

FOXM1 mediates the response to 5-fluorouracil via thymidylate synthase in colorectal cancer

Thesis submitted by
Vidhya Varghese

To

Imperial College London
For the degree of Doctor of Philosophy

Department of Surgery and Cancer
1st Floor ICTEM
Hammersmith Hospital Campus
Du Cane Road
London W12 0NN

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DECLARATION OF WORK

I hereby declare that this thesis and the work reported herein was composed and originated entirely by myself, with the exception of the ChIP sequencing studies, immunohistochemistry scoring, and statistical support which were carried out by Dr Luca Magnani, Dr Franscesco Mauri, and Richard M. Szydlo respectively.

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ABSTRACT

5-Fluorouracil (5-FU) is the most commonly used drug in the treatment of colorectal cancer (CRC). Tumour resistance to chemotherapy is a major challenge in cancer therapeutics, which ultimately leads to treatment failure and disease progression. Studies which investigate the mechanisms underpinning drug resistance may provide opportunities for the development of novel agents, and allow the early prediction of tumour sensitivity to specific agents. Resistance to 5-FU has been linked to elevated expression of the main 5-FU target, thymidylate synthase (TYMS), which catalyses the de novo pathway for production of deoxythymidine monophosphate. FOXM1 is a critical regulator of the G1/S and G2/M cell cycle transitions. Emerging evidence suggests that elevated tumour levels of FOXM1 are associated with a variety of aggressive human cancers and chemotherapy resistance, but little is known about their role in colorectal cancer and specifically in respect to 5-FU. In this study, I investigated the role of FOXM1 in CRC 5-FU drug resistance.

This study reveals a significant role of FOXM1 in 5-FU drug resistance. Over-expression and knock-down of FOXM1 in colon cancer cells suggests that FOXM1 regulates TYMS. ChIP data also confirms that FOXM1 mediates 5-FU drug resistance mainly through the regulation of TYMS, and the additional 5-FU targets thymidine kinase 1 (TK-1) and thymidine phosphorylase (TYMP). In human colorectal cancer tissue specimens, a strong correlation of FOXM1 and TYMS staining were observed. Elevated FOXM1 and TYMS expression were also observed in acquired 5-FU resistant colon cancer cells (HCT116 5-FU Res). A synergistic effect was observed following treatment of CRC cells with an antagonist of FOXM1, thiostrepton in combination with 5-FU. The combination treatment decreases colony

formation and migration, and induces cell cycle arrest, DNA damage, and caspase 3/7 dependent apoptosis in CRC cell lines.

I also studied the effect of E2F transcription activators (E2F1, E2F2 and E2F3) on 5-FU resistance as E2F1 is known to regulate FOXM1 and TYMS independently. I observed that in addition to E2F1, E2F2 and E2F3 can regulate TYMS and FOXM1. Furthermore I identified the binding sites of FOXM1 on E2F1 and E2F2 transcription activators, and knock-down of E2F1 increases resistance towards 5-FU in p53 mutant and HCT116 5-FU Res cells, indicating the importance of E2F1 in apoptosis.

In summary, this research demonstrated that FOXM1 plays a pivotal role in TYMS mediated 5-FU resistance. Direct targeting of FOXM1 in combination with 5-FU would be a better strategy than 5-FU alone in circumventing 5-FU resistance and provide a platform for further evaluation of FOXM1 targeted therapies as a mechanism to overcome 5-FU resistance. This finding should have important clinical implications for exploiting FOXM1 to prevent 5-FU drug resistance and subsequent metastasis in colorectal cancer patients.

PUBLICATIONS AND CONFERENCES ATTENDED

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FOXM1 mediates the response to 5-Fluorouracil via thymidylate synthase in colorectal cancer. (under review).
- Vidhya Varghese, Luca Magnani, Narumi Harada, Lam W. Eric, and Laura Kenny Forkhead box transcription factor M1 (FOX M1) plays a critical role in colorectal cancer resistance by regulating thymidylate synthase (TS). (2014), AACR Annual meeting.
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- Narumi Harada, Vidhya Varghese, Lingmin Xie and Laura M Kenny
Targeting lactate metabolism as a novel therapeutic target in platinum-resistant triple negative breast cancer (TNBC). (2014), SABCS, AACR.

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TABLE OF CONTENTS

DECLARATION OF WORK.....	1
COPYRIGHT DECLARATION.....	2
ABSTRACT.....	3
PUBLICATIONS AND CONFERENCES ATTENDED	5
ACKNOWLEDGMENTS.....	6
TABLE OF CONTENTS.....	7
LIST OF FIGURES.....	11
LIST OF TABLES.....	13
ABBREVIATIONS	14
CHAPTER 1	17
INTRODUCTION.....	17
1.1 Cancer- an overview	18
1.1.1 Oncogenes and tumour suppressor genes	21
1.1.2 Epigenetic modification	26
1.2 Cell cycle.....	26
1.3 DNA damage and repair.....	29
1.4 Cell death	32
1.4.1 Apoptosis	33
1.4.2 Non-apoptotic cell death	34
1.5 Colorectal cancer	36
1.5.1 Genomic instability and colorectal cancer.....	38
1.6 Chemotherapy treatment for colorectal cancer.....	41
1.6.1 5-Fluorouracil (5-FU).....	42
1.7 Thymidylate synthase (TYMS).....	48
1.8 E2F transcription factors.....	51
1.8.1 E2F family members and role in cell proliferation.....	52
1.8.2 Role of E2F in apoptosis.....	53
1.9 Forkhead box transcription factor	56
1.9.1 FOXO1.....	56
1.10 Thesis Aims.....	65

CHAPTER 2	67
MATERIALS AND METHODS	67
2.4.2 Cell culture	68
2.2 Cell maintenance	69
2.3 Cell preservation	69
2.4 Cell seeding	70
2.5 Drug treatment	70
2.6 Transient transfections	70
2.6.1 Plasmids	70
2.6.2 Transfection of plasmid DNA	71
2.6.3 RNA interference with SiRNA	72
2.7 Protein analysis by western blots	73
2.7.1 Preparation of whole cell extracts	73
2.7.2 Bio-Rad DC protein concentration assay	74
2.7.3 Western blots	74
2.7.4 Immunoblotting	76
2.8 Reverse transcription polymerase chain reaction (RT-PCR)	78
2.8.1 RNA isolation and quantification	78
2.8.2 cDNA synthesis	78
2.8.3 Primer design	78
2.8.4 RT-PCR	79
2.9 Sulforhodamine B (SRB) assay	80
2.9.1 Data analysis	80
2.10 Clonogenic assay	81
2.11 Cell cycle analysis via flow cytometry using propidium iodide	81
2.12 Immunofluorescent staining (IF)	82
2.13 Chromatin Immunoprecipitation (ChIP)	83
2.14 ChIP-Seq	86
2.15 Caspase glow assay	86

2.16	Wound healing assay	86
2.17	Migration assay	87
2.18	Immunohistochemistry staining	87
CHAPTER 3		89
RESULTS 3.1.....		89
IDENTIFYING THE ROLE OF FOXM1 IN 5-FU DRUG RESISTANCE.....		89
3.1.1	Introduction	90
3.1.2	Sensitivity of colon cancer cell lines to 5-FU.....	93
3.1.3	Overexpression of FOXM1 increases resistance to 5-FU	95
3.1.4	Effect of 5-FU on FOXM1, TYMS, and E2F1 in CRC	97
3.1.5	TYMS and TK-1: potential FOXM1 targets in colon cancer	100
3.1.6	Elevated FOXM1 and TYMS in 5-FU resistant HCT116 cells.....	104
3.1.7	γH2AX and cell cycle analysis after 5-FU treatment	107
3.1.8	FOXM1: A key regulator of TYMS.....	110
3.1.9	Genome wide distribution of FOXM1 binding in colon cancer cells.....	113
3.1.10	TYMS and TK-1 expression and its direct association with FOXM1 in clinical samples	125
3.1.11	Discussion.....	128
3.1.12	Future Work	132
RESULT 3.2		133
TARGETING FOXM1 BY PROTEASOME INHIBITION IN COLON CANCER CELLS		133
3.2.1	Introduction	134
3.2.2	SRB assay shows the average cell viability of colon cancer cells treated with thiostrepton 136	
3.2.3	Thiostrepton inhibits FOXM1 and its downstream targets	138
3.2.4	Thiostrepton can alter 5-FU resistance in colon cells through the down-regulation of FOXM1	140
3.2.5	Thiostrepton inhibits FOXM1-DNA binding	142
3.2.6	Thiostrepton synergise with 5-FU	145
3.2.7	Combination of thiostrepton and 5-FU increases DNA damage in colon cancer cells	149
3.2.8	Combination treatment significantly decreases colony formation in colon cancer cells ...	159

3.2.9 Combination treatment inhibit the migration and transformation ability of colon cancer cells	165
3.2.10 Thiostrepton enhances 5-FU induced caspase-dependent apoptosis in colorectal cancer cells	167
3.2.11 Discussion.....	173
3.2.12 Future work.....	176
RESULT 3.3	177
ROLE OF E2F1 IN 5-FU DRUG RESISTANCE.....	177
3.3.1 Introduction	178
3.3.2 Role of E2F1 in TYMS regulation.....	180
3.3.3 E2F transcription activators regulate TYMS and TK-1 in HCT116	181
3.3.4 Silencing E2F1 in HCT116 cells increases chemo-sensitivity.....	189
3.3.5 Silencing E2F1 in p53 mutant cells decreases chemo-sensitivity	194
3.3.6 Silencing E2F1 in 5-FU resistant and FOXM1 over expressed cells decreases chemo-sensitivity	199
3.3.7 Discussion.....	205
3.3.8 Future work.....	211
CHAPTER 4	212
FINAL DISCUSSION AND CONCLUSION	212
CHAPTER 5	228
SUPPLEMENTAL DATA.....	228
CHAPTER 6	257
REFERENCES.....	257

LIST OF FIGURES

Figure 1. 1 Schematic representation of the stepwise transformation observed in the cancer development of normal colonic mucosal tissues to carcinoma (Pitot, Goldsworthy et al. 1981).	21
Figure 1. 2 Schematic representation of the mammalian cell cycle.....	28
Figure 1. 3 Transformation of normal colonic mucosal tissue to carcinoma.....	37
Figure 1. 4 A: Structure of uracil B: Structure of 5-FU.....	43
Figure 1. 5 Mechanism of inhibition of TYMS by 5-FU (5-Fluorouracil).	45
Figure 1. 6 Schematic representation of role of E2F1 in apoptosis and the cell cycle.	55
Figure 1. 7 Schematic representation of splice variants of human FOXM1 protein.	58
Figure 1. 8 Schematic representation of the aim of the study.	66
Figure 2. 1 TMA microarray panel obtained from US Biomax inc®.....	88
Figure 3. 1 SRB assay shows the average cell viability of colon cancer cells.....	94
Figure 3. 2 Over-expression and knock-down of FOXM1 affect 5-FU sensitivity in CRC.	96
Figure 3. 3 A Western blot analysis of 5-FU treated CRC cells.....	98
Figure 3. 4 HCT116 cells transfected with pCDNA3 empty vector and pCDNA3 FOXM1 plasmid...	102
Figure 3. 5 HCT116 cells transfected with SiRNA mediated silencing of FOXM1.....	103
Figure 3. 6 HCT116 5-FU Res cells have elevated FOXM1 and TYMS protein levels.	105
Figure 3. 7 Cell cycle analysis in 5-FU treated HCT116 wt and HCT116 5-FU Res cells.	108
Figure 3. 8 γH2AX staining in 5-FU treated HCT116 wt and HCT116 5-FU Res cells.....	109
Figure 3. 9 FOXM1 is a key regulator of TYMS.	112
Figure 3. 10 Shared FOXM1 peaks between HCT116 and DLD1 cells.....	115
Figure 3. 11 FOXM1 binding in HCT116 cells.....	116
Figure 3. 12 FOXM1 binding in DLD1 cells.	117
Figure 3. 13 CEAS analysis showing genomic distribution of FOXM1 binding events	120
Figure 3. 14 A. The distance to TSS graphs show the gap in base pairs between	121
Figure 3. 15 Heat map showing FOXM1 binding events in HCT116 (right) and DLD1.....	122
Figure 3. 16 FOXM1 binding in HCT116 and DLD1 cells.	123
Figure 3. 17 RT-PCR to assess FOXM1 binding sites in HCT116 and DLD1.	124
Figure 3. 18 Correlation between FOXM1, TK-1 and TYMS in CRC tissue	127
Figure 3. 19 SRB assay showing the cell viability of CRC treated with thiostrepton.	137
Figure 3. 20 Thiostrepton inhibits FOXM1 and its downstream targets.....	139
Figure 3. 21 Thiostrepton can alter 5-FU resistance through the inhibition of FOXM1.	141
Figure 3. 22 Thiostrepton inhibits FOXM1 binding on the TYMS promoter.....	143
Figure 3. 23 The combination of thiostrepton and 5-FU is synergistic in cancer cells.	146
Figure 3. 24 The combination of thiostrepton and 5-FU is synergistic in cancer cells.	147
Figure 3. 25 The combination of thiostrepton and 5-FU is synergistic in cancer cells.	148
Figure 3. 26 Combination treatment increases DNA damage in HCT116 wt cells.	152
Figure 3. 27 Combination treatment increases DNA damage in HCT116 5-FU Res cells.	153
Figure 3. 28 Combination treatment increases DNA damage in DLD1 cells.....	154
Figure 3. 29 Combination treatment increases DNA damage in HT29 cells.	155
Figure 3. 30 Cell cycle analysis to determine cell death and cell cycle distribution.....	156
Figure 3. 31 Cell cycle analysis to determine cell death and cell cycle distribution in cells.	158
Figure 3. 32 Combination treatment decreases colony formation in HCT116 wt cells.....	161
Figure 3. 33 Combination treatment decreases colony formation in 5-FU Res cells.....	162
Figure 3. 34 Combination treatment decreases colony formation in DLD1 cells.....	163
Figure 3. 35 Combination treatments decreases colony formation in HT29 cells.	164
Figure 3. 36 The combination treatment reduces migration activity in HCT116 cells.....	166
Figure 3. 37 Combination treatment induces caspase-dependent apoptosis in HCT116 cells.	169
Figure 3. 38 Combination treatment induces caspase-dependent apoptosis in DLD1 cells.	170

Figure 3. 39 Combination treatment induces caspase-dependent apoptosis in HT29 cells.....	171
Figure 3. 40 Combination treatment induces caspase-dependent apoptosis in HCT116	172
Figure 3. 41 E2F1 regulates TYMS.	180
Figure 3. 42 E2F transcription activators regulate FOXM1, TYMS, and TK-1 in HCT116 cells.	183
Figure 3. 43 E2F transcription activators regulate FOXM1, TYMS and TK-1.....	184
Figure 3. 44 HCT116 cells transfected with E2F1, E2F2 and E2F3 plasmids.....	187
Figure 3. 45 HCT116 cells transfected with E2F1, E2F2 and E2F3 plasmids.....	188
Figure 3. 46 Effect of 5-FU in E2F transcription activators in HCT116 WT cells.....	192
Figure 3. 47 Effect of 5-FU in E2F transcription activators in HCT116 WT cells.....	193
Figure 3. 48 Effect of 5-FU on E2F transcription activators in HT29 cells.....	197
Figure 3. 49 Effect of 5-FU on E2F transcription activators in HT29 cells.....	198
Figure 3. 50 Effect of 5-FU in HCT116 5-FU Res cells following E2F1 knock-down.....	201
Figure 3. 51 Effect of 5-FU in E2F transcription activators in HCT116 5-FU Res cells.	202
Figure 3. 52 Effect of 5-FU in E2F1 knock-down in MCF7 FOXM1 over-expressed cells.	204
Figure 3. 53 Schematic representation of E2F transcription activators in cell cycle progression and 5-FU mediated apoptosis.....	210
Figure 4. 1 Schematic representation of the role of FOXM1 in 5-FU drug resistance.....	219
Figure 4. 2 Schematic representation of the combination of FOXM1 and 5-FU.....	226

LIST OF TABLES

Table 1.1 Most commonly diagnosed cancers worldwide 2008, estimates (Ferlay, Shin et al. 2010).	18
Table 1.2 Staging of colorectal cancer (Wickham and Lassere 2007, Sameer 2013).	38
Table 1.3 Examples of genes involved in genomic instability in CRC.	41
Table 1.4 Common chemotherapy combination regimens for colorectal cancer.	42
Table 2.1 Components for the SDS-phage gel	75
Table 2.2 List of antibodies used in western blots	77
Table 2.3 List of primers used in RT-PCR analysis	79
Table 2.4 Primers used for ChIP were	84
Table 2.5 Reagents used for ChIP assay is listed below	85

ABBREVIATIONS

5-FU- 5-Fluorouracil
Akt - Protein kinase B or (PKB)
APS - Ammonium persulfate
ARF - Alternative reading frame
APC- Adenomatous polyposis coli
ATM - Ataxia telangiectasia mutated
ATR - Ataxia telangiectasia and Rad3 related
BER - Base excision repair
BRCA1/2 - Breast cancer gene 1/2
BSA - Bovine serum albumin
bp- Base pair
CICD- Caspase-independent cell death
CIN – chromosomal instability
CIMP- CpG island methylator phenotype
CDKs - Cyclin-dependent kinases
CENPA/B - Centromere protein A/B
cDNA - Complementary DNA
ChIP- chromatin immunoprecipitation
CpG- cytosine–phosphate-guanine
CRC- Colorectal cancer
CKS1 – cdk-subunit1
DAPI - 4',6-diamidino-2-phenylindole
DNMTs- DNA methyltransferases
DDR - DNA damage response
DFUR – deoxy-5-fluorouridine
DFCR – deoxy-5-fluorocytidine
DMEM - Dulbecco's Modified Eagle Medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DPD -dihydropyrimidine dehydrogenase
dNTPs - Deoxynucleotide triphosphates
dTMP- deoxythymidine monophosphate
dUTP- Deoxyuridine triphosphates
dTTP - Deoxythymidine triphosphate
DOX - Doxorubicin
DSB - DNA Double strand break
DTT - Dithiothreitol
ECL - Enhanced chemiluminescence
EDTA - Ethylenediaminetetraacetic acid
EGFR- The epidermal growth factor receptor (ErbB-1)
FACs - Fluorescence-activated cell sorting
FADD- FAS-associated death domain protein
FCS - Foetal calf serum
Fkh - Forkhead gene
FKHL16 - Forkhead drosophila homolog-like 16
FOX - Forkhead transcriptional factor box
FdUMP- fluorodeoxyuridine monophosphate
FdUTP- fluorodeoxyuridine, triphosphate
GPCRs -G-protein-coupled receptors

h - Hour
 HER2 - Human epidermal growth factor2
 HR - Homologous recombination
 HRP - Horseradish peroxidase
 HNPCC- hereditary nonpolyposis colon cancer
 IgG - Immunoglobulin G
 JNK1 - c-Jun N-terminal kinase1
 KRAS- Kirsten Rat Sarcoma Viral Oncogene
 M - Molar (mole per litre)
 miRNA- micro RNA
 mRNA – messenger Ribonucleic acid
 MCF-7 - Michigan Cancer Foundation-7 cells
 MEF - Mouse embryonic fibroblasts
 MMP - Matrix metalloproteinase
 MMR - Mismatch repair
 MOMP- mitochondrial outer membrane permeabilisation
 IMS - mitochondrial inner membrane space
 MPP2 - M-phase phosphoprotein2
 MSH2- mutS homolog2
 MSS- microsatellite stable
 MLH1- MutL homolog1
 MSI- Microsatellite instability
 M-phase - Mitosis phase
 NRD - N-terminus repressing domain
 NaCl - Sodium chloride
 NaF - Sodium fluoride
 NBS1 - Nijmegen breakage syndrome1
 NER - Nucleotide excision repair
 NHEJ - Non-homologous end joining
 NSC - Non-specific control
 OD - Optical density
 OligoDT - Oligo deoxythymidine
 OS - Overall survival
 PCR- polymerase chain reaction
 PBS - Phosphate buffered saline
 PFA – Paraformaldehyde
 PI3K- phosphatidylinositol-3-kinases
 PDGF-R -Platelet-derived growth factor receptors,
 PLK - Polo-like kinase
 PMSF - Phenylmethylsulfonyl fluoride
 pRb - Phospho Retinoblastoma protein
 PSG - Penicillin/Streptomycin and L-glutamine
 PTEN- Phosphatase and tensin homolog
 Rb - Retinoblastoma protein
 RNA - Ribonucleic acid
 RPM - Round per minute
 RT-PCR - Reverse transcription polymerase chain reaction
 RTKs- Receptor tyrosine kinases
 PCNA - proliferating cell nuclear antigen
 SDS - Sodium dodecyl sulphate
 SDS-PAGE - Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis

SMC – Smooth muscle cells
S-phase - Synthesis phase
SRB - Sulphorhodamine-B assay
SSB - Single strand break
ssDNA - Single-stranded DNA
TAD - Transactivation domain
TBS-T - Tris-buffered saline with tween
TCA - Trichloroacetic acid
TEMED - N,N,N',N'-Tetramethylethylenediamine
SRB- Sulforhodamine-B assay
TK-1- Thymidine kinase-1
TYMS- Thymidylate synthase
TP- Thymidine phosphorylase
TNF- Tumour necrosis factor
VEGF - Vascular endothelial growth factor
WIN - Winged helix
XRCC4 - X-ray repair cross-complementing protein 4
XPA- Xeroderma pigmentosum complementation group A
XPD- Xeroderma pigmentosum complementation group D
XPG- Xeroderma pigmentosum complementation group G
XPF- Xeroderma pigmentosum complementation group F

CHAPTER 1

INTRODUCTION

1.1 Cancer- an overview

Cancer is a disease characterised by uncontrolled cellular proliferation, and metastasis which is a leading cause of death worldwide. Based on the GLOBOCAN (global cancer) project, conducted by the International Agency for Research on Cancer (IARC), 12.7 million new cancer cases were diagnosed and 7.6 million cancer deaths occurred in 2008. The worldwide number of cancer survivors within 5 years of diagnosis has been estimated to be almost 28.8 million (Romito, Goldzweig et al. 2013). The most commonly diagnosed cancers worldwide are lung (1.61 million, 12.7% of the total), breast cancer, which is the leading cause of cancer death among females (1.38 million, 10.9%) and colorectal cancers (1.23 million, 9.7%) (Table 1.1). Cancer survival rates tend to be less in developing countries, which could be due to a delayed diagnosis and limited access to standard treatment (Ferlay, Shin et al. 2010).

Table 1.1 Most commonly diagnosed cancers worldwide 2008, estimates (Ferlay, Shin et al. 2010).

Cancer site	Cases (%)	Total in million
Lung	13	1,608,055
Female breast	11	1,384,155
Colorectum	10	1,235,108
Stomach	8	988,602
Prostate	7	899,102
Liver	6	749,744
Cervix	4	530,232
Oesophagus	4	481,645
Bladder	3	382,660
Non-Hodgkin Lymphoma	3	356,431
Leukaemia	3	350,434
Uterus	2	288,387
Pancreas	2	278,684
Kidney	2	273,518
Lip and oral	2	263,020
Larynx	1	150,677
Other sites	20	875,466

Several hallmarks of cancer have been identified inside a cell such as: excessive proliferative signalling, unlimited proliferation, down-regulation of growth suppressors, resistance to cell death, induction of angiogenesis, activation of invasion and metastasis, and altered immune response (Hanahan and Weinberg 2011). The incidence of cancer is increasing which is thought to be due to external factors such as unhealthy lifestyle factors including smoking, low level of physical activity, poor diet, environmental pollutants, industrial effluents, and mutagenic agents including ionizing radiation. Pre-existing risk factors include age, hereditary factors, and hormonal status of individual (Kolman and Chovanec 2000, Rushton 2003). All these external and pre-existing factors act together to initiate or promote cancer (Jemal, Bray et al. 2011).

Cancer development is understood to be a multistep procedure, and three main phases are observed in carcinogenesis: initiation, promotion, and progression (figure1.1) (Pitot, Goldsworthy et al. 1981). Initiation is characterised by genomic changes within a cell such as mutations, (mutations can increase the activity of oncogenes or inactivate function of tumour suppressor genes), epigenetic modification, chromosomal rearrangements, which cause permanent cellular changes. Promotion of tumour development is stimulated by the survival and cell proliferation of these transformed cells. Progression involves a significant growth in tumour size and metastasis (Largillier, Etienne-Grimaldi et al. 2006).

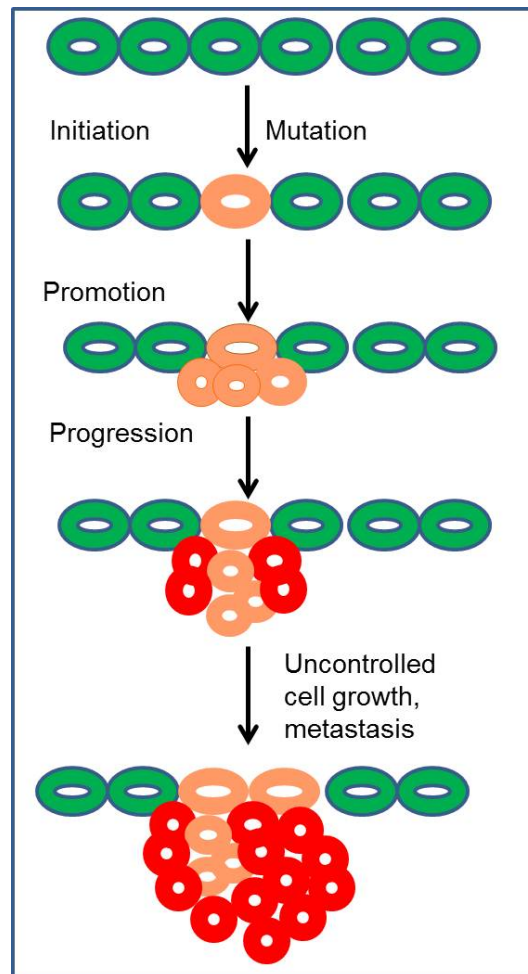


Figure 1. 1 Schematic representation of the stepwise transformation observed in the cancer development of normal colonic mucosal tissues to carcinoma (Pitot, Goldsworthy et al. 1981).

1.1.1 Oncogenes and tumour suppressor genes

The accumulation of mutations, as well as epigenetic changes, activate oncogenes or down regulate tumour-suppressor genes and lead to uncontrolled cell division (clonal expansion).

More than 100 proto-oncogenes and tumour suppressor genes have been identified. Oncogenes are mutated versions of normal cellular genes, oncogene activation is implicated in the positive control of cellular growth, and mutations in oncogenes are

generally dominant. In contrast, tumour suppressor genes function as negative regulators of cellular growth. Mutations in tumour-suppressors usually inactivate gene function. Thus, inactivation of both oncogene and tumour-suppressor genes work together for tumour development. The most well-known examples of oncogenes are PI3K, Ras, PTEN and tumour suppressors are, p53, and Rb (Chalhoub and Baker 2009).

1.1.1.1 PI3K pathway

In recent years, it has been reported that alterations to the Phosphatidylinositol-3 kinases- Protein kinase B (PKB), also known as the Akt signalling pathway, are frequent in human cancer (Vara, Casado et al. 2004). There are three classes of PI3Ks grouped according to structure and function. Class I PI3Ks are most clearly implicated in human cancer and are activated directly by cell surface receptors. Class I PI3K are further divided into class IA PI3K, and Class IB PI3K enzymes, regulated exclusively by G-protein-coupled receptors GPCRs. There are three class 2 PI3K isoforms, PI3KC2 α , PI3KC2 β and PI3KC2 γ , which can be activated by receptor tyrosine kinases (RTKs), cytokine receptors, and integrin. Class 3 PI3K consists of a single catalytic Vps34 subunit. In response to growth factor stimulation and the subsequent activation of RTKs, PI3K is recruited to the membrane via interaction of its p85 subunit to tyrosine phosphate motifs on activated receptors directly (e.g. Platelet-derived growth factor receptors, PDGF-R) or to adaptor proteins associated with the receptors (e.g. insulin receptor substrate 1, IRS1).

1.1.1.2 PTEN

The phosphatase and tensin homology, deleted on chromosome ten (PTEN) tumour suppressor is mutated or lost in both heritable and spontaneous cancers. The cellular level of PIP3 is tightly regulated by the activity of PTEN. PTEN, an important tumour suppressor, functionally antagonizes PI3K activity and reduces PIP3 by converting PI (3, 4, and 5) P3 back to PI (4, 5) P2. Loss of PTEN will result in unrestrained signalling by the PI3K pathway leading to cancer (Lee, Shan et al. 2013).

1.1.1.3 EGFR

The epidermal growth factor receptor (EGFR) is a growth factor receptor that prompts cell differentiation and proliferation upon activation through the binding of one of its ligands. The receptor is located at the cell surface, where the binding of a ligand activates a tyrosine kinase in the intracellular region of the receptor. This tyrosine kinase phosphorylates a number of intracellular substrates that activates pathways leading to cell growth, DNA synthesis and the expression of oncogenes (Voldborg, Damstrup et al. 1997). This group of receptor proteins comprises the c-erbB family of receptor tyrosine kinases. EGFR, which is also known as HER-1 or c-erbB-1, was the first member of this group to be described. Other members of the c-erbB group include HER2 (c-erbB-2), HER3 (c-erbB-3), and HER4 (c-erbB-4) (Herbst 2004). Overexpression of HER2, which occurs in 25% to 30% of breast cancers, is associated with a poor prognosis and shorter survival. (Herbst 2004).

1.1.1.4 RAS

Ras proteins are proto-oncogenes that are frequently mutated in human cancers (Prior, Lewis et al. 2012). The RAS family of GTPases divided in to HRAS, NRAS, and KRAS; the proteins are highly conserved across species. The human oncogenic members of the Ras family generally regulate cell proliferation, differentiation, morphology, and apoptosis (Rojas, Fuentes et al. 2012). Phosphatidylinositol 3-kinase (PI3K) is one of the main effector pathways of RAS. PI3K is necessary for RAS-induced transformation in vitro (Castellano and Downward 2011). Studies showed that Ras directly bound p110, and provided a direct link between Ras and PI3K (Pacold, Suire et al. 2000). Even though PI3K activation is necessary for K-Ras-induced tumourigenesis, studies suggest that inhibition of PI3K signalling alone is not sufficient to prevent tumour growth (Engelman, Chen et al. 2008) (Courtney, Corcoran et al. 2010).

1.1.1.5 AKT

The serine-threonine kinase Akt (also known as protein kinase B, PKB) is a member of the AGC family of protein kinases. Activation of all three Akt isoforms is dependent on PI3K. Activation of PI3K results in the generation of 3'-phosphorylated phosphatidylinositols in the cell membrane, which recruit Akt to the cell membrane (Davies 2011).

1.1.1.1 TP53

TP53 (p53) is the most frequently mutated gene in human cancers. Loss of TP53 activity may cause genetic instability, prevents cellular senescence and apoptosis.

Wild type TP53 acts mainly as a transcription factor, it can be activated by number of factors including DNA damage and hypoxia (Lowe 1999). Activation of TP53 can induce cell cycle arrest, apoptosis, senescence, and inhibition of angiogenesis in tumours by activating or repressing genes that regulate the formation of new blood vessels (Balint and Vousden 2001, Vousden and Lu 2002), which are all important processes associated with tumour suppression.

Inactivation of p53 occurs mainly through point mutations, although small deletions/insertions in the gene also have been detected. Mutant p53s can also initiate cell migration, invasion, and metastasis (Muller, Vousden et al. 2011).

In colorectal cancers, approximately 50% show TP53 gene mutations. Several in vitro, animal, and clinical studies have shown that wild type TP53 is required for the response of colorectal cancers to 5-fluorouracil based chemotherapy (Iacopetta 2003). In colorectal cancers, it was observed that tumours with TP53 mutations in the conserved regions of the gene were poorly differentiated and more aggressive (Jernvall, Mäkinen et al. 1997).

1.1.1.2 Rb

The retinoblastoma (Rb) tumour suppressor is a key component that regulates cell cycle entry and progression in mammalian cells. Rb family members associate with the transcription factor E2F, negatively regulating E2F (discussed in chapter 1:10) dependent transcription. E2F activity plays critical roles in cell cycle progression, including genes involved in cell cycle regulation, DNA replication, and mitosis (DeGregori 2006).

1.1.2 Epigenetic modification

Cancer initiation and progression is also controlled by epigenetic changes. Epigenetics refers to the study of mechanisms that alter gene expression without altering the primary DNA sequence. Epigenetic mechanisms are heritable and reversible, and include changes in DNA methylation, histone modifications, and small noncoding microRNAs (miRNA). Changes in epigenetic routes can lead to altered gene function and malignant cellular transformation (Kanwal and Gupta 2012).

1.2 Cell cycle

The basic mission of cell division is to ensure DNA replication and production of identical daughter cells during M phase, which is a fundamental to most forms of life. The eukaryotic cell cycle is divided into four stages: G1, S, G2, and M (Fueyo, Gomez-Manzano et al. 2005) (figure 1:2). G1 is the gap phase during which cells prepare for the process of DNA replication. It is a critical stage, as this stage checks are made to ensure that the DNA is capable for S-phase entry.

If any damage is identified, cell cycle inhibitors and tumour suppressors (p53, p21^{Cip}) get activated and cause cell cycle arrest or cell death. Cell cycle arrest ensures that DNA damage can be removed or repaired before division to avoid defected (mutated) DNA synthesis. S phase is the stage where DNA synthesis occurs. Between S and M phases is the G2 phase, during which the cell checks the completion of DNA replication and the genomic integrity before cell division starts (Gonzalez, Tachibana et al. 2005, Novak, Tyson et al. 2007).

This highly complex process is controlled by extracellular signals, regulatory proteins and involves multiple checkpoints. In response to cellular stress, activation of checkpoints at G1-S transition, S-phase, G2-M transition, and M-phase are essential for maintaining genetic stability. In response to DNA damage, mammalian cells arrest at different points in the cell cycle and initiate repair. For example, arrest in G1 phase prevents damaged DNA from being duplicated, and arrest in G2 avoids segregation of damaged chromosomes. Failures in cell cycle checkpoints can lead to the acquisition and accumulation of genetic alterations and karyotype abnormalities. These changes may result in the activation of oncogenes and/or the inactivation of tumour suppressor genes and ultimately result in tumourigenesis (Xu, Weaver et al. 1999).

The progression of cells through the various stages of the cells cycle is controlled by cyclin-dependent kinases (CDKs), which complex with cyclin proteins. CDK4 and CDK6 associated with D-type cyclins (CyclinD) and get activated. This complex is important for G1 cell cycle phase progression. The cyclin E–CDK2 complex is important during transition from G1 into S-phase.

Cyclin A–CDK2 and cyclin A–CDK1 are important during S and G2-phase progression respectively and, progression of cells through mitosis is dependent on cyclin B–CDK1 (Neganova and Lako 2008). These proteins regulate progression through the stages of the cell cycle and are in turn regulated by numerous proteins, including p53, p21^{Cip}, p16, and cdc25 (Schafer 1998).

The cyclin dependent kinase inhibitor p21^{Cip} inhibits cell cycle progression primarily through the inhibition of CDK activity, which is required for the phosphorylation of tumour suppressor Rb. Tumour suppressor gene Rb, blocks cell cycle progression in

G1 phases by binding to E2F1. In G1 phase CDK4/6 and cyclin D complex gets activated and cause phosphorylation of Rb and release of E2F1 in G1 phase. Free E2F1 activates cyclin E-cdk2 complex and helps the entry to S-Phase. The cell cycle is often dysregulated in cancer cells due to alterations either in oncogenes that indirectly affect the cell cycle or in tumour suppressor genes or oncogenes that directly impact cell cycle regulation, such as pRb, p53, p16, cyclin D1, or mdm-2 (Schafer 1998).

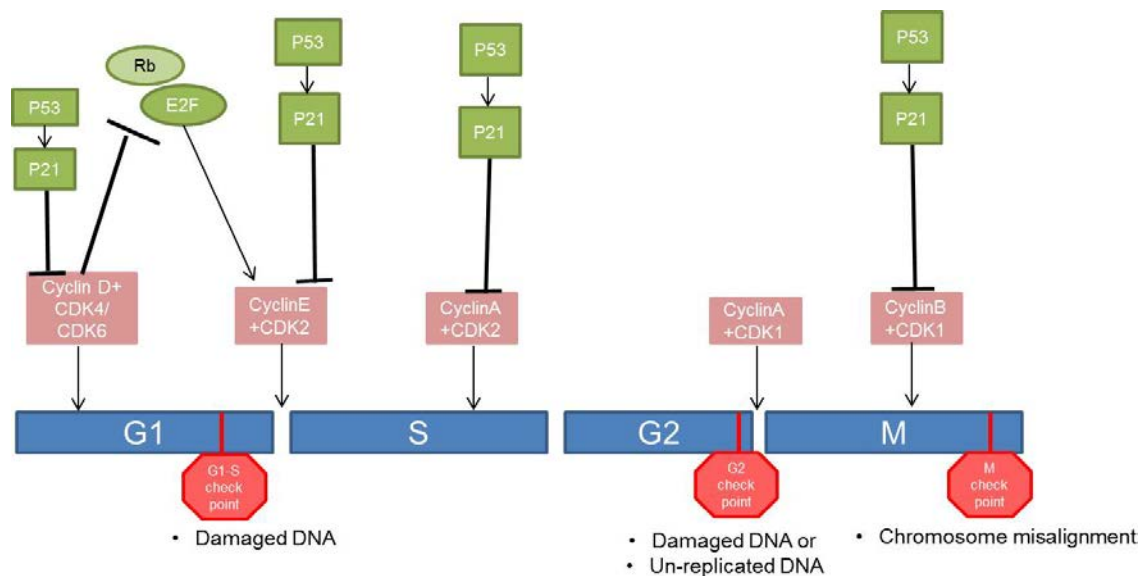


Figure 1. 2 Schematic representation of the mammalian cell cycle.

The cell cycle is divided into four stages: G1, S, G2, and M, and is mainly controlled by cyclin dependent kinase (CDKs) and cyclins (A, B, D, and E). In G1-phase cells get ready for DNA synthesis. In S-phase DNA replication occurs. In G2 phase cells prepare for mitosis that occurs in M-phase. Various checkpoints (G1-S, G2, and M) are used to identify possible defects during DNA synthesis or chromosomal segregation. Rb binds to E2F1 and blocks progression through the cell cycle. CDK4/6 and cyclin D complex are activated by external signals and cause deactivation of Rb and E2F1 in G1-phase. Free E2F1 activates the Cyclin E-cdk2 complex and helps the entry to S-Phase. P53 can block cell cycle progression via p21^{Cip} (Fueyo, Gomez-Manzano et al. 2005).

1.3 DNA damage and repair

DNA damage is a relatively common event in the life of a cell. Exogenous exposure to chemicals from the environment, UV light, X rays, genotoxic agents, such as alkylating agents, reactive oxygen species, errors that occur during normal DNA metabolism, aberrant DNA processing reactions can cause DNA damage (Povey 2000). Reduced DNA repair capacity may lead to genetic instability and carcinogenesis,

DNA damage response (DDR) reactions include:

- a) Removal of DNA damage and restoration.
- b) Activation of a DNA damage checkpoint, which arrests cell cycle progression to allow for repair or prevention of the damaged cells to replicate.
- c) Transcriptional response, which causes changes in the transcription profile that may be beneficial to the cell.
- d) Cell death, which eliminates heavily damaged or seriously deregulated cells (Sancar, Lindsey-Boltz et al. 2004).

DNA double strand breaks (DSBs) initially activate a number of DDR genes, including ATM (ataxia telangiectasia mutated), members of the phosphoinositide 3-kinase–related kinase family (PI3K), and phosphorylation of H2AX (γ H2AX). γ H2AX recruits proteins involved in DNA repair. The DNA double strand breaks can be repaired either by homologous recombination repair (HRR) or non-homologous end

joining (NHEJ) (Srinivas, Dyczkowski et al. 2015). Checkpoint kinases 1 and 2 (Chk1 and Chk2) are cell cycle checkpoint kinases that become phosphorylated and activated by ATM and send DNA damaging signals to other molecules involved in cell cycle progression, apoptosis, gene transcription. For example, the p53 tumour suppressor protein (Bartek and Lukas 2003), the Cdc25 phosphatase family (Falck, Mailand et al. 2001), the BRCA1 tumour suppressor (Zhang, Willers et al. 2004), and transcription factors: E2F1 (Stevens, Smith et al. 2003) and FOXM1 (Tan, Raychaudhuri et al. 2007). ATM can also directly phosphorylate and activate p53 and inactivate cyclin-dependent kinases to inhibit cell cycle progression from G1 to S (the G1/S checkpoint), DNA replication (the intra-S checkpoint), or G2 to mitosis (the G2/M checkpoint) (Hirao, Cheung et al. 2002).

The major DNA repair mechanisms include base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end-joining (NHEJ) (Houtgraaf, Versmissen et al. 2006).

BER is the primary DNA repair pathway that corrects base lesions, removing and replacing incorrect (e.g.: uracil) or damaged bases arising due to oxidative stress, alkylation, deamination, environmental chemicals, radiation, or treatment with cytostatic drugs. BER takes place in nuclei, as well as in mitochondria, largely using different isoforms of proteins or genetically distant proteins (Puebla-Osorio, Lacey et al. 2006, Krokan and Bjørås 2013). The major enzymatic activities involved in BER are:

- a) Recognition and removal of an incorrect or damaged part by DNA glycosylase.
- b) A basic site incision by an apurinic/apyrimidinic (AP) endonuclease or AP lyase
- c) Removal of the remaining sugar fragment by a lyase or phosphodiesterase

- d) Gap filling by a DNA polymerase
- e) Sealing of the nick by a DNA ligase (Kim and Wilson 2012).

NER is the most important repair system to remove bulky DNA lesions that can be caused by UV radiation (thymidine dimers), chemicals, and cross-links. It consists of several steps:

- a) Lesion sensing by a complex of bound proteins, including xeroderma pigmentosum, complementation group A (XPA).
- b) Opening of a denaturation bubble by a complex including xeroderma pigmentosum complementation group D (XPD).
- c) Incision of a damaged part and removal of the damaged nucleotide fragment by molecules including ERCC1 (excision repair cross-complementing group) xeroderma pigmentosum complementation group F (XPF), and group G (XPG).
- d) The final step is DNA synthesis with polymerases (Crew, Gammon et al. 2007, Nospikel 2009).

Studies show that inherited defects in the NER pathway lead to xeroderma pigmentosum, a rare autosomal recessive disease characterised by an extreme sensitivity to sunlight and increased risk of skin cancer (Eveno, Bourre et al. 1995).

MMR corrects DNA mismatches created during DNA replication. Defects in this pathway or defects of genes in this pathway increase the spontaneous mutation rate. Mutations in the main genes involved in this pathway are mutS homolog 2 (MSH2), mutL homolog 1 (MLH1), PMS2, proliferating cellular nuclear antigen (PCNA) and MSH6 (Li 2008, Martin, Lord et al. 2010). Mutations in these MMR genes cause failure to repair errors in short repetitive DNA sequences (microsatellites) leading to microsatellite instability (MSI) (Jiricny 2006). Tumours

with defective genes controlled by MMR generally exhibit MSI. MSI can occur in tumours of many organs, but it is mainly one of the hallmarks of colorectal cancer. A high frequency of instability in microsatellites (MSI-H) is common in hereditary non-polyposis (HNPCC) colon cancer, also known as Lynch syndrome and MSI is observed in 15-20% of sporadic colorectal cancer (Martin, Lord et al. 2010, Poulogiannis, Frayling et al. 2010).

Non-homologous end joining (NHEJ) and homologous recombination (HR) are pathways that repair DNA double-strand breaks (DSBs). NHEJ repairs DSBs through an imprecise pathway that joins ends of DNA together. There are six proteins known to be important for NHEJ in mammals: Ku80, Ku70, DNA-PKCS, Artemis, and Xrcc4. There are many proteins important for HR, including Rad51 that forms a filament on the single-strand ends at the DSB to promote strand exchange. Also important for HR is the breast cancer associated gene, Brca2, that associates with Rad51 in two regions and BLM. Both NHEJ and HR are critical for maintaining genomic stability (Yaneva, Li et al. 2005). $ku80^{-/-}$ (Zhu, Bogue et al. 1996) and $lig4^{-/-}$ (Frank, Sekiguchi et al. 1998) mice survive to adulthood but $rad51^{-/-}$ and $brca2^{-/-}$ cells cannot survive as the resultant mutant embryos arrest soon after implantation (Sharan, Morimatsu et al. 1997).

1.4 Cell death

Resistance to cell death is an important part of both tumourigenesis and the development of resistance to anticancer drugs. Apoptosis or programmed cell death is the most commonly studied pathway of cell death, however there has been recent interest in the physiological roles and molecular pathways that are involved in non-apoptotic cell death.

1.4.1 Apoptosis

Apoptosis is active, programmed cell death, characterised by typical morphological and biochemical changes including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner 2000).

In mammalian cells apoptosis is mediated by a family of cysteine proteases known as caspases. Caspases can get activated by extrinsic and intrinsic apoptotic pathways (Wong 2011). In the intrinsic pathway, mitochondrial outer membrane permeabilization (MOMP) releases pro-apoptotic proteins from the mitochondrial intermembrane space (IMS), which then leads to activation of an initiator caspase, caspase 9, which, in turn, cleaves and activates caspase 3 and caspase 7. In the extrinsic pathway, death receptor ligation causes the recruitment of adaptor molecules, such as FAS-associated death domain protein (FADD), that activate an initiator caspase, caspase 8. Activated caspase 8 directly cleaves and activates the executioner caspases, caspase 3 and caspase 7 (Tait and Green 2010). Recently caspase-independent cell death (CICD) has been identified, in which apoptosis occurs when activation of caspases fails. Even though CICD often shares common characteristics with caspase dependent cell death, the common caspase-dependent events such as phosphatidylserine externalization and chromatin condensation are absent during CICD. Cells undergoing CICD often display large-scale cytoplasmic vacuolization, autophagosome accumulation and peripheral nuclear condensation (Tait and Green 2010).

