuroectodermal cells and smooth muscle cells of blood vessels, and intestine and oesophagus [134-136]. Although its expression is not usually detectable in terminally differentiated adult cells, FOXM1 expression is reactivated and enhanced for tissue repair in the event of injury [91, 137-140]. This indicates a significant role of FOXM1 in regulating cell proliferation during tissue regeneration. Furthermore, FOXM1 has a role in initiating angiogenesis by transcriptionally activating the vascular endothelial growth factor (VEGF) expression [141-143]. VEGF is a major angiogenic activator secreted by cancer cells, which plays key roles in orchestrating the formation of new blood vessels. Additionally, FOXM1 has shown to be closely associated with cancer cell migration and invasion. During cancer cell invasion, the matrix metalloproteinases (MMPs) are a family of enzymes that are upregulated for degrading of the extracellular matrix (ECM). FOXM1 has been

demonstrated to directly binds to the *MMP2* promoter, while indirectly regulates *MMP9* through its downstream target JNK1 [144, 145]. The ectopic overexpression of FOXM1 subsequent activation of MMPs (i.e. *MMP2* and *MMP9*) have been observed in many malignancies, which is associated with a global enhancement of cancer cell migration, invasion and angiogenesis [113, 133, 144]. Another study by using the liver carcinogenesis model shows that transgenic expression of FOXM1 combined with the loss of its inhibitor p19^{ARF} could induce liver fibrosis and metastasis [146].

To summarise, dysregulation of FOXM1 is associated with not only the tumour initiation but also throughout the cancer progression. This highlights the value of targeting FOXM1 and/or FOXM1-mediated signalling pathway as an effective anti-cancer strategy.

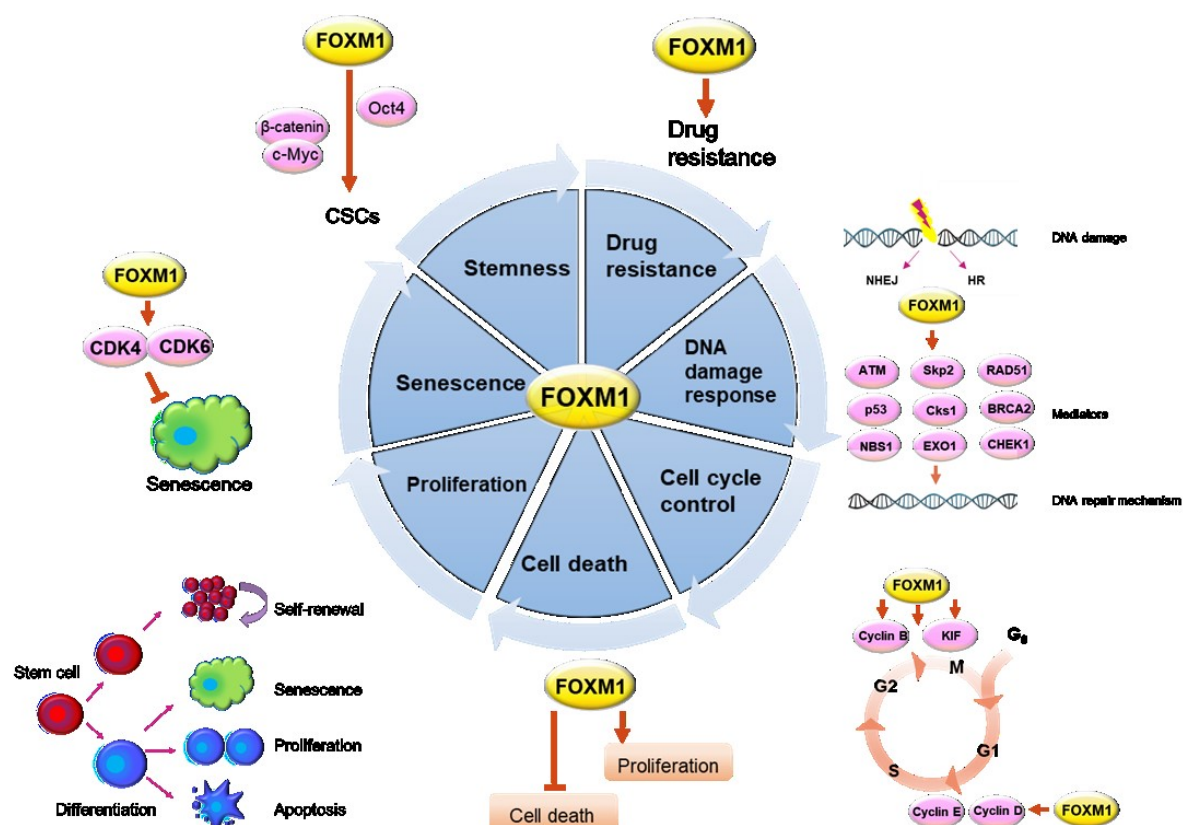


Figure 1. 2 Functional roles of FOXM1 in cancer progression. Adapted from the published journal (2018) [147].

1.3.5 FOXM1 in chemotherapy sensitivity and resistance

To date, the most commonly used cancer therapeutics are DNA damaging agents and mitotic inhibitors which arrest cancer growth and progression, leading to cell cycle arrest and eventually apoptosis [148, 149]. The most two commonly used chemotherapeutic agents are anthracyclines (e.g. Epirubicin and Doxorubicin) and taxanes (e.g. Paclitaxel and Docetaxel) in cancer treatment, and others include 5-fluorouracil (5-FU), cyclophosphamide and carboplatin. Most often, these chemotherapeutic drugs are administered in combination for treatment. These genotoxic agents, such as epirubicin, doxorubicin and 5-FU can cause DNA damage in forms of single-strand breaks (SSBs) and double-strand breaks (DSBs), which result in apoptosis and senescence of cancer cells. At the same time, cells have intrinsic mechanisms for preventing the accumulation of damaged DNA through repairing the damaged DNA, or undergoing senescence or apoptosis if the damage cannot be repaired in time. Some cancer cells acquired drug resistance by boosting their DNA repair mechanisms. Moreover, paclitaxel functions by inducing microtubule dysfunction. It has been shown that paclitaxel downregulates FOXM1 and mediates the mitotic catastrophe, which eventually results in cell death. Conversely, overexpression of FOXM1 is often observed with the development of paclitaxel resistance in cancer. Taken together, the development of resistance to both conventional and molecularly targeted therapies is a major challenge for current cancer treatment in the clinic. Intriguingly, several studies have linked the deregulated FOXM1 signalling pathway to the mechanism of therapeutic resistance, in which aberrant activation or expression of FOXM1 promotes the development of acquired drug resistance [147, 150, 151]. In recent years, it has become clear that FOXM1 has important roles in the DNA damage response and senescence pathways and these

are closely related to the abilities developed by cells to escape DNA damage-induced cell death.

The detailed FOXM1-mediated mechanisms of the DNA damage response have been extensively reviewed by Professor Eric W-F Lam and his colleagues [152]. In brief, FOXM1 has been demonstrated to induce DNA repair through transcriptionally regulating a network of genes which involved in DNA damage sensing, mediating, signalling and repair. Moreover, FOXM1 also participates directly and indirectly in the regulation of many chromatin structure-modifying genes, and therefore, plays a key role in chromatin structure remodelling and DNA repair. Furthermore, FOXM1 has also been shown to play a role in 'DNA damage checkpoint' signalling network. The 'DNA damage checkpoint' mechanism is activated to stall cell cycle upon DNA damage detection, allowing extra time for DNA repair to take place. In summary, FOXM1 is a key modulator that plays a fundamental role in many aspects of DNA damage response. Thus, it is not surprising that a wide variety of chemotherapeutic agents target FOXM1 signalling pathway in the treatment of cancer. In this respect, several chemotherapeutic agents including tyrosine kinase inhibitors, PI3K and Akt inhibitors, anthracyclines, platinum compounds, mitosis modulators and endocrine therapy have been discussed profoundly in my previous review article [147].

1.4 Kinesin: KIF20A

Kinesins (KIFs) are a superfamily of motor proteins that drive unidirectional movement of microtubules in a multitude of cellular processes, such as organelle transport and cell division. Since the first kinesin was discovered in neural tissue in 1985, many more have since been identified based on the sequence conservation of their motor domain [153]. Structurally, kinesins are classified into three major groups according to the

position of the motor domain: N-terminal KIFs, C-terminal KIFs and internal KIFs. These groups are further subdivided according to their polypeptide chain composition (monomers, homodimers, heterodimers, etc.) and to their directionality (moving toward the plus-end of microtubules or toward the minus-end) [154]. Moreover, since the kinesin family has become so large in recent years, and different naming schemes have emerged. Subsequently, researchers have since then determined a standardised classification of kinesins into 14 classes [155]. Kinesin motors that do not involve into any of these categories are known as ‘orphans.’

KIF20A (a.k.a. MKLP2), a member of kinesin-6 subfamily, with the other two members KIF20B (MPP1) and MKLP1, are essential mitotic kinesins functioning in anaphase and cytokinesis during cell division [156-159]. Many KIF20A homologues have been found from different species, including *Drosophila*, zebrafish, rat and human, and the highly conserved motor domain indicates the partially conserved functions of kinesin-6 KIF20A in diverse organisms [160-163]. The *KIF20A* gene locates on chromosome 5q31 and its mRNA encodes an 890-amino acid protein named KIF20A, which plays an important role in intracellular transport and microtubule organisation during cell division [164, 165]. The KIF20A protein is composed of three major functional domains. The first is the N-terminal motor domain which mediates the motor activity driven by ATP hydrolysis. The second is a central coiled-coil domain, containing RAB6 and Myosin II binding motifs, and is involved in the fission process. The third is the C-terminal tail domain which is essential for vesicle transport and interactions with partner proteins [160]. In addition, it also contains a PLK1 phosphorylation site, an Aurora B phosphorylation site and an INCENP binding site.

During interphase, KIF20A, RAB6 and Myosin II are co-localized mainly at the Golgi fission hotspots, where KIF20A and Myosin II are responsible for the movement of

RAB6-positive vesicles along microtubules during the early cell cycle events of transport biogenesis [166]. In G2 phase, KIF20A proteins are sequestered in the nucleus, thereby preventing undesirable binding to the cytoplasmic microtubules [167]. While the nuclear envelope breaks down at early stages of mitosis, KIF20A is released into cytoplasm where CDK1/Cyclin B complex actively phosphorylates KIF20A and prevents premature central spindle formation. After CDK1/Cyclin B degradation at anaphase, KIF20A is dephosphorylated and released [168]. PLK1 then phosphorylates KIF20A and regulates the binding activity of KIF20A to microtubules. At late stages of mitosis, KIF20A interacts with the Aurora B-INCENP complex and mediates the chromosomal passenger complex (CPC) from centromere localization to central spindle. During telophase, the KIF20A proteins accumulate at the midbody which is important in cleavage furrow formation at late stages of cytokinesis [169]. Moreover, KIF20A has a consensus mitotic arrest deficient 2 (Mad2) binding motif, and the binding by Mad2 inhibits the interaction of KIF20A with microtubules. During cell division, Mad2 negatively regulates KIF20A from loading to the mitotic spindle at early stages of mitosis, as well as preventing prematurely translocation of CPC at late stages of mitosis. Collectively, KIF20A exhibits unique properties in mitosis, including microtubule assembly, spindle formation, and cytokinesis, in addition to meiosis.

Furthermore, KIF20A has been shown to be regulated by transcription factors, such as FOXM1 and Glioma-associated oncogene 2 (Gli2) in breast cancer and hepatocellular carcinoma [170, 171]. It has been observed that aberrant activation of KIF20A leads to defects in cell division, which is highly correlated with tumour cell proliferation, angiogenesis, metastasis, poor clinical outcome and poor survival rate. Certainly, a variety of studies have revealed that KIF20A expression is significantly increased in many types of cancers including glioma, malignant melanoma,

nasopharyngeal carcinoma, hepatoma, hepatocellular carcinoma, intrahepatic cholangiocarcinoma, non-small cell lung cancers, gastric cancer, colorectal cancer, pancreatic cancer, cervical cancer and breast cancer [170, 172-183]. Therefore, KIF20A could serve as a potential prognostic biomarker and might be a novel target for anti-cancer treatment.

1.5 Autophagy adaptor: p62

Autophagy is a dynamic process of cellular degradation of its own components which not only providing nutrients to maintain cellular functions during starvation but also removing misfolded proteins, damaged organelles, and invading microorganisms. The self-digestion process is potentially triggered by various stress stimuli, such as nutrient deprivation (fasting), growth factor depletion, infection and hypoxia. To date, there are three types of autophagy including macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [184]. Macroautophagy is the main autophagic pathway, and it is characterised by using the intermediate organelle 'autophagosome'. An isolated membrane, called phagophore, enwraps a small portion of the cytoplasm that may include soluble materials and organelles, to form the autophagosome. The autophagosome fuses with the lysosome to become an autolysosome where the internal materials are degraded. In microautophagy, the lysosome directly engulfs small components of the cytoplasm by inward invagination of the lysosomal membrane. The third type of autophagy is chaperone-mediated autophagy in which the substrates translocate across the lysosomal membrane without membrane reorganisation. It is achieved by the chaperone protein heat shock cognate 70 (Hsc70) and co-chaperones that specifically recognise cytosolic substrates that contain a KFERQ-like

pentapeptide, and the Hsc70-containing complexes then translocate into the lysosomal lumen after binding with the lysosomal transmembrane receptor Lamp2A. Eukaryotic cells have two major degradation systems involved the proteasome and the lysosome. The proteasome serves a basic housekeeping function as an amino acid recycling machine under normal nutrient-rich conditions, whereas in conditions when there is a limitation of various types of nutrients, such as amino acids, growth factors, oxygen and energy, the lysosome and subsequent autophagic processes are induced. Autophagy regenerates amino acid products and contributes cellular nutrients. Restoration of cellular levels of amino acids reactivates the serine/threonine protein kinase and mammalian target of rapamycin complex 1 (mTORC1) consequently terminates autophagy [185]. Taken together, autophagy constitutes a negative feedback loop that governs nutrient sensing and lysosome homeostasis during starvation [186-188]. Given that the autophagy is a normal physiological process in the body, it acts as the quality-control machinery for cytoplasmic components and is fundamental for cellular homeostasis and health. Approximately 1%-1.5% of proteins are catabolized per hour by autophagy, even under nutrient-rich conditions. It has long been thought that autophagy is a non-selective bulk degradation process, but recent evidence has highlighted the selective elimination by autophagy of undesirable components such as misfolded protein aggregates, lipid droplets, dysfunctional organelles and invading pathogens [189].

One of the most established mediators of selective autophagy is p62, which is also known as sequestosome 1 (SQSTM1). p62 is a multifunctional protein consisting of an N-terminal Phox-BEM1 domain (PB1), a ZZ-type zinc finger domain, nuclear localization signals (NLS), an export motif (NES), a TRAF6-binding domain (TB), an LC3- interacting region (LIR), PEST1 domains, a Keap1-interacting region (KIR) and

a C-terminal ubiquitin-associated domain (UBA) [190, 191]. Briefly, through the PB1 domain, p62 can homo- or hetero-oligomerize with itself or with NBR1, respectively. The PB domain can also interact with other PB domain-containing proteins such as α PKC, MEKK3, MEK5, ERK1 and Rpt1, which are involved in activation of NF- κ B signalling for adipogenesis and proteasomal clearance [192-194]. The ZZ domain interacts with RIP kinase, which is implicated in the inflammation response. The TB binds with the TNF receptor-associated factor 6 (TRAF6) proteins to trigger protein polyubiquitination [195]. The NLS and NES are involved in the nuclear-cytoplasmic translocation of p62. PEST1 serves as a proteolytic signal for rapid degradation of short-lived proteins and as targets for caspases. KIR binds to Keap1 proteins that result in deactivation of Keap1 and activation of NRF2 [196]. NRF2 serves as a 'master regulator' that is responsible for activating antioxidant enzymes or survival genes in response to oxidative stress. Under normal conditions, NRF2 is degraded by the Keap1-Cullin3-dependent mechanism. In response to oxidative stress, NRF2 induces the expression of p62, the Keap1-Cullin3 complex is then recruited to p62 via its KIR motif, and Keap1 is degraded along with p62 in the autophagosome [197]. Most significantly, through the UBA and the LIR, p62 assists the autophagic degradation of ubiquitinated proteins. The UBA domain of p62 serves as a receptor which interacts with ubiquitinated proteins. The LIR of p62 then interacts with LC3 (microtubule-associated protein light chain 3) on the isolated membrane, phagophore. Subsequently, p62 and its associated cargos are incorporated into the autophagosome and then degraded [198, 199].

Considering of the major role of p62-mediated autophagy is preserving cellular survival under stresses, p62 seems beneficial for the cell. However, autophagy has also been implicated in many pathophysiological processes, including metabolic and

neurodegenerative disorders, cardiovascular and pulmonary diseases, as well as cancer development and progression [200, 201]. Therefore, the role of autophagy in cancer is strongly context-dependent. Its degradation function of unwanted cellular components is believed to have tumour-suppressive potential before the onset of tumorigenesis, and loss of function has been linked to enhanced risk of cancer [202]. Several studies have shown that the activation of autophagy in cancer therapies is advantageous by inducing immunogenic cell death. In this context, autophagy-induced tumour cells release ATP and HMGB1, which recruit immune effectors to the tumour and trigger a tumour-specific immune response [203-207]. In this point of view, activation of autophagy rather than its inhibition is beneficial for cancer treatment. On the other hand, it has been shown to allow premalignant cells to escape therapy-induced stresses and inflammation, as well as assisting the growth of tumours with low nutrients. In this regard, many types of advanced cancers have been shown to associated with hyperactivated autophagy [208]. Moreover, pharmacological inhibition of autophagy is an effective strategy to enhance the therapeutic efficacy in certain types of cancer [209-211]. Taken together, autophagy seems to be a double-edged sword in the context of cancer therapies.

In addition, beyond the autophagic role of p62, its multiple domains can interact with various binding partners and therefore is involved in multiple cellular processes including regulation of inflammatory responses, redox homeostasis and EMT [212-214]. Studies have found that several EMT-promoting transcription factors are downregulated in an autophagy-dependent manner, suggesting that p62 has an inhibitory effect on EMT [215-217]. Moreover, p62 directly degrades the actin dynamics regulator transforming protein RHOA in a ubiquitylation-dependent manner, thus inhibiting cell migration [218]. On the other hand, p62 facilitates the disassembly

of focal adhesions by degrading several focal adhesion components, thus contributing to cell mobility [219]. Taken these evidences together, p62 can either promote or suppress cancer progression and metastasis at different stages. This complicates the autophagy-mediated therapeutic intervention where various aspects, including types of tumour cells, stages of progression and tumour microenvironment, are required to be clarified.

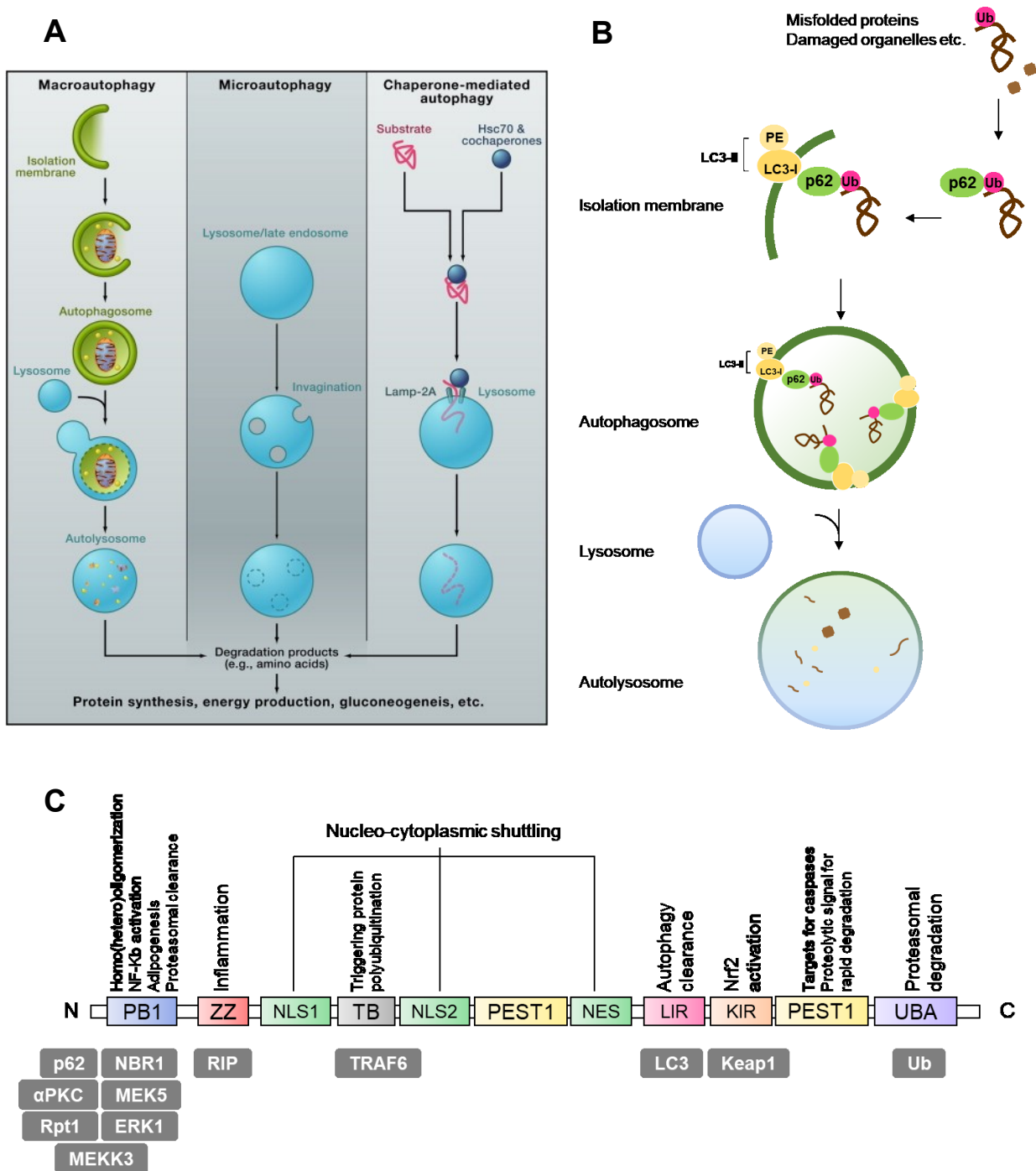


Figure 1. 3 (A) Three types of autophagy. Reprinted from Noboru Mizushima and Masaaki Komatsu (2011) with permission [184]. (B) p62-mediated autophagic process. (C) The functional domains of p62.

1.6 Histone mobile group proteins

1.6.1 General overview

Chromatin is the highly organised form of DNA which consists of nucleic acid and histones that attribute to the majority of total chromatin. The rest consists of other non-histone proteins, which include scaffold proteins, DNA polymerase, Heterochromatin Protein 1, and Polycomb, in which the most prevalent of non-histone proteins are high mobility group (HMG) proteins. The significant difference between histone and non-histone proteins is the structure they provide, in which histone proteins are the coils about which DNA winds, whereas non-histone proteins provide the scaffolding structure. Besides, non-histone proteins also affect the activity of DNA and its gene expression.

1.6.2 HMG proteins

The non-histone HMG proteins were discovered in proliferating HeLa cells in 1983 and named according to their rapid mobility property in polyacrylamide gel separations [220, 221]. Remarkably, HMG proteins can bind to specific structures in DNA in a sequence-independent manner, while they recognise structure rather than a nucleotide sequence. The HMG proteins are subdivided into three families, HMGA (a.k.a. HMGI/Y), HMGB (a.k.a. HMG-1 and -2) and HMGN (a.k.a. HMG -14 and -17), each of which has a characteristic functional sequence motif [220, 222]. The functional motif of the HMGA family is called the 'AT-hook'; that of the HMGB family is called the 'HMG box'; and that of the HMGN family is called the 'nucleosomal binding domain'. Proteins that contain any of these motifs embedded in their sequence are so called HMG proteins. Through these distinguishable functional motifs, HMG proteins can

modify the structure of their binding partners, generating conformational changes that modulating various DNA-dependent activities, such as transcription, replication, recombination and DNA repair [223]. Although the three families participate in many of the complementary and partially overlapping functions in nuclear processes, each family plays a unique role in modulating chromatin structure and regulating genomic function.

1.6.3 HMGA protein: structure and function

At present, the mammalian HMGA family consists of two functional subfamilies which are HMGA1 (HMGI/Y) and HMGA2 (HMGI-C), apart from that, there are numerous inactive pseudogenes in both mice and human [220]. HMGA1 is located at human chromosomal locus 6p21 which contains 8 exons across a genomic region about 10 kb; whereas HMGA2 is located at chromosomal locus 12q14-15 in humans and contains at least 5 exons that distributed over a region more than 160 kb [224, 225]. The *HMGA1* gene gives rise to three isoforms *HMGA1a* (a.k.a. HMG-I), *HMGA1b* (a.k.a. HMG-Y) and *HMGA1c* (a.k.a. HMG-I/R) that derived from alternatively splicing [224, 226, 227]. The *HMGA1a* (HMG-I) and *HMGA1b* (HMG-Y) are identical in their common sequences, except that *HMGA1a* has 107 amino acids while *HMG1b* has 96 amino acids. The two isoforms encoded by the most abundant splice variants of *HMGA1* gene in mammalian cells. The genomic sequence of *HMGA2* is much larger than *HMGA1*, which is mainly because of its longer 5' and 3' untranslated regions and the extremely long third intron [227]. In normal cells, *HMGA2* gene code primarily for the full-length HMGA2 (HMGI-C) protein. Recently, a more complex splicing pattern of *HMGA2* has also been reported [228, 229].

As described previously, the three HMG subfamilies have distinguishable functional sequence motifs, HMGA family has a palindromic amino motif called the 'AT-hook' that binds preferentially to the minor groove of short stretches of AT-rich β form DNA through recognition of structure rather than nucleotide sequence [230]. All HMGA proteins comprise three 'AT-hooks' except for the HMGA1c variant which only has two.

There is an unusual feature of HMGA which makes this group of proteins outstand all the others is that, unlike other proteins, they possess a high degree of intrinsic flexibility due to a lack of significant secondary structure prior to interactions with DNA or other substrates [222]. Following binding to substrates, HMGA proteins undergo specific disordered-to-ordered structural transitions and induce conformational alterations of the substrates themselves. This intrinsic flexibility allows them to actively participate in a wide variety of nuclear activities, including DNA replication, chromatin remodelling, gene transcription and DNA repair [231].

Although HMGA proteins are not transcription factors and have no intrinsic ability to activate transcription, they can modulate gene transcription by altering local chromatin structure. One of the best characterised biological roles of HMGA proteins is in assembling or modulating the multi-subunits, enhanceosome, a stereospecific multiprotein- DNA complex that bind to AT-rich promoter regions of specific inducible genes during transcription activation. One of the best-studied examples is that, upon virus infection, HMGA1a assembled in the nucleosome-free enhancer region of the gene interferon- β (IFN- β) along with other factors such as NF- κ B, IRFs and ATF2/c-Jun [232]. The assembled enhanceosome then recruits GCN5/PCAF that acetylates the nucleosome and also HMGA1a at Lys⁶⁵, the acetylated HMGA1a is vital in stabilising inter-protein interactions of the enhanceosome [233]. Meanwhile, transcriptional machineries, such as RNA polymerase, are recruited to the promoter

region to initiate gene transcription. Later, another acetyltransferase CBP modifies HMGA1a at Lys⁷¹ in which destabilising the enhanceosome and consequently, repressing transcription [234]. This recruitment of multi-subunits to promoter region of inducible gene is thought to be widespread; indeed, HMGA1 has also been shown to participate in activation of the interleukin-2 receptor α (IL-2R α) and the insulin receptor (IR) genes, in a manner very similar to its regulation of the IFN- β gene [235, 236].

Another notable role of HMGA is one which involves virus integration and viral genome expression in host cells. One remarkable strategy of virus to maximise its replication and attenuate the immune response of the host is that, they utilise the host's proteins for their own benefits. In the case of human immunodeficiency virus type 1 (HIV-1), several host factors have been identified to be necessary for the processes of viral gene expression and viral protein synthesis [237]. HMGA is one of them that has been found to be required for the integration of viral cDNA into host DNA. The HIV-1 cDNA and viral encoded enzyme, integrase, together with viral matrix, nucleocapsid, reverse transcriptase and VPR proteins, can be found *in vivo* in nucleoprotein complexes termed 'preintegration complex' (PIC). HMGA has found as an accessory factor for the function of HIV-1 PICs [238]. Although the mechanism of HMGA to promote integration is unclear, it is thought that HMGA binds to the HIV cDNA at the AT-rich sequences and alter the conformation of HIV cDNA so as to promote the formation and/or the function of the catalytically active complex. Other supportive evidence of HMGA proteins involving in the regulation of viral genes has been observed in herpes simplex virus type 1, JC papovavirus, and human papillomavirus type 18 (HPV18) [239-243]. In these cases, HMGA mainly induces conformational alterations of DNA and promotes formation of enhanceosome on promoter or enhancer regions.

HMGA proteins can modulate gene transcription not only by participating in the recruitment of enhanceosome, but also through direct protein-protein interactions with transcription factors and thus, inducing changes in their DNA binding affinities. Chin *et al.* and his colleagues have reported that HMGA enhances the ability of transcription factor serum response factor (SRF) binding to the *c-fos* and *SM22 α* promoters [244].

Moreover, HMGA has been demonstrated to displace Histone H1 and thus unpack the condensed chromatin by enabling transcription activation [245]. As described earlier, histone H1 is one of the five histone protein families who play fundamental roles in the compaction of chromatin, transcription regulation, nucleosome spacing and chromosome spacing. It has been observed that H1 binding to naked DNA inhibits transcription, and the addition of H1 increases the transcriptional repression [246]. One mechanism whereby H1 could repress transcription is by condensing chromatin structures, thus limiting the access of the transcription machinery to gene promoter and enhancer regions. Zhao *et al.* and his colleagues have developed an experimental assay to search for proteins capable of antagonising histone H1-mediated transcription repression [245]. They found that H1 proteins are not only binding to linker DNA between nucleosomes but also tightly binding to the scaffold/matrix attachment regions (SARs/MARs). The SARs/MARs are specific highly AT-rich (>70%) sequences in the genomic DNA which serving the chromatin to anchor onto the nuclear matrix. HMGA proteins have also been found to bind the SARs/MARs as well as displacing histone H1 in chromatin, thus leading to significant alterations in gene expression [247].

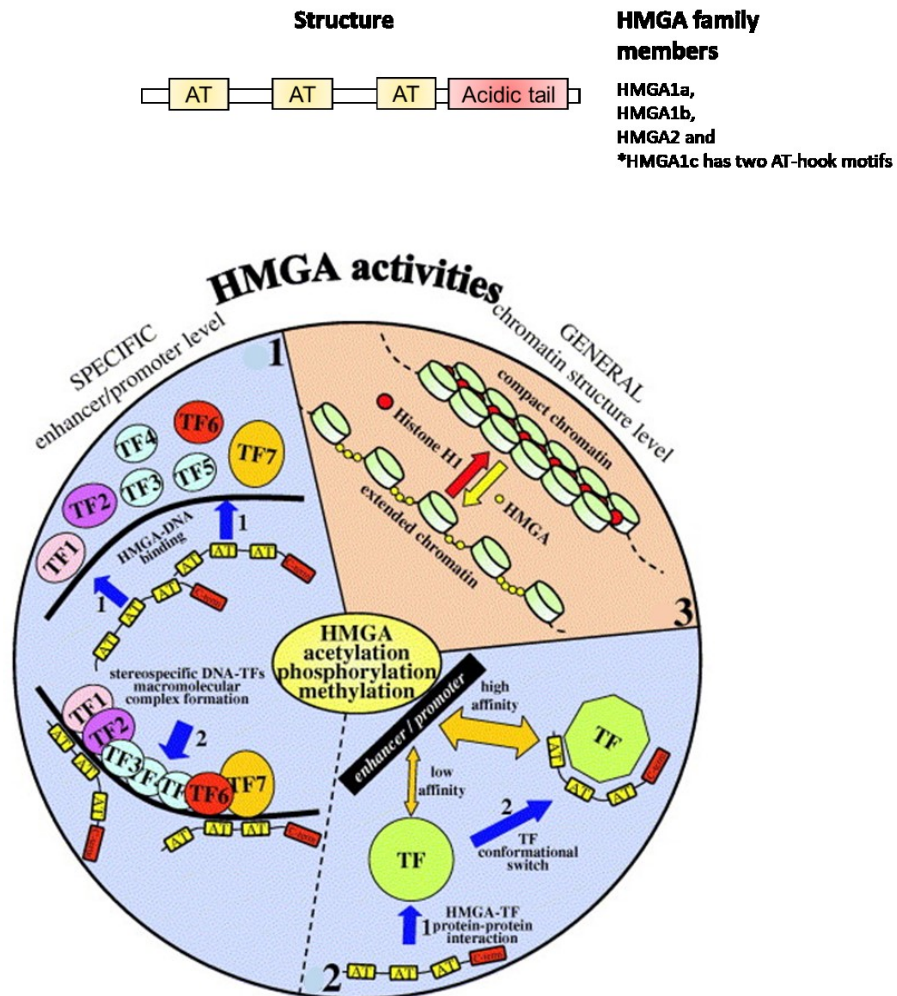


Figure 1. 4 Multiple functions of HMGA protein. Reprinted from Riccardo Sgarra *et al.* (2004) with permission [234].

1.6.4 Post-translational modifications of HMGA protein

Protein post-translational modifications (PTMs) increase the functional diversity of the proteome by modulating its folding, stability, conformation, distribution, localisation, half-lives, and interaction with DNA or other proteins. Remarkably, there are over 200 different types of PTMs, and such high diversity and their reversible nature add on complexity to their roles in many cellular activities. The biological activities of HMGA proteins are highly regulated by their PTMs, including phosphorylation, acetylation,

methylation and poly-ADP-ribosylation reactions [222]. These secondary biochemical modifications are dynamic and rapidly in responding to both intra- and extracellular signalling stimuli, which modulate their multi-interacting properties with both DNA and proteins.

It has long been known that HMGA proteins are among the most highly phosphorylated proteins in the nucleus [248, 249]. Studies have revealed that HMGA1 proteins could be phosphorylated by several kinases including cyclin-dependent kinase 1 (Cdk1), protein kinase C (PKC), casein kinase II (CK2) and homeodomain-interacting protein kinase 2 (HIPK2) [250-255]. For instance, it has been shown that Cdk1 induces the phosphorylation of Thr⁵², Thr⁷⁷ and Ser³⁵ in HMGA1a, which takes place at G2/M phase of the cell cycle and causes a more than 20-fold reduction in DNA-binding affinity of the modified proteins to AT-rich DNA substrates [250, 251, 256]. In addition, Cdk1 can catalyse the phosphorylation of the corresponding Thr⁴¹ and Thr⁶⁶ in HMGA1b [256]. Moreover, HIPK2, a newly identified serine/threonine kinase, was found to induce phosphorylation of the same sites, but with different site preferences (Thr⁷⁷ > Thr²² > Ser³⁵), whereas the preferred order for Cdk1 phosphorylation is Thr⁵² > Thr⁷⁷ > Ser³⁵. Consistent with the consequence of Cdk1 - induced phosphorylation, HIPK2 induces HMGA1 phosphorylation and results in reduced binding affinity of HMGA1 to DNA sequence. In fact, the major phosphorylation sites Thr⁵² and Thr⁷⁷ reside close to the second and third AT-hooks of HMGA1, which probably implies that they can modulate DNA binding affinity. Likewise, the phosphorylation of HMGA1a at Thr²¹, Ser⁴⁴ and Ser⁶⁴ by protein kinase PKC can attenuate its DNA binding affinity to the promoter regions of PKC γ and neurogranin/RC3 genes [252]. Another study has reported that CK2 constitutively phosphorylates HMGA1a and HMGA1b at residues Ser¹⁰² and Ser¹⁰³ of the C-terminal

end. Although the C-terminal domain of HMGA1 is not directly involved in DNA binding, an indirect effect of this phosphorylation on DNA binding has also been reported. It has been suggested that phosphorylation at the C-terminus could represent an additional way to modulate HMGA DNA-binding properties [234, 257, 258].

Both hyper- and de-phosphorylation of HMGA1a have been observed during apoptosis in leukaemic cell lines. Hyper-phosphorylation (at least five phosphate groups) occurs at the early apoptotic stages and is probably related to the displacement of HMGA1a from DNA and chromatin, therefore releasing from the scaffold/matrix attachment regions (SARs/MARs). De-phosphorylation (one phosphate or no phosphate group) of HMGA1a mainly appears in the highly condensed chromatin of apoptotic bodies [258]. Taken together, these findings suggest the direct link between apoptosis and the degree of phosphorylation in HMGA1a.

In addition to phosphorylation, HMGA proteins are subjected to acetylation at several lysine residues and the N-termini of HMGA1a and HMGA1b [259]. One of the best-characterised examples is the recruitment of HMGA1 proteins to the enhancer region of *interferon* - β (*IFN*- β), in which the acetylation of lysine residues in HMGA1 proteins governs the transcription of *IFN*- β . In particular, two molecules of HMGA1 contact with four binding sites at the enhancer in a highly cooperative fashion, thus altering the structure of the DNA and allowing recruitment of the *IFN*- β gene activators such as, ATF-2, c-Jun, IRF-3/IRF-7, NF-KB on PRDIV-PRDIII regions, assemble into a remarkably stable high order nucleoprotein complex termed the IFN- β enhanceosome [260, 261]. After the formation of the enhanceosome, the GCN5/PCAF complex is recruited and acetylates Lys⁶⁵ of HMGA1, hereby stabilising the structure. Subsequently, the acetyltransferase, CBP, acetylates Lys⁷¹ of HMGA1, which results

in destabilisation and disassembly of the enhanceosome, leading to transcriptional turn-off of the human *IFN-β* gene [262]. In addition to its role in regulating *IFN-β* transcription, an *in vivo* study has revealed that elevated acetylation is related to advanced metastatic potential in breast cancer cells [263].

More recently, mass spectrometry analysis has uncovered that HMGA1a is mono- and di-methylated at arginine²⁵ located in the first AT-hook motif [264]. Methylation of this residue appears to be progressively increased during apoptosis, and reaches the highest level at later stages of the apoptotic process in different tumour cells [258]. Blocking methylation by using adenosine dialdehyde (ADOX), which is an inhibitor that preventing methyltransferase activity, does not inhibit apoptotic process. This suggests that HMGA1 is not one of the critical molecules that causes apoptosis but instead as an architectural factor that modulates the chromatin of cell death-induced genes [265]. Segarra *et al.* have also demonstrated that methylation parallels the de-phosphorylation process during apoptosis, suggesting that the combination of these PTM events affects the formation of protein complexes involved in regulating chromatin remodelling and gene activity. Moreover, the same research group has identified that arginine methyltransferase, PRMT6 methylates HMGA1a on Arg⁵⁷ and Arg⁵⁹, which locate within the second AT-hook region. This region is critical for both DNA binding and protein-protein interactions, and modifications may cause profound alterations in the HMGA network [266]. Besides, Arg⁸³ and Arg⁵⁹ located in the third AT-hook, can also be methylated by PRMT6 but in a much lower extent [266].

It has been extensively noticed that overexpression of HMGA protein is related to malignant neoplasia of a variety of cancers, highlighting the oncogenic properties of HMGA proteins. Recent studies have identified that the high regulated PTMs may also be involved in tumorigenic progression. Indeed, different types of PTMs of HMGA1

have been found *in vivo* in human breast epithelial cells of different stages of neoplastic progression [259, 263]. In particular, HMGA1a proteins are highly acetylated and methylated in metastatic breast cancer cell lines (HA7C and HA8A) than those in non-metastatic parental MCF-7 cells [263]. In addition, acetylation and di-methylation of specific residues (i.e. Lys⁷³ and Lys⁴⁵ respectively) can only be found in these more malignant cells types but not in the parental ones. Furthermore, HMGA1a and HMGA1b proteins are more highly phosphorylated in the tumorigenic MCF-7/PKC- α cells than in the non-metastatic MCF-7 cells [259]. Zou *et al.* have also demonstrated that mono-methylation at Lys³⁰ and Lys⁵⁴ as well as mono-phosphorylation at Ser⁴³ and Ser⁴⁸ are correlated with more aggressive human breast cancer [267]. These cumulative results provide strong support to the notion that different patterns of HMGA1 PTMs reflect the distinct physiology or phenotype of the cells. Therefore, other than the overexpression of HMGA proteins as a potential cancer biomarker for tumorigenic progression, the types and sites of PTMs may also serve as an indicator for cancer cells.

1.6.5 Role of HMGA during development

The role of HMGA protein in embryogenesis, cell proliferation, differentiation, apoptosis and cancer development has been profoundly studied [268]. HMGA proteins have been demonstrated to play their major physiological role during embryogenesis, where their expression reaches very high levels during embryonic development and drop to very low levels and often undetectable in adult tissue. Specifically, HMGA proteins are highly expressed in all embryonic tissues at 8.5 dpc (days-post-coitum) [269]. In the later stages, even though the gene is still expressed in all tissues, *HMGA1* is more restricted to specific organs of all three germ layers, the ectoderm, mesoderm

and endoderm. In most adult tissues, its expression is relatively higher in testis, skeletal muscle and thymus [269]. Haematologic malignancies, cardiac hypertrophy and human type 2 diabetes were observed in *HMGA1* knockout and heterozygous mice, suggesting that it is required for cardiomyocyte growth and insulin signalling [270, 271]. In addition, both *HMGA1* and *HMGA2* are required for normal sperm production in mice [272, 273]. Moreover, loss of *HMGA1* gene function affects lymphohematopoietic differentiation and adipocyte differentiation [274, 275].

On the other hand, HMGA2 protein is predominantly expressed in proliferating, undifferentiated mesenchymal cells during embryogenesis. In normal adult tissues, its expression is hardly detectable and mainly limited to the mesenchymal cells in testis, skeletal muscle and adipose tissues [273, 276, 277]. In 1995, a study has demonstrated that disrupted *HMGA2* leads to repression of cell proliferation and results in reduced sizes of all the tissues except for the brain in the pygmy mice [278]. Almost ten years later, it has been uncovered that depletion of *HMGA2* leads to reduced expression of the insulin-like growth factor 2 mRNA binding protein 2 (*Igf2bp2*) gene, which results in pygmy phenotype in mice [279-281]. Likewise, knocking down both *HMGA1* and *HMGA2* resulted in a so called 'superpygmy' phenotype that the mouse showed a significant reduction of body size by 80% [282]. Furthermore, evidence shows that mice with partial or complete deficiency of *HMGA2* are characterised in a significant reduction of fat tissue which is capable of resisting diet-induced obesity in mice [276]. Consistently, another study has demonstrated that transgenic mice with overexpression of truncated version of *HMGA2* show not only an obese phenotype but also developed lymphomas [283, 284].

Furthermore, HMGA1 and HMGA2 have been shown to play an essential role in the self-renewal potential of hematopoietic stem cells [285]. More recently, a study has

reported that both HMGA1 and HMGA2 proteins are highly expressed in neural precursor cells (NPCs) only during the early stage of neocortical development in mice, which is essential for contributing to the neurogenic potential. It has been found that knockdown of HMGA in early stage results in reduced NPCs neurogenic potential, while overexpression of HMGA proteins confers neurogenic potential and self-renewal capacity on late stage NPCs, possibly through antagonising histone H1 and inducing global opening of the chromatin [286].

In summary, HMGA protein is essential factors for embryonic development and progenitor cell differentiation during adipogenesis, cardiogenesis, spermatogenesis, haematopoiesis, lymphohaematopoiesis, neurogenesis and myogenesis.

1.6.6 HMGA proteins in cancer

As described in the last section, HMGA proteins are expressed ubiquitously in early embryonic development and almost absent in normal adult tissues. In normal cells, HMG proteins are highly regulated and influenced by both developmental and environmental factors. Absence, mutations or aberrant expressions of HMG proteins can alter cell phenotype and lead to developmental abnormalities and diseases. Since the initial discovery of HMG proteins, HMGA has captured a lot of attention because of their oncogenic properties in several malignant neoplasias as well as the role in several other diseases like obesity and atherosclerosis [276, 287].

Since the first study in 1985 revealing the elevated phosphorylated HMGA protein expression in the virally transformed rat thyroid cells in comparison with those of normal thyroid cells [288], many subsequent studies have supported the idea that HMGA is overexpressed in malignancies, including colorectal, prostate, lung, thyroid,

cervical, oesophageal and breast carcinoma [289]. Together, these observations imply that HMGA proteins are closely associated with tumorigenesis and thus, they can be ideal biomarkers for cancer patient prognosis and diagnosis.

HMGA proteins contribute to tumour formation through several different mechanisms. For instance, it has been reported that HMGA proteins inhibit the apoptotic function of the transformation-related protein 53 (TRP53, a.k.a. p53). The p53 protein is a tumour suppressor involved in the regulation of cell cycle and cell death. HMGA1 antagonizes p53 and the p53-mediated transcriptional targets, cyclin-dependent kinase inhibitor 1A (*Cdkn1a*, a.k.a. *p21^{Kip1}*), B cell leukaemia/lymphoma 2 (*Bcl2*), and Bcl2-associated X protein (*Bax*), while enabling the transcription of the p53 inhibitor protein, E3 ubiquitin-protein ligase/mouse double minute 2 homologue (MDM2) [290, 291]. HMGA also induces inactivation of the p53 proapoptotic activator homeodomain-interacting protein kinase 2 (HIPK2) by relocating of nuclear HIPK2 to cytoplasm [292].

Another mechanism by which HMGA proteins exert their tumorigenic activity is through deregulating the pRB/E2F1 pathway. A critical role of the pRB/E2F1 pathway is to regulate the initiation of DNA replication, and deregulation of this pathway has been observed in virtually all human cancers [293]. Alike the role of p53, the retinoblastoma protein (pRB) is established as a tumour suppressor due to its growth inhibitory capability. It has been demonstrated that pRB interacts with the transcription factor E2F1 and in turn, represses the transcription of its target genes that encode DNA replication activities and cell cycle regulatory activities. In addition, the pRB recruits chromatin remodelling factors such as histone deacetylase 1 (HDAC1), methyltransferase, SWI/SNF factors or polycomb proteins that act on the nearby nucleosome [294-298]. Fedele *et al.* and colleagues have reported that HMGA2 binds to pRB and prevents it from repressing the E2F1 mainly by displacing HDAC1 from

pRB. In this way, the histone acetyltransferase EP300 is subsequently recruited to acetylate E2F1 and associated histones, resulting in enhanced E2F1 activity which is observed in pituitary adenomas of transgenic mice [299]. Interestingly, another research group has found that E2F1 can bind directly to the *HMGA1* promoter and regulate its expression [300]. Therefore, elevated HMGA2 can deregulate the pRB/E2F1 pathway, which subsequently induces HMGA2 to trigger tumorigenic formation in a positive feedback loop.

In addition, HMGA proteins also interfere with cell cycle by interacting with the activator protein 1 (AP1). AP1 is a heterodimeric complex consisting of a Fos protein [i.e. cFOS, Fos-B or Fos related antigen-1 and -2 (FRA1, FRA2)] and a Jun protein [i.e. JUN, JUNB, JUND or activating transcription factor (ATF)]. Upon various stimuli, such as cytokines, growth factors and stress signals, the MAP kinases ERK and JNK will phosphorylate the Jun and Fos proteins to induce the AP1 activity [301]. AP1 is a transcription factor that regulates the expression of diverse genes involved in cell proliferation, cell death and differentiation [302]. Therefore, it is not surprising that deregulation of AP1 is critical in development of carcinogenesis. In 1985, HMGA was first discovered to be essential for the establishment of the neoplastic phenotype in virally transformed thyroid cells [288]. Later in 1997, they found that elevated HMGA induce *junB* and *fra1* transcription to enhance AP1 activity to mediate neoplastic transformation.

Interestingly, although numerous studies have indicated that HMGA proteins act through opening the chromatin or promoting formation of enhanceosome, data have also shown that HMGA can play an inhibitory role by sterically blocking the binding of transcription factors to their recognition sites in gene promoters [303]. Indeed, HMGA2 has been demonstrated to compete with p120E4F for target promoter binding and thus,

inactivate its repressing effects on the cell cycle regulator, cyclin A [304]. On the other hand, HMGA2 can also facilitate the binding of ATF/CREB family of transcription factors, as well as transcription factors, such as E2F1 and AP1, to promote cyclin A expression [304-306]. Taken together, overexpression of HMGA proteins promote deregulation of E2F1 and AP1 activity, along with with increased cyclin A expression, disrupt the normal cell cycle progression and lead to tumorigenesis.

Not only interfering with the cell cycle, but HMGA also contributes to tumour progression through promoting cellular processes involved epithelial-mesenchymal transition (EMT). Although EMT occurs during normal embryonic development, when cells of the epithelial lineage undergo conversion to the mesenchymal lineage, it has also been implicated as a critical hallmark of carcinogenesis. This biological process, EMT, allows polarised epithelial cells to undergo multiple physiological changes that enable the cells to assume mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, resistance to apoptosis and chemotherapy, as well as increased production of extracellular matrix components. Several signalling pathways are involved in the regulation EMT, such as those mediated through bone morphogenic protein (BMP), fibroblast growth factor (FGF), epidermal growth factor receptor (EGFR), hepatocyte growth factor (HFG), Wnt/ β -catenin, Notch, and transforming growth factor-beta (TGF- β) pathways [307]. The TGF- β pathway is a major inducer of the EMT during tumour development. Recently, it has been shown that HMGA2 can increase the expression of TGF- β type II receptor, thereby promoting TGF- β signalling [308]. This suggests that HMGA2 can potentially play a vital role in promoting EMT and inducing invasion and metastasis in metastatic cancers. Interestingly, TGF- β also upregulates HMGA2 expression by derepressing SMAD3-mediated let-7 repression in a positive feedback loop. Let-7, one of the members of

the microRNA (miRNA) family, which are a group of small noncoding regulatory RNAs that regulates gene expression by base-pairing to mRNAs, resulting in mRNA decay and translational inhibition. Let-7 was originally discovered in *Caenorhabditis elegans* and was associated with controlling of timing of stem cell division and differentiation [309, 310]. Subsequently, it has also been found in humans and is widely considered to be a tumour suppressor, whereby the reduction of let-7 leads to a less differentiated cellular state and the development of cancer [311]. In mammals, let-7 expression levels are low during embryonic development, but increase after cellular differentiation and in mature tissues [312]. Let-7 has also been found to directly inhibit HMGA2 by binding to its 3' untranslated region (UTR) [313]. This is in agreement with the observation that HMGA2 shows a converse expression pattern to let-7, where it is widely expressed in undifferentiated embryonic tissues but almost undetectable in normal adult tissues. Taken together, TGF- β inhibits let-7 expression through SMAD3-mediated inhibition of let-7 expression, thereby resulting in upregulation of HMGA2 expression; enhanced HMGA2 further activating TGF- β signalling. Moreover, HMGA2 was found to cooperate with TGF- β pathway in regulating the expression SNAIL1, SNAIL2 and Twist, which are the key modulators for induction of EMT process [314, 315].

In addition, the RNA-binding protein Lin-28, a key developmental regulator, has found to inhibit let-7 biogenesis and in turn, upregulates the expression of HMGA2 [285, 316]. The balance between Lin-28 and let-7 affects the HMGA2 expression level which is essential for self-renewal and differentiation of foetal haematopoietic stem cells (HSC). Alterations in the Lin28-let7-HMGA2 axis lead to disrupted HSC self-renewal capacity and abnormal development. Indeed, activation of Lin-28 results in global post-transcriptional downregulation of let-7 expression. This is accompanied by an increase

in its oncogene targets, such as HMGA2, K-RAS, C-MYC and cell cycle factors, in different types of cancer, such as glioblastoma, ovarian, gastric, prostate and breast cancer [317]. Moreover, Lin-28 also contributes to development and progression of breast cancer through its direct regulation of translation of the oncogenes, HMGA1 and human epidermal growth factor receptor (HER2) [318].