1.4.2 Non-apoptotic cell death

In addition to apoptosis, cancer cells can be destroyed by necrosis, mitotic catastrophe, and autophagy. Thus, non-apoptotic modes of cell death have also been taken into consideration as response to cytotoxic therapy (Okada and Mak 2004).

Necrosis is considered as unregulated process of cell destruction, which is followed by the release of intracellular components. A distinctive set of morphological features resulting from profound cellular damage is seen including membrane distortion, organelle degradation, and cellular swelling (Kerr, Wyllie et al. 1972). Tumour necrosis factor (TNF) can activate necrotic death in some cell lines, such as mouse L929 cells (Vercammen, Beyaert et al. 1998). In contrast to apoptotic cell death, the molecular mechanism of necrosis is not well understood, though several mechanisms including the release of lysosomal enzymes, or generation of toxic oxygen radicals, and the activation of calcium-dependent phospholipases, have been proposed (Matsumura, Shimizu et al. 2000).

Autophagy is a lysosome-dependent process and the morphology of autophagic cells is distinct from those that undergo either necrosis or apoptosis (Levine, Mizushima et al. 2011). Cell death that is caused by aberrant mitosis or resulting from mitotic failure is known as mitotic catastrophe. Mitotic catastrophe is accompanied by chromatin condensation, mitochondrial release of pro-apoptotic proteins (in particular cytochrome c and apoptosis initiating factor), caspase

activation and DNA degradation (Castedo, Perfettini et al. 0000, Okada and Mak 2004).

1.5 Colorectal cancer

Colorectal cancer (CRC) is a leading cause of cancer morbidity and mortality and affects men and women equally (Hagggar and Boushey 2009). These cancers are complex and heterogeneous solid tumours, exhibiting multiple genetic mutations. Individuals may carry germline (inherited) mutations and somatic (acquired during the life of the cell) mutations at various stages in the transition from normal mucosa, through adenomatous polyp, to invasive cancer (Ewing, Hurley et al. 2013).

The Vogelstein model for colorectal cancer proposed stepwise formation of CRC from normal mucosa to cancer. This model states that CRC is the result of mutations of genes with important functions in regulating cell proliferation or DNA damage repair, with mutations in more than one gene; the sequence of mutations is important in determining the eventual formation of CRC (figure 1:3) (Hisamuddin and Yang 2006). Mutations in the tumour suppressor gene Adenomatous Polyposis Coli (APC), DPC4/SMAD4, p53, mutation in MMR repair genes, MSH2, MLH1, and PMS2 and mutational inactivation of oncogenes (COX-2 (cyclooxygenase-2), and *k-Ras*) are equally involved in this process, mutations in the APC gene chromosome are considered as one of the earliest events in the initiation and progression of colorectal cancer. Mutations in APC are associated with familial adenomatous polyposis (FAP) and also found in 60 to 80% of sporadic colorectal cancers and adenomas (Narayan and Roy 2003). MMR mutations are found to be associated with 15% sporadic colorectal cancer and also responsible for hereditary nonpolyposis colorectal cancers (HNPCC) or Lynch syndrome (Hisamuddin and Yang 2006).

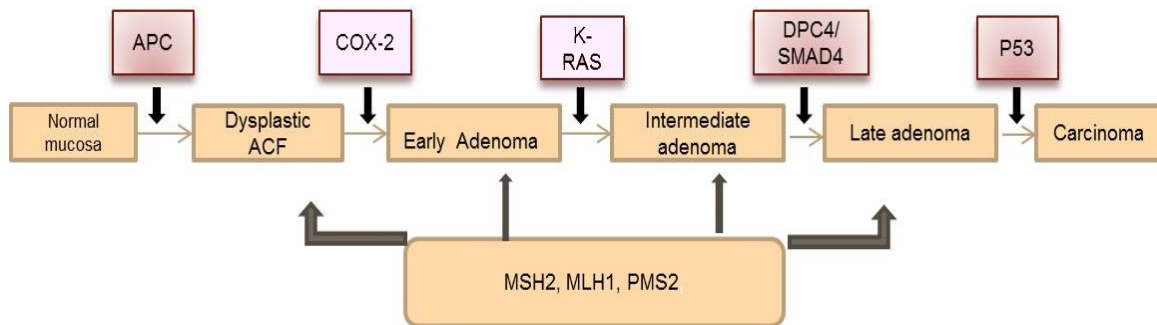


Figure 1. 3 Transformation of normal colonic mucosal tissue to carcinoma.

This model shows some of the key genetic changes that accompany the stepwise transformation of normal colonic mucosal tissues to carcinoma. The sequence of gene mutations in the APC pathway and those in the mismatch repair pathway is shown (Hisamuddin and Yang 2006).

Colorectal cancer survival is mainly dependent upon the stage of disease at diagnosis. The earlier the stage at diagnosis, the higher the chance of survival (Hagggar and Boushey 2009). Two types of staging system are used for colon cancer classification. The most common system is TNM, (tumour, node and metastasis), (T- how far the main tumour has grown into the wall of the intestine, N- degree of tumours spread to nearby lymph nodes. M - indicates whether the cancer has spread (metastasized) to other organs of the body). The other classification is Duke system, in which tumour categorised based on its depth, nodes and metastasis (table 1.2) (Wickham and Lassere 2007, Sameer 2013).

Table 1.2 Staging of colorectal cancer (Wickham and Lassere 2007, Sameer 2013).

Duke's staging	TNM staging	Description	5 years overall Survival (%)
	Stage 0	Carcinoma in situ	
A	Stage I	No nodal involvement, no metastases, tumour invades sub mucosa (T ₁ , N ₀ , M ₀); tumour invades muscularis propria (T ₂ , N ₀ , M ₀)	90-100
B	Stage II	No nodal involvement, no metastases, tumour invades sub mucosa (T ₃ , N ₀ , M ₀); tumour invades other organs (T ₄ , N ₀ , M ₀)	75-85
C	Stage III	Regional lymph nodes involved (any T, N ₁ , M ₀)	30-40
D	Stage IV	Distant metastases	<5

1.5.1 Genomic instability and colorectal cancer

Risk factors for colorectal cancer include personal or family history of colon cancer, (the best known familial form of colorectal cancers is FAP and HNPCC (Kinzler and Vogelstein 1996), increasing age, unhealthy diet or lifestyle, and loss of genetic or epigenetic factors leading to genomic instability. Three main pathways of genomic instability have been identified such as chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) (Pino and Chung 2010).

1.5.1.1 CIN

Chromosomal Instability (CIN) is characterised by changes in chromosome structure and number compared to a normal cells. The genomic changes include activation of proto-oncogenes such as K-Ras, inactivation of tumour suppression genes such as loss of APC and p53. CIN affects approximately 70% of colorectal cancer and has been observed in sporadic colorectal cancers (Bond, Umapathy et al. 2012).

1.5.1.2 MSI in colorectal cancer

Defects in the DNA mismatch repair system lead to microsatellite instability (discussed in 1.3). Microsatellite analysis is commonly used as the first diagnostic screening test for HNPCC because high frequencies of microsatellites instability MSI are observed in HNPCC colon cancer (Müller, Edmonston et al. 2004). Studies show that patients with stage II or stage III colon cancer with microsatellite-stable tumours or tumours exhibiting low-frequency microsatellite instability benefit from 5-Fluorouracil based chemotherapy as distinct from those with tumours exhibiting high-frequency microsatellite instability (Hemminki, Mecklin et al. 2000, Ribic, Sargent et al. 2003). “Microsatellite high” (MSI-H) is defined as the presence of instability in $\geq 30\%$ of the markers. “Microsatellite low” (MSI-L) is defined as the presences of instability in 10%–29% of markers, and “microsatellite stable” (MSS) is defined as no unstable markers (Ogino, Nosho et al. 2009). According to another study the mechanism for 5-FU resistance is due to the high level of thymidylate synthase (TYMS) enzyme (discussed in 1.4) in MSI colon cancer samples (Ricciardiello, Ceccarelli et al. 2005) but contradictory to this, a different study shows MSI status and TYMS expression were unrelated (Sinicrope, Rego et al. 2006).

1.5.1.3 CpG island methylation

DNA methylation is an epigenetic modification, in which addition of a methyl group to the 5' position of the cytosine ring occurs. In normal cells un-methylated GC-rich regions are mainly located in CpG Island; these regions are generally found in the promoter region of DNA. Insertion of methyl groups in these CpG island regions is associated with tumourigenesis (Cross and Bird 1995, Caiafa and Zampieri 2005).

DNA hypermethylation of promoter-associated CpG islands of tumour suppressor and DNA repair genes leads to transcriptional silencing. Widespread CpG island promoter methylation, also known as the CpG island methylator phenotype (CIMP), has been extensively studied in colorectal cancer (Hughes, Melotte et al. 2013). CIMP colorectal cancers are characterised by promoter CpG island hypermethylation of the mismatch repair gene, MLH1, resulting in its transcriptional inactivation. Once MLH1 is inactivated, the rate of progression to malignant transformation is rapid (Snover 2011, Hughes, Melotte et al. 2013). Some clinical studies show CIMP associated with number of diet factors (Curtin, Slattery et al. 2007, Hughes, van den Brandt et al. 2010). Examples of genes involved in genomic instability are mentioned below (table 1:3).

Table1.3 Types of genomic instability in CRC

Nature of genomic Instability	Example of gene mutations	Syndrome associated with germ line mutation	Comments
Chromosomal instability	Loss of function or mutation of the APC gene.	Familial adenomatous Polyposis	Somatic APC mutations are found in 85% of sporadic CRC
Microsatellite instability	Mismatch repair genes(MMR) MLH1,MSH2, MSH6 and PMS2	Lynch syndrome	Somatic inactivation of mismatch repair genes are found in 15% sporadic CRC
DNA base excision repair defect	MYH gene	MYH-associated Polyposis	No recognised somatic equivalent

1.6 Chemotherapy treatment for colorectal cancer

Colorectal cancer can be treated by surgery, radiotherapy, or chemotherapy based on the stage of disease.

Surgery can remove the tumour but undetected traces of tumour may remain either locally or at distant sites. If untreated, they could develop into life-threatening recurrence. Radiotherapy is the use of high energy rays to destroy cancer cells, (Sanche 2009) and has been shown to decrease the rate of local recurrence (Jagsi 2013). In colorectal cancer the use of adjuvant chemotherapy has been shown to improve patient survival rates and prevent tumour recurrence in stage III disease (Chau and Cunningham 2006).

The chemotherapy for colon cancer typically involves combination of several cytotoxic drugs to maximise tumour cell death. 5-Fluorouracil forms the backbone of many treatment regimens for both colorectal and breast cancer. Typical examples of regimens are shown below (table 1.4) (Peinert, Grothe et al. 2010).

Table 1.4 Common chemotherapy combination regimens for colorectal cancer.

Cancer site	Common Chemotherapy combination regimens
COLON	5-FU /leucovorin 5-FU/ oxaliplatin (Eloxatin) 5-FU/ irinotecan (Campto)

1.6.1 5-Fluorouracil (5-FU)

5-FU is one of the most commonly used chemotherapy drugs in the treatment of solid tumours including breast and colon cancer (Silverstein, González de Valdivia et al. 2011) and was first synthesized by Heidelberger et al in 1957 (Wilson, Danenberg et al. 2014). Capecitabine is a pro-drug of 5-FU which can be given orally with similar efficiency, and is increasingly being used as an alternative to 5-FU (Stockler, Harvey et al. 2011).

Capecitabine is first metabolized to 5'-deoxy-5-fluorocytidine (5'-DFCR) by carboxylesterase in the liver. 5'-DFCR is then converted to 5'-deoxy-5-fluorouridine (5'-DFUR) by cytidine deaminase in the liver and tumour tissue. Finally, 5'-DFUR is converted to 5-FU by thymidine phosphorylase (TYMP), which is significantly more active in malignant tissue than in adjacent healthy tissue. The cytotoxicity of

capecitabine and its metabolites 5'-DFCR, 5'-DFUR, and 5-FU was studied in cultures and found that capecitabine and its metabolites become active only after conversion to 5-FU (Blum 2001).

The structure of 5-FU is a pyrimidine analogue of uracil, with a fluorine atom at the C-5 position in place of hydrogen (Longley, Harkin et al. 2003) (figure 1.4).

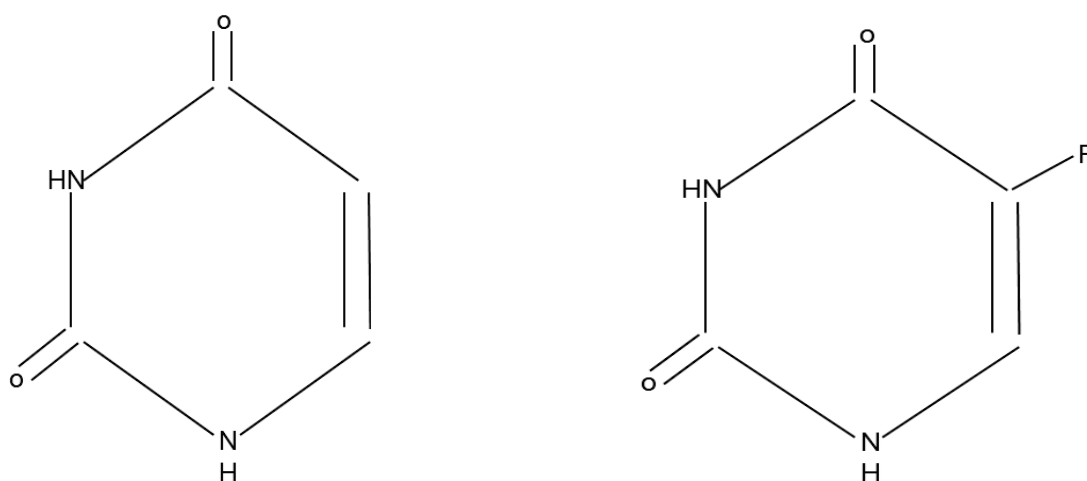


Figure 1. 4 A: Structure of uracil

B: Structure of 5-FU

5-FU directly interferes with DNA through direct incorporation of deoxyribonucleotide analogueFdUTP into DNA, incorporation of dUTP into DNA as a consequence of TYMS inhibition; enhanced mis-incorporation of dATP, dCTP, dGTP and dTTP, inhibition of DNA synthesis as consequence of DNA polymerase stalling; and interference with DNA repair enzymes causes DNA damage.

1.6.1.1 5-FU mechanism of action

5-FU is an S-phase specific active chemotherapy drug, with no activity in G0 or G1 phase. Studies show that treatment of cells with 5-FU causes DNA damage, specifically double-strand (and single-strand) breaks, through the inhibition of

thymidylate synthase (TYMS) (E.C.2.11.4.5). TYMS catalyses the de novo pathway, and converts deoxyuridylate (dUMP) to deoxythymidine-5'-monophosphate (dTMP), which is further phosphorylated by thymidine kinase (TK-1) (via the salvage pathway) to deoxythymidine-5'-triphosphate (dTTP), an important precursor for DNA synthesis.

When 5-FU is administered, it is converted to several active metabolites, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). These metabolites disrupt the action of TYMS and RNA synthesis. FdUMP binds to the dUMP binding site of TYMS and forms a complex structure, which blocks the binding of dUMP to TYMS and inhibits the synthesis of dTMP. This results in dNTP imbalance and an increase in dUTP levels, which causes DNA damage (figure 1.5) (Longley, Harkin et al. 2003).

Another 5-FU metabolite, FUTP is widely combined into RNA, disrupting normal RNA processing and function. Significant correlations between 5-FU mis-incorporation into RNA and loss of clonogenic potential have been shown in human colon and breast cancer cell lines (Kufe and Major 1981, Glazer and Lloyd 1982). 5-FU mis-incorporation can inhibit the synthesis of mature rRNA and also disrupts post-transcriptional modification of tRNAs (Glazer and Lloyd 1982, Kanamaru, Kakuta et al. 1986, Santi and Hardy 1987). It has been reported that both TYMS inhibition and mis-incorporation of 5-FU metabolites into RNA result in p53 stabilisation and the resulting loss of p53 function reduces cellular sensitivity to 5-FU (Longley, Harkin et al. 2003).

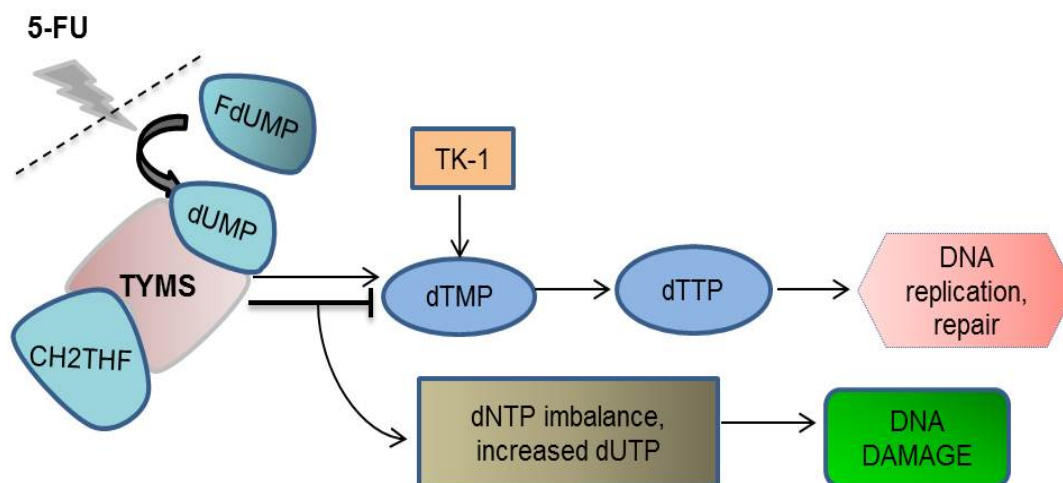


Figure 1. 5 Mechanism of inhibition of TYMS by 5-FU (5-Fluorouracil).

The metabolites of 5-FU bind to TYMS and reduce its activity, which inhibits the synthesis of DNA. The active metabolite fluorodeoxyuridine monophosphate binds to the nucleotide binding site of TYMS and forms a stable complex with TYMS and CH₂THF, blocking access of dUMP to the nucleotide-binding site and inhibiting dTMP synthesis. This causes dNTP imbalance and DNA damage. TK-1 from the salvage pathway can recover dTMP (Longley, Harkin et al. 2003).

1.6.1.2 5-FU sensitivity and resistance

Drug resistance is a major factor that limits the effectiveness of chemotherapy. The aetiology of drug resistance is complex. Previous studies have focused on major molecular factors that predict response to 5-FU based chemotherapy.

TYMS expression has been found to be the most important factor affecting 5-FU sensitivity (section 1.7), although other factors involved in thymidylate metabolism including thymidine phosphorylase (TYMP) and TK-1 may also be relevant. A study

on colorectal tumour samples indicates tumours with high TYMP expression have increased 5-FU resistance, and high TYMP expression correlates with poor prognosis (Metzger, Danenberg et al. 1998, Evrard, Cuq et al. 1999). Further studies show that tumour growth and chemosensitivity are independently related to TYMP expression (Maring, Groen et al. 0000). Dihydropyrimidine dehydrogenase (DPD) is the rate limiting enzyme in the catabolism of 5-FU and catalyses the formation of dihydrofluorouracil from 5-FU primarily in the liver, mutations in DPD can cause severe toxicity reactions to 5-FU and interestingly over expression of DPD in CRC cells has been associated with reduced cytotoxicity to 5-FU (van Kuilenburg, Haasjes et al. 2000).

Another molecule involved in 5-FU response is p53 (discussed in section 1.1.1.6), studies show that cells with wild-type p53 are more sensitive to 5-FU compared to p53 mutant cells which undergo significantly less apoptosis (Bunz, Hwang et al. 1999). Furthermore, a correlation between thymidylate synthase (TYMS) and p53 overexpression measured by immunohistochemistry (thought to correlate loosely with p53 mutation) was found in a tissue microarray study ($p=0.01$) (Ahnen, Feigl et al. 1998, Popat, Wort et al. 2006).

It is well known that treatment of cells with 5-FU causes DNA damage, specifically double strand (and single strand) breaks that occur during S phase due to the misincorporation of FdUTP into DNA (Matuo, Sousa et al. 2009). However, DNA damage and repair mechanisms can occur in different phases of the cell cycle.

Introduction of 5-FU in MMR proficient cells down regulates the expression of cdc25C, cyclins B1 and B2, histone H2A, H2B, and H3 compared to control. Along with this G1/S arrest, increased cyclin E and decreased cdc25C protein expression were observed, showing the influence of MMR gene in 5-FU associated DNA damage/ cell death (Chung, Chaudhry et al. 2010). In addition to correcting

replication errors, MMR is also shown to have an important role in apoptosis (O'Brien and Brown 2006). A number of studies have reported that cells deficient in MMR components, particularly MSH2 and MLH1, are resistant to 5-FU (Carethers, Chauhan et al. 1999, Meyers, Wagner et al. 2001, Meyers, Wagner et al. 2005), could be due to the role of MMR in apoptosis.

Although several studies have shown that MMR deficient cells are resistant to 5-FU, the results regarding clinical benefit have been mixed. Microsatellite instability (MSI) (discussed in sections 1.3 and 1.5.1.2) is caused by failure of the DNA mismatch-repair (MMR) machinery and studies show that for MSI-H patients 5-FU treatment might be beneficial (Hemminki, Mecklin et al. 2000). Other studies have found that patients with tumours lacking MSI (MMR proficient) benefit more significantly from 5-FU treatment while other studies report no obvious difference in 5-FU response and MSI status (Wyatt and Wilson 2009).

The uracil base excision repair (BER) pathway has also been reported to be involved in 5-FU resistance. When the active-metabolite of 5-FU, FdUMP inhibits TYMS, this leads to nucleotide pool imbalance and uridine incorporation into DNA. These DNA incorporations are recognised and removed by DNA glycosylase in BER pathway (discussed 1.3) (Kim and Wilson 2012).

Alterations in homologous recombination repair (HR) are also reported to be associated in 5-FU resistance. Two key mediators of HR, Rad51 and BRCA2, were found to be up regulated in CRC biopsies compared to normal mucosa. Inhibition of HR by targeting Rad51 enhanced DNA damage upon 5-FU treatment (Srinivas, Dyczkowski et al. 2015).

1.7 Thymidylate synthase (TYMS)

Thymidylate synthase (E.C.2.1.1.45) is a cytosolic enzyme, with a dimeric structure composed of two identical units of approximately 38 kDA (Van Triest, Pinedo et al. 2000). It is also known as TS, TYMS, TMS, and HST422, and is located at band 18p11. TYMS is a key enzyme in the folate pathway which catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5, 10-methylenetetrahydrofolate (CH₂H₄folate) as a cofactor. dTMP is metabolised to deoxythymidine triphosphate (dTTP), an essential precursor for DNA synthesis (Junior, Marson et al. 2012). As the sole source of dTMP, TYMS is a valuable target for cancer chemotherapeutic drugs, including the fluoropyrimidines and various anti-folate analogues (Wu and Dolnick 2003).

1.7.1 Thymidylate synthase as a predictive marker

Several new generation TYMS inhibitors have been clinically evaluated such as quinazoline antifolates, raltitrexed (Tomudex, ZD1694), ZD9331, and MTA but as yet, none of these compounds have successfully replaced 5-FU in cancer therapy. Over-expression of TYMS is linked to resistance to TYMS targeted drugs such as 5-FU in both breast and colorectal cancer (Marsh 2005). Similarly, low levels of TYMS in CRC predicted a very high response rate to 5-FU and a significantly longer survival in patients with advanced colorectal carcinoma (Sasaki, Watanabe et al. 2013). Studies have shown that significantly higher TYMS expression is found in resistant colon cancer cells compared to sensitive colon cancer cell lines (Saga, Suzuki et al. 2003, Popat, Matakidou et al. 2004). Patients with tumours expressing

high levels of TYMS appeared to have a poorer OS (overall survival) compared with tumours expressing low levels of TYMS (Saga, Suzuki et al. 2003, Popat, Matakidou et al. 2004). Furthermore tumour samples with high TYMS levels are more likely to be resistant to 5-FU (Kamoshida, Suzuki et al. 2007). Both high TYMS mRNA and high TYMS protein expression have been shown to predict for a poor response to fluoropyrimidine-based therapy in colorectal and gastric cancer (Johnston, Lenz et al. 1995). The relation between TYMS expression and the cell cycle has been examined in normal and cancer cells and it has been reported that cell proliferation and the TYMS expression is correlated, (Derenzini, Montanaro et al. 2002). The routine use of TYMS in the clinic as a predictive biomarker is not yet routine, as some studies have failed to fully demonstrate its utility, this is thought to be due to the methodology used for TYMS testing (Wilson, Danenberg et al. 2014).

1.7.2 Molecular mechanism of TYMS action

It has been reported that the protein expression of TYMS is initially induced following treatment with 5-FU or other TYMS inhibitors (Junior, Marson et al. 2012). Studies show that translation of TYMS protein is autoregulatory, i.e. TYMS protein binds tightly to TYMS mRNA preventing further translation of TYMS protein (Chu, Koeller et al. 1991, Kitchens, Forsthoefel et al. 1999). During DNA replication, TYMS binds to dUMP and the folate cofactor. Hence TYMS is no longer able to bind to TYMS mRNA, thereby allowing translation. A similar effect is seen when TYMS binds to an inhibitor with the result that TYMS can no longer bind to TYMS mRNA leading to up-regulation of TYMS protein. Another study proposed that the up-regulation of TYMS

protein after exposure to TYMS inhibitors is due to a stabilisation of the TYMS protein rather than an increase in translation (Keyomarsi, Samet et al. 1993, Welsh, Titley et al. 2000).

The TYMS gene is polymorphic, its promoter region comprises a 28-bp sequence, that is usually presented as a double-tandem repeat (2R) or a triple-tandem repeat (3R) (Pullarkat, Stoehlmacher et al. 2001). A TYMS polymorphism has been associated with the 5' promoter enhancer region (TSER). A polymorphism within the 5'-untranslated region of the TYMS gene has been associated with modulating TYMS mRNA expression. In addition, the 3R allele can be sub classified according to the presence of a single nucleotide polymorphism (SNP) replacing a cytosine with a guanine (G/C) in 3R (3C or 3G) (Kaneda, Takeishi et al. 1987). Preliminary studies indicate that 3R/3R homozygous patients are less likely to respond to 5-FU-based chemotherapy than 2R/ 2R homozygous and 2R/3R heterozygous patients. The presence of triple repeats (3R) in the TYMS promoter enhancer region is associated with a 2-4 fold increase in protein expression in comparison to double repeats (2R) in colon cancers. Therefore, the lack of response to 5-FU in 3R/3R patients might be due to higher levels of TYMS expression in the tumour of these patients compared with patients with the 2R/ 2R and 2R/ 2R genotypes (Longley, Harkin et al. 2003).

A polymorphism in the 3'-untranslated region (3'-UTR) of the TYMS gene has been identified as a deletion of the 6 base pair and this polymorphism is associated with decreased mRNA stability and a lower TYMS protein expression. A clinical study shows that the second group of patients get the most benefit from 5-FU chemotherapy (Fujishima, inui et al. 2010). Another study states that the TYMS 3'-UTR polymorphism affects TYMS mRNA expression, in the same manner as TYMS promoter polymorphism. When these polymorphisms were tested individually for

association with the toxicity and efficacy of 5-FU treatment in patients with colorectal cancer, only the TYMS promoter polymorphism was shown to be associated with a high risk of severe side effects to 5-FU (Lecomte, Ferraz et al. 2004).

Many studies have been carried out to identify the role of TYMS polymorphisms to predict the toxicity and efficiency in 5-FU treatment. A study reported that no significant associations were observed between the number of TYMS tandem repeats and toxicity (Curtin, Slattery et al. 2007) but conflicting with this, another study shows the role of TYMS 5' gene polymorphism on capecitabine toxicity and efficiency (Largillier, Etienne-Grimaldi et al. 2006, Loganayagam, Arenas Hernandez et al. 2013). Expression of TYMS and sensitivity to TYMS inhibitors such as 5-FU, nolatrexed, raltitrexed and pemetrexed in p53 wild-type (wt) and mutant (mt) colorectal cancer cells indicate that changes in the status of p53 can alter sensitivity to TYMS inhibitors by affecting TYMS levels (Giovannetti, Backus et al. 2008).

1.8 E2F transcription factors

The E2F family of transcription factors play a crucial role in the control of cell cycle progression, regulating the expression of genes required for G1/S transition. It has been reported that genes involved in S-phase, such as cell proliferation and DNA replication genes (including TYMS) are regulated by E2F1. A study shows that cells overexpressing E2F1 have up-regulation of TYMS expression and are resistant to 5-FU (Banerjee, Schnieders et al. 1998). Analysis of TYMS and E2F1 gene expression in tumour samples from 23 colon cancer patients show a close relationship between TYMS gene expression and E2F1 gene, suggesting that the TYMS gene may be regulated by E2F1 (Kasahara, Takahashi et al. 2000).

1.8.1 E2F family members and role in cell proliferation

The E2F family of transcription factors involves an E2F subunit and a differentiation regulated transcription factor proteins (DP) subunit. The E2F subunit consists of 8 members (E2F1-E2F8) and 2 DP members (DP1, DP2). E2F1, E2F2, and E2F3 are known as transcriptional activators. Their activities peak during G1/S phase of the cell cycle and when overexpressed they can drive quiescent cells into S-phase. In a previous microarray study E2F1–3 factors have been shown to promote the expression of genes involved in DNA replication, DNA repair, mitosis, and interestingly, some of these E2F regulated genes were identified, such as cdc2, cyclin B1, and mini-chromosome maintenance (MCM) members, that are also transcriptional targets of FOXM1 (Myatt and Lam 2007, Blanco-Bose, Murphy et al. 2008, Millour, de Olano et al. 2011).

In contrast, E2F4-8 factors act as inhibitors of transcription (Timmers, Sharma et al. 2012) (Tsai, Hu et al. 1998). The expression of various E2Fs is differently regulated throughout the cell cycle. E2F1–E2F3 are highly regulated during cell cycle progression whereas E2F4 and E2F5 are constitutively expressed (Crosby and Almasan 2004). E2F1 is the best-characterised member of the E2F family. E2F1 is widely described as a factor involved in promoting cell cycle progression, however recently it has been found to play an important role in apoptosis (Field, Tsai et al. 1996, Garcia, Murga et al. 2000)

The E2F transcription factors are regulated by the retinoblastoma (Rb) family of proteins (pRb, p107, and p130, also known as pocket proteins) (Rubin, Gall et al. 2005). E2F1, E2F2 and E2F3 can specifically interact with Rb. Rb binds to E2F

during the G1 phase of the cell cycle. This complex inhibits the transcriptional activity of E2F and actively represses E2F and its downstream targets. E2F4 and E2F5 interact with Rb family proteins, p107 and p130, but others appear to act independently of RB (Gaubatz, Lindeman et al. 2000). During cell cycle progression (G1/S transition), cyclin-dependent kinases cdk4/6 and cdk2 initiate phosphorylation of Rb family proteins, which results in the release of E2Fs. E2F1 coupled with heterodimer DP initiates S-phase transition and activation of E2F downstream targets (figure 1.2, 1.6) (Engelmann and Pützer 2010). A study in MEF (mouse embryonic fibroblasts) shows that combined loss of three E2F transcription activators (E2F1, E2F2 and E2F3) severely affects E2F target expression and completely abolishes the ability of MEF to enter into S phase, progression through mitosis and the ability to proliferate (Wu, Timmers et al. 2001).

1.8.2 Role of E2F in apoptosis

Despite the clear role in cell cycle progression, several studies have suggested the role of E2F in apoptosis. Among the E2F family members, E2F1 has a unique apoptotic function in the presence of cellular stress or when over-expressed via p53 and p53-independent mechanisms (Tsai, Hu et al. 1998, Phillips and Vousden 2001). Although recent research has reported that E2F3 can also function in the process of apoptosis induction it may still mainly depend on the presence of E2F1 (Lazzerini Denchi and Helin 2005, Martinez, Goluszko et al. 2010). The overexpression of E2F1 in transgenic mice promotes tumourigenesis and the absence of E2F1 reduces tumour phenotypes resulting from the inactivation of pRb. On the other hand, E2F1 knock-out mice developed different types of tumours, probably due to the lack of E2F1 induced apoptosis (Dyson 1998).

In addition, it has recently been shown that E2F1 levels increase following DNA damage and many reports have described E2F1 stabilisation following various DNA damaging agents and chemotherapeutic drugs and this stabilisation is mediated by ATM dependent phosphorylation (Lin, Lin et al. 2001).

As discussed in section 1.3, during DNA damage ATM is recruited to DNA double strand breaks, and these phosphorylation events can stabilise p53 (Valentine, Kumar et al. 2011). Furthermore, like p53, E2F1 can be phosphorylated at Ser31 by ATM and Chk2, resulting in stimulation of p73. Like its family member p53, p73 can also promote apoptosis and it has been demonstrated that the induction of p73 contributes to the apoptotic activity of E2F1 (figure 1.6) (Lin, Lin et al. 2001).

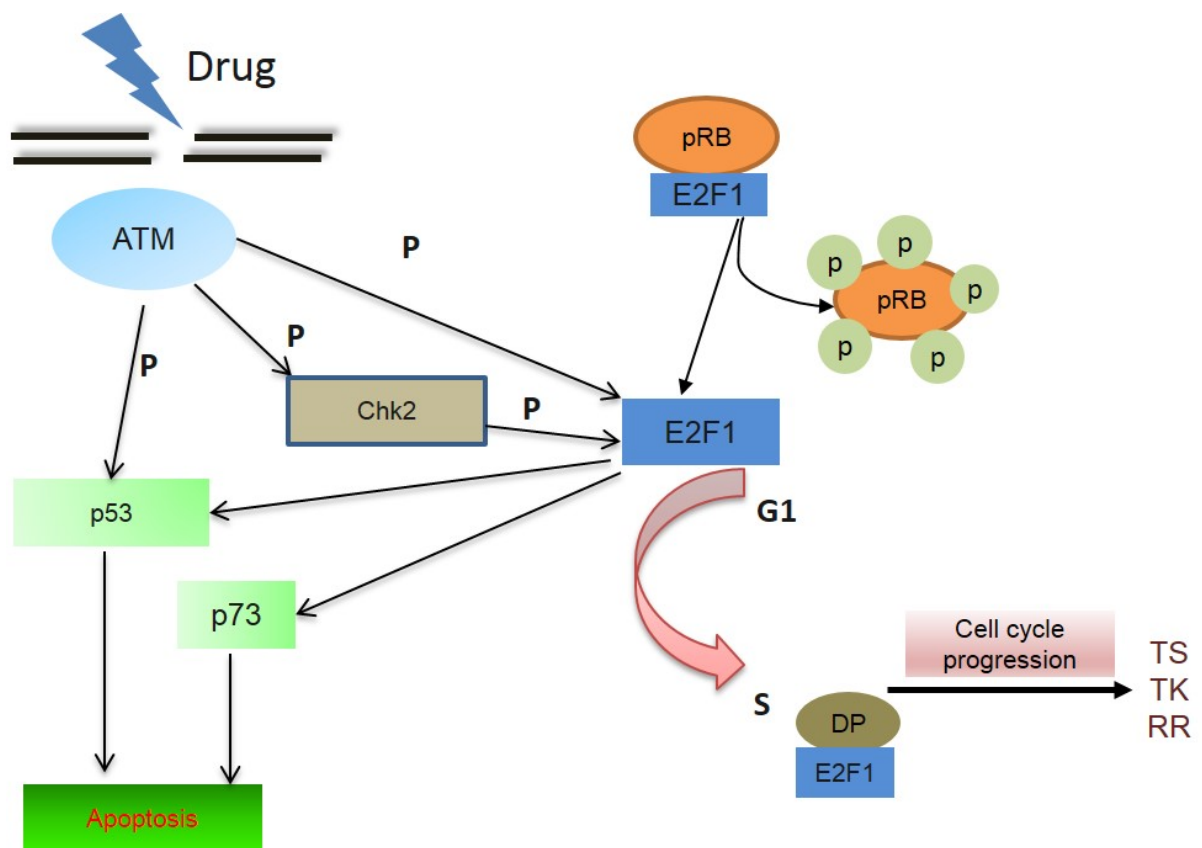


Figure 1. 6 Schematic representation of role of E2F1 in apoptosis and the cell cycle. Phosphorylation of Rb results in the dissociation of E2F1/Rb, complex and the release of E2F1 that contributes to S phase entry and apoptosis. E2F1 is phosphorylated after DNA damage by ATM/ Chk2. Phosphorylated E2F1 promotes apoptosis directly or indirectly through p53 and p73 (Powers, Hong et al. 2004)

1.9 Forkhead box transcription factor

Forkhead box (FOX) proteins are a family of evolutionarily conserved transcriptional regulators defined by a common DNA-binding domain (DBD) termed the forkhead box or winged helix domain. The first mammalian forkhead protein was identified in *Drosophila* (Kaestner, Knöchel et al. 2000). Fifty forkhead genes have been identified in the human genome and they further classified in to 19 subgroups (FOXA to FOXS) based on sequence homology (Jackson, Carpenter et al. 2010).

The FOX gene family encodes proteins that regulate the transcription of genes participating in a number of functions such as metabolism, development, differentiation, proliferation, apoptosis, migration, invasion and longevity (Lehmann, Sowden et al. 2003, Myatt and Lam 2007). Several Fox subfamilies such as FOXO, FOXM, FOXP, FOXC and FOXA have been linked to tumourigenesis and the progression of certain cancers (Teufel, Wong et al. 2003, Laoukili, Stahl et al. 2007, Habashy, Powe et al. 2008, Maiese, Hou et al. 2009, Jackson, Carpenter et al. 2010, Sizemore and Keri 2012).

1.9.1 FOXM1

FOXM1 has also been previously known as Trident (in mouse), HFH-11 (in human), WIN, INS-1 (in rat), MPP-2 (partial human cDNA) or FKHL-16 (Laoukili, Stahl et al. 2007). The human FOXM1 gene consists of 10 exons spanning approximately 25kb on the 12p13-3 chromosomal region. Among the 10 exons, two (Va or A1 and VIIa or A2) are alternatively spliced and this gives three classes of variants, FOXM1a, FOXM1b and FOXM1c. FOXM1a contains both spliced exons,

FOXM1b has none of the spliced exons, and FOXM1c contain exon A1 only. Both FOXM1b and c are transcriptionally active, whereas FOXM1a is transcriptionally inactive, due to the insertion of both exons Va and VIIa in the C-terminal transactivation domain, as FOXM1a can bind to DNA it is considered to be a dominant negative regulator of other FOXM1 isoforms (Ye, Kelly et al. 1997, Habashy, Powe et al. 2008). In addition to this, a recent study in pancreatic cancer identified two more FOXM1 isoforms, FOXM1b1 and FOXM1b2 because they were closely related to FOXM1b (Kong, Li et al. 2013).

FOXM1 protein contains three major domains:

- A) Trans-activation domain (TAD) within its C-terminal region.
- B) N-terminal repressor domain (NRD). NRD is important for the autoregulatory activity of FOXM1
- C) Conserved Forkhead box DNA binding domain (DBD) in its N-terminal region, (figure 1.7). FOXM1 binds DNA in vitro through the consensus site TAAACA. This motif shares the core sequence recognised by other members of the forkhead family (Laoukili, Stahl et al. 2007).



Figure 1. 7 Schematic representation of splice variants of human FOXM1 protein. Among the 10 exons, two of which (Va or A1 and VIIa or A2) are alternatively spliced and gives three FOXM1 isoforms, FOXM1a, FOXM1b and FOXM1c. The FOXM1 protein contains three regions: an N-terminal autorepressor domain (NRD), a conserved forkhead DNA Binding domain (FKH) and a transactivation domain (TAD). Diagram adapted from (Laoukili, Stahl et al. 2007).

1.9.1.1 FOXM1: Proliferation, cell cycle and embryogenesis

A study in mouse embryos shows expression of the FOXM1 gene in all tissues, but in adults the expression of the gene was detected only in the thymus. Further analysis revealed that FOXM1 expression strictly correlated with the cell cycle. (Korver, Roose et al. 1997, Xue, Chiang et al. 2010). In addition to this, during T cell differentiation FOXM1 expression peaked in the actively dividing immature single positive cells. In mature lymphocytes, expression of FOXM1 mRNA was absent (Korver, Roose et al. 1997). Both mRNA and protein expression were strongly up-regulated upon entry into the S phase of the cell and FOXM1 deficient cells exhibit delayed S phase entry and defect in the expression of many cell cycle regulatory proteins are also observed such as cyclin A, cyclin B1, cdc2, cdk2, p27 and the RB family members p107 and p130 (Korver, Roose et al. 1997, Xue, Chiang et al. 2010). FOXM1 knock-down human osteosarcoma U2OS cells and FOXM1^{-/-} MEFs were unable to significantly grow in culture due to a failure in mitosis progression and accumulated p21^{Cip1} and p27^{Kip1}. FOXM1 is essential for transcription of the mitotic regulatory genes Cdc25B, Aurora B kinase, survivin, centromere protein A (CENPA), and CENPB (Wang, Chen et al. 2005). Liver regeneration studies with transgenic mice demonstrated that FOXM1b regulates the hepatocyte DNA replication and mitosis by stimulating expression of cell cycle genes (Wang, Kiyokawa et al. 2002) (Wang, Chen et al. 2005).

FOXM1 deficient (-/-) mouse embryonic fibroblasts (MEFs) display abnormal chromosome segregation and accumulation of polyploid cells (Laoukili, Kooistra et al. 2005). These studies support the importance of FOXM1 in cell cycle regulatory proteins critical for S-phase and M-phase progression.

Another study in FOXM1^{-/-} mice found that embryos die due to severe defects in multiple organ systems including lungs, liver, heart and blood vessels - indicating that FOXM1 is essential for organ morphogenesis (Krupczak-Hollis, Wang et al. 2004, Ustiyan, Wang et al. 2009). Deletion of FOXM1 in the development of smooth muscle cells (SMC) (smFoxm1^{-/-} mice) caused the death of smFoxm1^{-/-} mice due to severe pulmonary hemorrhage, and structural defects in arterial walls and the esophagus. Although FOXM1 deletion did not influence SMC differentiation, decreased proliferation of SMC was found. FOXM1 deficiency in vitro and in vivo has been shown to be associated with reduced expression of cell cycle regulatory genes, including cyclin B1, Cdk1-activator Cdc25b phosphatase, Polo-like 1 and JNK1 kinases, and cMyc transcription factor. FOXM1 is critical for proliferation of smooth muscle cells and is essential for embryonic development of blood vessels and esophagus (Ustiyan, Wang et al. 2009).

1.9.1.2 FOXM1: DNA damage repair, senescence

The role of FOXM1 in DNA repair genes was initially identified in FOXM1 deficient (FOXM1^{-/-}) mouse embryonic fibroblasts and FOXM1 depleted osteosarcoma U2OS cells. In this study increased DNA breaks and reduced expression of the base excision repair factor X-ray cross-complementing group 1 (XRCC1) and breast cancer-associated gene 2 (BRCA2), was observed (Tan, Raychaudhuri et al. 2007). This study confirms that during DNA damage FOXM1 protein is phosphorylated by Chk2, causing increased stability of FOXM1, which in turn stimulates transcription of genes encoding DNA repair enzymes (Tan, Raychaudhuri et al. 2007). A recent report has shown that FOXM1 (-/-) MEFs (FOXM1 knock-out mouse embryonic

fibroblast) are hypersensitive to different DNA damaging insults, such as epirubicin (a topoisomerase II inhibitor), or γ -irradiation (IR), suggesting again a role of FOXM1 in DNA damage repair. In the same study, it was demonstrated for the first time that FOXM1 is required for DNA double strand break (DSB) repair by HR but not for NHEJ repair (Monteiro, Khongkow et al. 2013). In addition, FOXM1^{-/-} MEFs display premature senescence (Wang, Chen et al. 2005). A recent study in osteosarcoma U2OS cells and MEFs cells identified an induction of cell migration and invasion through transcriptional activation of the MAPK kinase JNK1, MMP-2 and MMP-9, these are the critical targets of FOXM1. Knock-down of FOXM1 inhibits tumour cell migration, invasion, and anchorage-independent growth (Wang, Chen et al. 2008).

1.9.1.3 FOXM1: Cancer initiation and progression

The FOXM1 transcription factor plays an essential role in the regulation of a wide spectrum of biological processes, including cell proliferation, cell cycle progression, cell differentiation, DNA damage repair, apoptosis, and cell survival, which are essential features for tumour growth. Consistent with this idea, over expression of FOXM1 is found in many types of cancers including liver (Kopanjan, Pandey et al.), prostate (Kalin, Wang et al. 2006), breast (Madureira, Varshochi et al. 2006), renal (Xue, Xiao et al. 2012) colon (Yoshida, Wang et al. 2007), and pancreas (Quan, Wang et al. 2013), and ovarian (2011). One of the earliest studies found up-regulation of FOXM1 in basal cell carcinoma (BCC) compared to normal skin samples (Teh, Wong et al. 2002). A microarray analysis identified FOXM1 is one of the most commonly overexpressed genes in solid tumours of the prostate, lung, bladder, ovary, colon, liver, breast, kidney, stomach and pancreas (Pilarsky, Wenzig

et al. 2004). A study in renal cell carcinoma (RCC), shows up-regulated FOXM1 at both the protein and mRNA levels in clinical samples. Furthermore, the same study reported that FOXM1 expression was significantly correlated with primary tumour stage, lymph node metastasis, distant metastasis, and that high FOXM1 expression was associated with poor prognosis (Xue, Xiao et al. 2012).

In patients with gastric cancer, FOXM1 overexpression and its direct association with poor prognosis have been identified and inhibition of FOXM1 by FOXM1 siRNA significantly suppresses the growth and metastasis of human gastric cancer cells. Further analysis on the effect of FOXM1 expression on tumour angiogenesis in vivo in nude mice shows promotion of gastric tumourigenesis by FOXM1 is directly and significantly correlated with transactivation of vascular endothelial growth factor and angiogenesis (Li, Zhang et al. 2009).