One of the examples for HMGA participating in EMT is a study of NIH-60 (human tumour cell lines screen), in which the low expression levels of let-7 and thus induced HMGA2 expression are closely associated with more advanced cancers [319]. In pancreatic cancer, HMGA2 maintains oncogenic EMT by modulating the RAS/MEK signalling pathway, rather than the TGF- β pathway. It has been reported that knockdown of HMGA2 inhibits cell proliferation, and leads to an epithelial state transition which restores cell-to-cell interaction via the upregulation of E-cadherin [320]. Consistently, in aggressive gastric cancers, it has been identified that HMGA2 induces EMT by activating the Wnt/ β -catenin signalling pathway. In this case, HMGA2 regulates several genes, such as *AXIN1* and *TWIST1*, by directly binding to their promoters. Subsequently, AXIN1 activates β -catenin via phosphorylation and TWIST1 promotes the nuclear translocation of β -catenin, by which the Wnt/ β -catenin pathway is activated, resulting in oncogenic EMT in gastric cancer [321]. Moreover, HMGA2 has also been found to participate in EMT in human high-grade papillary serous carcinoma (HG-PSG) [322]. Overexpression of HMGA2 is sufficient to induce ovarian surface epithelial cell transformation both *in vitro* and *in vivo*, and can result in aggressive tumour cell growth. A ChIP-based global gene and microRNA profiling analysis of gene expression in ovarian cell lines with and without HMGA2 overexpression has revealed that 5 (16%) HMGA2 target genes were associated with EMT, suggesting an active role of HMGA2 in EMT [322].

Furthermore, chromosomal rearrangements have long been found in a variety of benign tumours. The rearrangements of *HMGA* at 12q14-15 and several partner chromosomes are commonly observed in tumours of mesenchymal origin, which including lipomas, endometrial polyps, uterine leiomyomas, pulmonary chondroid hamartomas, fibroadenomas of the breast, pleomorphic adenomas of the salivary glands and aggressive vulvar angiomyxoma [268, 323-331]. Chromosomal aberrations of the *HMGA2* gene can also result in its expression deregulation, truncation, or generation of fusion genes encoding chimeric transcripts containing only the first three exons of *HMGA2* fused to sequences from other genes [332, 333]. Therefore, in most of the cases, the rearranged *HMGA2* genes encode for a truncated or a chimeric HMGA2 protein that retains its capacity to bind to DNA and interact with other proteins but loses its carboxy terminus, including the 3' UTR. As described earlier, the tumour suppressor let-7 directly binds to HMGA2 3'UTR and thereby inhibiting its function. Following truncation or fusion with ectopic sequences, HMGA2 loses the sites previously recognised by let-7, resulting in unregulated induction in HMGA2 levels which contribute to neoplastic diseases.

In addition to HMGA2, chromosomal rearrangement of *HMGA1* gene at 6p21 has also been frequently described in human mesenchymal tumours. However, in contrast to *HMGA2* in which the breakpoint is in the third intron, the breakpoints of *HMGA1* are located either upstream or downstream of the coding sequences, and sometimes within an 80kb region surrounding *HMGA1*, but no intragenic rearrangements have been documented [334].

Several studies propose a vital role of HMGA proteins in impairing DNA repair, which further emphasises its functions in tumorigenesis. It is widely accepted that one of the key steps in carcinogenesis is the impairment of DNA repair mechanisms. In human

cells, both normal metabolic activities and environmental factors, such as mutagenic chemicals and radiation, can cause DNA damage, accounting for several thousand to a million damaging events per cell per day. Many of these lesions in the genome generate errors which can alter or eliminate the transcription of DNA, translation of proteins, and even signalling and cellular functions. Other lesions induce genomic mutations that can be carried over into generations of daughter cells if the mutation is not repaired prior to mitosis. Thus, the correction of DNA sequence errors is significant in all types of cells. Cells have developed multiple repair processes, including mismatch, base excision and nucleotide excision repair, and failures in one or more of the DNA repair systems can lead to senescence, apoptosis or carcinogenesis.

It is well-known that the tumour suppressor, p53, can recognise certain types of DNA lesions and subsequently induce DNA repair in response to DNA damage. Likewise, the mismatch repair proteins share some standard features with p53, in recognising lesions such as Holliday junctions and extra-base bulges and thus, inhibiting DNA recombination [335-338]. HMGA1 has been found to compete with p53 and human mismatch repair proteins (hMSH2 - hMSH6) for Holliday junction binding, exerting adverse effects on DNA mismatch repair [339]. Moreover, cDNA microarray analyses revealed that HMGA proteins regulate the transcription of a number of genes that are involved in DNA repair. These genes involved in double-strand break repair (e.g. ATM, XRCC4, BRCA1, etc.), homologous recombination (e.g. RAD54, RECQL5, etc.), base excision repair (e.g. DNA ligase III, MBD4, XRCC1) and nucleotide excision repair (e.g. XPA, ERCC1). These HMGA1 target genes are directly or indirectly down-regulated by overexpression of HMGA1 in MCF-7 human breast cancer cells, suggesting that the HMGA family can affect DNA repair by regulating the transcription of genes involved in various aspects of DNA damage recognition and removal [340-342].

Moreover, it has been shown that HMGA1 downregulate BRCA1 expression, of which is involved in double-strand break (DSB) repair and nucleotide excision repair (NER), by direct binding to its promoter region [342, 343]. In this manner, HMGA proteins enhance genotoxic stress induced by different DNA-damaging agents by causing DSBs, such as cisplatin, bleomycin, doxorubicin and X-ray irradiation [344, 345]. It is to be noted that these DNA-damaging agents, such as cisplatin and doxorubicin, are commonly used in breast cancer treatment and have achieved impressive clinical results.

Furthermore, another mechanism that accounts for the role of HMGA proteins in carcinogenesis is its ability to stimulate inflammatory proteins, which in turn promote cancer progression by inducing proliferation, angiogenesis, invasion and metastasis, and suppressing apoptosis [346]. It is extensively accepted that our immune systems fight back against threats like cancer, but unexpectedly specialised cells from the adaptive immune system also orchestrate the inflammation response that promotes tumour progression. Epidemiological studies have shown that chronic inflammation makes individuals susceptible to many types of cancers, and most precancerous and cancerous tissues display signs of inflammation. In the tumour environment, the B lymphocytes (adaptive immune system) release antibodies that target cells such as neutrophils, macrophages and mast cells (innate immune system), these cells subsequently secrete specific inflammatory signalling molecules, thereby promoting growth and spreading of the tumour. Conversely, anti-inflammatory agents protect against several tumours. HMGA1 is known to contribute to the regulation of a number of genes that are involved in inflammation, including inducible nitric oxide (iNOS), cyclooxygenase 2 (COX2), E-selectin, immunoglobulin E (IgE), IL-2, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [347-354]. The

induction of these genes by HMGA1 occurs primarily through NF-KB signalling pathway. It is found that both HMGA1 and HMGA2 proteins function as architectural factors, facilitating the activity of the transcription factor, NF-KB, through the PRDII element of the β -interferon enhancer [354, 355].

NF-KB and NF-KB-mediated inflammation have a double-edged role in cancer. On the one hand, activation of NF-KB induces the activity of cytotoxic immune cells against cancer cells during the acute inflammatory response. On the other hand, NF-KB exerts oncogenic effects and its constitutive activation (chronic inflammatory response) results in enhanced EMT, angiogenesis and metastasis [356-358]. In this way, deregulated overexpression of HMGA promotes the oncogenic activities of NF-KB which contribute towards tumour progression.

Taken all the evidence together, through different mechanisms, HMGA protein expression correlates not only with tumorigenesis, but also with the highly malignant phenotype, and it is a poor prognostic marker as its overexpression strongly correlates with metastasis and reduced patient survival. Therefore, blocking HMGA function could be an excellent strategy for cancer treatment because of their close association with human neoplastic diseases.

A striking dual role of HMGA proteins is that they are not only potent oncogenes but also tumour suppressors. Given that HMGA1 exerts a direct role on cardiomyocytic growth in mice, both heterozygous and homozygous *Hmga1*-null mice develop cardiac hypertrophy, in addition to myelolymphoid malignancies, also including B cell lymphomas and myeloid leukaemia. The malignant phenotype may be due to the role of HMGA1 in the regulation of specific cytokine gene transcription. These HMGA1 targets include IFN- β , E-selectin, TNF- β , IL-2 receptor α , IL-2, GM-CSF and CXCL1,

which are positively regulated by HMGA proteins. Some other immune function genes that are negatively regulated by HMGA include IL-4, ϵ -IgG and NOX2 [359]. In the presence of reduced or null amounts of HMGA1, the T cell expansion modulators, IL-2 and IL-2R α are down-regulated, while decreased IL-2 and increased IL-6 results in B cell expansion and the consequent development of B cell neoplasia [270]. This suggests a potential onco-suppressor role of HMGA1 in lymphoproliferative malignancies.

Another study has also revealed that HMGA proteins contribute to anti-tumour activity through regulation of cellular senescence [360]. Senescence is defined as an irreversible state of cell cycle arrest that promotes tissue remodelling during development and in response to different damaging stimuli [361]. Upon different mechanisms in various cell type and conditions, cellular senescence has been categorised into several subtypes, including 'replicative senescence', 'DNA-damage-induced senescence', 'stress-induced senescence' and 'oncogene-induced senescence'. It is widely accepted that senescence is an important mechanism for preventing the proliferation of potential cancer cells. In addition to suppressing tumorigenesis, it is also participated in promoting cancer progression, tissue repair and ageing [362]. Some of the opposing effects of senescence are thought to associated with diverse cellular activities with ageing.

Interestingly, it has been shown that HMGA proteins accumulate on the chromatin of senescent fibroblasts and are essential structural components of SAHF (senescence-associated heterochromatic foci). In general, upon environmental stress and DNA damage, p16^{INK4A} is induced and accompanied by activated pRB, causing formation of SAHF. Heterochromatin is characterised by chromatin compaction and gene silencing. HMGA proteins cooperative with the p16^{INK4A} to promote SAHF formation

which represses the transcription of proliferation-associated genes, and therefore results in proliferative arrest and stabilised cellular senescence [360]. However, these HMGA anti-proliferative activities are no longer functional in the presence of the *HDM2* and *CDK4* oncogenes, which are often co-expressed with HMGA2 in human cancers. Moreover, suppression of both HMGA and p16^{INK4A} acts synergistically to enable cells to elude senescence, probably by reactivating of some proliferation-associated genes and inducing S phase re-entry in senescent cells [360]. Thus, in this context, HMGA proteins are considered as tumour suppressors in terms of their ability to initiate and maintain cellular senescence. Although HMGA proteins are more commonly considered as the 'architectural factors' that open chromatin and activate transcription, the fact that the activity of HMGA proteins can be modulated, perhaps like histones, by PTMs which control their ability to promote open or closed chromatin states in different cellular contexts [222]. As described in the earlier PTMs section, HMGA proteins can be modulated through phosphorylation, acetylation and methylation. Indeed, acetylation on different lysine residues of HMGA can alter the stability of enhanceosome recruited by HMGA1 and result in either initiation or cease transcription [232, 262]. Furthermore, Li *et al.* have also reported a dual role of HMGA proteins in which the Ubc9 interacting sites in HMGA proteins determine distinct cellular activates. Ubc9 is the E2 conjugase that transfers activated SUMO (small ubiquitin-related modifier) to protein substrates, through which modulates many aspects of cell physiology, including transcription, nucleocytoplasmic transport, DNA replication and repair, chromosome dynamics, cell cycle regulation, apoptosis and ribosome biogenesis [363]. It has been shown that the interaction of Ubc9 with the proline-rich HMGA1b domain can result in increased anchorage-independent growth of Rat1a cell lines, and an increase in average colony size. In contrast, Ubc9

interaction with only the C-terminus of HMGA1b can lead to a significant loss in clonogenic capacity [364]. Therefore, HMGA1 proteins can exert either positive or negative effects on proliferation and transformation by interacting with the same protein Ubc9 but at different domains.

This is not surprising given the diverse mechanisms by which HMGA proteins are regulated at the post-translational level, that HMGA proteins are endowed with both oncogenic and anti-oncogenic properties. Notably, the protein partners might be varied depending on the cell type and cellular context, and they will exert different effects on the HMGA proteins in cell transformation [268].

1.6.7 HMGN protein: structure and function

Among the three different subfamilies of HMG proteins, HMGNs are non-histone chromosomal proteins that specifically bind to nucleosomes and modulate the structure and function of chromatin [365]. The global and local structures of chromatin fibres are dynamic and continuously changed by many enzymatic activities and structural factors. HMGN proteins compete for chromatin binding sites with the linker histone H1 and hence reduce H1 mediated chromatin compaction and facilitate the accessibility of various regulatory factors. Therefore, HMGN proteins are capable of modulating cellular epigenetic profile, affecting DNA-dependent nuclear activities such as transcription, replication, recombination and repair, and impacting biological processes, such as cell cycle, differentiation and development [365-367].

HMGN protein families are only found in vertebrates which consist of HMGN1 (HMG-14), HMGN2 (HMG-17), HMGN3a, HMGN3b, HMGN4 and HMGN5 (NSBP1 or NBP-45). HMGN1, 2, 3a and 4 exhibits similar structure and are around 90 amino acids in

length, while HMG3b lacks the C-terminal domain (77 amino acids) and HMGN5 possesses a long acidic tail that contains about 300 negatively charged amino acid repeats [368, 369]. *HMGN* genes are located on different chromosomes. HMGN1 through 5 are located on 21q22.3, 1p36.1, 6q14.1, 6p21.3 and Xp13.3, respectively of the human genome [370]. These HMGN proteins are characterised by the presence of three distinct functional domains which are the bipartite nuclear localisation signal (NLS), the nucleosomal binding domain (NBD) and the acidic C-terminal chromatin regulatory domain (RD). There is a highly conserved sequence 'RRSARLSA' embedded in the NBD, called the 'NBD core', which specifically recognises the generic structure of the 147 base pair nucleosome core particles [371]. Unlike other nuclear proteins that bind to chromatin by targeting certain DNA sequences or specific histone modifications, HMGN proteins bind to nucleosome particles better than isolated DNA or histones [372]. In the nucleus, rather than being statically and permanently associated with specific genes, HMGN proteins are highly mobile among nucleosomes in a 'stop-and-go' manner, in which a molecule binds to a binding site for a limited time and then dissociates and transfers rapidly to another. Fluorescence recovery after photobleaching (FRAP) analysis shows that this whole process ensues extremely fast residence time of about 5 to 20 seconds [373, 374]. Besides, it is known that the residence time of an HMGN molecule stays on a nucleosome is longer than that when it moves to another. Therefore, although the amount of HMGNs in the nucleus is sufficient to bind about 1% of the nucleosomes, the dynamic binding of HMGNs ensures more than 99% HMGN proteins are bound to chromatin fibres at any given moment [373, 375]. The dynamic behaviour of HMGN proteins also facilitates potential functional redundancy among HMGN variants of which one is missing or altered, and another variant can rapidly compensate for it by binding to the same site. Yet, these

HMGN proteins are not fully redundant and each has its unique properties, which will be further discussed in the later section.

Another unique characteristic of HMGNs is that they possess a disordered secondary structure. An intrinsically disordered protein lacks a stable or fixed three-dimensional structure of which freely interact with many proteins and form macromolecules. It has been established that both HMGN1 and HMGN2 form multiple metastable protein complexes in Hela cells, and the nucleosome binding ability of HMGNs in the complex is stronger than that of the free HMGNs [376]. In this respect, since HMGN binding to nucleosome core particles is independent of the underlying DNA sequence, the inclusion of HMGN proteins in multiprotein complexes serves to sharpen the specificity of HMGN-nucleosome interaction. In addition, the association of HMGN proteins in complexes is relatively unstable and with the mixing of subunits of the macromolecular complexes provide diverse gene regulations in response to changing metabolic states. Several studies have provided direct evidence that HMGNs interact with other protein factors in chromatin. For instance, HMGN2 incorporates with the sequence-specific activator GAL4-VP16 and initiates gene transcription [377]. The Cockayne syndrome protein A (CSA) recruits HMGN1 to the RNA polymerase II-containing complexes at UV-induced chromatin lesions for nucleotide excision repair [378, 379]. Moreover, HMGN1 affects the induction of several estrogen-regulated genes by explicitly interacting with estrogen receptor alpha (ER α) both *in vitro* and *in vivo*. The estrogen signal recruits HMGN1 proteins to ER α via protein-protein interaction at the TFF1 promoter region and activates its gene transcription [380]. Likewise, HMGN3 and transcription factor PDX1 mutually reinforce their binding to the promoter of the Glut2 gene and affect glucose homeostasis in pancreatic beta cells [381].

Furthermore, it is known that the binding of these HMGN variants to nucleosome core particles is highly dependent on ionic strength. *In vitro* analysis showed that at low ionic strength, nucleosome core particles could bind either one or two HMGN variants with high affinity. However, under physiological conditions with high ionic strength, HMGN molecules bind to chromatin in a highly specific manner, in which HMGN1 and HMGN2 bind to nucleosomes cooperatively to form complexes containing homodimers of either one or the other variant and not mixed pairs [382]. Thus, studies have shown that in living cells, the HMGN containing nucleosome domains are homogeneous in their content of HMGN molecules, in which the isolated nucleosome core particles contain either HMGN1 or HMGN2 but not a mixture of both proteins [383].

Specifically, structural studies demonstrate that the 'NBD core' anchors the HMGN1 and HMGN2 proteins to nucleosome through interaction with the acidic patch on the histone H2A/H2B surface, where the N-terminal of NBD interacts with DNA near the edge of the dyad axis of nucleosome core and the negatively charged RD domain contacts a restricted region in the N-terminal tail of histone H3 [372, 384, 385]. Once bound to nucleosomes, the RD domain functions to decondense chromatin fibres to promote DNA-dependent nuclear processes, such as transcription, DNA replication and DNA repair. More significantly, HMGN proteins disrupt the H1-mediated stabilisation of condensed chromatin by competing for binding at the entry/exit site of DNA on the nucleosome [373]. *In vivo*, the amount of HMGN proteins is significantly less than that for H1 (the ratio is about 1:100) [386]. However, HMGN1 might be enriched at specific genomic regions where the local concentrations are significantly higher [387, 388]. To note, most recent studies have revealed that, rather than directly replacing H1 from nucleosomes, HMGN1 diminishes the extent to which H1 stabilises

higher-order chromatin structures by directly altering H3 and H4 tail interactions and the C-terminal domain structure of H1 [389]. Besides, the unique HMGN5 protein possesses a long and negatively charged C-terminus and interacts with the positively-charged H1 tail more efficiently than other HMGN variants. Taken together, HMGNs reduce chromatin compaction not only by interacting with histone subunits such as H2A, H2B, H3 and H4, but also by destabilising the binding of H1 to chromosome, resulting in chromatin decompaction and increased access of the regulatory factors to nucleosomal DNA. Therefore, HMGN-mediated decompaction can either enhance or repress transcription by permitting access of either activators or suppressors.

1.6.8 Histone modifications and post-translational modifications of HMGNs

The histones are modified by covalent PTMs which include phosphorylation, acetylation, methylation, ubiquitylation and SUMOylation. These modifications to histones can alter chromatin structure or recruit histone modifiers and thus affect gene expression, replication and DNA repair. Consequently, they play an essential role in biological processes, including development and differentiation as well as in cellular responses to various internal and external stimuli. In this respect, it is not surprising that there are more than 100 different PTMs of nucleosomal core histones and their levels and patterns are continually changing.

Interestingly, although HMGN proteins have no enzymatic activity, they can mediate histone modifications by binding to the nucleosome, thereby modifying chromatin structure and affecting epigenetic processes. Studies on *Hmgn1*^{-/-} mice have demonstrated that loss of HMGN1 affects the global levels of H3K14ac, H3K9ac, H3K9me, H3S10ph, H3S28ph, H4S1ph and H2AS1ph [365, 390]. More specifically,

studies revealed that HMGN1 elevates the action of histone acetyltransferases (HATs) such as PCAF (P300/CBP), and results in enhanced H3K14 acetylation and subsequent transcriptional activation [391]. Significantly, HMGN1 modulates these modifications, not only H3K14ac, but also the other ones such as H3S10ph and H3S28ph, through binding to chromatin. The modification of free, non-nucleosomal histones is not affected by HMGN proteins [390]. Moreover, different HMGN proteins drive specific histone modifications, inferring these variants are not fully functional redundant [392]. For example, although HMGN proteins affect the acetylation and phosphorylation of H3, only HMGN1 and not HMGN2 can modulate the phosphorylation of H3S10 and H3S28. Similarly, HMGN2 induces H3K14 acetylation more significantly (3.5-fold increase) than the action of HMGN1 (2-fold increase) [392].

Several different mechanisms have been proposed in related to the effects of HMGN1 on histone modifications. One possibility is that HMGN1 competes with H1 for chromatin-binding sites or that it alters the entry/exit angle of the nucleosomal DNA, and leads to structural transformation of chromatin that preferentially recruits HATs to their specific targets. The other possibility is that the binding of HMGN1 to nucleosomes induces steric changes in the nucleosomes, which eventually increases the enzymatic activities on the histone tails. An additional possible mechanism is that HMGNs affect the activity of ATP-driven nucleosome remodelling enzymes and hence the chromatin dynamics. Nevertheless, two independent studies have obtained different results, and the mechanism involved is not yet clear [393, 394]. Most likely, these three possible mechanisms can be interrelated and can act together.

HMGNs play important roles in histone modifications and as a consequence, they impact numerous biological processes in the cell. Indeed, several studies have demonstrated that HMGN-mediated histone modifications are physiologically

significant. One study has indicated that the heat shock mediated induction of Hsp70 gene is impaired in *Hmgn*^{-/-} mice embryonic fibroblasts (MEFs). Heat shock response protects cells against adverse insults by upregulating the expression of HSPs (e.g. HSP70, HSP90, HSP60 etc.) in response to stress. HMGN1 has been shown to enhance the levels of heat shock-induced H3K14 acetylation on the *Hsp70* promoter, thereby promoting chromatin remodelling and the activation of *Hsp70* transcription [395]. Loss of HMGN1 significantly reduces the levels of H3K14ac on the *Hsp70* promoter and the amount of *Hsp70* transcripts. Another study has revealed that HMGN1 induces protein kinase ataxia-telangiectasia (ATM) activation and recruitment to the sites of double-stranded DNA breaks in order to initiate DNA repair system. Again, HMGN1-mediated activation of ATM through enhanced levels of acetylation H3K14 [396]. Furthermore, loss of HMGN1 reduces the ionising radiation-induced auto-phosphorylation on H3S10 and decreases the expression of several ATM targets, such as p53, MDM2, SMC1, CHK1 and CHK2 [390]. In addition, a study has established that loss of HMGN3 leads to reduced acetylation and affects the transcription of several genes involved in glucose-stimulated insulin secretion, including that of the Glut2 glucose transporter in the pancreatic beta cells [381]. In agreement, the expression of HMGN3 is closely correlated with glucose homeostasis and its impaired function may lead to type-2 diabetes.

Despite HMGNs having a role in histone modifications, PTMs on HMGNs themselves are also crucial in influencing the accessibility of DNA and the regulatory networks. To date, acetylation and phosphorylation are the only PTMs identified on HMGN proteins. While acetylation has been observed at different residues Lys³⁰, -37, -41, -54, -58 and -60 *in vitro* and Lys², and -4 *in vivo*, phosphorylation has been detected at six residues which are Ser⁶, -20, and -24 inducible by specific stimuli and Ser⁸⁵, -88, and -98 not

related with any specific induction [397]. Up to now, the only enzymes established to acetylate HMGNs are PCAF and p300 [398]. These histone acetyltransferase proteins are not only involved in modulating histones or transcription factors but also modifying the structural non-histone HMGN proteins and thus impact on chromatin-related activities. Interestingly, the recombinant p300 acetylates both HMGN1 and HMGN2 at precise sites, whereas PCAF specifically acetylates HMGN2 but not HMGN1, even when HMGN1 is expressed at higher levels [399]. The biological function of such acetylation has been identified by equilibrium dialysis, in which acetylation reduces the affinity of HMGNs bound to nucleosome. Studies have indicated that p300 can acetylate free HMGN1 in the NBD, loosening the interaction of HMGN1 with nucleosomes and generating a pool of non-chromosomal-binding HMGNs [398].

Several kinases including PKA, PKC, cGK, CK2, RSK2, MSK1/2 are involved in phosphorylating HMGN1. PKA and cGK phosphorylate HMGN1 mainly on Ser⁶ and/or Ser²⁴; PKC phosphorylates HMGN1 on Ser²⁰ and/or Ser²⁴ in the NBD, RSK2 on Ser⁶ and CK2 on Ser⁸⁵, Ser⁸⁸ and Ser⁹⁸ within the RD [400]. *In vivo* studies show PKA phosphorylates HMGN1 on Ser⁶, MSK1/2 phosphorylate Ser⁶, Ser²⁰ and/or Ser²⁴ in stimulated mouse fibroblasts [390, 401, 402]. All these phosphorylation sites located within three functional domains of HMGN1, the N-terminal NLS, the NBD and the C-terminal RD. The biological significance of PTMs of HMGNs is linked to two primary contexts, the regulation of immediate-early (IE) gene activation and cell-cycle-specific regulation of the subcellular localisation. The IE genes (e.g. *c-fos*, *fosB*, *c-jun*, *junB*, *c-myc*, *junD*) are activated rapidly and transiently in response to signalling events without any requirement for new protein synthesis. Phosphorylation of HMGNs may affect subsequent PTMs on H3, thereby affecting the accessibility of kinases (i.e. MSK1/2) during IE gene (i.e. *fosB*) induction [390].

On the other hand, HMGN modifications also play a vital role in protein subcellular distribution during mitosis. One unique feature of the nucleus is that its chromosomes condensed and decondensed during mitosis. At the beginning of cell division, histones and other chromosome condensation factors remain associated with chromatin fibres, while transcription proteins and chromatin remodelling factors lose their association. Studies have observed that phosphorylation-induced heterochromatin protein 1 (HP1) displacement facilitates proper chromosome condensation and segregation at early mitotic stages [403, 404]. Likewise, it has also been observed that phosphorylation of HMGNs abolishes their nucleosome binding during mitosis [405]. After being displaced from chromosomes, HMGNs are restored in the nucleus, but the highly phosphorylated HMGN1s are retained in the cytoplasm [406, 407]. Furthermore, HMGN1 NBD contains recognition motifs for 14-3-3 protein binding, and phosphorylation of HMGN1 on Ser⁶, Ser²⁰ and Ser²⁴ enhances interactions with 14-3-3 proteins. This interaction is involved in maintaining phosphorylated HMGN1 outside of the nucleus, whereas only unphosphorylated HMGN1 can re-enter the nucleus after mitosis [407, 408]. Altogether, these findings suggest that interactions of HMGNs with nucleosomes are significantly reduced, if not entirely abrogated, during mitosis.

In conclusion, PTMs, such as acetylation and phosphorylation, on HMGN proteins have profound impacts on various cellular biological processes, involving not only in modifying chromatin accessibility for transcription activation but also in regulating proper chromatin conformation during cell division.

1.6.9 Role of HMGNs during development

The HMGN protein family play a key role in cellular differentiation and development, where the expression levels of HMGNs vary between different tissues and developmental stages. HMGN1 and HMGN2 proteins are strongly expressed during

embryogenesis, when their expression is highly regulated in a developmental stage-specific manner. In early-embryogenesis, HMGN1 and HMGN2 are detectable throughout oogenesis, and their transcripts accumulated beyond the two-cell stage. Transient depletion of HMGN1 and HMGN2 proteins in mouse oocytes delayed cell cleavage and the onset of the blastocyst stage [409]. Likewise, during bovine early-stage embryonic development, HMGN1 and HMGN2 are expressed in oocytes but are downregulated after fertilisation and almost disappear by the eight-cell stage [410]. Further studies with *in vitro* fertilisation experiments have demonstrated the incapability of HMGN2 downregulation can lead to failure of blastocyst development. It is thought to be due to altered chromatin remodelling which may be caused by hyperacetylation of H3 at Lys¹⁴ [411]. Moreover, in *Xenopus* development, researchers have found that the synthesis of HMGN1 and HMGN2 only starts in blastula stages and progresses throughout the neurula and tadpole embryos. Only after mid-blastula transition (MBT), overexpression of either HMGN1 or HMGN2 causes imperfectly closed blastopore, distorted body axis and abnormal head structures [412]. In addition, depletion of *Hmgn1* or *Hmgn2* resulted in similar developmental defects and re-introduced HMGN2 expression rescued these phenotypes. These observations imply the importance of proper regulation of HMGN1 and HMGN2 expression in embryogenesis.

Moreover, it has been observed *in vitro* that overexpression of either HMGN1 or HMGN2 inhibits myogenesis and chondrogenesis, suggesting that proper downregulation of HMGNs is required for normal cellular differentiation [413, 414]. Indeed, further experiments with mouse models have confirmed that HMGN1 and HMGN2 expression levels are downregulated during myogenesis, erythropoiesis, and chondrogenesis, as well as during embryonic organ formation, such as during kidney

development [413, 415-417]. Furthermore, the role of HMGNs in development has been studied in *Hmg1*^{-/-} mice. Results have revealed that these *Hmg1*^{-/-} mice appear normal during development but show abnormal phenotypes in response to stresses. In particular, *Hmg1*^{-/-} mice are hypersensitive to both UV and ionising radiation, implying an essential role of HMGNs in DNA repair, and the absence of HMGN1 significantly restricts the chromatin accessibility of DNA repair modulators [379, 418]. For example, it has been observed that Cockayne syndrome protein B (CSA) recruits HMGN1 to the RNA polymerase II-containing complex stalled at the UV damaged sites, in which HMGN1 is involved in transcription-coupled DNA repair [379]. Furthermore, HMGN1 has been demonstrated to be involved in affecting ATM activation by modifying the levels of histone modifications. The *Hmg1*^{-/-} mice and the cells derived from these mice show an enhanced tumorigenic potential in response to ionising radiation [396].

Another piece of evidence for the involvement of HMGNs in development has been observed in *Hmg1*^{-/-} mice. These mice display slight defects in the development of corneal epithelium [419]. The abnormal development of corneal epithelium might be mediated through altered expression of cell adhesion molecules and of p63, a key regulator of epithelial cell differentiation, through the absence of HMGN1 proteins. However, the phenotype of these *Hmg1*^{-/-} mice was not severe, which might be because of the functional redundancy between HMGN1 and HMGN2.

The other variants, such as HMGN3 and HMGN5, also exhibit distinct developmental and tissue-specific expression. In particular, HMGN3 has two splice variants, HMGN3a and HMGN3b, and the structure of HMGN3a is similar to HMGN1 and HMGN2, HMGN3b lacks C-terminal regulatory domain. However, their distribution and function have not displayed any differences. In mouse and human tissues, HMGN3 is

highly expressed in the eye and the brain [420, 421]. It is also detected in presumptive corneal epithelium and lens fibre during embryonic eye development as well as the lens fibre and inner nuclear layer of retina in the adult eye [422]. In addition, specific expression of HMGN3 has also observed in adult pancreatic islet cells [381]. Studies with *Hmgn3*^{-/-} mice have demonstrated that HMGN3 proteins are important in regulating the transcription of several genes, such as Glut2 and Kir6, in pancreatic-beta islet cells, and therefore may have a role in influencing glucose-dependent insulin secretion and thus diabetes pathogenesis. On the other hand, HMGN5 is detected in trophoblast giant cells, spongiotrophoblast and trophoblast cells located in the placenta labyrinth, implying the involvement of HMGN5 in trophoblast development and placenta formation [423]. Indeed, HMGN5 expression is upregulated during the Rcho-1 trophoblast stem cell line differentiation. Alterations in HMGN5 expression in Rcho-1 affect the differentiation-related expression of several prolactin-related genes, without affecting either the expression of their transcription factors or the morphology of the cell. Given the role of HMGNs in chromatin binding, the modulation of placental lactogen gene expression by HMGN5 might be mediated through inducing changes in chromatin structure. In addition, HMGN5 expression is also involved in the reproductive system development. It is known that exposure of neonatal mice to diethylstilbestrol (DES) and genistein (GEN) induces uterine adenocarcinoma in ageing mice and causes significant deficits in the sexual behaviour of male offspring [424, 425]. Further studies have demonstrated that such exposure also caused aberrant overexpression of HMGN5 in adult ovaries at 6 and 18 months, hence inferring the expression of HMGN5 is highly regulated during development.

In summary, the expression of HMGNs is developmentally regulated and alterations in the expression of these proteins can lead to developmental abnormalities. It is

possible that HMGN variants partially compensate for the loss of a specific variant and rescues severe defects; however, each HMGN member has specific function and distribution. Furthermore, loss of any HMGN variants shows significant effects on the ability of a cell to resist stress.

1.6.10 HMGN proteins and cancer

HMGN proteins modulate the chromatin structure and function through altering the levels of histone modifications and incorporating with other chromatin remodelling factors. DNA can be constantly damaged in exposure to the insulting agents generated from internal metabolic processes or external sources, such as ultraviolet light (UV) and ionising irradiation (IR). The damaged DNA influences gene transcription and replication, as well as promotes genetic mutations and chromosomal aberrations, which are the main causes leading to uncontrolled cell growth and eventually tumour formation. The chromatin-binding proteins, HMGNs, are found as a key participant in DNA repair mechanisms.

As described in earlier sections, 1.6.8 and 1.6.9, HMGNs play an important role in the cellular response to UV- and IR-induced DNA damage. Briefly, studies have established that loss of HMGN1 leads to hyper-UV sensitivity in *Hmgn1*^{-/-} mice, thus results in skin deformations such as acanthosis and hyperkeratosis [426]. This phenotype can be rescued by ectopic expression of HMGN1, which suggest the ability of HMGN1 in sustaining cell survival following UV exposure. Furthermore, this study has also demonstrated that ectopic expression of mutated forms of HMGN1 cannot bind to nucleosomes and thus unable to rescue the hypersensitive phenotype. Besides, recent studies have found that HMGN1 proteins are recruited to the sites of

transcription-coupled repair (TCR) [379]. The TCR and global genome nucleotide excision repair (GG-NER) are two sub-pathways of the NER process, which are activated by UV light and capable of repairing UV-induced DNA lesion. UV light induces genomic lesion which can impose a blockage on transcription. Once the RNA polymerase encounters a transcription-blocking lesion, the RNA polymerase cofactor, CSB, initiates the TCR pathway and recruits additional factors, such as p300, CSA, XAB2 and HMGN1, to eventually activate the repair machinery. Although the exact mechanism of how HMGN1 accelerates the repair of the DNA damage is not clear, the recruitment of HMGN1 to the damage sites suggests that it may have a direct role in DNA repair.

On the other hand, IR induced DNA lesions, including the double-stranded breaks (DSB), are hazardous insults for the genome. The cellular responses to IR include changes in the organisation of the chromatin both at the damage sites and globally. In the cellular DNA damage response to IR, the ternary protein complex MRN is initially recruited to the damaged sites, and then facilitates the recruitment of the key transducer of damage signal, ATM kinase. Among the local changes, HMGNs are found to be involved in de-condensation of the chromatin in the cellular response to IR. Studies have demonstrated that HMGN1 modules the ATM bound to chromatin which is essential for the activation of ATM-mediated cellular repair response [396]. Before DNA damage occurs, a sufficient amount of HMGN1 is required for proper nuclear organisation of ATM, during which HMGN1 may prevent incorrect retention of ATM to chromatin fibre [396, 427]. Once DSB is formed, there is a global HMGN1-mediated induction of H3K14 acetylation and subsequent transcriptional activation and gene expression. In the absence of HMGN1, the activation of ATM is reduced by 2- to 3-fold in *Hmg1*^{-/-} cells. To summarise, HMGNs, especially HMGN1, play an

important role in DNA repair machinery in response to UV and IR-induced genomic damage.

In addition to the global effects of HMGN1 in the cellular response to DNA lesions, HMGN1 also has roles in the transcriptional regulation of proto-oncogenes and tumour suppressor genes. In this manner, HMGN1 is thought to be closely related to cancer progression. Interestingly, it has found that HMGN proteins modulate the expression of only a small set of genes, which infers that HMGNs might target specific genes. Given that HMGN exhibits no binding preference to the underlying DNA sequences, it is unlikely that HMGNs regulate gene transcription through the direct recognition of specific DNA sequences. It is known that certain HMGN variants are recruited to specific gene promoter regions where they are incorporated into various multiprotein complexes, thereby affecting the structure of the chromatin fibre [376]. In this respect, they can be recruited to gene regulatory regions through direct interactions with transcription factors, such as ER α , SRF and PITX2 [380, 428]. Given that HMGN1 and HMGN3 interact with thyroid hormone receptor, and that HMGN1 and HMGN2 can interact with ER α , binding to specific transcription factors is likely a general property of all HMGN proteins [380, 429]. The advantage of such protein-protein interactions between HMGNs and transcription factors could be related to the higher affinity and stability binding to specific promoter regions, in contrast to the dynamic low-affinity binding of HMGNs to chromatin. Consequently, HMGNs can either specifically activates or represses the expression of particular genes, in rapid response to specific stimuli.

Estrogens control multiple physiological processes and have been implicated in several diseases, especially breast cancer. It is well known that high levels of serum estrogens correlate with an increased risk of breast cancer [430]. Furthermore,

emerging data revealed that ER α is closely associated with the formation and/or progression of breast cancer. In MCF-7 breast cancer cell line, cell proliferation and increased tumour formation only occur when ER α is present [431]. HMGN1 has been found to reduce ER α -dependent transcription in the MCF-7 cell line, through its ability to interact with the ER α and SRF, thus affecting subsequent transcription activities [380]. In this respect, HMGN1 is involved in suppressing breast cancer progression. In addition, HMGN2 has been observed to be involved in inhibiting the DNA binding ability of PITX2 through direct interacting with the PITX2 homeodomain. PITX2 is a transcriptional activator which plays a major role in the development of eye, tooth and abdominal organs during embryogenesis; as well as regulating the transcription of hormone-regulated activity of prolactin. Emerging evidence suggests that aberrant upregulation of PITX2 may be associated with tumour progression [432]. Research has demonstrated that HMGN2 forms an inhibitory complex with PITX2 to limit its transcriptional activity. This inhibitory mechanism can be activated by canonical Wnt/ β -catenin signalling upon stimulation. Therefore, this evidence supports the idea that HMGN proteins involved in regulating cancer-related genes and possibly in inhibiting cancer progression.

Furthermore, HMGN-modulated chromatin remodelling can lead to transcriptional activation or repression under different circumstances [380, 395, 428]. In particular, it is shown that HMGN1 binds to the promoter region of the proto-oncogene *fosB* to repress its expression by inhibiting H3S10 phosphorylation [390]. In the other studies with *Hmgn1*^{-/-} cells, the proto-oncogenes and pro-metastatic genes, such as *c-fos*, *BCL3* and *N-cadherin*, are found to be upregulated [390]. In agreement with these results, the pro-apoptotic gene *NGFR* is downregulated in *Hmgn1*^{-/-} cells. On the other

hand, some other proto-oncogenes, such as *JunB* and *c-Jun*, are downregulated in the absence of HMGN1 [390].

Taken together, HMGN1 represses cell proliferation and cancer development through regulating the transcription of multiple cancer-related genes [428]. Indeed, *Hmgn1*^{-/-} mice have developed 2-fold more spontaneous tumours compared to their *Hmgn1*^{+/+} littermates. The cells derived from *Hmgn1*^{-/-} mice tend to proliferate much faster than the normal cells and enter senescence stage after more passages. Significantly, nude mice develop 5.6 times more tumours when injecting of *Hmgn1*^{-/-} cells than transformed *Hmgn1*^{+/+} cells [418]. In agreement, HMGN1 expression is reduced by 2-fold in highly metastatic breast cancer cells compared to the low metastatic cell lines [433]. In summary, HMGN proteins seem to be important for repression of cancer progression through repairing DNA lesions and regulating transcription of cancer-related genes.

	HMGA	HMGN
Subfamily	HMGA1a, HMGA1b, HMGA1c, HMGA2	HMGN1, HMGN2, HMGN3a, HMGN3b, HMGN4, HMGN5
Functional motif	AT-hook: binds to the minor groove of short stretches of AT-rich β form DNA	Nucleosomal binding domain with conserved motif 'RRSARLSA': binds to the 147-base pair nucleosome core particle
Biological function	- displace Histone H1 and decondense chromatin - stabilise inter-protein interactions of enhanceosome and induce transcription activation - interact with transcription factors and induce changes in their DNA binding affinities - as accessory factor for the integration of viral cDNA into host DNA	- highly mobile in a 'atop-and-go' manner - interact with histone subunits H2A/B, H3, H4 and diminish H1 stabilization to decondense chromatin fibres - interact with other proteins into metastable multiprotein complexes in the nucleus - Posttranslational modifications on HMGNs affect chromatin conformation during mitosis
Expression during development	Express at high level during embryogenesis and deficient in adult tissues	Highly regulated in a stage-dependent manner
Role in cancer	- tumour expansion and progression - epithelial-mesenchymal transition - promote metastatic progression - stem-like and cancer stem cell properties - initiate and maintain cellular senescence - impact DNA repair mechanisms in a cellular-dependent context	- enhance DNA repair response to resist UV- and IR- induced DNA damage - affect transcription of proto-oncogenic gene transcription and tumour suppressor genes

Table 1. 2 Summary of differences between HMGA and HMGN proteins

1.7 Aims

FOXM1 is a master regulator that associated with tumour initiation, progression, metastasis, angiogenesis and drug sensitivity in breast cancer. Besides, HMG proteins, especially HMGA1 and HMGN1, possess similar roles in different types of cancer. Moreover, The PIPs database has defined the potential interactions between FOXM1 and HMG proteins, but there was no further experimental evidence developed. In the project, I aimed to determine the links between FOXM1, HMGA1 and HMGN1, and the effects of their interactions on the regulation of FOXM1 activities. In this respect, *KIF20A* and *SQSTM1/p62*, as two putative FOXM1 transcriptional targets, were selected to examine for FOXM1 transcriptional activities. Moreover, the functions of these targets were further identified. In particular, the role of p62 in tumour-induced angiogenesis and cell migration were explored in the zebrafish model. Furthermore, KIF20A inhibitor (BKS0349 compound) was used to investigate the role of KIF20A in terms of drug response, cell migration and invasion, and stemness.

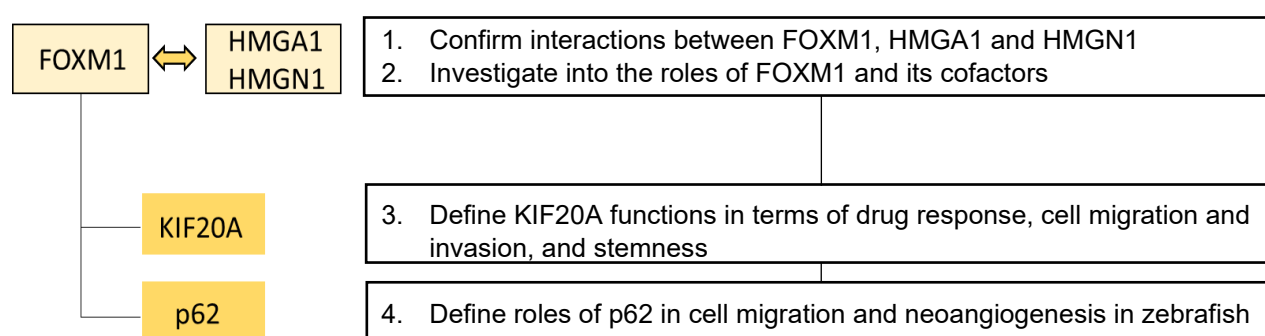


Figure 1. 5 Flow-chart of research aims.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Cell lines

Human breast cancer cell line MCF-7 was acquired from the Cell Culture Service, Cancer Research UK, where it was verified and authenticated. All the cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) and supplemented with 10% foetal calf serum (FCS, Gibco), 100 units/ mL penicillin/streptomycin (Sigma-Aldrich), 2 mM L-glutamine, under 10% CO₂ humidified atmosphere at a constant temperature of 37 °C. The paclitaxel resistant MCF-7 (MCF-7 TaxR) and epirubicin resistant MCF-7 (MCF-7 EpiR) cells were previously established in Professor Eric Lam's laboratory by chronic exposure of the parental MCF-7 cells to stepwise increased concentrations of corresponding drug until they acquired resistance to 0.05 µM paclitaxel and 10 µM epirubicin, respectively [434].

2.1.2 Cell maintenance

Adherent cells were cultured and passaged at approximately 80% confluency (80% of the flask surface covered by cell monolayer). To subculture, the culture medium was aspirated from the flask and the cells were gently washed once with sterile 1× PBS (Phosphate Buffered Saline) to get rid of the residual culture media. Cells were detached by incubation with 1× trypsin diluted in EDTA (Sigma-Aldrich, UK) at 37 °C. After detached, the cells were then resuspended in the completed media to inactivate the trypsin. An appropriate amount of cells was transferred into the new flask or the cell culture dishes.

2.1.3 Cell storage

For long term maintenance, the healthy and low passage cells were trypsinised, as described above, resuspended in culture media and transferred into a sterile Falcon tube. The cells were collected by centrifuging at $300\times g$ for 5 min. The cell pellet was then resuspended in 1 mL of a solution containing 10% di-methyl sulfoxide (DMSO) in FCS (Sigma- Aldrich, UK), and then transferred into suitable cryotubes. The vials were placed into the isopropanol freezing box (VWR International, Lutterworth, UK) at -80°C overnight in advance of liquid nitrogen storage. When defrosting cells, the cryotubes were defrosted in a water bath maintained at 37°C for a short period of time (approximately 1-2 min), and the defrosted solution was added to 1 ml completed media and spun at $300\times g$ for 5 min. The DMSO containing supernatant was removed and the pellet resuspended in fresh supplemented media and pipetted into an appropriate flask for recovering and proliferation.

2.2 Drug treatment

Paclitaxel (6 mg/mL) (Teva UK Limited, East Sussex, UK) and Epirubicin (2 mg/mL) (Medac, Germany) were obtained from Imperial College Healthcare, UK. Both chemotherapeutic agents were stored at 4°C . BKS0349 (Biokinesis, Paris, France) was diluted in 100% DMSO and stored at -20°C with protection from light. The stock solution was aliquoted to avoid freeze/thaw steps.

2.3 Plasmid Constructs

2.3.1 Plasmids

The pcDNA3-FOXM1 expression plasmid has been previously described [435, 436]. The pFN21A-HMGA1-Halotag (FHC08399) and pFN21A-HMGN1-Halotag (FHC07825) were generated and purchased from the Kazusa Genome Technologies Inc., Chiba, Japan [437].

2.3.2 Plasmid Miniprep and Maxiprep

The Miniprep Plasmid Purification Kit (Qiagen) was used for small-scale DNA preparation. For isolation a large amount of plasmid DNA, Maxiprep Plasmid Purification Kit (Qiagen) was used and the manufacturer's instructions were followed.

2.4 Transfection

2.4.1 Gene Silencing with Small Interfering RNAs (siRNA)

Transient transfection was performed with siRNA SMARTpool reagents purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA) via oligofectamine transfection (Invitrogen) according to the manufacturer's instruction. All siRNA used in this study were ON-TARGET^{plus} SMARTPool siRNA, which comprised a mixture of four distinct siRNA species against the target gene. This provided up to 90% reduced off-target effects compared to single siRNA. All the siRNAs were diluted to 20 μ M in 1 $\times$ siRNA buffer and stored at -20 °C. One day before transfection, cells were counted and plated in 6-well plate so that they were 50-60% confluent the day of transfection. Once the cells reached the desired confluency, the oligo solution was prepared on the day for transfection. For each well, 5 μ L oligofectamine (Invitrogen, UK) was diluted

with 60 μ L Opti-MEM (Life Technology, UK), mixed gently and incubated at room temperature for 5 min. The siRNA solution was then prepared by adding 7.5 μ L of 20 μ M siRNA stock and 7.5 μ L of 1 $\times$ siRNA in 235 μ L of Opti-MEM. The diluted oligofectamine solution was then combined with the siRNA solution, and vortexed briefly before incubated for 20 min at room temperature. This allowed the siRNA/oligofectamine complexes to form. Opti-MEM was added to make final volume of 500 μ L. Before the oligofectamine/siRNA mixture added, the culture media was removed and the cells washed once with 1 $\times$ PBS. After the mixture being added, the plate was returned to incubate at 37°C. 4-6 h post transfection, the completed media was added to each well. The cells were incubated at 37°C in a CO₂ incubator for 24-72 h until they are ready to assay for gene knockdown.

The SMARTPool siRNA used were FOXM1 siRNA (L-009762-00-0005), KIF20A siRNA (L-004957-00-0005), HMGA1 siRNA (L-004597-00-0005), HMGN1 siRNA (L-015567-00-0005) and the NS control siRNA (D-001810-10-05), which have minimal targeting of known genes.

2.4.2 FuGENE 6 transfection

The cells were transfected with DNA plasmids using the FuGENE 6 transfection reagent (Roche Diagnostics, West Sussex, UK) at a ratio of 1:3 (1 μ g of DNA plasmid: 3 μ L of FuGENE 6). One day before the transfection experiment, cells were seeded with optimized cell concentrations and plated overnight to achieve the desired density of 50%-60% confluency at the time of transfection. FuGENE 6 was diluted in serum-free DMEM medium (Sigma-Aldrich, Poole, UK) and incubated for 5 min at room temperature. One μ g of DNA plasmids were added into the diluted FuGENE 6,

vortexed for one second and incubated for 15-45 min at room temperature to allow the DNA/FuGENE 6 complex to form. The DNA/FuGENE 6 complex solution was then added into the cells in a drop-wise manner. Cells were then incubated at 37 °C in a CO₂ incubator for 24-48 h until they were ready to be assayed for gene expression.

2.5 Western blotting

2.5.1 Protein extraction and quantification

Cells were trypsinised and collected in eppendorf tubes. The cell pellets were lysed in lysis buffer containing 1% (v/v) Nonidet P40, 50 mM Tris-HCL (pH7.4), 0.5 mM EDTA, 150 mM NaCl, 50 mM HEPES, 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na₃VO₄), 2 mM phenylmethysulfonyl fluoride (PMSF), 100 mM N-Ethylmaleimide (NEM) and complete protease inhibitors mixture (Roche Applied Science), and kept on ice for 10 min. Vortexing and pipetting were performed throughout to facilitate the lysis. The lysed cell pellets were then centrifuged at 800 ×g for 10 min at 4 °C. Supernatants were collected in a new eppendorf tube and they concentrations determined using the Pirce BCA protein assay according to manufacturer's protocol (Thermo Fisher Scientific, UK). Two µl of protein solution were combined to 200 µl of a mixture of reagent A and B (1:50 = B:A ratio). After 30 min incubation at 37°C, absorbance was read at 526 nm using the Sunrise spectrophotometer (Tecan, Reading, UK). The protein concentrations of each sample were then determined using the absorbance readings multiplied by the values of the standardized regression coefficient, which were established by assaying 5 dilutions of protein standard within the range of 0.2 mg/ml to 1.5 mg/ml protein.

2.5.2 Subcellular Fractionation

Subcellular fractionation was performed according to the manufacture's protocol using NER-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, UK). The cell pellets were resuspended in 200 μ l of the ice-cold CER I solution with protease inhibitors, followed by vigorous vortexing for 15 s to fully suspend the cell pellets. After incubation on ice for 10 min, 11 μ l CER II was added and vortexed for 5 s at maximum speed and incubated on ice for 1 min. The samples were then vortexed for another 5 s before centrifuged at 13,000 $\times g$ for 5 min. Supernatants (cytoplasmic fractions) were collected and transferred to the new pre-chilled microcentrifuge tubes. The insoluble fraction was mixed with 100 μ l ice-cold NER solution with protease inhibitors. The samples were then vortexed for 15 s for every 10 min over a period of 40 min. At the end, the tubes were centrifuged at maximum speed (13,000 $\times g$, 4 $^{\circ}$ C) for 10 min before the supernatants (nuclear fraction) were collected in the new microcentrifuge tubes. Both the cytoplasmic and nuclear fractions were stored in the -80 $^{\circ}$ C freezer until further use.

2.5.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Western blotting was used to detect protein expression. Proteins were separated according to their molecular weights using SDS-PAGE. The SDS-PAGE gel was made of two different layers, an upper stacking gel layer and a lower running layer. The resolving gel was made with varying amounts of bis-acrylamide solution of which ranged from 5% to 14%. A lower percentage of gels allowed better visualisation of proteins of higher molecular weights. The bis-acrylamide with ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) were the catalysts for gel

polymerisation. Table 2.1 summarises the components of the different percentages of gels.

Reagents	Resolving gel					Stacking gel
	5%	7%	10%	12%	14%	5%
dH ₂ O-mL	6.13	5.02	4.02	3.35	2.68	3.67
1.5 M Tris pH 8.8-mL	2.5	2.5	2.5	2.5	2.5	—
1.5 M Tris pH 6.8-mL	—	—	—	—	—	0.42
30% Acrylamide-mL	1.16	2.33	3.33	4.00	4.67	0.83
10% SDS- μ L	100	100	100	100	100	50
25% APS- μ L	40	40	40	40	40	20
TEMED- μ L	10	10	10	10	10	10
Total-mL	10	10	10	10	10	5

Table 2. 1 Composition of resolving and stacking gel.

To prepare protein samples, 20 μ g of protein extracts were mixed with 6 $\times$ SDS protein dye (0.375 M Tris pH 6.8, 12 % SDS, 60 % glycerol, 0.6 M DTT, 0.06 % bromophenol blue), heated at 100 °C for 10 min. Samples were then loaded to SDS-PAGE gels, alongside with the pre-stained protein standard (Invitrogen, Paisley, UK) to recognise the molecular weights of the separated proteins. The gels were placed in running buffer (25 mM Tris-Base, 109 mM Glycine, and 0.1 % (w/v) SDS) and run at a constant voltage of 90 volts. After electrophoresis, the proteins were then electro-transferred onto the nitrocellulose membranes (Bio-Rad, Hemel Hempstead, UK) using a wet tank blotting system (Bio-Rad Laboratories Trans-Blot Cell) in transfer buffer at 90 volts for 90 min at room temperature. The transfer buffer contained 25 M Tris-Base, 190 mM glycine and 20 % (v/v) ethanol.

2.5.4 Immunoblotting

The membranes were blocked in 5% bovine serum albumin (BSA) in Tris buffered solution with 0.05% Tween-20 (TBST, pH 7.5) for 30 min at RT and then incubated with specific primary antibodies overnight at 4°C. The following day, membranes were

washed three times with TBST over 15 min. Secondary antibodies obtained from (DAKO, Ely, UK) were diluted 1:2000 in TBST and incubated with the membranes on a slow shaker for 45 min at room temperature. Subsequently the membranes were washed five times with TBST for at least 2 h. Immunoblots were visualised using Western Lightning® ECL detection system (chemiluminescence peroxidase substrate) according to the manufacturer's instructions (Amersham Biosciences, Pollards Woods, UK) and the signals detected using Hyperfilm ECL (GE healthcare) on SRX-101A X-ray developer or using the chemiluminescence imager (Fusion Solo S).

2.5.5 Antibodies

Table 2.2 below shows all the antibodies used in this study, with the dilutions used. FOXM1 (C-20) was discontinued and replaced by FOXM1 (G-5) by late 2017. Since then, all the experiments were performed using the other two FOXM1 antibodies in different circumstances. Both FOXM1 (G-5) and FOXM1 (NBP1) were used in Duolink PLA assay, and FOXM1 (NBP1) for Chromatin immunoprecipitation (ChIP).

Antibody	Catalogue #	Company	Species	Dilution
FOXM1	c-20/sc-502	Santa Cruz	Rabbit	1:1000
FOXM1	G-5/sc-376471	Santa Cruz	Mouse	1:1000
FOXM1	NBP1-30961	Novus Biologicals	Rabbit	1:1000
KIF20A	ab104118	Abcam	Rabbit	1:1000
KIF20A	D-3/sc-374087	Santa Cruz	Mouse	1:1000
p62/SQSTM1	PA5-27247	ThermoFisher	Rabbit	1:2000
HMGA1	Ab168260	Abcam	Mouse	1:500
HMGN1	5692s	Cell Signalling	Rabbit	1:1000
E-cadherin	3195s	Cell Signalling	Rabbit	1:2000
N-cadherin	14215s	Cell Signalling	Mouse	1:1000
Vimentin	5741s	Cell Signalling	Rabbit	1:1000
β-tubulin	2146s	Cell Signalling	Rabbit	1:1000
Lamin B	119D5	Abcam	Mouse	1:1000
Rabbit HRP	P0448	Dako	Goat	1:2000
Mouse HRP	P0447	Dako	Goat	1:2000

Table 2. 2 List of antibodies used for Western blotting.

2.6 Real-time quantitative PCR (RT-qPCR)

2.6.1 RNA extraction and quantification

Total RNA was extracted from cell pellets using the RNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's instruction. RNA purity and concentration were determined by measuring the spectrophotometric absorbance at 260 nm and 280 nm with an ND-1000 NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware USA).

2.6.2 Complementary DNA (cDNA) synthesis

RNA was reverse transcribed into first strand cDNA using SuperScript III First-Strand Synthesis System (Life technologies, UK). Briefly, 2 µg of RNA was mixed with 1 µL of 50 µM oligo (dT)₂₀ primer, 1 µL of 10 mM dNTPs mix and DEPC-treated water up to 13 µL. Samples were denatured at 65 °C for 5 min and then placed on ice for 1 min. Subsequently, 6 µL of cDNA synthesis solution, containing 1 µL of the reverse transcriptase superscript III (200U/ µL), 1 µL of 0.1 M DTT, 1 µL of RNaseOUT recombinant inhibitor and 4 µL of 1× first strand buffer, was added to the denatured RNA. It then mixed gently and centrifuged briefly to collect the liquid at the bottom of the PCR tube. All the cDNA synthesis reagents were obtained from Life technologies, UK. The PCR tube was placed into a thermocycler and ran the following programme: incubated at 25 °C for 10 min at the initial step, then 50 min at 50°C for the reverse transcription step, and 70 °C for 15 min to inactivate the reverse transcription enzyme. The cDNA mixture was diluted 1:4 with DEPC-treated water and used immediately for RT-qPCR or stored at -20 °C.

2.6.3 Primer design and optimization

All the primers used were designed using Primer Express 3 software (Applied Biosystems) and purchased from Life technologies, UK.

Target gene	5' - Forward -3'	5' -Reverse – 3'
<i>L19</i>	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
<i>FOX M1</i>	TGCAGCTAGGGATGTGAATCTTC	GGAGCCCAGTCCATCAGAACT
<i>KIF20A</i>	TAACAAGGGCCTAACCCTCA	TGCTCTGTCGTCTCTACCTCC
<i>p62</i>	CTGGGACTGAGAAGGCTCAC	GCAGCTGATGGTTTGAAAT
<i>HMGA1</i>	GGAAGATGAGTGAGTCGAGCTCGAAG	TGGGTCACTGCTCCTCCTCCG
<i>HMG N1</i>	AACCTCCTGCAAAAGTGGAA	CATCAGAGGCTGGACTCTCC

Table 2. 3 List of human RT-qPCR primers used in this study

2.6.4 Real time quantitative PCR (RT-qPCR)

RT-qPCR analysis was carried out on either StepOnePlus (Life technologies, UK) or 7900HT Fast (Applied BioSystem, UK), using PowerUp SYBR Green Master Mix (Life technologies, UK). SYBR Green is a DNA binding dye that intercalates tightly to double-stranded DNA. This intercalation induces SYBR fluorescence which is detected by the Real time PCR system. The machine reads the fluorescence and calculates Ct values from the intensity of the fluorescence. Transcript levels were quantified using the relative standard curve method. All analyses were carried out in triplicates, and the target gene expression levels were normalized to a control mRNA level of ribosomal protein L19.

Briefly, a mixture containing an optimised concentration of gene-specific forward and reverse primer pairs with 5 µL PowerUp SYBR Green Master mix was added to 1 µL

(50 ng) of the cDNA. For each reaction, DEPC-treated water was added to adjust the final volume to 10 μ L. Transcript levels were quantified using the relative standard curve method. The standard curve was made by mixing 5 μ L from each cDNA sample into a single tube and diluted 1:4 for serial dilutions. The standard dilution included 1, 1:4, 1:16, 1:64, 1:256 and NTC (negative control which only contains DEPC-treated water). The PCR program was set to 95 °C for 10 min for the enzyme activation, followed by 40 cycles of denaturing at 95 °C for 3 s, and primer annealing at 55 °C for 30 s. After the Real-time qPCR run, dissociation curves were generated to confirm the absence of non-specific amplification.

2.7 Co-immunoprecipitation

Immunoprecipitation (IP) is used to enrich or purify a specific protein from a complex, while Co-IP is used to detect protein-protein interactions. The detailed protocol was previously described by my colleagues and myself in the 'Methods in Molecular Biology' book series [438]. Briefly, the lysates were added to the Dynabeads (Life Technologies, UK) and incubated for 4- 6 h at 4 °C under rotary agitation. Dynabeads A was used for rabbit primary antibody and beads G were used for mouse primary antibody. Following pre-cleaning, the lysates were transferred to the new microcentrifuge tubes with fresh Dynabeads A/G, while using DynaMag magnetic rack (Life Technologies, UK). For each sample, the lysate was separated into 3 volumes (input, IgG control and IP at a ratio of 1:10:10). Two to five hundred μ g of cell lysates were used for each IP sample and equivalent amounts were used per IgG control. Specific antibodies (0.5-2 μ g) were then added to the lysate-beads mixture, and incubated together overnight at 4°C under rotary agitation. The following day, the supernatants were removed, and the beads washed with 1 $\times$ PBS for 3 times to wash

off the unbound proteins. The bound proteins were eluted from Dynabeads under denaturing conditions (samples were mixed with 2 × SDS loading dye and boiled for 5 mins at 100 °C), and kept in the new microcentrifuge tubes at -20 °C till the time of SDS-PAGE gel separation.

2.8 Duolink proximity ligation assay (PLA)

This assay is used to detect and quantify protein expression, protein interaction and specific posttranslational protein modification. In the case of detecting the protein-protein interactions, two primary antibodies are required to be raised from different species. The PLA oligonucleotides- conjugated secondary antibodies (anti-rabbit PLA MINUS and anti-mouse PLA PLUS) bind to the corresponding primary antibodies. Only when the two PLA probes are in close proximity (≤ 40 nm), the oligonucleotides are hybridised and combined to a closed circle. The ligated circles as templates are then amplified and subsequently generated several-hundredfold repeated sequence products. Since the amplification solution consists of fluorescent labelled oligonucleotides, the replicated product can be visualised as a distinct fluorescent spot when observed with fluorescence microscopy.

Cells were cultured on chamber culture slides, fixed with 4% paraformaldehyde (Thermo Fisher Scientific) for 10 min at 37°C and permeabilised with PBS containing 0.1% [v/v] Triton X-100 for 10 minutes at room temperature. The samples were then blocked and incubated with respective antibodies (FOX M1-G5, FOX M1-NBP1, HMGA1 and HMGN1, see *table 2.2*), with Duolink in Situ Kit (Sigma-Aldrich) according to the manufacturer's instructions. The detailed step-by-step protocol also has been defined in the 'Methods in Molecular Biology' book series [438]. In the end, cells were

stained with DAPI and mounted with Vectashield mounting solution (Vector Laboratories). Images were captured with LAS-AF software using a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) with a 63× oil immersion objective.

2.9 Chromatin immunoprecipitation (ChIP)

The chromatin immunoprecipitation (ChIP) is used to evaluate the interactions between proteins and DNA in the cells. This assay enables an analysis of the association of regulatory molecules to specific genomic regions.

2.9.1 Cell harvest and lysis

Cells were grown to 90-95% confluency (150 mm dish) and cross-linked with 1% formaldehyde (1% FA in fixation buffer: 50mM HEPES-KOH pH 7.5, 100mM NaCl, 1mM EDTA, 0.5mM EGTA) for 10 min at room temperature, 2.5 mM of glycine was added for 5 min to quench cross-linking. Following three times washes with PBS, cells were then scraped using scrapping buffer (100 mM Tris-HCl (pH 9,4) and 0.1% SDS) and collected in microcentrifuge tubes. The pellets obtained were then washed with PBS, Buffer I [0.25% Triton x-100, 10mM EDTA, 0.5 mM Ethylene Glycol Tetraacetic Acid (EGTA) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 6.5)] and Buffer II [200 mM NaCl, 10 mM EDTA, 0.5 mM EGTA and 10 mM HEPES (pH 6.5)], all with 1× proteinase inhibitor. Five minute-centrifugation of 3000 ×g at 4 °C was performed after each wash. The final cell pellets were suspended in lysis buffer (50mM HEPES-KOH pH 7.5, 140mM NaCl, 1mM EDTA, 10% Glycerol, 0.5% Igepal CA-630, 0.25% Triton X-100) and incubated for 10 minutes on ice.

2.9.2 Beads preparation, sonication and chromatin immunoprecipitation

To prepare the Dynabeads, beads A/G were washed with TSE I buffer (0.1% w/v SDS, 1% v/v Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8,1) and 150 mM NaCl) three times. One μg of corresponding antibodies (FOX M1, HMGN1, HMGA1 and IgG control), was added to the beads and buffer D [1% Triton x-100, 2 mM EDTA, 20 mM Tris-HCl (pH 9,1) and 150 mM NaCl], and the mixture of antibody-beads-buffer D then rotated overnight at 4°C.

DNA was fragmented using the Bioruptor (Diagenode, Denville, USA) with the setting 4 cycles with 5 min duration. After sonication, the lysates were centrifuged at 13,000 $\times g$ at 4 °C. Fifty μl of each protein sample was taken and used as input samples. The rest of supernatants were transferred to the pre-prepared mixture of antibody-beads-buffer D, and rotated at 4°C overnight.

2.9.3 Purification of ChIP DNA

The following day, the supernatants were removed and the Dynabeads washed with RIPA buffer (50mM HEPES-KOH pH 7.5, 500mM LiCl, 1mM EDTA, 1% Igepal CA-630, 0.7% sodium deoxycholate) for 5 times on a rotating platform (10 min for each wash), followed by the last wash with cold TBS (20mM Tris-HCL pH 7.6, 150mM NaCl). Then, 100 μl of elution buffer (50mM Tri-HCl pH 8.0, 10 mM EDTA, 1% SDS) was added to the beads and the mixture rotated at room temperature for 1 h. The supernatants were transferred to the new microcentrifuge tubes, and the beads taken to rotated with another 100 μl of elution buffer. After 1 h, the supernatants were combined with previous eluted ones. The eluted samples were incubated at 65 °C for 6-8 h to reverse the crosslink. The DNA samples were then purified using the PCR purification kit (Qiagen), according to the manufacturer's instruction. In the end, the

input samples were diluted to 0.1% with elution buffer before the real-time qPCR was performed. Data were presented as % Input using the formula: % Input = $100 \times 2^{\Delta}$ (CT adjusted Input sample – CT immunoprecipitated sample).

Target gene	5' - Forward -3'	5' -Reverse – 3'
<i>KIF20A</i> Set1	GCTGCAGGCTTCCCTATCTG	TACTCACACCTAGTCGCCGA
<i>KIF20A</i> Set2	TTCCTTACGCGGATTGGTAG	AGCCGCAGAGCACAACCTC
<i>p62</i> Set1	CTCCGCGTTCGCTACAAAAG	CGTCCTCCTTGCCCAGAAG
<i>p62</i> Set2	CTCAGGGGACTCACGGTGA	GTGAGTCAGCGTCCCATGAC
<i>HMGA1</i> Set1	CTCCAAGAAGGCGTGAGTTC	CCGCGGGGTATTTATGTGT
<i>HMGA1</i> Set2	CTGGGTCGCTCTTTTAAAGC	CGCGGCCTCAATAAATAAAT
<i>HMGA1</i> Set3	GAGGGGGCTTTCTTCCTCT	GGGTGACTCTGCAAGTGTCTG
<i>HMGA1</i> Set4	AGGGGGCTTTCTTCCTCTC	GTGACTCTGCAAGTGTCTGC
<i>HMGN1</i> Set1	ATAGCCTTTCTTGCCGACAG	CCACCCACAATTCTTCTGGA
<i>HMGN1</i> Set2	CCCCTGGATAGCCTTTCTTG	CCTTCAGGCCACTGGTATCT
<i>HMGN1</i> Set3	ACGACACAACGAGACCCTGT	AGACTGGGAAGGGAGAAGGA
<i>HMGN1</i> Set4	ACGACACAACGAGACCCTGT	GACTGGGAAGGGAGAAGGAT
<i>HMGN1</i> Set5	GGTCCGGGATCAATTAAGG	GGGAGAACCGGATGGAAC
<i>HMGN1</i> Set6	GGTCCGGGATCAATTAAGG	GGAGAACCGGATGGAACC
<i>Cyclin B1</i>	CGCGATCGCCCTGGAAACGCA	CCCAGCAGAAACCAACAGCCGT
<i>Actin</i>	AGCGCGGCTACAGCTTCA	CGTAGCACAGCTTCTCCTTAATGT
<i>PLK1</i>	CGTCCGTGTCAATCAGGTTT	GCTGGGAACGTTACAAAAGC
<i>Cyclin D1</i>	TGCCACACACCAGTGACTTT	ACAGCCAGAAGCTCCAAAAA

Table 2. 4 List of primer sequences used for quantitative real-time PCR following ChIP.

*CCNB1, ACTB and CCND1 control primer sequences are obtained from Sanders' publication [439]. Others were designed by using the IGV (interactive genome viewer) data [440].

2.10 Sulphorhodamine B (SRB) assay

The sulphorhodamine B (SRB) assay was used to measure drug-induced cytotoxicity and cell survival. SRB, is an anionic aminoxanthene dye that forms the electrostatic complex with the amino acid residues of proteins under moderately acidic conditions. The cell density is achieved through the stoichiometric SRB binding, in which the amount of dye extracted from stained cells is proportional to the total protein mass [441]. Approximately 3,000 cells were plated in each well of the 96-well plates (Falcon, USA). After 24 h culture, cells were treated with different concentration of drugs for 0, 24, 48 and 72 h. After the appropriate incubation period, the monolayer of cells was fixed with 100 μ L of 40% (w/v) trichloroacetic acid (TCA) and incubated for 1 h at 4°C. The plates were then rinsed with water before staining with 0.4% SRB solution (0.4% SRB in 1% acetic acid) for 1 h at room temperature. The excess dye is removed by washing with 1% (v/v) acetic acid and the plated were left to dry overnight. The SRB dye was dissolved in 10 M-Tris base solution for 30 min at room temperature with agitation. Absorbance was measured at 492 nm using a Sunrise spectrophotometer (Tecan, Reading, UK). The results were normalized to 0 h or untreated wells.

2.11 Clonogenic assay

The clonogenic assay is long-term *in vitro* cell survival assay used to examine the effectiveness of cytotoxic agents on the ability of the cell colony formation. Cells were seeded in 6-well plates at approximately 2,000 cells/well. The following day, cells were treated with different concentrations of drugs (paclitaxel, epirubicin or BKS0349 component) for 72 h. The original culture was removed and replaced with fresh completed media with the corresponding drugs. The surviving cells were left to form

colonies and stored in the incubator for 14 days. Fresh completed media were replaced every 72 h. After 14 days of treatment, cell colonies were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by rinsing with PBS for three times. The fixed cells were then stained using 0.5% crystal violet in PBS solution for visualisation. After 30 min staining, the excess crystal violet was removed, and the plates washed with water and left to dry overnight. Once the images of each plate were captured, the crystal violet stained colonies were solubilised with 1 ml 33% (w/v) acetic acid for 30 min while shaking. The quantification of the colony intensity was obtained by measuring the absorbance of the dye/acetic acid solution at 592 nm with the Tecan microplate reader (Tecan, Reading, UK).

2.12 Transwell migration assay

For the migration assay, cells were collected and suspended at a density of 2×10^4 cells in 200 μ L of the serum-free medium after transfection for 24 h, and seeded in upper chamber of the 6.5 mm transwell plate (Corning Inc.). Then, 500 μ L of complete medium containing 10% FBS was added into the lower compartment of the chamber. After incubation for 48 h, the chambers were removed and the cells on the upper surface of membrane wiped off with cotton swabs. Then, the cells that had migrated into the microporous membrane (the reverse side of the membrane) were washed three times with PBS, followed by fixation with 4% paraformaldehyde solution for 20 min at room temperature. The membranes were then cut off carefully and placed on the slide for 4,6-diamidino-2-phenyl indole (DAPI) staining. Finally, the cells were observed with a fluorescent microscope (EVOS), and images recorded. Cell counting was achieved using Image J particle analysis.

2.13 Transwell invasion assay

In tumour cell invasion assay, transwell chambers with 8.0 µm pores were obtained from Corning. A density of 1×10^4 cells was placed on the upper surface of membrane which was pre-coated with a 60 µL Matrigel layer. Subsequent procedures were similar to the migration assay and cells were incubated for 4 days.

2.14 Mammosphere formation assay

Five-hundred cells were plated with 1ml of mammosphere media per well in 24-well ultra-low attachment plates (Costar, USA). The mammosphere media consisted of phenol red-free serum-free minimal essential media, supplemented with B27 (Invitrogen), 20ng/ml human basic fibroblast growth factor (Invitrogen) and 20ng/ml human epidermal growth factor (Invitrogen). The media also contained 20 nM of BKS0349 or without drug treatment as a control. The plates were incubated in a humidified atmosphere at 37°C and 10% CO₂ for 7 days, after which the number of mammospheres developed was counted ($\times 4$ magnification) and pictures were taken ($\times 10$ magnification) under a phase-contrast microscope (EVOS).

2.15 Zebrafish

2.15.1 Zebrafish husbandry

Zebrafish were maintained using the established system following the standard guidelines depicted in Nusslein-Volhard and Dahm [442]. Zebrafish were kept in a self-recirculating aquarium at a constant temperature of 28 °C with a 14 h light and 10 h dark cycle. Adult specimens were fed twice a day on a diet of Hikari micropellets

(Kyorin) and brine shrimp. Zebrafish embryos (1–7 dpf) were kept in a solution composed of system water (chlorine deprived tap and distilled water mixture) with the addition of 0.0003% (v/v) methylene blue (antifungal) at 28°C.

2.15.2 CM-Dil labelling

Cancer cell lines were pre-seeded to obtain a culture with 80% level of confluency on the day of the CM-Dil (Invitrogen) cell labelling. CM-Dil dye was diluted in DMEM without solutes according to the concentrations defined during the Dil-labelling optimisation. Cell lines were individually trypsinised and centrifuged to obtain a pellet. Cell pellets were resuspended in 5 µl/ml CM-Dil dilutions. Cells were incubated in the dye at 37°C at 10% CO₂ for 10 min. Following incubation, cells were rinsed once with phosphate-buffered saline (PBS) and centrifuged twice to remove all excess media before injection. The final pellet was then used for the zebrafish microinjection procedure.

2.15.3 Microinjection

The zebrafish embryos of *Casper* strain and *Tg (fli:GPF)* strain were dechorionated with a pair of sharp-tip forceps at 24 hpf before injection. Cell lines were pre-labelled with CM-Dil and centrifuged to obtain a dry pellet. The embryos were anaesthetized in a solution containing 0.003% tricaine methanesulfonate (Sigma-Aldrich) 10 min before injection. Zebrafish embryos were embedded in a thin mould of 3% agarose to stabilise the fish in a lateral position. Injections were carried out on the mould in a solution of pre-warmed phosphate-buffered saline (+Mg, +Ca) 0.003 % tricaine methanesulfonate, using a 12 mm borosilicate glass capillaries needle fixed on a

Narishige microinjector. Approximately 150 cancer cells were injected in the yolk sac region in proximity of the embryos sub-intestinal vessels. Following injection, fish were carefully removed from the agarose mould and transferred to the petri dish with a solution of system water with methylene blue, and maintained at 28°C for the duration of the experiment.

Embryos were placed in 96-well plates and were imaged individually at 3 days post-implantation under a wide-field fluorescent microscope (Olympus CKX41). Tricaine methanesulfonate (0.005 %) was added to their water to prevent movement during the live imaging procedure. Pictures were captured with Q Capture-Pro (QImaging). Any alteration of the original picture was performed with the aid of ImageJ. The corrected total cell fluorescence (CTCF) was measured according to the formula: $CTCF = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings})$ for each imaged zebrafish. Normally, experimentally used embryos (xenotransplanted zebrafish) were euthanized at the end of experiments (about 4-5 dpf) by tricaine overdose [442].

2.16 Statistical analysis

The statistical differences between two groups were evaluated by two-tailed unpaired Student's t-test using the Microsoft Excel. For comparisons between more than two groups, one-way ANOVA or two-way ANOVA followed by Tukey's multiple comparison methods as a posthoc test was performed using GraphPad Prism software (Version 6.0; GraphPad Software Inc., La Jolla, CA, USA) in different circumstances. In all cases, differences were considered to be significant when * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, and n.s. denotes non-significant.

CHAPTER 3

FOX M1 and HMG interaction networks

3.1 Introduction

The transcription factor FOX M1 has significant impacts on breast cancer progression, and deregulated HMGA1 and HMGN1 have also been reported in association with tumorigenesis in different types of cancer [427, 433, 443-446]. Besides, a PIPs database has reported a potential connection between FOX M1 and HMG proteins [447]. Most importantly, the HMG proteins as chromatin remodelling modulators that recognise structure rather than particular nucleotide sequences, and do not possess intrinsic transcriptional activity, while having the ability to modulate transcription of their targets by altering the chromatin structure or incorporating with other proteins. The question which is raised that whether the HMG proteins affect breast cancer progression through interacting with FOX M1. Moreover, although HMGA1 and HMGN1 are the most well-studied HMG proteins, the mechanisms underlying their functions in cancer progression remain largely unknown. In the project, I aimed to uncover the interconnections between FOX M1, HMGA1 and HMGN1, as well as the subsequent consequences of their interactions on the effectors, *KIF20A* and *SQSTM1/p62*.

3.2 Basal expression levels of FOXM1, HMGA1 and HMGN1

Our lab has previously shown that the paclitaxel resistant (TaxR) and epirubicin resistant (EpiR) MCF-7 breast cancer cell lines have enhanced potentials for metastasis, self-renewal and drug resistance, which are properties synonymous with the metastatic breast cancer cells [170, 434, 448]. In this project, the two resistant cell lines and their parental chemosensitive MCF-7 cell line were used to investigate the underlying mechanisms of FOXM1, HMGA1 and HMGN1.

At first, the basal expression levels of FOXM1, HMGA1 and HMGN1 were identified in the three cell lines. Results showed that FOXM1 protein and mRNA were overexpressed in the resistant cells; HMGA1 proteins were significantly highly expressed in the resistant cell lines but were almost undetectable in MCF-7. Consistently, the mRNA levels of HMGA1 were significantly high in resistant cells compared to the MCF-7. In contrast, another subfamily of HMG protein, HMGN1, expressed in a distinct pattern which was lower in resistant cells and higher in sensitive MCF-7. To summarise, FOXM1 and HMGA1 are highly overexpressed in TaxR and EpiR cell lines, whereas HMGN1 are expressed at a relatively low level in the resistant cells.

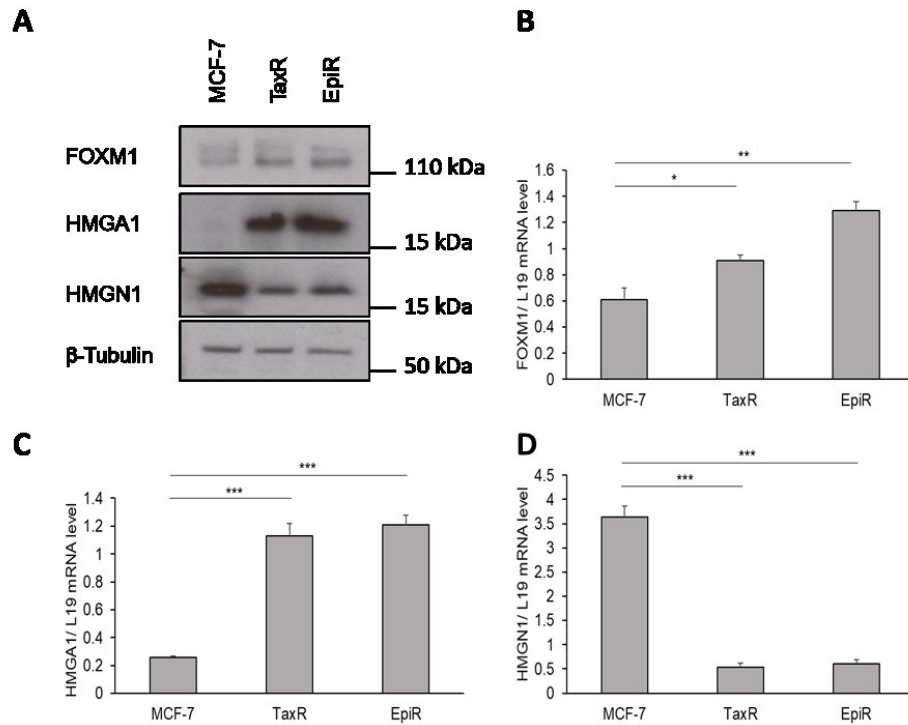


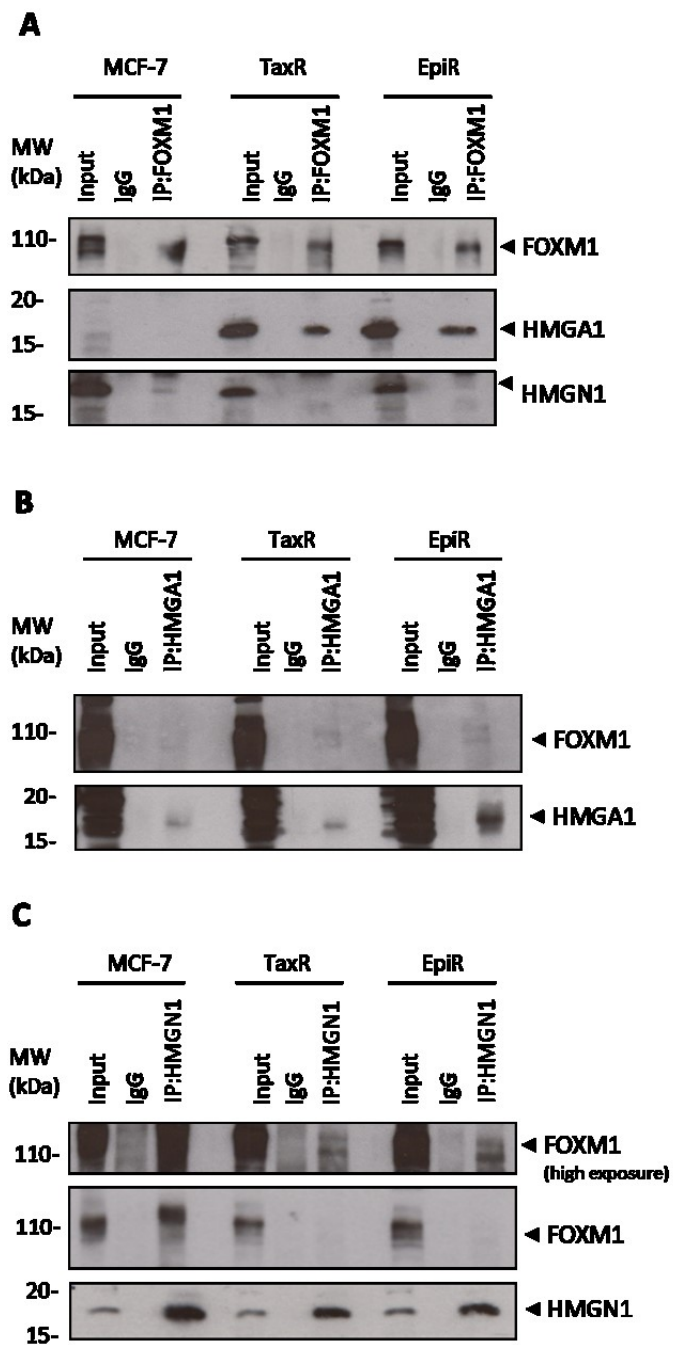
Figure 3. 1 The basal expression levels of FOXM1, HMGA1 and HMGN1 in MCF-7, TaxR and EpiR cell lines.

(A) Western blot was performed in three breast cancer cell lines and the protein expression levels of FOXM1, HMGA1 and HMGN1 are shown. β -tubulin was used as a loading control. (B, C, D) RT-qPCR analysis determining the relative mRNA expression levels. The experiments were repeated three times independently and results were normalized against L19 housekeeping gene. All RT-qPCR values are presented as bars representing means $\pm$ s.d. and significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) was analysed by Student's t-test. (n=3)

3.3 FOXM1 interacts with HMGA1 and HMGN1 at protein level

To identify the correlations between FOXM1, HMGA1 and HMGN1 proteins, I first performed co-immunoprecipitation (Co-IP) to examine if FOXM1 interacts with HMGA1 and HMGN1 as protein-protein complex. Figure 3.2 A shows that FOXM1 was immunoprecipitated using anti-FOXM1 antibody in three cell lines and was immunoblotted with anti-HMGA1 and anti-HMGN1 antibodies separately. The results demonstrated HMGA1 binds to FOXM1 by direct protein-protein interaction. However, this interaction was weak, almost undetectable in MCF-7 in comparison to the resistant cells of which might because of the low HMGA1 expression in MCF-7. Furthermore, reverse co-IP was performed by pulling down HMGA1 and immunoblotting with the anti-FOXM1 antibody. In agreement, the result confirmed that HMGA1 interacts with FOXM1 in all three cell lines and with stronger interactions in the resistant cells. On the other hand, results showed that HMGN1 cooperates with FOXM1 and vice versa. In here, the FOXM1-HMGN1 interactions were observed much stronger in MCF-7 cells compared to that in the TaxR and EpiR cell lines.

In the meantime, a highly specific and sensitive immunohistochemical technique-proximity ligation assay (PLA) was conducted along with co-IP. PLA is a powerful tool to detect protein-protein interactions in which a distinct fluorescent spot will be visualized only when the two proteins are in close proximity (< 40 nm). The PLA results confirmed previous co-IP findings suggesting that FOXM1 interacts with HMGA1 and HMGN1 through direct protein-protein interactions in MCF-7, TaxR and EpiR cell lines.



D

**FOXM1-
HMGA1**

MCF-7

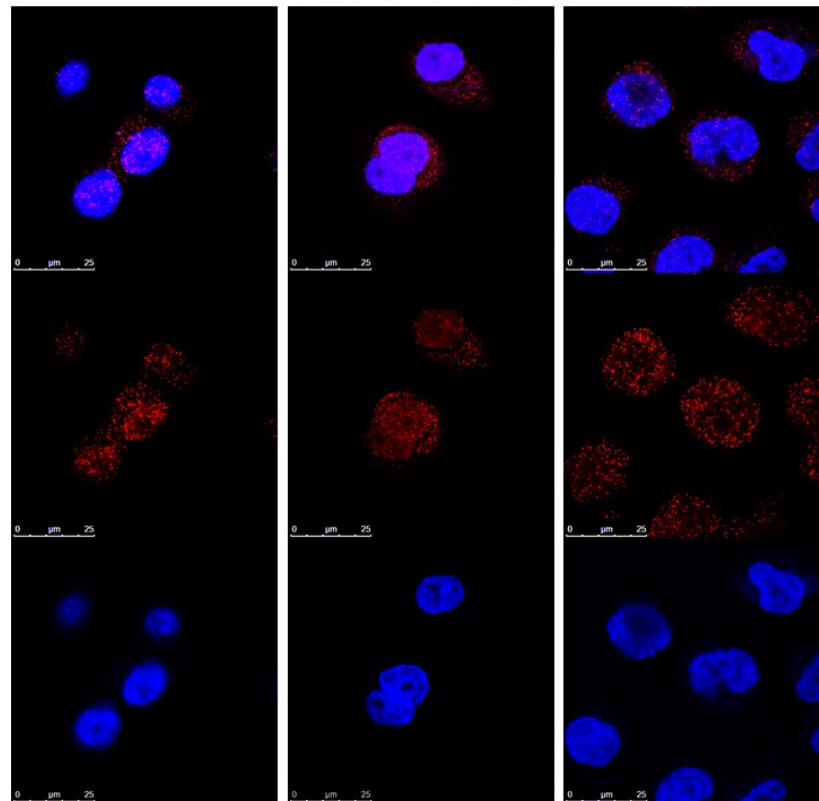
TaxR

EpiR

Merge

PLA

DAPI



**FOXM1-
HMGN1**

MCF-7

TaxR

EpiR

Merge

PLA

DAPI

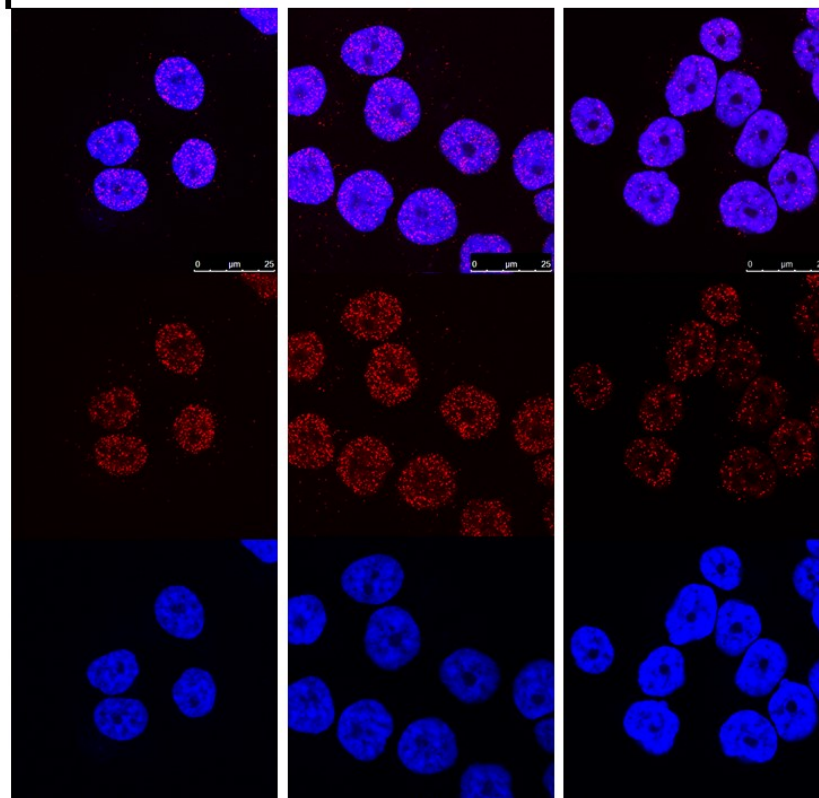


Figure 3. 2 FOXM1 interacts with HMGA1 and HMGN1.

Co-immunoprecipitation (co-IP) was performed with an IgG antibody control, an anti-FOXM1 antibody (A), an anti-HMGA1 antibody (B) and an anti-HMGN1 antibody (C) on lysates from MCF-7, MCF-7 TaxR and MCF-7 EpiR cell lines. Input (1/10 of IP), and IP products with IgG and specific antibodies were resolved on western blot and probed for FOXM1, HMGA1 and HMGN1. The specific bands were indicated by arrows. (n=2) (D) PLA was conducted and interactions were visualised as red spots with confocal microscopy. The nuclei of the cell were stained with 4,6-diamidino-2-phenyl indole (DAPI) in blue. Six fields were randomly captured of each condition and representative image are shown here.

3.4 FOXM1 binds to *KIF20A* and *p62* gene promoter regions

Previous results revealed the associations between FOXM1, HMGA1 and HMGN1. To further explore the roles of their associations on chromatin, I first demonstrated that *KIF20A* and *p62* are FOXM1 transcriptional targets. To determine whether FOXM1 regulates these genes at promoter regions, chromatin immunoprecipitation (ChIP) assay was performed in the three cell lines. Figure 3.3.1 displays the potential binding sites of FOXM1 on *KIF20A* and *p62* promoters, and primers are designed correspondingly. Figure 3.3.2 shows both *KIF20A* and *p62* promoter regions were significantly enriched by FOXM1 in MCF-7, TaxR and EpiR cells. This suggested that FOXM1 directly regulates *KIF20A* and *p62* at the transcription level. Notably, although the ectopic high expression of FOXM1 was observed in the resistant cells, the FOXM1 binding ability on *KIF20A* and *p62* was relatively weaker in these cells compared to the MCF-7.

To confirm the ChIP findings, FOXM1 overexpression and knockdown experiments were performed in the three MCF-7 cell lines. Western blotting (Figure S. 3) and RT-qPCR (Figure 3.3.3) were used to analyze the effects of FOXM1 modulation on the relevant protein and mRNA levels. The results showed that overexpression of FOXM1 was much more efficient in the sensitive MCF-7 cell line, which might due to its low basal expression in MCF-7. Moreover, the RT-qPCR results showed that *KIF20A* was upregulated and *p62* was downregulated upon FOXM1 overexpression, whereas the protein levels did not vary significantly which might due to the transient transfection and their high protein stability. On the other hand, the FOXM1 depletion experiments demonstrated the transfection efficiency was high in all three cell lines. Results showed that *p62* was significantly induced and *KIF20A* reduced at mRNA levels with FOXM1 knockdown. which suggested that *p62* might be negatively regulated by

FOXO1. Taken together with ChIP findings, results suggested that FOXO1 indeed binding to *KIF20A* and *p62* promoters and modulating their transcription.

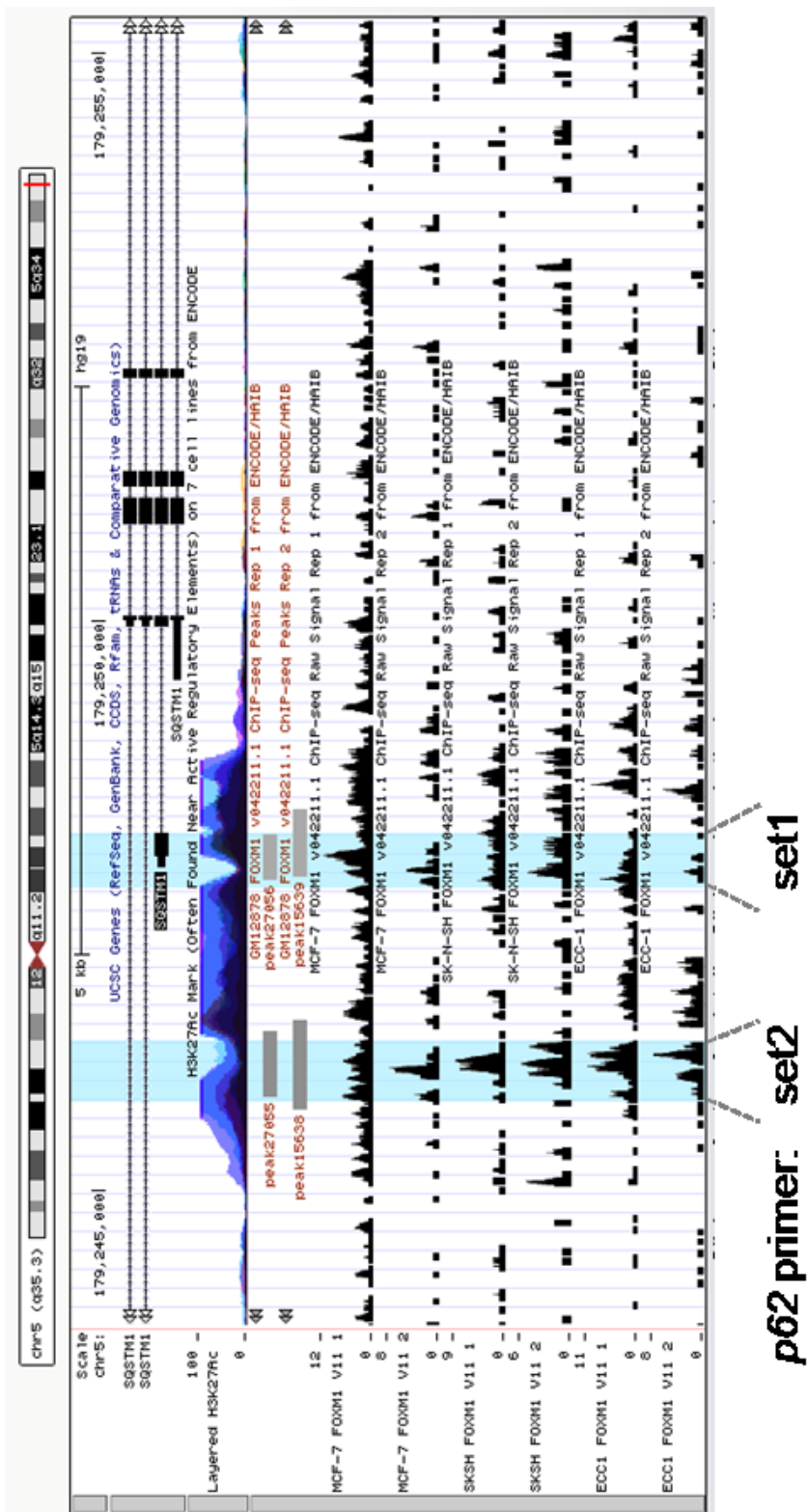


Figure 3. 3.1 FOXM1 binding sites on human *KIF20A* and *p62*.

ENCODE/HAIB ChIP-sequencing data of FOXM1 binding in the MCF-7, SKSH and ECC1 cells were used for predicting global genome-binding profiles for FOXM1. The analyses were using the University of California Santa Cruz (UCSC) Genome Browser ([http:// genome.ucsc.edu](http://genome.ucsc.edu)) and the Integrative Genomics Viewer (Version 2.3.83). The primers were designed from the highlighted (blue) regions.

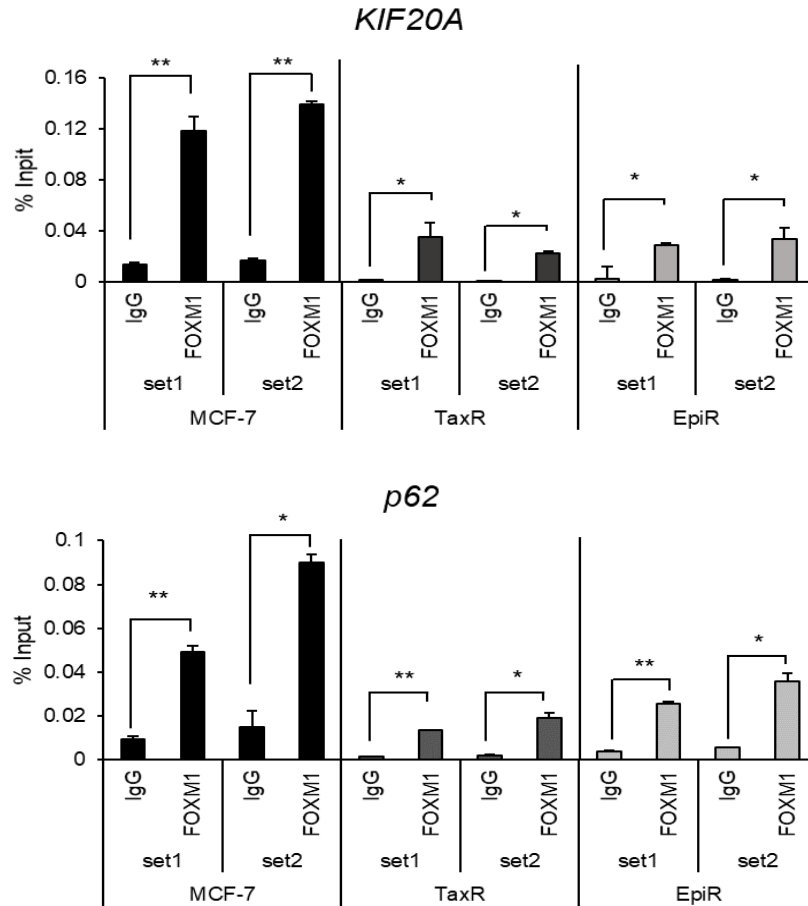


Figure 3. 3.2 FOXM1 directly binds to the *HMGA1*, *HMGN1*, *KIF20A* and *p62* at promoter regions.

Chromatin immunoprecipitation (ChIP) assay was performed. The chromatin obtained from cells were immunoprecipitated with either FOXM1 or IgG negative control antibodies. The binding of FOXM1 at either *HMGA1* or *HMGN1* sequence was then quantified by RT-qPCR analysis. The binding of FOXM1 to the human *HMGA1*, *HMGN1*, *KIF20A* and *p62* promoter regions was examined by ChIP-qPCR. The results were normalized to 0.1% input. Value is means $\pm$ s.d. (n=3). Statistical significance was determined by two-tailed unpaired Student's t-test, with* <0.05; ** P<0.01; *** P<0.001; ns, non-significant.

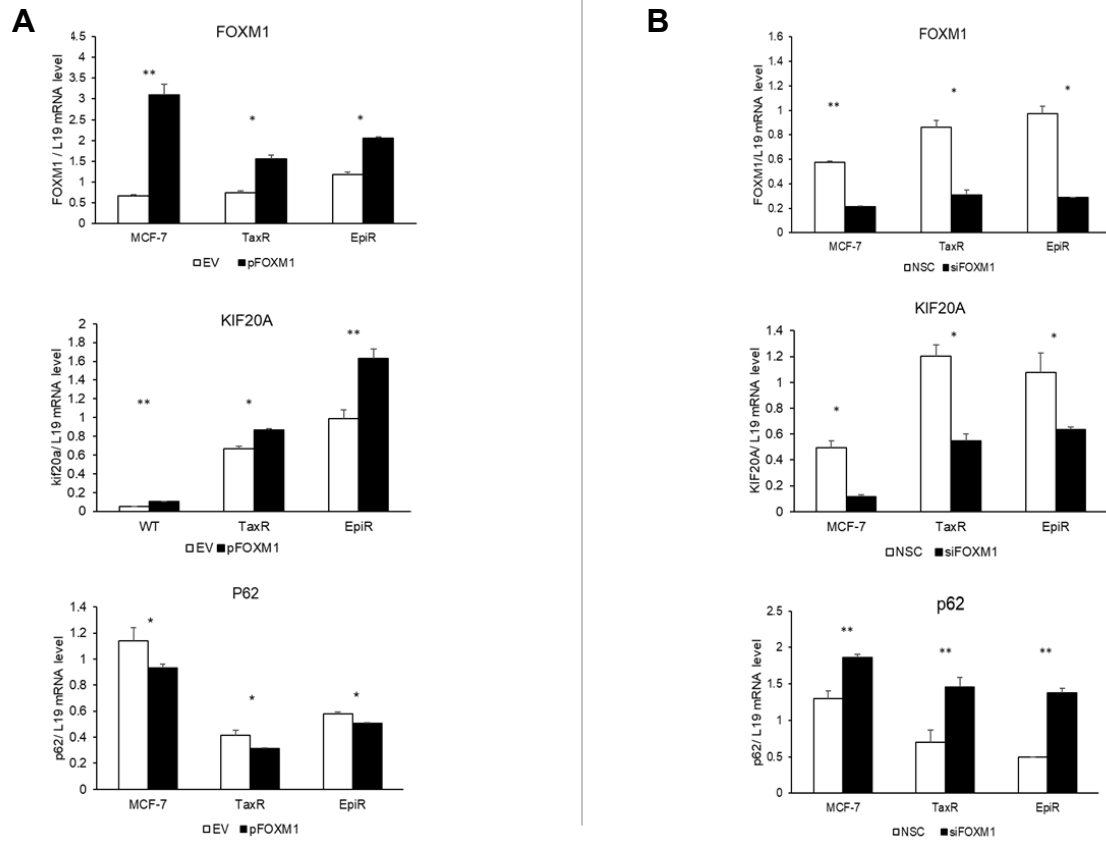


Figure 3. 3.3 Regulations of FOXM1 on KIF20A and p62.

MCF-7, TaxR and EpiR cells were transfected with pCDNA3-FOXM1 or pCDNA3 control plasmids and Smart pool siRNA against *FOXM1* or non-silencing controls (NSC) for overexpression and silencing transfection, respectively. *FOXM1*, *KIF20A*, *p62* mRNA levels were determined by RT-qPCR and normalized to the ribosomal protein L19 RNA expression. **(A)** FOXM1 overexpression and **(B)** FOXM1 knockdown results are shown. Values are means $\pm$ s.d. (n=3). Statistical significance was determined by two-tailed unpaired Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant).

3.5 HMGA1 attenuates FOXM1 promoter binding ability whereas HMGN1 promotes FOXM1 binding

Since the HMG proteins are not transcription factors and do not possess transcriptional activity unless through cooperating with other proteins. It is likely that they interact with FOXM1 to alter the FOXM1 transcription activity on its downstream target genes, such as *KIF20A* and *p62*. To further assess the associations between FOXM1 and HMG proteins at the transcription level, HMGA1 and HMGN1 knockdown experiments were performed to examine whether these cofactors could affect the binding of FOXM1 to transcriptional targets *cyclin B1*, *KIF20A* and *p62*. Two sets of primers were designed for each gene at the FOXM1 ChIP-Seq binding peaks (Figure 3.3.1). ChIP results demonstrated that HMGA1 knockdown significantly increased FOXM1 binding at the promoter regions of *cyclin B1*, *KIF20A* and *p62* in MCF-7 cells, implying that HMGA1 might prevent FOXM1 from binding to its targets or compete with FOXM1 for binding in MCF-7. Interestingly, this scenario was not observed in the resistant cell lines, in which HMGA1 knockdown reduced FOXM1 binding to the same target genes. Hence, HMGA1 may play different roles in MCF-7 and resistant cell lines. In contrast, depletion of HMGN1 significantly reduced FOXM1 binding at the promoter regions of *cyclin B1*, *KIF20A* and *p62* in MCF-7 cells. This suggested that HMGN1 might play a role in enhancing or stabilizing FOXM1 promoter binding ability in MCF-7 cells. Yet, this was not observed in the TaxR and EpiR cell lines, in which knockdown HMGN1 did not affect FOXM1 binding significantly.