1.9.1.4 FOXM1: Drug resistance

The role of FOXM1 in cancer drug resistance has also been identified mainly in breast cancer (Carr, Park et al. 2010, Millour, de Olano et al. 2011, Park, Jung et al. 2012, Park, Jung et al. 2012). Silencing FOXM1 expression led breast cancer cells to become more sensitive to DNA topoisomerase II α (TOP2A), doxorubicin (Dox), a microtubule stabilizer (paclitaxel), a DNA alkylating agent (cisplatin) (Carr, Park et al. 2010, Millour, de Olano et al. 2011, Park, Jung et al. 2012, Park, Jung et al. 2012). Silencing FOXM1 in a mouse xenograft model significantly sensitised the cells to doxorubicin. This study has also identified that FOXM1 dependent resistance to doxorubicin is mediated by regulating DNA repair genes (Park, Jung et al. 2012). Another study has shown that epirubicin resistant MCF7 cells display higher FOXM1

levels compared to sensitive MCF7 breast carcinoma cells and that FOXM1 expression is down-regulated in sensitive MCF7 cells, but induction observed in resistant cells treated with epirubicin (Millour, de Olano et al. 2011). A study on MCF7 cisplatin resistant cells proposed that the role of FOXM1 in cisplatin resistance is mediated through the DNA damage repair targets of FOXM1, BRCA2 and XRCC1 (Kwok, Peck et al. 2010). In a gastric cancer study, expression of FOXM1 was found to be high compared to normal gastric cells and FOXM1 was shown to mediate docetaxel resistance in these cancers (Li, Qiu et al. 2013). In human breast cancer samples, FOXM1 overexpression confers resistance to trastuzumab (a targeted therapy for HER2 positive breast cancer) and the microtubule-stabilising drug paclitaxel, both as single agents and in combination. Inhibiting FOXM1 expression by siRNA increased the drugs sensitivity (Carr, Park et al. 2010).

1.9.1.5 Targeting FOXM1

Over the years it became apparent that FOXM1 appears to be an attractive target for the development of novel anticancer therapies. Down-regulation of FOXM1 expression by FOXM1 specific siRNA decreased cell proliferation, invasion, migration, invasion, angiogenesis (Li, Zhang et al. 2009, Wu, Liu et al. 2010), and cellular senescence (Zeng, Wang et al. 2009). FOXM1 knock-down decreases tumour growth in mouse models (Bhat, Jagadeeswaran et al. 2011, Wang, Park et al. 2011). All these have led to the discovery of numerous novel agents for targeting FOXM1 for anti-cancer therapy. Using a high throughput cell based assay system small molecule inhibitors against FOXM1 have been identified such as the antibiotic thiazole compound Siomycin A. A study reports that Siomycin A was able to down-

regulate the transcriptional activity as well as the protein and mRNA expression of FOXM1 and the downstream target genes of FOXM1, such as Cdc25B, Survivin, and CENPB, were repressed. Furthermore, Siomycin A was shown to induce apoptosis in U2OS cancer cells (Radhakrishnan, Bhat et al. 2006). Other proteasome inhibitors MG115, MG132 and bortezomib are also found to repress FOXM1 protein expression.

Similarly, another thiazole antibiotic thiostrepton is also found to reduce the expression of FOXM1 and induce apoptosis in melanoma cells (Bhat, Zipfel et al. 2008). In breast cancer cells thiostrepton selectively induces cell cycle arrest and cell death through down-regulating FOXM1 expression (Kwok, Myatt et al. 2008). Thiostrepton is a natural product isolated from *Streptomyces azureus* (Bagley, Dale et al. 2005). In human breast cancer cells, thiostrepton interacts directly with the FOXM1 protein. In cellular experiments, thiostrepton inhibits the binding of FOXM1 to genomic target sites. Other forkhead family members, such as FOXA1, FOXO1 and FOXO3a, share a common DNA binding domain with FOXM1, but are resistant to thiostrepton (Hegde, Sanders et al. 2011) indicating that FOXM1 might be the key target of thiostrepton.

1.10 Thesis Aims

5-FU is one of the most commonly used chemotherapy drugs to treat many types of cancer including colorectal cancer. Despite its many advantages, the development of drug resistance is the leading cause of treatment failure. The early clinical successes with TYMS inhibitors such as 5-FU highlighted the importance of the thymidylate biosynthesis pathway as a viable therapeutic target in cancer, and prompted the search for more effective approaches to inhibit TYMS. Drug resistance in cancer can arise from a complex range of biochemical and molecular events, which eventually result in tumour cell survival. Therefore, identifying key genes and pathways involved in the molecular mechanisms of drug resistance can help to develop new drug targets, improve treatments and patient outcomes.

The FOXM1 transcription factor plays important roles in tumourigenesis through the regulation of several genes involved in cell proliferation, cell cycle progression, DNA damage repair, apoptosis, cancer initiation, metastasis, and cell survival. In this study, I aimed to investigate the role of FOXM1 in 5-FU drug resistance, and questioned the direct or indirect involvement (through E2F transcription activators) of FOXM1 in TYMS regulation in colorectal cancer. I also planned to study the role of E2F transcription activators, E2F1, E2F2 and E2F3 in cell cycle progression and apoptosis in response to 5-FU treatment. I also looked at role of E2F transcription activators in FOXM1 and TYMS regulation. FOXM1 is already identified as a transcriptional target of E2F1. The figure below illustrates the schematic representation of the aims of this study (figure 1.8).

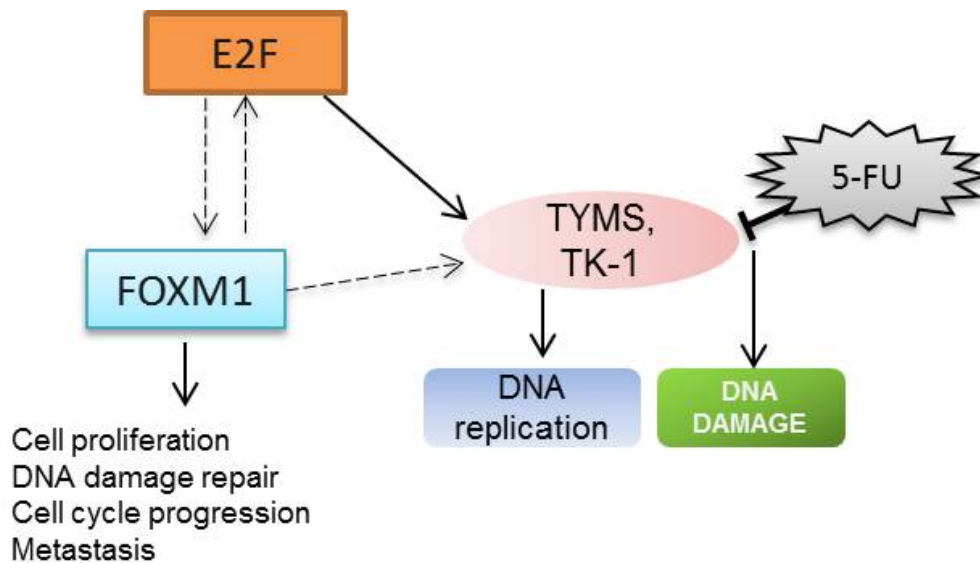


Figure 1. 8 Schematic representation of the aim of the study.

5-FU blocks DNA synthesis through the inhibition of TYMS and TK-1 and cause DNA damage.

E2F transcription activators plays a central role in regulating cellular proliferation by regulating genes required for cell cycle progression and DNA synthesis including TYMS, TK-1. FOXM1, another transcription factor has important role in cell cycle progression, cell proliferation, and chemotherapy drug resistance. It is already known that E2F1 transcription activators can regulate TYMS, and TK-1. Here I investigated the role of FOXM1 in TYMS, TK-1 regulation directly or indirectly through E2F I also studied if E2F and FOXM1 can regulate each other.

CHAPTER 2

MATERIALS AND METHODS

2.4.2 Cell culture

The human colorectal cancer cell line HT29 (P53 point mutation, Arg273-His p53) (purchased from DSMZ Braunschweig, Germany), was maintained in DMEM (Dulbecco's Modified Eagle's Medium), (Sigma®) and supplemented with 10% foetal calf serum, 200mM L-glutamine, and penicillin/streptomycin. Other colorectal cancer cell lines HCT116 (p53 wt) and DLD1 (p53 point missense mutation Ser241- Phe), were obtained from American Type Culture Collection (ATCC) and maintained in RPMI-1640 (Sigma®), (RPMI-1640 was developed by Moore et. al. at Roswell Park Memorial Institute, hence the abbreviation RPMI) and supplemented with 10% foetal calf serum, 200mM L-glutamine, penicillin/streptomycin.

The human breast carcinoma MCF-7 cell line was originated from the American Type Culture Collection (ATCC) and was acquired from the Cell Culture Service were maintained in DMEM (Sigma®), which is supplemented with 10% Foetal Calf Serum, 200mM L-glutamine, penicillin/streptomycin. MCF-7 FOXM1 over expressed cells was generated in Prof Eric Lam's laboratory by Lara Monteiro by transfecting MCF-7 cells with the pCMV-FOXM1 plasmid. The cells were maintained in DMEM (Sigma®), which is supplemented with 10% Foetal Calf Serum, 200mM L-glutamine, and penicillin/streptomycin.

The 5-FU drug-resistant HCT116 sub-line was a gift from Dr Daniel Longley, department of Oncology, Queen's University Belfast. HCT116 5-FU resistant cells were developed by repeated exposure to stepwise increasing concentrations of 5-FU over a period of ~10 months. Drug-resistant HCT116 cell lines were grown in McCoy's 5A medium, supplemented with 10% dialyzed FCS, 50 µg/ml penicillin/streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate

(all from Invitrogen Life Technologies Corp., Paisley, Scotland). HCT116 5-FU Res cells were maintained in 12uM 5-FU every time cells are split (Boyer, McLean et al. 2004). All the above cell lines were maintained in a humidified incubator at 37°C in an atmosphere of 10% CO₂.

All the cells were routinely checked for mycoplasma contamination. HCT116, DLD1 and HT29 cell lines were authenticated at DNA diagnostics centre (DDC), Hammersmith.

2.2 Cell maintenance

Cells were grown and split at ~80% confluency, generally twice a week. Medium was aspirated and cells washed once with warm PBS (Oxoid Ltd, Basingstoke, UK). Cells were removed using 1x trypsin in EDTA (both Sigma Aldrich) at 37°. After detaching, cells were centrifuged at 1300 rpm for 3 min to remove trypsin, then re-suspended in the appropriate medium and seeded into a new flask or dish.

2.3 Cell preservation

Cells were washed in PBS, trypsinised, washed in complete media and spun down at 1300 rpm for 3 min. The cell pellet was then re-suspended in FCS with 10% DMSO (dimethyl sulphoxide). 1ml aliquots were transferred to cryo-vials and frozen at -80°C overnight before transfer to storage in liquid nitrogen. To defrost cells, cryo-vials were placed into a 37° water bath until melted. Cell suspension was mixed with fresh, fully supplemented medium, and then

centrifuged at 1200 rpm for 2 min. Supernatant was discarded and the cells were re-suspended in fresh DMEM and seeded into a flask.

2.4 Cell seeding

Cells for the experiments were trypsinised. Once re-suspended in the appropriate medium, the cells were counted using a haemocytometer to confirm the confluence. Appropriate numbers of cells were seeded in plates or in flasks. Cells were treated with drug or transfected or harvested for 24, 48 or 72 hours after the seeding.

To find the growth rate of each cell lines, cells were seeded in 96 well plates with different seeding density, ranging from 1000 cells/ well to 6000 cells/ well. After 24 hours incubation, the plates were checked for 50% cell confluence.

2.5 Drug treatment

5-FU (F6627-10G) was dissolved in DMSO to a stock concentration of 1mg/mL and DMEM/RPMI was used for further dilutions. Thiostrepton (T8902) was dissolved in DMSO to a stock concentration of 25mM and DMEM/RPMI was used for further dilutions. Both drugs were purchased from Sigma®, Poole, UK. 5-FU was stored at room temperature and thiostrepton was stored at -20°C.

2.6 Transient transfections

2.6.1 Plasmids

The pcDNA3-FOXM1 and pcMV-E2F1 plasmids were provided by Prof. Eric W Lam. pCMVHA E2F2, pCMV-Neo-Bam E2F3a plasmids were purchased from Addgene,

pcDNA3-FOXM1 and pcMV-E2F1 were amplified in One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, UK). Firstly, competent cells were thawed on ice for 30 min. Up to 1g of DNA was added to the competent cells and thoroughly mixed, followed by incubation for 15 min. The competent cells were then transferred to an Eppendorf tube and placed in a 42°C water bath for 1 min. The mixture was then incubated for 2 min on ice. 1 ml of Luria broth (LB) was added and the bacteria were outgrown for 1 hour in water bath at 37°C. Bacterial cells were then centrifuged at 8000 rpm for 1 min. The supernatant was then removed and bacteria were then spread out on a LB agar plate with the appropriate antibiotic concentration and incubated at 37°C overnight. For pCMVHA E2F2, pCMV-Neo-Bam E2F3a, bacteria were gathered from bacterial stabs using an inoculating loop and spread out on a LB agar plate with the appropriate antibiotics and incubated at 37°C overnight. A single colony was chosen from the streaked LB agar plate and transferred to the liquid LB + antibiotic and left on a shaking incubator at 37°C for 12-18h. A bacterial stock was prepared using glycerol. 500µL of bacterial stock was mixed with 500µL of 50% glycerol in a cryovial and kept at -80°C for long-term storage. Plasmid DNA was extracted from the bacterial culture using a QIAGEN plasmid MAXI kit (QIAGEN, UK) according to the manufacturer's protocol.

2.6.2 Transfection of plasmid DNA

Cells were seeded into 6 well plate to a confluency of approximately 60%. Plasmid DNA was transfected using XtremeGENE HP DNA transfection reagent (Roche Diagnostics, UK). The ratio 1:1 (µl of fugene: µg of DNA) was used, as recommended by the manufacturer. To 1 µg (1 µl) plasmid DNA, 200 µl of optimum

and 1 µl of XtremeGENE HP reagent were added. After 30 min of incubation the mixture was pipetted drop wise into the dish while gently being shaken, ensuring that the mixture dispersed evenly. Overexpression of the desired proteins was confirmed 24 hours after transfection, after cell harvesting and western blot analysis.

2.6.3 RNA interference with SiRNA

RNA interference was used to specifically repress the expression of a chosen gene or genes. In this study, all SMARTpool siRNAs used were purchased from Dharmacon, RNA technologies.

The SMARTpool siRNAs used were: FOXM1 (L-(009762-00), E2F1 (L-003259-00), E2F2 (L-003260-00), E2F3 (L-003261-00), and non-specific siRNA control (NSC) (D-001810-10-20-00). Oligofectamine (Invitrogen LT) was used as the transfection reagent. Prior to siRNA transfection, cells were grown in appropriate culture dishes until they reached 50 – 60% confluence. 20,000 cells per well of a 6-well plate were plated 24h prior to transfection. For each well, 76µl of Optimem, was mixed with 5µl of oligofectamine and incubated at room temperature for 10min. Next, 250µl of Optimem, 7.5µl (100nM final concentration) of siRNA, and 7.5 µl of siRNA buffer were mixed and the solution was incubated for another 20 min at room temperature to allow the oligofectamine-DNA complexes to form. After 25min incubation, 160µl Optimem was added to each mixture, reaching a final volume of 500µl, which was then added to the wells that had been previously washed with warmed PBS. The plates were then incubated for 4 h at 37°C, after which the appropriate medium containing 10%FCS was added to each well and the plates were incubated for a further 24-48 h. Cells were harvested, protein and mRNA expression was performed or the transfected cells were treated with cytotoxic agents and SRB assays or cell

cycle analysis was performed. This ensured that any effect the transfection procedure had on the cells would be detected. Also cells were transfected with a non-specific siRNA (D-001210-01-05, Dharmacon), which does not target any known proteins, ensuring that any non-targeting effects of siRNA delivery and interaction with cellular machinery would be measured.

2.7 Protein analysis by western blots

2.7.1 Preparation of whole cell extracts

Cells were washed in PBS harvested by trypsinisation or by scraping in radioimmunoprecipitation assay buffer (RIPA) (Thermo Fisher scientific, Waltham, USA) (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1 % NP40, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate). In order to preserve protein phosphorylation and to protect the cell lysates from protein degradation, the RIPA buffer was supplemented with 1 X protease and phosphatase inhibitor cocktail (1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 800 nM aprotinin, 50 µM bestatin, 15 µM E-64, 5µM EDTA, 20 µM leupeptin, 10 µM pepstatin A; Thermo scientific). Detached cells were collected into an Eppendorf, placed on ice for 15 minutes and centrifuged at 14 000 rpm for 10 min.

Trypsinised cells were washed in 1 ml of PBS and transferred to an Eppendorf tube, the supernatant was discarded and RIPA lysis buffer including inhibitors was used to lyse cell pellets. The cells were left on ice for 15 min and centrifuged at 14 000 rpm for 10min. The supernatant was transferred to a new Eppendorf tube and stored at -80°C until next use.

2.7.2 Bio-Rad DC protein concentration assay

The concentrations of the protein lysates were determined using the Bio-Rad Dc Protein Assay kit (Bio-Rad, UK). Following the manufacturer's protocol, the protein concentration was determined by a microtitre plate reader (Sunrise, Tecan, Reading, UK) at 700 nm wavelength.

2.7.3 Western blots

Western blotting was used to investigate protein expression. Whole cell lysates were separated via SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) according to the weight of the protein. The protein containing SDS gel was then transferred on to nitrocellulose membranes for immunoblotting.

2.7.3.1 SDS page and nitrocellulose membrane transfer

Protein lysate was prepared by mixing 25 µg of lysate with 2x SDS loading dye [2% (w/v) SDS, 350 mM DTT, 62.5 mM Tris buffer pH 6.8, 25% (v/v) glycerol and 0.001% (w/v) bromphenol blue]. Protein samples were denatured at 100°C for 5 min. SDS-PAGE consists of two parts: a stacking gel (upper part) and a resolving gel (lower part). In this study I used 7, 10 and 12 % resolving gels. The percentages of resolving gel (varying the amount of bis-acrylamide solution) were adjusted depending on the size of the protein of interest. For example, the smaller the protein, the higher the percentage of bis-acrylamide solution would be used to make the SDS-PAGE gels. The polymerisation of the gel was catalysed by ammonium

persulphate (APS) and tetramethylethylenediamine (TEMED). Components for the SDS-phage gel are mentioned in the table below (Table 2:1).

Table 2.1 Components for the SDS-phage gel

	Resolving gel					Stacking gel
	5%	7%	10%	12%	14%	
ddH ₂ O (ml)	5.68	5.02	4.02	3.35	2.68	3.67
1.5M Tris pH 8.8 (ml)	2.5	2.5	2.5	2.5	2.5	
1.5M Tris pH 6.8 (ml)						0.42
30% Acrylamide (ml)	1.67	2.33	3.33	4	4.67	0.83
10% SDS (μl)	100	100	100	100	100	50
25% APS (μl)	40	40	40	40	40	20
TEMED (μl)	10	10	10	10	10	5

Samples were loaded into the wells of the stacking gel. Novex sharp protein standard (Invitrogen, UK) served as a marked for determining molecular sizes. The Bio-Rad Mini-Protean system was used (Bio-Rad, Hemel Hempstead, UK). SDS-PAGE gels were run using SDS-PAGE running buffer (25 mM Tris base, 250 mM glycine, 0.1 % SDS) at 120 V for separation of proteins. The proteins on the gel were then transferred to Protran nitrocellulose membrane (Whatman International, Kent, UK) using a Bio-Rad Trans-Blot cell wet transfer system (Bio-Rad, Hempstead, UK)

in transfer buffer (25 mM Tris, 190 mM glycine and 20% Ethanol) at 90 V for 90 min on ice.

2.7.3.2 SDS page and membrane transfer with pre cast gel

Protein lysates were prepared by addition of 10 x reducing agent (Invitrogen) and 4x NuPAGE® LDS Sample Buffer (4X) (lithium dodecyl sulphate) (Invitrogen). The protein lysates were denatured by heating at 70 °C for 10min. 15-25 µg of the protein samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V. For this 4-15 and 10 % polyacrylamid Mini-PROTEAN TGX precast gels (Bio-Rad, Hercules, USA) were used. Samples were transferred to polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo system from Bio-Rad®.

2.7.4 Immunoblotting

After removing the membranes, protein transfer was confirmed by staining the membranes with Ponceau S (Sigma-Aldrich, UK). The red stain from Ponceau S was washed out by Tris-buffered saline with tween (TBST, 20 mM Tris pH 7.6, 136 mM NaCl, 0.01% (v/v) tween). After removing the stain, the membranes were blocked with 5% (w/v) BSA (BSA, Sigma) in TBS-T for 45 to 60 min. Membranes were incubated with (1:1000) primary antibodies in 5% BSA-TBST and 0.02% (v/v) azide overnight, shaking at 4°C (see table 2.2 for the list of antibodies used). On the following day, the membranes were washed with TBST for 10 min 3 times by

shaking. The membranes were then incubated on the shaker with the appropriate (rabbit or mouse) secondary antibody conjugated with horseradish peroxidase (HRP) (Dako, Ely, UK) in TBS-T (1:2000 dilution) for 1 h at room temperature, and then the membranes were again washed 3 times (10 min per each wash). Once washed, an enhanced chemiluminescence detection system (ECL, Perkin Elmer, UK) was added to enable visualisation. The luminescence signal was detected using Amersham Hyperfilm ECL (GE Healthcare, UK) before being developed with a SRX-101A X-ray developer (Konica Minolta, Japan) or an Optimax 2010 X-Ray film processor (IGP, UK).

Table 2.2 List of antibodies used in western blots

Antibody	Company	species	Catalogue No.
FOXMI(C-20)	Santa Cruz	Rabbit	SC502
E2F1 (C-20)	Santa Cruz	Rabbit	SC193
E2F2 (C-20)	Santa Cruz	Rabbit	SC633
E2F2 (TFE-25)	Santa Cruz	Mouse	SC9967
E2F3 (N-20)	Santa Cruz	Rabbit	SC879
TYMS	Abcam	Mouse	ab58287
TK-1	Abcam	Mouse	ab57757
P53	Santa Cruz	Mouse	SC6243
p21 ^{Cip} (187)	Santa Cruz	Mouse	SC817
RB (D20)	Cell signalling	Rabbit	9313
pRB (S807/S811)	Cell signalling	Rabbit	9308
ATM (AM9)	Millipore	Mouse	05-513
pATM	Millipore	Mouse	DR1002
Chk2	Cell signalling	Rabbit	2662
pChk2	Cell signalling	Rabbit	2669
β -tubulin (H-235)	Santa Cruz	Rabbit	SC9104

2.8 Reverse transcription polymerase chain reaction (RT-PCR)

2.8.1 RNA isolation and quantification

Cells were harvested by trypsinisation. Total RNA was extracted using the RNeasy Mini kit (Qiagen). The concentration of RNA was assessed by measuring the absorbance at 260 nm on the nanodrop ND1000 spectrophotometer (Labtech International, UK). Extracted RNA was stored at – 80 °C.

2.8.2 cDNA synthesis

500 nanograms of RNA were retro-transcribed into cDNA using the QuantiTech reverse transcription kit (Qiagen-cat no.205313) following the product protocol. cDNA was diluted 1 in 5 with RNase and DNase free water and was stored at -20 °C.

2.8.3 Primer design

In this study, all primers were designed using Primer 3.0 (Applied Biosystems, UK). Fasta formatted nucleotide sequences were selected and run on Primer 3®. 100-200 bp length primers were selected. Newly designed primers were tested using a mixture of cDNAs as template; a dissociation curve was also performed for each primer set to ensure only one product was produced. Primer pairs resulting in Ct values below 25 were deemed acceptable. The list of primers used for this study is shown in Table 2.3

2.8.4 RT-PCR

RT-PCR was performed using SybrGreen mastermix (Applied Biosystems, UK) and run in a 7900HT Fast Real-Time PCR system (Applied Biosystems).

A master mix was prepared for each set of primers. For one reaction, a mixture of 2 µl of cDNA, 12.5µl of SYBR-green master mix, 7.5µl of DEPC treated water and 1µl of (10µM) forward and reverse primers were used. The standard curve was produced from the mixture of 5µl cDNA from every sample, and then diluted 1:4 for 4 serial dilutions. DEPC-treated water was used as a template control. 7900HT fast Real-time PCR system was set as: 95°C for 10 min for enzyme activation and followed by 40 cycles at 95°C for 10 s (denaturation) and 60°C for 30s (primer annealing/extension). Gene expression was quantified using a standard curve and normalised to the L19, a non-regulated ribosomal housekeeping gene, which served as an internal control. All measurements were in triplicate.

Table 2.3 List of primers used in RT-PCR analysis

Gene	Species	Sense	Antisense
L19	Human	GCGGAAGGGTACAGCCAAT	GCGGAAGGGTACAGCCAAT
FOXM1	Human	TGCAGCTAGGGATGTGAATCTTC	GGAGCCCAGTCCATCAGAACT
TYMS	Human	GCCTCGGTGTGCCTTTCA	GATGTGCGCAATCATGTACGT
TK-1	Human	ATGAGCTGCATTAACCTGCCCACT	ATGTGTGCAGAAGCTGCTGC
E2F1	Human	ATGTTTTCTGTGCCCTGAG	ATCTGTGGTGAGGGATGAGG
E2F2	Human	GTTTGGAAGCAGCTTTCTGG	GCAAGGTTCTTCAGCTCAC
E2F3	Human	CTTAAGCTGGCAGGAACAGG	GAGACACCCTGGCATTGTTT

2.9 Sulforhodamine B (SRB) assay

Cells were seeded in 96 well plates (3000-5000 cells/well) and left to grow at 37°C with 10% CO₂ overnight. On the following day, the cells were treated with the drug of interest. After incubation (24h, 48h or 72h) cells were fixed by adding 100µl of ice-cold 40% (w/v) trichloroacetic acid (TCA, Sigma-Aldrich, UK) to each well and incubated at 4°C for 1 h. Plates were rinsed 4-5 times with slow running water before being stained with 100µl of 0.4% (w/v) Sulphorhodamine-B (SRB, Sigma-Aldrich, UK) in 1% (v/v) acetic acid (VWR International, UK), followed by incubation for 1 h at room temperature. Excess dye was removed upon washing with 1% acetic acid (Sigma) solution four times. Plates were dried and bound protein was solubilised upon addition of 150µL of 10mM Tris base buffer. Absorbance was measured at 540 nm using a Thermo Multiskan-Ex plate reader.

2.9.1 Data analysis

Cell viability vs compound concentration curves were plotted as a percentage of control cells. The concentration of drug that gave 50 % inhibition in cell growth (IC₅₀) was extrapolated from these curves using the sigmoid curve fit with variable slope in GraphPad prism (version 5.01, La Jolla California, USA). Three to six replicate wells were used for each drug concentration and experiments were carried out on five separate occasions for each compound.

2.10 Clonogenic assay

Tumour colony formation assays were conducted in 6 well plates. 2000-3000 cells/well were seeded, after 24 hours cells were treated with 5-FU (1 µg/mL), thiostrepton (1 µM) alone or both drugs at the same concentration in combination for 72 hours. After 72 hours drug free medium was added and the plates were maintained for 12-14 days with medium changes every 3 days, plates were then washed in cold PBS and colonies were fixed with 4% formaldehyde. They were then stained with crystal violet (0.05%) (0.05g crystal violet 2.5ml 25% methanol 7.5ml water) for 30-60 min in room temperature and wash thoroughly with tap water. Stained plates were photographed, and the colonies were dissolved by shaking the plates on a Perkin Elmer Delfia plate shaker with 15% acetic acid. Optical density at 540 nm was measured in a microplate reader (Sunrise, Tecan). Results were normalised to untreated or control wells.

2.11 Cell cycle analysis via flow cytometry using propidium iodide

The distribution of cells within the different phases of the cell cycle and cell death, were assessed by performing DNA staining with propidium iodide (PI). Stained cells were then analysed via Flow cytometry. Cells were trypsinised and pelleted by centrifugation at 1800rpm for 3 min. Cells were washed with 1 mL of ice cold cell staining buffer (biolegend). 70% of 2ml ethanol was then added to fix the cells. Samples were then incubated at -20°C overnight. Cells were centrifuged at 600 g

and the supernatant was discarded. The cell pellet was twice re-washed in cell staining buffer (biolegend). Cells were stained with Propidium iodide (biolegend).

Samples were then incubated in the dark at room temperature for 15 min. The BD FACS Cantos machine (BD Biosciences) was then used to record and analyse the results. For each condition 10,000 cells were counted.

Data analysis was performed using Flowing software version 2.5.1 (University of Turku, Finland).

2.12 Immunofluorescent staining (IF)

8000-10000 cells were seeded in 4 chamber/slides. The cells were treated with the appropriate drug for different time points. The cells were then washed with 200µl of PBS for 2 times at room temperature. 4% formaldehyde (Ultrapure Polysciences) was used to fix the cells and then prepared in PBS for 15 min. The fixed cells were washed 3 times with PBS and permeabilised with 0.2% (v/v) Triton X-100 (Sigma-Aldrich, UK) in PBS for 10min. 100 µl PBST-BSA was used for blocking the slides at 1h in room temperature. The cells were then incubated overnight at -20⁰ C with 1:200 dilution of primary rabbit anti-phospho H2AX (Ser139) antibody (Cell Signaling). Following three washes with 0.5 ml of PBS, cells were incubated with secondary antibodies: Alexa Fluor 594 (Red) and 488 (green) at 1:500 (Invitrogen). Cells were then washed 3 times with PBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories Inc.). Slides were visualized with a Leitz Laborlux UV microscope (Wetzlar, Germany) using a ×40 objective fitted with a Spot Insight 4 camera (Diagnostic Instruments, Sterling Heights, Michigan). The intensity of fluorescence was evaluated with ImageJ software.

2.13 Chromatin Immunoprecipitation (ChIP)

ChIP assay was conducted by using HCT116, DLD1 and HCT116 5-FU Res cell lines. Cells were grown to 90-95% confluency (150mm dish) and cross-linked with 1% formaldehyde for 10 min at the room temperature. 125 mM of glycine was added for 5 minutes to stop the cross-linking. The cells were washed with cold PBS and harvested using scrapping buffer with 1X PI (protease inhibitor). Wash the cells pellet with 1ml of PBS+PI, Buffer1+PI, Buffer2+PI. Then add 300µl of lysis buffer+ (1X) PI, leave it on ice and sonicate.

Sonication time was optimised in these cell lines by sonicating the cells at 0, 4, 8, 10, 12 and 14 min. Samples are sheared at high power setting (position H) for several rounds of sonication cycles of 30 sec ON/30 sec OFF with the Bioruptor® PLUS combined with the Bioruptor® Water cooler & Single Cycle Valve. The sheared chromatin was decross-linked and 10 µl of DNA (equivalent to 500 ng as measured with nanodrop) was analysed on a 1.5% agarose gel. The samples were run on E-gel Electrophoresis systems (Life technologies), a complete buffer less system for agarose gel electrophoresis of DNA samples. The gels were visualised using a UV transilluminator. Optimal DNA size range for ChIP and ChIP-seq analysis ranged from 200 bp to 1 kb with a peak around 500 bp. Based on agarose gel electrophoresis the best sonication time was identified. (10 minutes- HCT116 and 14 minutes- DLD1).

After sonication, the samples were cleared by centrifugation (14,000rpm/10 min/ 4°C), supernatants were transferred in to new tubes and diluted with buffer 300µl D+PI (1:1). From this mixture store 100µl in -80 as input. The antibodies were bound

(FOX M1 C-20 X, Santa Cruz biotechnology) with dynabeads (IgG protein-A and IgG protein-G (Invitrogen) by incubating in a rotator for overnight in 4°C. The dynabeads were washed with TSE I + PI. The DNA was eluted with elution buffer and the samples were decross linked (including input) overnight at 65°C or 6 hours. Extracted the DNA with Qiagen PCR purification kit and the samples were run in a 7900 HT Fast Real-time PCR System (Applied Biosystems). List of primers used for ChIP were listed below.

Table 2.4 Primers used for ChIP were

TYMS promoter (bp)	Sense	Anti-sense
0 to-500	CCTGGCGGTTTTTAATCAAG	CACAGTTCCCACGTTTTTCCT
0 to -500	CCTGGCGGTTTTTAATCAAG	ACAGTTCCCACGTTTTTCCTG
-500 to 1000	GTAGTCCCAGCTACGCGAGA	AGGGCTTTTCCGAGGTTTT
-1000 to -1500	CTGCTGAGGGCTCTATCACC	CTCGGCCCCAAGTTTTTAAT
-1500 to -2000	GACCACTCCTCTGGGTCAAA	ACCTGTCTTGAGGCCTGTGT
-1500 to -2000	TTCTACGCCCAGAACCATCT	GGCCTGTGTTGATGTCAATG
-2000 to -3000	GGCACAGGAAGAAAGGTCTG	TTGTGGTGCTTGCTGTACTTG
-2000 to -3000	GGGCACAGGAAGAAAGGTCT	GTGGTGCTTGCTGTACTTGG
-3000 to -4000	GGCACAGGAAGAAAGGTCTG	CCTTGTGGTGCTTGCTGTACT

Table 2.5 Reagents used for ChIP assay is listed below

Buffer I
0.25% Triton X-100
10mM EDTA
0.5mM EGTA
10mM HEPES pH6.5
H2O

Buffer II
200mM NaCl
10mM EDTA
0.5mM EGTA
10mM HEPES pH6.5
H2O

Lysis Buffer
1% SDS
10mM EDTA
50mM Tris-HCL pH8.1
H2O

Buffer D
1 % Triton X-100
2mM EDTA
20mM Tris-HCL pH8.1
150mM NaCl
H2O

TSE I
0.1% SDS
1 % Triton X-100
2mM EDTA
20mM Tris-HCL pH8.1
150mM NaCl
H2O

TE Buffer
10mM Tris-HCL pH8
1mM EDTA
H2O

Scraping Buffer
100mM Tris-HCL pH9.4
0.1% SDS
H2O
Protease inhibitor (1X)

Elution Buffer
0.1M NaHCO ₃
1% SDS
H2O

2.14 ChIP-Seq

The ChIPed DNA and input DNA were amplified as described above. Libraries were generated, and sequencing was performed on Illumina Hi Seq 2000 analyser according to the manufacturer's protocols (NEBNext Multiplex Oligos for Illumina, New England Biolabs). IGV (interactive genome viewer) (Thorvaldsdóttir, Robinson et al. 2012) was used to visualise ChIP sequenced data. The basic bed file format was used to visualise ChIP- seq peaks. The CEAS function in Cistrome was used to functionally annotate binding sites (Shin, Liu et al. 2009).

2.15 Caspase glow assay

Cellular apoptosis induced by drug treatment was detected using a Caspase 3/7-Glo assay (Promega, Fitchburg, USA) on cells seeded in white 96 well plates, according to the manufacturer's instructions. This luminescence assay kit, measures levels of activity of caspase 3 and 7, both of which play a key role in apoptosis.

2.16 Wound healing assay

Cells were seeded to complete confluence in a monolayer on a 6 well plate. A wound was created by scratching firmly with a 2µl pipette tip. Cells were treated with 5-FU (1µg/mL), thiostrepton (1 µM) alone or in combination. Images were taken at 0, 6, 24, 48 and 72 hours. Images were taken on an Olympus DP-70/ Evos AMG microscope.

2.17 Migration assay

Cell migration assays measure the movement of cells from one area to another. This technique can be used to assess response to a chemical signal for drug treatment, and is commonly used to study metastatic potential in vitro.

HCT116 cells were seeded in 6 well plates and treated with 5-FU (1 μ g/mL), thiostrepton (1 μ M) alone or combination 5-FU and thiostrepton at the same concentration for 72 hours. The cells were then harvested and seeded to the upper chamber of the inserts (10000 cells/ well in 200ul of serum free RPMI). (Corning transwell –sigma, diam. 6.5 mm, pore size 8.0 μ m, sterile) 750ul of RPMI medium with 10% FBS was added to the lower chamber. The cells were allowed to migrate for 48 hours at 37 °C. The cells were removed from the upper side of the transwell with a cotton swab. The wells were washed with cold PBS. The invading cells on the underside of the transwell were fixed with 4% formaldehyde in PBS and stained with crystal violet (0.05g crystal violet, 2.5ml 25% methanol, 7.5ml water). The stained invasive cells were photographed under a microscope and then the cells were dissolved with 15% acetic acid. Optical density at 540 nm was measured in a microplate reader (Sunrise, Tecan).

2.18 Immunohistochemistry staining

Human CRC tissue microarray samples containing 110 tumours were purchased from Biomax USA (product number BC051110). FOXM1, TK-1 and TYMS immunoreactivity was evaluated on TMA sections using polyclonal rabbit anti human FOXM1 (C20) antibody (Santa Cruz Biotechnology) TYMS antibody (Abcam) and

TK-1 (Abcam). Briefly, the sections were rehydrated and heated in a microwave oven at 900W for 20 min in citrate buffer at pH6. Primary antibodies were used at 1:100 (FOXm1), 1:150 (TYMS), and 1:150 (TK-1) dilution for 1 hour at room temperature and then processed with Polymer-HRP Kit (BioGenex, San Ramon CA) with Diaminobenzidine development and Mayer haematoxylin counterstaining. External positive controls were used as suggested by the company. Negative controls were obtained by omitting the primary antibody. A semi-quantitative immunohistochemical score (IHS) was calculated by an independent accredited histopathologist Dr Francesco Mauri, Imperial College Healthcare NHS Trust using the intensity of staining and the percentage of positive cells as previously described. TMA micro array panel display and specification sheet is included in the supplemental data.

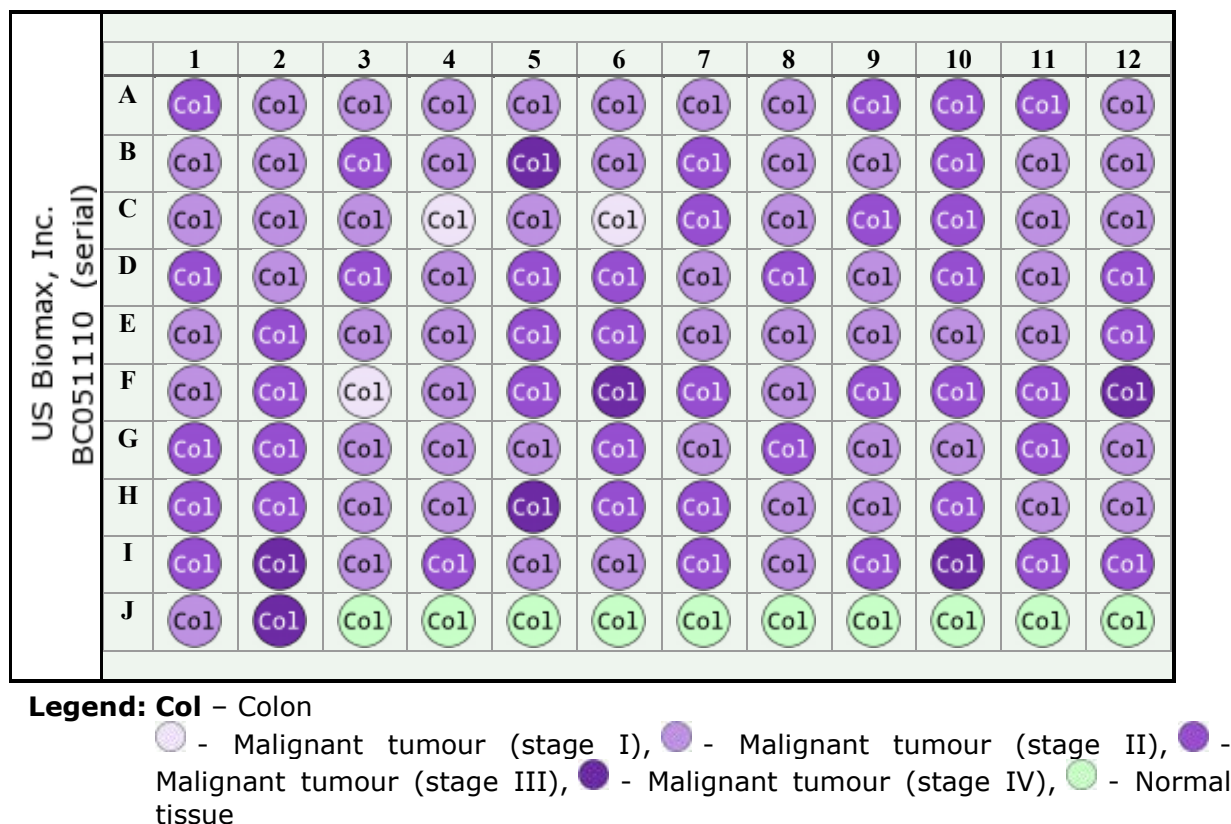


Figure 2. 1 TMA microarray panel obtained from US Biomax inc®

CHAPTER 3

RESULTS 3.1

IDENTIFYING THE ROLE OF FOXM1 IN 5-FU DRUG RESISTANCE

3.1.1 Introduction

5-FU is one of the most widely used agents in the first-line therapy of colorectal carcinoma, although various regimens have been developed over the past 30 years, no single agent has yet been able to replace 5-FU in the treatment of CRC (Loehrer, Turner et al. 1988, Pinedo and Peters 1988, Poon, O'Connell et al. 1989). Studies have shown that 5-FU exerts its anticancer effects mainly through inhibition of TYMS and also TK-1 (Foekens, Romain et al. 2001). TK-1 plays an important role in salvage pathway mediated 5-FU resistance (Lee, Kim et al. 2010).

TYMS over-expression is widely accepted as a major molecular mechanism responsible for 5-FU resistance. Significantly higher TYMS expression has been found in resistant cancer cells compared to sensitive colon cancer cells and tumours expressing high levels of TYMS appeared to have a poorer overall survival (OS) compared with tumours expressing low levels of TYMS (Saga, Suzuki et al. 2003, Popat, Matakidou et al. 2004). Furthermore, tumours from patients with high TYMS levels are more likely to be resistant to 5-FU (Kamoshida, Suzuki et al. 2007).

It is well known that the E2F1 transcription factor regulates the cell cycle by controlling G1-S regulatory genes, including TYMS. E2F1 is also known to regulate FOXM1 (Kasahara, Takahashi et al. 2000, Wu, Zheng et al. 2009, Millour, de Olano et al. 2011).

Elevated expression of FOXM1 has been detected in a broad range of cancer cell lines and cancer types examined so far, suggesting that FOXM1 is essential for tumour cell proliferation (Ahmad, Wang et al. 2010, Kwok, Peck et al. 2010, Park, Jung et al. 2012, Halasi and Gartel 2013). In a preclinical CRC model, Rosa26-FOXM1 transgenic mice displayed increased growth and number of tumours

compared to wild type, and similarly FOXM1 depletion was associated with reduced CRC carcinogenesis and growth after exposure to a carcinogen (Yoshida, Wang et al. 2007). Elevated expression of FOXM1 is associated with tumour progression and metastasis; inactivation of FOXM1 leads to inhibition of cell cycle progression in breast cancer cells (Blanco-Bose, Murphy et al. 2008, Yang, Chen et al. 2013).

Another study shows that FOXM1 deficient cells have increased levels of DNA damage. Mouse embryonic fibroblasts (MEFs) derived from FOXM1 knock-out mouse displayed high levels of the γ H2AX marker compared to wild-type MEFs, confirming the role of FOXM1 in DNA damage pathways (Tan, Raychaudhuri et al. 2007). Further studies have revealed the multiple facets of FOXM1 in oncogenesis. Deletion of FOXM1 reduced migration, invasion, or the angiogenic potential of pancreatic (Wang, Banerjee et al. 2007), gastric (Li, Zhang et al. 2009), breast (Ahmad, Wang et al. 2010), colon cancer cells (Chu, Zhu et al. 2012), glioma (Dai, Kang et al. 2007) and osteosarcoma cells (Wang, Chen et al. 2008) in vitro.

The role of FOXM1 in chemotherapy drug resistance has been also identified most notably in breast cancer. A recent study shows that epirubicin resistant cells display higher FOXM1 levels compared to sensitive MCF7 breast carcinoma cells and that FOXM1 expression is down regulated in sensitive MCF7 cells. (Millour, de Olano et al. 2011); knock-down of FOXM1 induces apoptosis in MCF7 cisplatin resistant cells (Kwok, Peck et al. 2010). Another study on breast cancer shows overexpression of FOXM1 confers resistance to herceptin and paclitaxel. A study in gastric cancer identifies role of FOXM1 in docetaxel drug resistance (Li, Qiu et al. 2013).

The role of FOXM1 in CRC drug resistance has not been established. Due to its central role in oncogenesis and proliferation, I planned to investigate the potential role of FOXM1 with respect to TYMS and other 5-FU target genes involved in 5-FU resistance.

3.1.2 Sensitivity of colon cancer cell lines to 5-FU

I first investigated the effect of 5-FU on colon cancer cell lines. I chose three colon cancer cell lines for this experiment, p53 wt HCT116, p53 mutant DLD1, and p53 mutant HT29 cell lines (Figure 3.1). Cells were treated with different concentrations of 5-FU ranging from 0.04 µg/ml to 100 µg/ml for 72 hours.

5-FU treatment reduced cell viability in a concentration dependent manner. HT29 cells were more resistant than HCT116 or DLD1 cells (IC_{50} mean $\pm$ SD=3.09 µg/ml $\pm$ 0.39, 0.6 µg/ml $\pm$ 0.23, 0.7 $\pm$ 0.42 µg/ml, respectively). Based on the IC_{50} values, I chose 0.5 µg/ml of 5-FU as a suitable concentration for further experiments. Although the IC_{50} values for HCT116 and DLD1 were similar, at higher concentrations of 5-FU, DLD1 cells were less sensitive than HCT116.