Figure 3.4.3 shows the ChIP analysis of HMGA1 and HMGN1 binding abilities at the same target regions. In concordance, the results indicated that FOXM1 knockdown significantly enhanced the binding of HMGA1 at the *KIF20A* and *p62* promoters in MCF-7 cells. This further confirmed the previous suggestion where FOXM1 and

HMGA1 competed on the same binding sites in MCF-7. When FOXM1 was silenced, HMGA1 binding was about 2 times increased, whereas when HMGA1 was knocked down the FOXM1 binding was approximately 4 times enriched. Again, this was only observed in the MCF-7 cell line but not in the resistant cells, implying a different regulatory mechanism of FOXM1 and HMGA1 in the resistant cells. Besides, Figure 3.4.3 B shows the binding of HMGN1 at *KIF20A* and *p62* promoters under different transfection conditions. The results revealed that knockdown of FOXM1 did not affect HMGN1 chromatin binding ability. Taken together with the ChIP results of pulling down FOXM1, it suggested that HMGN1 binds to chromatin and then recruits FOXM1 to the promoter regions, in such a way it enhances the FOXM1 binding ability. In addition, knockdown HMGA1 showed an increased HMGN1 binding at *KIF20A* and *p62* promoters which suggested that HMGA1 and HMGN1 might antagonize one another of chromatin binding.

Hereby, the results showed that these chromatin-associated HMG proteins, HMGA1 and HMGN1, influence the binding ability of FOXM1 at *KIF20A* and *p62* gene promoters in MCF-7. Besides, the HMG protein might play different roles in regulating FOXM1 activities in other cell lines.

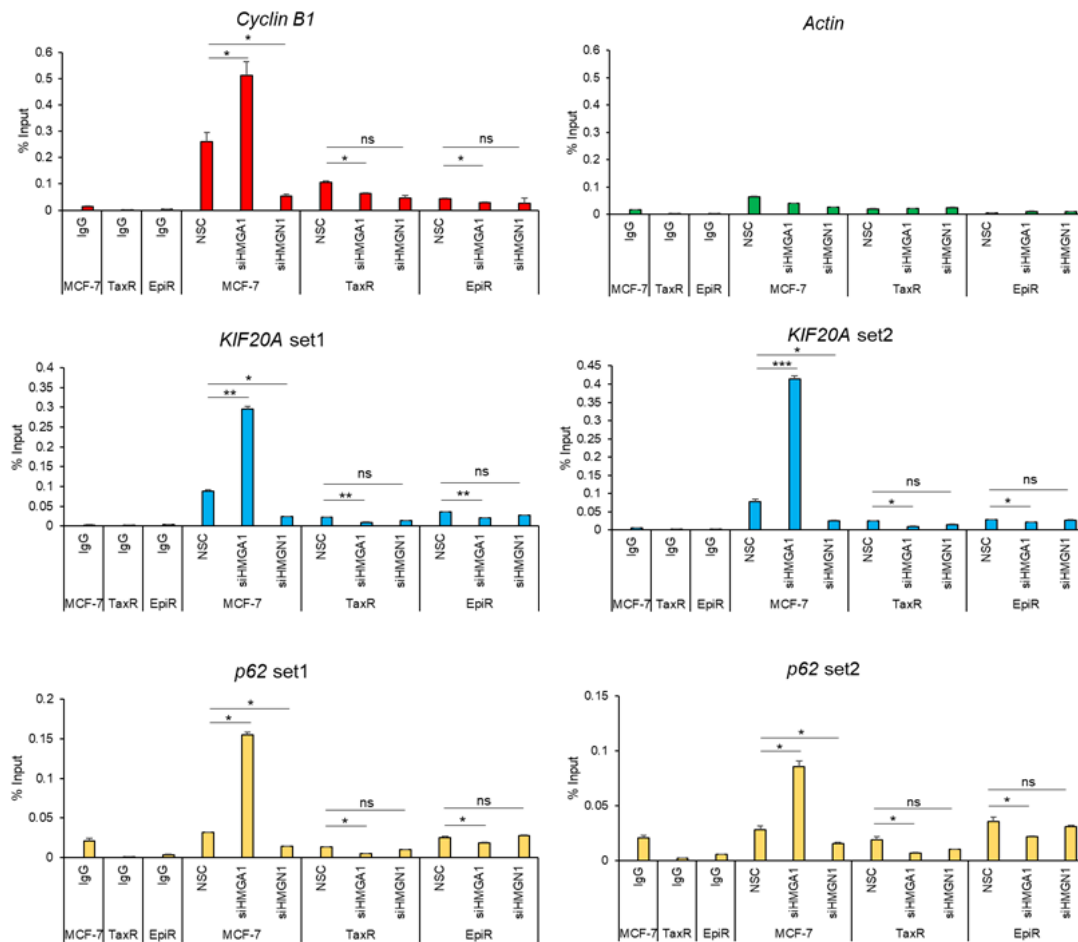


Figure 3. 4.1 HMG1 restricts FOXM1 binding ability in MCF-7 cells, whereas HMGN1 is required for FOXM1 binding.

ChIP for FOXM1 binding was assessed following siRNA treatment in MCF-7, TaxR and EpiR cells. The three cell lines were transfected with HMG1 siRNA, HMGN1 siRNA and non-silencing control siRNA and harvested after 48 h. ChIP-qPCR was used to quantify FOXM1 binding ability on cyclin B1 (positive control), actin (negative control), *KIF20A* and *p62*. Two sets of *KIF20A* and *p62* primers were designed along the FOXM1 binding peak regions. The results were normalized to 0.1% input. Value is means $\pm$ s.d. (n=3). Statistical significance was determined by two-tailed unpaired Student's t-test, with * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant.

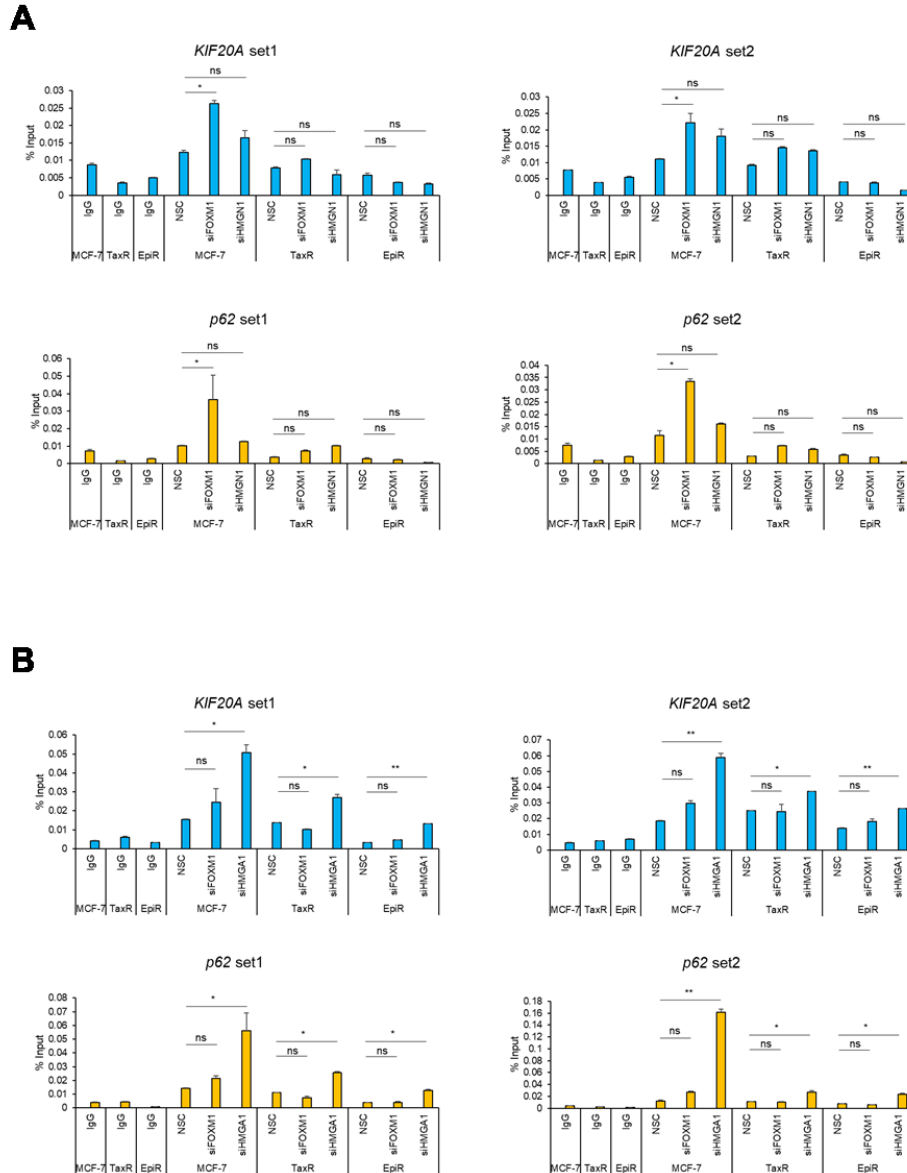


Figure 3.4.2 Binding of HMGA1 and HMGN1 at *KIF20A* and *p62* promoter regions.

Chromatin Immunoprecipitation analysis for (A) HMGA1 and (B) HMGN1 binding on *KIF20A* and *p62* was assessed following siRNA treatment in MCF-7, TaxR and EpiR cells. The three cell lines were transfected with HMGA1 siRNA, HMGN1 siRNA, FOXM1 siRNA and non-silencing control siRNA and harvested after 48 h. ChIP-qPCR was used to quantify their binding ability at *KIF20A* and *p62*. The results were normalized to 0.1% input. Value is means $\pm$ s.d. (n=3). Statistical significance was

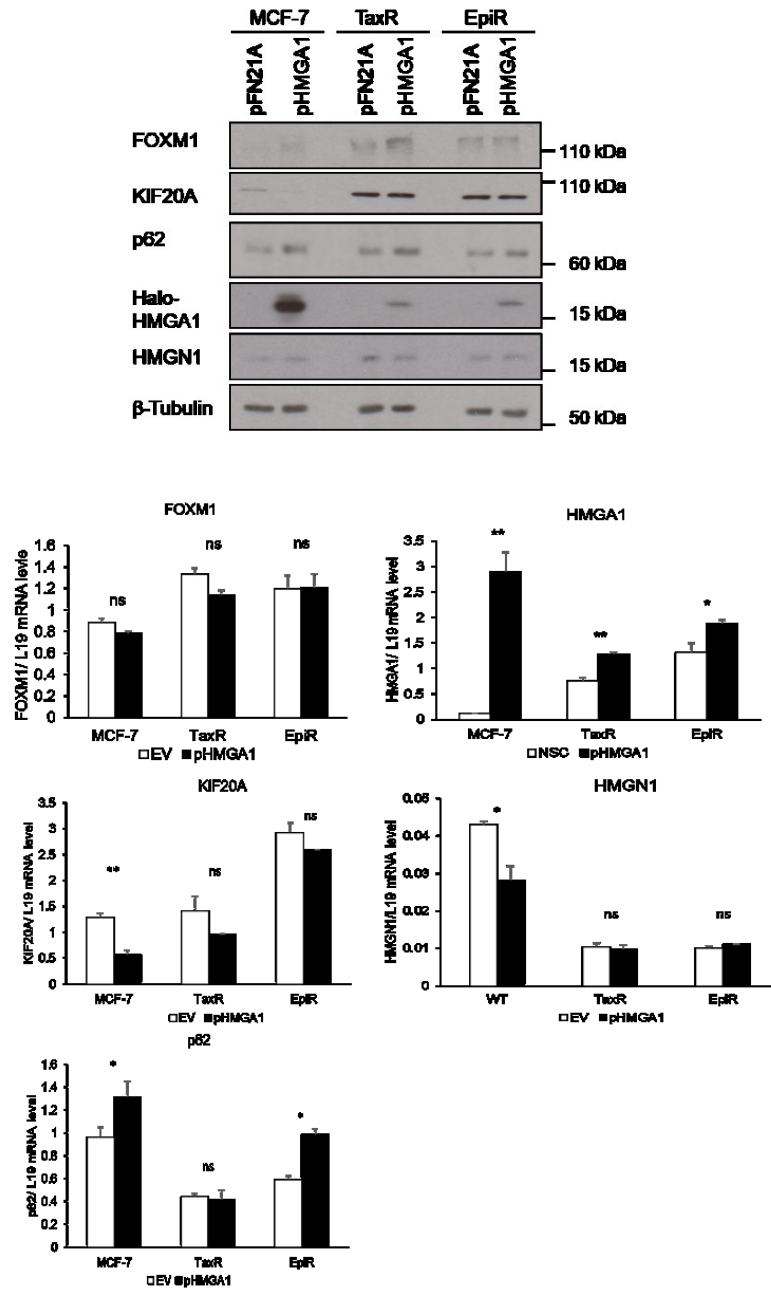
determined by two-tailed unpaired Student's t-test, with * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant.

3.6 HMGA1 modulates the expression of FOXM1 target genes- *KIF20A* and *p62*

To further clarify the hypothesized mechanisms of HMG proteins in modulating FOXM1 transcription activities, the overexpression and knockdown of HMGA1 were conducted independently using transient transfection. The expression of relevant genes was examined using RT-qPCR and western blotting for mRNA and protein analysis, respectively. *KIF20A* and *p62* have been demonstrated as FOXM1 transcriptional targets in MCF-7, TaxR and EpiR cell lines. In here, the results showed that HMGA1 overexpression did not overtly alter the FOXM1 expression levels, but resulted in a significant decrease of *KIF20A* and increase of *p62* mRNA expression in MCF-7 cells. On the other hand, Figure 3.5 B shows the HMGA1 depletion resulted in increased *KIF20A* expression and reduced *p62* in MCF-7 cells. These observations confirmed the previous hypothesis that HMGA1 blocks FOXM1 transcriptional activities by binding to the same promoter sites, therefore overexpression and knockdown of HMGA1 affect FOXM1-modulated gene expression. Besides, the ChIP results revealed that HMGA1 was no longer able to affect FOXM1 binding in TaxR and EpiR cells, we have observed the consistent outcome that *KIF20* and *p62* expressions were not altered significantly upon HMGA1 modulation in the resistant cell lines. This further implied that HMGA1 functions distinctively in various cell lines.

Taken together, the results showed that alterations of HMGA1 significantly affect the expression pattern of FOXM1 downstream targets, *KIF20A* and *p62* in MCF-7 cells, which might through competing against FOXM1 transcriptional binding ability.

A



B

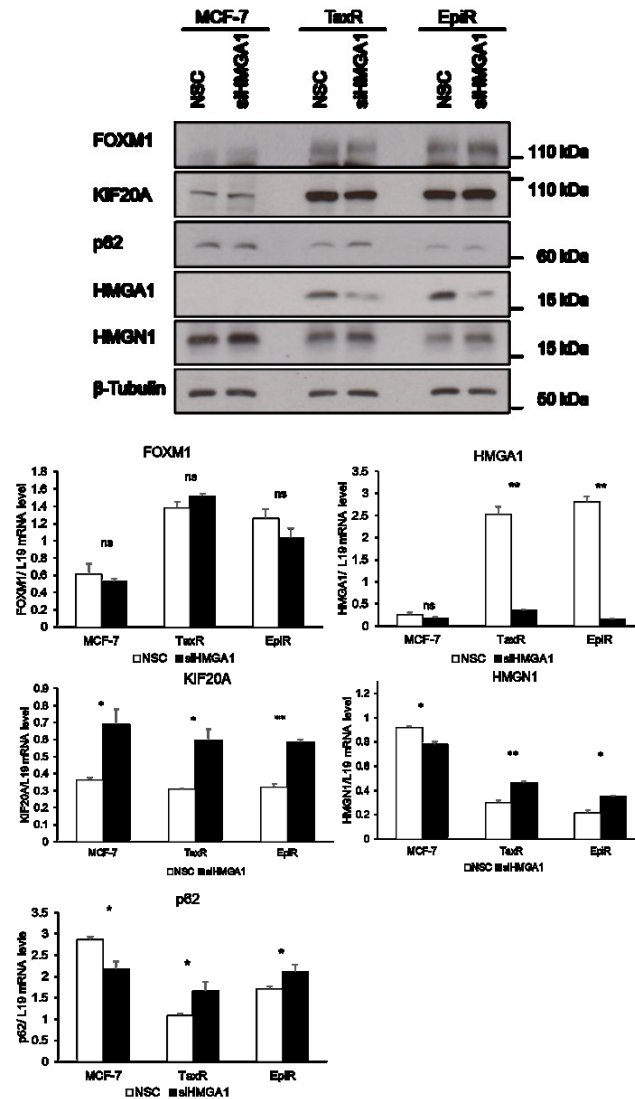


Figure 3. 5 Altercations of HMGA1 affect KIF20A and p62 expression.

MCF-7, TaxR and EpiR cells were transfected with (A) pFN21A-HaloTag- HMGA1 plasmid (pHMGA1) and pFN21A-HaloTag vector control for HMGA1 overexpression; and transiently transfected with (B) HMGA1 siRNA and non-specific siRNA (siNSC) for HMGA1 silencing. Protein expression levels of FOXM1, HMGA1 and HMGN1 and two FOXM1 downstream targets KIF20A and p62, were examined by western blotting analysis. β -tubulin was used as loading control. RT-qPCR analysis of these proteins' mRNA levels. All values were normalized to L19 mRNA values. Bars represent means $\pm$ s.d. Statistical significance was determined by two-tailed unpaired Student's t-test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant. (n=3)

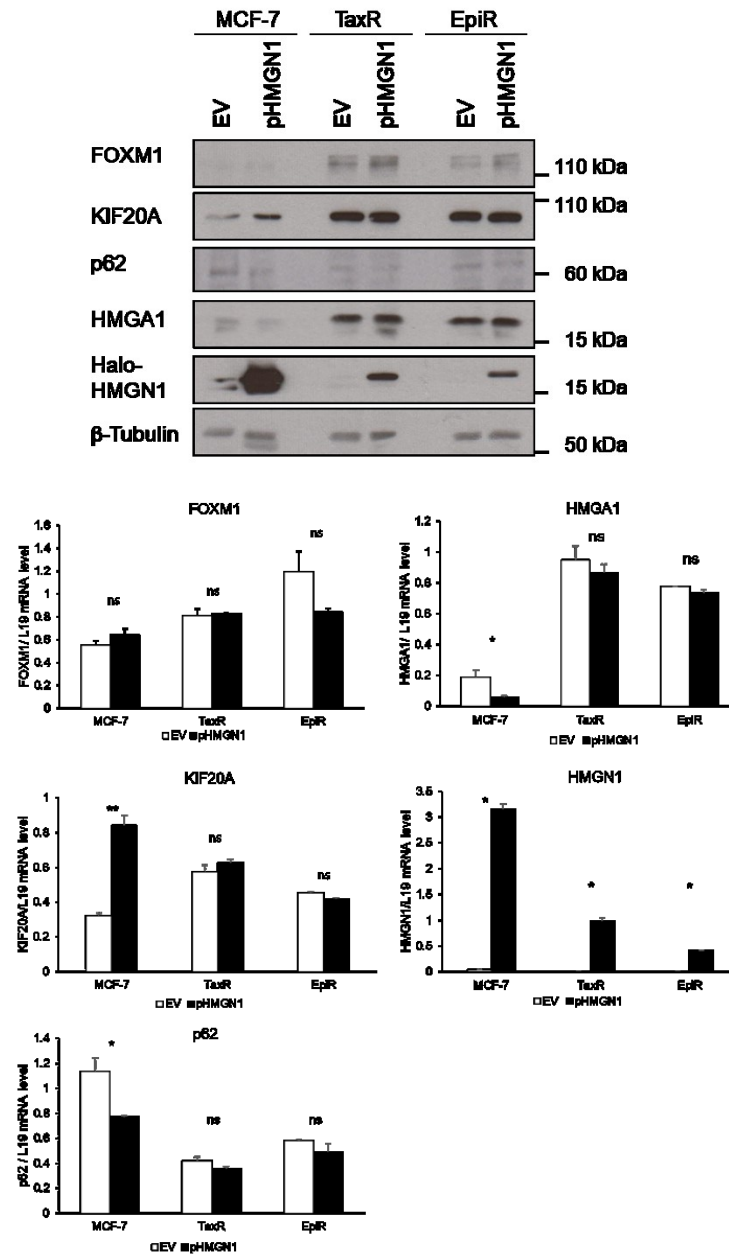
3.7 Alteration of HMGN1 expression affects *KIF20A* and *p62* expression

To further study the role of FOXM1-HMGN1 interactions on FOXM1 activity in terms of its regulation of downstream targets, overexpression and knockdown HMGN1 experiments were performed, and both Western blotting and RT-qPCR analysis were used to investigate their effects.

Figure 3.6 A shows overexpression of HMGN1 did not alter FOXM1 mRNA levels. *KIF20A*, the downstream target of FOXM1, was significantly upregulated by HMGN1 overexpression in MCF-7. Another putative downstream target, *p62*, that was considered to be negatively controlled by FOXM1, displayed a significant downregulation in expression in MCF-7. In agreement, HMGN1 knockdown experiment (Figure 3.6 B) showed HMGN1 depletion lead to reduced *KIF20A* and induced *p62* expression in MCF-7 cells. These results further confirmed the previous ChIP findings that HMGN1 may assist and stabilize FOXM1 transcriptional activities of *KIF20A* and *p62*. Interestingly, results showed HMGN1 overexpression significantly decreases HMGA1 protein and mRNA expression in MCF-7 cells. Likewise, HMGA1 overexpression downregulated HMGN1 expression levels. Taken together, these suggested that HMGA1 and HMGN1 might antagonize each other's activity in MCF-7 cells.

In summary, HMGN1 overexpression and knockdown results demonstrated that HMGN1 might incorporate with FOXM1 on promoter regions of certain genes, thus alterations of HMGN1 expression affect the expression levels of the *KIF20A* and *p62* in the MCF-7 cell line.

A



B

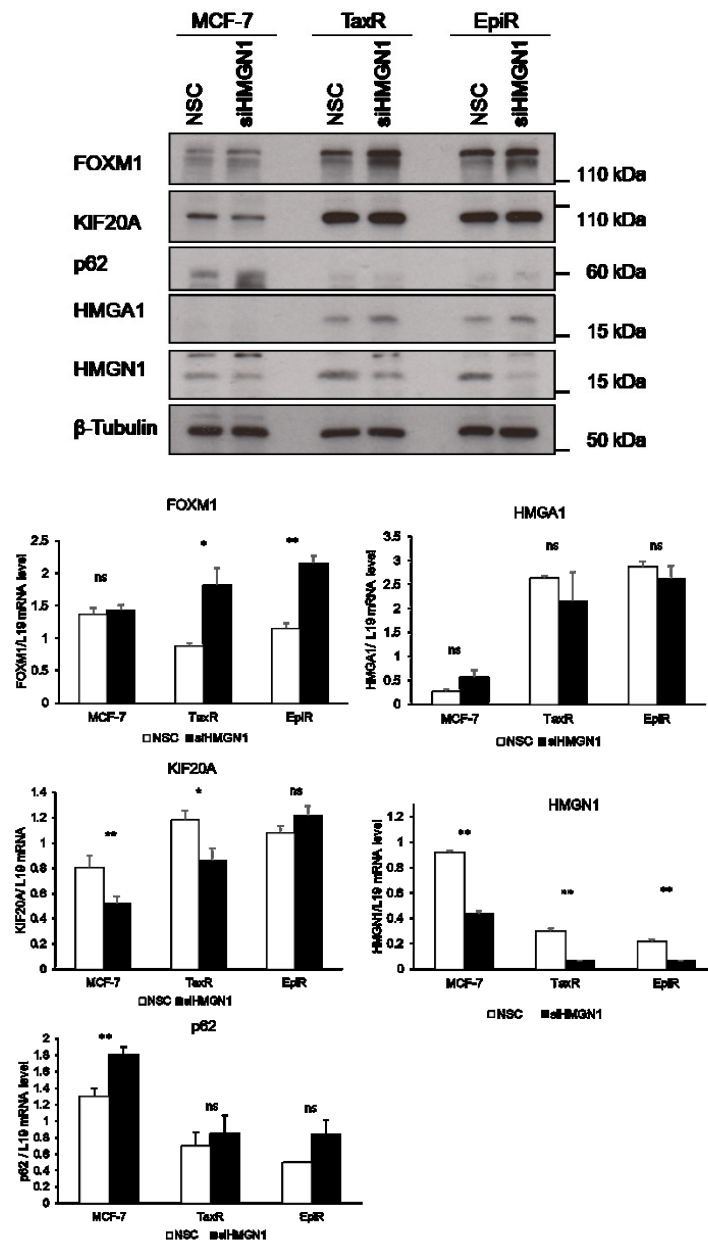


Figure 3. 6 HMGN1 overexpression and knockdown significantly affect KIF20 and p62 expression in MCF-7 cells.

MCF-7, TaxR and EpiR cells were transfected with (A) pFN21A-HaloTag- HMGN1 plasmid (pHMGN1) or empty vector control (EV); or transfected with (B) HMGN1 siRNA and non-specific siRNA (siNSC). Cells were then harvested to probe with antibodies: FOXM1, KIF20A, p62, HMGA1 and HMGN1. β -tubulin was used as loading control for Western blotting. mRNA levels of the same genes were analyzed using RT-

qPCR. Expression of each gene was normalized relative to L19. The assay was carried out in triplicated, and data are presented as means $\pm$ s.d. All statistical analysis was performed via Student t-test, with * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant.

3.8 FOXM1 interacts with HMGA1 and HMGN1 in response to drug treatment

Hitherto, the results elucidated the potential regulatory roles of FOXM1-HMGA1 and FOXM1-HMGN1 interactions on the *KIF20A* and *p62* target genes. Since FOXM1 is a master regulator that plays essential roles in multiple hallmarks of cancer progression, it was then aimed to investigate whether the interactions between FOXM1, HMGA1 and HMGN1 have impacts associated with drug sensitivity, cancer cell migration and invasion.

First, the co-IP and PLA experiments were conducted to demonstrate if the FOXM1-HMGA1 and FOXM1-HMGN1 interactions have altered in response to drug treatment. To assess this concept, experiments were performed with the treatment of paclitaxel and epirubicin at 0h, 6h and 24h. Consistent with co-IP results (Figure S.3, S.4), PLA results confirmed that FOXM1-HMGA1 and FOXM1-HMGN1 interactions are presented in all three MCF-7 cell lines, in which their interactions were reduced in response to paclitaxel and epirubicin treatment in MCF-7 cells; whereas the interactions remained constant in the resistant cell lines. Moreover, although HMGA1 proteins are mainly located in the nucleus, the FOXM1-HMGA1 interactions were observed abundantly in the cytoplasm. This suggested there might be a cytoplasmic translocation of their proteins after in contact with FOXM1. In contrast, FOXM1-HMGN1 interactions were mainly observed in the nucleus in all three cell lines.

Taken together, FOXM-HMGA1 and FOXM1-HMGN1 interactions were reduced in response to paclitaxel and epirubicin treatment in MCF-7 cell lines. These interactions remain constant upon drug treatment in TaxR and EpiR cell lines. These observations

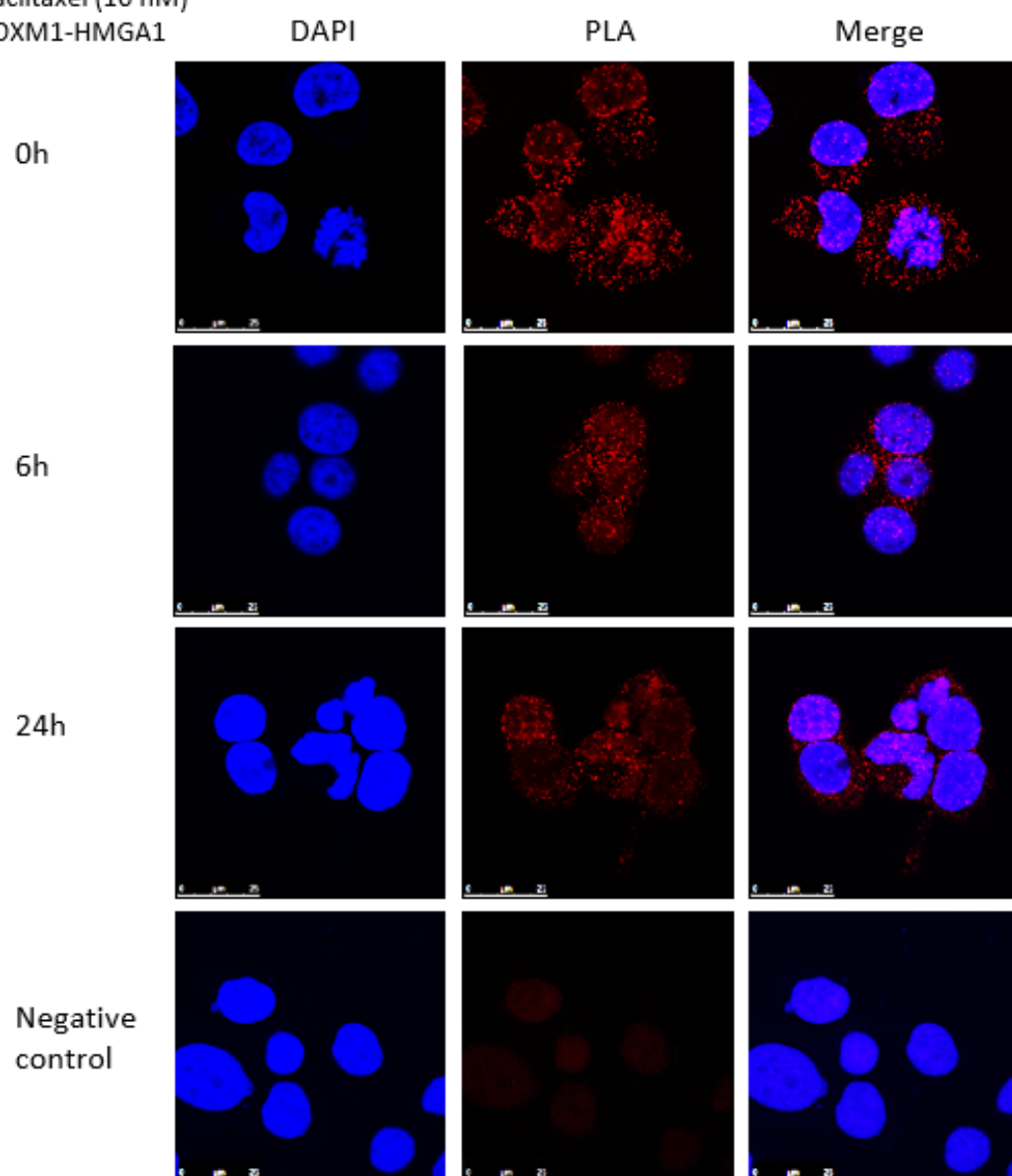
implied their interactions might be associated with drug sensitivity in breast cancer cell lines.

A

MCF-7

Paclitaxel (10 nM)

FOXM1-HMGA1

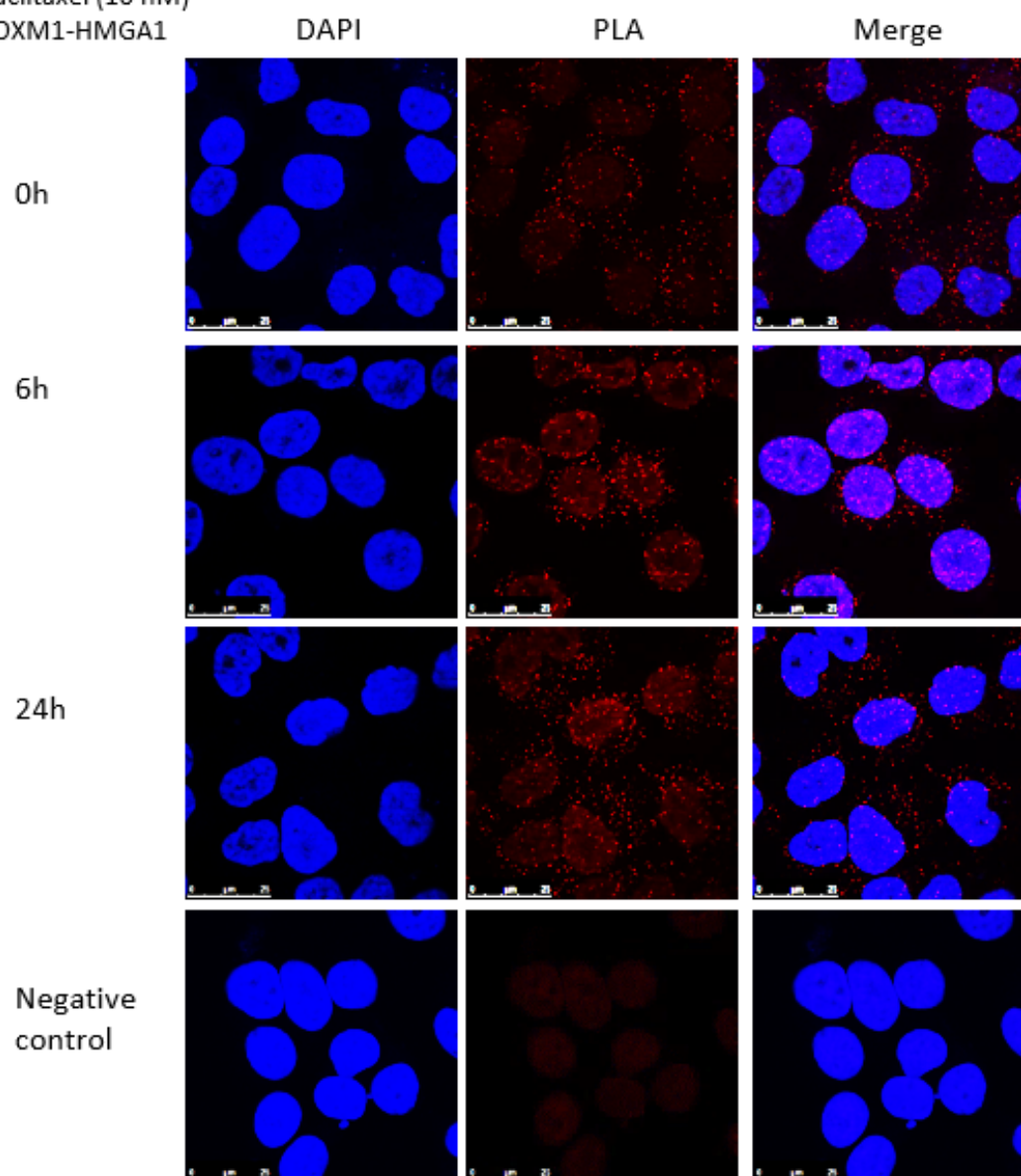


B

MCF-7 TaxR

Paclitaxel (10 nM)

FOXM1-HMGA1



C

MCF-7

Epirubicin (1 μ M)

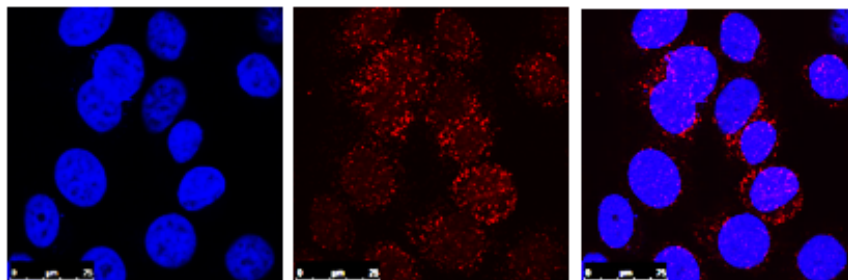
FOXM1-HMGA1

DAPI

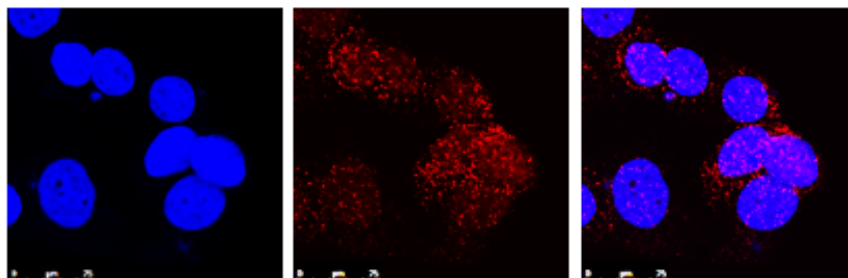
PLA

Merge

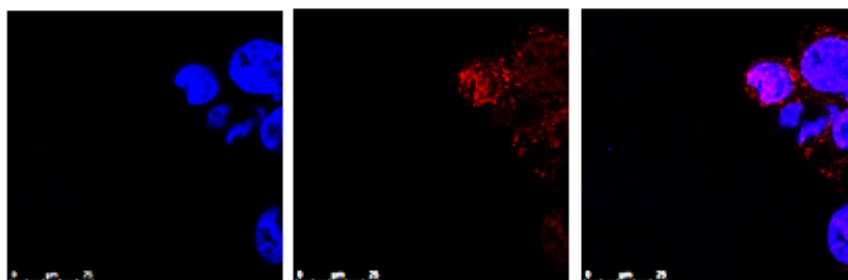
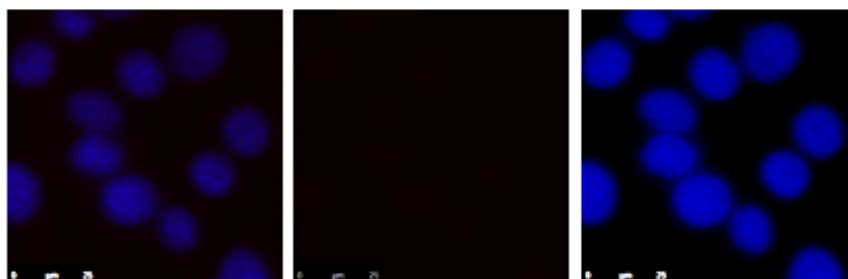
0h



6h

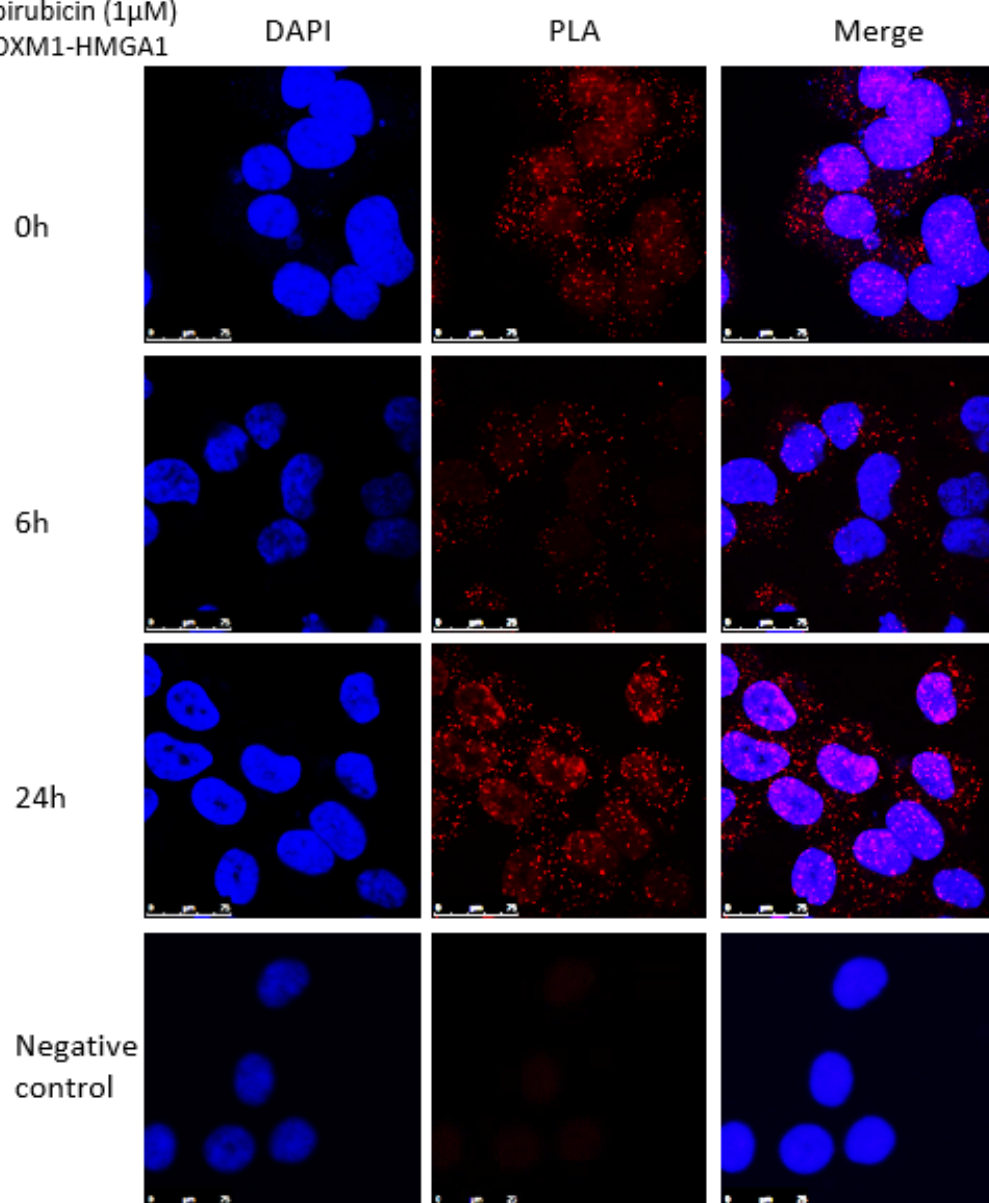


24h

Negative
control

D

MCF-7 EpiR
Epirubicin (1 μ M)
FOXM1-HMGA1



E

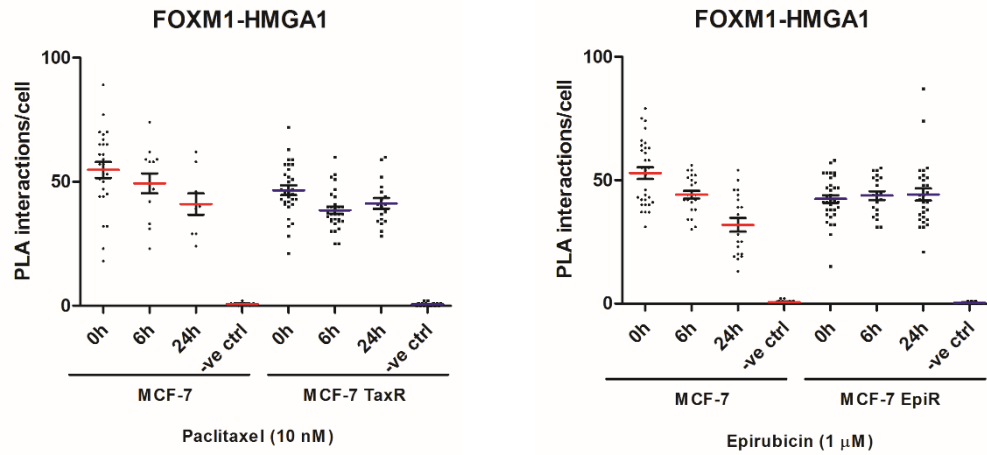
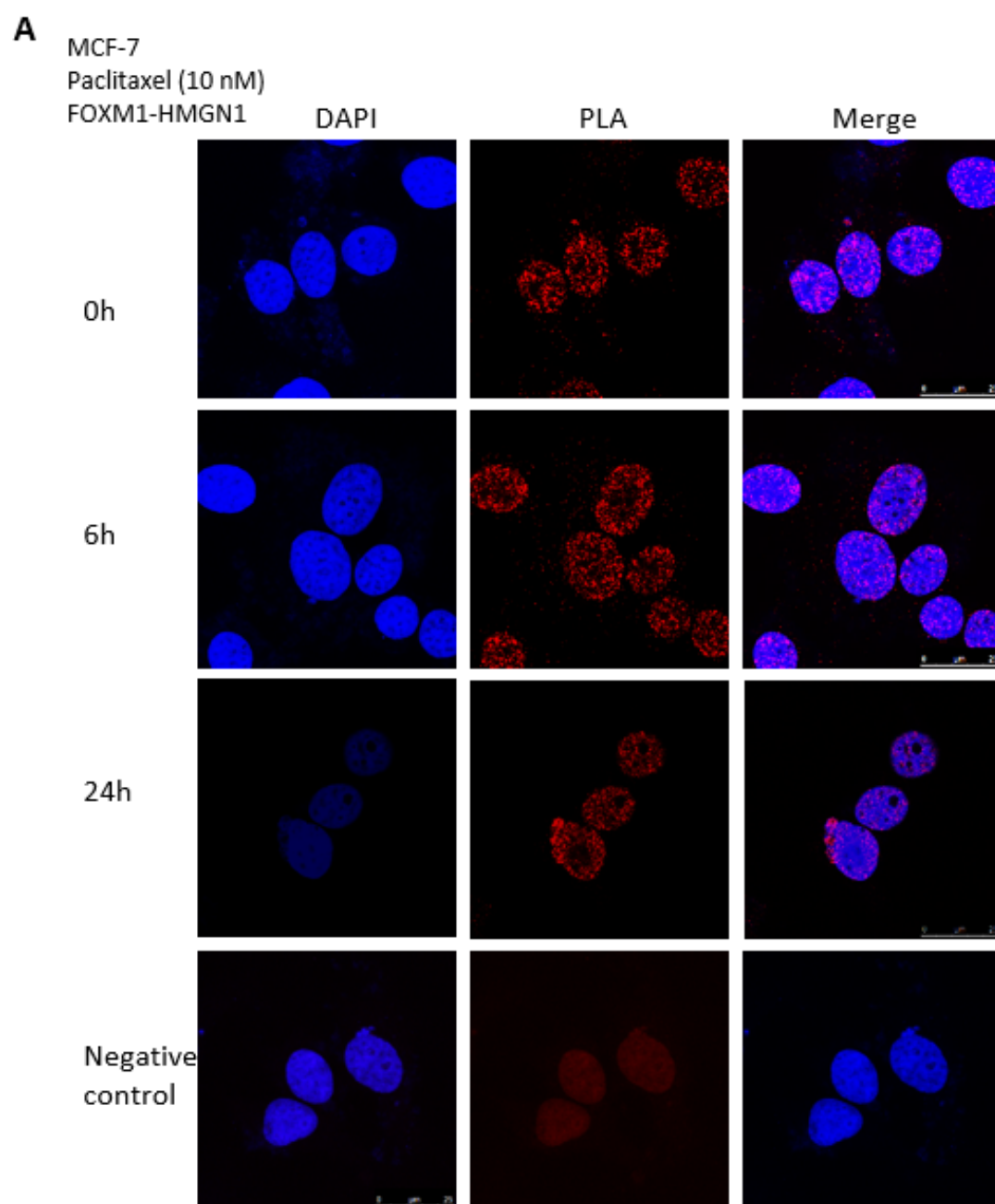
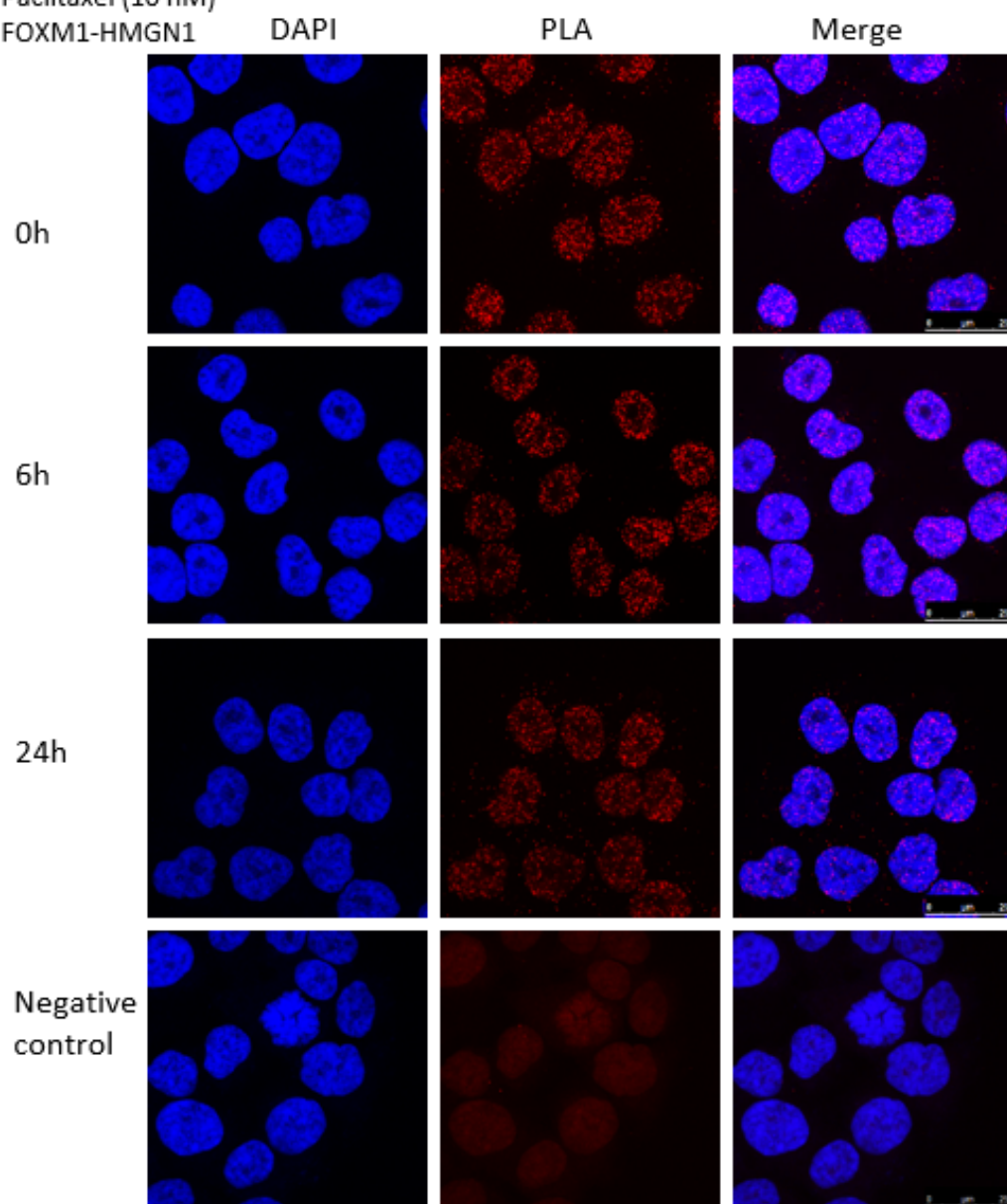


Figure 3. 7.1 Protein-protein interactions of FOXM1 and HMGA1 in breast cancer MCF-7, MCF-7 TaxR and MCF-7 EpiR cell lines. Cells were visualised with a Leica TCS SP5 confocal microscope equipped with a 63× oil immersion objective and LAS-AF software. Each red spot represents for a single interaction and DNA was stained with 4,6-diamidino-2-phenyl indole (DAPI) in blue. **(A)** MCF-7 and **(B)** MCF-7 TaxR cells were treated with 10 nM paclitaxel with time points 0, 6 and 24 hours. **(C)** MCF-7 and **(D)** MCF-7 EpiR cells were treated with 1 μM epirubicin with different time points indicated. Cells without probing with primary antibodies were used as negative control. Representative confocal images are shown. **(E)** The plot represents the number of positive PLA signals per cell that were counted from each image with Image J. Six images were taken account for each condition. Data exhibited as means ± s.d.



B

MCF-7 TaxR
Paclitaxel (10 nM)
FOXM1-HMGN1



C

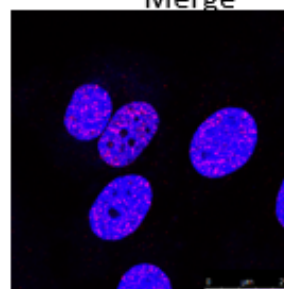
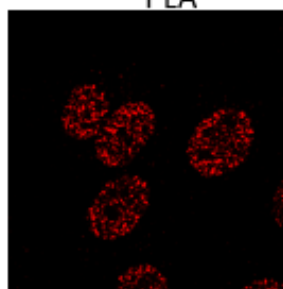
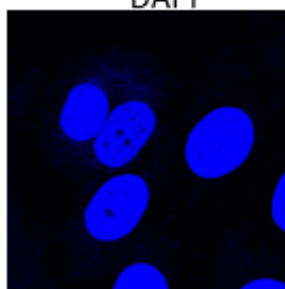
MCF-7
Epirubicin (1 μ M)
FOXM1-HMGN1

DAPI

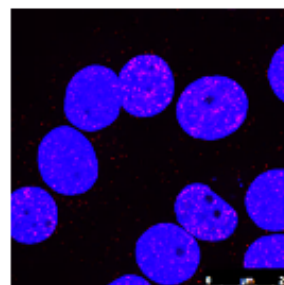
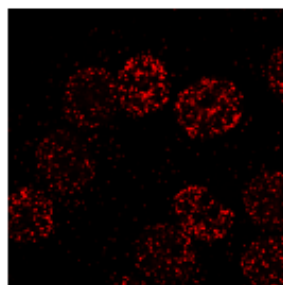
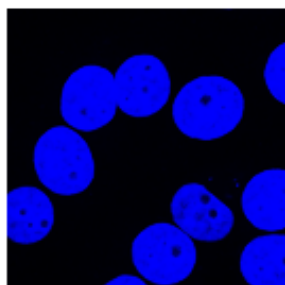
PLA

Merge

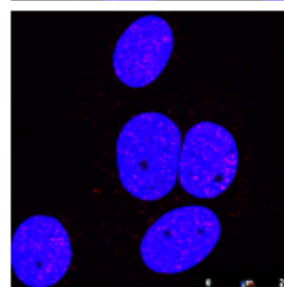
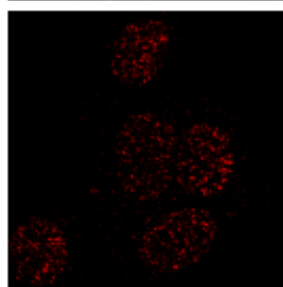
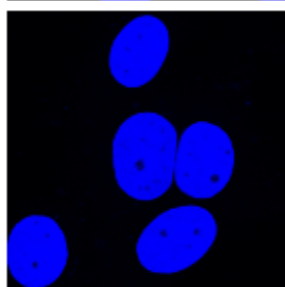
0h



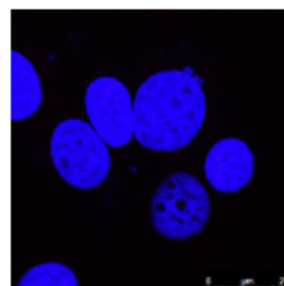
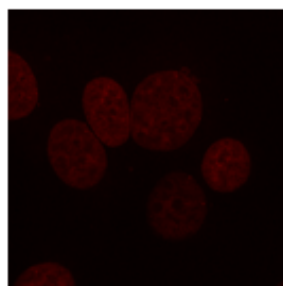
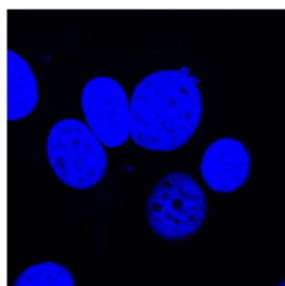
6h

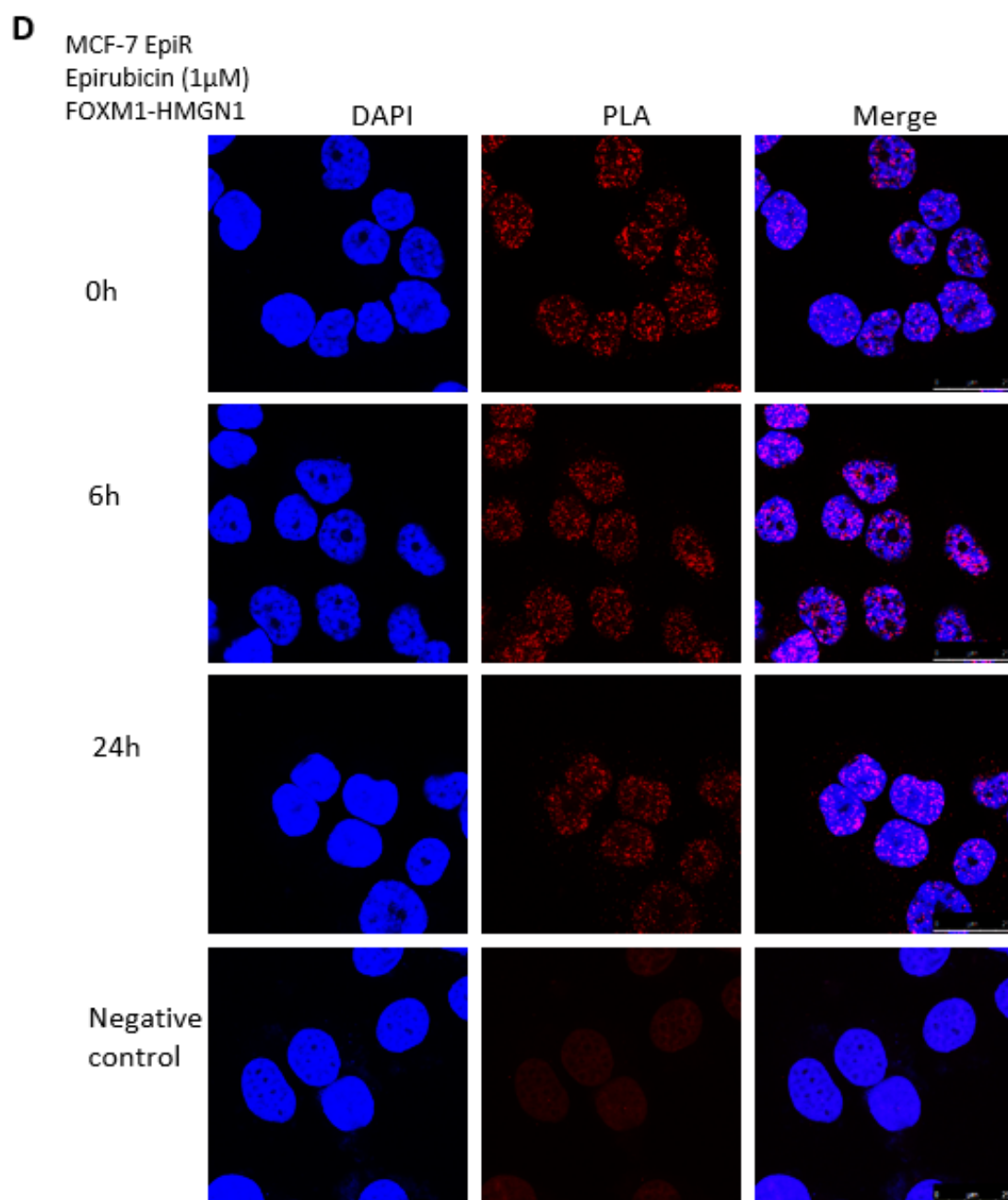


24h



Negative
control





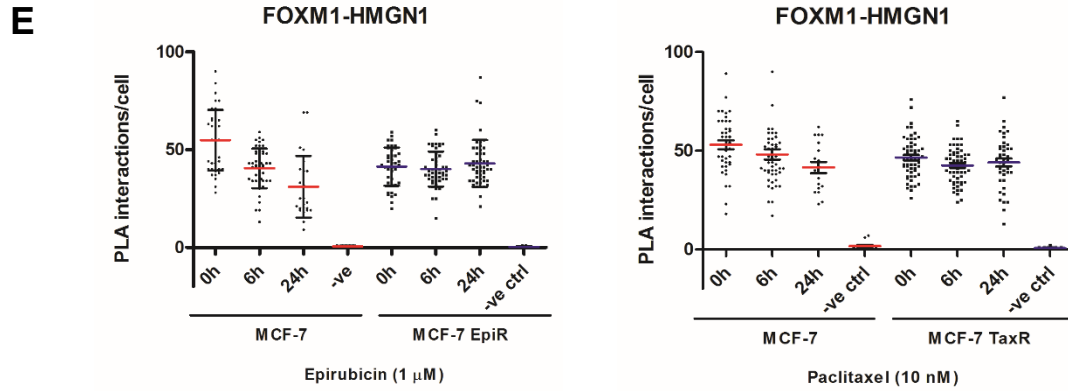


Figure 3. 7.2 *In situ* PLA for protein-protein interactions between FOXM1 and HMGN1.

PLA in MCF-7, TaxR and EpiR cells were fixed at 0h, 6h and 24h following treatment of either 10 nM paclitaxel or 1 μ M epirubicin treatment respectively. DNA was stained with 4,6-diamidino-2-phenyl indole (DAPI) in blue. The PLA-positive red dots represent the amplified FOXM-HMGN1 interactions. For the control, cells were without adding the primary antibodies. Representative confocal images are shown. MCF-7 (**A**) and TaxR (**B**) were treated with paclitaxel; MCF-7 (**C**) and EpiR (**D**) were treated with epirubicin for different time points. (**E**) Positive PLA signals were captured from z-stack confocal images and the amount of PLA signals were counted using Image J. The plot represents the number of FOXM1-HMGN1 interaction per cell, six images that each contains about 10 cells were taken account for each condition. Data displayed as means $\pm$ s.d.

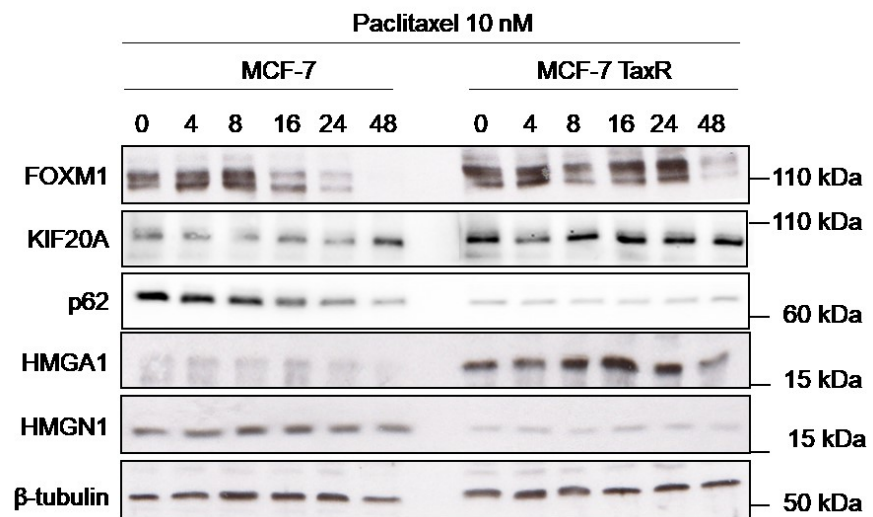
3.9 Distinct expression patterns of FOXM1 and HMG proteins upon drug treatments

Since both co-IP and PLA results demonstrated that FOXM1-HMGA1 and FOXM1-HMGN1 interactions were decreased upon drug treatment in MCF-7 cells, I then examined if this reduction is caused by interruption of individual protein expression in response to paclitaxel and epirubicin drug treatment. The results showed that, upon either 10 nM paclitaxel or 1 μ M epirubicin drug treatment, FOXM1 expression was gradually decreased in sensitive MCF-7 cells and remained at high levels in resistant cells over the time course. Besides, HMGA1 and HMGN1 expression levels did not vary significantly. Furthermore, the mRNA levels of FOXM1, HMGA1 and HMGN1 were measured using RT-qPCR and the results showed similar expression patterns as protein expression (Figure 3.8 C). Moreover, the results showed that KIF20A displayed a similar expression pattern as FOXM1 upon drug treatment of which was reduced in MCF-7 and remained consistent high levels in the resistant cells. Whereas, p62 was downregulated to some extent in MCF-7, and maintained the same level over the time-course in the resistant cell lines. Notably, the p62 mRNA levels showed a distinct expression pattern in which it was gradually increased. This might be due to p62 is a multifunctional protein that involved in multiple cellular processes. The protein reduction could be caused by increased autophagic cell death upon drug treatment as p62 protein was degraded during the process; whereas the increased mRNA level may be related to loss of FOXM1 inhibitory control at the transcription level in MCF-7 cells.

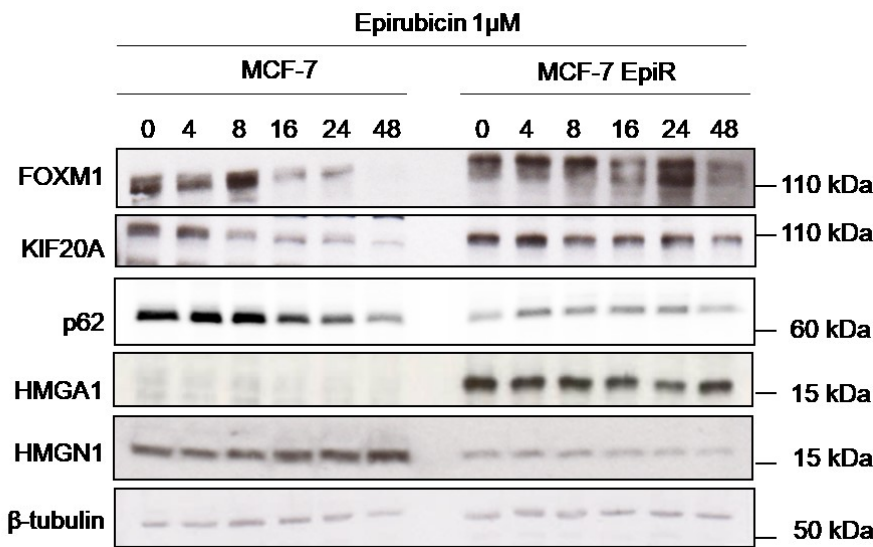
In conclusion, results suggested that paclitaxel and epirubicin drug treatment downregulates FOXM1 and HMGA1 protein and mRNA levels in the sensitive MCF-7 cells but not in the resistant ones, therefore previous co-IP and PLA results which

indicated the diminished interactions upon drug treatment might due to the FOXM1 expression reduction in MCF-7 cells.

A



B



C

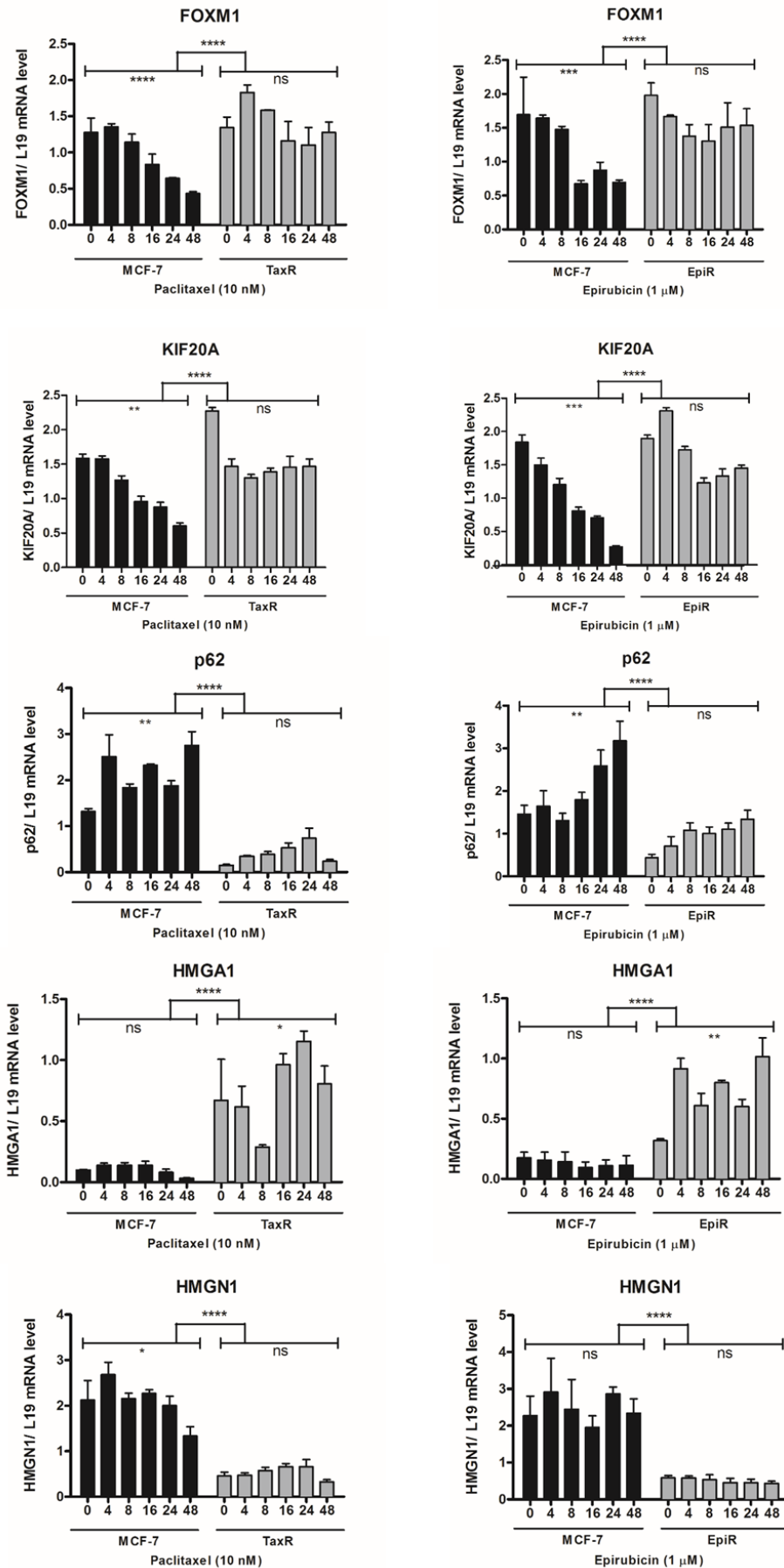


Figure 3. 8 The expression levels of FOXM1 and relevant proteins in response to drug treatments at different time points.

(A) Representative western blot determining the protein expression levels of FOXM1, KIF20A, p62, HMGA1 and HMGN1 in MCF-7 and MCF-7 TaxR cell lines after being treated with 10 nM of paclitaxel at different time points 0, 4, 8, 16, 24 and 48 h. β -tubulin was used as loading control. (n=3) (B) MCF-7 and MCF-7 EpiR cell lines were treated with 1 μ M epirubicin in MCF-7 and MCF-7 EpiR for time points indicated. (C) RT-qPCR analysis shows the relative mRNA expression levels of the five genes in MCF-7 and TaxR or MCF-7 and EpiR cell lines as indicated. Results were normalized against L19 gene values. Data were analysed by two-way ANOVA with the Turkey's multiple comparisons post-test, and $P^* < 0.05$; $** P < 0.01$; $*** P < 0.001$; $**** P < 0.0001$; ns, non-significant. (n=3)

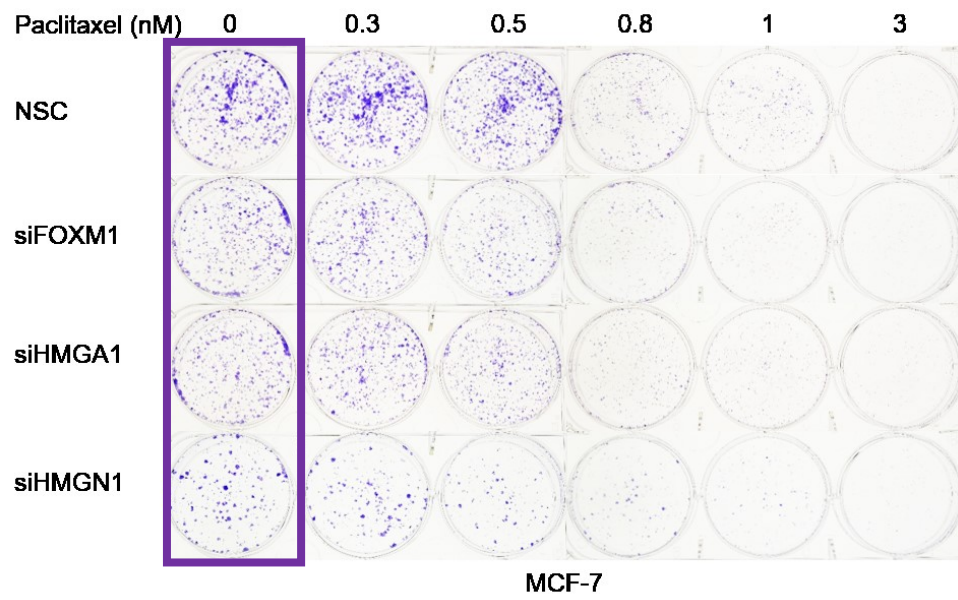
3.10 Silencing FOXM1, HMGA1 or HMGN1 sensitize breast cancers cells in response to drug treatment

Previous results suggest the reduced interactions of FOXM1-HMGA1 and FOXM1-HMGN1 upon drug treatment might be due to the diminished FOXM1 expression with treatment in the parental MCF-7 cells. Nevertheless, both FOXM1 expression and its associated interactions remain consistent in the TaxR and EpiR cell lines which implies the expression and interactions of FOXM1, HMGA1 and HMGN1 might be related to chemotherapeutic drug sensitivity. Clonogenic assay was then performed to examine whether disruption of their protein interactions could affect drug response in breast cancer cells. Hence, FOXM1, HMGA1 and HMGN1 were silenced using corresponding siRNAs in MCF-7, TaxR and EpiR cells. Cells were then treated with different concentrations of either paclitaxel or epirubicin and grown for approximately 10 days. The results demonstrated that interruption of FOXM1, HMGA1 and HMGN1 by the corresponding siRNA resulted in a significant reduction of cell colony formation even prior to drug treatment in all three cell lines. This observation suggested that FOXM1, HMGA1 and HMGN1 might play essential roles in cell proliferation. Following paclitaxel and epirubicin treatment, plots (Figure 3.9.1 C and 3.9.2 C) show that knockdown of FOXM1, HMGA1 and HMGN1 lead to enhanced sensitization to drug treatment in both parental MCF-7 and EpiR cells. In addition, the sensitization effect was not statistically significant in the TaxR cells.

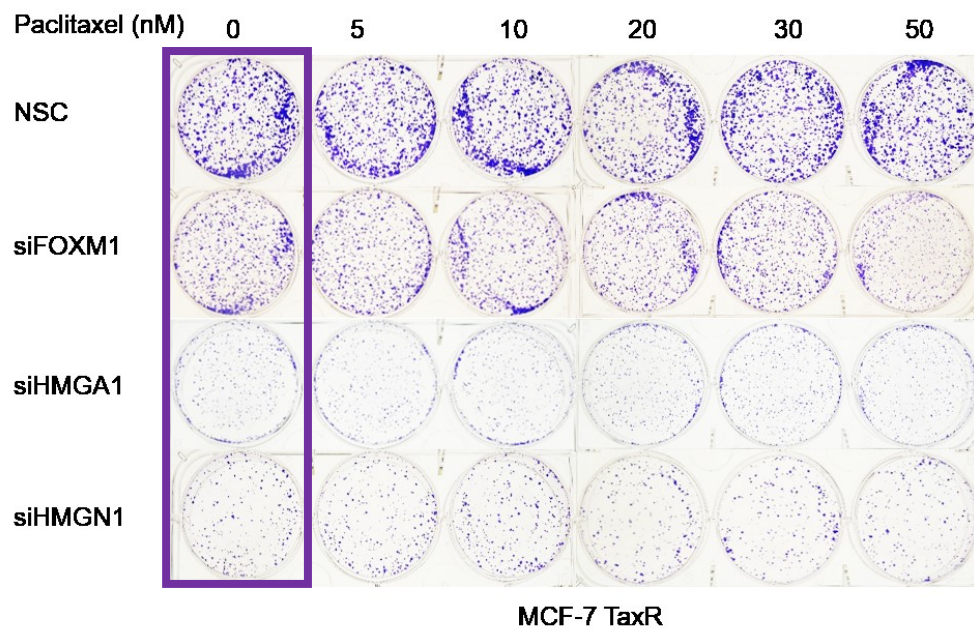
Collectively, the results demonstrated that silencing of FOXM1, HMGA1 and HMGN1 proteins significantly affects cell colony formation and also further sensitizes the cell to paclitaxel and epirubicin treatment in MCF-7, TaxR and EpiR cells. These findings suggested that FOXM1, HMGA1 and HMGN1 are involved in cell survival, and

disruption of their expression could enhance drug efficiency in the breast cancer patient.

A



B



C

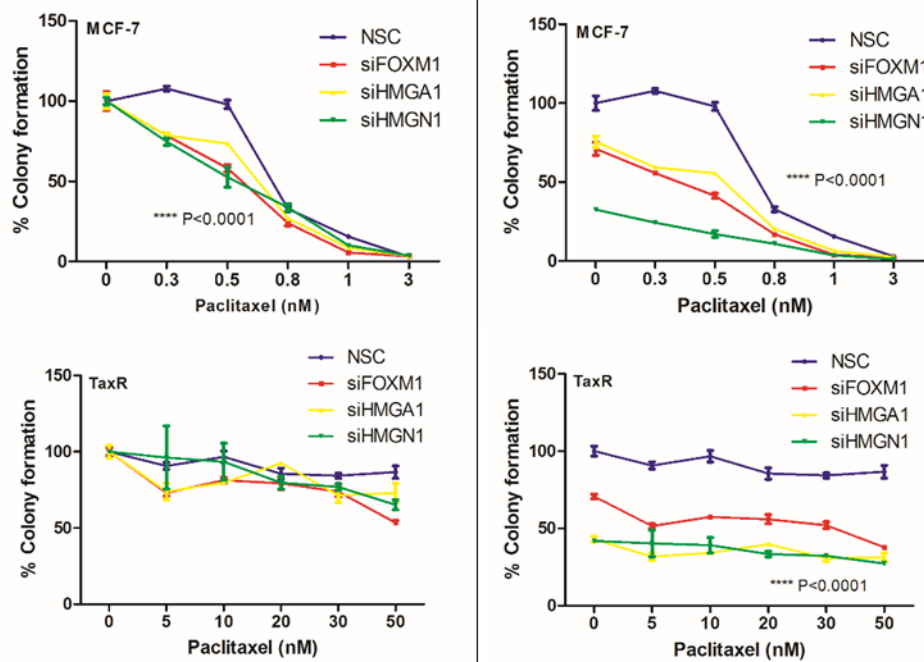
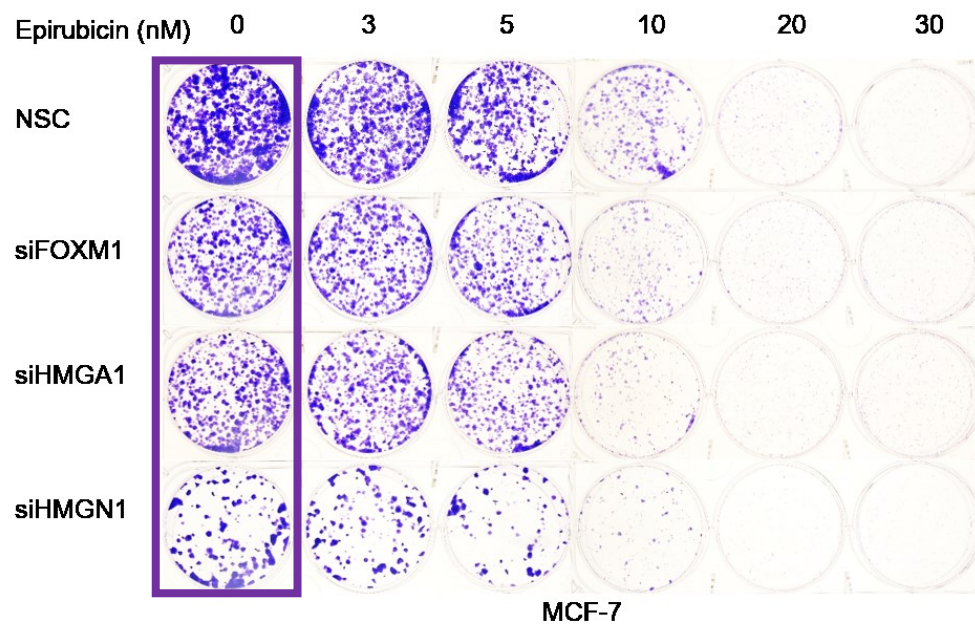


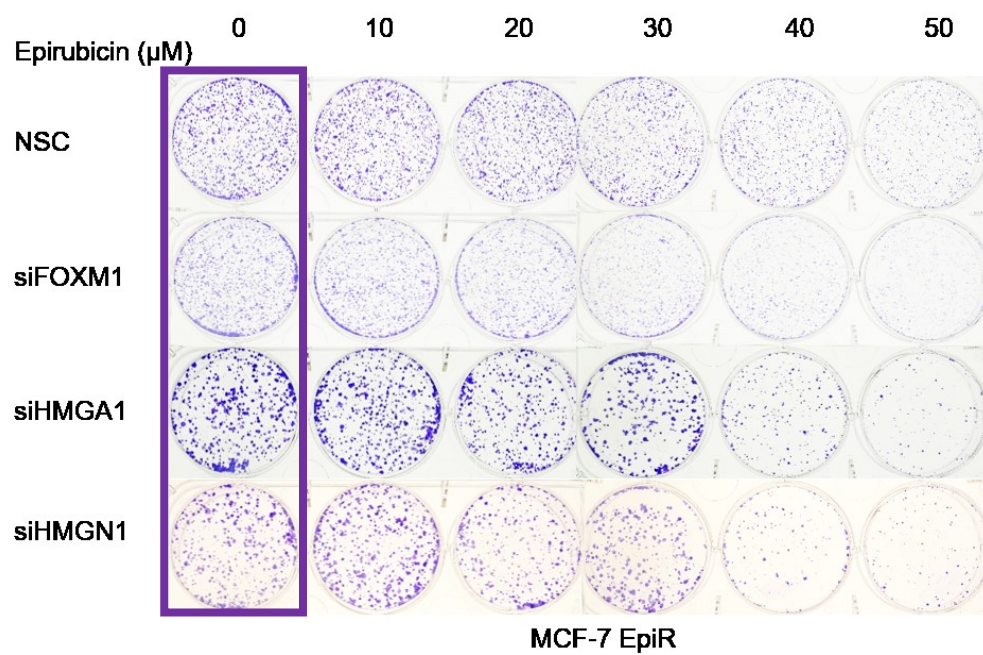
Figure 3. 9.1 Knockdown of FOXM1, HMGA1 and HMGN1 reduces colony formation.

In the clonogenic assay, MCF-7 and TaxR cells were transfected with FOXM1 siRNA, siHMGA1 siRNA, HMGN1 siRNA and non-silencing control (NSC) respectively. Then, 2000 cells were seeded per well in the six-well plate. Cells were treated with different concentrations of paclitaxel the following day. After 48 h of incubation with the drug, cells were cultured in fresh DMEM media, and grown for approximately 10 days. The experiment was carried out in duplicates and representative images are shown. **(A)** MCF-7 and **(B)** TaxR were treated with different concentrations of paclitaxel as indicated. **(C)** Percentage of colony formation was measured using Image J. Left panel represents measurement normalized to NSC non-treated control and right panel shows the values normalized to the non-treated control of each condition. MCF-7 and TaxR cell lines are labelled with each graph. Data were analysed by two-way ANOVA with Turkey's multiple comparisons test post-hoc test (n=3). The asterisks (****) indicate significant difference at p<0.0001 in comparison to the NSC group.

A



B



C

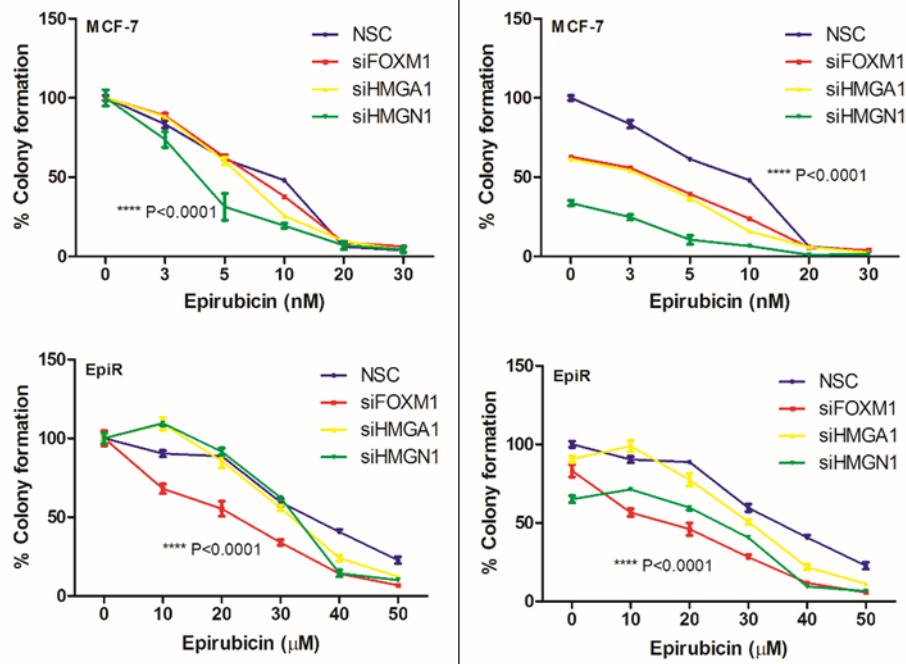


Figure 3. 9.2 Knockdown of FOXM1, HMGA1 and HMGN1 significantly reduces cell colony formation and sensitizes cellular response to epirubicin treatment.

FOXM1, HMGA1 and HMGN1 were silenced using the corresponding siRNA in MCF-7 and EpiR cells, non-silencing siRNA (NSC) was used as control. After 24 h transfection, 2000 cells/well were seeded for clonogenic assay. (A) MCF-7 and (B) EpiR cells were then treated with different dosages of epirubicin as indicated. The following procedures were the same as the cells treated with paclitaxel. Representative images are shown (n=3). (C) The percentage of colony formation was analysed using Image J. Left panel shows the clonogenic results that normalized to non-treated control with NSC whereas right panel represents data normalized to non-treated control of each condition. Different cell lines were indicated in each graph. Data were analysed by two-way ANOVA with Turkey's multiple comparisons post-test (n=3). The asterisks (****) indicate significant difference at $p < 0.0001$ in comparison to the NSC group.

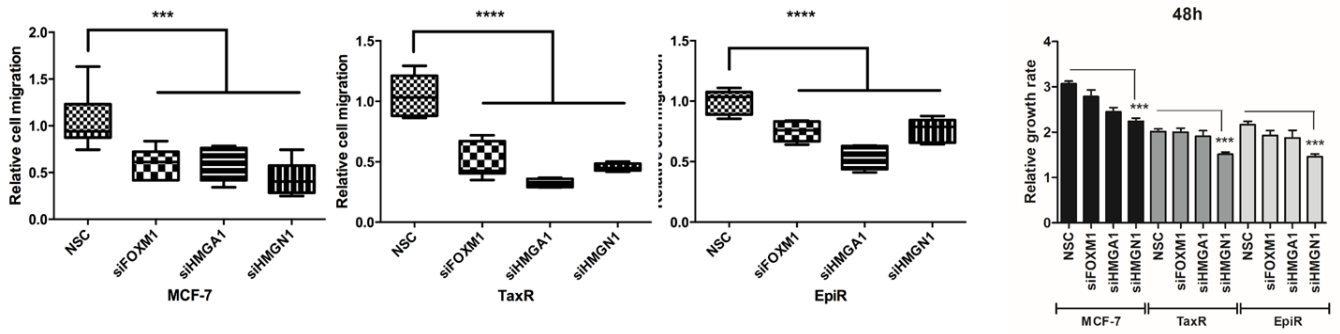
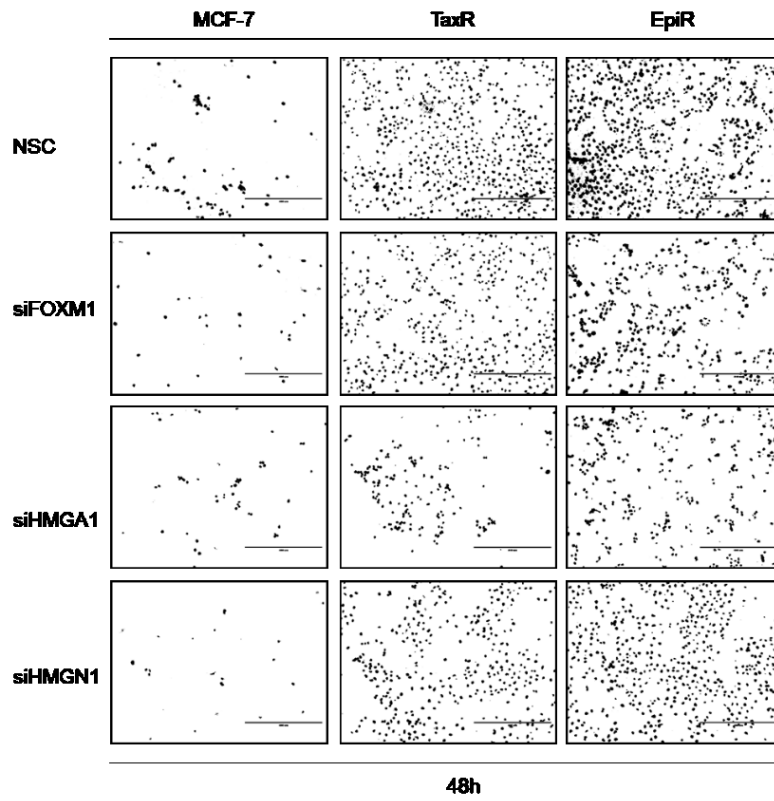
3.11 Silencing of FOXM1, HMGA1 or HMGN1 reduces cell migration and invasion

To further evaluate the role of the interactions between FOXM1, HMGA1 and HMGN1 in breast cancer, migration and invasion assays were performed. Since we have observed a significant reduction in colony formation after silencing FOXM1, HMGA1 and HMGN1, thus a proliferation assay was also conducted alongside to evaluate whether the effects are caused by decreased protein depletion or cell proliferation. Only the cells that enable to migrate through the membrane after 48 h incubation has taken account for migration. Results demonstrated that silencing of either one of these three genes significantly reduced the cell migration in all three cell lines, especially with knockdown of HMGA1 in the resistant cells. The proliferation results demonstrated that knockdown of either FOXM1 or HMGA1 did not significantly affect cell proliferation, which suggested that the FOXM1 and HMGA1 expression are involved in the regulation of cell migration. Alternatively, the proliferation results showed that HMGN1 depletion reduced cell proliferation when compared to the cells without transfection, which suggested that the inhibited migratory effect after HMGN1 knockdown might partially due to the reduced proliferation rate.

Consistent with these findings, invasion results also showed that FOXM1 knockdown significantly decreased cell invasion in MCF-7 cells. In addition, silence of HMGA1 or HMGN1 expression resulted in decreased cell invasion in the resistant cell lines but was not statistically significant in the MCF-7 cell. Moreover, the proliferation assay revealed that depletion of HMGA1 and HMGN1 significantly reduced cell proliferation rate at 96 h post-transfection. Nevertheless, silencing of FOXM1 did not affect cell proliferation at this time point, which suggested that that FOXM1 is a key factor involved in cancer cell invasion.

Taken together, it suggested that FOXM1 is a key factor in cell migration and invasion, depletion of FOXM1 significantly inhibits cancer cell mobility; on the other hand, HMGA1 and HMGN1 are essential for cell proliferation, silencing of their expression critically reduced cell growth rate at 48 h post-transfection and onwards. Moreover, HMGA1 and HMGN1 may be also involved in regulating these malignant processes through interacting with FOXM1.

A



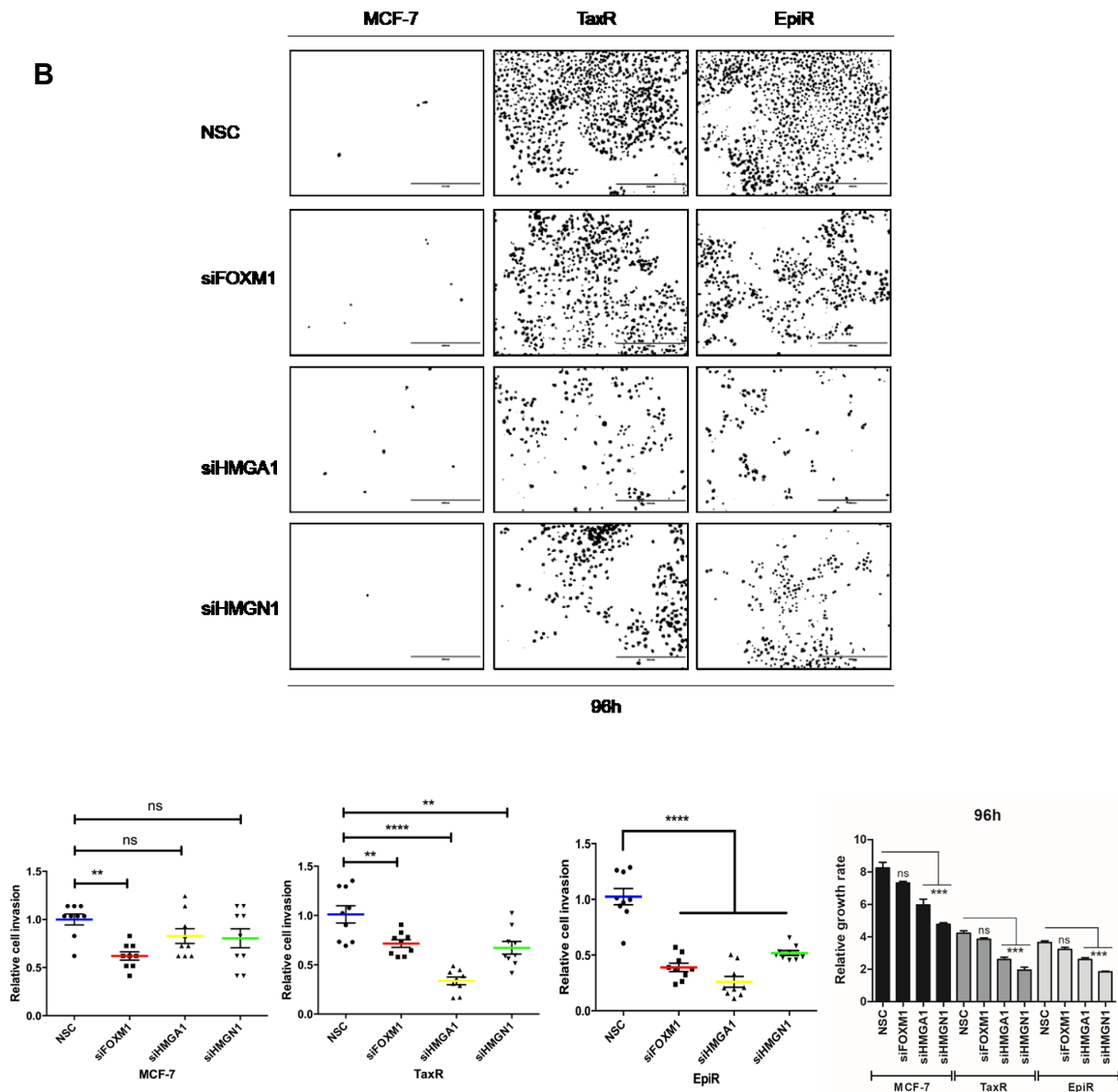


Figure 3. 10 Silencing of FOXM1, HMGA1 or HMGN1 significantly reduces breast cancer cell migration and invasion.

MCF-7, TaxR and EpiR cell lines were transiently transfected with siRNA (siFOXM1, siHMGA1 or siHMGN1) and non-silencing siRNA as control (NSC). After 24 h, (A) 10,000 cells were reseeded in each well and grown for 48 h for migration assay; (B) and 20,000 cells were reseeded on transwell membrane pre-coated with Matrigel for invasion assay. Following incubation, cells that migrated through the membrane were fixed and captured in 9 fields under a 10 × objective lens. The number of migrated

cells was then measured using Image J. Representative images are shown and relative cell invasion were calculated by normalising to NSC of each cell line. Proliferation assay was performed alongside and absorbance was read at 492 nm. Data were analysed by one-way ANOVA with the Turkey's multiple comparisons test, and **** $P < 0.0001$ and *** $P < 0.001$. (n=2)

3.12 Discussion

In this project, I have demonstrated that FOXM1 interacts with HMGA1 and HMGN1 as protein-protein complexes. FOXM1 regulates the transcription of *KIF20A* and *p62* by binding directly to their promoter regions, and affects subsequent protein expression levels. In particular, results suggest that FOXM1 activates *KIF20A* and inhibits *p62* at the mRNA level. Subsequent ChIP analyses reveal that FOXM1 and HMGA1 compete for the same binding sites on *KIF20A* and *p62* promoters, whereas HMGN1 is required for stabilising FOXM1 binding to the promoter regions. Moreover, the functional effects in terms of their interactions in response to drug treatment, proliferation, cell migration and invasion were further investigated. Following paclitaxel and epirubicin drug treatment, the FOXM1-HMGA1 and FOXM1-HMGN1 interactions are reduced in MCF-7 cells but remained constant in the TaxR and EpiR cell lines. Simultaneously, the expression level of each individual protein was examined in a time-course drug treatment assay. Results showed that drug treatment reduces FOXM1 expression in the sensitive MCF-7 cells but not in the resistant ones, implying the reduced interactions might due to the diminished FOXM1 expression. Furthermore, clonogenic assays further demonstrated that knockdown any of FOXM1, HMGA1 or HMGN1 significantly reduces colony formation upon drug treatment which suggested that these proteins are involved in the modulation of chemotherapeutic drug sensitivity. In addition, silencing of FOXM1 attenuates cell migration and invasion, whereas HMGA1 and HMGN1 might be partially involved in the regulation of cell mobility through incorporating with FOXM1.

In this study, I have demonstrated for the first time that FOXM1 physically binds to *p62* promoter and inhibits its expression. *p62*, along with Beclin-1, LC3II are major autophagy mediators and often used as autophagy markers. These proteins are

ultimately degraded after autophagosome fused with lysosome and the degradation products may be recycled to produce energy for cell survival [449]. Over the last few years, FOXM1 has been reported to be involved in regulation of the autophagic survival pathway, yet the molecular mechanism remains largely unknown. Earlier this year, a group has reported that FOXM1 regulates the expression of the major autophagy proteins, Beclin-1 and LC3/II, through binding to their promoters in human triple-negative breast cancer cells (TNBC) and hence promoting autophagic activity [450]. It is also found that disruption of FOXM1 by miR-365-5p resulted in decreased Beclin-1 and LC3/II expression and increased p62 [451, 452]. Given that the level of p62 is considered to be inversely correlated to autophagy, increased p62 may indicate the inhibition of autophagy, therefore the finding further confirms the involvement of FOXM1 in autophagy. Moreover, miR-361-5p overexpression significantly suppresses chemoresistance in gastric cancer, and this is thought to be achieved by inhibiting FOXM1-modulated autophagy process. This provides new insights into the mechanisms that FOXM1 promotes tumorigenesis and chemoresistance may partially through modulating autophagy process. My results reveal that FOXM1 directly binds to p62 at the promoter and may inhibit its mRNA expression, yet the luciferase reporter assay must be conducted to draw the conclusion. In here, the decreased p62 expression upon FOXM1 overexpression could be a consequence of FOXM1-induced autophagic flux in which the p62 is degraded. On the other hand, the regulation of FOXM1 on *p62* may be involved in other biological signalling processes since both of them are multifunctional proteins. The functional role of p62 is further examined in Chapter 4.

Furthermore, the interactions between FOXM1 and HMG proteins on *KIF20A* and *p62* gene promoters suggest a possible mechanism of which their interactions modulates

FOXM1 transcriptional activity. In MCF-7 cells, although the expression level of HMGA1 is relatively low, it appears to play a predominant role in modulating the FOXM1 binding. When HMGA1 binds to the promoter regions of *KIF20A* or *p62* gene, it simultaneously restricts FOXM1 transcriptional activity. This is probably mediated through two mechanisms. First, they might compete to same DNA binding sites, as the ChIP results showed that knockdown FOXM1 promotes HMGA1 binding to the *KIF20A* and *p62* promoters, while knockdown HMGA1 enriches FOXM1 binding. A second potential mechanism is that HMGA1 binds to FOXM1 as a complex and consequently prevents its binding ability. The PLA results revealed that the majority of the FOXM1-HMGA1 interactions were in the cytoplasm, it is therefore that HMGA1 interacts with FOXM1 and eventually induces cytoplasmic translocation. In this way, HMGA1 sequesters FOXM1 from the nucleus and limits FOXM1 transcription activity in the nucleus. In contrast, these two mechanisms might be, to some extents, limited in the TaxR and EpiR resistant cells, where HMGA1 no longer against FOXM1 in binding to the chromatin. Further investigation is needed to elucidate the precise mechanism of FOXM1-HMGA1 axis. On the other hand, HMGN1 plays an essential role in enhancing the transcriptional activity of FOXM1. Results demonstrated that FOXM1 knockdown does not affect HMGN1 binding to the chromatin, whereas silencing of HMGN1 significantly reduces FOXM1 binding to the *KIF20A* and *p62* promoter regions. In consequence, HMGN1 is required for FOXM1 transcription in MCF-7 cells. Given the main function of HMGN1 is to decompact chromatin and promote accessibility for transcription factors, it is likely that HMGN1 binds first to the chromatin and then recruits FOXM1 to the promoter regions, and to eventually initiate gene transcription. However, this proposed mechanism is not applicable in the TaxR and EpiR resistant cell lines, where HMGN1 does not influence FOXM1 binding. In the

resistant cells, FOXM1 may have other binding partners instead of HMGN1, in which FOXM1 transcriptional activity is no longer limited by the expression of HMGN1. This might be due to the deregulated FOXM1 signalling in resistant cells.

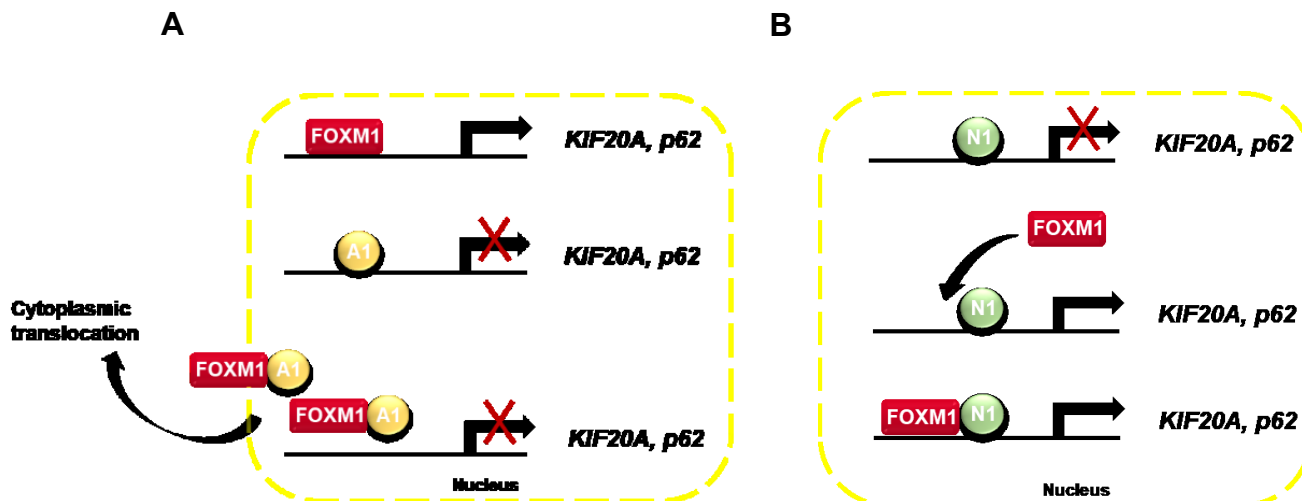


Figure 3. 11 The proposed mechanism of interactions between FOXM1, HMGA1 and HMGN1 in breast cancer MCF-7 cells.

(A) When HMGA1 exists, HMGA1 inhibits FOXM1 transcriptional activity by competing to the same binding sites; alternatively HMGA1 binds to FOXM1 and restricts its binding ability by translocating the complex to the cytoplasm. (B) HMGN1 binds first to the chromatin and recruits FOXM1 to its targets; such interactions enhance FOXM1 transcriptional activity.

In addition, the interactions of FOXM1-HMGA1 and FOXM1-HMGN1 are reduced upon drug treatment in MCF-7 cells as observed with both co-IP and PLA experiments. The drug treatment time-course experiments reveal that the FOXM1 expression is downregulated gradually in response to paclitaxel and epirubicin treatment in the MCF-7, and the significant reduction of the interactions between FOXM1 and HMGA1/HMGN1 might due to the reduced expression levels of FOXM1. Consistent with this idea, FOXM1 expression levels remain constant after drug treatment in the resistant cell lines and their interactions are also maintained at the same levels. This implies that FOXM1 is the essential regulator in modulating chemotherapeutic drug

sensitivity. Further clonogenic results indicate that silencing of HMGA1 or HMGN1 sensitize the cell response to paclitaxel and epirubicin treatment. It is known that these two HMG proteins are involved in DNA repair machinery as loss of their expression significantly impacts cell survival. The sensitized response may also be attained by disruption of HMG-associated FOXM1 activities. Moreover, HMGN1 is a critical factor for cell proliferation as the cell viability is hugely decreased when silencing of HMGN1. This implies that HMGN1 is not only important in modulating FOXM1-mediated activities but is also critical for other cellular processes, such as clonogenicity and cell proliferation.