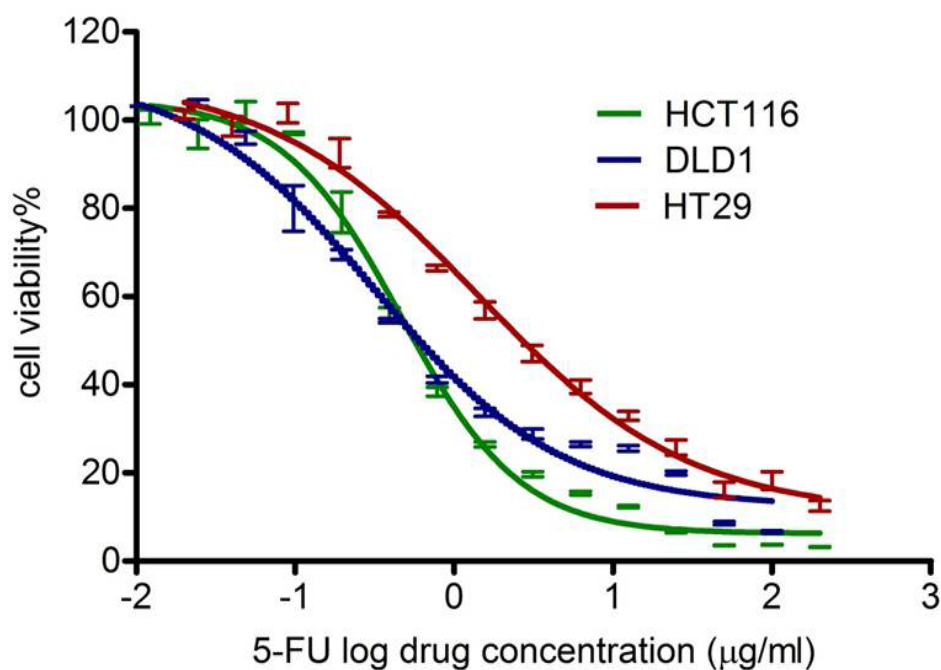


Figure 3. 1 SRB assay shows the average cell viability of colon cancer cells. HCT116, DLD1, and HT29 colon cancer cells were treated with 5-FU, ranging from 0 to 100 $\mu\text{g/ml}$ for 72 h. IC₅₀ values were: HCT116 0.6 ± 0.23 , DLD1 0.7 ± 0.22 , and HT29 $3.09 \pm 0.39 \mu\text{g/ml}$. Results shown are mean and SD of three independent experiments, each with three replicates. IC₅₀ values were determined by fitting a sigmoidal dose-response curve to the data using Graph pad Prism®.

3.1.3 Overexpression of FOXM1 increases resistance to 5-FU

To investigate the role of FOXM1 in mediating 5-FU drug sensitivity, HCT116 cells were transiently transfected with SiFOXM1 or FOXM1 pcDNA3 plasmid and treated with 5-FU, HCT116 cells transfected with SiNSC (non-specific control) or empty vector pcDNA3 were used as controls (figure 3.2). I examined the protein expression of FOXM1 in transiently transfected cells by western blotting to confirm the transfection efficacy in HCT116 cells (figure 3.2 A). This figure is spliced. The original un-spliced film is included in the supplementary data (figure 5.2).

Subsequently, I assessed the sensitivity of HCT116 FOXM1pcDNA3 and HCT116 SiFOXM1 cells to 5-FU (figure 3.2B). Cell viability assays showed that FOXM1 knock-down cells were significantly more sensitive towards 5-FU compared to control ($p < 0.001$) and likewise, transient over-expression of FOXM1 in HCT116 cells increased resistance to 5-FU than control ($p < 0.05$). These results indicate the role of FOXM1 in 5-FU sensitivity in colon cancer cell lines.

I also confirmed this effect on an established FOXM1 over expressed model, MCF7 pCMV FOXM1 and found a 4 fold increase in the 5-FU resistance in the FOXM1 over expressed cells compared to MCF7 wt cells (Supplementary data figure 5.1).

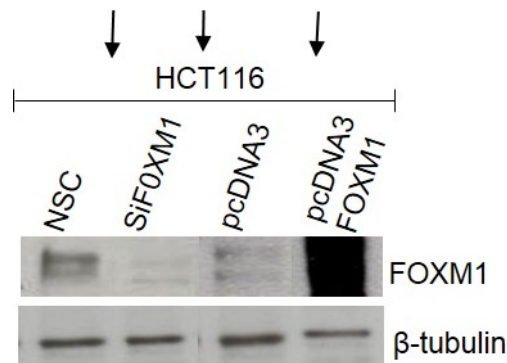
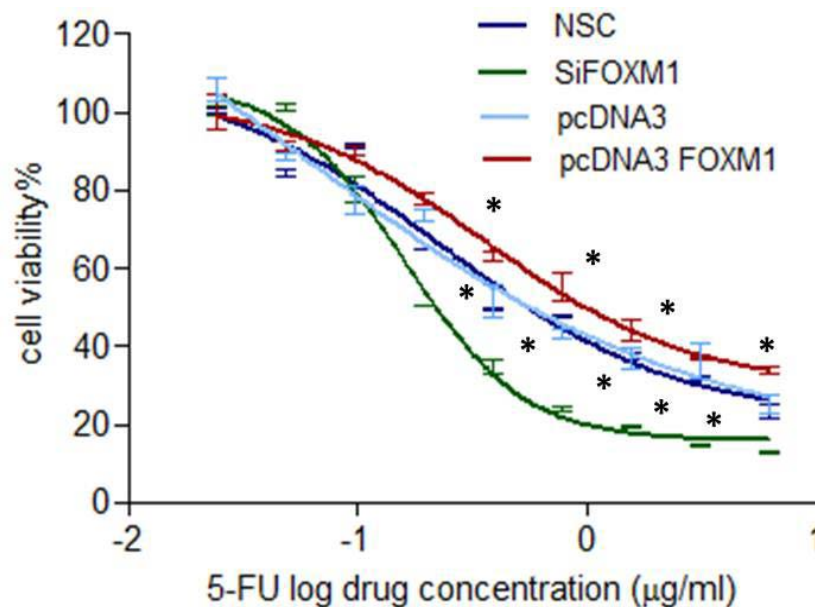
A**B**

Figure 3. 2 Over-expression and knock-down of FOXM1 affect 5-FU sensitivity in CRC. HCT116 cells were transiently transfected with SiNSC/SiFOX M1/ FOXM1 pcDNA3 plasmid/empty vector pcDNA3. 24h after the transfection cells were reseeded in to 96 well plates at a density of 3000 cells/well and exposed to various concentrations of 5-FU. After 48h, cells were fixed and optical density was measured at 540 nm. (A) Transfection efficiency was confirmed by western blot. The arrows indicate that the gels were spliced. The original un-spliced film is included in the supplementary data (figure 5.2). (B) Overexpression of FOXM1 increases 5-FU chemo-resistance in HCT116 cells and SiFOX M1 HCT116 cells were more sensitive to the treatment. Significance testing was performed using student t test SiNSC to SiFOX M1 $p < 0.001$, pcDNA3 to pcDNA3 FOX M1 $p < 0.05$.

3.1.4 Effect of 5-FU on FOXM1, TYMS, and E2F1 in CRC

Having identified the role of FOXM1 in 5-FU drug resistance, I next sought to investigate possible downstream mechanisms of FOXM1 that mediate reduced cytotoxicity. I carried out western blots to screen and detect the expression of different proteins, which could have potential roles in 5-FU resistance (figure 3.3 A, B).

In p53 mutant HT29 (p53/Arg²⁷³His) and DLD1 (mutant p53/Ser²⁴¹Phe) cells, up-regulation of E2F1, FOXM1 and TYMS persisted to 48hrs in both protein and mRNA. Although p53 protein is expressed in DLD1 and HT29 cells, it is not fully functional since these cell lines are p53 mutant (Russo, Malacarne et al. 2002). Expression of FOXM1, TYMS, and E2F1 protein (figure 3.3 A) were found to be induced initially and decreased at 48h after 5-FU treatment in p53 wt HCT116 cells and a similar trend was observed in mRNA (figure 3.3.B). FOXM1 0h versus 48h (P value = 0.0068), TYMS 0h versus 48h (P value = 0.0172) and E2F1 0h versus 48h (P value=0.0008). Raw mRNA values of L19, FOXM1, TYMS and E2F1 are included in the supplementary section (Table 5.2). Treatment with 5-FU has been shown to induce TYMS expression initially, this induction seems to be due to inhibition of the auto regulatory mechanism of TYMS by FdUMP (Junior, Marson et al. 2012). The double band observed after 5-FU treatment in TYMS (ternary complex-upper band and the free- TYMS lower band) is due to the active metabolite of 5-FU, FdUMP, forming a ternary complex with TYMS and its reduced-folate cofactor (de la Cueva, Ramírez de Molina et al. 2013). ATM, phosphorylated ATM (p-ATM), Chk2, and phosphorylated Chk2 (p-Chk2) are the proteins involved in DNA damage response. Interestingly, the expression levels of these proteins were generally found to be higher in p53 mutant cells compared to p53 wt cells.

A

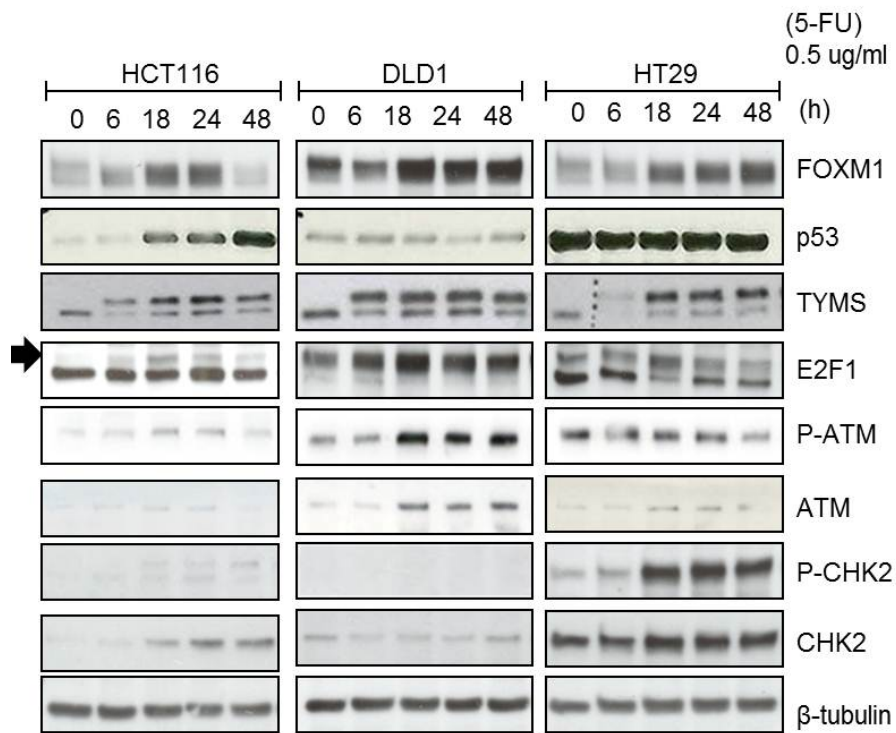


Figure 3. 3 A Western blot analysis of 5-FU treated CRC cells. HCT116, DLD1, and HT29 cells were seeded at a cell density of 1×10^6 / 100 mm plate. At 60-70% confluence, the cells were treated with 0.5 g/ml 5-FU for 0, 6, 18, 24, and 48 h. Cells were trypsinised and harvested at the indicated time points for western blot analysis (A) and RT-PCR (B)

B

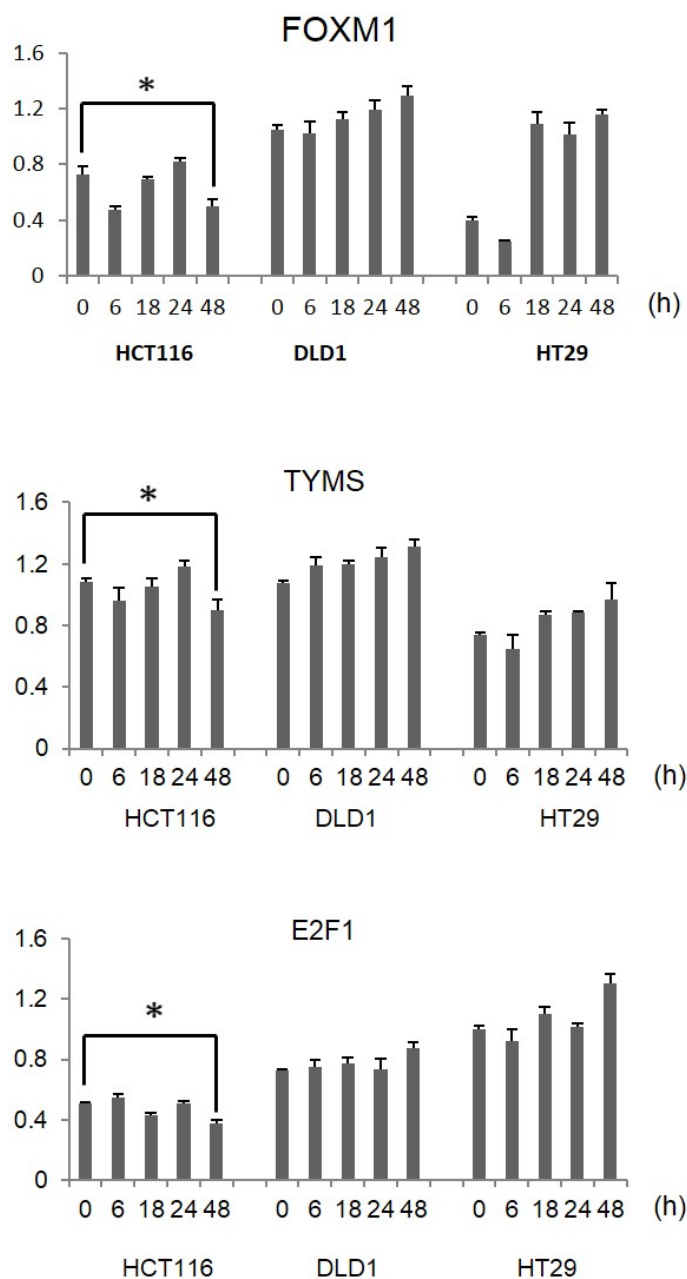


Figure 3.3 B RT-PCR analysis of 5-FU treated CRC cells.

Total RNA was extracted using the RNeasy mini kit. Quantitative real-time PCR (RT PCR) was used to determine mRNA levels of FOXM1, E2F1, and TYMS. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. This experiment was done in triplicate and statistically significant decreases were observed in HCT116 FOXM1, TYMS and E2F1 mRNA levels at 48h. Error Bars represent standard deviation. Statistical significance was determined by student's T-test (*p value < 0.05 0h versus 48h in HCT116 cells). Gene expression was quantified using a standard curve and normalised to the L19.

3.1.5 TYMS and TK-1: potential FOXM1 targets in colon cancer

HCT116 cells were used to check the involvement of FOXM1 transcription factor in regulation of main 5-FU targets, TYMS and TK-1. I also investigated the expression of E2F transcription activators E2F1 (a transcription factor widely known to regulate TYMS and TK-1), E2F2 and E2F3. HCT116 cells were transiently transfected with pcDNA3 FOXM1 (figure 3.4 A, B) and SiFOXM1 (figure 3.5 A, B). I have used spliced western blot figures. The arrow indicate spliced region of the gel. The original un-spliced film is included in the supplementary data (figure 5.3, 5.4).

Over-expression of FOXM1 [Figure 3.4 A, B] significantly increased both protein and mRNA expression of TYMS. In TK-1, slight increase (not statistically significant) observed in mRNA level. Raw mRNA values of L19 TYMS and TK-1 are included in the supplementary section (Table 5.5, 5.6). But no changes observed in E2F1, E2F2, and E2F3 protein or mRNA level. Cell cycle regulatory protein, p21^{Cip} is found to be decreased in HCT116 FOXM1 over expressed cells. This indicates, down-modulation of p21^{Cip} leads to the inactivation of cell cycle control and uncontrolled S phase entry.

Knock-down in FOXM1 (figure 3.5 A, B) shows no changes in the protein levels of TYMS, TK-1, E2F1, E2F2 and E2F3. It could be due to the stability of these proteins is very high. Some studies reports that the mRNA and protein levels are not similar after the transfection. mRNA knock-down will occur early (24-48hrs) while protein knockdown will occur at 48-96 hours due to the siRNAs not targeting proteins directly. In my experiments I have used 48h transfection. This could be a reason for

different mRNA and protein expression. The best way to avoid this issue is to do a time course to analyse the best termination times for mRNA and protein.

In contrast, significant decreases were observed in the TYMS, TK-1, E2F3 ($P < 0.05$), mRNA expression. FOXM1 knock-down slightly decreased E2F1 and E2F2 mRNA expression but no statistical significance observed. Raw mRNA values of L19, TYMS, TK-1, E2F1, E2F3 and E2F2 are included in the supplementary section (Table 5.7, 5.8, 5.9, 5.10).

This results might indicate that FOXM1 could be a sole regulator of TYMS and TK-1. Transient over expression of FOXM1 did not change E2Fs expression but transient knock down of FOXM1 shows slight changes in mRNA expression of E2F transcription activators could indicate the direct regulation of FOXM1 on E2F transcription activators.

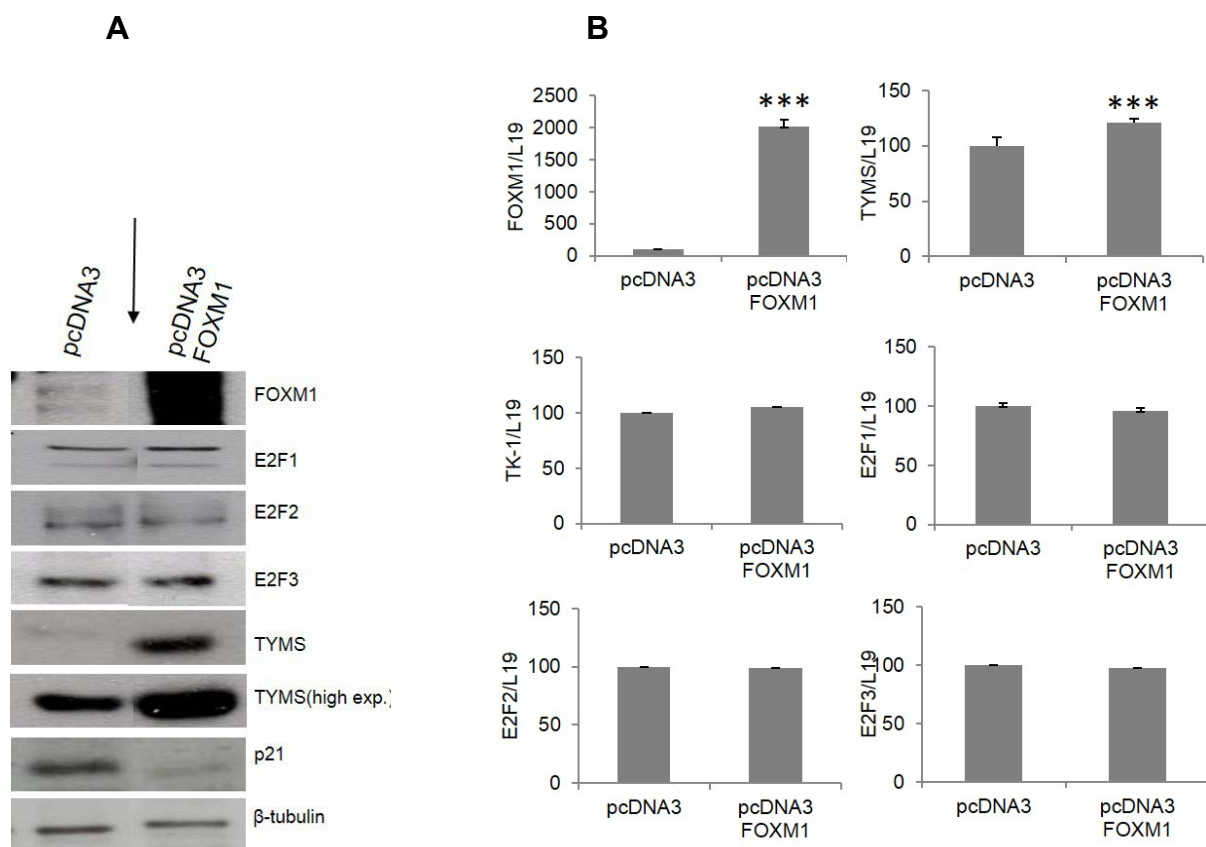


Figure 3. 4 HCT116 cells transfected with pcDNA3 empty vector and pcDNA3 FOXM1 plasmid.

All transfections were optimised and mediated by X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol. After 48h of transfection, cells were harvested and analysed by western blot (A) and RT-PCR (B), β -tubulin was used as a loading control. I have used spliced western blot figures. The arrow indicate spliced region of the gel. A). Overexpression of FOXM1 increases TYMS protein expression and decreases p21^{Cip} expression but E2Fs expression remains unchanged. B) Total RNA was extracted using the RNeasy Mini Kit. Real-time PCR (RT PCR) was used to determine FOXM1, E2F1, E2F2, E2F3, TYMS, and TK-1 mRNA expression. A housekeeping gene L19 was used as an internal control to normalise input complementary DNA. TYMS mRNA expression found

higher compared to control pcDNA3 (p value <0.05). Statistical significance was determined by a student's T-test. Error Bars represent standard deviation.

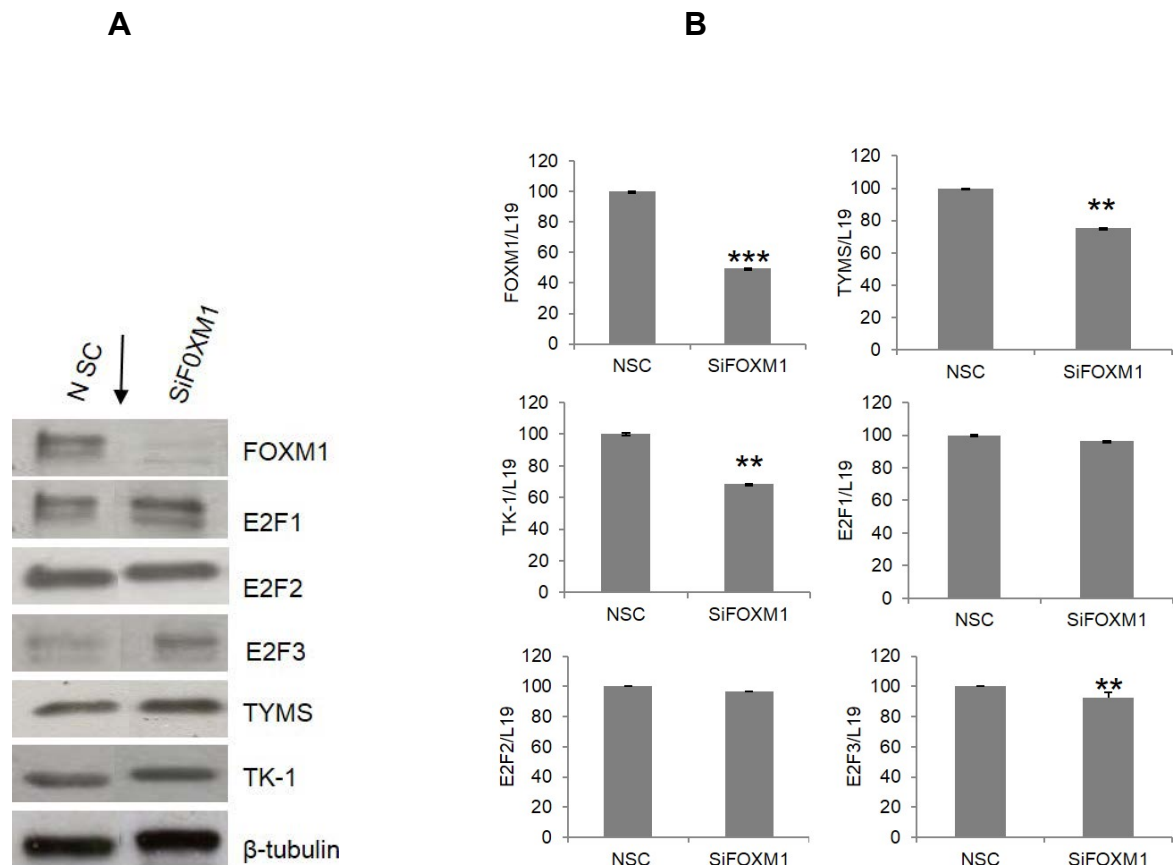


Figure 3. 5 HCT116 cells transfected with SiRNA mediated silencing of FOXM1.

All transfections were optimised and mediated by oligofectamine following the manufacturer's protocol. After 48 hours of transfection, cells were harvested and analysed by western blot and RT-PCR to detect FOXM1, E2F1, E2F2, E2F3, TYMS and TK-1. A). Western blot results shows no changes in the TYMS, TK-1, E2F1, or E2F2. There was a slight increase in E2F3 protein expression compared to NSC, β -tubulin was used as a loading control. B). Total RNA was extracted using the RNeasy Mini Kit. Real-time PCR (RT PCR) was used to determine mRNA expression. The housekeeping gene L19 was used as an internal control to normalise input complementary DNA. TYMS, TK-1, and E2F3 mRNA expression decrease following FOXM1 knock-down (p<0.05) Statistical significance was

determined by a student's T-test. FOXM1 knock-down shows a trend in decreasing E2F1 and E2F2 gene expression but no statistical significance observed.

3.1.6 Elevated FOXM1 and TYMS in 5-FU resistant HCT116 cells

My previous results indicate that over-expression of FOXM1 increases 5-FU resistance in colon cancer (figure 3.2) cell lines. My studies also indicate that FOXM1 could regulate TYMS directly.

HCT116 5-FU resistant cells were a gift from Dr Daniel Longley's laboratory, Queen's University, Belfast. The cells were maintained in 2 μ M 5-FU to maintain their resistant properties each time they were split. Before starting the experiment the cells were maintained in the medium for 1 week without drug and SRB assays were carried out to analyse cell viability of HCT116 5-FU Res cells compared to HCT116 wt cells. HCT116 5-FU Res cells were 10 fold less sensitive than HCT116 wt cells (Figure 3.6 A), HCT116 wt mean IC₅₀, 0.54 μ g/ml $\pm$ SD 0.03, n=2 and HCT116 5-FU Res mean IC₅₀, 5.33 μ g/ml $\pm$ SD 0.30, n=3.

Subsequent western blot analysis revealed that HCT116 5-FU Res cells expressed a higher basal level of FOXM1 and that the expression increased following 1 μ g/ml of 5-FU treatment (0-72h). Furthermore, HCT116 5-FU Res cells also show high basal TYMS protein expression that is persistent following 5-FU treatment (Figure 3.6 B, C). However, HCT116 wt cells treated with 5-FU (0-72h) showed initial induction followed by down regulation of FOXM1 and TYMS. A top band (shift in TYMS protein) was observed after 5-FU treatment and, as stated earlier in 3.1.4, is due to the active metabolite of 5-FU, FdUMP, forming a ternary complex with TYMS and its reduced-folate cofactor (de la Cueva, Ramírez de Molina et al. 2013). FOXM1 and

TYMS protein were quantified using ImageJ software (figure 3.6 C). These results also suggest that FOXM1 has a role in 5-FU sensitivity and resistance in colorectal cancer in vitro and that FOXM1 is a key regulator of TYMS.

A

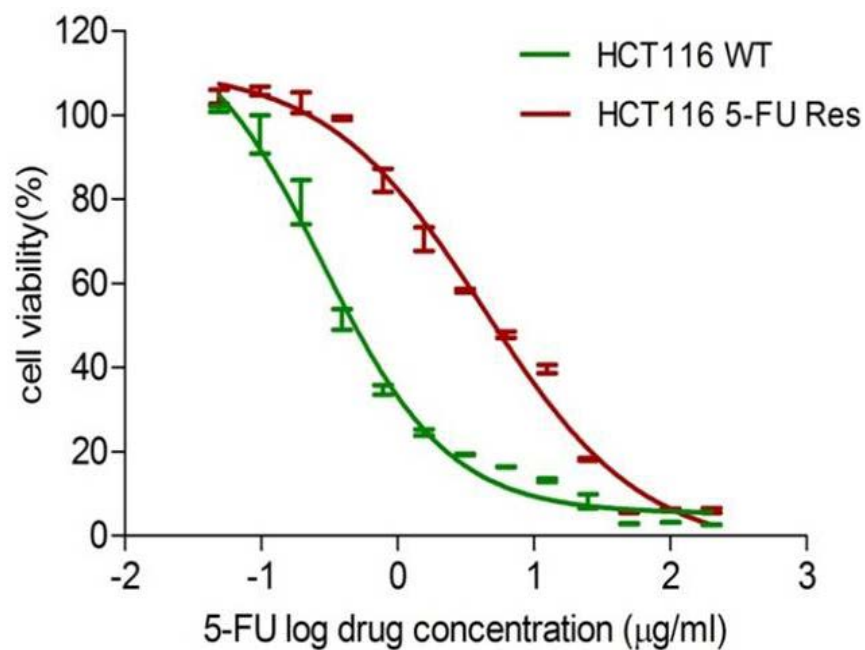


Figure 3. 6 HCT116 5-FU Res cells have elevated FOXM1 and TYMS protein levels.

A. HCT116 wt and HCT116 5-FU Res cells were treated with increasing concentrations of 5-FU and their rates of proliferation measured by SRB assay. HCT116, (mean IC₅₀, 0.54 µg/ml ± SD 0.03, n=2) and HCT116 5-FU Res cells (mean IC₅₀, 5.33 µg/ml ± SD 0.30, n=3).

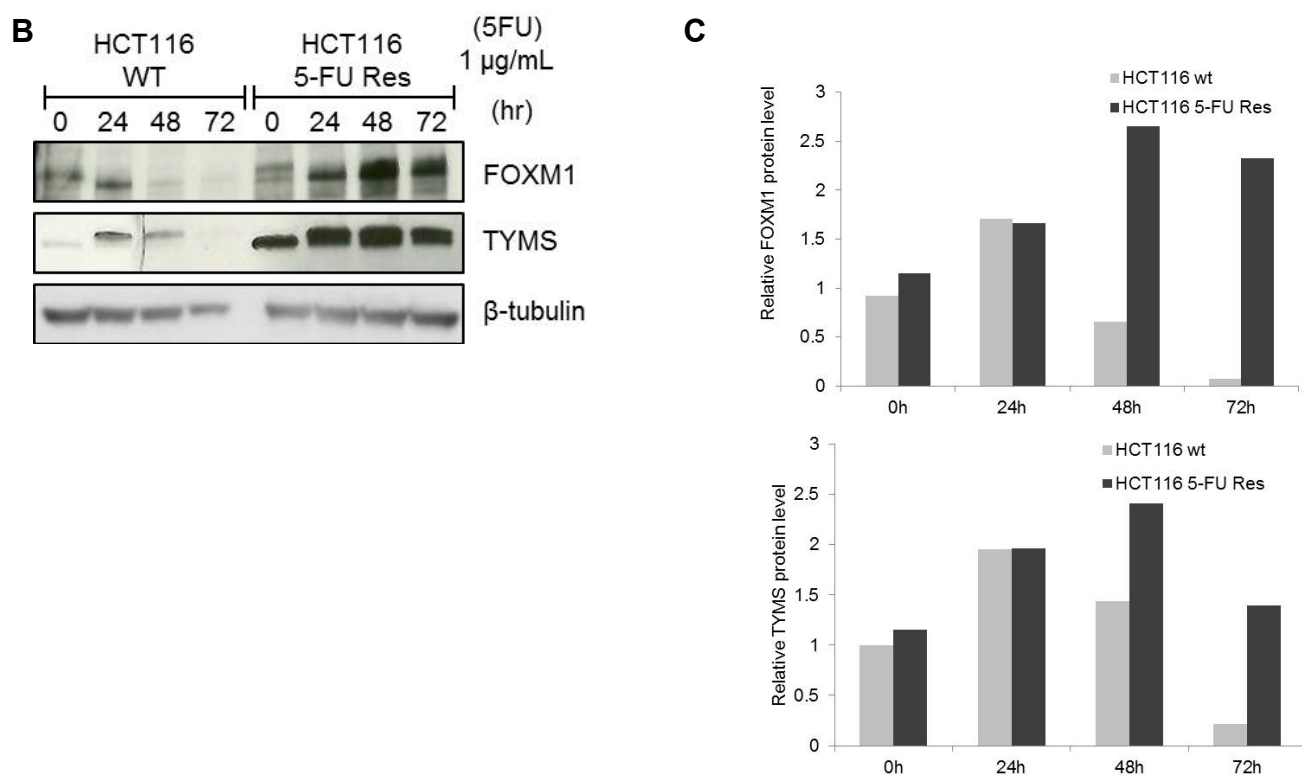


Figure 3.6 B. Western blot analysis determining the relative protein expression levels of FOXM1 and TYMS in HCT116 wt and HCT116 5-FU Res. C. FOXM1 protein expression level was quantified using ImageJ normalized to β -tubulin levels. Statistical significance was determined by student T-test using graph pad prism. In HCT116 wt significant decrease observed in FOXM1, TYMS (P value<0.0001 0h vs 72h). In HCT116 5-FU Res cells significant up-regulation of FOXM1, TYMS (P value<0.0001 0h vs 72h).

3.1.7 γ H2AX and cell cycle analysis after 5-FU treatment

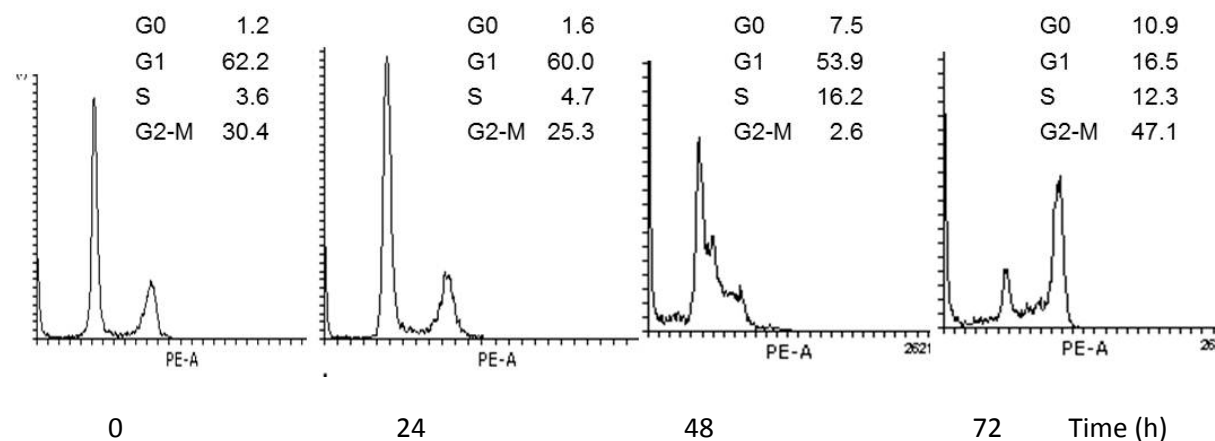
Having identified that over-expressed FOXM1 is associated with 5-FU drug resistance in colon cancer cells, I next sought to carry out cell cycle analysis and γ H2AX staining to identify cell cycle distribution and cell death in HCT116 wt and FOXM1 over expressed HCT116 5-FU Res cells (figure 3.7, 3.8).

A study has shown that HCT116 cells exposed to 5-FU are targeted to two different pathways which are dose dependent, G1-S phase arrest and apoptosis reported at 10ng/ml of 5-FU, or G2-M arrest and mitotic catastrophe at 100ng/ml (Yoshikawa, Kusunoki et al. 2001). Concordant with that idea, my FACS data finds that HCT116 wt cells (figure 3.7 A) treated with 5-FU (1 μ g/ml) for 48h show accumulation of cells at 48h in G0, G1-S phase and reduction of cells in G2-M phase. At 72 hours, G0 and G2-M accumulation indicate cell death, whilst as expected at this concentration (1 μ g/ml) no significant changes were observed in HCT116 5-FU Res cells treated with 5-FU (figure 3.7 B).

I next performed immunostaining of phosphorylated histone H2AX loci to assay for DNA damage in response to 5-FU in HCT116 cells (figure 3.8). γ H2AX staining was examined to detect cell death as a result of DNA damage induced by 5-FU. γ H2AX staining demonstrated that HCT116 wt cells had significantly higher levels of DNA damage after 5-FU treatment compared to HCT116 5-FU Res cells, indicating that HCT116 5-FU Res are more efficient than HCT116 wt cells in the repair of damaged DNA induced by 5-FU, which correlates with a much lower amount of apoptosis. These results might also indicate that higher levels of FOXM1 protect HCT116 5-FU Res cells from DNA damage and apoptosis.

A

HCT116 WT

**B**

HCT116 5-FU Res

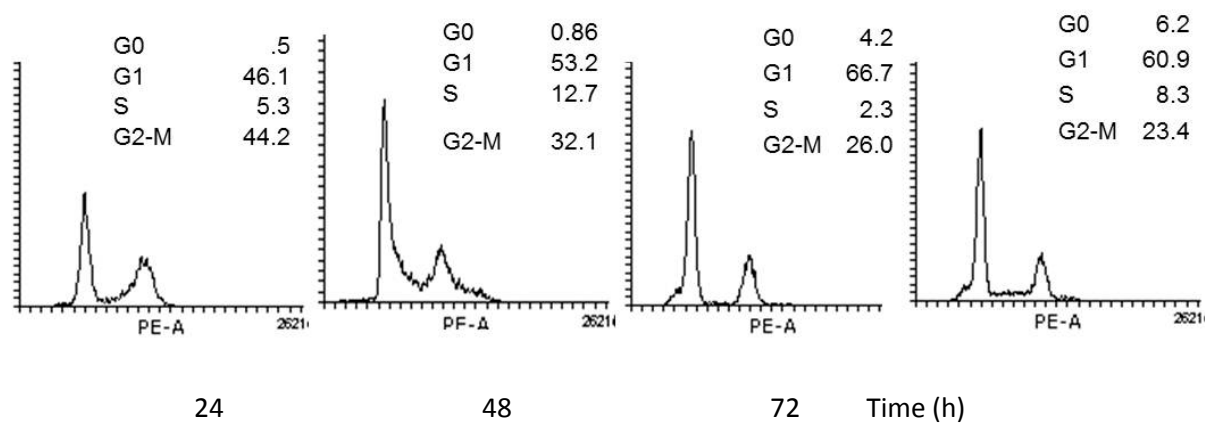
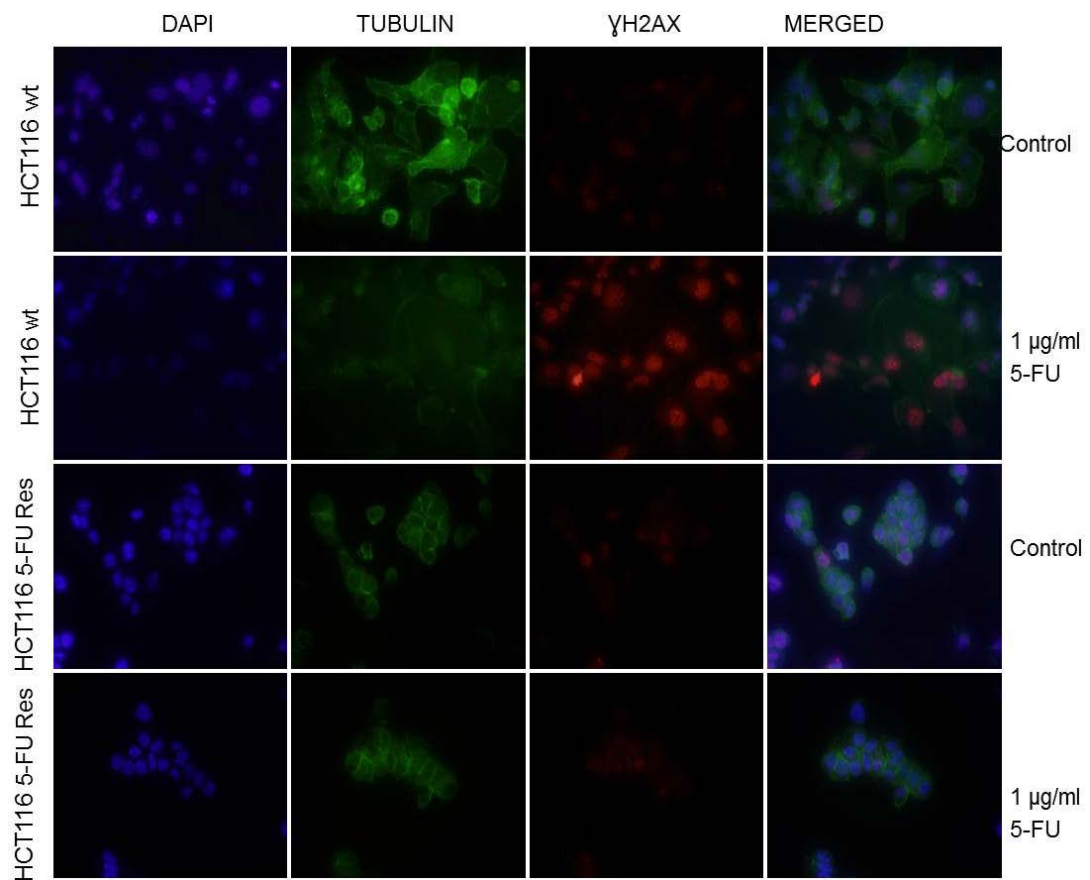


Figure 3. 7 Cell cycle analysis in 5-FU treated HCT116 wt and HCT116 5-FU Res cells.

Cells were treated with 1µg/ml of 5-FU for 72h, fixed with 70% ethanol and stained with PI (propidium iodide) and cell cycle phase distribution was analysed by flow cytometry. The percentage of the cells in each phase of the cycle is indicated (G0 (sub G1), G1, S and G2-M). Data analysis was performed using flowing software.

A



B

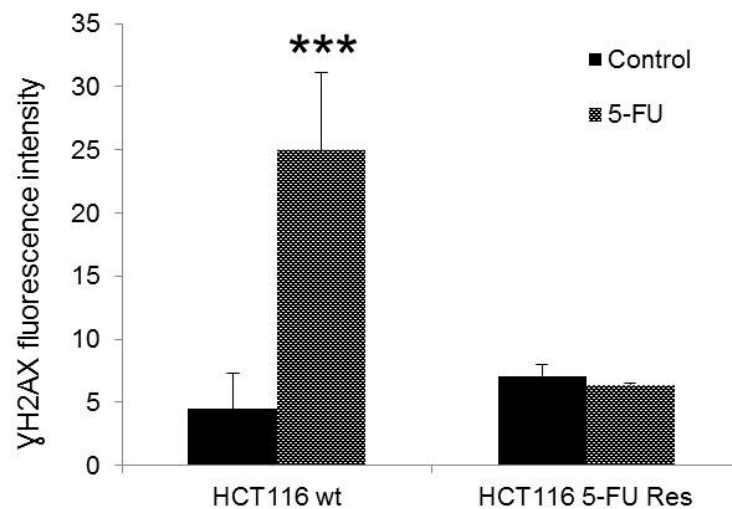


Figure 3. 8 γH2AX staining in 5-FU treated HCT116 wt and HCT116 5-FU Res cells.

Cells were treated with 1μg/ml of 5-FU for 0h and 24h and stained with γH2AX antibodies and DAPI. Images were visualized by confocal microscopy and the average integrated fluorescence intensity quantified by ImageJ software. Images: original magnification X 40. A significant amount of γH2AX staining observed in HCT116 wt. B Average integrated fluorescence intensity $p < 0.0001$.

3.1.8 FOXM1: A key regulator of TYMS

Next, I performed a ChIP assay (chromatin immuno precipitation) to detect if FOXM1 can directly bind to the TYMS promoter region in HCT116, DLD1 and HCT116 5-FU Res cell lines.

Evaluation of the TYMS promoter sequence revealed FOXM1 consensus sites (TAAACA) found between position -1053 and -894 and the 5'- flanking region of TYMS, details are included in the supplementary section (figure 5.6).

Six TYMS primers from 0 to 2000 base pair (bp) upstream and 3 negative primers from 2000bp to 4000 bp upstream of TYMS transcription start site were designed.

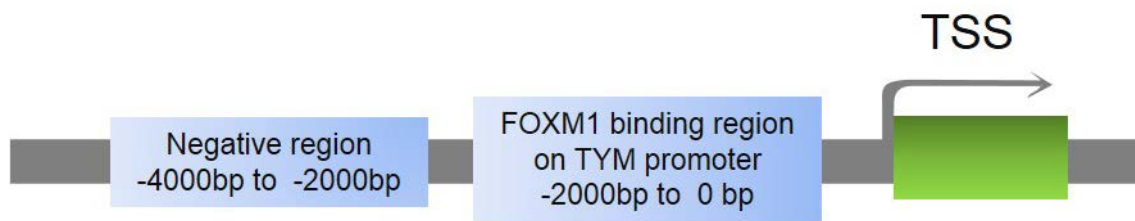
Among the six 0- 2000 bp upstream TYMS primers, I have chosen 2 sets of primers from the same region (0-500 bp and 1500-2000 bp) for fine mapping of TYMS in FOXM1 promoter region.

FOXM1 binding was analysed by RT-PCR, and normalised to the input sample. Data were presented as a fold enrichment relative to negative primers that were designed from 2000-4000 bp length. Enrichment of FOXM1 binding was observed in 0-2000 bp upstream of the TYMS promoter region in HCT116 wt (figure 3.9 B), and DLD1 cell lines (figure 3.9 C) supporting that FOXM1 can directly regulate TYMS. No enrichment was observed in the negative control (2000-4000 bp). Interestingly, expression of FOXM1 in the TYMS promoter region was significantly higher in acquired 5-FU resistant HCT116 cells than HCT116 wt and DLD1 cells (figure 3.9 D).