To conclude, in this chapter, I confirmed the protein-protein interactions between FOXM1 and its cofactors HMGA1 and HMGN1, as well as the effects of their associations on FOXM1 transcriptional activity. Moreover, FOXM1 is a master regulator that involved in multiple stages of cancer progression, disruption of FOXM1 significantly promotes drug sensitivity and reduces cancer cell migration and invasion. As FOXM1's cofactors, HMGA1 and HMGN1 affect these processes may through regulating FOXM1 transcriptional activities. The functions of FOXM1's targets *KIF20A* and *p62* in terms of drug sensitivity, cell migration and invasion are further investigated and discussed in following chapters.

CHAPTER 4

The angiogenic and metastatic functions of p62

4.1 Introduction

The adaptor protein p62 is a well-known autophagy marker that its expression level is inversely related to autophagic process. Given its multiple domains can interact with many signalling molecules such as PKC, raptor and Keap1, p62 is thought to be involved in various signalling pathways [197, 453, 454]. Interestingly, our collaborator (Professor Quentin Liu's lab) in Dalian, China, has identified p62 interacts with vimentin protein and thus promoting invasive phenotypes of breast cancer cells. They have examined the relevant functions in the mouse model. In the meantime, I used the zebrafish as an *in vivo* model to examine whether alterations of p62 affect breast cancer progression in terms of angiogenesis and metastasis.

Metastasis is a complex multistep process that involves the spread of cancer cells from the primary tumour to surrounding tissues and distant organs, which is responsible for up to 90% deaths associated with breast cancer [455]. In recent years, the usage of zebrafish for cancer research in related to angiogenesis and metastasis, has increased dramatically because of several advantages. These include high genetic homology to humans, transparent embryos that facilitate observations, high fecundity which allows large number of replicates in each assay, and relatively low husbandry costs [22-24]. The transparent embryos provide live tracking and non-invasive imaging which significantly aid the observation without having to sacrifice the animals. Moreover, like humans, zebrafish have both innate and adaptive immune systems, in which the adaptive immunity is morphologically and functionally matured only after 4-6 weeks post-fertilization [34, 35]. This time period allows most

transplanted tumour cells to survive and metastasize in the body of zebrafish. Additionally, it has been identified that the tumour microenvironment of zebrafish is strikingly similar to humans [46]. Hence, zebrafish is a valuable tool for investigating the fundamental mechanisms underlying cancer progression.

4.2 Silencing of p62 significantly diminishes tumour-induced angiogenesis in zebrafish

To determine if p62 is associated with angiogenic development, MCF-7 breast cancer cells were transfected with p62 siRNA to silence p62 expression and were injected into the yolk sac of *casper* Tg (*fli:GFP*) zebrafish embryos. The transgenic *casper* fish line has been genetically engineered to present GFP-labelled blood vessels with a completely transparent body. This line is ideal for angiogenesis study as it allows to monitor the development of the blood vessels following tumour injection without requirement of invasive procedures. Prior to injection, cells were labelled with CM-Dil, which is a lipophilic red fluorescent dye that is retained by the cell membranes. Injection of MCF-7 cells in the yolk sac enabled the cells to be kept in a compartmentalized environment with sufficient source of nourishment, as its endogenous maternally deposited yolk provides nutrients for the embryo to survive for the first five days of life [456]. Injections were performed 2 days post-fertilization (dpf) of the embryos. At this stage, the embryo lacks the adaptive immune system and has slowly developed the innate immunity, thus providing time for the implanted cells to be examined. Moreover, the sub-intestinal vessel (SIV) complex resides in yolk sac and is proximity to injected tumour. The pattern of SIV was further analysed for angiogenic effect.

The results demonstrated that embryos injected with p62 silenced MCF-7 cells showed a significant reduction ($P < 0.001$) of the new sprouting blood vessels when compared to the non-silencing control group (Figure 4.1 E). Consistent with this, the percentage of sprouting vessels in p62 depleted cell implantation was about two times lower than the control group (40%: 82%). These findings suggest that p62, may be involved in modulating angiogenesis during cancer progression.

of a non-injected zebrafish. **(C)** Transgenic *casper* (*fli:GFP*) zebrafish embryos at 2 dpf transplanted with NSC siRNA. Blood vessels of the embryos are in green and implanted tumour mass in red. Presence of two blood vessels sprouting from the SIV is indicated with white arrows. **(D)** Zebrafish embryos at 2 dpf transplanted with MCF-7 cells with silencing of p62 shows one sprouting blood vessel (indicated by white arrow). **(E)** Dot-plot indicating the number of sprouting blood vessels present in each zebrafish injected with NSC and p62 siRNA cells. Each was normalized to the non-injected control to account for natural variation in the SIV morphology. Statistical significance was analysed by one-way ANOVA, and *** $P < 0.001$. **(F)** Percentage of zebrafish with the presence of sprouting blood vessels in the total number of injected zebrafish. (n=2)

4.3 Knockdown of p62 attenuates cell migration in zebrafish

To further assess the role of p62 in breast cancer progression, the effects of its depletion on cell migration were studied in the zebrafish embryo xenograft assay. In the experiment, MCF-7 cells were transfected with either p62 siRNA or non-specific silencing siRNA at 24 h before implantation. Here, I used an optically transparent non-pigmented mutant line (TraNac), which allows clear visualization of the movement of pre-labelled tumour cells [457]. At 3 days post-implantation, silencing of p62 expression in MCF-7 cells showed a significant reduced invasive behaviour when compared to the control group (Figure 4.2 A). Moreover, zebrafish embryos engrafted with p62 siRNA MCF-7 cells had a lower migration incidence (20/90, 22.2%) compared to control (49/111, 44.1%) (Figure 4.2 B). Quantification of the correlated fluorescence intensity of invasive cells showed a significant decrease elicited by p62 knockdown (Figure 4.2 C). Taken together, these results suggest that alterations of p62 expression affect cancer cell migration.

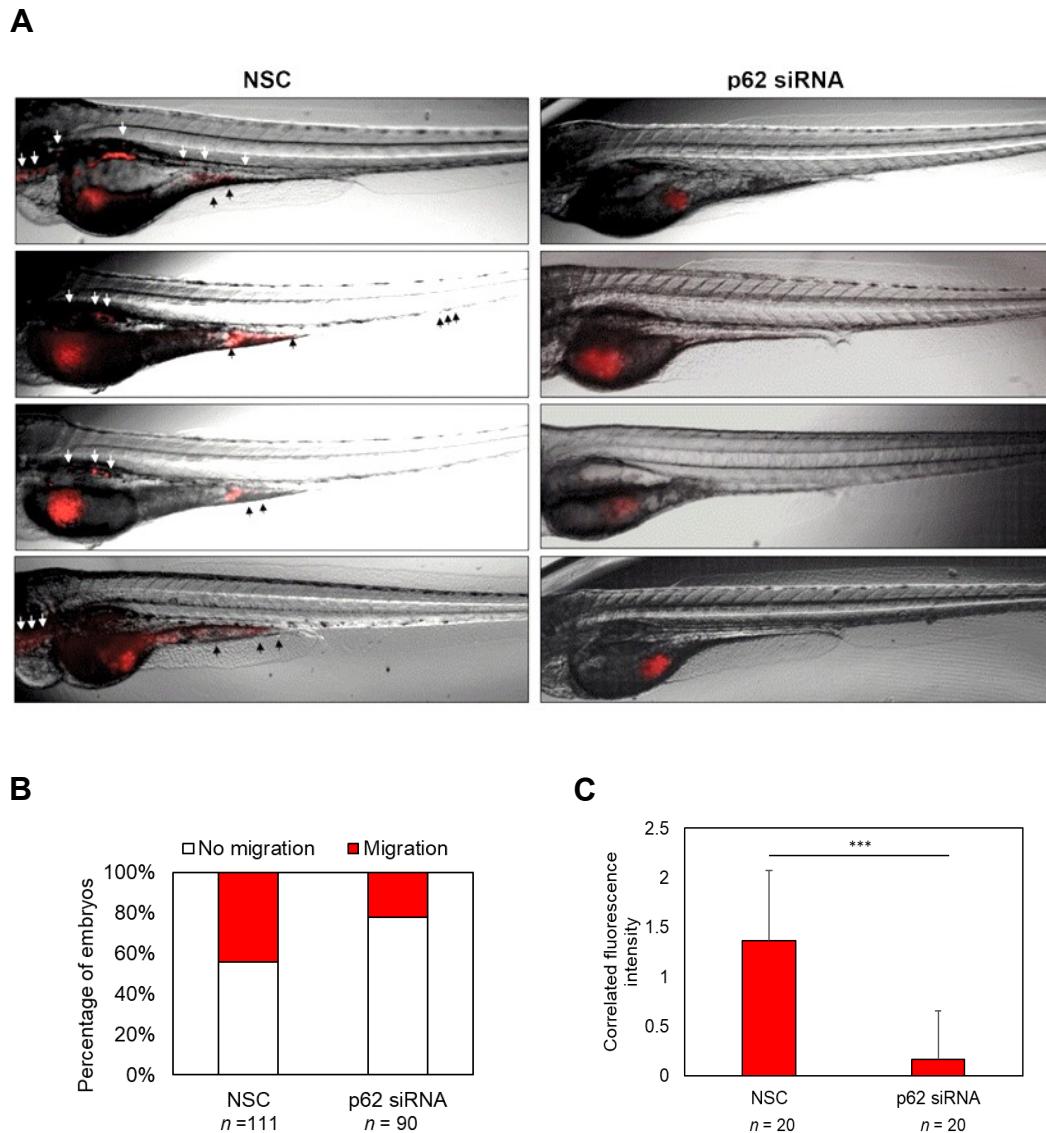


Figure 4. 2 Depletion of p62 reduces cell migration in Tra/Nac zebrafish.

MCF-7 cells were transfected with p62 siRNA or non-specific silencing control (NSC) respectively. Approximately 150 CM-Dil labelled MCF-7 cells were injected into each Tra/Nac zebrafish embryo at 2 dpf. Cell migration was observed under the fluorescent microscope at 3 dpi. Some of the embryos died at this stage were not counted. **(A)** Tra/Nac zebrafish embryos at 2 dpf transplanted with MCF-7 NSC and MCF-7 p62 siRNA. The implanted cells were detected in red under fluorescent microscope. Presence of migrated tumour cells from yolk sac to the tail or brain is indicated with black or white arrows, respectively. **(B)** Percentage of embryos of which presented any

migratory cells in the total number of injected zebrafish. (C) The corrected total cell fluorescence (CTCF) was measured using the formula: $CTCF = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings})$ for each imaged specimen. Data were analysed by one-way ANOVA, and *** $P < 0.001$. Error bars represented means $\pm$ s.d. (n=3)

4.4 Discussion

To summarize, in this chapter, I demonstrated an autophagy-independent role of p62 which is involved in modulating angiogenesis and cell migration in the zebrafish model. In agreement, elevated p62 expression has been found to be correlated with tumours exhibiting the aggressive features and poor prognosis [458-462]. In parallel, our collaborator in Quentin Liu's lab has showed that genetic ablation of p62 significantly reduces the size of metastatic nodules in immunodeficient mouse models. Moreover, it is thought that p62 promotes breast cancer invasion through interacting with vimentin [458]. Vimentin protein is a type III intermediate filament that participated in various processes including cytoskeleton remodelling, EMT, TGF β signalling and Wnt signalling pathways, which are fundamental for tumorigenesis and metastasis [463]. It has been found as one of the most elevated proteins in multiple metastatic cell lines. In our model, p62 exerts a specific, autophagy-independent role that facilitates angiogenesis and cancer cell migration through interacting with vimentin. This finding adds on the multifunctional nature of p62.

Other studies have been reported p62 is able to interact with multiple partner proteins and to participate in different mechanisms in several human cancers [217, 459, 460, 464]. A study has found that in the autophagy-competent cells, oncogenic transcription factor Twist1 can be degraded with autophagic process, whereas its expression is stabilized by the autophagy mediator p62 in autophagy-defective cells [217]. Given the essential role of p62 is to serve as a shuttling protein that delivers ubiquitinated substrates to the lysosome for degradation, it was unexpected to find that p62 interacts with ubiquitinated Twist1 and stabilizes the protein from degradation, and thus promoting cell proliferation and migration. These findings not only confirm the critical role of p62 in promoting cell proliferation and metastasis, but also render a new point

of view that p62 possesses multiple functions in diverse cellular contexts. Besides, p62 has also been reported to promotes EMT and further tumour growth and metastasis through sustaining histone deacetylase 6 (HDAC6) and impairing autophagy flux [465]. Herein, again, it suggested that p62 not only passively acts as an autophagy mediator but also functions as an autophagy suppressor. However, the precise mechanisms underlying the cellular context-dependent modulation of p62 remain unknown and require further investigation.

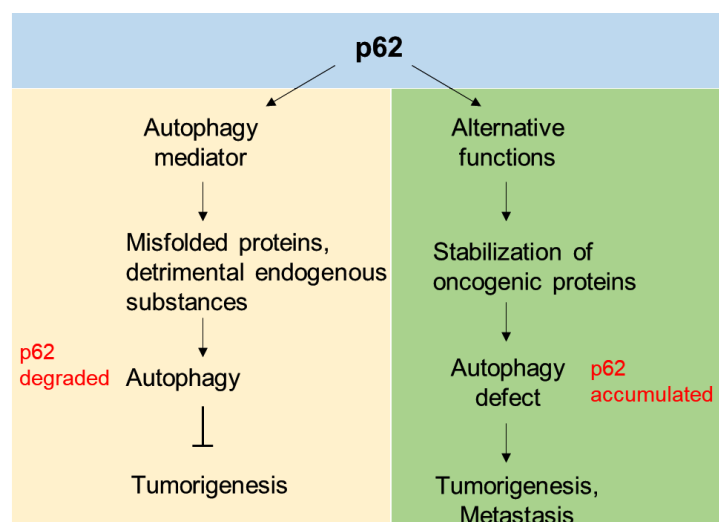


Figure 4. 3 Multifunctional nature of p62 impacts on tumorigenesis and metastasis.

In the project, I used zebrafish model to examine angiogenesis and cell migration and obtained equivalent results as our collaborators did in mice, which suggested that zebrafish and mouse *in vivo* models have equivalent effects in investigating cancer progression in terms of angiogenesis and cell migration. Moreover, observation of blood vessel development is not applicable in mouse model. This is an outstanding

advantage of zebrafish in cancer research. Furthermore, zebrafish live at 28 °C in nature, which is different from the human body temperature of 37 °C. The previous lab member Dr. Laura Bella, has demonstrated that any alterations in the temperature between 28 – 34 °C does not significantly affect the development of embryos, or the tumour induced cell migration and angiogenesis till 7 dpf stage. In addition, this study also provides an standard procedure to examine angiogenesis and cell migration in zebrafish, which can be used for other molecular mechanisms research.

To summarize, this chapter confirmed an alternative role of p62 that rather than mediating autophagy, it can interact with other protein partners and participate in diverse biological processes. In particular, p62 stabilizes vimentin, the major cytoskeletal component of mesenchymal cells, thus promoting tumourigenesis and metastasis. Increasing evidence of specific and autophagy-independent regulation of p62 adds on the complexity of this protein. Therefore, understanding of this protein may shed new lights on cancer prevention and treatment.

CHAPTER 5

Evaluation of the functions of KIF20A in metastatic breast cancer

5.1 Introduction

The mitotic kinesin KIF20A (a.k.a MKLP-2) plays a vital role in spindle formation and cytokinesis. Its abnormal overexpression has been well documented in several cancers, hence is identified as a potential target for cancer treatment [170]. Our previous findings in the lab suggested that the FOXM1-KIF20A axis is important in modulating mitotic spindle formation and deregulated FOXM1 and KIF20A expression may confer drug resistance. In this chapter, the regulation of KIF20A on other hallmarks of cancer progression such as cell migration, invasion and cancer stem-like cell formation were further investigated. Herein, a newly invented KIF20A inhibitor was used.

The first established KIF20A inhibitor is named Paprotrain, which is a reversible inhibitor uncompetitive with ATP and inhibits ATPase activity of KIF20A over 12 other members of the kinesin superfamily [466, 467]. ATPase is a class of enzymes that catalyse ATP to turn into ADP and a free phosphate ion which releases energy. Kinesins, including KIF20A, can use the energy to move along microtubule filaments. The new KIF20A inhibitor, named BKS0349 compound, is an analogue of paprotrain with introduced a methoxy group in the 5-position of indole ring of the original chemical structure [466]. It has been reported that is 10 times more potent than paprotrain and has shown more restricted selectivity on KIF20A ATPase over a large panel of kinases [468]. In this study, I aimed to first evaluate the efficacy of BKS0349 in MCF-7, TaxR and EpiR cell lines, and then use this inhibitor to further investigate the role of KIF20A in terms of cell migration, invasion and self-renewal properties.

5.2 The cytotoxicity effect of BKS0349 in breast cancer cells

I first intended to establish the IC_{50} values of BKS0394 in MCF-7, TaxR and EpiR cell lines. This could provide an optimal dosage to be used for further investigations. To examine the cytotoxicity effects, sulforhodamine B (SRB) assay was used. Sulforhodamine B is an anionic aminoxanthene dye which can form an electrostatic complex with the basic amino acid residues of proteins under mild acidic conditions. Cell density can be analysed based on the measurement of cellular protein content [441]. In this study, the three breast cancer cell lines were treated with a range of BKS0349 concentrations for 0, 24, 48, 72 and 96 h. Surprisingly, I observed that the BKS0349 was more efficient in triggering cell death in the TaxR and EpiR cells when compared to the MCF-7 at all four time points. Moreover, IC_{50} was calculated of the concentrations of the drug that is required for 50% inhibition, which indicates directly the effectiveness of the drug in inhibiting cell viability. Therefore, the lower the IC_{50} , the more potent the drug is. The results are listed in figure 5.1, where the IC_{50} values of resistant cells are in a range of 0.005 μ M to 0.03 μ M (5 nM to 30 nM) which are much lower in comparison to the range 0.01 μ M to 2.40 μ M (10 nM to 240 nM) of MCF-7. This suggests that the BKS0349 compound is more efficient in targeting the drug-resistant TaxR and EpiR cells.

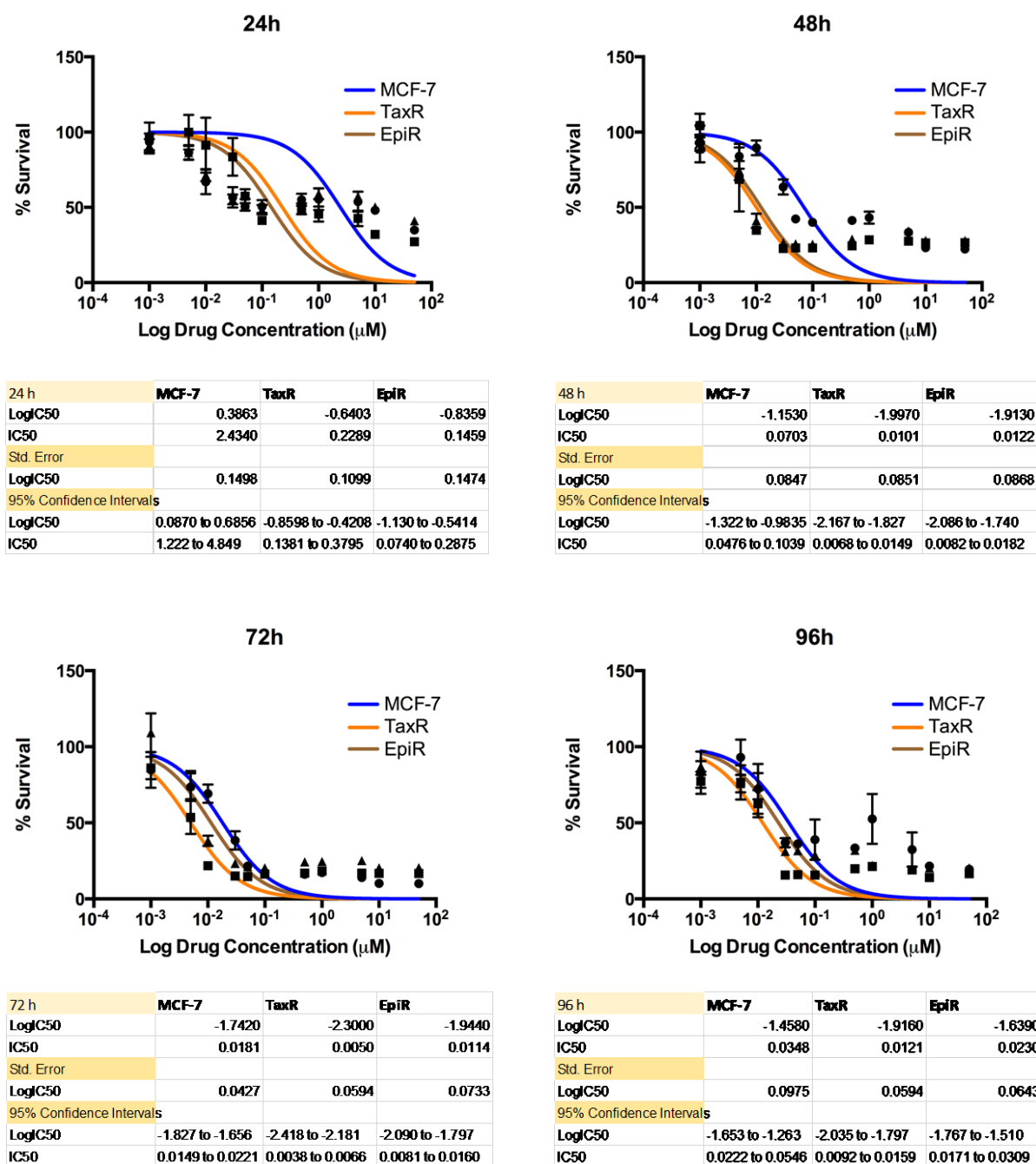


Figure 5. 1 The BKS0349 compound significantly reduces cell viability of TaxR and EpiR cells compared to MCF-7.

SRB assay was performed to examine the cell viability of MCF-7, TaxR and EpiR in response to BKS0349 treatment. Cells were treated with the BKS0349 compound at

concentrations of 0, 0.001, 0.005, 0.01, 0.03, 0.05, 0.1, 0.5, 1, 5, 10, 50 μ M for a different time as indicated. IC₅₀ and 95% confident intervals were determined using GraphPad Prism (Version 6.0) and corresponding values listed below each graph. Results shown are means $\pm$ s.d. of two independent experiments, each with six replicates.

5.3 The cytotoxic effect of BKS0349 following long-term exposure

In the previous SRB experiment, I observed a striking result in which the cytotoxic effect of BKS0349 was higher in the resistant cells. I then performed clonogenic assays to further examine how these breast cancer cell lines could be affected by BKS0349 over long-term treatment. In this assay, cells were continuously incubated with the drug over a period of 7 days. Consistent with the SRB findings, results showed that BKS0349 was more potent in triggering cell death in TaxR and Epi cells. The distinguishable effects began to be observed at concentrations as low as 0.005 μM and became more apparent at 0.01 μM , and significant cell death has observed at concentrations of 0.03 μM and upwards.

Taken together, both the short-term SRB and the long-term clonogenic assays demonstrated that the BKS0349 compound is highly cytotoxic to breast cancer cells lines, especially the resistant TaxR and EpiR cells. These findings confirm that KIF20A is a potential new target for breast cancer chemotherapy and BKS0349 is a putative candidate compound for cancer treatment. Furthermore, the concentration of 20 nM was selected for drug treatment in further experiments since it shows distinguishable inhibitory effects on three cell lines and is not too strong to kill all the cells.

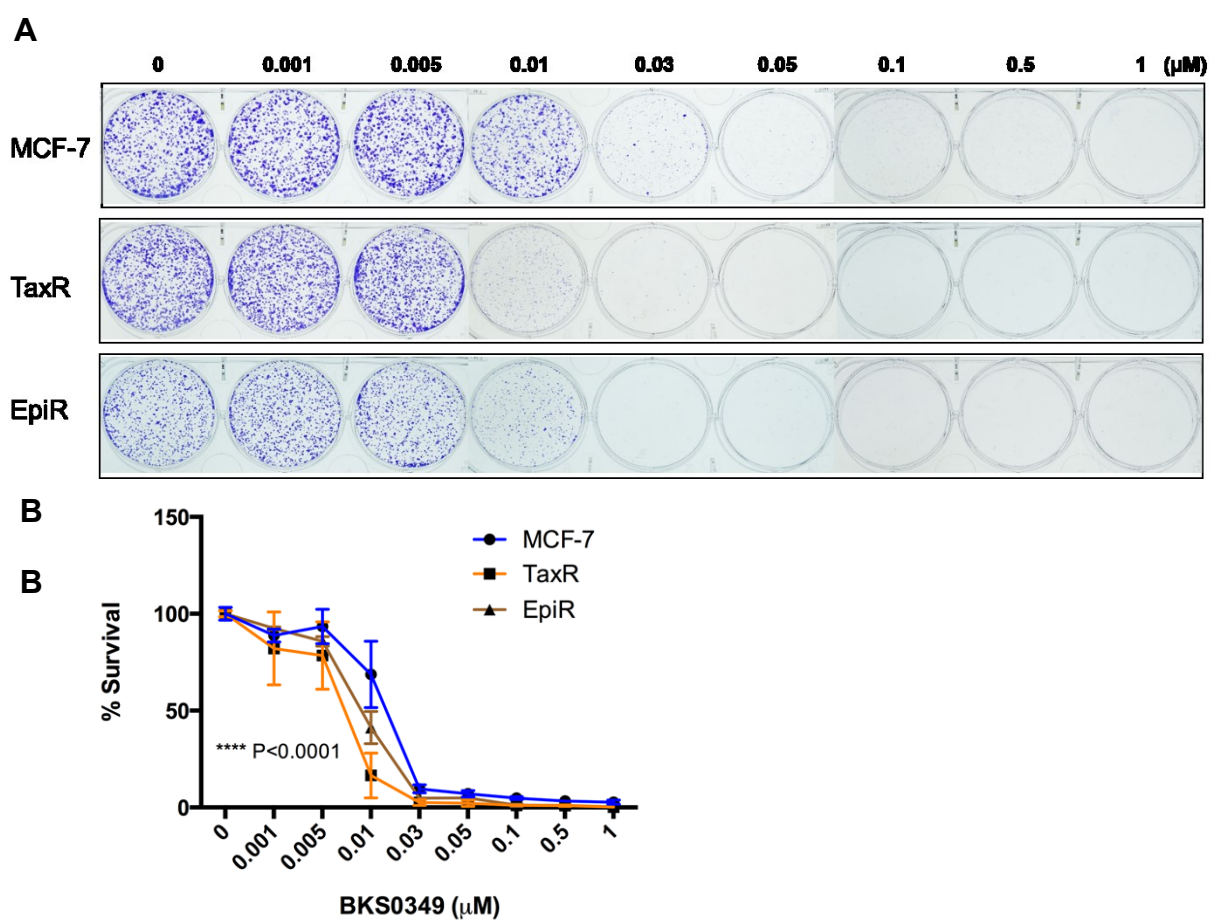


Figure 5. 2 BKS0349 acts more efficiently on TaxR and EpiR cells over a longer term.

MCF-7, TaxR and EpiR cells were seeded onto 6 well plates and treated with a range of drug concentrations as indicated. **(A)** Following 7 days incubation, cells were fixed and stained with crystal violet. Representative images are shown here. **(B)** The number of survived cells was analysed using Image J. Percentage of cell survival are shown with means $\pm$ s.d. Data were analysed by two-way ANOVA with Turkey's multiple comparisons post-test (n=3). The asterisks (****) indicate significant difference at $P < 0.0001$, representing the comparison between % cell survival at different dosages and non-treated control in each cell line.

5.4 BKS0349 significantly diminishes cell migration and invasion of TaxR and EpiR cells

To identify if KIF20A is involved in modulating cell migration and invasion, MCF-7, TaxR and EpiR cells were treated with BKS0349 compound in transwell assays. In the assay, cells were seeded on the upper layer of a permeable membrane. The lower compartment filled with media with or without the BKS0349. Following 16 h and 48 h incubation for migration and invasion respectively, the cells that had migrated through the membrane were taken for further analysis. In this study, 20 nM of BKS0349 was used to treat all three cell lines. This was based on the IC₅₀ values among three cell lines were ranged from 5 nM to 240 nM, with the TaxR and EpiR cells being around 5 nM to 30 nM. The chosen concentrations fell between this range that provides distinguishable effects among the three cell lines, without immediately killing all the cells.

Results revealed that KIF20A inhibition by BKS0349 treatment significantly reduces cell migration and invasion in TaxR and EpiR cells. In comparison to resistant cells, MCF-7 cells showed much lower migratory and invasive activities. Taken together, this suggests that KIF20A plays key role in regulating cell migration and invasion, disruption of KIF20A activity critically diminishes the progress. Furthermore, the compound shows high efficiency which could be a novel candidate to be developed for further cancer treatment.

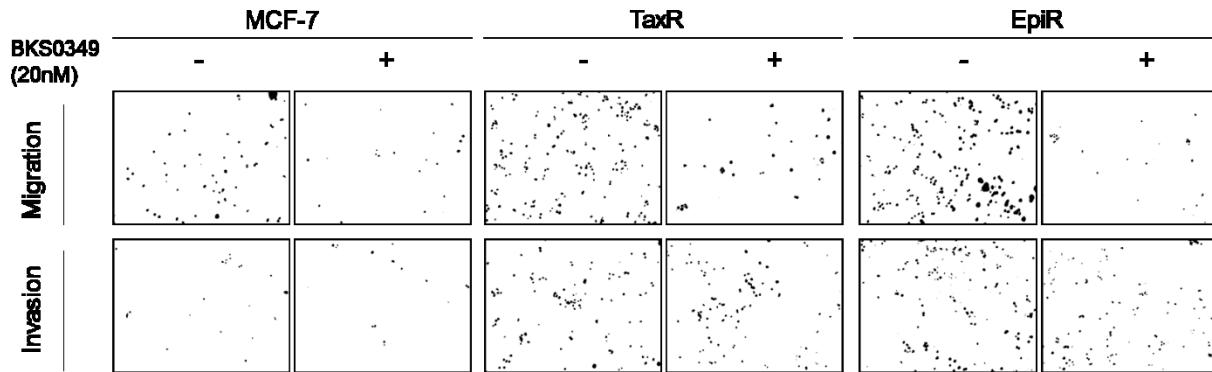
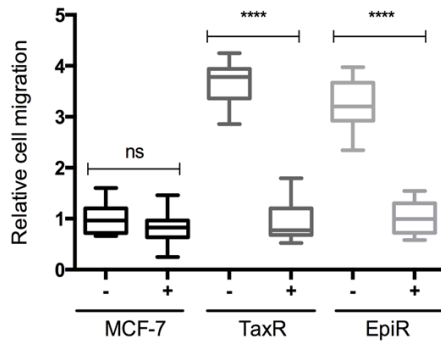
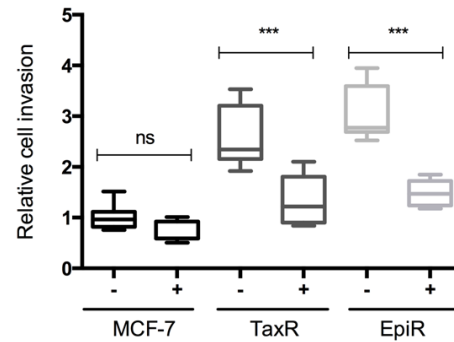
A**B****C**

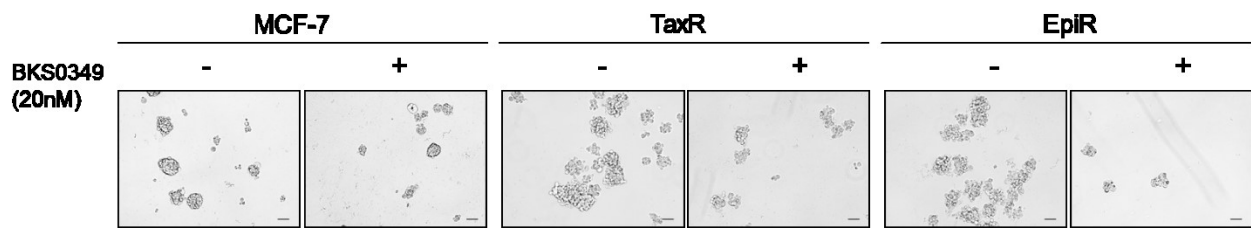
Figure 5. 3 BKS0349 compound attenuates cell migration and invasion.

20,000 cells were seeded into each transwell upper chamber, the lower chamber filled with either control media or 20 nM BKS0349 containing media. For migration assay, cells were incubated for 16 h. For invasion assay, the upper surface of membrane was pre-coated with a Matrigel layer, and cells incubated for 48 h. After the corresponding incubation time, non-migrating cells were removed, and only migrated cells were stained with DAPI. **(A)** Pictures were taken using a fluorescent microscope with a 10× objective. Representative images are examined using Image J and number of migrated cells was also measured. Quantification of migrated **(B)** or invaded **(C)** cells were analysed by two-way ANOVA with the Turkey's multiple comparisons test, and **** $P < 0.0001$, *** $P < 0.001$ indicate very significant and ns non-significant. (n=2)

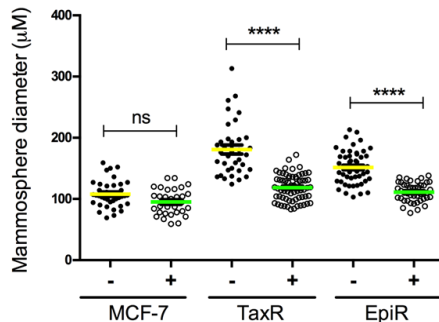
5.5 Inhibitory effects of BKS0349 compound on mammosphere formation

Emerging evidence indicated that FOXM1 directly contributes to cancer stem-like properties [469]. Cancer stem-like cells (CSCs) possess the characteristic of self-renewal, which has been shown to be closely associated with tumorigenesis [470]. KIF20A is essential for maintaining stem/progenitor state and potentially links to the regulation of cell fate determination [471]. It was then aimed to identify the role of KIF20A in development of CSCs as well as if the BKS0349 compound affects the activity. Mammosphere assay is widely used to investigate the CSC activity, with the formation of mammospheres demonstrating the presence of stem cell populations [472]. To evaluate whether BKS0349 has an effect on sphere-forming ability, I cultured MCF-7, TaxR and EpiR cell lines in established mammosphere-forming conditions and treated them with and without BKS0349 compound. The results indicated that KIF20A is involved in regulating of mammosphere formation. Disruption of KIF20A activity significantly reduced sphere formation in the resistant cells. Moreover, in comparison to the resistant cell lines, MCF-7 cells yielded fewer spheres which suggested their lower self-renewal abilities. Notably, the sphere formation rates were relatively higher with TaxR and EpiR cells, and BKS0349 significantly reduced both the number and size of spheres in these cell lines in a 7-day incubation period. The results suggested that BKS0349 has an inhibitory effect on mammosphere development and self-renewal ability of breast cancer cells. It is believed that BKS0349 would affect spheres-forming ability of MCF-7 cells at higher concentrations. Nevertheless, it has shown a strong effect on suppressing sphere formation in the resistant cells with a lower drug concentration (20 nM). This further implies the therapeutic potential of BKS0349 in the chemoprevention of breast cancer.

A



B



C

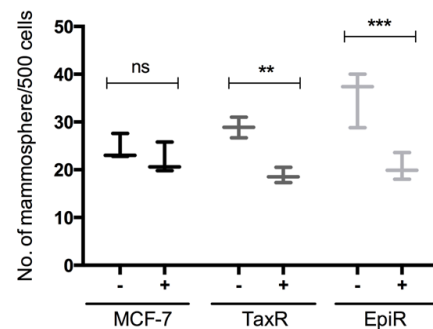


Figure 5. 4 Inhibitory effect of BKS0349 on mammosphere formation in MCF-7, TaxR and EpiR cells.

Five hundred cells were cultured in mammosphere-forming conditions, in which the culturing media contained 20 nM of BKS0349 or were without drug treatment as controls. Cells were allowed to grow on non-adherent plates for 7 days. **(A)** Representative images of spheres are shown. Experiments were independently repeated three times. Bar size is 100 μm. **(B)** The sizes of spheres were analysed using Image J. Each dot represents a measurement of a single sphere. **(C)** Number of mammosphere formation over 500 cells were counted under a microscope. All values were analysed by two-way ANOVA with the Turkey's multiple comparisons test, and **** P<0.0001, *** P<0.001, ** P<0.01 indicate significant and ns non-significant. (n=2)

5.6 Discussion

In this chapter, I have demonstrated that inhibition of KIF20A activity significantly reduced cell migration, invasion and mammsphere formation, which suggested that KIF20A plays important functions in these hallmarks during cancer progression. Moreover, the efficacy of BKS0349 was examined in breast cancer MCF-7, TaxR and EpiR cell lines. The results reveal that BKS0349 is more potent in the TaxR and EpiR breast cancer cell lines, which with a lower IC₅₀ value and reduced cell viability over a long exposure time. Moreover, the inhibitor also has significant effects on cell migration, invasion and mammosphere formation in the breast cancer cell lines. These findings provide a solid rationale for clinical evaluation of BKS0394 in the future.

It was surprising to observe a compound acts more efficient on resistant cell lines rather than the parental ones. The results from migration, invasion and mammosphere assays support the idea that BKS0349 has significant impacts on tumorigenesis in the TaxR and EpiR cells. To understand how this compound works in this manner, the role of KIF20A in the resistant cells has to be addressed. It has been well documented that KIF20A is highly overexpressed in the TaxR and EpiR resistant cell lines when compared with the parental drug sensitive MCF-7 cells [170, 473, 474]. As also shown in my previous time course drug treatment results in chapter 3, paclitaxel and epirubicin are capable of reducing FOXM1 and KIF20A expression in MCF-7 cells, but show no impacts on their expression in the resistant cells. This suggests the possibility that KIF20 has an essential role in mediating drug resistance in TaxR and EpiR cells. Moreover, the chemotherapeutic drug, paclitaxel, targets FOXM1 to downregulate the KIF20A expression which results in disruption of the structure of mitotic spindles, ultimately causing cell cycle arrest, cellular senescence and cell death [170]. Depletion of KIF20A can readily induce senescence in the resistant cells which suggests that the

TaxR and EpiR cells may have become excessively dependent on KIF20A for their long-term survival and renewal. As a consequence, downregulation of KIF20A activity in the resistant cells renders the cells more sensitive to drug induced senescence. Cellular senescence is a tumour suppressive mechanism that restricts cell proliferation and thus prevents cancer initiation and progression [475]. Moreover, BKS0349 is known to selectively target the ATPase activity of KIF20A. In the same manner, disruption of KIF20A activity might stress the resistant cells to enter premature senescence. Therefore, BKS0349 shows a more potent effect in TaxR and EpiR cells compared to the MCF-7 cells.

Furthermore, the resistant cells exhibit an aggressive phenotype with high migratory abilities, while BKS0349 shows a profound effect on limiting cell migration and invasion in the TaxR and EpiR cell lines. In the assay, 20 nM of BKS0349 was used to treat all three breast cancer cells, but it did not have a substantial effect on the MCF-7 cells. One reason is probably because that MCF-7 has much lower migratory abilities compared to the resistant cells. Another reason might be that the drug concentrations are too low for MCF-7 cells to show the effect. However, higher concentration would be too strong to the resistant cells as those concentrations trigger cell death rather than inhibit cell movement. More concentrations should be used to valid the compound in future experiments. More potent derivatives of the present compound should also be sought.

Over the last decades, accumulating evidence has suggested that CSCs play important roles in tumour initiation, progression and they are also responsible for treatment failure [476]. CSCs possess characteristics associated with normal stem cells, which may act as reservoirs to generate numerous progenitor/daughter cells through the stem cell functions of self-renewal and 'dedifferentiation'. Therefore, the

concept of CSCs has profound clinical implications for cancer therapy and prevention. In this project, I observed that disruption of KIF20A activity can limit both the number and size of mammospheres. Mammosphere formation is one of the gold standards used to evaluate the CSC activity *in vitro*. This finding not only suggests a potential role of KIF20A in promoting CSCs but also provides evidence of BKS0349 as an efficient anti-cancer compound by preventing CSC development. In addition, I demonstrated that BKS0349 restricts mammosphere formation in conditions with parallel drug treatment. It would be more valuable to test if BKS0349 can induce CSC cell death or senescence. This can be verified by administering the compound to the growing solution after CSCs have developed.

In conclusion, these findings show that KIF20A is a potential new target and BKS0349 a promising compound to be developed for breast cancer treatment in the future.

CHAPTER 6 FINAL DISCUSSION

In summary, I demonstrated the direct interactions between FOXM1-HMGA1 and FOXM1-HMGN1 in MCF-7, TaxR and EpiR cell lines and their associations significantly affect FOXM1 transcriptional activities on targets: *KIF20A* and *p62*. Specifically, the transcription cofactor HMGA1 inhibits FOXM1 activity either by competing the same binding sites or through forming a complex and transporting to the cytoplasm. Besides, HMGN1 binds to the promoters of *KIF20A* and *p62*, in turn recruits FOXM1 to the same sites and facilitates subsequent transcription. It is known that HMGA1 possesses a feature that can bind to other substrates, and following binding to the target molecules, HMGA1 undergoes specific disordered-to-ordered structural transition and induces conformational change to the binding substrates [231]. According to this characteristic, HMGA1 can interact with transcription factors and induce changes in their DNA binding affinities [244]. Therefore, although HMGA1 is not a transcription factor on its own, it can modulate gene transcription and many other nuclear activities. In agreement, in this project, HMGA1 interacts with FOXM1, may induce a conformational alteration of FOXM1 which weakens or inhibits its DNA binding affinity. Moreover, the FOXM1-HMGA1 interaction may trigger a nucleus-to-cytoplasm translocation, which further restricts FOXM1 activity in the nucleus. It has previously been observed that HMGA1 transports from nucleus to the cytoplasm in a cell cycle-dependent manner in NIH3T3 cells and transgenic (HMGA1 overexpressing) MCF-7 cells [477]. Such subcellular translocation of HMGA1 is rapid and reversible. However, the precise mechanism is still unclear, some speculations are made based on the protein characteristics. As described in the introduction, HMGA1 is extensively regulated by PTM which alterations of the posttranslational state might regulate

HMGA1 subcellular relocation [248, 249, 477]. It is also possible that chaperone proteins are involved in the transport process.

HMGA1 contains three short repeats of AT-hook motifs which determines its binding preference to the minor groove of AT-rich DNA stretches [478, 479]. On the other hand, FOXM1 can *transactivate* its target genes not only through the conventional FOXM1 binding sites (consensus sequence 5'-A(C/T) AAA(C/T) AA-3' sequence) upstream of core promoter, but also via binding with its DNA binding domain (DBD) to TATA-boxes. The TATA-box is an 8 bp AT-rich promoter sequence that is the binding site for the TATA-box binding protein (TBP). FOXM1 has been demonstrated to be a 'TATA-box binding protein' which transactivates *c-fos*, *hsp20* and histone *H2B/a* through binding to the TATA-boxes [451]. Taken these facts together, it appears that both HMGA1 and FOXM1 have similar features of binding to AT-rich sequences on promoter, therefore it is likely that they are competing for the binding sites of one another. Indeed, my ChIP results demonstrated that either of their presence restricts another protein binding to the promoter regions of *KIF20A* and *p62*. However, further experiments are needed to elucidate whether HMGA1 and FOXM1 compete for the same AT-rich regions. In addition, it is of interest to note that the AT-hook motifs of HMGA1 bind with a higher affinity to DNA regions, and as a consequence, will out-compete the binding for another substrate [480, 481]. Consistent with this, it was observed in my results that HMGA1 is the predominant binding factor to the promoters of *KIF20A* and *p62* in MCF-7 cells. Furthermore, HMGA1 is generally expressed at high levels during embryogenesis and at low levels or absent in adult normal tissues [482]. Its overexpression has been frequently reported in advanced cancers, with the presence of metastasis and poor prognosis [268, 483]. Most of the mechanisms proposed for the oncogenic role of HMGA1 are based on transcriptional regulation of genes that are

involved in cell proliferation and DNA repair [268]. Likewise, FOXM1 is shown to be involved in promoting cell proliferation, metastasis, angiogenesis, stemness which are the main hallmarks of cancer progression. The question then arises that is it contradictory if HMGA1 competes with FOXM1 for binding as both of them are oncogenic proteins. Firstly, HMGA1 itself is a master regulator of tumour progression by reprogramming cancer cells through a variety of cellular networks. It can facilitate DNA repair, cell proliferation, EMT, angiogenesis, and stemness in cooperation with other molecules or independently [268, 444, 484]. Hence, it is possible that the two proteins compete one another so as to primarily control similar signalling pathways during cancer progression. Secondly, this competitive phenomenon is not applicable to every cell line. In agreement with this idea, I did not observe the competition between FOXM1 and HMGA1 in the TaxR and EpiR resistant cells. Yet, there is no clear mechanistic explanation for this observation. One possible suggestion is made based on the understanding of PTMs on HMGA1. Several phosphorylation, methylation and acetylation sites of HMGA1 are located in the AT-hooks regions, any alterations of these PTMs could therefore significantly disrupt HMGA1 DNA binding affinity [252, 254, 275]. Indeed, hyper-phosphorylation and hyper-methylation of HMGA1 have been observed especially in metastatic breast cancer cell lines (HA7C and HA8A) compared to those in non-metastatic parental MCF-7 cells [263]. In my study, both TaxR and EpiR resistant cell lines possess metastatic properties as highly mobile and resistant to anticancer drugs. It is likely that HMGA1 protein is highly phosphorylated and/or methylated in the resistant cells, and altered AT-hook domains of which no longer able to bind DNA sequence. As a consequence, HMGA1 mostly functions in cooperation with other partner proteins rather than regulating transcription through binding to chromatin. Furthermore, two uncompetitive, highly expressed

oncogenic proteins may play a vital role for sustaining metastatic and drug-resistant properties of the TaxR and EpiR cells. Inhibition of either FOXM1 or HMGA1 expression would have high inhibitory potentials of breast cancer progression.

Most recently, a study revealed an alternative mechanism of transcription factor recruitment through binding directly to different sets of cofactors on DNA sequences [485, 486]. This is thought to increase regulatory flexibility under different circumstances in the cell. In concordance, I demonstrated for the first time that HMGN1 is a FOXM1 cofactor that binds to DNA sequence and recruits FOXM1 binding as a protein-protein complex. In support, enhanced HMGN1 expression facilitates FOXM1 transcription activity on *KIF20A* and *p62*, but HMGN1 binding is not obligatory for FOXM1's activity. There are three proposed mechanisms of FOXM1 binding to chromatin. Firstly, FOXM1-regulated *transactivation* is mediated through its highly conserved DNA binding domain recognising and binding to DNA sequences containing an FKH motif (RYAAAYA) [487, 488]. Second, FOXM1 is recruited by protein complexes and itself does not directly bind to the DNA. Third, FOXM1 binds directly to DNA sequences facilitated by other proteins. Herein, my results support the third theory and contribute to the understanding of an alternative mechanism of FOXM1 induced transcription. Furthermore, I found silencing either one of the three genes have inhibitory effects on metastasis and drug resistance in breast cancer. Notably, HMGN1 plays a critical role in chromatin de-condensation and transcription activation [393]. Thus, HMGN1 inhibition might have critical global impacts in the cell, which may not be suitable for systemic cancer treatment. However, HMGA1 is expressed at a low level or absent in the normal tissues, and is only highly expressed in cancer cells. This suggests that HMGA1 is a powerful biomarker and a promising

target for cancer treatment. My result also suggests that targeting HMG proteins might be an alternative way to modulate the oncogenic activities of FOXM1.

In the project, I also demonstrated that the oncogenic transcription factor FOXM1 binds directly to p62 promoter. The autophagy regulatory protein p62 is a selective autophagy substrate which is degraded via the ubiquitination/proteosome pathway during autophagy, therefore its downregulation is often used as an indicator of autophagic activation. It is important to clarify if the downregulation of p62 is due to the FOXM1 inhibitory regulation or the autophagic flux. In chapter 3, I described the results that alterations of FOXM1 expression affect p62 expression, in which overexpression of FOXM1 reduces p62 expression. It is unclear whether the reduction is caused by the predominant inhibitory effect of FOXM1 since no further luciferase reporter assay was conducted; or is caused by increased FOXM1 and subsequent enhanced autophagic flux. Since FOXM1 upregulates the major autophagy proteins, Beclin-1 and LC3 and thus inducing autophagy, it is thought that increased FOXM1 is accompanied with reduced p62. Taken together, the regulation of FOXM1-p62 axis requires further experiments to make the conclusion. Moreover, autophagy is a double-edged sword that can promote and prevent tumour progression [201]. In regard to the beneficial effects, autophagy acts as tumour suppressive mechanism which can limit cell proliferation and remove damaged organelles which may generate free radicals and genetic mutations. Conversely, autophagy allows cancer cell survival in limited nutrient environments and under stresses, thereby preventing cell elimination and protects them against cancer therapeutic treatments [489, 490]. Considering the complexity of autophagy, a better understanding of FOXM1-modulated autophagy is urgently needed, and an equilibrium between the promotion and prevention effects is required for further investigation in improving current cancer treatment regimes.

I further identified that p62 exerts an autophagy-independent role by which interacting with vimentin and promoting angiogenesis and metastasis in zebrafish model. Considering that p62 is implicated in different cellular processes and is involved in several molecular mechanisms of tumour initiation and development, my results suggest p62 plays a pro-tumorigenic role in breast cancer. Likewise, p62 has been found to stabilize oncogenic transcription factor Twist1 and thus enhancing tumorigenesis [217, 458]. Hence, silencing of p62 might disrupt these interactions and suppress cancer progression. In these regards, specific and targeted knockdown or downregulation of the p62 gene would be an exciting application to prevent breast cancer tumorigenesis and progression. The results also validate the zebrafish as an effective and practical model to examine angiogenesis and cell migration *in vivo*. In comparison, zebrafish provides extensive advantages including high genetic homology to humans, transparent embryos for straight-forward observations, high fecundity with less time consuming and relatively low husbandry costs [22-24].

In addition, I investigated the functions of KIF20A by using a new KIF20A inhibitor, named the BKS0349 compound. This chemical shows profound inhibitory effects on cancer cell migration and cancer stem-like cell development which suggest KIF20A is essential in these processes. KIF20A is a kinesin protein that plays a vital role in spindle formation and cytokinesis. Besides the role in mitosis, it also has been shown in participating tumour development and progression, as well as in the development of drug resistance [491]. Selective inhibition of this protein shows critical impacts in the hallmarks of breast cancer progression. Although additional validation studies are required to confirm these findings in other breast cancer subtypes and in different cancer cell lines, the current results provide a solid rationale for evaluation of BKS0394 compound in future clinical trials.

Overall, my study identified that HMGA1 and HMGN1 are genuine FOXM1 cofactors which can be recruited to FOXM1 targets' promoters and affect its transcription activity. Although the precise mechanism is yet elucidated, the current understanding reveals the cofactors contribute to modulating FOXM1 transcriptional activities by either activating or repressing FOXM1 target gene expression. Both *KIF20A* and *p62* as FOXM1 downstream targets, are involved in multiple hallmarks of cancer progression. Therefore, blocking their interactions between FOXM1, HMGA1 and HMGN1 by specific small molecules (peptides) might be a new strategy to interfere FOXM1-modulated oncogenic activities.