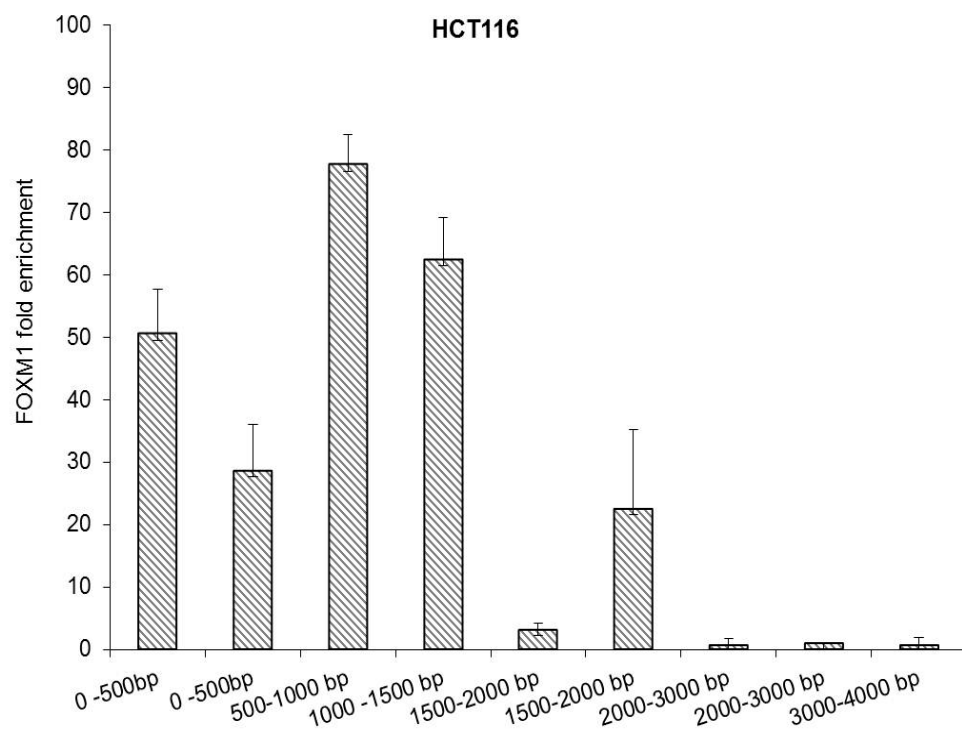
This experiment confirms direct regulation of TYMS by FOXM1, and that FOXM1 is an important mediator of 5-FU drug resistance acting through TYMS.

A schematic representation of the primers that were designed and the FOXM1 binding sites on the TYMS transcription start site (TSS) is shown in figure 3.9 A.

A.



B.



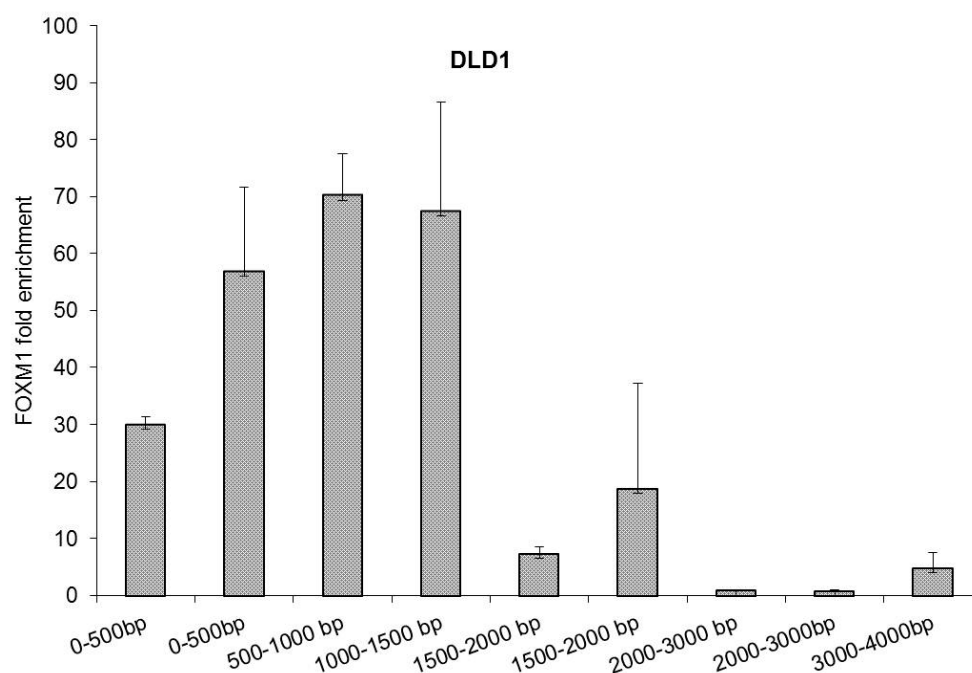
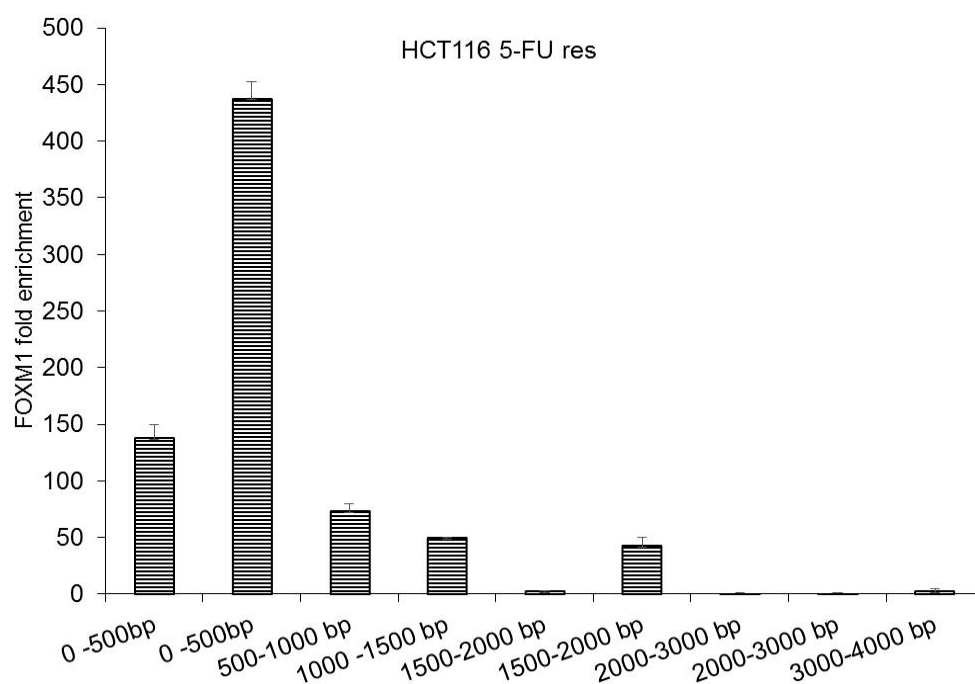
C**D**

Figure 3. 9 FOXM1 is a key regulator of TYMS.

ChIP assay for FOXM1 binding on TYMS promoter was done on B. HCT116, C. DLD1 and D. HCT116 5-FU Res cell lines. FOXM1 binding in the TYMS promoter region was analysed by RT-PCR. Enrichment of FOXM1 in the TYMS promoter was observed (0-2000 base pair upstream) and no enrichment observed in the negative control. (A) Schematic representation of FOXM1 binding sites in 0 to 2000 base pair upstream of TYMS transcription start site (TSS). All the experiments were done in triplicate and the data shown here is the average of those results.

3.1.9 Genome wide distribution of FOXM1 binding in colon cancer cells

To gain further insight in to FOXM1 binding on other possible targets, we performed ChIP-seq analysis of HCT116 and DLD1 cells.

Single-end 50bp long reads were obtained on a HiSeq 2000 instrument (Illumina). The MACS peak-calling algorithm was used to call significantly enriched peaks using default settings ($P < 10^{-5}$). In order to visualize the raw profiles on the UCSC Genome Browser, wiggle files were generated with MACS v1.4 and converted to bigWig. Overall number of peaks identified in HCT116 input is 1848872 and HCT116 chromatin is 7636560. Overall number of peaks identified in DLD1 input is 8640633 and DLD1 chromatin is 44773456.

Total number of shared FOXM1 reads between HCT116 and DLD1 was determined using web-based CEAS software of the Cistrome/Galaxy platform. The venn-diagram shows intersect of FOXM1 peaks in HCT116 and DLD1 (Figure 3.10 A). The number of reads, observed in HCT116 is 26962, but in DLD1 few number of reads were observed, 2124. Total number of shared reads observed between HCT116 and DLD1 was 1525. I chose this common FOXM1 peaks and analysed gene ontology. Gene Ontology (GO) was determined by utilizing web-based GREAT (Genomic Regions Enrichment of Annotations Tool) software (Figure 3.10 B, C). Graphs displaying statistics about the association of input genomic regions to the TSS of all the genes putatively regulated by the genomic regions. Among them 1475 genomic regions shows the association with one or more genes and 66 genomic region shows no association with any genes. As per my data the most significantly enriched GO term were associated with gene transcription regulatory region

sequence-specific DNA binding and neural precursor cell proliferation. Unfortunately no pathways were observed from the HCT116, DLD1 shared peaks. This might be some enrichment might in DLD1 input sample. Further to this I have analysed gene ontology in HCT116 and DLD1 individually.

In HCT116 cells (Figure 3.11 A) 27673 genomic regions shows the association with one or more genes and 814 genomic region shows no association with any genes. The most significantly enriched GO terms and pathway analysis are included in figure 3.11 C, D. In DLD1 cells (Figure 3.12 A). 3546 genomic regions shows the association with one or more genes and 104 genomic region shows no association with any genes. The most significantly enriched GO terms and pathway analysis are included in figure 3.12 C, but no pathway analysis is identified in DLD1.

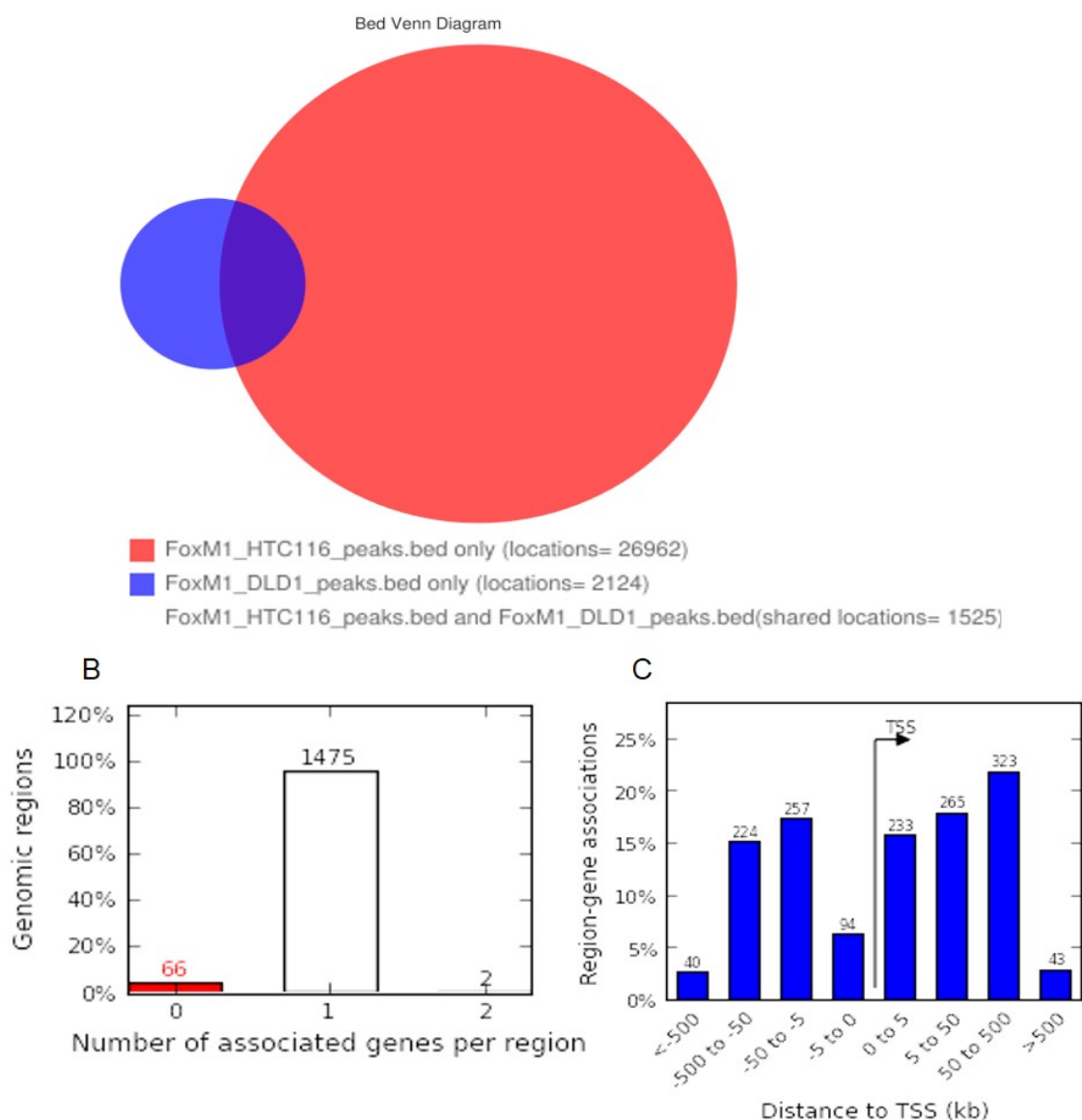


Figure 3. 10 Shared FOXM1 peaks between HCT116 and DLD1 cells.

Venn diagram representing the shared FOXM1 peaks between HCT116 and DLD1 cells. B. Graphs displaying statistics about the association of input genomic regions to the TSS of all the genes putatively regulated by the genomic regions. The 'Number of associated genes per region' graph shows how many genes each genomic region is assigned as putatively regulating based on the association rule used. C. The distance to TSS graphs show the distance between input regions and their putatively regulated genes. The distances are divided into four separate bins: one from 0 to 5 kb, another from 5 kb to 50 kb, a third from 50 kb to 500 kb, and a final bin of all associations over 500Kb.

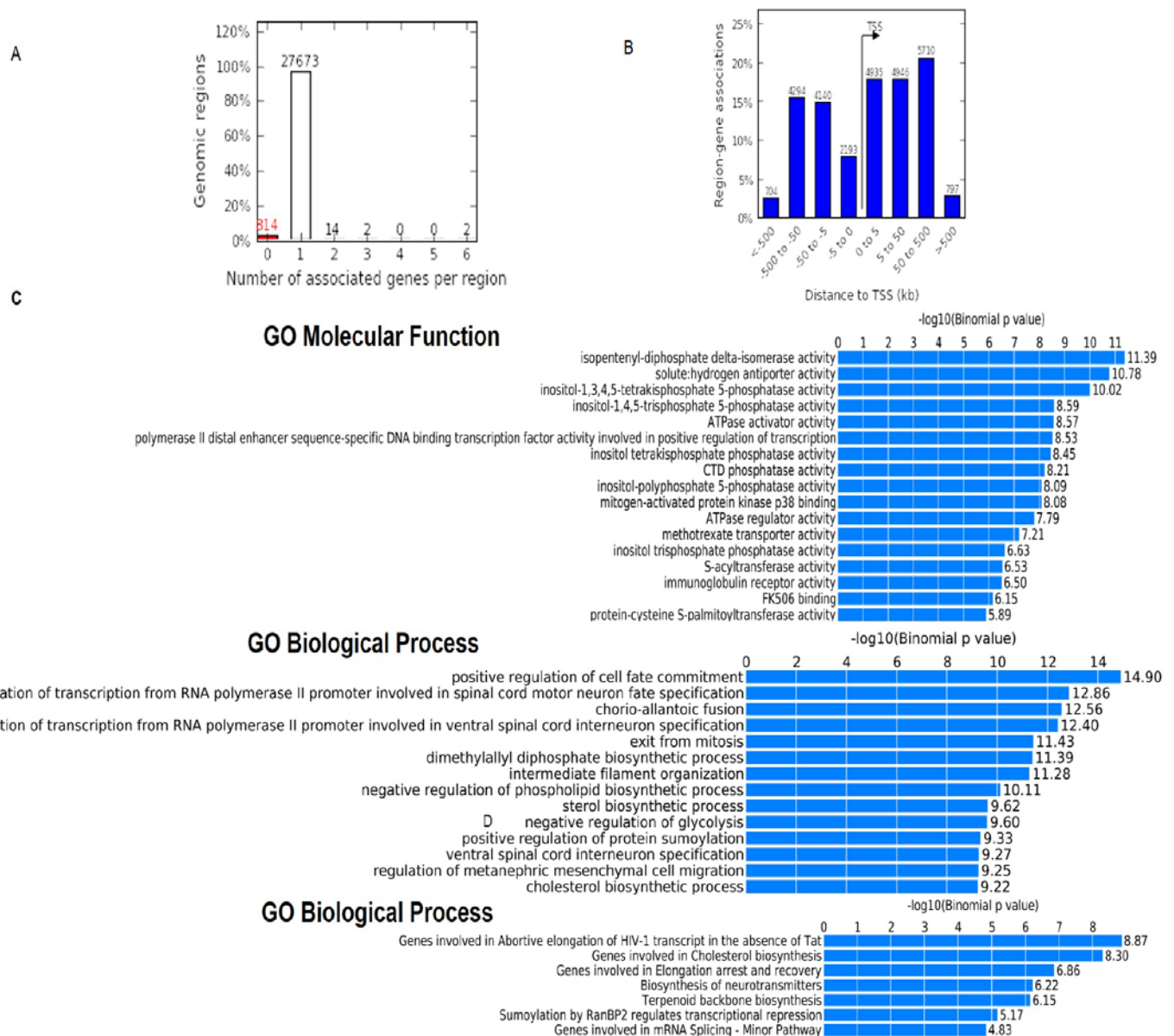


Figure 3. 11 FOXM1 binding in HCT116 cells.

A The 'Number of associated genes per region' graph shows how many genes each genomic region is assigned as putatively regulating based on the association rule used. B. The distance to TSS graphs show the distance between input regions and their putatively regulated genes. The distances are divided into four separate bins: one from 0 to 5 kb, another from 5 -50 kb, a third from 50 kb to 500 kb, and a final bin of all associations over 500 kb. C, D. The top gene ontology terms in genes and pathways associated with FOXM1 binding regions in HCT116.

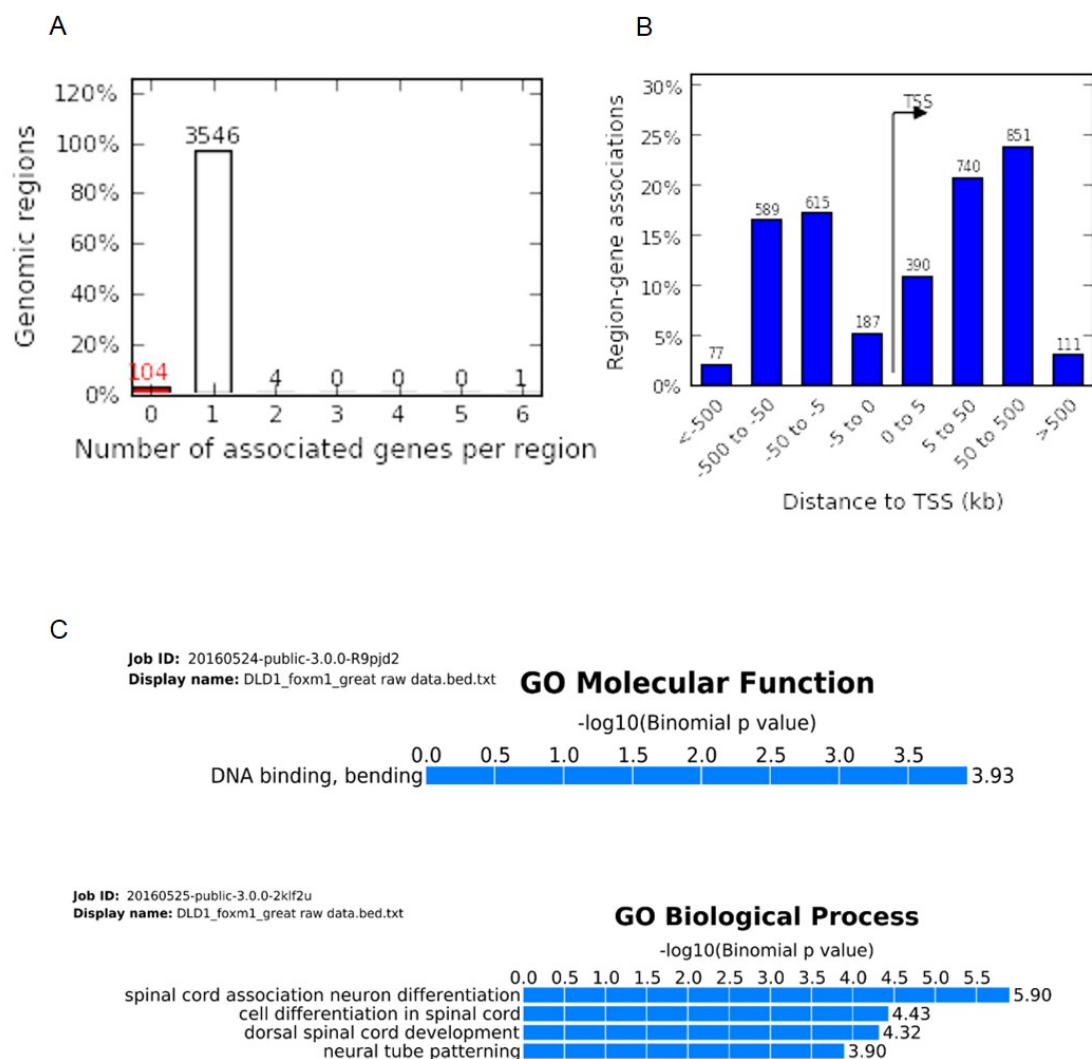


Figure 3. 12 FOXM1 binding in DLD1 cells.

A The 'Number of associated genes per region' graph shows how many genes each genomic region is assigned as putatively regulating based on the association rule used. B. The distance to TSS graphs show the distance between input regions and their putatively regulated genes. The distances are divided into four separate bins: one from 0 to 5 kb, another from 5 -50 kb, a third from 50 kb to 500 kb, and a final bin of all associations over 500 kb. C. The top gene ontology terms in genes and pathways associated with FOXM1 binding regions in DLD1.

The distribution of FOXM1 ChIP-seq regions in HCT116 (bottom) compared to the total genomic DNA distribution (top) is shown (figure 3.13). The bottom panel shows a detailed representation of FOXM1 binding 3kb upstream from the promoter region (figure 3.13). 12.4% of binding observed in 3kb region and 4% binding observed in 5'UTR, these are the places where most of the TSS present and 37% of introns identified and this is the region where mainly enhancers present. The FOXM1 ChIP-seq showed 12.6% FOXM1 binding in 0 to 3kb upstream of the promoter region compared to the total genome (2.5%). The distance to TSS graphs show the number of putatively FOXM1 regulated genes, with distances divided into four separate groups, one from 0 to 5 kb, another from 5 kb to 50 kb, a third from 50 kb to 500 kb, and the final is associations over 500 kb (figure 3.14 A, B). A heat map showing (figure 3.15) FOXM1 binding events in HCT116 and DLD1 cells. Next, I analysed DNA binding motifs using MEME. Three main motifs were identified (supplementary section Figure 5.5), matches to these motifs were found in >60%, and the motifs are located very close to the FOXM1 binding peak. List of few genes associated with this motifs are, CPEB1, FOXJ3, FOXO1, HNF6, FOXL1, FUP1, and BRAC. But no FOXM1 is identified in the list.

Next, the Integrative Genomics Viewer (IGV) was used to visualise the binding of FOXM1 to other potential targets of 5-FU (figure 3.16). Blue peaks represent FOXM1 enrichment in 5-FU targets: TYMS, TYMP, and TK-1. E2F transcription activators: E2F1, E2F2, E2F3. Cell cycle regulatory genes: Cyclins B1, Cyclins D1, CDKN1A (p21^{Cip}), CDKN1C (p57). Mismatch repair gene: MLH1, DNA damage repair gene: RAD51, RAD54, XRCC1, BRCA2 and matrix metalloproteinase (involved in progression and metastasis): MMP9, MMP2 in HCT116 and DLD1 cells. Input track is not included because input is subtracted from the data before running on IGV.

FOX M1 binding was detected in the promoter regions of two other 5-FU targets: thymidine phosphorylase (TYMP) and thymidine kinase 1 (TK-1), confirming that FOX M1 has a central role in the control of thymidylate biosynthesis. The E2F transcription activators are also found to be regulated by FOX M1. In addition, Cyclin B1, Cyclin D1, CDKN1A (p21^{Cip}), and DNA damage repair genes, RAD51, RAD54, XRCC1, BRCA2 and matrix metalloproteinases (involved in progression and metastasis) MMP9 and MMP2 were found to be regulated by FOX M1 in our ChIP-seq data.

To confirm the IGV ChIP-seq data, expression of these genes in HCT116 and DLD1 were validated by RT-PCR analysis (figure 3.17). The results confirm significant enrichment in 5-FU targets such as TK-1 and TYMP, and enrichment was also observed in E2F1, E2F2, MMP2, and MLH1 (mismatch repair gene) (figure 3.17).

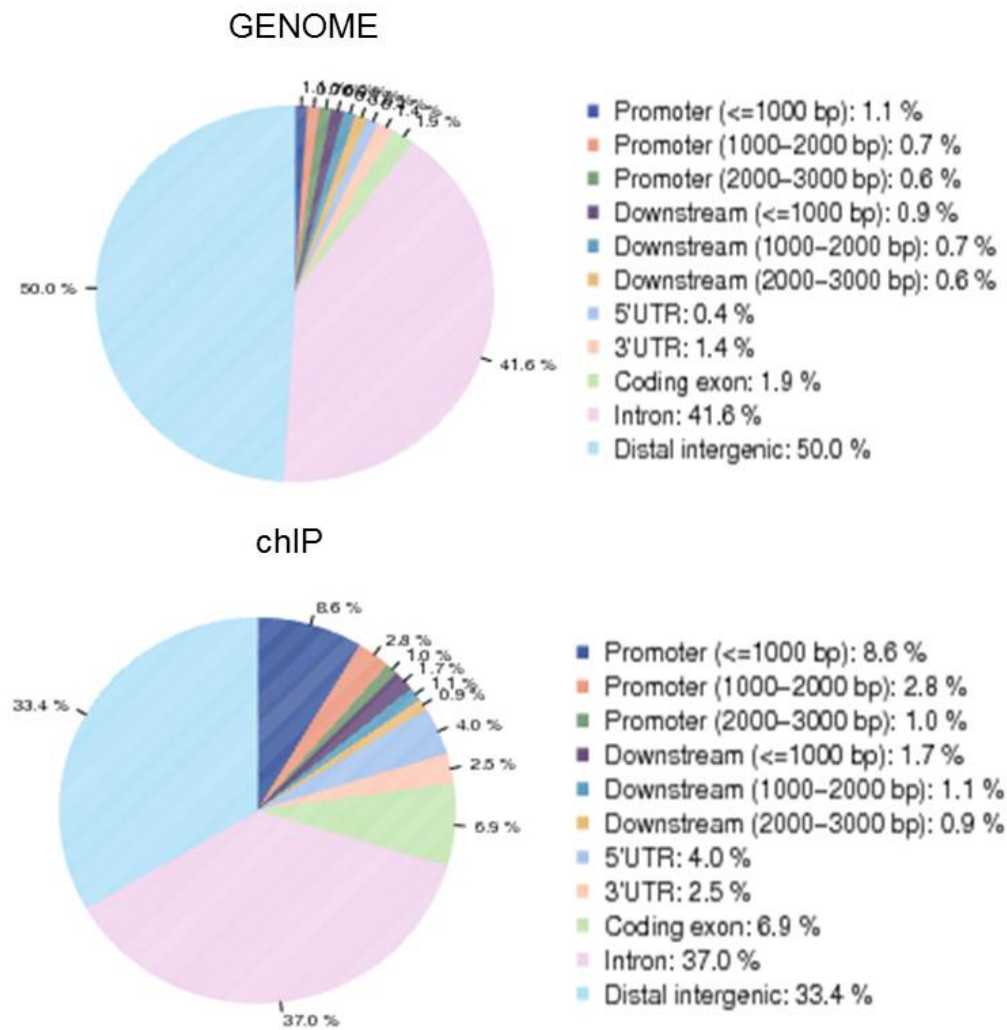
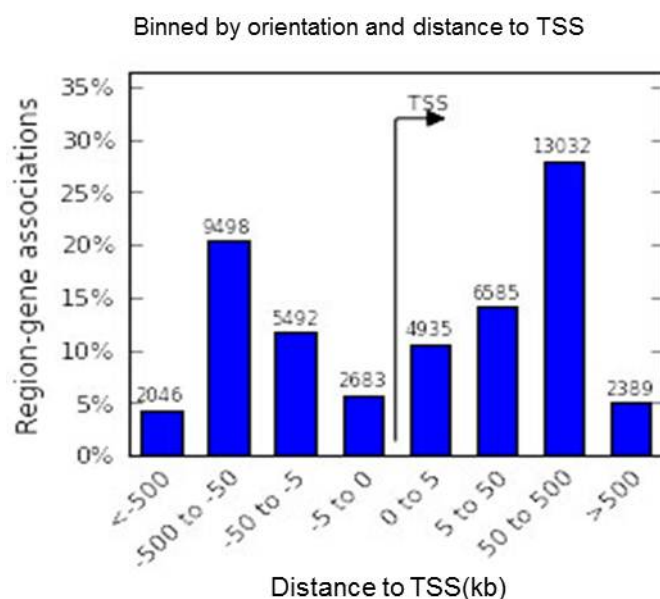


Figure 3. 13 CEAS analysis showing genomic distribution of FOXM1 binding events in HCT116 cells.

Distribution of FOXM1 ChIP-seq regions (bottom) compared to the total genomic DNA distribution (top).

A



B

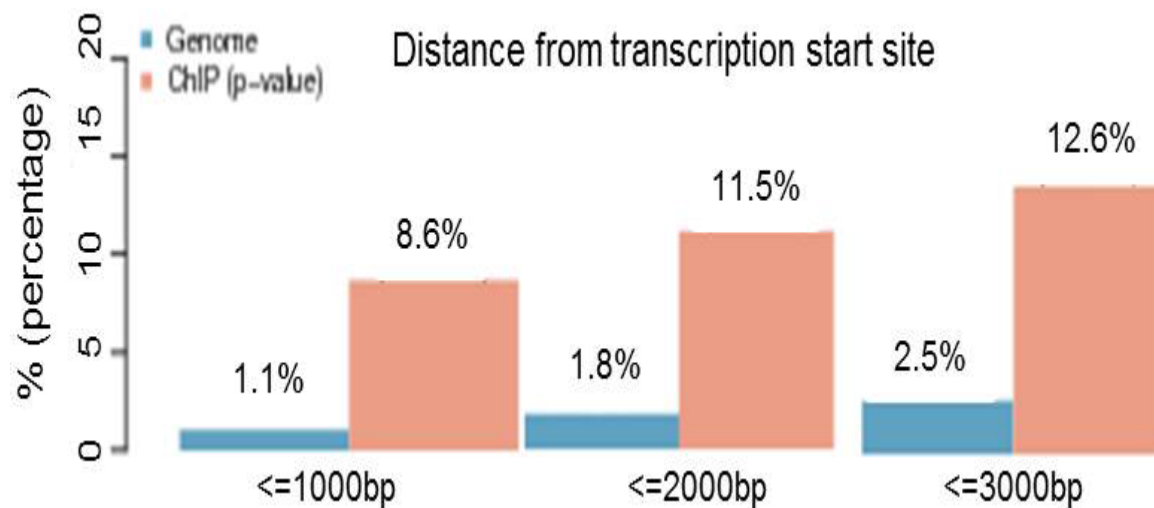


Figure 3. 14 A. The distance to TSS graphs show the gap in base pairs between input regions and their putatively regulated genes, divided into four separate groups 0-5kb, 5-50kb, 50-500kb, and >50kb. Figure B. The bottom panel shows genomic distribution of FOXM1 binding events in 0 to 3000bp upstream in the promoter region of HCT116 cells.

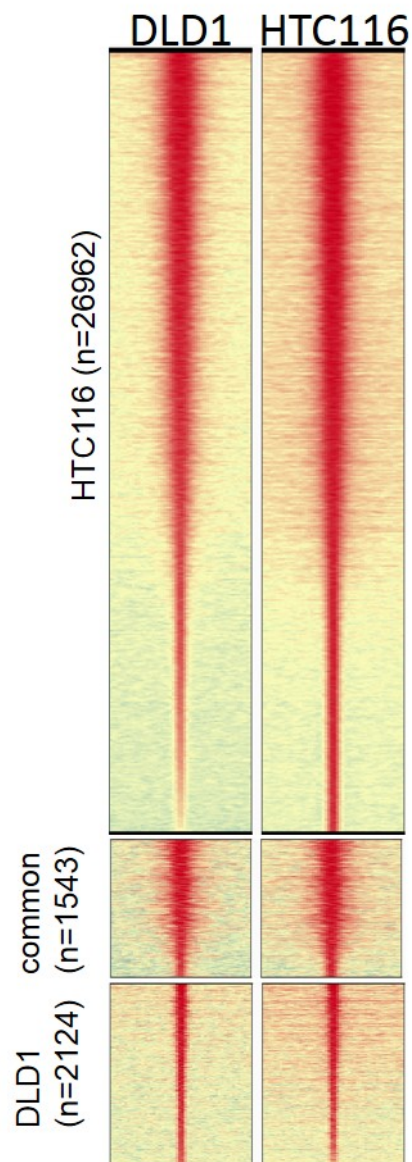


Figure 3. 15 Heat map showing FOXM1 binding events in HCT116 (right) and DLD1 (left) and HCT116 DLD1 shared reads.

Heatmaps were generated using ChAsE (Chromatin Analysis and Exploration) platform. Settings (peak extensions 5 kb upstream and 5 kb downstream of the peak summit and bin size 50 bp).

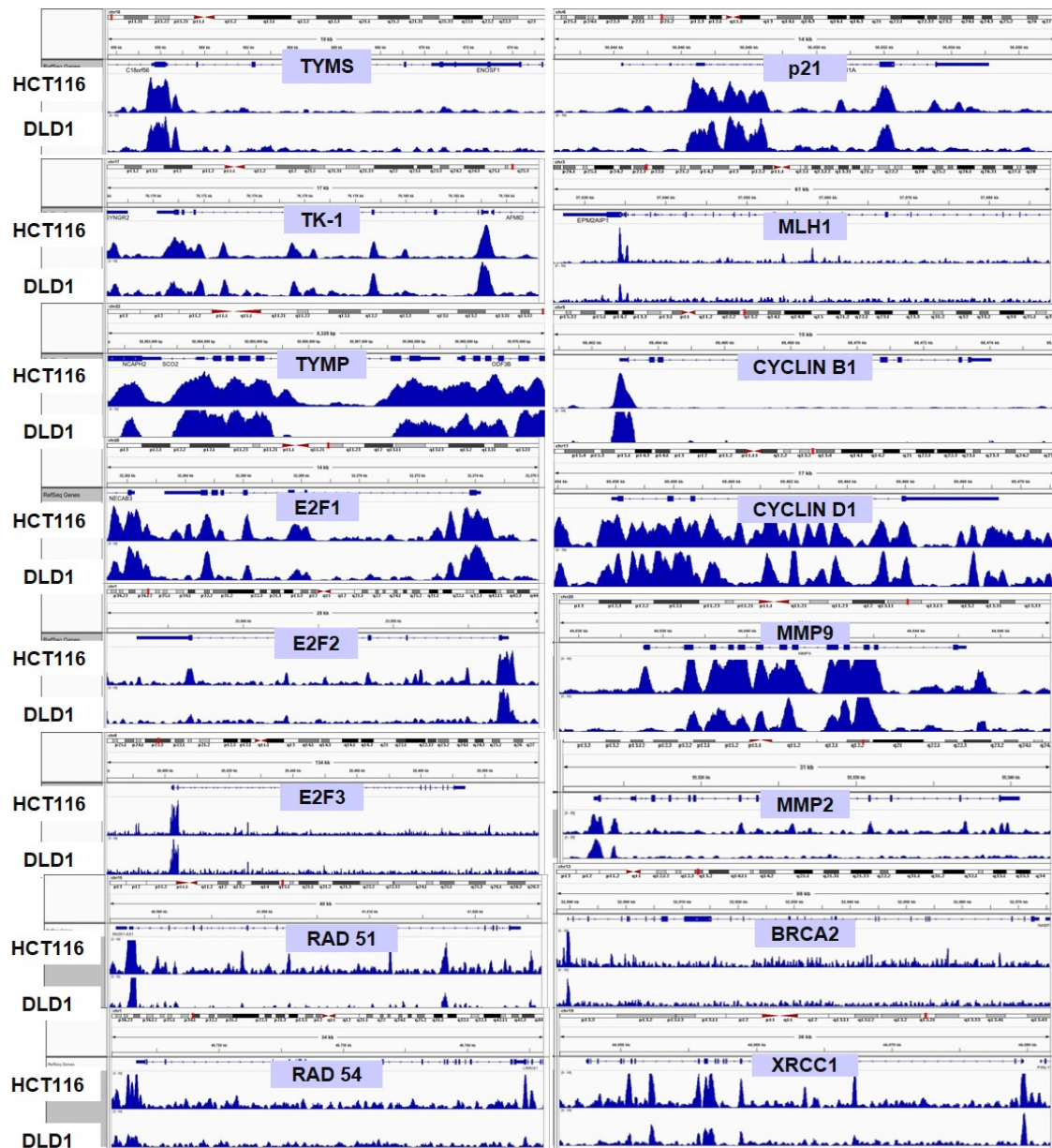
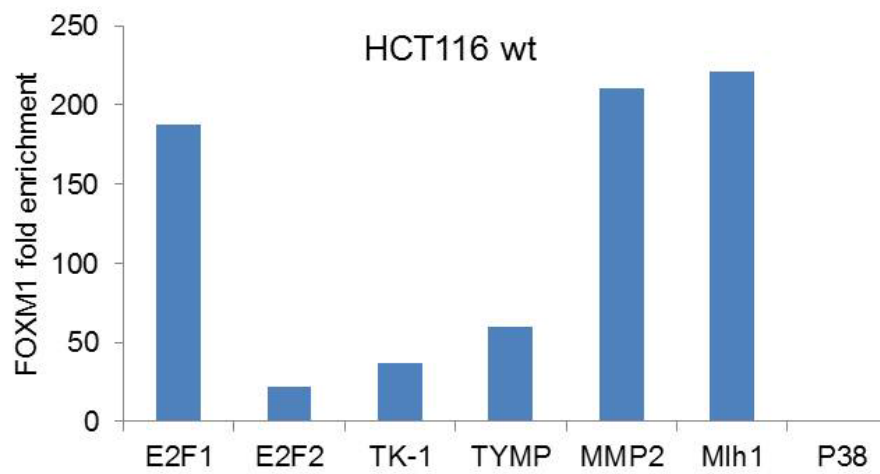


Figure 3. 16 FOXM1 binding in HCT116 and DLD1 cells.

The Integrative Genomics Viewer (IGV) browser was used to visualise the FOXM1 binding in down-stream targets. The input track has been subtracted from the data shown above. Blue peaks represent FOXM1 enrichment in 5-FU targets: TYMS, TYMP, and TK-1. E2F transcription activators: E2F1, E2F2, E2F3. Cell cycle regulatory genes: Cyclins B1, Cyclins D1, CDKN1A (p21^{Cip}), CDKN1C (p57). Mismatch repair gene: MLH1, DNA damage repair gene: RAD51, RAD54, XRCC1, BRCA2 and matrix metalloproteinase (involved in progression and metastasis): MMP9, MMP2 in HCT116 and DLD1 cells.

A



B

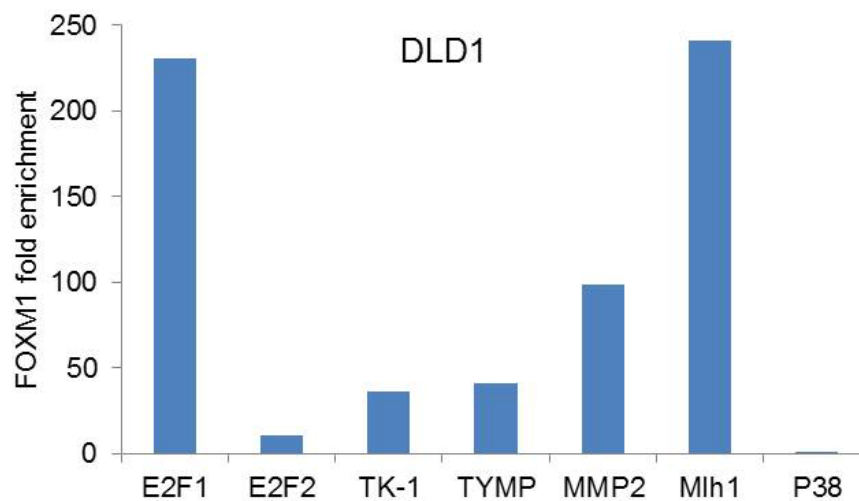


Figure 3. 17 RT-PCR to assess FOXM1 binding sites in HCT116 and DLD1.

To confirm the IGV ChIP-seq data of FOXM1 binding, RT-PCR was carried out on HCT116 (A) and DLD1 (B) cells. The result shows FOXM1 binding on TK-1, TYMP, E2F1, E2F2, MMP2, and MLH1. P38, which is not a FOXM1 regulator, does not show any FOXM1 enrichment.

3.1.10 TYMS and TK-1 expression and its direct association with FOXM1 in clinical samples

After establishing that TYMS and TK-1 are direct targets of FOXM1 in colorectal cancer cell lines, the correlation of FOXM1, TYMS and TK-1 expression were assayed by immunohistochemistry in a colorectal tumour tissue microarray of 111 colon cancer samples (figure 3. 18). Initially I optimised the conditions and antibody dilutions in human colorectal cancer tissue samples obtained from Charing Cross Hospital. Concentrations of 1:150 for FOXM1, and 1:100 for TYMS and TK-1 1:100 dilution were selected; this was supervised by Dr Francesco Mauri, consultant histopathologist.

We observed FOXM1 positive staining in both the cytoplasm and nuclei of the majority of cancer cells (>90%), indicating that FOXM1 is commonly overexpressed in human colon cancer. We further evaluated TYMS and TK-1 expression in the same cohort and observed strong TYMS positive staining in the cytoplasm of CRC cells. Furthermore, there was a significant correlation between FOXM1 and TYMS (Spearman $r=0.314$, $p=0.008$), the correlation was less strong for FOXM1 and TK-1 (Spearman $r=0.284$, $p=0.004$). These results provide the clinical evidence to support the relationship between FOXM1 and TYMS. Among the 111 samples (cores), one core is chosen from each slide (C8) as a representative image. A list of the 111 samples detailing the primary tumour characteristics is included in the supplemental section (Table 5.1).

The scoring was calculated based on percentage of positive cells and intensity of the staining in TYMS Cytoplasm, TK-1 cytoplasm and nucleus, FOXM1 cytoplasm and nucleus. I have only scored TYMS cytoplasm, based on previously published IHC

data and TYMS immunoreactivity was demonstrated mainly in the cytoplasm of cancer cells. The following scoring system was used: The staining intensity (0, 1, 2, and 3) and the fraction of positive tumour cells were recorded for each tissue spot. Negative scores had staining intensity of 0 (tissues missing or no staining), weak scores had percentage of positive cells in 10-40%, moderate score had percentage of positive cells in 40-70% and high score had percentage of positive cells in 70-100% of tumour cells. Two values were multiplied and calculated the score. Immunohistochemistry scoring is also included in the supplementary section (Table 5.12).

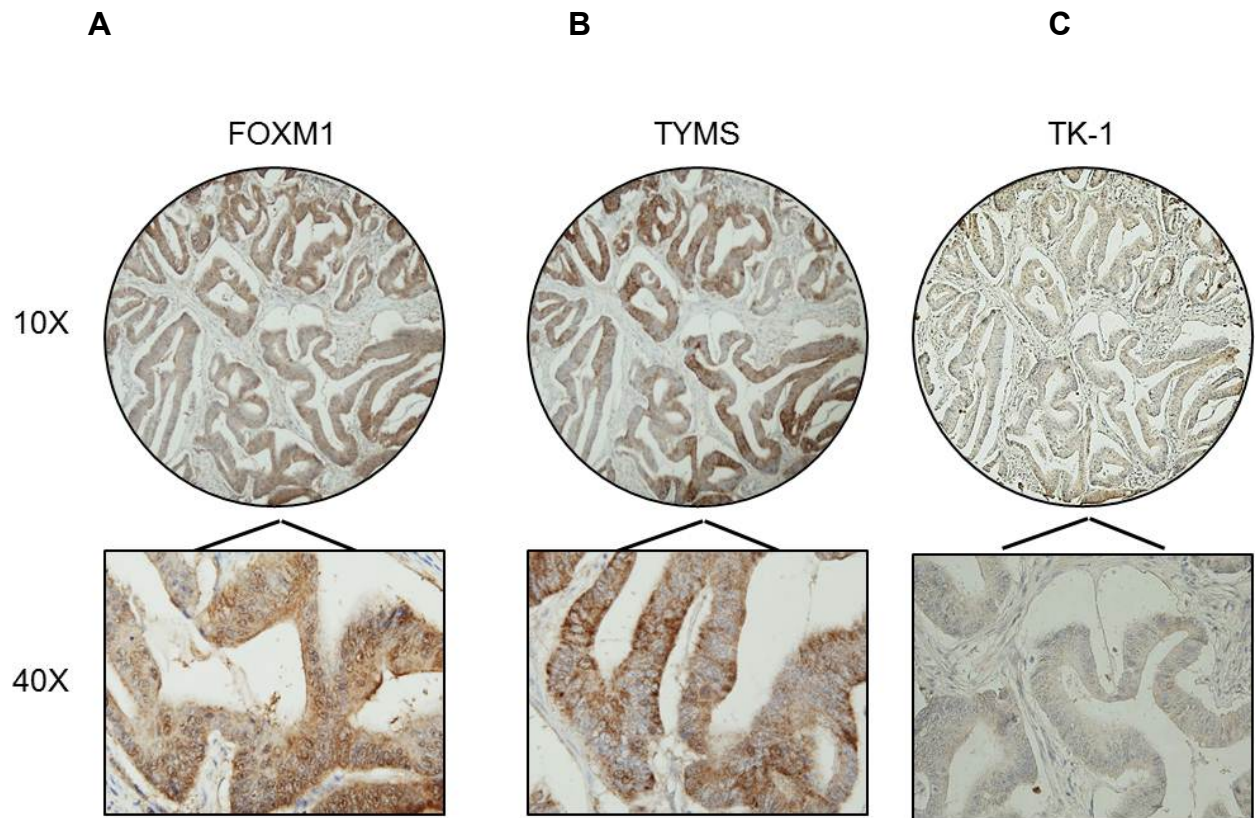


Figure 3. 18 Correlation between FOXM1, TK-1 and TYMS in CRC tissue

Representative photographs of: A. FOXM1, B. TYMS, and C. TK-1 staining in human CRC tissue showing close similarity in protein expression. Cytoplasmic FOXM1 expression levels were directly correlated with the TYMS expression in the TMA ($p=0.008$, $r=0.314$), TK-1 ($r=0.284$, $p=0.004$). The images above were taken from the C8 core: Colon Adenocarcinoma grade2, stage II, T4N0M0.

3.1.11 Discussion

My study confirms for the first time that higher levels FOXM1 are associated with 5-FU resistance in colon cancer. Furthermore, my studies also reveal that the 5-FU targets TYMS, TK-1, and TYMP are directly regulated by FOXM1.

Initially, I studied the cell viability of colon cancer cells (p53 wt HCT116, p53 mutant DLD1 and HT29) and observed that p53 wt cells are more sensitive to 5-FU. This is in agreement with previous studies that indicated the efficacy of 5-FU mainly depends upon p53 status and mutation or deletion of p53 can cause cells to become resistant to 5-FU; treatment with 5-FU leads to p53 stabilisation and G1/S arrest (Sun, Dai et al. 2007, Huang, Zhang et al. 2009).

According to my data, several observations suggest that FOXM1 expression is an important factor of 5-FU sensitivity and resistance. Firstly, SiRNA mediated FOXM1 knock-down in HCT116 cells led to higher 5-FU sensitivity compared to NSC control and likewise, overexpression of FOXM1 increased 5-FU resistance compared to pcDNA3 control cells. Screening of proteins, which might have potential roles in 5-FU resistance in CRC showed an initial induction followed by a decrease of FOXM1, TYMS, and E2F1 in p53 wt HCT116 cells compared to the p53 mutant cell lines where persistent up-regulation of FOXM1 was observed in both protein and mRNA. This is consistent with previous studies that demonstrated genotoxic drugs cause activation of DNA damage genes and stabilisation of p53. Stabilisation of p53 has been shown to decrease FOXM1 expression in p53 wt MCF7 cells (Barsotti and Prives 2009). DNA damage will result initial induction of FOXM1 expression followed by an eventual down-regulation, however in the absence of functional p53 up-

regulation of FOXM1 is observed during DNA damage (Pandit, Halasi et al. 2009, Millour, de Olano et al. 2011).

Along with protein, mRNA expression of FOXM1, E2F1 and TYMS are found to be down-regulated at 48h post 5-FU treatment in p53 wt HCT116 cells compared to p53 deficient cells. The expression levels of DNA damage response proteins including ATM and Chk2 were found to be up-regulated mainly in p53 mutant HT29 and DLD1 cells compared to p53 wt HCT116 cells, supporting a previous study that showed 5-FU treatment induces DNA damage and activation of ATM (Akasaka, Tsujii et al. 2015). In fact, a recent study showed that ATM is overexpressed in drug resistant cells compared to the sensitive cells and its expression is up-regulated in response to drug treatment. ATM is activated in response to drug treatment to enhance E2F1 activity and consequently FOXM1 expression to promote cell survival in drug resistant breast cancer cells (Millour, de Olano et al. 2011). My results could also indicate that p53 mutant cells can tolerate 5-FU mediated DNA damage through enhancing DNA damage repair genes and sustain proliferation due to high levels of ATM, Chk2, FOXM1 and E2F1.

Furthermore, in this study, I have demonstrated for the first time that FOXM1 is a key regulator of thymidylate metabolism, in particular TYMS, which confers resistance to the most commonly used anti-pyrimidine, 5-FU and that this occurs downstream of FOXM1. In my study, several lines of evidence confirmed the role of FOXM1 in TYMS regulation.

Initially, with 5-FU treatment the protein expression patterns of FOXM1, TYMS and E2F1, were found to be similar, potentially indicating a positive association between these genes. HCT116 cells transfected with pcDNA3 FOXM1 demonstrated increased mRNA and protein expression of TYMS, but no significant changes were

observed in other gene. Likewise, FOXM1 knock-down in HCT116 cells led to down-regulation of TYMS, TK-1, and E2F3 mRNA expression. These data could also indicate that TYMS and TK-1 are direct targets of the FOXM1 transcription factor.

HCT116 5-FU Res cells treated with 5-FU are 10 fold more resistant than HCT116 wt cells. During protein analysis, basal expression of FOXM1 and TYMS were high in HCT116 5-FU Res cells compared to HCT116 wt. Following 5-FU treatment, FOXM1 was ultimately down-regulated in the sensitive HCT116 cells, whereas in the 5-FU resistant HCT116 cells, persistent up-regulation of FOXM1 and TYMS protein expression was observed. This result suggests that elevated FOXM1 expression and concomitant TYMS is associated with 5-FU drug resistance, again demonstrating the crucial role of FOXM1 in 5-FU drug resistance and that the potential underlying mechanism is via modulation of TYMS.

Following 5-FU treatment, FACS analysis showed an increased number of sub G1 (G0) accumulation at 48 and 72h and γ H2AX staining in HCT116 wt cells, whereas no significant changes were observed in cell cycle analysis and γ H2AX staining in HCT116 5-FU Res cells. This is indicative of DNA fragmentation and cell death in HCT116 wt cells and an increase in DNA repair activities in FOXM1 over expressed HCT116 5-FU Res cells. This could be due to higher levels of FOXM1 promoting DNA repair mechanisms in HCT116 5-FU Res cells.

My ChIP seq data also confirms the direct binding of FOXM1 on DNA damage repair genes. This observation is also in accordance with previous study demonstrating that FOXM1 is directly regulates DNA damage repair genes (Park, Jung et al. 2012). Decreased FOXM1 levels are associated with increased DNA breaks, as evidenced by H2AX staining and cell cycle analysis (Tan, Raychaudhuri et al. 2007, Kwok, Peck et al. 2010, Zhang, Wu et al. 2012) and knock-down of FOXM1 sensitises

human cancer cells to DNA-damaging agents (Halasi and Gartel 2012). This result is also in an agreement with another study which shows high levels of ATM and FOXM1 protein and mRNA levels in MCF7 epirubicin cells compared with MCF7 wt cells suggesting a role of ATM in mediating the increase in DNA repair activity in the resistant cells (Millour, de Olano et al. 2011).

Moreover, the ChIP assay showed strong enrichment of FOXM1 in the TYMS promoter region in HCT116 and DLD1 cells. Interestingly, significantly highest FOXM1 binding was observed in the TYMS promoter of 5-FU resistant HCT116 cells, again confirming the significance of FOXM1 mediated 5-FU resistance through TYMS. Immunohistochemical staining further confirmed the clinical relevance between FOXM1 and TYMS, where expression was significantly correlated in human colorectal cancer samples.

The ChIP seq data confirms FOXM1 binding on E2F1 and E2F2 transcription activators. However, no significant binding was observed in E2F3. Our ChIP seq data also confirmed the direct regulation of FOXM1 on other 5-FU targets such as TK-1 and TYMP. Genes associated with DNA damage repair and cell cycle regulation were also showed enrichment on the Heat map.

In summary, I demonstrated that FOXM1 is a critical mediator of 5-FU sensitivity and resistance. Therefore, FOXM1 could be a potential biomarker for predicting and monitoring 5-FU response. FOXM1 directly regulates 5-FU targets, such as TYMS, TK-1 and TYMP in colon cancer, and hence 5-FU drug resistance. A strong correlation was observed between FOXM1 and TYMS in human colorectal tissue specimens. Even though E2F1 directly regulates FOXM1 and TYMS, my results could also indicate the regulation of FOXM1 on the E2F1 promoter and that targeting FOXM1 could possibly improve drug sensitivity, and more evidence is shown in the next chapters.

To conclude, in this study, I have demonstrated for the first time that FOXM1 possesses a crucial role in 5-FU drug resistance in colon cancer cells through the direct regulation of the main 5-FU target TYMS. FOXM1 also regulates TK-1 and TYMP, which are crucial enzymes for thymidylate biosynthesis. Therefore, FOXM1 could be a novel predictive biomarker in 5-FU therapy, and new treatment strategies that target FOXM1 should be developed to improve clinical response in CRC.

3.1.12 Future Work

My first step in continuing this work would be to look at the protein and mRNA expression of ATM, Chk2, E2F transcription activators, TK-1, and DNA damage repair genes in HCT116 5-FU Res cells to evaluate the changes induced by acquired 5-FU resistance.

In this study I have validated some FOXM1 binding sites by RT-PCR, so I would like to carry out the optimisation of the rest of the IGV data by the same method.

I would do ChIP sequencing on HCT116 5-FU Res cells to study the effect of FOXM1 binding on gene expression and map global changes in FOXM1 binding.

RESULT 3.2

TARGETING FOXM1 BY PROTEASOME INHIBITION IN COLON CANCER CELLS

3.2.1 Introduction

FOXO1 has recently been identified as an attractive molecular target in cancer therapy, but no inhibitor has yet entered the clinical arena. It is no surprise that there has been an intense focus in the development of potential FOXO1 inhibitors due to the mounting evidence of the roles of FOXO1 in carcinogenesis and drug resistance. Within the last decade, an emerging class of naturally occurring thiopeptide antibiotics have demonstrated a wide range of physiological activities including anti-bacterial, anti-parasitic, anti-fungal and anti-cancer properties. It is already well established that thiopeptides exert their antibacterial function via the inhibition of ribosomal protein synthesis (Just-Baringo, Albericio et al. 2014). Within the thiazole antibiotic family thiostrepton is most widely studied member, originally isolated from *Streptomyces azureus* (Mocek, Zeng et al. 1993) and has been reported to possess antitumour activities by direct inhibition of FOXO1 (Liao, Duan et al. 2009). Thiazole antibiotics carry out their antibacterial activity by blocking bacterial translation via binding to the 23S ribosomal RNA, but they do not affect eukaryotic protein synthesis (Bhat, Halasi et al. 2009).

Other forkhead family members, such as FOXA1, FOXO1 and FOXO3a, share a common DNA-binding domain with FOXO1, but are resistant to thiostrepton, which suggests that the natural product may bind at a distinct site of FOXO1 and act as an allosteric regulator (Hegde, Sanders et al. 2011). Normal cells (non-cancerous) that have wild-type p53 were resistant to the thiazole antibiotic, thiostrepton, suggesting that p53 plays an anti-apoptotic role in normal cells (Halasi, Schraufnagel et al. 2009)

A study on laryngeal squamous cell carcinoma (LSCC) shows targeting FOXM1 by thiostrepton inhibits tumour growth and induces apoptosis through mitochondrial and caspase-dependent intrinsic pathways, and treatment of LSCC cell line xenografts with thiostrepton resulted in inhibition of tumourigenesis in nude mice by reducing proliferation and inducing apoptosis (Jiang, Wu et al. 2014). Another study shows thiostrepton enhances sensitivity to cisplatin in ovarian cancer cells and enhances sensitivity to carboplatin in vivo (Zhang, Cheng et al. 2014). Thiostrepton induces apoptosis and significantly decreases the proliferation and metastasis in paediatric medulloblastoma, this study also showed that thiostrepton acts synergistically with cisplatin by enhancing cisplatin-induced apoptosis (Lin, Zheng et al. 2013). Likewise, the combination of thiostrepton and bortezomib in triple-negative MDA-MB-231-luc solid breast tumour xenografts reduced tumour growth compared to either drug alone (Wang and Gartel 2012). No reports have yet studied the effect of targeting FOXM1 in CRC.

During 5-FU treatment FOXM1 and its target genes were ultimately down-regulated in p53 wt cells, but in p53 mutant cells increased persistent FOXM1 expression was observed which was also apparent in acquired 5-FU resistant cells. Having established the key role of FOXM1 in 5-FU drug resistance and sensitivity, I next sought to investigate the anti-cancer potential of FOXM1 inhibitor thiostrepton in colorectal cancer cells.

3.2.2 SRB assay shows the average cell viability of colon cancer cells treated with thiostrepton

Thiostrepton is a thiazole antibiotic which specifically inhibits FOXM1 activity (Kwok, Myatt et al. 2008). I detected the sensitivity of HCT116, DLD1, and HT29 cell lines to thiostrepton by SRB. Thiostrepton treatment (0 to 200 μ M for 72 hours) significantly decreases cell viability in HCT116 (IC₅₀-0.7 $\pm$ 0.37 μ M), DLD1 (IC₅₀-1.43 $\pm$ 0.02 μ M) and HT29 (IC₅₀-2.1 $\pm$ 0.98 μ M) cell lines (Figure 3.19). This result demonstrates that thiostrepton can inhibit cell viability in colorectal cancer cells, with HCT116 wt being the most sensitive compared to p53 mutant DLD1 and HT29 cells.

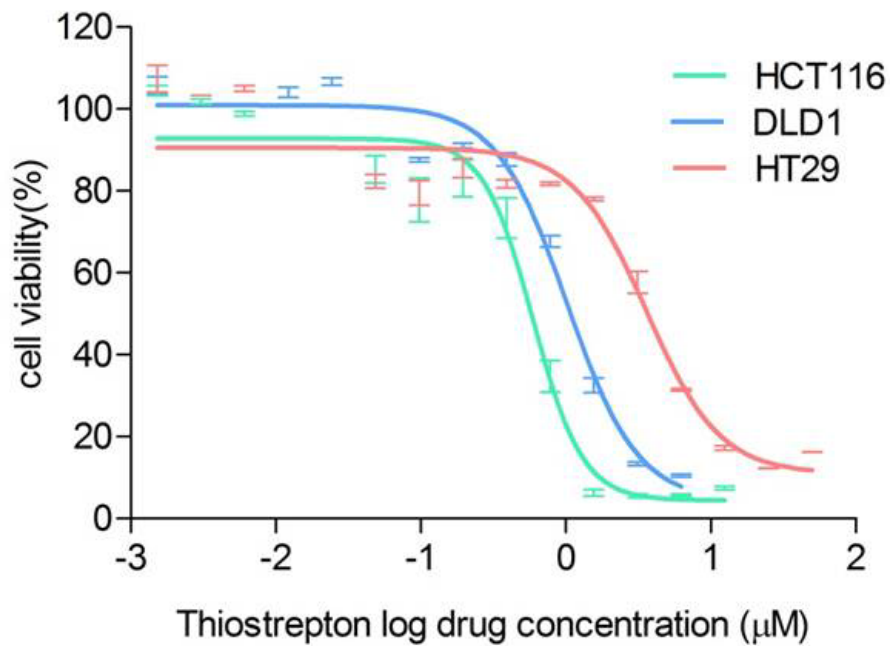


Figure 3. 19 SRB assay showing the cell viability of CRC treated with thioestrepton.

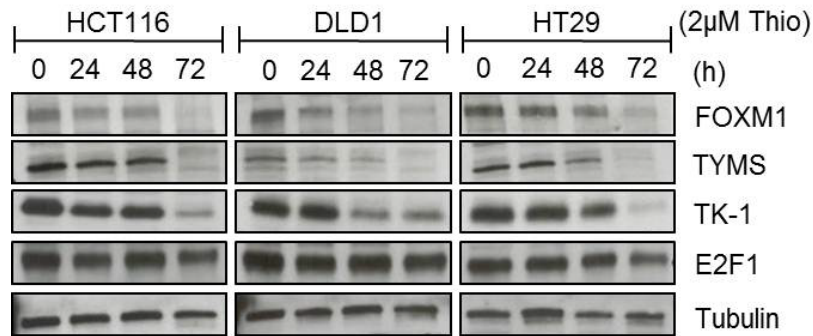
HCT116, DLD1, and HT29 cells were treated with thioestrepton ranging from 0-200μM for 72h. Thioestrepton significantly decreases cell viability in HCT116 ($IC_{50}=0.7\pm0.37\mu M$), DLD1 ($IC_{50}=1.43\pm0.02$) and HT29 cells ($IC_{50}=2.1\pm0.98$). Results were normalised to 0h, and depict the mean and SD values of three independent experiments, each with three replicates. IC_{50} values were determined by fitting a sigmoidal dose-response curve to the data.

3.2.3 Thiostrepton inhibits FOXM1 and its downstream targets

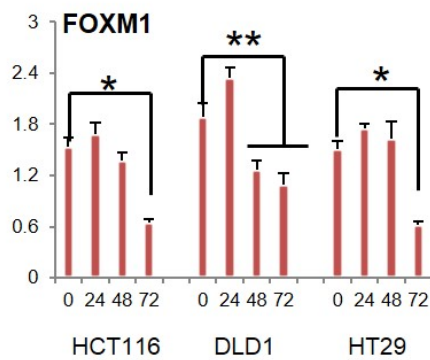
Studies show that thiostrepton directly interacts with FOXM1 and inhibits its binding with genomic target sites such as MYC, CDC25B, CCNB1, XBP1, CCNB1 and PLK1 (Hegde, Sanders et al. 2011, Buchner, Park et al. 2015).

In accordance with these findings, I decided to study the expression of FOXM1, TYMS, TK-1 and E2F1 in colon cancer cell lines following thiostrepton treatment (Figure 3.20 A-D). Cells were treated with 2 μ M of thiostrepton for 24, 48 and 72 hours. After thiostrepton treatment, significant reductions in FOXM1 protein and mRNA were observed including the p53 mutant cell lines at 72h. Similarly, the FOXM1 targets TYMS and TK-1 were also down-regulated. The mRNA expression patterns of TYMS and TK-1 were similar to FOXM1. Slight decreases were observed in E2F1 protein expression at 72h in HCT116 and HT29 cell lines, and significant down-regulation of E2F1 was observed in the mRNA of HCT116 and HT29 cells but no change observed in DLD1 (Figure 3.20 C), the reason for this is unclear. This result supports that thiostrepton can down-regulate the protein and transcriptional activity of FOXM1.

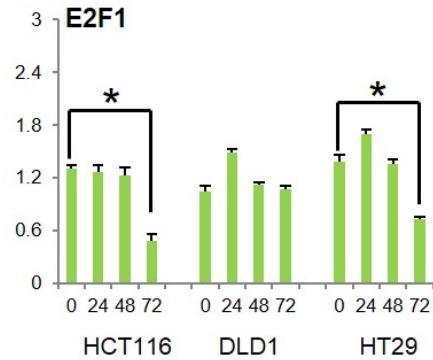
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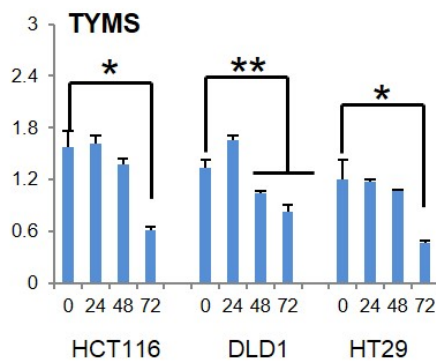
B



C



D



E

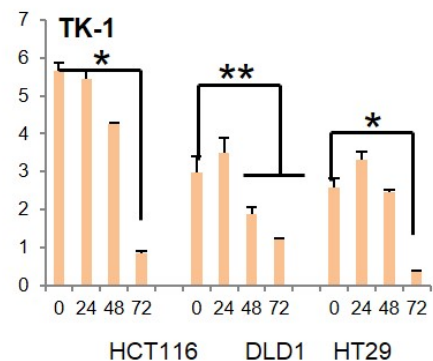


Figure 3. 20 Thiostrepton inhibits FOXM1 and its downstream targets. HCT116, DLD1, and HT29 colon cancer cells were treated with 2μM of thiostrepton for 0, 24, 48, and 72h; cells were trypsinised and harvested at the time points indicated for western blot analysis and RT-PCR. (A) Protein lysates were prepared and expression levels were analysed by western blotting using antibodies for FOXM1, E2F1, TYMS and TK-1. β-tubulin was used as a loading control. mRNA expression indicates a significant decrease in FOXM1, TYMS, E2F1 and TK-1 levels (B,C,D,E) at 72h. Error Bars represent standard deviation. Statistical significance was determined by student's T-test. (*p value < 0.05 versus control).

3.2.4 Thiostrepton can alter 5-FU resistance in colon cells through the down-regulation of FOXM1

Further to this, I sought to determine if inhibition of FOXM1 with thiostrepton could be a useful tactic to overcome 5-FU resistance. For this experiment I used HCT116 wt and HCT116 5-FU Res cells. The cells were treated with various concentrations of thiostrepton ranging from 0-200 μ M for 72h (Figure 3.21). HCT116 5-FU Res cells were previously shown to have elevated FOXM1 and 5-FU resistance (figure 3.6).

SRB assays show significant decreases in the viability of 5-FU resistant HCT116 cells (Figure 3.21). The IC₅₀ value for HCT116 5-FU Res cells to thiostrepton (0.8 μ M) was similar to the IC₅₀ of HCT116 wt, suggesting that resistance to proteasome inhibition does not develop in the 5-FU resistant phenotype. This result clearly indicates that FOXM1 is important in acquired 5-FU resistance and inhibiting FOXM1 alone can be cytotoxic even in highly 5-FU resistant cells.

I also confirmed this effect on an established FOXM1 over expressed model, MCF7 pCMV FOXM1. (Supplementary data: figure 5.7). The IC₅₀ values for thiostrepton were similar in both MCF7 FOXM1 over expressed cells and MCF7wt cells (MCF7 FOXM1 cells IC₅₀ 1.7 μ M MCF7 wt IC₅₀ 1.8 μ M) in contrast with a previous study on breast cancer cells that suggested thiostrepton has no effect on exogenous FOXM1 expression in the transfected cells where FOXM1 expression remained constant (Kwok, Myatt et al. 2008).

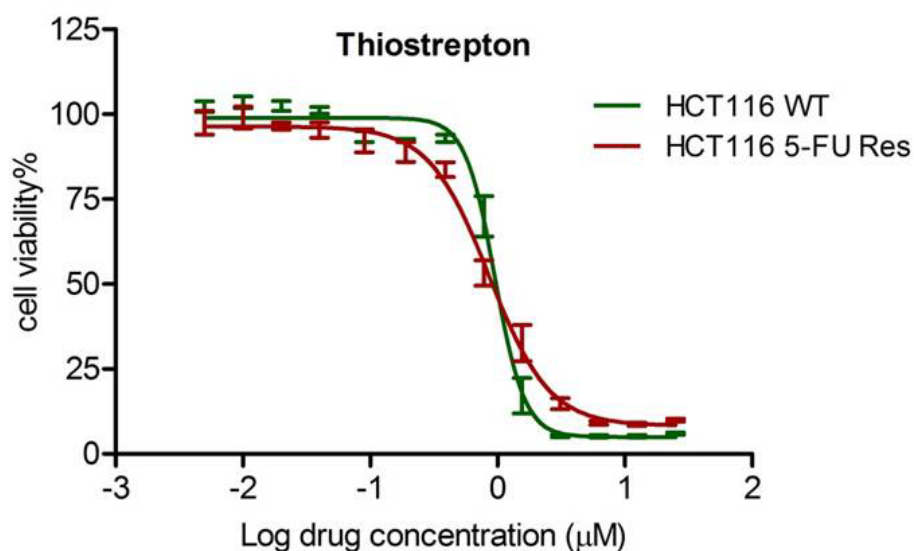
A**B**

Figure 3. 21 Thiostrepton can alter 5-FU resistance through the inhibition of FOXM1.

A. Western blots were carried out to detect the expression of FOXM1 after thiostrepton treatment. HCT116 wt, HCT116 5-FU res cells were treated with 2 μ M of thiostrepton for 0, 24, 48 and 72h. B. HCT116 wt, HCT116 5-FU Res cells were treated with thiostrepton ranging from 0-200 μ M for 72h. SRB assays indicate that the treatment decreases the cell viability of HCT116 5-FU Res along and HCT116 wt cells (IC₅₀ 0.8 μ M). Results were normalised to untreated. Results shown are mean and SD of three independent experiments, each with three replicates. IC₅₀ values were determined by fitting a sigmoidal dose-response curve to the data.

3.2.5 Thiostrepton inhibits FOXM1-DNA binding

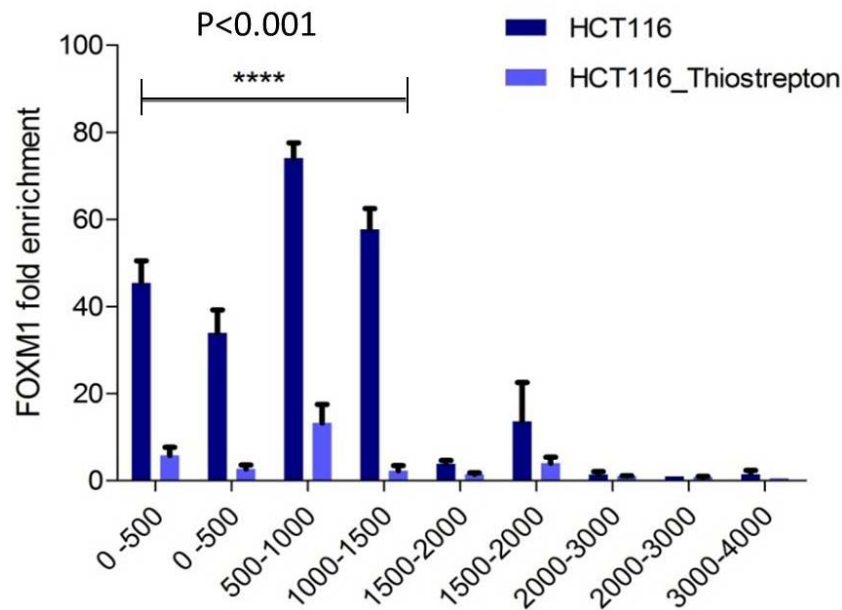
I previously confirmed that thiostrepton can inhibit FOXM1 and the proposed downstream targets TYMS and TK-1 at the protein and mRNA level. Furthermore, I wished to determine whether the thiostrepton inhibitor could inhibit FOXM1–DNA binding in vitro in colon cancer cells.

HCT116, DLD1, and HCT116 5-FU Res cell lines were treated with 2 μ M of thiostrepton for 24 hours and ChIP was performed using a FOXM1, followed by RTq–PCR antibody (Figure 3.22 A, B, C). Interestingly, the results show a significant ($P<0.001$) disruption in the FOXM1–DNA interactions, with associated substantial down regulation in binding of FOXM1 in TYMS promoter region in all the 3 cell lines. Decreases in FOXM1 binding of 80% in HCT116, 85% in DLD1, and 90% in HCT116 5-FU Res (Figure 3.22 A, B, C respectively) were observed in the TYMS promoter region.

Further to this I also analysed if the FOXM1 inhibitor, thiostrepton can decrease the expression of other FOXM1 targets. I analysed FOXM1 DNA binding on E2F1, E2F2, E2F3, MMP1, MMP2, MLH1, TK-1 and P38 (figure 3.22 D). Interestingly, the results show significant inhibition in 5-FU targets TK-1 and TYMP, and, in addition inhibition was observed in E2F1, E2F2, E2F3, MMP1, MMP2, and the DNA MMR gene MLH1. FOXM1 is known regulator of matrix metalloproteinase genes (Xue, Xiao et al. 2012). P38, which is not a FOXM1 target, does not show any enrichment (Behren, Muhlen et al. 2010).

According to these results, FOXM1 binding events were significantly decreased after thiostrepton treatment, indicating that thiostrepton can reduce FOXM1 binding to its downstream effectors.

A



B

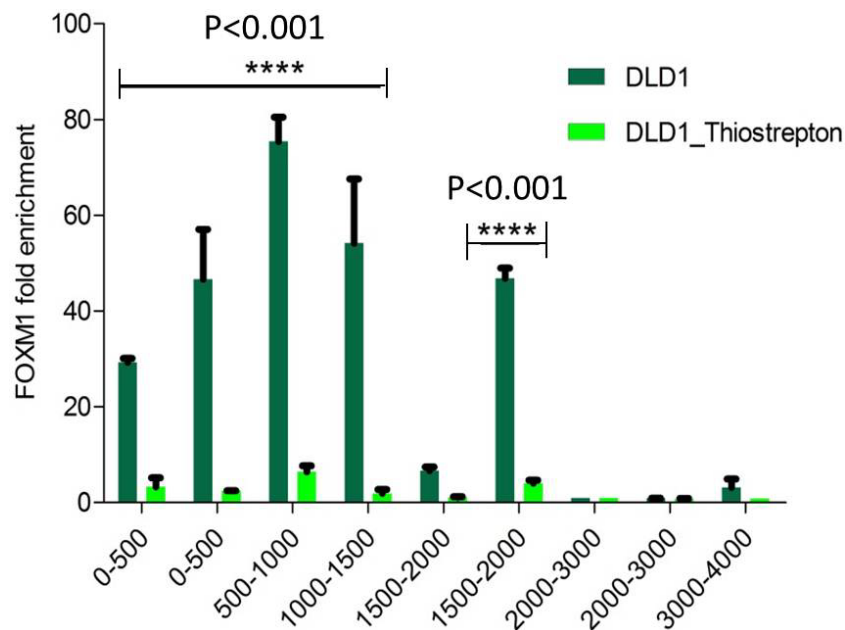
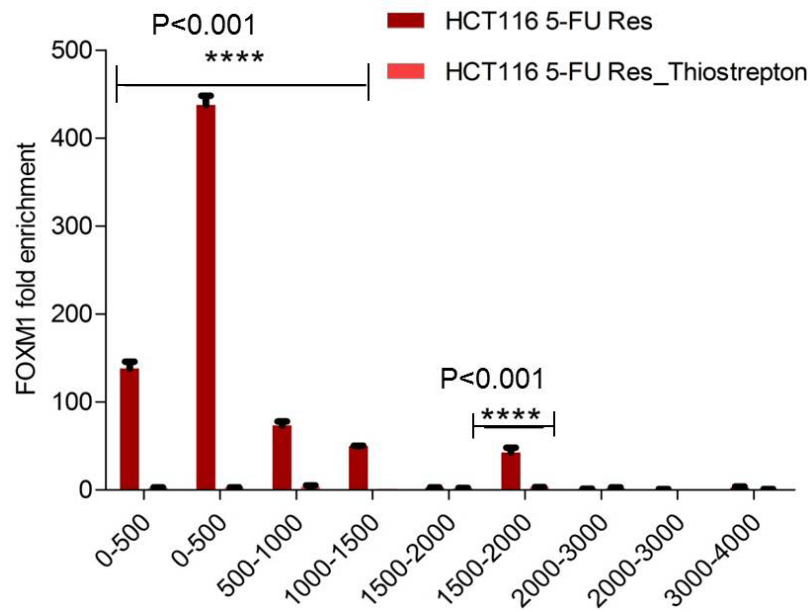


Figure 3. 22 Thiostrepton inhibits FOXM1 binding on the TYMS promoter. A HCT116 and B DLD1 cell lines were treated with 2 μ M of thiostrepton for 24h and ChIP was performed with FOXM1 antibody followed by RTQ-PCR). Results show that thiostrepton treatment significantly down-regulates FOXM1 binding on TYMS promoter. These experiments were done in triplicate. Error Bars represent standard deviation. Statistical significance between un-treated and thiostrepton treated cells was determined by 2way annova using graph pad prism (****p value < 0.001).

C



D

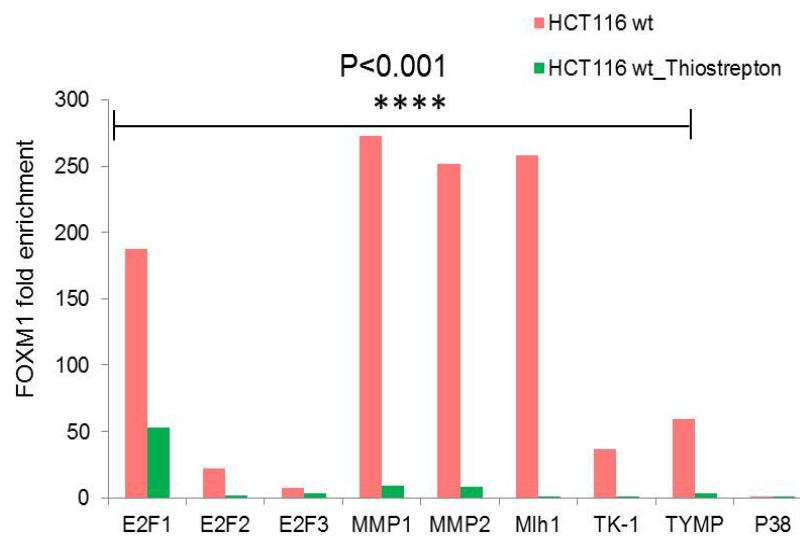


Figure 3. 22 C. HCT116 5-FU Res cells were treated with 2 μ M of thiostrepton for 24h and ChIP was performed with a FOXM1 antibody followed by RT-PCR. Thiostrepton treatment significantly down-regulate FOXM1 binding on TYMS promoter. D. HCT116 wt cells thiostrepton treatment significantly down-regulate FOXM1 binding on E2F1, E2F2, E2F3, MMP1, MMP2, MLH1, TK-1, TYMP promoter. These experiments were done in triplicate. Error Bars represent standard deviation. Statistical significance between untreated and thiostrepton treated cells was determined by 2 way annova using graph pad prism (p value < 0.001).

3.2.6 Thiostrepton synergises with 5-FU

According to previous studies thiostrepton can enhance sensitivity to chemotherapeutic agents like bortezomib (Pandit and Gartel 2011, Wang, Halasi et al. 2012), docetaxel (Li, Yao et al. 2014). This prompted me to investigate the potential synergistic effects of thiostrepton with 5-FU treatment in combination.

HCT116, DLD1 and HT29 cells were treated with single and combination drug treatments by using the Chou-Talalay method (Chou 2010). SRB assays were performed to measure the viability of cells following 72 hours treatment.

To evaluate the effect of combination treatment with 5-FU and thiostrepton, the combination index (CI) isobologram methods of Calcosyn software (Chou 2010) were used. Combination index values of 1, <1, and >1 indicate additive, synergistic and antagonistic effects respectively. The results using the combination treatment on p53 wt HCT116 cells (figure 3.23), p53 mutant DLD1 (figure 3.24) and HT29 (figure 3.25) cells indicate synergism of thiostrepton with 5-FU.

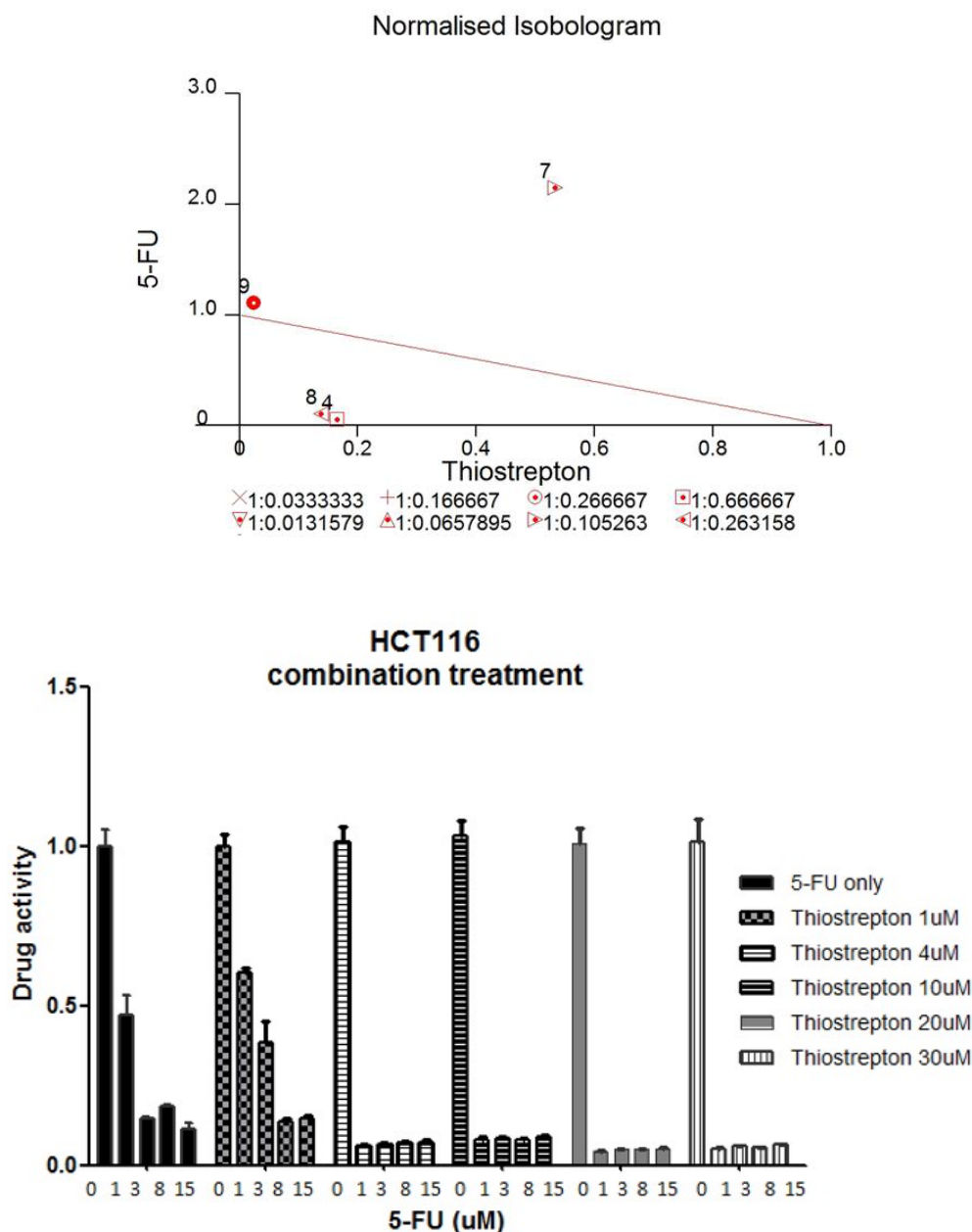


Figure 3. 23 The combination of thiostrepton and 5-FU is synergistic in cancer cells. Combination index chart for the thiostrepton/5-FU drug combination was plotted with Calcosyn software®. The drug combination is plotted using Isobologram (top). The points under 1 indicate synergism of 5-FU and thiostrepton. The bar chart indicates the effect of 5-FU alone and in combination with thiostrepton.

B

DLD1

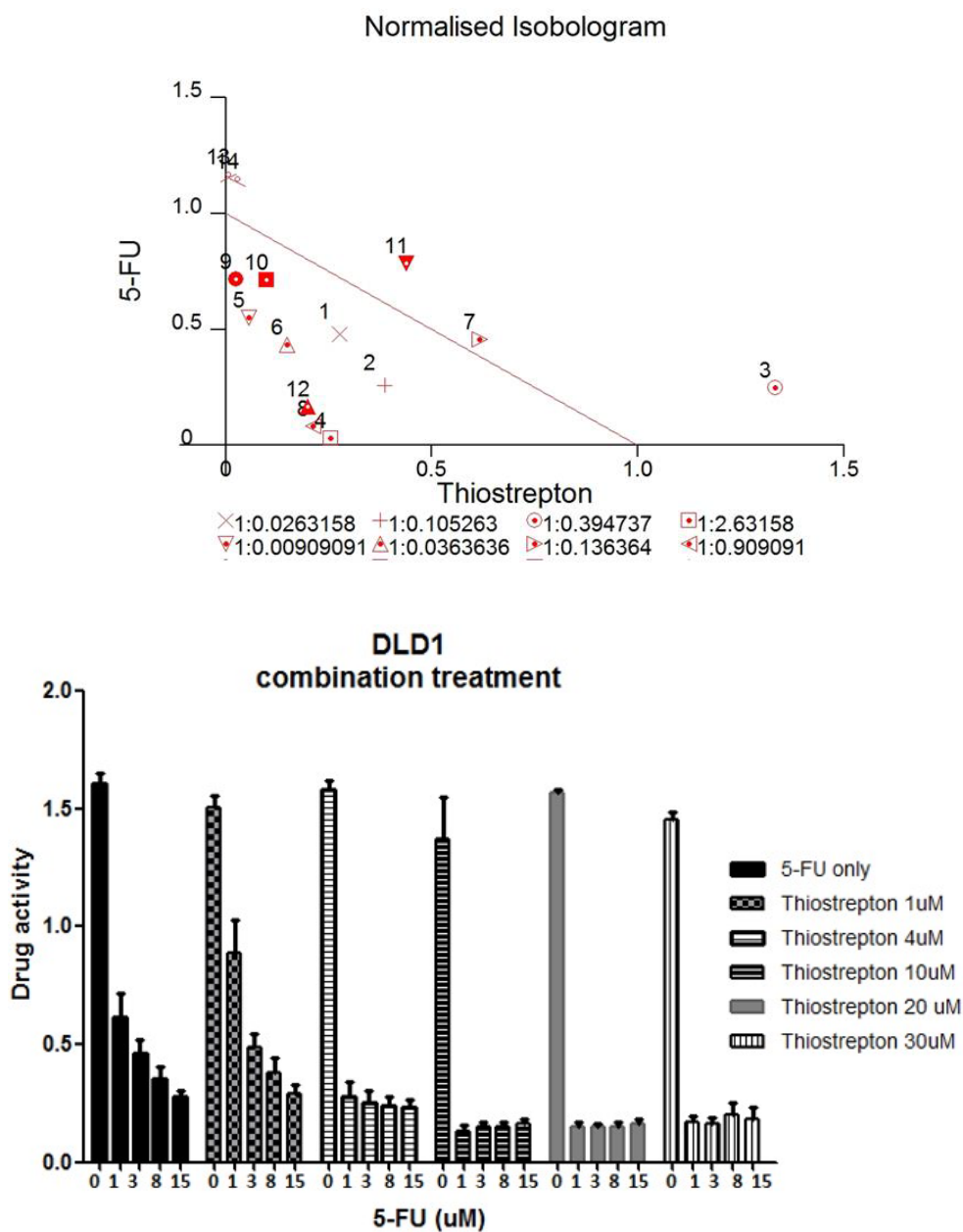


Figure 3. 24 The combination of thiostrepton and 5-FU is synergistic in cancer cells. Combination index chart for the thiostrepton/5-FU drug combination was plotted with calcsyn software. The drug combination is plotted using Isobologram (top). The points under 1 indicate synergism of 5-FU and thiostrepton. The bar chart indicates the effect of 5-FU alone and in combination with thiostrepton.

C

HT29

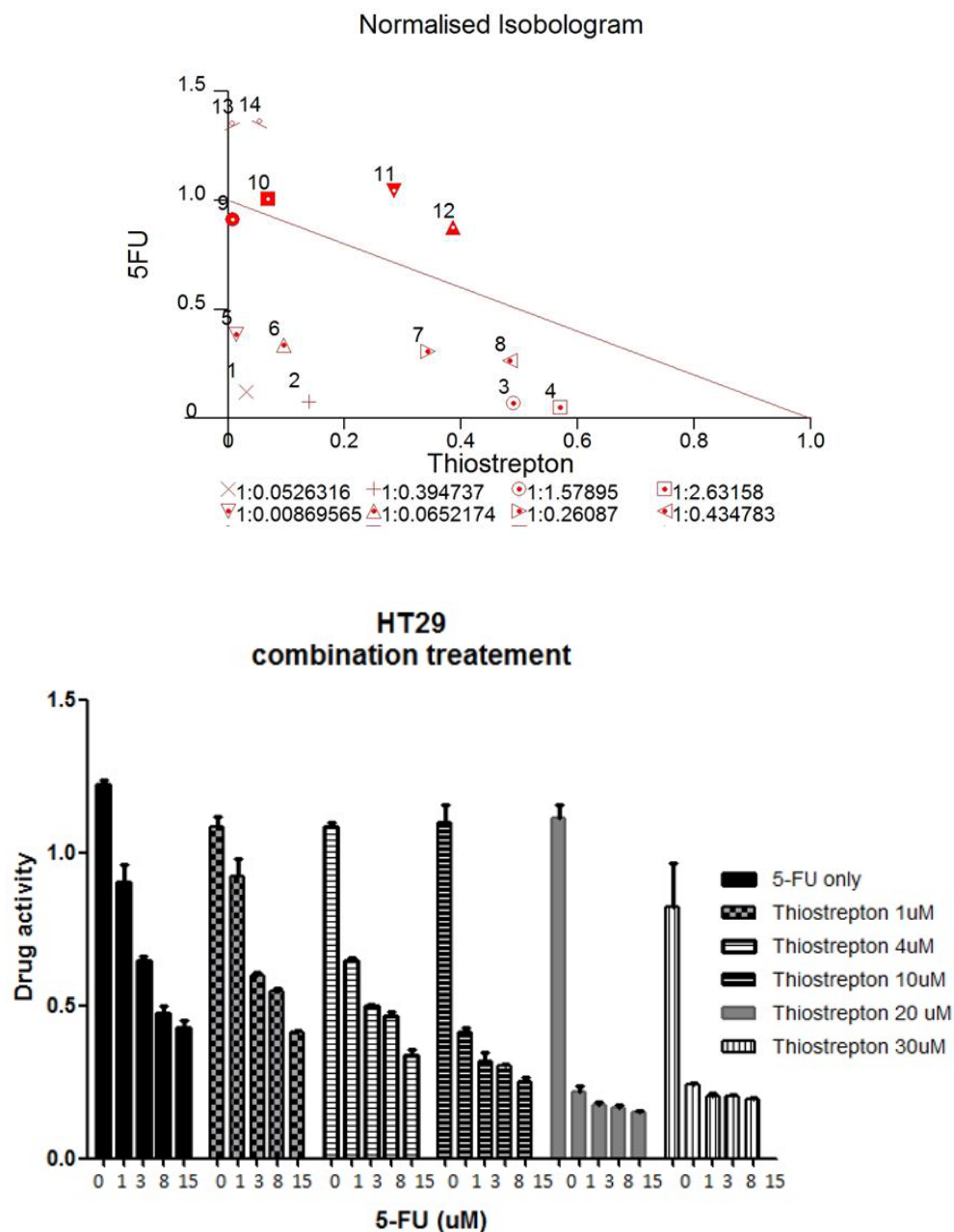


Figure 3. 25 The combination of thiostrepton and 5-FU is synergistic in cancer cells. Combination index chart for the thiostrepton/5-FU drug combination was plotted with calcosyn software. The drug combination is plotted using Isobologram (top). The points under 1 indicate synergism of 5-FU and thiostrepton. The bar chart indicates the effect of 5-FU alone and in combination with thiostrepton.