REFERENCES

1. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018*. Eur J Cancer, 2018.
2. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries*. CA Cancer J Clin, 2018. **68**(6): p. 394-424.
3. Sun, Y.S., et al., *Risk Factors and Preventions of Breast Cancer*. Int J Biol Sci, 2017. **13**(11): p. 1387-1397.
4. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. Cell, 2000. **100**(1): p. 57-70.
5. Waks, A.G. and E.P. Winer, *Breast Cancer Treatment: A Review*. JAMA, 2019. **321**(3): p. 288-300.
6. Frank, S.A., in *Dynamics of Cancer: Incidence, Inheritance, and Evolution*. 2007: Princeton (NJ).
7. Arvelo, F., F. Sojo, and C. Cotte, *Tumour progression and metastasis*. Ecancermedicallscience, 2016. **10**: p. 617.
8. Yokota, J., *Tumor progression and metastasis*. Carcinogenesis, 2000. **21**(3): p. 497-503.
9. Nishida, N., et al., *Angiogenesis in cancer*. Vasc Health Risk Manag, 2006. **2**(3): p. 213-9.
10. Folkman, J., *Role of angiogenesis in tumor growth and metastasis*. Semin Oncol, 2002. **29**(6 Suppl 16): p. 15-8.
11. Qi, X.L., L. Zhang, and X.J. Lu, *New Insights into the Epithelial-to-Mesenchymal Transition in Cancer*. Trends in Pharmacological Sciences, 2016. **37**(4): p. 246-248.
12. Witsch, E., M. Sela, and Y. Yarden, *Roles for growth factors in cancer progression*. Physiology (Bethesda), 2010. **25**(2): p. 85-101.
13. Christiansen, J.J. and A.K. Rajasekaran, *Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis*. Cancer Res, 2006. **66**(17): p. 8319-26.
14. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: diversity and escape mechanisms*. Nat Rev Cancer, 2003. **3**(5): p. 362-74.
15. Fidler, I.J., *Timeline - The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited*. Nature Reviews Cancer, 2003. **3**(6): p. 453-458.
16. Riggi, N., M. Aguet, and I. Stamenkovic, *Cancer Metastasis: A Reappraisal of Its Underlying Mechanisms and Their Relevance to Treatment*. Annual Review of Pathology: Mechanisms of Disease, Vol 13, 2018. **13**: p. 117-140.
17. Weigelt, B., J.L. Peterse, and L.J. van 't Veer, *Breast cancer metastasis: markers and models*. Nat Rev Cancer, 2005. **5**(8): p. 591-602.
18. Scully, O.J., et al., *Breast cancer metastasis*. Cancer Genomics Proteomics, 2012. **9**(5): p. 311-20.
19. Feng, Y., et al., *Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis*. Genes Dis, 2018. **5**(2): p. 77-106.
20. Harris, L., et al., *American society of clinical oncology 2007 update of recommendations for the use of tumor markers in breast cancer*. Journal of Clinical Oncology, 2007. **25**(33): p. 5287-5312.
21. Mandal, J. and S.C. Parija, *Ethics of involving animals in research*. Trop Parasitol, 2013. **3**(1): p. 4-6.
22. Zhao, S., J. Huang, and J. Ye, *A fresh look at zebrafish from the perspective of cancer research*. Journal of Experimental & Clinical Cancer Research, 2015. **34**.
23. Teng, Y., et al., *Evaluating human cancer cell metastasis in zebrafish*. BMC Cancer, 2013. **13**: p. 453.
24. Ignatius, M.S. and D.M. Langenau, *Zebrafish as a Model for Cancer Self-Renewal*. Zebrafish, 2009. **6**(4): p. 377-387.

25. Gore, A.V., et al., *Vascular development in the zebrafish*. Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006684.
26. Quaife, N.M., O. Watson, and T.J. Chico, *Zebrafish: an emerging model of vascular development and remodelling*. Curr Opin Pharmacol, 2012. **12**(5): p. 608-14.
27. Kalueff, A.V., A.M. Stewart, and R. Gerlai, *Zebrafish as an emerging model for studying complex brain disorders*. Trends in Pharmacological Sciences, 2014. **35**(2): p. 63-75.
28. Guyon, J.R., et al., *Modeling human muscle disease in zebrafish*. Biochim Biophys Acta, 2007. **1772**(2): p. 205-15.
29. Weinstein, B., *Vascular cell biology in vivo: a new piscine paradigm?* Trends Cell Biol, 2002. **12**(9): p. 439-45.
30. Lieschke, G.J., et al., *Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish*. Blood, 2001. **98**(10): p. 3087-96.
31. Howe, K., et al., *The zebrafish reference genome sequence and its relationship to the human genome*. Nature, 2013. **496**(7446): p. 498-503.
32. White, R.M., et al., *Transparent adult zebrafish as a tool for in vivo transplantation analysis*. Cell Stem Cell, 2008. **2**(2): p. 183-189.
33. Eimon, P.M., *Studying apoptosis in the Zebrafish*. Methods Enzymol, 2014. **544**: p. 395-431.
34. Novoa, B. and A. Figueras, *Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases*. Adv Exp Med Biol, 2012. **946**: p. 253-75.
35. Traver, D., et al., *The zebrafish as a model organism to study development of the immune system*. Advances in Immunology, Vol 81, 2003. **81**: p. 253-+.
36. Lee, L.M.J., et al., *The fate of human malignant melanoma cells transplanted into zebrafish embryos: Assessment of migration and cell division in the absence of tumor formation*. Developmental Dynamics, 2005. **233**(4): p. 1560-1570.
37. Yang, X.J., et al., *A novel zebrafish xenotransplantation model for study of glioma stem cell invasion*. PLoS One, 2013. **8**(4): p. e61801.
38. Hou, Y., et al., *Recombinant disintegrin domain of ADAM15 inhibits the proliferation and migration of Bel-7402 cells*. Biochem Biophys Res Commun, 2013. **435**(4): p. 640-5.
39. Moshal, K.S., et al., *Discriminating different cancer cells using a zebrafish in vivo assay*. Cancers (Basel), 2011. **3**(4): p. 4102-13.
40. Weiss, F.U., et al., *Retinoic acid receptor antagonists inhibit miR-10a expression and block metastatic behavior of pancreatic cancer*. Gastroenterology, 2009. **137**(6): p. 2136-45 e1-7.
41. Latifi, A., et al., *Cisplatin treatment of primary and metastatic epithelial ovarian carcinomas generates residual cells with mesenchymal stem cell-like profile*. J Cell Biochem, 2011. **112**(10): p. 2850-64.
42. Drabsch, Y., et al., *Transforming growth factor-beta signalling controls human breast cancer metastasis in a zebrafish xenograft model*. Breast Cancer Res, 2013. **15**(6): p. R106.
43. Wagner, D.S., et al., *The in vivo performance of plasmonic nanobubbles as cell theranostic agents in zebrafish hosting prostate cancer xenografts*. Biomaterials, 2010. **31**(29): p. 7567-7574.
44. Jo, D.H., et al., *Orthotopic transplantation of retinoblastoma cells into vitreous cavity of zebrafish for screening of anticancer drugs*. Molecular Cancer, 2013. **12**.
45. Zhang, B., et al., *Quantitative phenotyping-based in vivo chemical screening in a zebrafish model of leukemia stem cell xenotransplantation*. PLoS One, 2014. **9**(1): p. e85439.
46. Tobia, C., G. De Sena, and M. Presta, *Zebrafish embryo, a tool to study tumor angiogenesis*. Int J Dev Biol, 2011. **55**(4-5): p. 505-9.
47. Nicoli, S., et al., *Mammalian tumor xenografts induce neovascularization in zebrafish embryos*. Cancer Res, 2007. **67**(7): p. 2927-31.
48. He, S.N., et al., *Neutrophil-mediated experimental metastasis is enhanced by VEGFR inhibition in a zebrafish xenograft model*. Journal of Pathology, 2012. **227**(4): p. 431-445.

49. Jackson, B.C., et al., *Update of human and mouse forkhead box (FOX) gene families*. Hum Genomics, 2010. **4**(5): p. 345-52.
50. Weigel, D., et al., *The Homeotic Gene Fork Head Encodes a Nuclear-Protein and Is Expressed in the Terminal Regions of the Drosophila Embryo*. Cell, 1989. **57**(4): p. 645-658.
51. Kaestner, K.H., W. Knochel, and D.E. Martinez, *Unified nomenclature for the winged helix/forkhead transcription factors*. Genes Dev, 2000. **14**(2): p. 142-6.
52. Liao, G.B., et al., *Regulation of the master regulator FOXM1 in cancer*. Cell Commun Signal, 2018. **16**(1): p. 57.
53. Korver, W., et al., *The human TRIDENT/HFH-11/FKHL16 gene: structure, localization, and promoter characterization*. Genomics, 1997. **46**(3): p. 435-42.
54. Park, H.J., et al., *An N-terminal inhibitory domain modulates activity of FoxM1 during cell cycle*. Oncogene, 2008. **27**(12): p. 1696-704.
55. Littler, D.R., et al., *Structure of the FoxM1 DNA-recognition domain bound to a promoter sequence*. Nucleic Acids Res, 2010. **38**(13): p. 4527-38.
56. Bella, L., et al., *FOXM1: A key oncofoetal transcription factor in health and disease*. Semin Cancer Biol, 2014. **29**: p. 32-9.
57. Zhong, S., et al., *Downregulating forkhead box M1 inhibits proliferation by inhibiting autophagy in the sw480 cell line*. Biomed Rep, 2017. **7**(1): p. 47-50.
58. Zhang, J., Y. Niu, and C. Huang, *Role of FoxM1 in the Progression and Epithelial to Mesenchymal Transition of Gastrointestinal Cancer*. Recent Pat Anticancer Drug Discov, 2017. **12**(3): p. 247-259.
59. Wang, K., et al., *FoxM1 inhibition enhances chemosensitivity of docetaxel-resistant A549 cells to docetaxel via activation of JNK/mitochondrial pathway*. Acta Biochim Biophys Sin (Shanghai), 2016. **48**(9): p. 804-9.
60. Yu, C., et al., *Targeting FoxM1 inhibits proliferation, invasion and migration of nasopharyngeal carcinoma through the epithelial-to-mesenchymal transition pathway*. Oncology Reports, 2015. **33**(5): p. 2402-2410.
61. Jiang, L., et al., *Down-regulation of FoxM1 by thiostrepton or small interfering RNA inhibits proliferation, transformation ability and angiogenesis, and induces apoptosis of nasopharyngeal carcinoma cells*. Int J Clin Exp Pathol, 2014. **7**(9): p. 5450-60.
62. Wang, D., et al., *Aberrant activation of hedgehog signaling promotes cell proliferation via the transcriptional activation of forkhead Box M1 in colorectal cancer cells*. J Exp Clin Cancer Res, 2017. **36**(1): p. 23.
63. Ko, C.B., et al., *CTCF-FoxM1 axis regulates tumor growth and metastasis in hepatocellular carcinoma*. Cancer Science, 2018. **109**: p. 1117-1117.
64. Chen, P.M., et al., *MnSOD promotes tumor invasion via upregulation of FoxM1-MMP2 axis and related with poor survival and relapse in lung adenocarcinomas*. Mol Cancer Res, 2013. **11**(3): p. 261-71.
65. Mencalha, A.L., et al., *Forkhead Box M1 (FoxM1) Gene Is a New STAT3 Transcriptional Factor Target and Is Essential for Proliferation, Survival and DNA Repair of K562 Cell Line*. Plos One, 2012. **7**(10).
66. Xia, L.M., et al., *Upregulated FoxM1 expression induced by hepatitis B virus X protein promotes tumor metastasis and indicates poor prognosis in hepatitis B virus-related hepatocellular carcinoma*. Journal of Hepatology, 2012. **57**(3): p. 600-612.
67. Millour, J., et al., *ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance*. Mol Cancer Ther, 2011. **10**(6): p. 1046-58.
68. Qian, J.X., et al., *Twist1 Promotes Gastric Cancer Cell Proliferation through Up-Regulation of FoxM1*. Plos One, 2013. **8**(10).
69. Xia, L.M., et al., *Transcriptional Up-Regulation of FoxM1 in Response to Hypoxia Is Mediated by HIF-1*. Journal of Cellular Biochemistry, 2009. **106**(2): p. 247-256.

70. Xia, L., et al., *The TNF-alpha/ROS/HIF-1-induced upregulation of FoxM1 expression promotes HCC proliferation and resistance to apoptosis*. Carcinogenesis, 2012. **33**(11): p. 2250-9.
71. Dai, B.B., et al., *Forkhead Box M1 Is Regulated by Heat Shock Factor 1 and Promotes Glioma Cells Survival under Heat Shock Stress*. Journal of Biological Chemistry, 2013. **288**(3): p. 1634-1642.
72. Hu, C., et al., *LXRalpha-mediated downregulation of FOXM1 suppresses the proliferation of hepatocellular carcinoma cells*. Oncogene, 2014. **33**(22): p. 2888-97.
73. Barsotti, A.M. and C. Prives, *Pro-proliferative FoxM1 is a target of p53-mediated repression*. Oncogene, 2009. **28**(48): p. 4295-4305.
74. Kurinna, S., et al., *p53 regulates a mitotic transcription program and determines ploidy in normal mouse liver*. Hepatology, 2013. **57**(5): p. 2004-13.
75. Millour, J., et al., *FOXM1 is a transcriptional target of ERalpha and has a critical role in breast cancer endocrine sensitivity and resistance*. Oncogene, 2010. **29**(20): p. 2983-95.
76. Horimoto, Y., et al., *ERbeta1 represses FOXM1 expression through targeting ERalpha to control cell proliferation in breast cancer*. Am J Pathol, 2011. **179**(3): p. 1148-56.
77. Blanco-Bose, W.E., et al., *C-Myc and its target FoxM1 are critical downstream effectors of constitutive androstane receptor (CAR) mediated direct liver hyperplasia*. Hepatology, 2008. **48**(4): p. 1302-11.
78. Delpuech, O., et al., *Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression*. Mol Cell Biol, 2007. **27**(13): p. 4917-30.
79. Yang, N., et al., *FOXM1 recruits nuclear Aurora kinase A to participate in a positive feedback loop essential for the self-renewal of breast cancer stem cells*. Oncogene, 2017. **36**(24): p. 3428-3440.
80. Xie, X., et al., *Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals*. Nature, 2005. **434**(7031): p. 338-45.
81. Wang, J.M., et al., *MiR-214 inhibits cell migration, invasion and promotes the drug sensitivity in human cervical cancer by targeting FOXM1*. Am J Transl Res, 2017. **9**(8): p. 3541-3557.
82. Liu, X., et al., *MicroRNA-149 Increases the Sensitivity of Colorectal Cancer Cells to 5-Fluorouracil by Targeting Forkhead Box Transcription Factor FOXM1*. Cell Physiol Biochem, 2016. **39**(2): p. 617-29.
83. Li, X.R., et al., *miR-342-3p suppresses proliferation, migration and invasion by targeting FOXM1 in human cervical cancer*. FEBS Lett, 2014. **588**(17): p. 3298-307.
84. Wang, S.H., et al., *Long non-coding RNA H19 regulates FOXM1 expression by competitively binding endogenous miR-342-3p in gallbladder cancer*. J Exp Clin Cancer Res, 2016. **35**(1): p. 160.
85. Chen, Y.J., et al., *A Conserved Phosphorylation Site within the Forkhead Domain of FoxM1B Is Required for Its Activation by Cyclin-CDK1*. Journal of Biological Chemistry, 2009. **284**(44): p. 30695-30707.
86. Anders, L., et al., *A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells*. Cancer Cell, 2011. **20**(5): p. 620-34.
87. Ma, R.Y., et al., *Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c*. J Cell Sci, 2005. **118**(Pt 4): p. 795-806.
88. Luscher-Firzlaff, J.M., R. Lilischkis, and B. Luscher, *Regulation of the transcription factor FOXM1c by Cyclin E/CDK2*. Febs Letters, 2006. **580**(7): p. 1716-1722.
89. Laoukili, J., et al., *Activation of FoxM1 during G2 requires cyclin A/Cdk-dependent relief of autorepression by the FoxM1 N-terminal domain*. Mol Cell Biol, 2008. **28**(9): p. 3076-87.
90. Koo, C.Y., K.W. Muir, and E.W. Lam, *FOXM1: From cancer initiation to progression and treatment*. Biochim Biophys Acta, 2012. **1819**(1): p. 28-37.
91. Tan, Y.J., P. Raychaudhuri, and R.H. Costa, *Chk2 mediates stabilization of the FoxM1 transcription factor to stimulate expression of DNA repair genes*. Molecular and Cellular Biology, 2007. **27**(3): p. 1007-1016.

92. Fu, Z., et al., *Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression*. Nat Cell Biol, 2008. **10**(9): p. 1076-82.
93. Chen, Y., et al., *Wnt-induced deubiquitination FoxM1 ensures nucleus beta-catenin transactivation*. EMBO J, 2016. **35**(6): p. 668-84.
94. Zhang, J., et al., *Polo-like kinase 1-mediated phosphorylation of Forkhead box protein M1b antagonizes its SUMOylation and facilitates its mitotic function*. J Biol Chem, 2015. **290**(6): p. 3708-19.
95. Laoukili, J., et al., *FoxM1 is degraded at mitotic exit in a Cdh1-dependent manner*. Cell Cycle, 2008. **7**(17): p. 2720-2726.
96. Park, H.J., et al., *Anaphase-promoting complex/cyclosome-Cdh1-mediated proteolysis of the forkhead box M1 transcription factor is critical for regulated entry into S phase*. Molecular and Cellular Biology, 2008. **28**(17): p. 5162-5171.
97. Schimmel, J., et al., *Uncovering SUMOylation dynamics during cell-cycle progression reveals FoxM1 as a key mitotic SUMO target protein*. Mol Cell, 2014. **53**(6): p. 1053-66.
98. Lv, C.C., et al., *Acetylation of FOXM1 is essential for its transactivation and tumor growth stimulation*. Oncotarget, 2016. **7**(37): p. 60366-60382.
99. Cohn, O., et al., *Chromatin associated SETD3 negatively regulates VEGF expression*. Sci Rep, 2016. **6**: p. 37115.
100. Wierstra, I. and J. Alves, *Transcription factor FOXM1c is repressed by RB and activated by cyclin D1/Cdk4*. Biological Chemistry, 2006. **387**(7): p. 949-962.
101. Kalinichenko, V.V., et al., *Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19(ARF) tumor suppressor*. Genes & Development, 2004. **18**(7): p. 830-850.
102. Bhat, U.G., et al., *Nucleophosmin interacts with FOXM1 and modulates the level and localization of FOXM1 in human cancer cells*. J Biol Chem, 2011. **286**(48): p. 41425-33.
103. Khan, I., et al., *Nuclear FOXM1 drives chemoresistance in AML*. Leukemia, 2017. **31**(1): p. 251-255.
104. Jin, B., et al., *Anthelmintic Niclosamide Disrupts the Interplay of p65 and FOXM1/beta-catenin and Eradicates Leukemia Stem Cells in Chronic Myelogenous Leukemia*. Clin Cancer Res, 2017. **23**(3): p. 789-803.
105. Zhang, N., et al., *FoxM1 promotes beta-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis*. Cancer Cell, 2011. **20**(4): p. 427-42.
106. Maachani, U.B., et al., *FOXM1 and STAT3 interaction confers radioresistance in glioblastoma cells*. Oncotarget, 2016. **7**(47): p. 77365-77377.
107. Joshi, K., et al., *MELK-Dependent FOXM1 Phosphorylation is Essential for Proliferation of Glioma Stem Cells*. Stem Cells, 2013. **31**(6): p. 1051-1063.
108. Sullivan, C., et al., *Novel Interactions between FOXM1 and CDC25A Regulate the Cell Cycle*. Plos One, 2012. **7**(12).
109. Kruiswijk, F., et al., *Targeted inhibition of metastatic melanoma through interference with Pin1-FOXM1 signaling*. Oncogene, 2016. **35**(17): p. 2166-2177.
110. Xue, J.F., et al., *Sustained activation of SMAD3/SMAD4 by FOXM1 promotes TGF-beta-dependent cancer metastasis*. Journal of Clinical Investigation, 2014. **124**(2): p. 564-579.
111. Xu, M.D., et al., *A Positive Feedback Loop of lncRNA-PVT1 and FOXM1 Facilitates Gastric Cancer Growth and Invasion*. Clin Cancer Res, 2017. **23**(8): p. 2071-2080.
112. Pilarsky, C., et al., *Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data*. Neoplasia, 2004. **6**(6): p. 744-50.
113. Uddin, S., et al., *Genome-Wide Expression Analysis of Middle Eastern Colorectal Cancer Reveals FOXM1 as a Novel Target for Cancer Therapy*. American Journal of Pathology, 2011. **178**(2): p. 537-547.

114. Okabe, H., et al., *Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: Identification of genes involved in viral carcinogenesis and tumor progression*. Cancer Research, 2001. **61**(5): p. 2129-2137.
115. Gong, A. and S. Huang, *FoxM1 and Wnt/beta-catenin signaling in glioma stem cells*. Cancer Res, 2012. **72**(22): p. 5658-62.
116. Kalinina, O.A., et al., *Sustained hepatic expression of FoxM1B in transgenic mice has minimal effects on hepatocellular carcinoma development but increases cell proliferation rates in preneoplastic and early neoplastic lesions*. Oncogene, 2003. **22**(40): p. 6266-6276.
117. Kalin, T.V., et al., *Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice*. Cancer Res, 2006. **66**(3): p. 1712-20.
118. Kim, I.M., et al., *The Forkhead Box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer*. Cancer Res, 2006. **66**(4): p. 2153-61.
119. Madureira, P.A., et al., *The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells*. J Biol Chem, 2006. **281**(35): p. 25167-76.
120. Yoshida, Y., et al., *The forkhead box M1 transcription factor contributes to the development and growth of mouse colorectal cancer*. Gastroenterology, 2007. **132**(4): p. 1420-31.
121. Chan, D.W., et al., *Over-expression of FOXM1 transcription factor is associated with cervical cancer progression and pathogenesis*. J Pathol, 2008. **215**(3): p. 245-52.
122. Cancer Genome Atlas Research, N., *Integrated genomic analyses of ovarian carcinoma*. Nature, 2011. **474**(7353): p. 609-15.
123. Nakamura, S., et al., *The FOXM1 transcriptional factor promotes the proliferation of leukemia cells through modulation of cell cycle progression in acute myeloid leukemia*. Carcinogenesis, 2010. **31**(11): p. 2012-2021.
124. Huynh, K.M., et al., *FOXM1 expression mediates growth suppression during terminal differentiation of HO-1 human metastatic melanoma cells*. J Cell Physiol, 2011. **226**(1): p. 194-204.
125. Teh, M.T., et al., *Upregulation of FOXM1 induces genomic instability in human epidermal keratinocytes*. Mol Cancer, 2010. **9**: p. 45.
126. Vogiatzi, P., et al., *Deciphering the underlying genetic and epigenetic events leading to gastric carcinogenesis*. J Cell Physiol, 2007. **211**(2): p. 287-95.
127. Laoukili, J., M. Stahl, and R.H. Medema, *FoxM1: At the crossroads of ageing and cancer*. Biochimica Et Biophysica Acta-Reviews on Cancer, 2007. **1775**(1): p. 92-102.
128. Gartel, A.L., *FoxM1 inhibitors as potential anticancer drugs*. Expert Opinion on Therapeutic Targets, 2008. **12**(6): p. 663-665.
129. Adami, G.R. and H. Ye, *Future roles for FoxM1 inhibitors in cancer treatments*. Future Oncol, 2007. **3**(1): p. 1-3.
130. Zeng, J., et al., *FoxM1 is up-regulated in gastric cancer and its inhibition leads to cellular senescence, partially dependent on p27 kip1*. J Pathol, 2009. **218**(4): p. 419-27.
131. Wang, I.C., et al., *Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase*. Mol Cell Biol, 2005. **25**(24): p. 10875-94.
132. Li, S.K., et al., *FoxM1c counteracts oxidative stress-induced senescence and stimulates Bmi-1 expression*. J Biol Chem, 2008. **283**(24): p. 16545-53.
133. Ahmad, A., et al., *FoxM1 down-regulation leads to inhibition of proliferation, migration and invasion of breast cancer cells through the modulation of extra-cellular matrix degrading factors (Retraction of vol 122, pg 337, 2010)*. Breast Cancer Research and Treatment, 2016. **158**(3): p. 607-607.
134. Kalin, T.V., et al., *Forkhead Box m1 transcription factor is required for perinatal lung function*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(49): p. 19330-19335.

135. Ustiyany, V., et al., *Forkhead box M1 transcriptional factor is required for smooth muscle cells during embryonic development of blood vessels and esophagus*. Developmental Biology, 2009. **336**(2): p. 266-279.
136. Ueno, H., et al., *FoxM1-driven cell division is required for neuronal differentiation in early Xenopus embryos*. Development, 2008. **135**(11): p. 2023-2030.
137. Misfeldt, A.A., R.H. Costa, and M. Gannon, *beta-Cell Proliferation, but Not Neogenesis, Following 60% Partial Pancreatectomy Is Impaired in the Absence of FoxM1*. Diabetes, 2008. **57**(11): p. 3069-3077.
138. Kalinichenko, V.V., et al., *Ubiquitous expression of the forkhead box M1B transgene accelerates proliferation of distinct pulmonary cell types following lung injury*. J Biol Chem, 2003. **278**(39): p. 37888-94.
139. Wang, X.H., N.J. Hung, and R.H. Costa, *Earlier expression of the transcription factor HFH-11B diminishes induction of p21(CIP1/WAF1) levels and accelerates mouse hepatocyte entry into S-phase following carbon tetrachloride liver injury*. Hepatology, 2001. **33**(6): p. 1404-1414.
140. Ye, H.G., et al., *Premature expression of the winged helix transcription factor HFH-11B in regenerating mouse liver accelerates hepatocyte entry into S phase*. Molecular and Cellular Biology, 1999. **19**(12): p. 8570-8580.
141. Zhang, Y., et al., *FoxM1B transcriptionally regulates vascular endothelial growth factor expression and promotes the angiogenesis and growth of glioma cells*. Cancer Res, 2008. **68**(21): p. 8733-42.
142. Karadedou, C.T., et al., *FOXO3a represses VEGF expression through FOXM1-dependent and -independent mechanisms in breast cancer*. Oncogene, 2012. **31**(14): p. 1845-1858.
143. Li, Q., et al., *Critical role and regulation of transcription factor FoxM1 in human gastric cancer angiogenesis and progression*. Cancer Res, 2009. **69**(8): p. 3501-9.
144. Dai, B., et al., *Aberrant FoxM1B expression increases matrix metalloproteinase-2 transcription and enhances the invasion of glioma cells*. Oncogene, 2007. **26**(42): p. 6212-9.
145. Wang, I.C., et al., *Transgenic expression of the forkhead box M1 transcription factor induces formation of lung tumors*. Oncogene, 2008. **27**(30): p. 4137-49.
146. Park, H.J., et al., *Deregulation of FoxM1b leads to tumour metastasis*. EMBO Mol Med, 2011. **3**(1): p. 21-34.
147. Yao, S., L.Y. Fan, and E.W. Lam, *The FOXO3-FOXM1 axis: A key cancer drug target and a modulator of cancer drug resistance*. Semin Cancer Biol, 2018. **50**: p. 77-89.
148. Nardella, C., et al., *Pro-senescence therapy for cancer treatment*. Nat Rev Cancer, 2011. **11**(7): p. 503-11.
149. Lord, C.J. and A. Ashworth, *The DNA damage response and cancer therapy*. Nature, 2012. **481**(7381): p. 287-94.
150. Myatt, S.S. and E.W. Lam, *The emerging roles of forkhead box (Fox) proteins in cancer*. Nat Rev Cancer, 2007. **7**(11): p. 847-59.
151. Alvarez-Fernandez, M. and R.H. Medema, *Novel functions of FoxM1: from molecular mechanisms to cancer therapy*. Front Oncol, 2013. **3**: p. 30.
152. Zona, S., et al., *FOXM1: an emerging master regulator of DNA damage response and genotoxic agent resistance*. Biochim Biophys Acta, 2014. **1839**(11): p. 1316-22.
153. Vale, R.D., T.S. Reese, and M.P. Sheetz, *Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility*. Cell, 1985. **42**(1): p. 39-50.
154. Vale, R.D. and R.J. Fletterick, *The design plan of kinesin motors*. Annu Rev Cell Dev Biol, 1997. **13**: p. 745-77.
155. Lawrence, C.J., et al., *A standardized kinesin nomenclature*. J Cell Biol, 2004. **167**(1): p. 19-22.
156. Kamimoto, T., et al., *Identification of a novel kinesin-related protein, KRMP1, as a target for mitotic peptidyl-prolyl isomerase Pin1*. Journal of Biological Chemistry, 2001. **276**(40): p. 37520-37528.

157. Westendorf, J.M., P.N. Rao, and L. Gerace, *Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope*. Proc Natl Acad Sci U S A, 1994. **91**(2): p. 714-8.
158. Sellitto, C. and R. Kuriyama, *Distribution of a matrix component of the midbody during the cell cycle in Chinese hamster ovary cells*. J Cell Biol, 1988. **106**(2): p. 431-9.
159. Camlin, N.J., E.A. McLaughlin, and J.E. Holt, *Motoring through: the role of kinesin superfamily proteins in female meiosis*. Human Reproduction Update, 2017. **23**(4): p. 409-420.
160. Lai, F., et al., *cDNA cloning, expression pattern, genomic structure and chromosomal location of RAB6KIFL, a human kinesin-like gene*. Gene, 2000. **248**(1-2): p. 117-25.
161. Vaid, K.S., et al., *A kinesin is present at unique sertoli/spermatid adherens junctions in rat and mouse testes*. Biology of Reproduction, 2007. **77**(6): p. 1037-1048.
162. Louw, J.J., et al., *Compound heterozygous loss-of-function mutations in KIF20A are associated with a novel lethal congenital cardiomyopathy in two siblings*. PLoS Genet, 2018. **14**(1): p. e1007138.
163. Schupbach, T. and E. Wieschaus, *Female sterile mutations on the second chromosome of Drosophila melanogaster. I. Maternal effect mutations*. Genetics, 1989. **121**(1): p. 101-17.
164. Hirokawa, N., *Kinesin and dynein superfamily proteins and the mechanism of organelle transport*. Science, 1998. **279**(5350): p. 519-26.
165. Wu, W.D., et al., *Roles and mechanisms of Kinesin-6 KIF20A in spindle organization during cell division*. Eur J Cell Biol, 2018.
166. Miserey-Lenkei, S., et al., *Coupling fission and exit of RAB6 vesicles at Golgi hotspots through kinesin-myosin interactions*. Nature Communications, 2017. **8**.
167. Fontijn, R.D., et al., *The human kinesin-like protein RB6K is under tight cell cycle control and is essential for cytokinesis*. Molecular and Cellular Biology, 2001. **21**(8): p. 2944-2955.
168. Verhey, K.J. and J.W. Hammond, *Traffic control: regulation of kinesin motors*. Nat Rev Mol Cell Biol, 2009. **10**(11): p. 765-77.
169. Kitagawa, M., et al., *Targeting Aurora B to the Equatorial Cortex by MKlp2 Is Required for Cytokinesis*. Plos One, 2013. **8**(6).
170. Khongkow, P., et al., *Paclitaxel targets FOXM1 to regulate KIF20A in mitotic catastrophe and breast cancer paclitaxel resistance*. Oncogene, 2015.
171. Shi, C., et al., *Aberrantly activated Gli2-KIF20A axis is crucial for growth of hepatocellular carcinoma and predicts poor prognosis*. Oncotarget, 2016. **7**(18): p. 26206-26219.
172. Duan, J., W. Huang, and H. Shi, *Positive expression of KIF20A indicates poor prognosis of glioma patients*. Onco Targets Ther, 2016. **9**: p. 6741-6749.
173. Liu, S.L., et al., *Overexpression of Kinesin Family Member 20A Correlates with Disease Progression and Poor Prognosis in Human Nasopharyngeal Cancer: A Retrospective Analysis of 105 Patients*. PLoS One, 2017. **12**(1): p. e0169280.
174. Zhao, X., et al., *Overexpression of KIF20A confers malignant phenotype of lung adenocarcinoma by promoting cell proliferation and inhibiting apoptosis*. Cancer Med, 2018. **7**(9): p. 4678-4689.
175. Yan, G.R., et al., *Genistein-induced mitotic arrest of gastric cancer cells by downregulating KIF20A, a proteomics study*. Proteomics, 2012. **12**(14): p. 2391-9.
176. Kitahara, O., et al., *Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia*. Cancer Res, 2001. **61**(9): p. 3544-9.
177. Gasnereau, I., et al., *KIF20A mRNA and its product MKlp2 are increased during hepatocyte proliferation and hepatocarcinogenesis*. Am J Pathol, 2012. **180**(1): p. 131-40.
178. Lu, M.Q., et al., *Aberrant KIF20A expression might independently predict poor overall survival and recurrence-free survival of hepatocellular carcinoma*. Iubmb Life, 2018. **70**(4): p. 328-335.

179. Obama, K., et al., *Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinoma*. Hepatology, 2005. **41**(6): p. 1339-48.
180. Stangel, D., et al., *Kif20a inhibition reduces migration and invasion of pancreatic cancer cells*. J Surg Res, 2015. **197**(1): p. 91-100.
181. Taniuchi, K., M. Furihata, and T. Saibara, *KIF20A-mediated RNA granule transport system promotes the invasiveness of pancreatic cancer cells*. Neoplasia, 2014. **16**(12): p. 1082-93.
182. Zhang, W.J., et al., *High Expression of KIF20A Is Associated with Poor Overall Survival and Tumor Progression in Early-Stage Cervical Squamous Cell Carcinoma*. Plos One, 2016. **11**(12).
183. Yamashita, J., et al., *Kinesin Family Member 20A is a Novel Melanoma-associated Antigen*. Acta Dermato-Venereologica, 2012. **92**(6): p. 593-597.
184. Mizushima, N. and M. Komatsu, *Autophagy: renovation of cells and tissues*. Cell, 2011. **147**(4): p. 728-41.
185. Yu, L., et al., *Termination of autophagy and reformation of lysosomes regulated by mTOR*. Nature, 2010. **465**(7300): p. 942-6.
186. Sou, Y., et al., *The Atg8 Conjugation System Is Indispensable for Proper Development of Autophagic Isolation Membranes in Mice*. Molecular Biology of the Cell, 2008. **19**(11): p. 4762-4775.
187. Kuma, A., et al., *The role of autophagy during the early neonatal starvation period*. Nature, 2004. **432**(7020): p. 1032-6.
188. Komatsu, M., et al., *Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice*. J Cell Biol, 2005. **169**(3): p. 425-34.
189. Hubbard, V.M., et al., *Selective autophagy in the maintenance of cellular homeostasis in aging organisms*. Biogerontology, 2012. **13**(1): p. 21-35.
190. Wang, X. and E.J. Terpstra, *Ubiquitin receptors and protein quality control*. J Mol Cell Cardiol, 2013. **55**: p. 73-84.
191. Lin, X.L., et al., *Interaction Domains of p62: A Bridge Between p62 and Selective Autophagy*. DNA and Cell Biology, 2013. **32**(5): p. 220-227.
192. Moscat, J., et al., *Cell signaling and function organized by PB1 domain interactions*. Mol Cell, 2006. **23**(5): p. 631-40.
193. Sanz, L., et al., *The interaction of p62 with RIP links the atypical PKCs to NF-kappaB activation*. EMBO J, 1999. **18**(11): p. 3044-53.
194. Nakamura, K., et al., *PB1 domain interaction of p62/sequestosome 1 and MEKK3 regulates NF-kappaB activation*. J Biol Chem, 2010. **285**(3): p. 2077-89.
195. Wooten, M.W., et al., *The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination*. J Biol Chem, 2005. **280**(42): p. 35625-9.
196. Puissant, A., N. Fenouille, and P. Auberger, *When autophagy meets cancer through p62/SQSTM1*. American Journal of Cancer Research, 2012. **2**(4): p. 397-413.
197. Jain, A., et al., *p62/SQSTM1 Is a Target Gene for Transcription Factor NRF2 and Creates a Positive Feedback Loop by Inducing Antioxidant Response Element-driven Gene Transcription*. Journal of Biological Chemistry, 2010. **285**(29): p. 22576-22591.
198. Johansen, T. and T. Lamark, *Selective autophagy mediated by autophagic adapter proteins*. Autophagy, 2011. **7**(3): p. 279-96.
199. Weidberg, H., E. Shvets, and Z. Elazar, *Biogenesis and cargo selectivity of autophagosomes*. Annu Rev Biochem, 2011. **80**: p. 125-56.
200. White, E. and R.S. DiPaola, *The double-edged sword of autophagy modulation in cancer*. Clin Cancer Res, 2009. **15**(17): p. 5308-16.
201. Shintani, T. and D.J. Klionsky, *Autophagy in health and disease: a double-edged sword*. Science, 2004. **306**(5698): p. 990-5.
202. Liang, X.H., et al., *Induction of autophagy and inhibition of tumorigenesis by beclin 1*. Nature, 1999. **402**(6762): p. 672-676.

203. Michaud, M., et al., *Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice*. Science, 2011. **334**(6062): p. 1573-7.
204. Michaud, M., et al., *An autophagy-dependent anticancer immune response determines the efficacy of melanoma chemotherapy*. Oncoimmunology, 2014. **3**(7): p. e944047.
205. Martins, I., et al., *Premortem autophagy determines the immunogenicity of chemotherapy-induced cancer cell death*. Autophagy, 2012. **8**(3): p. 413-5.
206. Parodi, M., et al., *Natural Killer (NK)/melanoma cell interaction induces NK-mediated release of chemotactic High Mobility Group Box-1 (HMGB1) capable of amplifying NK cell recruitment*. Oncoimmunology, 2015. **4**(12).
207. Thorburn, J., et al., *Autophagy regulates selective HMGB1 release in tumor cells that are destined to die*. Cell Death and Differentiation, 2009. **16**(1): p. 175-183.
208. Rao, S., et al., *A dual role for autophagy in a murine model of lung cancer*. Nat Commun, 2014. **5**: p. 3056.
209. Guo, J.Y., et al., *Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis*. Genes Dev, 2011. **25**(5): p. 460-70.
210. Galluzzi, L., et al., *Activating autophagy to potentiate immunogenic chemotherapy and radiation therapy*. Nat Rev Clin Oncol, 2017. **14**(4): p. 247-258.
211. Hu, Y.L., et al., *Tumor Cell Autophagy as an Adaptive Response Mediating Resistance to Treatments Such as Antiangiogenic Therapy*. Cancer Research, 2012. **72**(17): p. 4294-4299.
212. Alegre, F., et al., *Role of p62/SQSTM1 beyond autophagy: a lesson learned from drug-induced toxicity in vitro*. Br J Pharmacol, 2018. **175**(3): p. 440-455.
213. Katsuragi, Y., Y. Ichimura, and M. Komatsu, *p62/SQSTM1 functions as a signaling hub and an autophagy adaptor*. Febs Journal, 2015. **282**(24): p. 4672-4678.
214. Bertrand, M., et al., *SQSTM1/p62 regulates the expression of junctional proteins through epithelial-mesenchymal transition factors*. Cell Cycle, 2015. **14**(3): p. 364-374.
215. Catalano, M., et al., *Autophagy induction impairs migration and invasion by reversing EMT in glioblastoma cells*. Mol Oncol, 2015. **9**(8): p. 1612-25.
216. Lv, Q., et al., *DEDD interacts with PI3KC3 to activate autophagy and attenuate epithelial-mesenchymal transition in human breast cancer*. Cancer Res, 2012. **72**(13): p. 3238-50.
217. Qiang, L., et al., *Regulation of cell proliferation and migration by p62 through stabilization of Twist1 in human SCC and melanoma cells*. Journal of Investigative Dermatology, 2014. **134**: p. S34-S34.
218. Belaid, A., et al., *Autophagy plays a critical role in the degradation of active RHOA, the control of cell cytokinesis, and genomic stability*. Cancer Res, 2013. **73**(14): p. 4311-22.
219. Sharifi, M.N., et al., *Autophagy Promotes Focal Adhesion Disassembly and Cell Motility of Metastatic Tumor Cells through the Direct Interaction of Paxillin with LC3*. Cell Reports, 2016. **15**(8): p. 1660-1672.
220. Bustin, M., *Revised nomenclature for high mobility group (HMG) chromosomal proteins*. Trends Biochem Sci, 2001. **26**(3): p. 152-3.
221. Mellon, I. and J.S. Bhorjee, *Two-Dimensional Gel-Electrophoretic Analysis of the High Mobility Group (Hmg) Non-Histone Chromosomal-Proteins*. Journal of Cell Biology, 1982. **95**(2): p. A69-A69.
222. Reeves, R., *Molecular biology of HMGA proteins: hubs of nuclear function*. Gene, 2001. **277**(1-2): p. 63-81.
223. Cleynen, I. and W.J. Van de Ven, *The HMGA proteins: a myriad of functions (Review)*. Int J Oncol, 2008. **32**(2): p. 289-305.
224. Friedmann, M., et al., *Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene*. Nucleic Acids Res, 1993. **21**(18): p. 4259-67.
225. Chau, K.Y., et al., *The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element*. Nucleic Acids Res, 1995. **23**(21): p. 4262-6.

226. Johnson, K.R., D.A. Lehn, and R. Reeves, *Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y*. Mol Cell Biol, 1989. **9**(5): p. 2114-23.
227. Nagpal, S., et al., *Retinoid-dependent recruitment of a histone H1 displacement activity by retinoic acid receptor*. J Biol Chem, 1999. **274**(32): p. 22563-8.
228. Cesana, M., et al., *A CLK3-HMGA2 Alternative Splicing Axis Impacts Human Hematopoietic Stem Cell Molecular Identity throughout Development*. Cell Stem Cell, 2018. **22**(4): p. 575-588 e7.
229. Hauke, S., et al., *Extensive expression studies revealed a complex alternative splicing pattern of the HMGA2 gene*. Biochim Biophys Acta, 2005. **1729**(1): p. 24-31.
230. Reeves, R. and M.S. Nissen, *The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure*. J Biol Chem, 1990. **265**(15): p. 8573-82.
231. Reeves, R., *HMGA proteins: flexibility finds a nuclear niche?* Biochem Cell Biol, 2003. **81**(3): p. 185-95.
232. Yie, J., et al., *The role of HMG I(Y) in the assembly and function of the IFN-beta enhanceosome*. EMBO J, 1999. **18**(11): p. 3074-89.
233. Munshi, N., et al., *Coordination of a transcriptional switch by HMGI(Y) acetylation*. Science, 2001. **293**(5532): p. 1133-1136.
234. Sgarra, R., et al., *Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer*. Febs Letters, 2004. **574**(1-3): p. 1-8.
235. Reeves, R., W.J. Leonard, and M.S. Nissen, *Binding of HMG-I(Y) imparts architectural specificity to a positioned nucleosome on the promoter of the human interleukin-2 receptor alpha gene*. Mol Cell Biol, 2000. **20**(13): p. 4666-79.
236. Brunetti, A., et al., *Transcriptional regulation of human insulin receptor gene by the high-mobility group protein HMGI(Y)*. FASEB J, 2001. **15**(2): p. 492-500.
237. Maddon, P.J., et al., *The T4 Gene Encodes the Aids Virus Receptor and Is Expressed in the Immune-System and the Brain*. Cell, 1986. **47**(3): p. 333-348.
238. Farnet, C.M. and F.D. Bushman, *HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes in vitro*. Cell, 1997. **88**(4): p. 483-92.
239. French, S.W., M.C. Schmidt, and J.C. Glorioso, *Involvement of a high-mobility-group protein in the transcriptional activity of herpes simplex virus latency-active promoter 2*. Mol Cell Biol, 1996. **16**(10): p. 5393-9.
240. Panagiotidis, C.A. and S.J. Silverstein, *The host-cell architectural protein HMG I(Y) modulates binding of herpes simplex virus type 1 ICP4 to its cognate promoter*. Virology, 1999. **256**(1): p. 64-74.
241. Leger, H., et al., *Functional Interaction between the Pou Domain Protein Tst-1/Oct-6 and the High-Mobility-Group Protein Hmg-I/Y*. Molecular and Cellular Biology, 1995. **15**(7): p. 3738-3747.
242. Bouallaga, I., et al., *An enhanceosome containing the Jun B/Fra-2 heterodimer and the HMG-I(Y) architectural protein controls HPV18 transcription*. Embo Reports, 2000. **1**(5): p. 422-427.
243. Bouallaga, I., et al., *HMG-I(Y) and the CBP/p300 coactivator are essential for human papillomavirus type 18 enhanceosome transcriptional activity*. Mol Cell Biol, 2003. **23**(7): p. 2329-40.
244. Chin, M.T., et al., *Enhancement of serum-response factor-dependent transcription and DNA binding by the architectural transcription factor HMG-I(Y)*. J Biol Chem, 1998. **273**(16): p. 9755-60.
245. Zhao, K., et al., *SAR-dependent mobilization of histone H1 by HMG-I/Y in vitro: HMG-I/Y is enriched in H1-depleted chromatin*. EMBO J, 1993. **12**(8): p. 3237-47.
246. Owen-Hughes, T. and J.L. Workman, *Experimental analysis of chromatin function in transcription control*. Crit Rev Eukaryot Gene Expr, 1994. **4**(4): p. 403-41.

247. Galande, S., *Chromatin (dis)Organization and Cancer: BUR-binding Proteins as Biomarkers for Cancer*. Current Cancer Drug Targets, 2002. **2**(2): p. 157-190.
248. Lund, T., J. Holtlund, and S.G. Laland, *On the phosphorylation of low molecular mass HMG (high mobility group) proteins in Ehrlich ascites cells*. FEBS Lett, 1985. **180**(2): p. 275-9.
249. Elton, T.S. and R. Reeves, *Purification and postsynthetic modifications of Friend erythroleukemic cell high mobility group protein HMG-I*. Anal Biochem, 1986. **157**(1): p. 53-62.
250. Nissen, M.S., T.A. Langan, and R. Reeves, *Phosphorylation by cdc2 kinase modulates DNA binding activity of high mobility group I nonhistone chromatin protein*. J Biol Chem, 1991. **266**(30): p. 19945-52.
251. Reeves, R., T.A. Langan, and M.S. Nissen, *Phosphorylation of the DNA-binding domain of nonhistone high-mobility group I protein by cdc2 kinase: reduction of binding affinity*. Proc Natl Acad Sci U S A, 1991. **88**(5): p. 1671-5.
252. Xiao, D.M., et al., *Phosphorylation of HMG-I by protein kinase C attenuates its binding affinity to the promoter regions of protein kinase C gamma and neurogranin/RC3 genes*. Journal of Neurochemistry, 2000. **74**(1): p. 392-399.
253. Schwanbeck, R. and J.R. Wisniewski, *Cdc2 and mitogen-activated protein kinases modulate DNA binding properties of the putative transcriptional regulator Chironomus high mobility group protein I*. J Biol Chem, 1997. **272**(43): p. 27476-83.
254. Palvimo, J. and A. Linnala-Kankkunen, *Identification of sites on chromosomal protein HMG-I phosphorylated by casein kinase II*. FEBS Lett, 1989. **257**(1): p. 101-4.
255. Pierantoni, G.M., et al., *High mobility group I (Y) proteins bind HIPK2, a serine-threonine kinase protein which inhibits cell growth*. Oncogene, 2001. **20**(43): p. 6132-6141.
256. Zhang, Q.C. and Y.S. Wang, *Homeodomain-interacting protein kinase-2 (HIPK2) phosphorylates HMGA1a at Ser-35, Thr-52, and Thr-77 and modulates its DNA binding affinity*. Journal of Proteome Research, 2007. **6**(12): p. 4711-4719.
257. Wang, D.Z., P. Ray, and M. Boothby, *Interleukin 4-Inducible Phosphorylation of Hmg-I(Y) Is Inhibited by Rapamycin*. Journal of Biological Chemistry, 1995. **270**(39): p. 22924-22932.
258. Diana, F., et al., *A link between apoptosis and degree of phosphorylation of high mobility group A1a protein in leukemic cells*. Journal of Biological Chemistry, 2001. **276**(14): p. 11354-11361.
259. Banks, G.C., Y. Li, and R. Reeves, *Differential in vivo modifications of the HMGI(Y) nonhistone chromatin proteins modulate nucleosome and DNA interactions*. Biochemistry, 2000. **39**(28): p. 8333-46.
260. Yie, J.M., et al., *Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter*. Molecular and Cellular Biology, 1997. **17**(7): p. 3649-3662.
261. Panne, D., T. Maniatis, and S.C. Harrison, *An atomic model of the interferon-beta enhanceosome*. Cell, 2007. **129**(6): p. 1111-23.
262. Munshi, N., et al., *Acetylation of HMG I(Y) by CBP turns off IFN beta expression by disrupting the enhanceosome*. Mol Cell, 1998. **2**(4): p. 457-67.
263. Edberg, D.D., et al., *In vivo posttranslational modifications of the high mobility group A1a proteins in breast cancer cells of differing metastatic potential*. Biochemistry, 2004. **43**(36): p. 11500-15.
264. Sgarra, R., et al., *During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry*. Biochemistry, 2003. **42**(12): p. 3575-85.
265. Sgarra, R., et al., *Increase of HMGA1a protein methylation is a distinctive characteristic of leukaemic cells induced to undergo apoptosis*. Cell Death and Differentiation, 2003. **10**(3): p. 386-389.

266. Sgarra, R., et al., *The AT-hook of the chromatin architectural transcription factor high mobility group A1a is arginine-methylated by protein arginine methyltransferase 6*. Journal of Biological Chemistry, 2006. **281**(7): p. 3764-3772.
267. Zou, Y. and Y.S. Wang, *Mass spectrometric analysis of high-mobility group proteins and their post-translational modifications in normal and cancerous human breast tissues*. Journal of Proteome Research, 2007. **6**(6): p. 2304-2314.
268. Fusco, A. and M. Fedele, *Roles of HMGA proteins in cancer*. Nature Reviews Cancer, 2007. **7**(12): p. 899-910.
269. Chiappetta, G., et al., *High level expression of the HMGI (Y) gene during embryonic development*. Oncogene, 1996. **13**(11): p. 2439-46.
270. Fedele, M., et al., *Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice*. Cancer Res, 2006. **66**(5): p. 2536-43.
271. Foti, D., et al., *Lack of the architectural factor HMGA1 causes insulin resistance and diabetes in humans and mice*. Nat Med, 2005. **11**(7): p. 765-73.
272. Liu, J., et al., *Hmga1 is required for normal sperm development*. Mol Reprod Dev, 2003. **66**(1): p. 81-9.
273. Chieffi, P., et al., *HMGA1 and HMGA2 protein expression in mouse spermatogenesis*. Oncogene, 2002. **21**(22): p. 3644-50.
274. Battista, S., et al., *Loss of Hmga1 gene function affects embryonic stem cell lympho-hematopoietic differentiation*. FASEB J, 2003. **17**(11): p. 1496-8.
275. Esposito, F., et al., *Interaction between HMGA1 and Retinoblastoma Protein Is Required for Adipocyte Differentiation*. Journal of Biological Chemistry, 2009. **284**(38): p. 25993-26004.
276. Anand, A. and K. Chada, *In vivo modulation of Hmgic reduces obesity*. Nat Genet, 2000. **24**(4): p. 377-80.
277. Caron, L., et al., *A new role for the oncogenic high-mobility group A2 transcription factor in myogenesis of embryonic stem cells*. Oncogene, 2005. **24**(41): p. 6281-91.
278. Zhou, X., et al., *Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C*. Nature, 1995. **376**(6543): p. 771-4.
279. Brants, J.R., et al., *Differential regulation of the insulin-like growth factor II mRNA-binding protein genes by architectural transcription factor HMGA2*. FEBS Lett, 2004. **569**(1-3): p. 277-83.
280. Cleyne, I., et al., *HMGA2 regulates transcription of the Imp2 gene via an intronic regulatory element in cooperation with nuclear factor-kappaB*. Mol Cancer Res, 2007. **5**(4): p. 363-72.
281. Li, Z., et al., *An HMGA2-IGF2BP2 axis regulates myoblast proliferation and myogenesis*. Dev Cell, 2012. **23**(6): p. 1176-88.
282. Federico, A., et al., *Hmga1/Hmga2 double knock-out mice display a "superpygmy" phenotype*. Biol Open, 2014. **3**(5): p. 372-8.
283. Baldassarre, G., et al., *Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway*. Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7970-5.
284. Fedele, M., et al., *Role of the high mobility group A proteins in human lipomas*. Carcinogenesis, 2001. **22**(10): p. 1583-91.
285. Copley, M.R., et al., *The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells*. Nat Cell Biol, 2013. **15**(8): p. 916-25.
286. Kishi, Y., et al., *HMGA regulates the global chromatin state and neurogenic potential in neocortical precursor cells*. Nat Neurosci, 2012. **15**(8): p. 1127-33.
287. Schlueter, C., et al., *HMGA1 proteins in human atherosclerotic plaques*. Pathol Res Pract, 2005. **201**(2): p. 101-7.
288. Giancotti, V., et al., *Changes in nuclear proteins on transformation of rat epithelial thyroid cells by a murine sarcoma retrovirus*. Cancer Res, 1985. **45**(12 Pt 1): p. 6051-7.