3.2.7 Combination of thiostrepton and 5-FU increases DNA damage in colon cancer cells

After identifying the synergism between thiostrepton and 5-FU in colon cancer cells, I next sought to analyse the effect of combination treatment on cell cycle distribution and cell death/ apoptosis.

Initially, I examined if the combination of 5-FU and thiostrepton treatment increased DNA damage. HCT116, HCT116 5-FU Res, DLD1 and HT29 colon cancer cell lines were treated with 1 μ g/ml 5-FU and 1 μ M thiostrepton alone or in combination for 24 hours and immunostained for γ H2AX, which is a known marker to detect the presence of DNA breaks, induced by the combination treatment.

HCT116 cells (p53 wt) show high levels of DNA damage after 5-FU which was increased further by the combination treatment (Figure 3.26). Treatment with 5-FU alone in HCT116 5-FU Res cells did not cause γ H2AX induction while the combination treatment shows significant γ H2AX staining in HCT116 5-FU Res cells (Figure 3. 27). p53 mutant HT29 and DLD1 cells show higher levels of DNA damage following combined 5-FU and thiostrepton (Figure 3. 28, 3.29) compared to either drug alone. My previous results show high levels of DNA damage response proteins in HT29 and DLD1 cells compared to HCT116 wt cells (figure 3.3 A), suggesting that all these proteins contribute to the resistant nature of these cells, enabling them to withstand DNA damage (Millour, de Olano et al. 2011).

Quantification of γ H2AX foci showed that combination treatment induced the accumulation of significantly more foci observed in all of the cell lines compared to control, or either drug alone. This is likely to be due to inhibition of FOXM1 target

genes including DNA damage repair genes, caused by thiostrepton, leading to enhanced DNA damage and apoptosis in the presence of 5-FU.

Further to these experiments, I carried out cell cycle analysis to determine cell death and cell cycle distribution in colon cancer lines (Figure 3.30, 3.31). In HCT116 cells, 5-FU treatment led to G2-M arrest and G0 (sub G1) accumulation (7.3%). Thiostrepton also increased the number of cells in G0 (18.5%), and reduced the proportion of cells in G2/M compared to control, indicating cell death and apoptosis. Notably, the combination treatment resulted in significant G0 (25.5%) accumulation compared to either drug alone, which indicates that the combination treatment can enhance cell death and apoptosis and the FACS data also correlates with γ H2AX staining in HCT116 wt cells (Figure 3.30 A).

Meanwhile, in HCT116 5-FU Res cells significant changes were observed following treatment with thiostrepton and the combination, similar to γ H2AX staining, no significant changes were observed in 5-FU resistant cells following 5-FU treatment. But treating the cells with thiostrepton alone shows cell death in 5-FU resistant cells. This could indicate that HCT116 5-FU Res cells are sensitive to thiostrepton. Treatment with thiostrepton alone resulted in 17% of HCT116 5-FU Res cells in sub G1 (Figure 3.30 B). The most remarkable effect of the combination treatment was accumulation of cells in G0 (sub G1) (47%) population.

In p53 mutant DLD1 cells, no significant changes were observed after treatment with 5-FU alone. However, combination treatment decreased the number of cells in G1 higher than either control or 5-FU treatment. An accumulation of cells in G0 was observed following the combination treatment (Figure 3.31 A).

In HT29 cells, 5-FU treatment alone caused G1 arrest and G2/M accumulation, whilst using combination treatment an accumulation of cells in G0 and G2/M and a

decrease of cells in G1 phase were also observed, indicating cell death and apoptosis (Figure 3.31 B).

These results indicate that the combination treatment can effectively increase DNA damage and cell death in colon cancer cells.

HCT116

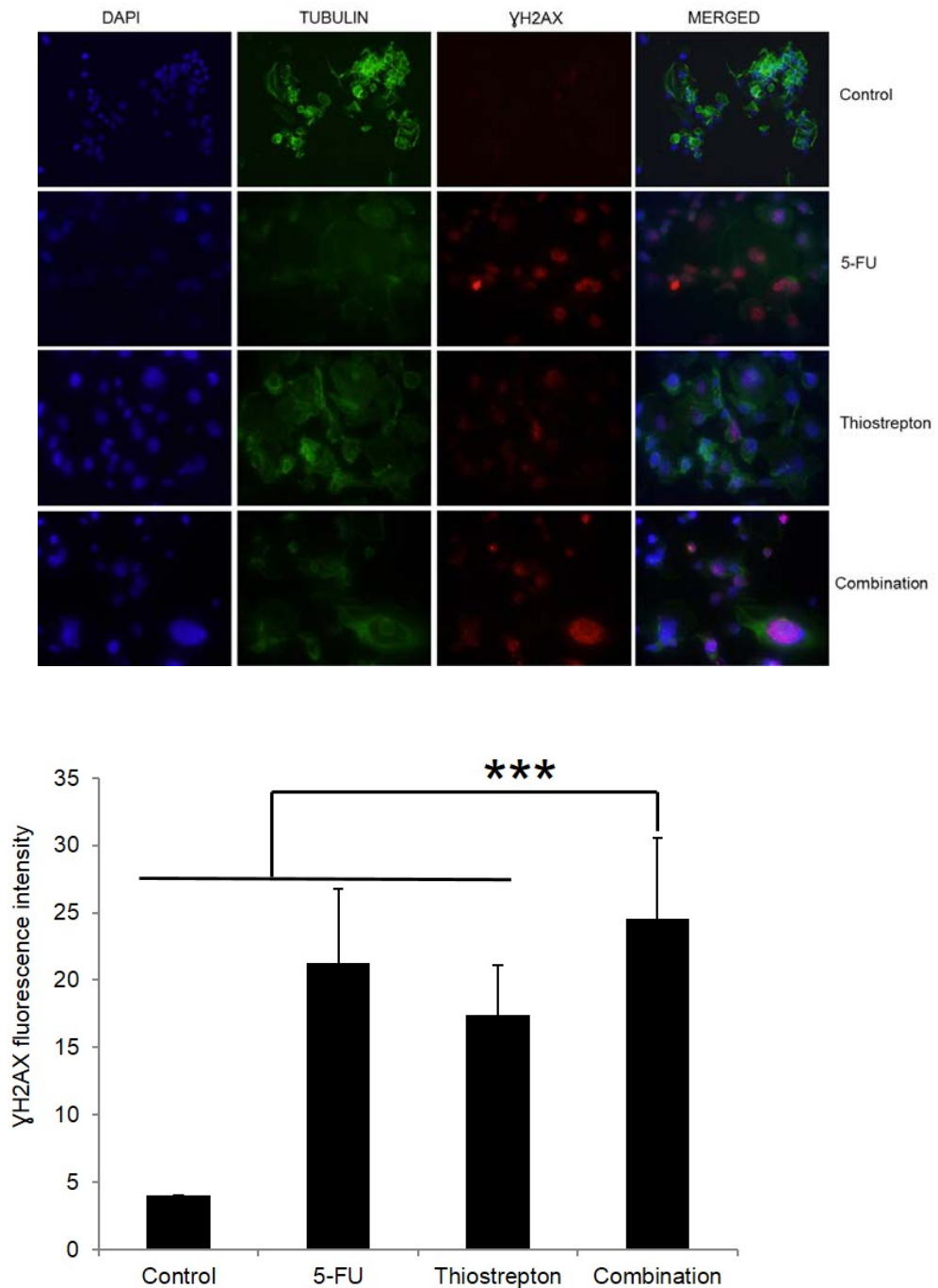


Figure 3. 26 Combination treatment increases DNA damage in HCT116 wt cells. HCT116 cells were treated with 1 μ g/ml of 5-FU, 1 μ M of thiostrepton and combination for 24h and stained with DAPI, tubulin and γ H2AX antibodies. Images were visualized by microscopy and are shown at original magnification X 40. Images were quantified using ImageJ software. The combination of 5-FU and thiostrepton significantly increases γ H2AX staining in HCT116 cells compared to control and either drugs alone *** p <0.001.

HCT116 5-FU Res

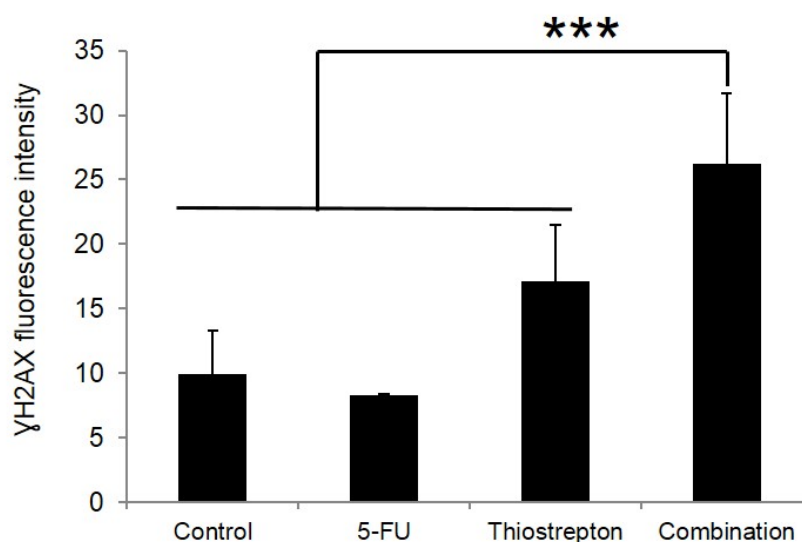
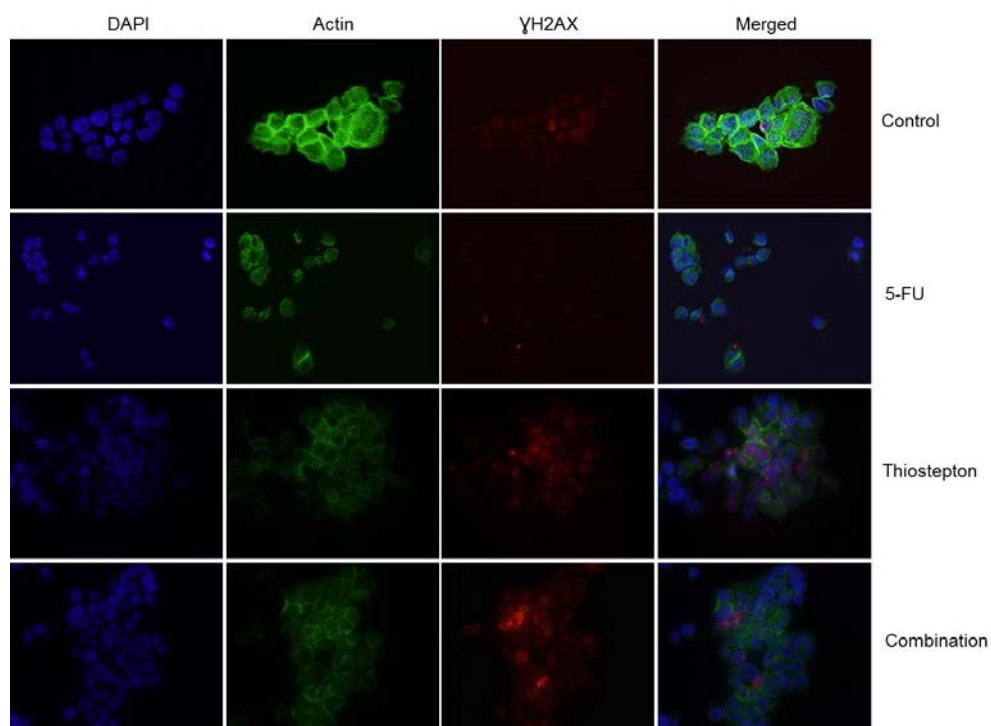


Figure 3. 27 Combination treatment increases DNA damage in HCT116 5-FU Res cells. Cells were treated with 1 $\mu\text{g/ml}$ of 5-FU, 1 μM of thiostrepton and combination for 24h and stained with DAPI, tubulin and γH2AX antibodies. Images were visualized by microscopy and are shown at original magnification X 40. Images were quantified using ImageJ software.

The combination of 5-FU and thiostrepton significantly increases γH2AX staining in HCT116 5-FU Res cells compared to control and either drugs alone *** $p < 0.001$.

DLD1

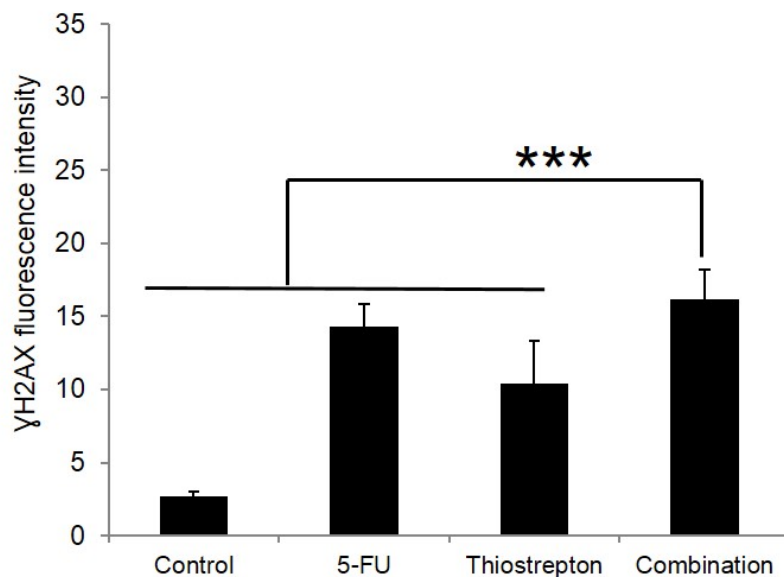
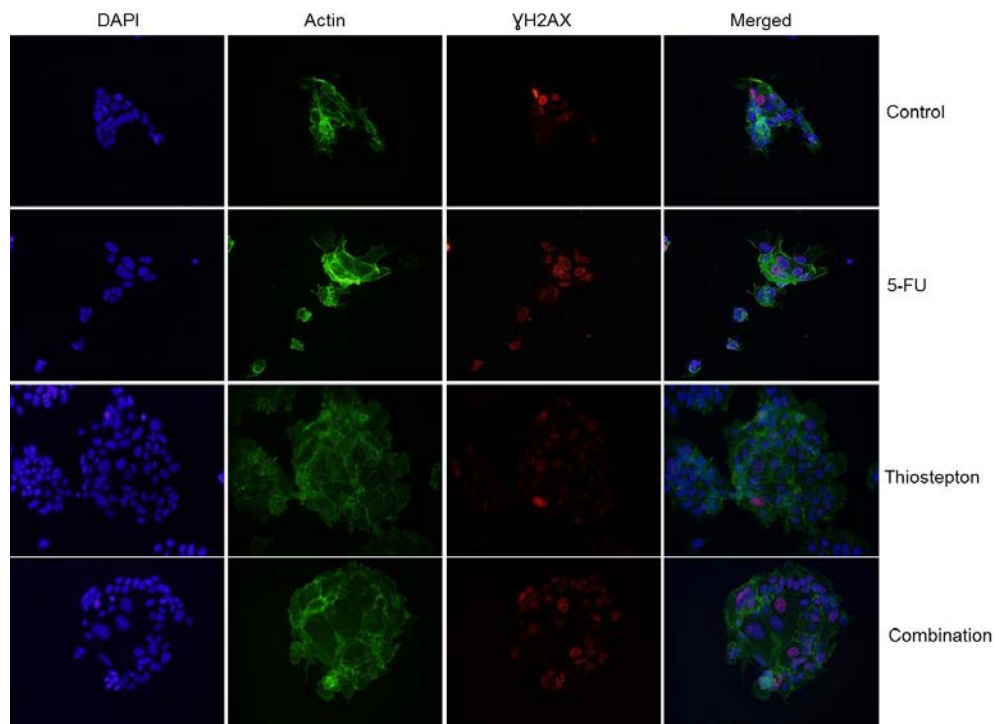


Figure 3. 28 Combination treatment increases DNA damage in DLD1 cells.

DLD1 cells were treated with 1 $\mu\text{g/ml}$ of 5-FU, 1 μM of thiostrepton and combination for 24h and stained with DAPI, tubulin and γH2AX antibodies. Images were visualized by microscopy and are shown at original magnification X 40. Images were quantified using ImageJ software.

The combination of 5-FU and thiostrepton significantly increases γH2AX staining in DLD1 cells compared to control and either drugs alone *** $p < 0.001$.

HT29

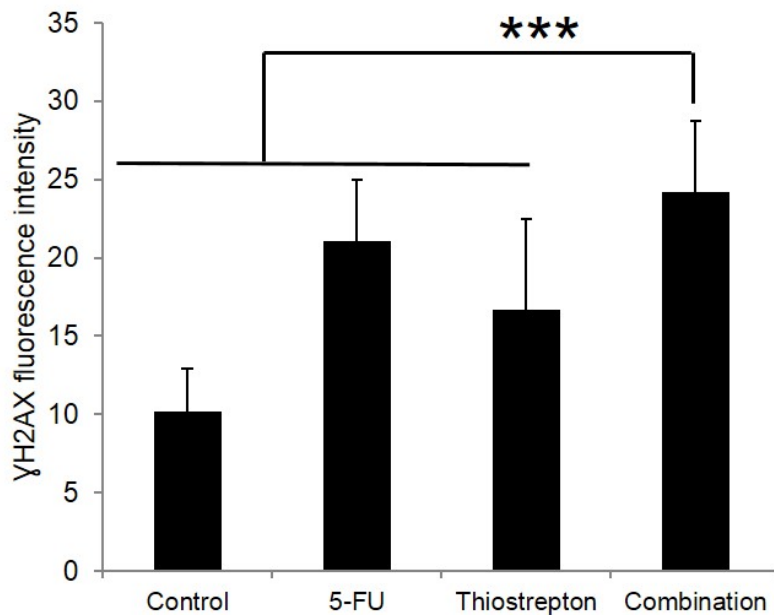
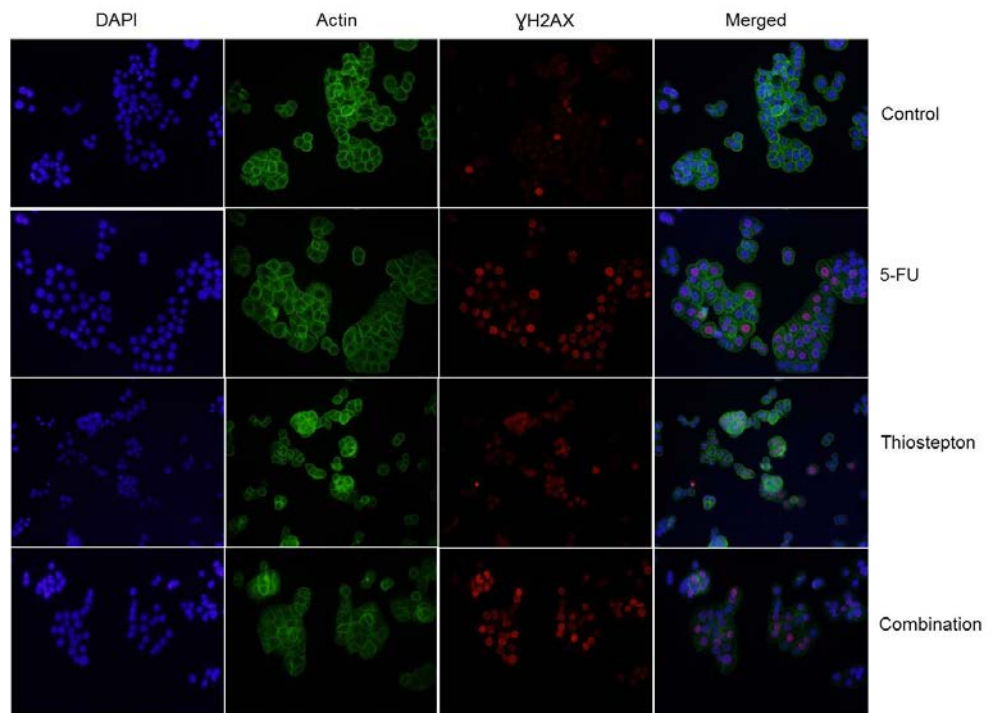


Figure 3. 29 Combination treatment increases DNA damage in HT29 cells. HT29 cells were treated with 1 $\mu\text{g/ml}$ of 5-FU, 1 μM of thioestpton and combination for 24h and stained with DAPI, tubulin and γH2AX antibodies. Images were visualized by microscopy and are shown at original magnification X40. Images were quantified using

ImageJ software. The combination of 5-FU and thioistrepton significantly increases γ H2AX staining in HT29 cells compared to control and either drugs alone *** $p < 0.01$.

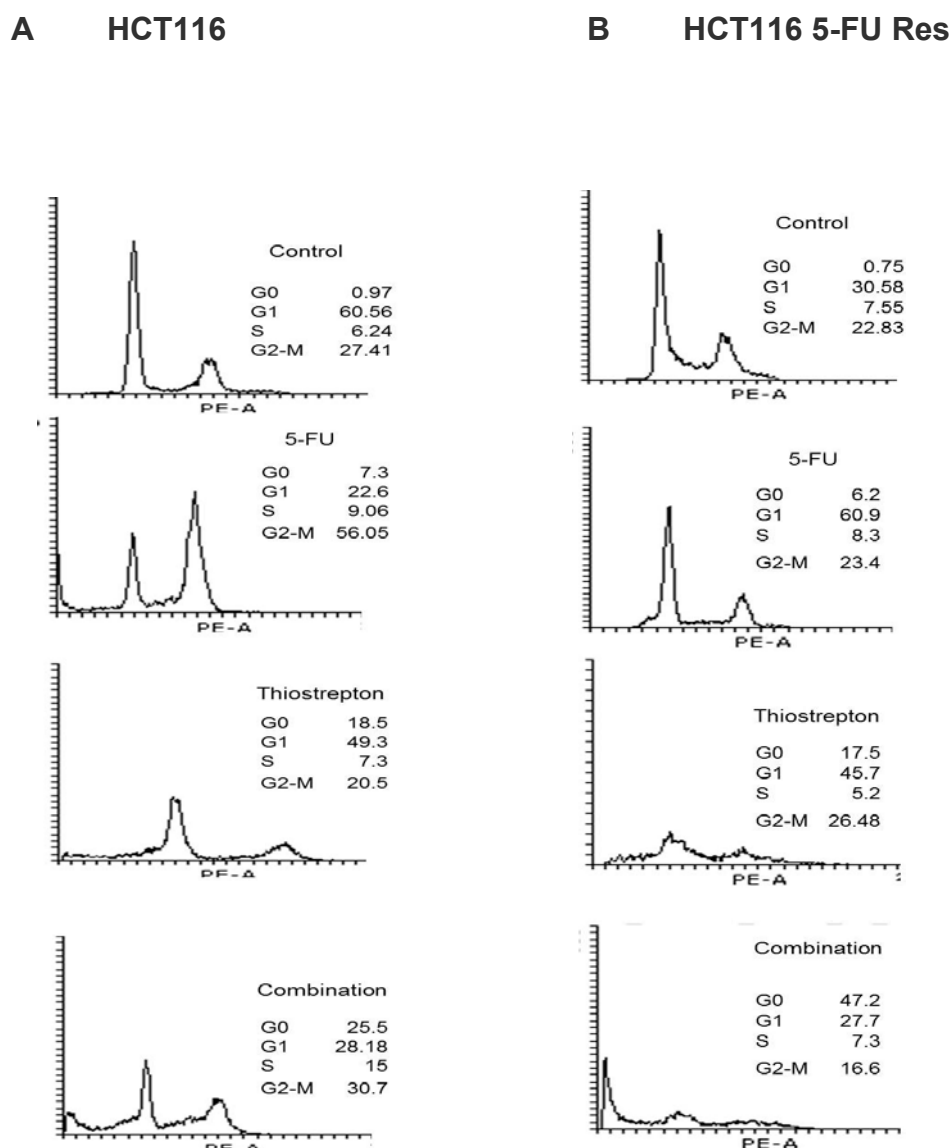


Figure 3. 30 Cell cycle analysis to determine cell death and cell cycle distribution in HCT116 wt and HCT116 5-FU Res cells.

(A) HCT116 wt (B) HCT116 5-FU res cells were treated with 1 μ g/ml of 5-FU, 1 μ M thioistrepton, and combination drugs for 72h. Cells were fixed with 70% ethanol and stained with PI (propidium iodide) and cell cycle phase distribution was analysed by flow cytometry. The percentage of the cells in each phase of the cycle is indicated (G0 (sub G1), G1, S and G2-M).

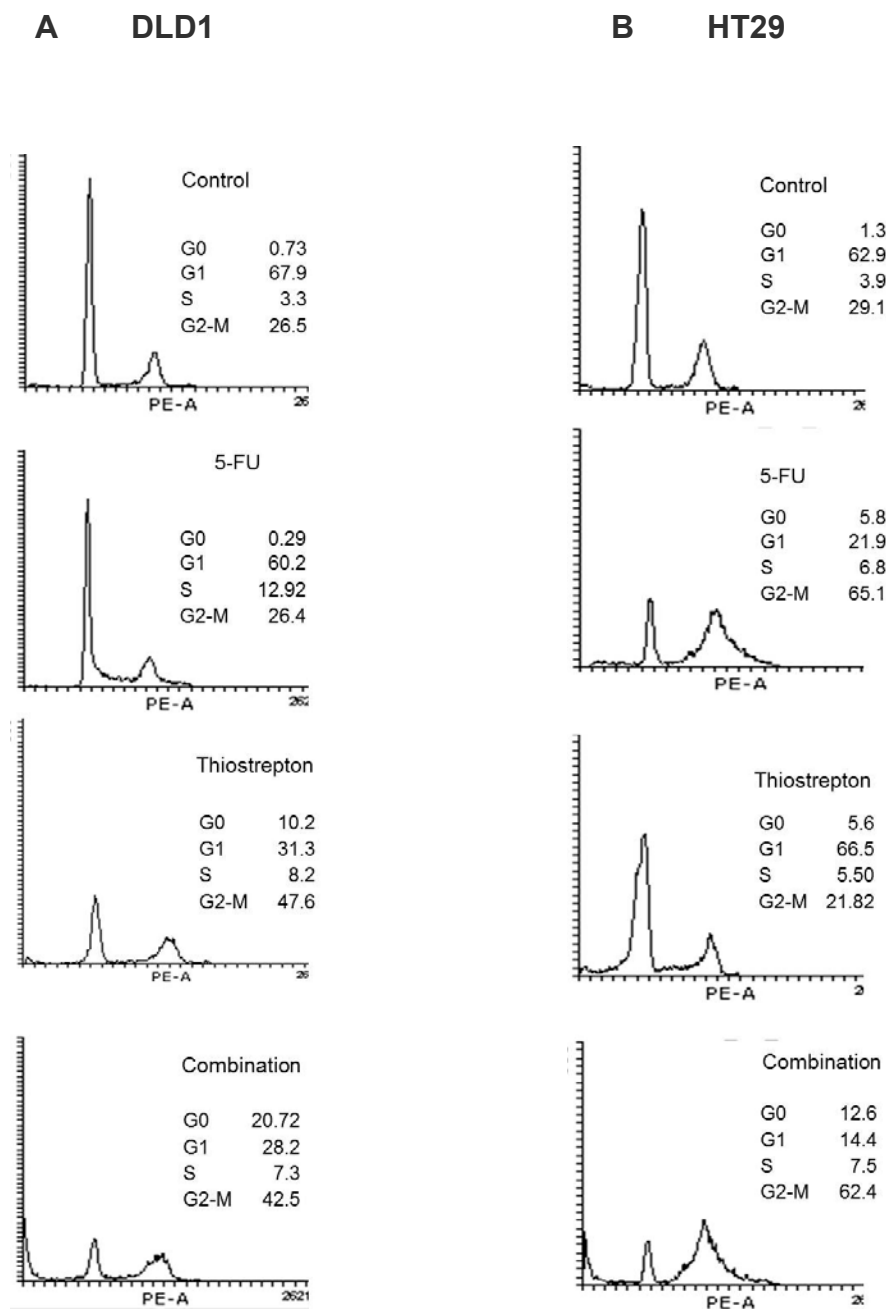


Figure 3. 31 Cell cycle analysis to determine cell death and cell cycle distribution in DLD1 and HT29 cells.

(A) DLD1 (B) HT29 cells were treated with 1 μ g/ml of 5-FU, 1 μ M thiostrepton, and combination drugs for 72 h. cells were fixed with 70% ethanol and stained with PI (propidium iodide) and cell cycle phase distribution was analysed by flow cytometry. The percentage of the cells in each phase of the cycle is indicated (G0 (sub G1), G1, S and G2-M).

3.2.8 Combination treatment significantly decreases colony formation in colon cancer cells

Next, I decided to detect the effect of the drug combination on long-term cell survival by performing colony formation assays in colorectal cancer cell lines.

HCT116, DLD1, and HT29 cells were treated with 1 μ M thiostrepton and 1 μ g/ml 5-FU alone or in combination. After 72 hours, drug free medium was added and the cells were grown for 12-14 days. In HCT116 wt cells, tumour cell colony formation was significantly inhibited by thiostrepton alone and the combination of 5-FU and thiostrepton compared to 5-FU alone or control. Although there was significant activity of 5-FU alone in this cell line (where colony formation was reduced by 82%), it was less potent than either thiostrepton or combination therapy (colony formation reduced by 96.3% and 96% respectively). The drug combination effect was most notable for DLD1 (Figure 3.34) and HT29 (Figure 3.35) cells. Combination treatment demonstrated 94.6% reduction in DLD1 and 91.2% reduction in HT29 compared to control, supporting our earlier findings of synergy. 5-FU and thiostrepton were less potent in these cells when used as single agents, and reduced the colony formation by 57% and 30% compared to control respectively in DLD1, while in HT29, 5-FU and thiostrepton reduced the colony formation by 67% and 24% compared to control respectively.

HCT116 5-FU Res cells were treated with 2 μ M thiostrepton and 2 μ g/ml 5-FU alone or in combination for 72 hours (figure 3.33). For HCT116 5-FU Res cells, higher concentration of drugs were used as there were no significant changes (6% reduction in combination) observed using lower drug concentrations during the optimisation test. As expected there were no changes were observed in 5-FU treated

cells compared to control. Importantly, similar results to the parental HCT116 cells were observed, showing marked sensitivity to both thiostrepton (96%) and combination (97%) treatment (Figure 3.32).

HCT116

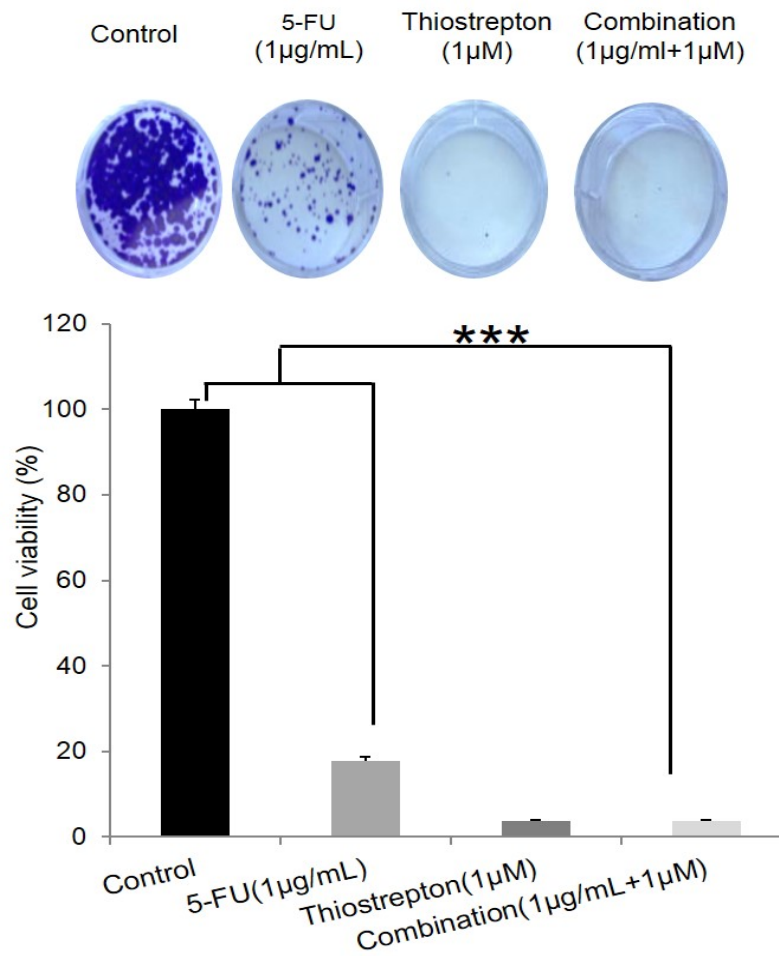


Figure 3. 32 Combination treatment decreases colony formation in HCT116 wt cells. Clonogenic assays showing HCT116 wt cells treated with 5-FU (1 µg/ml) and thiostrepton (1 µM) alone or in combination for 72h, followed by media replacement for 14 days. The combination of 5-FU and thiostrepton significantly decreases colony formation in HCT116 compared to control and 5-FU alone. Data were represented as mean $\pm$ SD (n=3). ***p<0.0001.

HCT116 5-FU Res

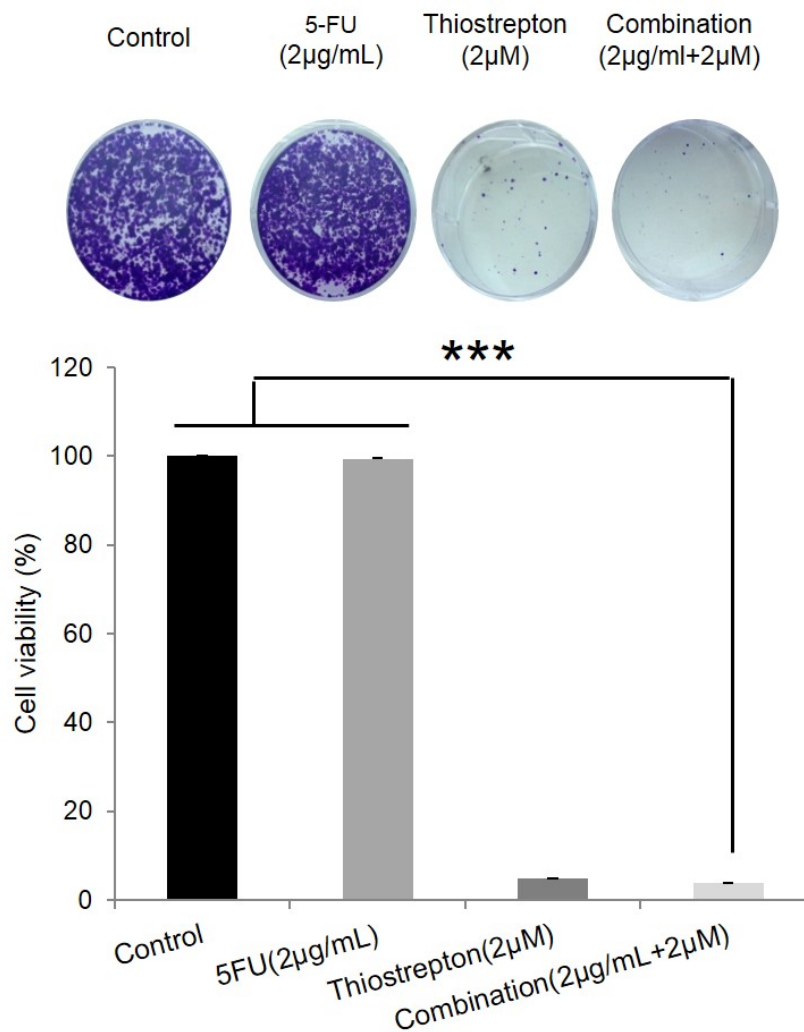


Figure 3.33 Combination treatment decreases colony formation in 5-FU Res cells. Clonogenic assays showing HCT116 5-FU Res cells were treated with 5-FU (2µg/ml) and thiostrepton (2µM) alone or in combination for 72h, followed by media replacement for 14 days. The combination of 5-FU and thiostrepton significantly decreases colony formation in HCT116 5-FU Res cells compared to control and 5-FU alone. Data were represented as mean $\pm$ SD (n=3). ***p<0.0001.

DLD1

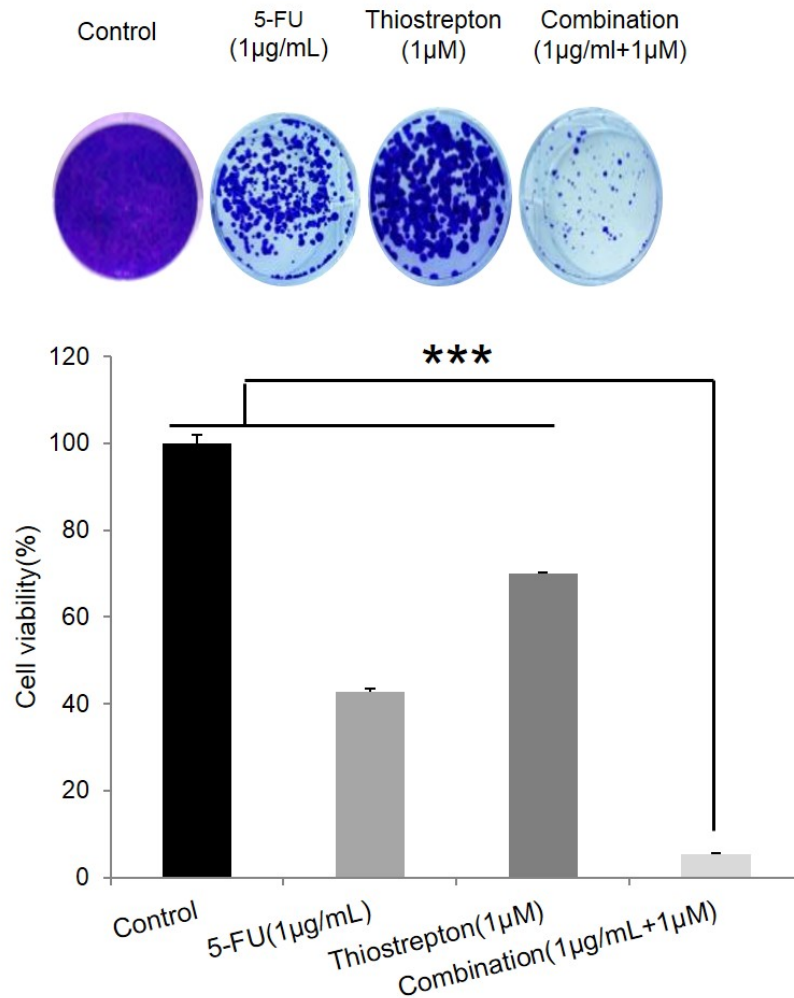


Figure 3. 34 Combination treatment decreases colony formation in DLD1 cells. Clonogenic assays showing DLD1 cells were treated with 5-FU (1 µg/ml) and thiostrepton (1 µM) alone or in combination for 72h, followed by media replacement for 14 days. The combination of 5-FU and thiostrepton significantly decreases colony formation in DLD1 compared to control, 5-FU, and thiostrepton alone. Data were represented as mean $\pm$ SD (n=3). ***p<0.0001.

HT29

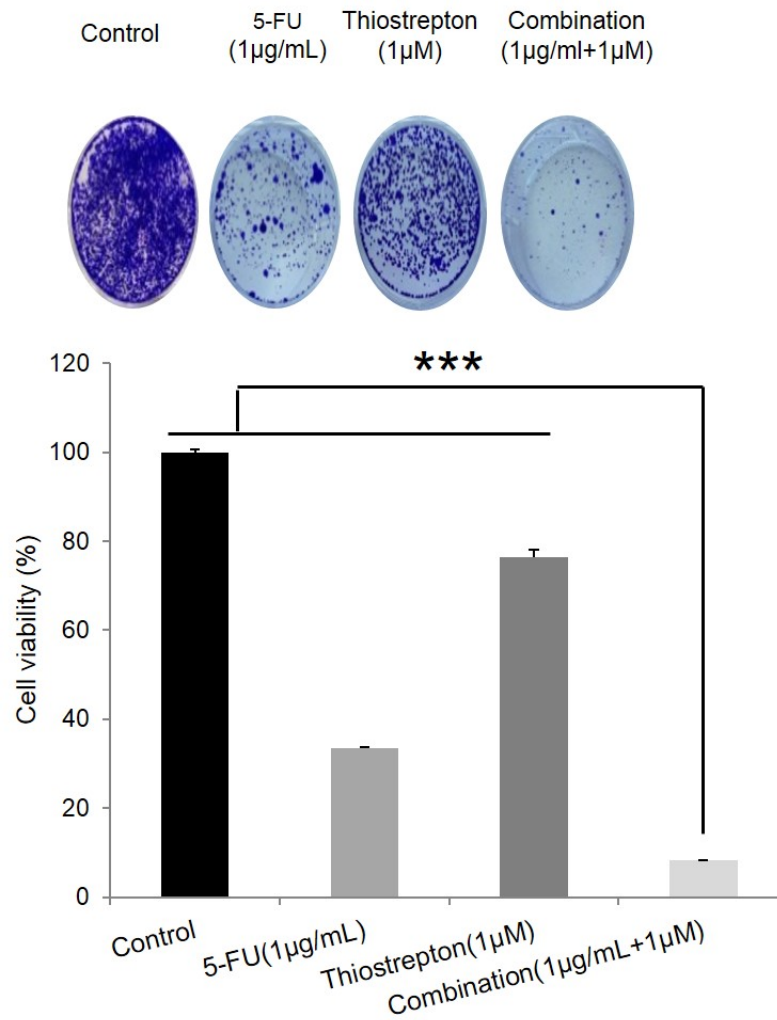


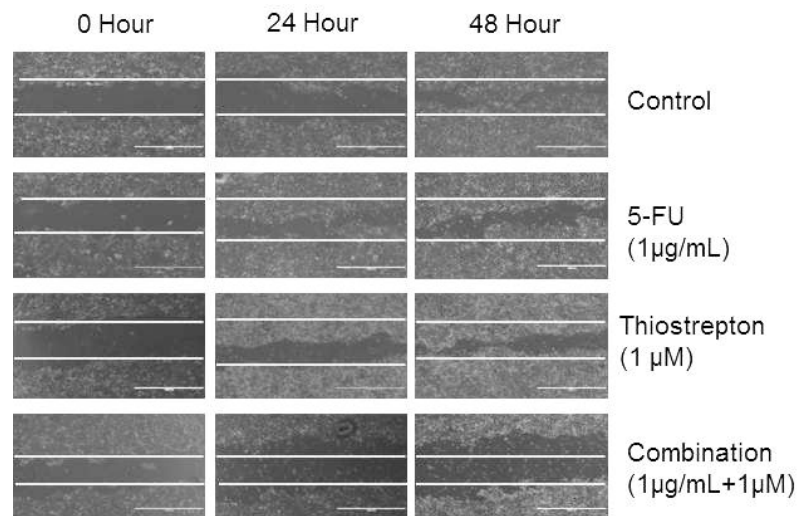
Figure 3. 35 Combination treatments decreases colony formation in HT29 cells. Clonogenic assays showing HT29 cells were treated with 5-FU (1µg/ml) and thiostrepton (1 µM) alone or in combination for 72h, followed by media replacement for 14 days. The combination of 5-FU and thiostrepton significantly decreases colony formation in HT29 compared to control, 5-FU, and thiostrepton alone. Data were represented as mean $\pm$ SD (n=3). ***p<0.0001.

3.2.9 Combination treatment inhibit the migration and transformation ability of colon cancer cells

Besides cell proliferation, FOXM1 plays a critical role in tumour metastasis (Raychaudhuri and Park 2011) and thiostrepton has been reported to inhibit cell migration and transformation in breast cancer cells through the down-regulation of FOXM1 expression (Kwok, Myatt et al. 2008). Therefore, I decided to examine the effects of 5-FU and thiostrepton on colorectal cancer cell metastasis. Cellular invasiveness and migration were analysed by wound-healing and transwell migration assays, respectively. I performed wound-healing assays on HCT116 colon cancer cells in the presence of 1µg/ml 5-FU, 1µM thiostrepton alone, or in combination using the same concentrations for 24 and 48 hours. The wound-healing assays revealed that HCT116 cells treated with thiostrepton, 5-FU, and control covered the wound effectively more than the combination treatment. These wound-healing assays suggest that the combination can indeed inhibit colon cancer cell mobility and migration in vitro (Figure 3.36 A).

To further confirm this observation, I performed migration assays and the results suggest that thiostrepton and 5-FU can significantly reduce colon cancer cell migration by 75% compared to 5-FU alone (Figure 3.36 B). This could suggest that thiostrepton inhibition of FOXM1 expression down-regulates the key FOXM1 targets involved in metastasis, MMP2 and MMP9 (Raychaudhuri and Park 2011). My ChIP seq data also revealed the association of FOXM1 with MMP1 and MMP2, and furthermore, inhibition of FOXM1 by thiostrepton significantly decreases FOXM1 binding on MMP1 and MMP2.