289. Pallante, P., et al., *High mobility group a proteins as tumor markers*. *Frontiers in Medicine*, 2015. **2**.
290. Frasca, F., et al., *HMGA1 inhibits the function of p53 family members in thyroid cancer cells*. *Cancer Res*, 2006. **66**(6): p. 2980-9.
291. Esposito, F., et al., *High-mobility group A1 proteins regulate p53-mediated transcription of Bcl-2 gene*. *Cancer Res*, 2010. **70**(13): p. 5379-88.
292. Pierantoni, G.M., et al., *High-mobility group A1 inhibits p53 by cytoplasmic relocation of its proapoptotic activator HIPK2*. *J Clin Invest*, 2007. **117**(3): p. 693-702.
293. Nevins, J.R., *The Rb/E2F pathway and cancer*. *Hum Mol Genet*, 2001. **10**(7): p. 699-703.
294. Luo, R.X., A.A. Postigo, and D.C. Dean, *Rb interacts with histone deacetylase to repress transcription*. *Cell*, 1998. **92**(4): p. 463-73.
295. Zhang, H.S., et al., *Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF*. *Cell*, 2000. **101**(1): p. 79-89.
296. DeLuca, A., et al., *A unique domain of pRb2/p130 acts as an inhibitor of Cdk2 kinase activity*. *Journal of Biological Chemistry*, 1997. **272**(34): p. 20971-20974.
297. Dahiya, A., et al., *Linking the Rb and polycomb pathways*. *Mol Cell*, 2001. **8**(3): p. 557-69.
298. Nielsen, S.J., et al., *Rb targets histone H3 methylation and HP1 to promoters*. *Nature*, 2001. **412**(6846): p. 561-5.
299. Fedele, M., et al., *HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity*. *Cancer Cell*, 2006. **9**(6): p. 459-71.
300. Ozturk, N., et al., *HMGA proteins as modulators of chromatin structure during transcriptional activation*. *Front Cell Dev Biol*, 2014. **2**: p. 5.
301. Whitmarsh, A.J. and R.J. Davis, *Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways*. *J Mol Med (Berl)*, 1996. **74**(10): p. 589-607.
302. Odabaei, G., et al., *Raf-1 kinase inhibitor protein: structure, function, regulation of cell signaling, and pivotal role in apoptosis*. *Adv Cancer Res*, 2004. **91**: p. 169-200.
303. Reeves, R. and L. Beckerbauer, *HMGI/Y proteins: flexible regulators of transcription and chromatin structure*. *Biochim Biophys Acta*, 2001. **1519**(1-2): p. 13-29.
304. Tessari, M.A., et al., *Transcriptional activation of the cyclin A gene by the architectural transcription factor HMGA2*. *Mol Cell Biol*, 2003. **23**(24): p. 9104-16.
305. Casalino, L., et al., *Fra-1 promotes growth and survival in RAS-transformed thyroid cells by controlling cyclin A transcription*. *EMBO J*, 2007. **26**(7): p. 1878-90.
306. Schulze, A., et al., *Cell-Cycle Regulation of the Cyclin-a Gene Promoter Is Mediated by a Variant E2f Site*. *Proceedings of the National Academy of Sciences of the United States of America*, 1995. **92**(24): p. 11264-11268.
307. Said, N.A. and E.D. Williams, *Growth factors in induction of epithelial-mesenchymal transition and metastasis*. *Cells Tissues Organs*, 2011. **193**(1-2): p. 85-97.
308. Morishita, A., et al., *HMGA2 Is a Driver of Tumor Metastasis*. *Cancer Research*, 2013. **73**(14): p. 4289-4299.
309. Sulston, J.E. and H.R. Horvitz, *Post-embryonic cell lineages of the nematode, Caenorhabditis elegans*. *Dev Biol*, 1977. **56**(1): p. 110-56.
310. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans*. *Nature*, 2000. **403**(6772): p. 901-6.
311. Boyerinas, B., et al., *The role of let-7 in cell differentiation and cancer*. *Endocr Relat Cancer*, 2010. **17**(1): p. F19-36.
312. Thomson, J.M., et al., *A custom microarray platform for analysis of microRNA gene expression*. *Nat Methods*, 2004. **1**(1): p. 47-53.
313. Lee, Y.S. and A. Dutta, *The tumor suppressor microRNA let-7 represses the HMGA2 oncogene*. *Genes Dev*, 2007. **21**(9): p. 1025-30.
314. Thuault, S., et al., *HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition*. *J Biol Chem*, 2008. **283**(48): p. 33437-46.

315. Tan, E.J., et al., *Regulation of transcription factor Twist expression by the DNA architectural protein high mobility group A2 during epithelial-to-mesenchymal transition*. J Biol Chem, 2012. **287**(10): p. 7134-45.
316. Thornton, J.E. and R.I. Gregory, *How does Lin28 let-7 control development and disease?* Trends in Cell Biology, 2012. **22**(9): p. 474-482.
317. Balzeau, J., et al., *The LIN28/let-7 Pathway in Cancer*. Front Genet, 2017. **8**: p. 31.
318. Feng, C., et al., *Lin28 regulates HER2 and promotes malignancy through multiple mechanisms*. Cell Cycle, 2012. **11**(13): p. 2486-2494.
319. Shell, S., et al., *Let-7 expression defines two differentiation stages of cancer*. Proc Natl Acad Sci U S A, 2007. **104**(27): p. 11400-5.
320. Watanabe, S., et al., *HMGA2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells*. Am J Pathol, 2009. **174**(3): p. 854-68.
321. Zha, L., et al., *HMGA2 Elicits EMT by Activating the Wnt/beta-catenin Pathway in Gastric Cancer*. Digestive Diseases and Sciences, 2013. **58**(3): p. 724-733.
322. Wu, J.J., et al., *HMGA2 Overexpression-Induced Ovarian Surface Epithelial Transformation Is Mediated Through Regulation of EMT Genes*. Cancer Research, 2011. **71**(2): p. 349-359.
323. Schoenmakers, E.F., et al., *Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours*. Nat Genet, 1995. **10**(4): p. 436-44.
324. Ashar, H.R., et al., *Disruption of the Architectural Factor Hmgi-C - DNA-Binding at Hook Motifs Fused in Lipomas to Distinct Transcriptional Regulatory Domains*. Cell, 1995. **82**(1): p. 57-65.
325. Heim, S., et al., *A specific translocation, t(12;14)(q14-15;q23-24), characterizes a subgroup of uterine leiomyomas*. Cancer Genet Cytogenet, 1988. **32**(1): p. 13-7.
326. Hennig, Y., et al., *HMGIC expressed in a uterine leiomyoma with a deletion of the long arm of chromosome 7 along with a 12q14-15 rearrangement but not in tumors showing del(7) as the sole cytogenetic abnormality*. Cancer Genetics and Cytogenetics, 1997. **96**(2): p. 129-133.
327. Kazmierczak, B., et al., *HMGI-C rearrangements as the molecular basis for the majority of pulmonary chondroid hamartomas: A survey of 30 tumors*. Oncogene, 1996. **12**(3): p. 515-521.
328. Staats, B., et al., *A fibroadenoma with a t(4;12) (q27;q15) affecting the HMGI-C gene, a member of the high mobility group protein gene family*. Breast Cancer Res Treat, 1996. **38**(3): p. 299-303.
329. Walter, T.A., et al., *inv(12)(p11.2q13) in an endometrial polyp*. Cancer Genet Cytogenet, 1989. **41**(1): p. 99-103.
330. Wanschura, S., et al., *Regional fine mapping of the multiple-aberration region involved in uterine leiomyoma, lipoma, and pleomorphic adenoma of the salivary gland to 12q15*. Genes Chromosomes Cancer, 1995. **14**(1): p. 68-70.
331. Rabban, J.T., P. Dal Cin, and E. Oliva, *HMGA2 rearrangement in a case of vulvar aggressive angiomyxoma*. Int J Gynecol Pathol, 2006. **25**(4): p. 403-7.
332. Kazmierczak, B., et al., *Molecular characterization of 12q14-15 rearrangements in three pulmonary chondroid hamartomas*. Cancer Res, 1995. **55**(12): p. 2497-9.
333. Kools, P.F. and W.J. Van de Ven, *Amplification of a rearranged form of the high-mobility group protein gene HMGIC in OsA-CI osteosarcoma cells*. Cancer Genet Cytogenet, 1996. **91**(1): p. 1-7.
334. Kazmierczak, B., et al., *HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors*. Genes Chromosomes & Cancer, 1998. **23**(4): p. 279-285.
335. Lee, S.M., L. Cavallo, and J. Griffith, *Human p53 binds Holliday junctions strongly and facilitates their cleavage*. Journal of Biological Chemistry, 1997. **272**(11): p. 7532-7539.
336. Lee, S., et al., *p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches*. Cell, 1995. **81**(7): p. 1013-20.

337. Fishel, R., A. Ewel, and M.K. Lescoe, *Purified human MSH2 protein binds to DNA containing mismatched nucleotides*. *Cancer Res*, 1994. **54**(21): p. 5539-42.
338. Alani, E., et al., *Saccharomyces cerevisiae MSH2, a mispaired base recognition protein, also recognizes Holliday junctions in DNA*. *Journal of Molecular Biology*, 1997. **265**(3): p. 289-301.
339. Subramanian, D. and J.D. Griffith, *Interactions between p53, hMSH2-hMSH6 and HMG I(Y) on Holliday junctions and bulged bases*. *Nucleic Acids Research*, 2002. **30**(11): p. 2427-2434.
340. Reeves, R. and J.E. Adair, *Role of high mobility group (HMG) chromatin proteins in DNA repair*. *DNA Repair (Amst)*, 2005. **4**(8): p. 926-38.
341. Borrmann, L., et al., *High mobility group A2 protein and its derivatives bind a specific region of the promoter of DNA repair gene ERCC1 and modulate its activity*. *Nucleic Acids Res*, 2003. **31**(23): p. 6841-51.
342. Baldassarre, G., et al., *Negative regulation of BRCA1 gene expression by HMGA1 proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma*. *Mol Cell Biol*, 2003. **23**(7): p. 2225-38.
343. Hartman, A.R. and J.M. Ford, *BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair*. *Nat Genet*, 2002. **32**(1): p. 180-4.
344. Baldassarre, G., et al., *HMGA1 protein expression sensitizes cells to cisplatin-induced cell death*. *Oncogene*, 2005. **24**(45): p. 6809-6819.
345. Boo, L.M., et al., *High mobility group A2 potentiates genotoxic stress in part through the modulation of basal and DNA damage-dependent phosphatidylinositol 3-kinase-related protein kinase activation*. *Cancer Res*, 2005. **65**(15): p. 6622-30.
346. Mantovani, A., *Cancer: inflammation by remote control*. *Nature*, 2005. **435**(7043): p. 752-3.
347. Perrella, M.A., et al., *High mobility group-I(Y) protein facilitates nuclear factor-kappa B binding and transactivation of the inducible nitric-oxide synthase promoter/enhancer*. *Journal of Biological Chemistry*, 1999. **274**(13): p. 9045-9052.
348. Ji, Y.S., Q. Xu, and J.F. Schmedtje, Jr., *Hypoxia induces high-mobility-group protein I(Y) and transcription of the cyclooxygenase-2 gene in human vascular endothelium*. *Circ Res*, 1998. **83**(3): p. 295-304.
349. Whitley, M.Z., et al., *A Striking Similarity in the Organization of the E-Selectin and Beta-Interferon Gene Promoters*. *Molecular and Cellular Biology*, 1994. **14**(10): p. 6464-6475.
350. Kim, J., et al., *The non-histone chromosomal protein HMG-I(Y) contributes to repression of the immunoglobulin heavy chain germ-line epsilon RNA promoter*. *Eur J Immunol*, 1995. **25**(3): p. 798-808.
351. Chuvpilo, S., et al., *Multiple Closely-Linked Nfat-Octamer and Hmg I(Y) Binding-Sites Are Part of the Interleukin-4 Promoter*. *Nucleic Acids Research*, 1993. **21**(24): p. 5694-5704.
352. Himes, S.R., et al., *High mobility group protein I(Y) is required for function and for c-Rel binding to CD28 response elements within the GM-CSF and IL-2 promoters*. *Immunity*, 1996. **5**(5): p. 479-489.
353. Kim, H.P., J. Kelly, and W.J. Leonard, *The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: Importance of two widely separated IL-2 response elements*. *Immunity*, 2001. **15**(1): p. 159-172.
354. Mantovani, F., et al., *NF-kappa B mediated transcriptional activation is enhanced by the architectural factor HMGI-C*. *Nucleic Acids Research*, 1998. **26**(6): p. 1433-1439.
355. Thanos, D. and T. Maniatis, *The High Mobility Group Protein Hmg-I(Y) Is Required for Nf-Kappa-B-Dependent Virus Induction of the Human Ifn-Beta Gene*. *Cell*, 1992. **71**(5): p. 777-789.
356. Huber, M.A., et al., *NF-kappa B is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression*. *Journal of Clinical Investigation*, 2004. **114**(4): p. 569-581.
357. Xie, T.X., et al., *Constitutive NF-kappa B activity regulates the expression of VEGF and IL-8 and tumor angiogenesis of human glioblastoma*. *Oncology Reports*, 2010. **23**(3): p. 725-732.

358. Yoshida, A., et al., *Suppression of retinal neovascularization by the NF-kappa B inhibitor pyrrolidine dithiocarbamate in mice*. Investigative Ophthalmology & Visual Science, 1999. **40**(7): p. 1624-1629.
359. Bustin, M. and R. Reeves, *High-mobility-group chromosomal proteins: Architectural components that facilitate chromatin function*. Progress in Nucleic Acid Research and Molecular Biology, Vol 54, 1996. **54**: p. 35-100.
360. Narita, M., et al., *A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation*. Cell, 2006. **126**(3): p. 503-14.
361. Munoz-Espin, D. and M. Serrano, *Cellular senescence: from physiology to pathology*. Nat Rev Mol Cell Biol, 2014. **15**(7): p. 482-96.
362. Rodier, F. and J. Campisi, *Four faces of cellular senescence*. J Cell Biol, 2011. **192**(4): p. 547-56.
363. Wang, Y.G. and M. Dasso, *SUMOylation and deSUMOylation at a glance*. Journal of Cell Science, 2009. **122**(23): p. 4249-4252.
364. Li, Y., J. Lu, and E.V. Prochownik, *Dual role for SUMO E2 conjugase Ubc9 in modulating the transforming and growth-promoting properties of the HMGA1b architectural transcription factor*. J Biol Chem, 2007. **282**(18): p. 13363-71.
365. Postnikov, Y. and M. Bustin, *Regulation of chromatin structure and function by HMGN proteins*. Biochim Biophys Acta, 2010. **1799**(1-2): p. 62-8.
366. Hock, R., et al., *HMG chromosomal proteins in development and disease*. Trends Cell Biol, 2007. **17**(2): p. 72-9.
367. Bustin, M., *Chromatin unfolding and activation by HMGN(*) chromosomal proteins*. Trends Biochem Sci, 2001. **26**(7): p. 431-7.
368. Rochman, M., et al., *The interaction of NSBP1/HMGN5 with nucleosomes in euchromatin counteracts linker histone-mediated chromatin compaction and modulates transcription*. Mol Cell, 2009. **35**(5): p. 642-56.
369. Shirakawa, H., et al., *NBP-45, a novel nucleosomal binding protein with a tissue-specific and developmentally regulated expression*. J Biol Chem, 2000. **275**(9): p. 6368-74.
370. Strichman-Almashanu, L.Z., M. Bustin, and D. Landsman, *Retroposed copies of the HMG genes: a window to genome dynamics*. Genome Res, 2003. **13**(5): p. 800-12.
371. Richmond, T.J. and C.A. Davey, *The structure of DNA in the nucleosome core*. Nature, 2003. **423**(6936): p. 145-50.
372. Alfonso, P.J., et al., *The footprint of chromosomal proteins HMG-14 and HMG-17 on chromatin subunits*. J Mol Biol, 1994. **236**(1): p. 189-98.
373. Catez, F., et al., *Competition between histone H1 and HMGN proteins for chromatin binding site*. Embo Reports, 2002. **3**(8): p. 760-766.
374. Phair, R.D., et al., *Global nature of dynamic protein-chromatin interactions in vivo: three-dimensional genome scanning and dynamic interaction networks of chromatin proteins*. Mol Cell Biol, 2004. **24**(14): p. 6393-402.
375. Catez, F. and R. Hock, *Binding and interplay of HMG proteins on chromatin: lessons from live cell imaging*. Biochim Biophys Acta, 2010. **1799**(1-2): p. 15-27.
376. Lim, J.H., et al., *Metastable macromolecular complexes containing high mobility group nucleosome-binding chromosomal proteins in HeLa nuclei*. J Biol Chem, 2002. **277**(23): p. 20774-82.
377. Paranjape, S.M., A. Krumm, and J.T. Kadonaga, *Hmg17 Is a Chromatin-Specific Transcriptional Coactivator That Increases the Efficiency of Transcription Initiation*. Genes & Development, 1995. **9**(16): p. 1978-1991.
378. Fousteri, M. and L.H. Mullenders, *Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects*. Cell Res, 2008. **18**(1): p. 73-84.

379. Fousteri, M., et al., *Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo*. Mol Cell, 2006. **23**(4): p. 471-82.
380. Zhu, N. and U. Hansen, *HMGN1 modulates estrogen-mediated transcriptional activation through interactions with specific DNA-binding transcription factors*. Mol Cell Biol, 2007. **27**(24): p. 8859-73.
381. Ueda, T., et al., *The nucleosome binding protein HMGN3 modulates the transcription profile of pancreatic beta cells and affects insulin secretion*. Mol Cell Biol, 2009. **29**(19): p. 5264-76.
382. Postnikov, Y.V., et al., *Homodimers of chromosomal proteins HMG-14 and HMG-17 in nucleosome cores*. J Mol Biol, 1995. **252**(4): p. 423-32.
383. Postnikov, Y.V., et al., *Clusters of nucleosomes containing chromosomal protein HMG-17 in chromatin*. J Mol Biol, 1997. **274**(4): p. 454-65.
384. Kato, H., et al., *Architecture of the high mobility group nucleosomal protein 2-nucleosome complex as revealed by methyl-based NMR*. Proc Natl Acad Sci U S A, 2011. **108**(30): p. 12283-8.
385. Brawley, J.V. and H.G. Martinson, *HMG proteins 14 and 17 become cross-linked to the globular domain of histone H3 near the nucleosome core particle dyad*. Biochemistry, 1992. **31**(2): p. 364-70.
386. Woodcock, C.L., A.I. Skoultchi, and Y. Fan, *Role of linker histone in chromatin structure and function: H1 stoichiometry and nucleosome repeat length*. Chromosome Res, 2006. **14**(1): p. 17-25.
387. Cuddapah, S., et al., *Genomic Profiling of HMGN1 Reveals an Association with Chromatin at Regulatory Regions*. Molecular and Cellular Biology, 2011. **31**(4): p. 700-709.
388. Deng, T., et al., *HMGN1 modulates nucleosome occupancy and DNase I hypersensitivity at the CpG island promoters of embryonic stem cells*. Mol Cell Biol, 2013. **33**(16): p. 3377-89.
389. Murphy, K.J., et al., *HMGN1 and 2 remodel core and linker histone tail domains within chromatin*. Nucleic Acids Research, 2017. **45**(17): p. 9917-9930.
390. Lim, J.H., et al., *Chromosomal protein HMGN1 modulates histone H3 phosphorylation*. Mol Cell, 2004. **15**(4): p. 573-84.
391. Lim, J.H., et al., *Chromosomal protein HMGN1 enhances the acetylation of lysine 14 in histone H3*. EMBO J, 2005. **24**(17): p. 3038-48.
392. Ueda, T., Y.V. Postnikov, and M. Bustin, *Distinct domains in high mobility group n variants modulate specific chromatin modifications*. Journal of Biological Chemistry, 2006. **281**(15): p. 10182-10187.
393. Hill, D.A., C.L. Peterson, and A.N. Imbalzano, *Effects of HMGN1 on chromatin structure and SWI/SNF-mediated chromatin remodeling*. J Biol Chem, 2005. **280**(50): p. 41777-83.
394. Rattner, B.P., T. Yusufzai, and J.T. Kadonaga, *HMGN proteins act in opposition to ATP-dependent chromatin remodeling factors to restrict nucleosome mobility*. Mol Cell, 2009. **34**(5): p. 620-6.
395. Belova, G.I., et al., *Chromosomal protein HMGN1 enhances the heat shock-induced remodeling of Hsp70 chromatin*. Journal of Biological Chemistry, 2008. **283**(13): p. 8080-8088.
396. Kim, Y.C., et al., *Activation of ATM depends on chromatin interactions occurring before induction of DNA damage*. Nature Cell Biology, 2009. **11**(1): p. 92-U192.
397. Pogna, E.A., A.L. Clayton, and L.C. Mahadevan, *Signalling to chromatin through post-translational modifications of HMGN*. Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms, 2010. **1799**(1-2): p. 93-100.
398. Bergel, M., et al., *Acetylation of novel sites in the nucleosomal binding domain of chromosomal protein HMG-14 by p300 alters its interaction with nucleosomes*. J Biol Chem, 2000. **275**(15): p. 11514-20.

399. Herrera, J.E., et al., *Specific acetylation of chromosomal protein HMG-17 by PCAF alters its interaction with nucleosomes*. Mol Cell Biol, 1999. **19**(5): p. 3466-73.
400. Jiang, X.G. and Y. Wang, *Phosphorylation of human high mobility group N1 protein by protein kinase CK2*. Biochem Biophys Res Commun, 2006. **345**(4): p. 1497-503.
401. Walton, G.M., et al., *Thyrotropin-stimulated phosphorylation of high mobility group protein 14 in vivo at the site catalyzed by cyclic nucleotide-dependent protein kinases in vitro*. J Biol Chem, 1984. **259**(1): p. 601-7.
402. Soloaga, A., et al., *MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14*. EMBO J, 2003. **22**(11): p. 2788-97.
403. Cheung, P., C.D. Allis, and P. Sassone-Corsi, *Signaling to chromatin through histone modifications*. Cell, 2000. **103**(2): p. 263-271.
404. Hirota, T., et al., *Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin*. Nature, 2005. **438**(7071): p. 1176-80.
405. Prymakowska-Bosak, M., et al., *Mitotic phosphorylation prevents the binding of HMGN proteins to chromatin*. Mol Cell Biol, 2001. **21**(15): p. 5169-78.
406. Hock, R., U. Scheer, and M. Bustin, *Chromosomal proteins HMG-14 and HMG-17 are released from mitotic chromosomes and imported into the nucleus by active transport*. Journal of Cell Biology, 1998. **143**(6): p. 1427-1436.
407. Prymakowska-Bosak, M., et al., *Mitotic phosphorylation of chromosomal protein HMGN1 inhibits nuclear import and promotes interaction with 14.3.3 proteins*. Mol Cell Biol, 2002. **22**(19): p. 6809-19.
408. Pallier, C., et al., *Association of chromatin proteins high mobility group box (HMGB) 1 and HMGB2 with mitotic chromosomes*. Mol Biol Cell, 2003. **14**(8): p. 3414-26.
409. Mohamed, O.A., M. Bustin, and H.J. Clarke, *High-mobility group proteins 14 and 17 maintain the timing of early embryonic development in the mouse*. Dev Biol, 2001. **229**(1): p. 237-49.
410. Vigneault, C., et al., *Transcription factor expression patterns in bovine in vitro-derived embryos prior to maternal-zygotic transition*. Biol Reprod, 2004. **70**(6): p. 1701-9.
411. Bastos, G.M., P.B. Goncalves, and V. Bordinon, *Immunolocalization of the high-mobility group N2 protein and acetylated histone H3K14 in early developing parthenogenetic bovine embryos derived from oocytes of high and low developmental competence*. Mol Reprod Dev, 2008. **75**(2): p. 282-90.
412. Korner, U., et al., *Developmental role of HMGN proteins in Xenopus laevis*. Mech Dev, 2003. **120**(10): p. 1177-92.
413. Furusawa, T., et al., *Down-regulation of nucleosomal binding protein HMGN1 expression during embryogenesis modulates Sox9 expression in chondrocytes*. Molecular and Cellular Biology, 2006. **26**(2): p. 592-604.
414. Pash, J.M., P.J. Alfonso, and M. Bustin, *Aberrant Expression of High-Mobility Group Chromosomal Protein-14 Affects Cellular-Differentiation*. Journal of Biological Chemistry, 1993. **268**(18): p. 13632-13638.
415. Lehtonen, S. and E. Lehtonen, *HMG-17 is an early marker of inductive interactions in the developing mouse kidney*. Differentiation, 2001. **67**(4-5): p. 154-163.
416. Crippa, M.P., J.M. Nickol, and M. Bustin, *Developmental-Changes in the Expression of High Mobility Group Chromosomal-Proteins*. Journal of Biological Chemistry, 1991. **266**(5): p. 2712-2714.
417. Pash, J.M., et al., *Persistence of Chromosomal-Proteins Hmg-14/-17 in Myotubes Following Differentiation-Dependent Reduction of Hmg Messenger-Rna*. Journal of Biological Chemistry, 1990. **265**(8): p. 4197-4199.
418. Birger, Y., et al., *Increased tumorigenicity and sensitivity to ionizing radiation upon loss of chromosomal protein HMGN1*. Cancer Research, 2005. **65**(15): p. 6711-6718.
419. Birger, Y., et al., *A role for chromosomal protein HMGN1 in corneal maturation*. Differentiation, 2006. **74**(1): p. 19-29.

420. West, K.L., et al., *HMGN3a and HMGN3b, two protein isoforms with a tissue-specific expression pattern, expand the cellular repertoire of nucleosome-binding proteins*. J Biol Chem, 2001. **276**(28): p. 25959-69.
421. Ito, Y. and M. Bustin, *Immunohistochemical localization of the nucleosome-binding protein HMGN3 in mouse brain*. J Histochem Cytochem, 2002. **50**(9): p. 1273-5.
422. Lucey, M.M., et al., *Differential expression of the HMGN family of chromatin proteins during ocular development*. Gene Expr Patterns, 2008. **8**(6): p. 433-7.
423. Shirakawa, H., et al., *The Nucleosomal Binding Protein NSBP1 Is Highly Expressed in the Placenta and Modulates the Expression of Differentiation Markers in Placental Rcho-1 Cells*. Journal of Cellular Biochemistry, 2009. **106**(4): p. 651-658.
424. Whitten, P.L., et al., *Potential adverse effects of phytoestrogens*. J Nutr, 1995. **125**(3 Suppl): p. 771S-776S.
425. Tang, W.Y., et al., *Persistent hypomethylation in the promoter of nucleosomal binding protein 1 (Nsbp1) correlates with overexpression of Nsbp1 in mouse uteri neonatally exposed to diethylstilbestrol or genistein*. Endocrinology, 2008. **149**(12): p. 5922-31.
426. Birger, Y., et al., *Chromosomal protein HMGN1 enhances the rate of DNA repair in chromatin*. EMBO J, 2003. **22**(7): p. 1665-75.
427. Gerlitz, G., *HMGNs, DNA repair and cancer*. Biochim Biophys Acta, 2010. **1799**(1-2): p. 80-5.
428. Amen, M., et al., *Chromatin-associated HMG-17 is a major regulator of homeodomain transcription factor activity modulated by Wnt/beta catenin signaling*. Nucleic Acids Research, 2008. **36**(2): p. 462-476.
429. Amano, T., et al., *Thyroid hormone regulation of a transcriptional coactivator in Xenopus laevis: implication for a role in postembryonic tissue remodeling*. Dev Dyn, 2002. **223**(4): p. 526-35.
430. Duffy, M.J., *Estrogen receptors: role in breast cancer*. Crit Rev Clin Lab Sci, 2006. **43**(4): p. 325-47.
431. Paruthiyil, S., et al., *Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest*. Cancer Res, 2004. **64**(1): p. 423-8.
432. Fung, F.K., et al., *Increased expression of PITX2 transcription factor contributes to ovarian cancer progression*. PLoS One, 2012. **7**(5): p. e37076.
433. Li, D.Q., et al., *Gene expression profile analysis of an isogenic tumour metastasis model reveals a functional role for oncogene AF1Q in breast cancer metastasis*. Eur J Cancer, 2006. **42**(18): p. 3274-86.
434. de Moraes, G.N., et al., *Forkhead box K2 modulates epirubicin and paclitaxel sensitivity through FOXO3a in breast cancer*. Oncogenesis, 2015. **4**.
435. Kwok, J.M., et al., *FOX M1 confers acquired cisplatin resistance in breast cancer cells*. Mol Cancer Res, 2010. **8**(1): p. 24-34.
436. Leung, T.W., et al., *Over-expression of FoxM1 stimulates cyclin B1 expression*. FEBS Lett, 2001. **507**(1): p. 59-66.
437. Nagase, T., et al., *Exploration of human ORFeome: high-throughput preparation of ORF clones and efficient characterization of their protein products*. DNA Res, 2008. **15**(3): p. 137-49.
438. Yao, S., et al., *Characterization of FOXO Acetylation*. Methods Mol Biol, 2019. **1890**: p. 77-90.
439. Sanders, D.A., et al., *Genome-wide mapping of FOXM1 binding reveals co-binding with estrogen receptor alpha in breast cancer cells*. Genome Biol, 2013. **14**(1): p. R6.
440. Robinson, J.T., et al., *Integrative genomics viewer*. Nature Biotechnology, 2011. **29**(1): p. 24-26.
441. Vichai, V. and K. Kirtikara, *Sulforhodamine B colorimetric assay for cytotoxicity screening*. Nat Protoc, 2006. **1**(3): p. 1112-6.
442. Nüsslein-Volhard, C. and R. Dahm, *Zebrafish : a practical approach*. 2002, Oxford: Oxford University Press. xviii, 303 p., 8 p. of plates.

443. Pegoraro, S., et al., *HMGA1 promotes metastatic processes in basal-like breast cancer regulating EMT and stemness*. *Oncotarget*, 2013. **4**(8): p. 1293-308.
444. Shah, S.N., et al., *HMGA1: a master regulator of tumor progression in triple-negative breast cancer cells*. *PLoS One*, 2013. **8**(5): p. e63419.
445. Evans, A., T.W. Lennard, and B.R. Davies, *High-mobility group protein 1(Y): metastasis-associated or metastasis-inducing?* *J Surg Oncol*, 2004. **88**(2): p. 86-99.
446. Ji, S.Q., et al., *Knockdown of the nucleosome binding protein 1 inhibits the growth and invasion of clear cell renal cell carcinoma cells in vitro and in vivo*. *J Exp Clin Cancer Res*, 2012. **31**: p. 22.
447. McDowall, M.D., M.S. Scott, and G.J. Barton, *PIPs: human protein-protein interaction prediction database*. *Nucleic Acids Res*, 2009. **37**(Database issue): p. D651-6.
448. Zhao, F., et al., *Overexpression of forkhead box protein M1 (FOXM1) in ovarian cancer correlates with poor patient survival and contributes to paclitaxel resistance*. *PLoS One*, 2014. **9**(11): p. e113478.
449. Yu, L., Y. Chen, and S.A. Tooze, *Autophagy pathway: Cellular and molecular mechanisms*. *Autophagy*, 2018. **14**(2): p. 207-215.
450. Hamurcu, Z., et al., *FOXM1 plays a role in autophagy by transcriptionally regulating Beclin-1 and LC3 genes in human triple-negative breast cancer cells*. *J Mol Med (Berl)*, 2019. **97**(4): p. 491-508.
451. Wierstra, I. and J. Alves, *FOXM1, a typical proliferation-associated transcription factor*. *Biol Chem*, 2007. **388**(12): p. 1257-74.
452. Tian, L., et al., *MiR-361-5p suppresses chemoresistance of gastric cancer cells by targeting FOXM1 via the PI3K/Akt/mTOR pathway*. *Oncotarget*, 2018. **9**(4): p. 4886-4896.
453. Moscat, J. and M.T. Diaz-Meco, *p62 at the crossroads of autophagy, apoptosis, and cancer*. *Cell*, 2009. **137**(6): p. 1001-4.
454. Duran, A., et al., *p62 Is a Key Regulator of Nutrient Sensing in the mTORC1 Pathway*. *Molecular Cell*, 2011. **44**(1): p. 134-146.
455. Seyfried, T.N. and L.C. Huysentruyt, *On the origin of cancer metastasis*. *Crit Rev Oncog*, 2013. **18**(1-2): p. 43-73.
456. Quinlivan, V.H. and S.A. Farber, *Lipid Uptake, Metabolism, and Transport in the Larval Zebrafish*. *Front Endocrinol (Lausanne)*, 2017. **8**: p. 319.
457. Krauss, J., et al., *transparent, a gene affecting stripe formation in Zebrafish, encodes the mitochondrial protein Mpv17 that is required for iridophore survival*. *Biology Open*, 2013. **2**(7): p. 703-710.
458. Li, S.S., et al., *p62/SQSTM1 interacts with vimentin to enhance breast cancer metastasis*. *Carcinogenesis*, 2017. **38**(11): p. 1092-1103.
459. Thompson, H.G., et al., *p62 overexpression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells*. *Oncogene*, 2003. **22**(15): p. 2322-33.
460. Inoue, D., et al., *Accumulation of p62/SQSTM1 is associated with poor prognosis in patients with lung adenocarcinoma*. *Cancer Science*, 2012. **103**(4): p. 760-766.
461. Kitamura, H., et al., *Cytosolic overexpression of p62 sequestosome 1 in neoplastic prostate tissue*. *Histopathology*, 2006. **48**(2): p. 157-61.
462. Li, L.J., et al., *SQSTM1 Is a Pathogenic Target of 5q Copy Number Gains in Kidney Cancer*. *Cancer Cell*, 2013. **24**(6): p. 738-750.
463. Kidd, M.E., D.K. Shumaker, and K.M. Ridge, *The Role of Vimentin Intermediate Filaments in the Progression of Lung Cancer*. *American Journal of Respiratory Cell and Molecular Biology*, 2014. **50**(1): p. 1-6.
464. Rolland, P., et al., *The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease*. *Endocrine-Related Cancer*, 2007. **14**(1): p. 73-80.

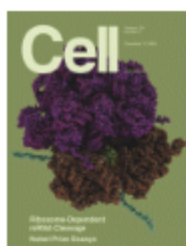
465. Jiang, X., et al., *Metastatic prostate cancer-associated P62 inhibits autophagy flux and promotes epithelial to mesenchymal transition by sustaining the level of HDAC6*. Prostate, 2018. **78**(6): p. 426-434.
466. Labriere, C., et al., *New MKLP-2 inhibitors in the paprotrain series: Design, synthesis and biological evaluations*. Bioorg Med Chem, 2016. **24**(4): p. 721-34.
467. Tcherniuk, S., et al., *Relocation of Aurora B and survivin from centromeres to the central spindle impaired by a kinesin-specific MKLP-2 inhibitor*. Angew Chem Int Ed Engl, 2010. **49**(44): p. 8228-31.
468. Collette, Y., et al., *Identification of a selective MKLP2/KIF20A inhibitor with high in-vivo antitumor activity*. Cancer Research, 2017. **77**.
469. Luo, W., et al., *FoxM1 Promotes Cell Proliferation, Invasion, and Stem Cell Properties in Nasopharyngeal Carcinoma*. Front Oncol, 2018. **8**: p. 483.
470. Shaw, F.L., et al., *A detailed mammosphere assay protocol for the quantification of breast stem cell activity*. J Mammary Gland Biol Neoplasia, 2012. **17**(2): p. 111-7.
471. Geng, A., et al., *KIF20A/MKLP2 regulates the division modes of neural progenitor cells during cortical development*. Nat Commun, 2018. **9**(1): p. 2707.
472. Dontu, G., et al., *Stem cells in normal breast development and breast cancer*. Cell Prolif, 2003. **36 Suppl 1**: p. 59-72.
473. Khongkow, P., et al., *FOX M1 targets NBS1 to regulate DNA damage-induced senescence and epirubicin resistance*. Oncogene, 2014. **33**(32): p. 4144-55.
474. Karunaratna, U., et al., *OTUB1 inhibits the ubiquitination and degradation of FOXM1 in breast cancer and epirubicin resistance*. Oncogene, 2015.
475. Collado, M. and M. Serrano, *Senescence in tumours: evidence from mice and humans*. Nat Rev Cancer, 2010. **10**(1): p. 51-7.
476. Reya, T., et al., *Stem cells, cancer, and cancer stem cells*. Nature, 2001. **414**(6859): p. 105-11.
477. Dement, G.A., et al., *Dynamic mitochondrial localization of nuclear transcription factor HMGA1*. Exp Cell Res, 2005. **307**(2): p. 388-401.
478. Cui, T. and F. Leng, *Specific recognition of AT-rich DNA sequences by the mammalian high mobility group protein AT-hook 2: a SELEX study*. Biochemistry, 2007. **46**(45): p. 13059-66.
479. Winter, N., et al., *Chromatin immunoprecipitation to analyze DNA binding sites of HMGA2*. PLoS One, 2011. **6**(4): p. e18837.
480. Hill, D.A. and R. Reeves, *Competition between HMG-I(Y), HMG-1 and histone H1 on four-way junction DNA*. Nucleic Acids Research, 1997. **25**(17): p. 3523-3531.
481. Hill, D.A., M.L. Pedulla, and R. Reeves, *Directional binding of HMG-I(Y) on four-way junction DNA and the molecular basis for competitive binding with HMG-1 and histone H1*. Nucleic Acids Res, 1999. **27**(10): p. 2135-44.
482. Cleynen, I., et al., *Transcriptional control of the human high mobility group A1 gene: basal and oncogenic Ras-regulated expression*. Cancer Res, 2007. **67**(10): p. 4620-9.
483. Hristov, A.C., et al., *HMGA1 correlates with advanced tumor grade and decreased survival in pancreatic ductal adenocarcinoma*. Mod Pathol, 2010. **23**(1): p. 98-104.
484. Liao, S.S., et al., *High mobility group AT-Hook 1 (HMGA1) is an independent prognostic factor and novel therapeutic target in pancreatic adenocarcinoma*. Cancer, 2008. **113**(2): p. 302-314.
485. Heldring, N., et al., *Multiple Sequence- Specific DNA-Binding Proteins Mediate Estrogen Receptor Signaling through a Tethering Pathway*. Molecular Endocrinology, 2011. **25**(4): p. 564-574.
486. Sanders, D.A., et al., *FOX M1 binds directly to non-consensus sequences in the human genome*. Genome Biol, 2015. **16**: p. 130.
487. Ye, H.G., et al., *Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues*. Molecular and Cellular Biology, 1997. **17**(3): p. 1626-1641.

- 488. Major, M.L., R. Lepe, and R.H. Costa, *Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators*. Mol Cell Biol, 2004. **24**(7): p. 2649-61.
- 489. Mathew, R., V. Karantza-Wadsworth, and E. White, *Role of autophagy in cancer*. Nat Rev Cancer, 2007. **7**(12): p. 961-7.
- 490. Karantza-Wadsworth, V. and E. White, *Role of autophagy in breast cancer*. Autophagy, 2007. **3**(6): p. 610-3.
- 491. Rath, O. and F. Kozielski, *Kinesins and cancer*. Nat Rev Cancer, 2012. **12**(8): p. 527-39.

APPENDIX

Page No.	Type of work:	Name of work	Source of work	Copyright holder and contact	permission requested on	I have permission yes /no	Permission note
38	Figure 1.2	Functional roles of FOXM1 in cancer progression	Yao, S., et al. (2018). "The FOXO3-FOXM1 axis: A key cancer drug target and a modulator of cancer drug resistance." <u>Semin Cancer Biol</u> 50: 77-89.	This article is available under the terms of the Creative Commons Attribution License (CC BY) . Permission is not required for this type of reuse.	01.06.2019	yes	Out of copyright
48	Figure 1.3 A	Three types of autophagy	N. Mizushima, M. Komatsu, Autophagy: renovation of cells and tissues, <u>Cell</u> 147(4) (2011) 728-41.	RightsLink® customercare@copy right.com	04.06.2019	yes	Licensed through Copyright Clearance Center's RightsLink® service
54	Figure 1.4	Multiple functions of HMGA protein	R. Sgarra, A. Rustighi, M.A. Tessari, J. Di Bernardo, S. Altamura, A. Fusco, G. Manfioletti, V. Giancotti, Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer, <u>Febs Lett</u> 574(1-3) (2004) 1-8.	RightsLink® customercare@copy right.com	12.06.2019	yes	Licensed through Copyright Clearance Center's RightsLink® service

Table S. 1 Copyright permissions summary.



Thank you for your order!

Dear Shang Yao,

Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

Order Summary

Licensee:	Imperial College London
Order Date:	Jun 4, 2019
Order Number:	4601971215322
Publication:	Cell
Title:	Autophagy: Renovation of Cells and Tissues
Type of Use:	reuse in a thesis/dissertation
Order Total:	0.00 GBP

View or print complete [details](#) of your order and the publisher's terms and conditions.

Sincerely,

Copyright Clearance Center

Tel: +1-855-239-3415 / +1-978-646-2777
customer@copyright.com
<https://myaccount.copyright.com>



RightsLink®

Figure S. 1 Copyright permission for Figure 1. 3 A.

WILEY

Thank you for your order!

Dear Shang Yao,

Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

Order Summary

Licensee: Imperial College London
Order Date: Jun 12, 2019
Order Number: 4606581371694
Publication: FEBS Letters
Title: Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer
Type of Use: Dissertation/Thesis
Order Total: 0.00 USD

View or print complete [details](#) of your order and the publisher's terms and conditions.

Sincerely,

Copyright Clearance Center

Tel: +1-855-239-3415 / +1-978-646-2777
customercare@copyright.com
<https://myaccount.copyright.com>



RightsLink®

Figure S. 2 Copyright permission for Figure 1. 4.

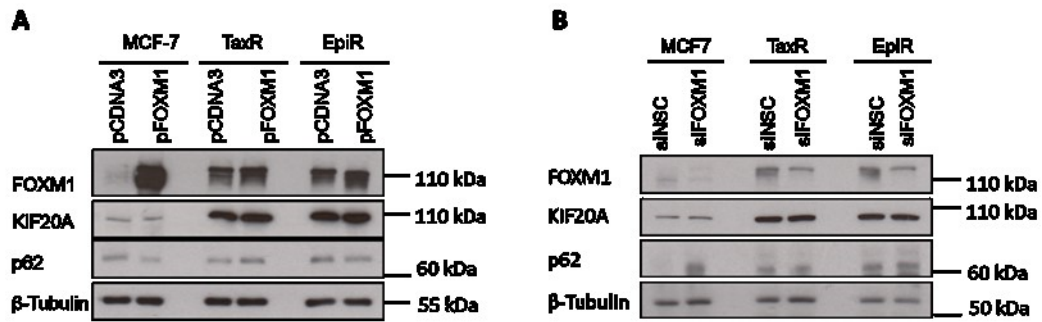


Figure S. 3 Regulations of FOXM1 on KIF20A and p62.

MCF-7, TaxR and EpiR cells were transfected with pCDNA3-FOXM1 or pCDNA3 control plasmids and Smart pool siRNA against FOXM1 or non-silencing controls (NSC) for overexpression and silencing transfection, respectively. Western blotting was used for FOXM1, KIF20A, p62 protein expression analysis. **(A)** FOXM1 overexpression and **(B)** FOXM1 knockdown results are shown. (n=3)

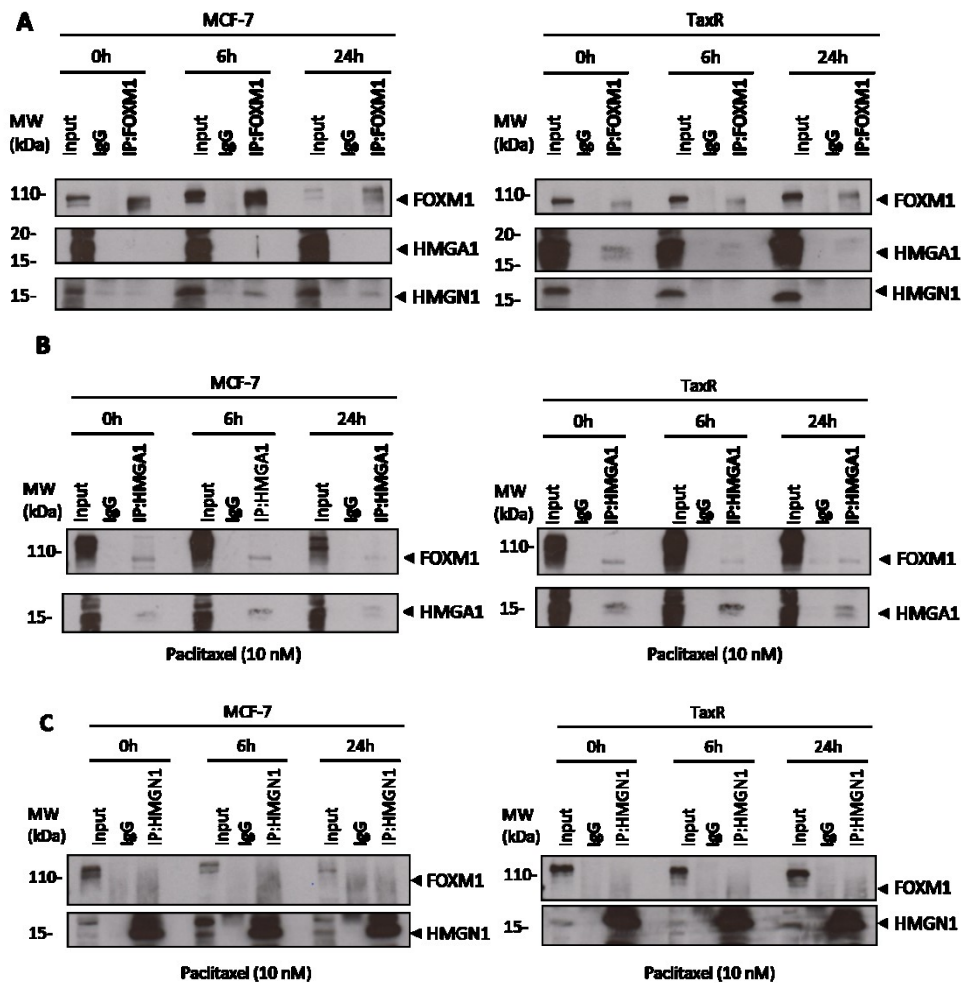


Figure S. 4 FOXM1, HMGA1 and HMGN1 interactions in response to paclitaxel treatment.

MCF-7 and TaxR cell lines were treated with 10 nM paclitaxel and harvested at 0h, 6h and 24h time points. Input (1/10 of IP), and IP products with IgG and specific antibodies were resolved on western blot and probed for specific antibodies. The specific bands were indicated by arrows. **(A)** Co-IP was performed using anti-FOXM1 antibody and immunoblotted with anti-HMGA1 and anti-HMGN1 antibodies. **(B)** Reverse co-IP of HMGA1 immunoprecipitation and probed with anti-FOXM1 antibody in MCF-7 and TaxR **(C)** Reverse co-IP of HMGN1 pulldown and immunoblotted with FOXM1. (n=2)

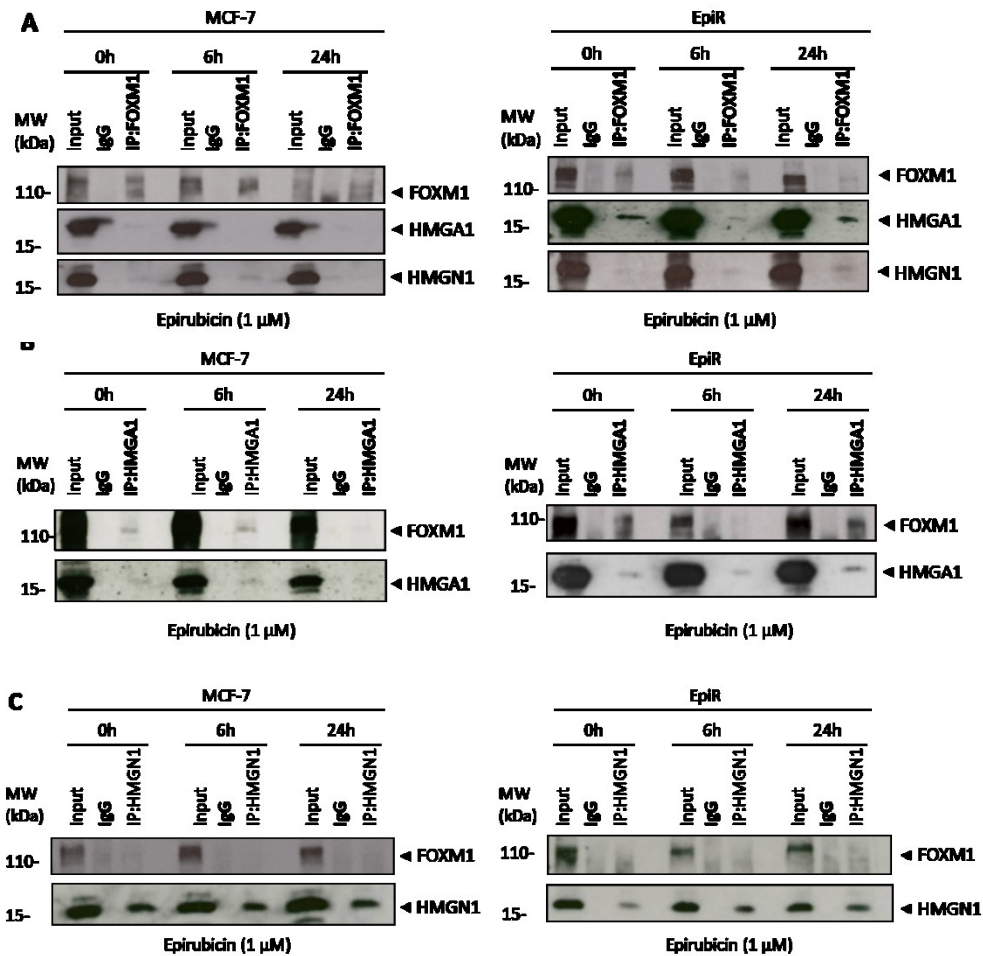


Figure S. 5 The interactions between FOXM1, HMGA1 and HMGN1 in response to epirubicin treatment.

MCF-7 and EpiR cells were treated with 1 μ M epirubicin and cell pellets were collected at 0h, 6h and 24h. Input (1/10 of IP), and IP products with IgG and FOXM1 (**A**), HMGA1 (**B**) and HMGN1 (**C**) were resolved on SDS-PAGE gels for immunoblotting analysis. Each arrow represents a specific antibody that has been probed. (**A**) Co-IP was performed using anti-FOXM1 antibody and immunoblotted with anti-HMGA1 and anti-HMGN1 antibodies. (**B**) Reverse co-IP of HMGA1 immunoprecipitation and probed with anti-FOXM1 antibody in MCF-7 and EpiR (**C**) Reverse co-IP of HMGN1 pulldown and immunoblotted with FOXM1. (n=2)

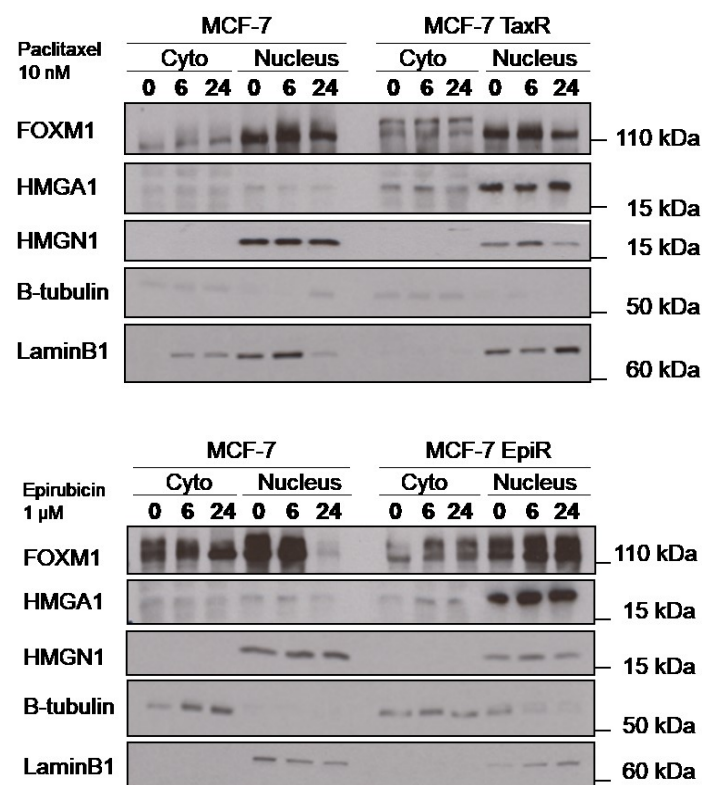


Figure S. 6 Subcellular localization of HMGA1 and HMGN1 upon drug treatment.

MCF-7 and two resistant cell lines TaxR and EpiR were treated with either 10nM paclitaxel (upper) or 1μM epirubicin (lower) respectively, harvested at different time points 0h, 6h or 24h, and fractionated into cytoplasmic and nuclear fractions. Cytoplasmic and nuclear fractions were loaded at the same amount onto SDS-polyacrylamide gel, and the proteins were resolved in the gel. FOXM1, HMGA1 and HMGN1 were detected by Western blotting with specific corresponding antibodies. β -tubulin (cytoplasmic marker) and LaminB1 (nuclear marker) were detected as fractionation and loading controls. (n=3)