A



B

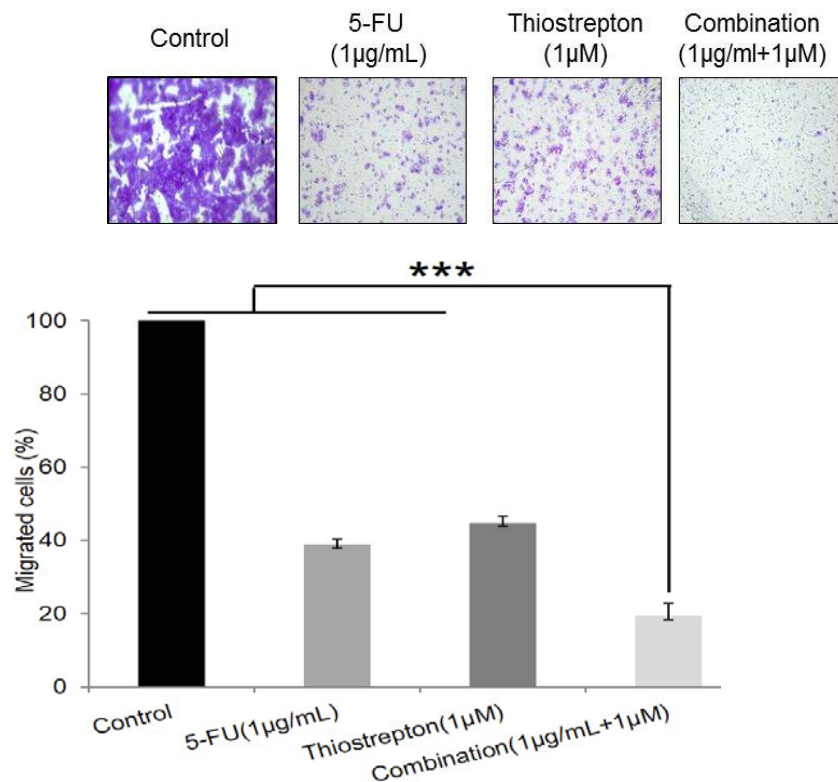


Figure 3. 36 The combination treatment reduces migration activity in HCT116 cells. A. HCT116 wt cells were seeded to complete confluence in a monolayer and a wound created by scratching firmly with a 2 µl pipette tip. Cells were then treated with 5-FU (1 µg/ml) thiostrepton (1 µM) and combination for 24 and 48 h. images taken and shown on the left. (B) 5-FU and thiostrepton combination significantly decreases migration of colorectal cancer cells, HCT116. Data were represented as mean $\pm$ SD (n=2) (5-FU alone, thiostrepton alone with combination $p < 0.0001$).

3.2.10 Thiostrepton enhances 5-FU induced caspase-dependent apoptosis in colorectal cancer cells

Next, caspase 3/7 assays were carried out to determine caspase-dependent cell death induced by 5-FU and thiostrepton alone or in combination. I found that combination treatment increased the expression of caspase3/7 activity in a time and concentration dependent manner.

I analysed caspase activity in HCT116, DLD1, HT29 and HCT116 5-FU Res cell lines. HCT116, DLD1 and HT29 cell lines were treated with 1µg/ml 5-FU, 1µM of thiostrepton, or the combination for 48 and 72 hours. In HCT116 cells, combination treatment significantly increased caspase 3/7 activity at 48 and 72 hours. But at later time points (72 hours) lesser caspase3/7 activity was observed in HCT116 cells (figure 3.37). This could be due to the length of exposure time, as some of the cells may have died already at 48h or cell death was due to necrosis or a caspase-independent pathway. In DLD1 (figure 3.38) and HT29 (figure 3.39) cells, the combination treatment induced caspase-dependent apoptosis at 48 and 72 hours, with higher caspase levels observed at 72 hours. Even at low thiostrepton concentrations, the combination treatment caspase-dependent apoptosis was evident in p53 mutant DLD1 and HT29 cells.

HCT116 5-FU Res cells were treated with different concentrations of 5-FU and thiostrepton (5-FU 1µg/ml, 2µg/ml and 4µg/ml, thiostrepton 1µM, 2µM and 4µM) for 48 hours. The results indicate that increasing concentration of these drugs is associated with increased caspase 3/7 activity in 5-FU resistant colon cancer cells. In summary, these experiments reveal that 5-FU and thiostrepton in combination significantly increased caspase-dependent apoptosis in all CRC cell lines compared

to either agent alone ($p < 0.0001$) (figure 3.40), and that thiostrepton induced apoptosis is mediated via a caspase 3/7-dependent pathway.

HCT116

- 1 Control
- 2 5-FU(1 μ g/ml)
- 3 Thiostrepton (1 μ M)
- 4 Combination (1 μ g/ml+ 1 μ M)

A

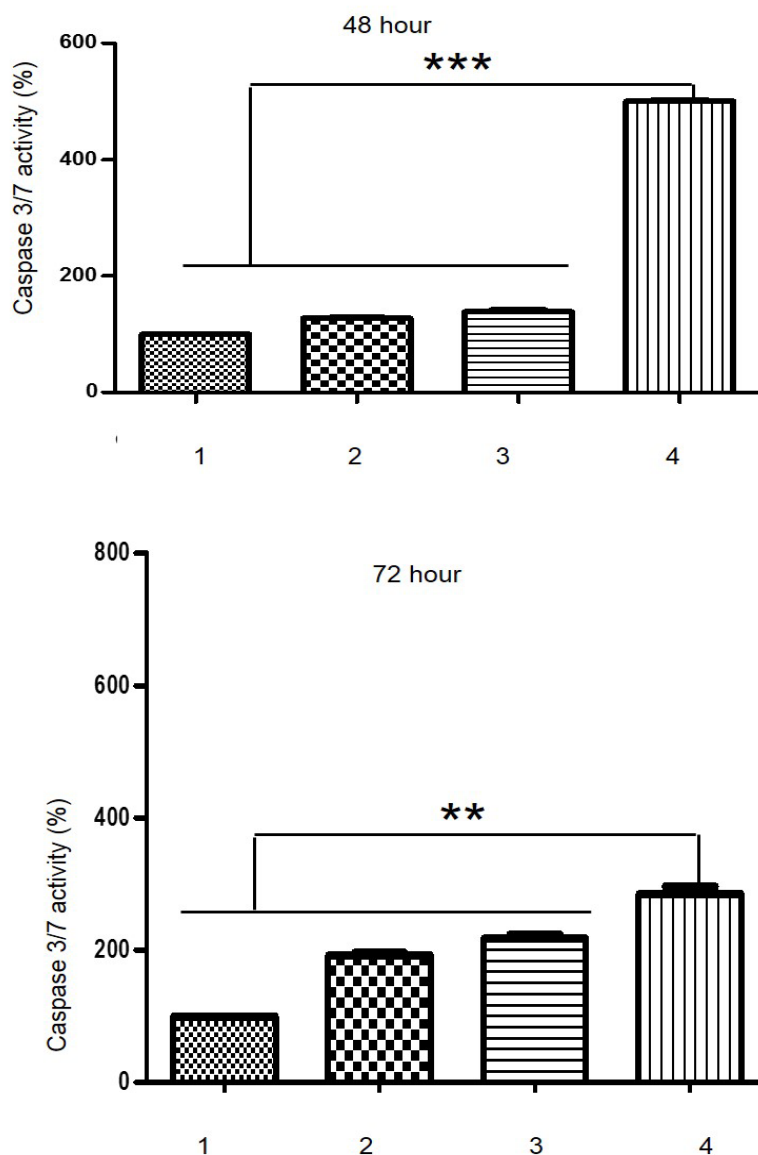
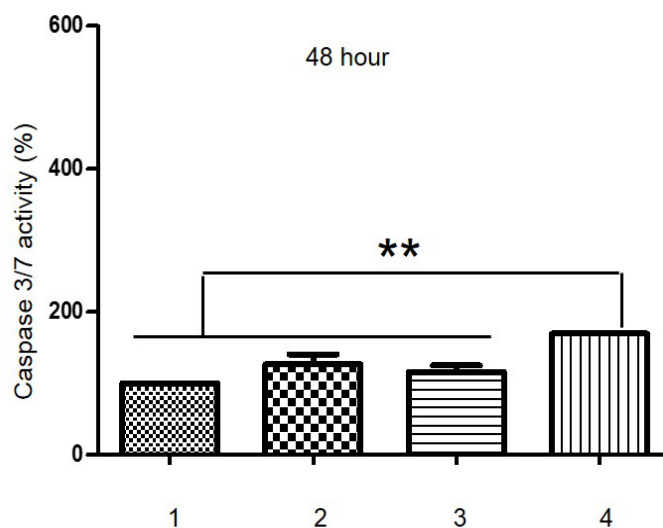


Figure 3. 37 Combination treatment induces caspase-dependent apoptosis in HCT116 cells. Cellular caspase 3/7 activity measured in HCT116 cells following treatment with 5-FU (1 μ g/ml) and thiostrepton (1 μ M) alone or in combination for 48 and 72 hours. Data were represented as mean $\pm$ SD (n=3). Figure A. 5-FU and thiostrepton combination significantly increased apoptosis compared to either agent alone or control (p<0.0001). Figure B. 5-FU and thiostrepton combination significantly increased apoptosis compared to either agent alone or control (p<0.006).

DLD1

- 1 Control
- 2 5-FU(1 μ g/ml)
- 3 Thiostrepton (1 μ M)
- 4 Combination (1 μ g/ml+ 1 μ M)

A



B

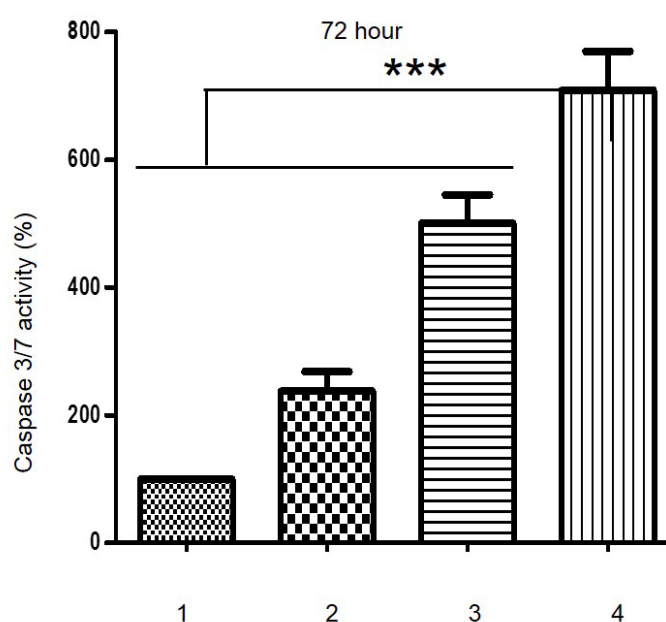
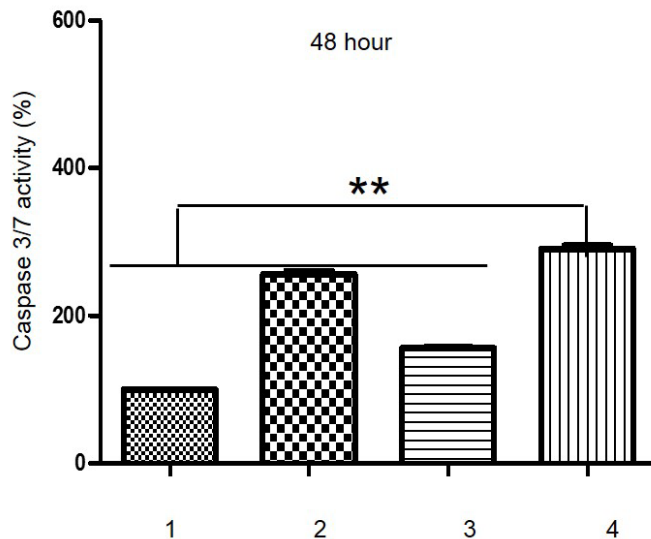


Figure 3. 38 Combination treatment induces caspase-dependent apoptosis in DLD1 cells. Cellular caspase 3/7 activity measured in DLD1 cells following treatment with 5-FU (1 μ g/ml) and thiostrepton (1 μ M) alone or in combination for 48 and 72 hours. Data were represented as mean $\pm$ SD (n=3). Figure A. 5-FU and thiostrepton combination significantly increased apoptosis compared to either agent alone or control ($p < 0.006$). Figure B. 5-FU and thiostrepton combination significantly increased apoptosis compared to either agent alone or control ($p < 0.0001$).

HT29

A



B

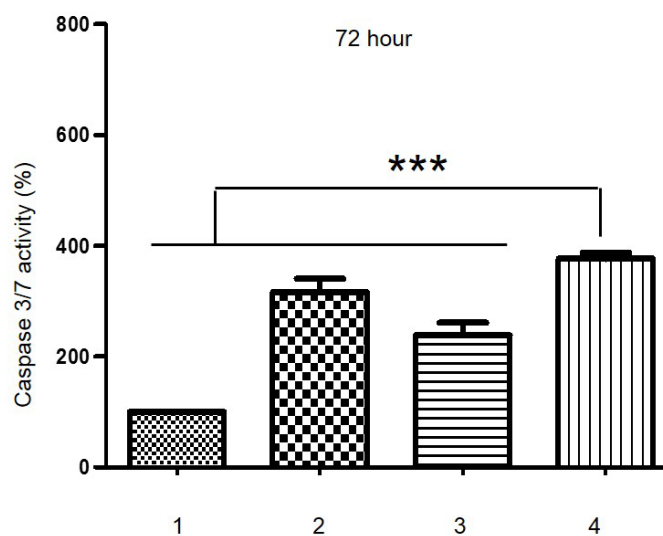
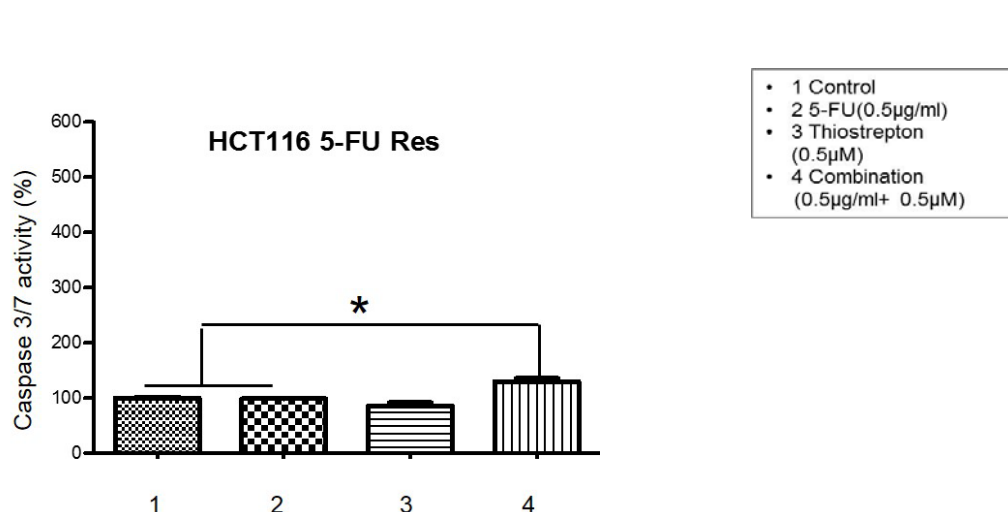


Figure 3. 39 Combination treatment induces caspase-dependent apoptosis in HT29 cells. Cellular caspase 3/7 activity measured in HT29 cells following treatment with 5-FU (1 μ g/ml) and thiostrepton (1 μ M) alone or in combination for 48 and 72 hours. Data were represented as mean $\pm$ SD (n=3). Figure A. 5-FU and thiostrepton combination significantly increased apoptosis compared to either agent alone or control (p<0.005). Figure B. 5-FU and thiostrepton combination significantly increased apoptosis compared to thiostrepton alone or control (p<0.0001).

A



B

C

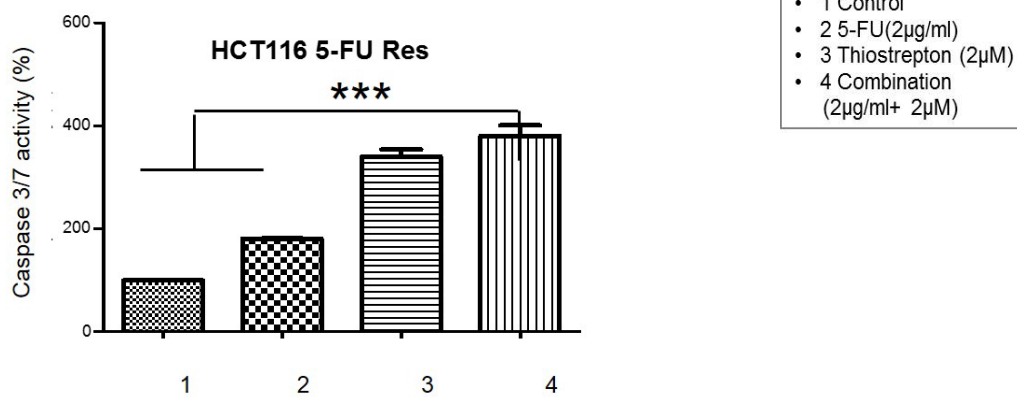


Figure 3. 40 Combination treatment induces caspase-dependent apoptosis in HCT116 5-FU Res cells. Cellular caspase 3/7 activity measured in HCT116 5-FU Res cells following treatment with 5-FU (0.5, 2, 4 µg/ml) and thiostrepton (0.5, 2, 4 µM) alone or in combination for 48 hours. Data were represented as mean $\pm$ (n=2). Figure B and C. 5-FU and thiostrepton combination significantly increased apoptosis compared to either agent alone or control ($p<0.0001$). Figure A. combination significantly increased apoptosis compared to control or 5-FU alone ($p<0.0001$).

- 1 Control
- 2 5-FU(4µg/ml)
- 3 Thiostrepton (4µM)
- 4 Combination (4µg/ml+ 4µM)

SD

3.2.11 Discussion

In my previous results section (Results 3.1) I demonstrated the critical role of FOXM1 in 5-FU drug resistance in CRC. As a natural progression, in this chapter I studied inhibition of FOXM1 by the thiostrepton in colorectal cancer. Previous studies have indicated that thiostrepton specifically inhibits FOXM1 activity and expression; it does not inhibit the activity or expression of other members of the Forkhead family (Gartel 2014, Buchner, Park et al. 2015).

I first assessed the cell viability of thiostrepton treated colon cancer cells (p53 wt HCT116, p53 mutant DLD1 and HT29) and observed that p53 wt cells are more sensitive to thiostrepton than DLD1 and HT29 cells. Thiostrepton treatment in p53 wt HCT116, p53 mutant DLD1 and HT29 cell lines significantly decreased protein and mRNA expression of FOXM1, and its downstream targets TYMS and TK-1. Down-regulation of E2F1 protein and mRNA was observed in HT29 and HCT116 cell lines, consistent with previous studies showing that thiostrepton treatment can inhibit FOXM1 and its downstream targets (Hegde, Sanders et al. 2011, Ahmed, Uddin et al. 2012, Buchner, Park et al. 2015). Interestingly, thiostrepton was initially reported to inhibit FOXM1 transcriptional activity (Bhat, Halasi et al. 2009). According to my data, this drug can inhibit both the mRNA and protein expression of FOXM1, which is in accordance with another study from the same laboratory and was explained by the discovery of a FOXM1 auto-regulatory loop whereby FOXM1 binds to its own promoter (Halasi and Gartel 2009).

Moreover, acquired 5-FU resistant HCT116 cells demonstrated the same IC₅₀ as the parental HCT116 cells with thiostrepton. This could demonstrate that thiostrepton mediated inactivation of FOXM1 can help to reverse 5-FU resistance and targeting

FOXM1 could potentially be a useful therapeutic strategy for overcoming the resistance to 5-FU.

Inhibition of FOXM1 binding by thiostrepton was seen in ChIP experiments in HCT116, DLD1, and HCT116 5-FU Res cells, confirming the importance of the FOXM1 transcription factor in the regulation of 5-FU targets such as: TYMS, TK-1, TYMP, E2F1/2/3, matrix metalloproteinases genes MMP1 and MMP2, and the MMR gene MLH1. My data also supports a direct mechanism of action whereby thiostrepton blocks the binding of FOXM1 to target DNA, preventing the normal activation of FOXM1 downstream effectors.

Furthermore, synergistic effects were identified in this study when thiostrepton was combined with 5-FU. Specifically, I found that the combination treatment could significantly increase γ H2AX foci in colon cancer cell lines, including p53 mutant and acquired 5-FU resistant cells. This may indicate that thiostrepton enhances 5-FU mediated DNA damage by inhibiting FOXM1 and its genomic targets involved in DNA repair pathways, which is in agreement with a previous study showing higher levels of γ H2AX staining in FOXM1^{-/-} MEFs compared with WT MEFs treated with epirubicin treatment or γ -irradiation. The same group found that FOXM1 is required for DNA repair by homologous recombination (HR), and also identified BRIP1 as a direct transcription target of FOXM1 an important factor for DSB repair (Monteiro, Khongkow et al. 2013).

In addition, the FACS data demonstrate that combination treatment significantly increases the G0 (sub G1) population, indicative of cell death and apoptosis in colon cancer cells. This suggests that FOXM1 contributes to 5-FU resistance in colon cancer, and inhibition of FOXM1 by thiostrepton down-regulates FOXM1 and its target regulatory genes involved in cell cycle progression leading to increases in cell

cycle arrest and cell death. These results support a study in renal cell carcinoma where knock-down of FOXM1 decreased cell cycle regulatory genes such as cyclin B1, cyclin D1, and cyclin-dependent kinase 2 (Cdk2) and caused accumulation of cells in the G0-G1 phase and a decrease in the S phase compared with control (Xue, Xiao et al. 2012). Another study on FOXM1 deficient mice shows the importance of FOXM1 in the regulation of many cell cycle proteins, including cyclin A, cyclin B1, cdc2, cdk2, p27 and the Rb family members p107 and p130 (Xue, Chiang et al. 2010). A different study on ovarian cancer cells also indicates knock-down of FOXM1 increase G0 (sub G1) population (Zhao, Siu et al. 2014). Furthermore, I found that combination treatment significantly increases caspase-dependent apoptosis in both p53 wt and p53 mutant colorectal cancer cell lines, in agreement with a previous studies where thiostrepton treatment induced p53 independent apoptosis in human cancer cells (Pandit and Gartel 2011).

In terms of long-term survival of tumour cells, the colony formation assays revealed that combination treatment strongly suppressed the ability colon cancer cells to form colonies compared to either drug alone or control. The combination of 5-FU and thiostrepton also significantly reduces migration in colon cancer cells, in accordance with a study showing that thiostrepton represses FOXM1 expression and down-regulates FOXM1 targets, MMP2 and MMP9. MMPs are crucial in the process of tumour invasion and metastasis (Ahmed, Uddin et al. 2012). My ChIP-seq data also showed a strong association between FOXM1 and MMPs.

In summary, my studies demonstrated for the first time that thiostrepton can exert synergistic effects with 5-FU and that combination treatment decreases cell viability, migration, and increases DNA damage and apoptosis in colon cancer cells. Thiostrepton can down-regulate FOXM1 expression and induce cytotoxicity in p53

mutant and acquired 5-FU Res cell lines. My data shows the benefit of thiostrepton in combination with 5-FU treatment, which could potentially allow us to target the p53 mutant and acquired drug resistant phenotype.

3.2.12 Future work

My first step in continuing this work would be to carry out thiostrepton treatment on HCT116 5-FU Res cells and examine FOXM1, E2F1, TYMS and TK-1 expression. I would also like to do migration assays in the same cells. Finally, I would like to investigate the effect of the combination of thiostrepton and 5-FU on in vivo CRC models using parental HCT116, HCT116 5-FU Res, DLD1, and HT29 xenografts.

RESULT 3.3

ROLE OF E2F1 IN 5-FU DRUG RESISTANCE

3.3.1 Introduction

E2F1, E2F2, and E2F3 belong to a subclass of E2F transcriptional activators that are important for progression through the G1/S phase. Loss of one of the E2F family transcription activators can be functionally compensated by other related members (Wu, Timmers et al. 2001). E2F transcription activators can specifically interact and are regulated by Rb. (Lees, Saito et al. 1993, Leone, DeGregori et al. 1998). During G0 and G1, Rb binds and inhibits the activity of E2Fs, as cells progress through G1, Rb gets phosphorylated, which then dissociates and activates E2F complexes during late G1 phase (Wells, Yan et al. 0000).

Therefore the E2F family of transcription factors play a crucial role in the control of cell cycle progression and regulate the expression of genes required for G1/S transition (Genovese, Trani et al. 0000). The regulatory role of E2Fs are complex due to the dual role of E2F1 in apoptosis and cellular proliferation, by controlling the transcription of a number of genes involved in G1/S transition, DNA replication, including the main targets of 5-FU, TYMS and TK-1 (Pan, Yin et al. 1998, Bertino and Banerjee 2003, Liu, Luo et al. 2004).

Earlier studies show that over expression of E2F2 or E2F3 cannot induce apoptosis, but over-expression of E2F1 can induce apoptosis; in addition combined over-expression of E2F1, and E2F2 or E2F1 and E2F3 led to apoptosis (DeGregori, Leone et al. 1997). Experiments using primary fibroblasts found that E2F1 expression is unable to induce cell proliferation except when combined with pRB or p53 mutations (Lin, Lin et al. 2001). Rb contains two binding sites for E2F proteins. One binding site (the G binding site) contacts E2F1, E2F2, E2F3, and E2F4, while the other (c-terminal, the S binding site) is exclusively used for interaction with E2F1, and acts as a potent inhibitor of E2F1 induced apoptosis (Dick and Dyson 2003).

Later studies found that activation of E2F2 or E2F3 can also induce apoptosis but E2F1 is still required for initiation of apoptosis (laquinta and Lees 2007). This suggests that E2F1 is the sole member of the E2F family that can directly stimulate apoptosis and that E2F2 and E2F3 act in an indirect manner by promoting the expression of E2F1 (laquinta and Lees 2007). Another report demonstrates that E2F1 induced apoptosis is mediated through both p53-dependent and p53-independent pathways (Wu and Levine 1994, Phillips, Bates et al. 1997). One example of E2F1 p53- dependent apoptosis involves stabilisation of p53 by E2F1 through the activation of ARF which then enhances the apoptosis (Ginsberg 2002).

A more recent study shows that increased levels of HDM2 and E2F1 following treatment with a p53 activating derivative, JNJ-26854165 in p53 mutant leukaemia cells. E2F1 target genes NOXA, PUMA, p73, p21^{Cip} and SIP were found to be up-regulated in this experiment. Knock-down of E2F1 in U937 cells significantly reduced apoptosis induced by JNJ-26854165. This study also confirms that p53 independent apoptosis may not be directly associated with S-phase delay (Kojima, Burks et al. 2010).

It has been reported that E2F activators mediate cell cycle progression by two mechanisms: first, they can directly activate cell-cycle dependent gene expression, and second, they can inhibit p21^{Cip1} protein accumulation, thereby promoting Rb phosphorylation and initiate the expression of Rb-E2F target genes (Wu, Timmers et al. 2001).

In this chapter I have investigated role of E2F transcription activators in FOXM1, and TYMS, regulation and 5-FU drug resistance. I have also studied the role of E2F1 in 5-FU mediated apoptosis.

3.3.2 Role of E2F1 in TYMS regulation

To confirm the previous studies, which showed that TYMS is directly targeted by E2F1, I studied the expression of TYMS in an established model system used frequently in our laboratory: MCF7 Doxorubicin resistant cells (figure 3.41). The doxorubicin-resistant MCF7 derived cells, (MCF7 Dox^R) were previously generated in Prof. Eric Lam laboratory by exposing MCF7 cells to increasing concentrations of doxorubicin. MCF7 Dox^R cells were transfected with E2F1 specific SiRNA and nonspecific SiRNA was used as a control. Cells were harvested 48 hours post-transfection. Western blot analysis confirmed efficient knock-down of E2F1 and significant decreases in TYMS protein expression. This supports previous studies (DeGregori, Kowalik et al. 1995) showing the role of E2F1 in TYMS regulation.

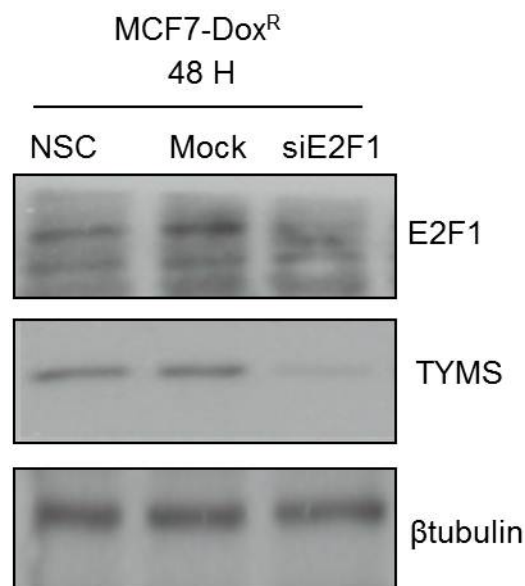


Figure 3. 41 E2F1 regulates TYMS.
Western blot analysis of MCF7-Dox^R transfected with SiRNA specifically targeting E2F1 and NSC (non-specific SiRNA) 48 hours post-transfection, this was performed to confirm efficient E2F1 knock-down and study the effects downstream on TYMS expression.

3.3.3 E2F transcription activators regulate TYMS and TK-1 in HCT116

I next focused on colon cancer cells to study the role of the E2F1 in FOXM1 and TYMS mediated 5-FU drug resistance. Along with E2F1, I also studied the role of E2F2 and E2F3 in FOXM1 and TYMS regulation.

HCT116 cells were transiently transfected with SiE2F1, or SiE2F2, or SiE2F3, or combination of all three. The controls were transfected with non-specific siRNA (NSC). Cells were harvested 48h post transfection (figure 3.42). Knock-down of E2F1 was associated with significant decreases in the protein expression of FOXM1 and E2F3 and a smaller but yet significant decrease in TYMS and TK-1 protein expression. Transient transfection of SiE2F3 led to broadly similar results, although no effect was observed on TK-1.

Knock-down of E2F2 was not successful, as no decrease in E2F2 or the other proteins were observed. I have repeated the experiment with different transfection reagents (lipofectamine), and increased the transfection time and amount of SiRNA to achieve the efficient knock-down. The first E2F2 antibody used was E2F2 C-20 (detect C-terminus of E2F-2 of human origin). As per the suggestion by Dr Ashish Lal, National Cancer Institute, Bethesda, Maryland, E2F2 protein is not very easy to detect because the antibody is not very sensitive. According to their suggestions, I then purchased E2F2 (TFE-25) antibody. Unfortunately, none of these methods were able to deliver an efficient knock-down in E2F2 protein and no changes were observed in its potential down-stream targets.

RT-PCR was performed to investigate the mRNA levels of E2F1, E2F2, E2F3, TYMS, TK-1 and FOXM1 in HCT116 SiE2F1, SiE2F2, and SiE2F3 cells (figure 3.43). Surprisingly, mRNA expression of E2F2 in HCT116 SiE2F2 cells decreased, as did the mRNA expression of E2F1, E2F3, TYMS, TK-1 and FOXM1 (figure 3.43). Efficient knock-down was observed in E2F2 mRNA but not in protein, this could be due to the high stability E2F2 protein.

Similar to E2F2, deletion of E2F3 was associated with reductions in the mRNA expression of E2F1, E2F2, TYMS, TK-1 and FOXM1, and SiE2F1 led to a reductions in the mRNA expression levels of E2F2, E2F3, TYMS, TK-1 and FOXM1 (figure 3.43). Triple knock-down of E2F transcription activators was not significantly superior to SiE2F1 in decreasing FOXM1, TYMS and TK-1 mRNA expression (figure 3.43).

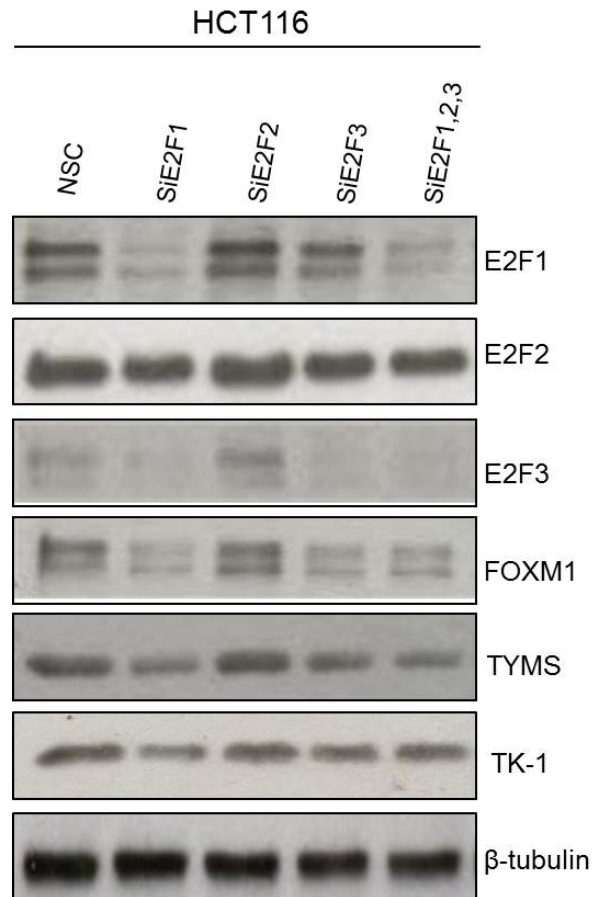


Figure 3. 42 E2F transcription activators regulate FOXM1, TYMS, and TK-1 in HCT116 cells.

HCT116 cells were transfected with siRNA E2F1, E2F2, E2F3, and triple knock-down of E2F1/2/3. All siRNA transfections were optimised and transfections were mediated by oligofectamine following the manufacturer's protocol. After 48h cells were harvested and analysed by western blot to determine protein expression of FOXM1, E2F1, E2F2, E2F3, TYMS and TK-1. β -tubulin was used as a loading control. The western blot results show that the knock-down of E2F1 and E2F3 reduces TYMS protein level, knock-down of E2F1 reduces TK-1 protein expression.

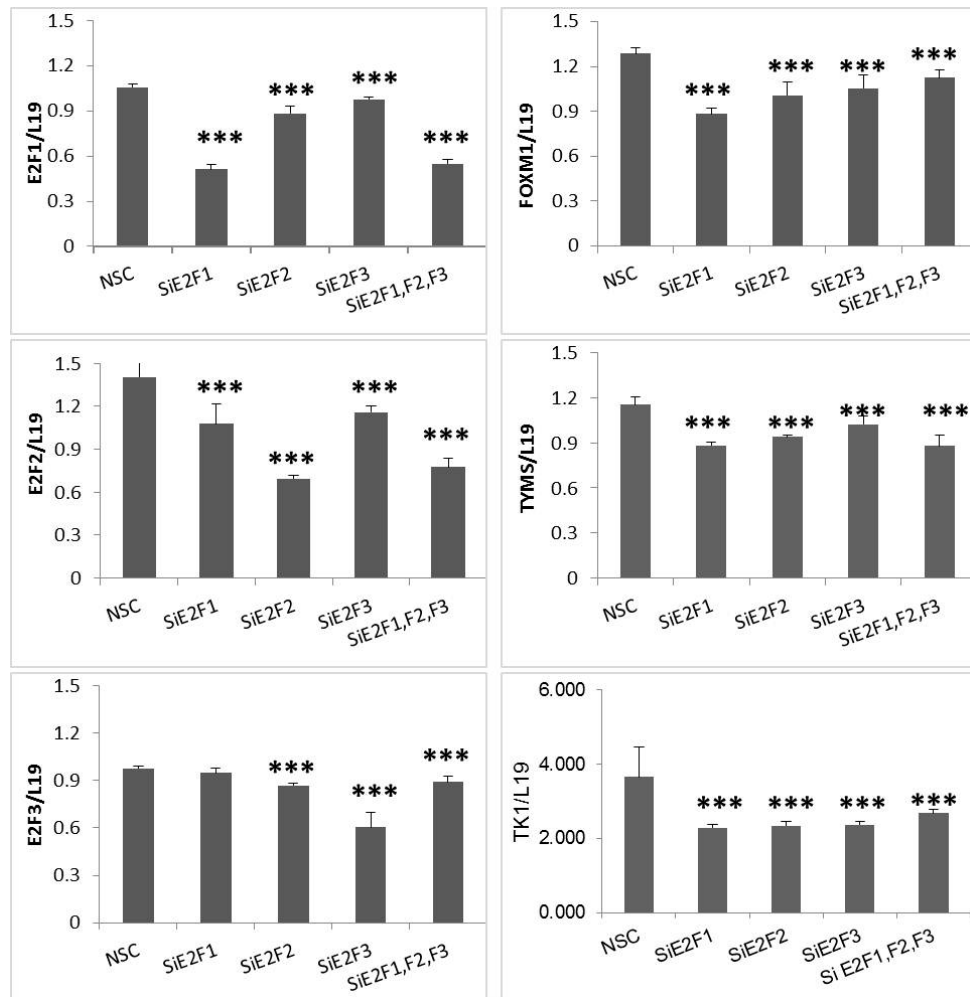


Figure 3. 43 E2F transcription activators regulate FOXM1, TYMS and TK-1. HCT116 cells were transfected with siRNA E2F1, E2F2, E2F3, and triple knock-down of E2F1, E2F2, and E2F3. All siRNA transfections were optimised and transfections were mediated by oligofectamine following the manufacturer's protocol. After 72h total RNA was extracted using the RNeasy mini kit. Quantitative real-time PCR (RT-PCR) was used to determine mRNA level of FOXM1, E2F1, E2F2, E2F3, and TYMS. A housekeeping gene L19 was used as an internal control to normalize input complementary DNA. This experiment was done in triplicate. Error Bars represent standard deviation. Statistical significance was determined by student's T-test (p value < 0.05 versus control).

Further to the knock-down experiments, HCT116 cells were transiently transfected with pCMVHA E2F1, pCMVHA E2F2 and pCMV-Neo-Bam E2F3 plasmids (figure 3.44). Over-expression of E2F1, E2F2, and E2F3 was associated with an increase in FOXM1 and TYMS protein levels, most notable for E2F1, and strong decreases in the cell cycle regulatory protein p21^{Cip} were also observed. Down-modulation of p21^{Cip} leads to repression in cell cycle regulatory genes and uncontrolled S phase entry. The status of pRb associated with E2F transcription activators was also measured, as pRb has a central role in the regulation of S-phase entry through the ability to repress E2F activity (Burkhart and Sage 2008). My experiments show over-expression of E2F1 increases the protein expression of E2F2 and E2F3. E2F2 increases the protein level of E2F1 and E2F3 and over-expression of E2F3 increases E2F1 protein but not E2F2.

Consistent with the western blot results, RT-PCR results show that over-expression of E2F1, E2F2, and E2F3 upregulate the mRNA expression of TYMS, TK-1 and FOXM1 (figure 3.45). In addition to this, over-expression of E2F1 increased the mRNA expression of E2F2 and E2F3, E2F2 increases the mRNA level of E2F1 and E2F3. But, unlike protein expression, over-expression of E2F3 shows an up-regulation in both E2F1 and E2F2 at the mRNA level. Even though the over-expression of E2F3 is not showing any changes in E2F2 protein, an increase in mRNA expression was observed. This could be due to the high protein stability of E2F2 or by the positive feedback mechanism of the other E2F transcription activators, which could help to increase the expression of E2F2 in mRNA level, or a suboptimal antibody. The results support a previous study where loss of one of the E2F family transcription activators can be functionally compensated by other related members (Wu, Timmers et al. 2001). These transient transfection data therefore

suggest that TYMS and TK-1 could be the targets of E2F transcription activators, my results confirm a previous study showing that FOXM1 is regulated by E2F1 (de Olano, Koo et al. 2012). My results also indicate, in addition to E2F1, E2F2 and E2F3 might also regulate FOXM1 and that the transcription activators E2F1, E2F2 and E2F3 can closely regulate each other.

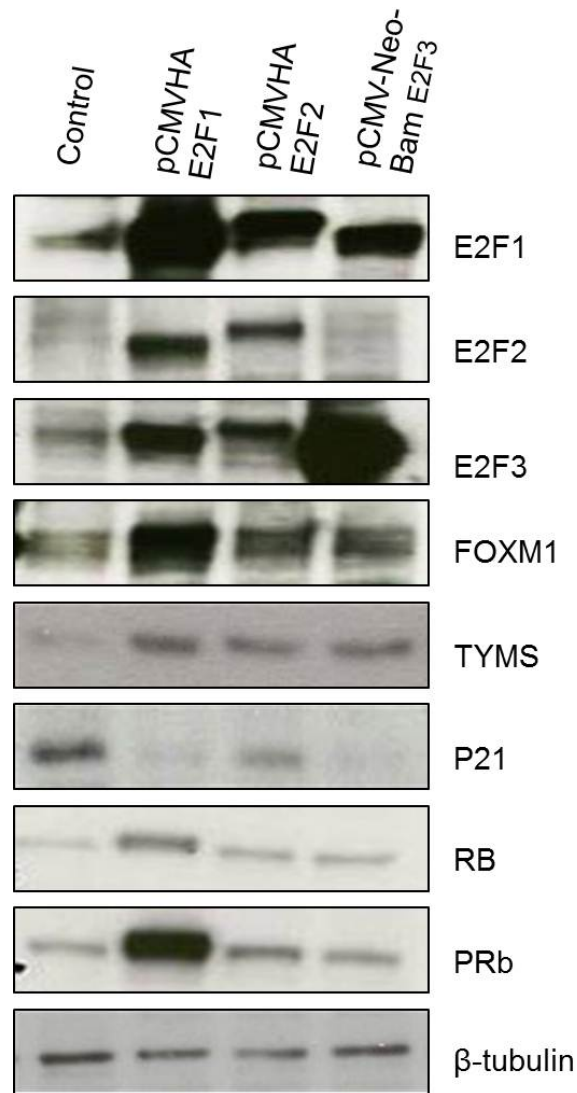


Figure 3. 44 HCT116 cells transfected with E2F1, E2F2 and E2F3 plasmids. All transfections were optimised and mediated by X-treme GENE HP DNA transfection reagent following the manufacturer's protocol. After 48h of transfection, cells were harvested and analysed by western blot to determine protein expression of FOXM1, E2F1, E2F2, E2F3, TYMS, p21^{Cip}, Rb and PRb. β-tubulin was used as a loading control. Over-expression of E2F1 and E2F3 reduces p21^{Cip} expression. Over-expression of E2F1 increases E2F3 expression and similarly, over-expression of E2F3 increases E2F1. Over-expression of E2F1, E2F2 and E2F3 led to an increase in TYMS protein and FOXM1 protein, most notable for pCMVHA E2F1 transfected cells.

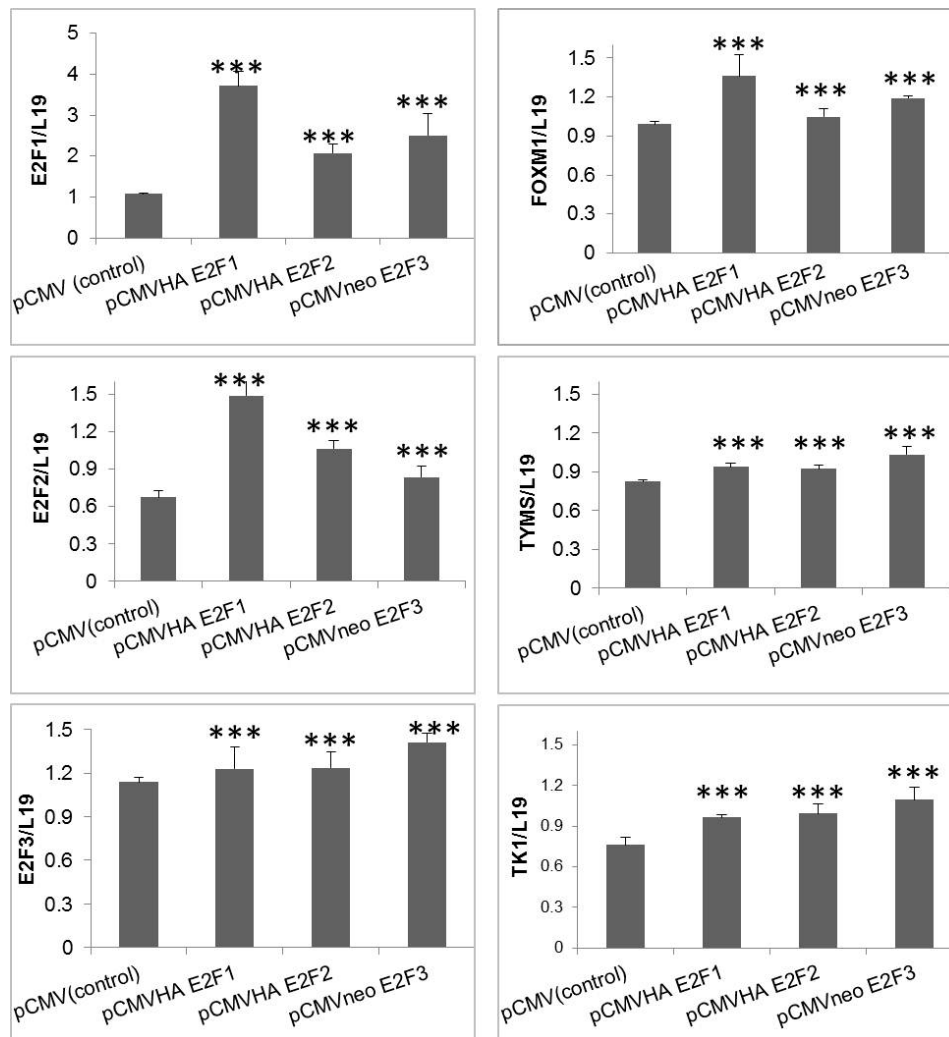


Figure 3. 45 HCT116 cells transfected with E2F1, E2F2 and E2F3 plasmids. All transfections were optimised and mediated by X-treme GENE HP DNA transfection reagent following the manufacturer's protocol. After 48 h of transfection, total RNA was extracted using the RNeasy mini kit. Real-time PCR (RT PCR) was used to determine mRNA level of FOXM1, E2F1, E2F2, E2F3, TK-1 and TYMS. A housekeeping gene L19 was used as an internal control to normalise input complementary DNA. This experiment was done in triplicate. Error Bars represent standard deviation. Statistical significance was determined by student's T-test (p value < 0.05 versus control).

3.3.4 Silencing E2F1 in HCT116 cells increases chemo-sensitivity

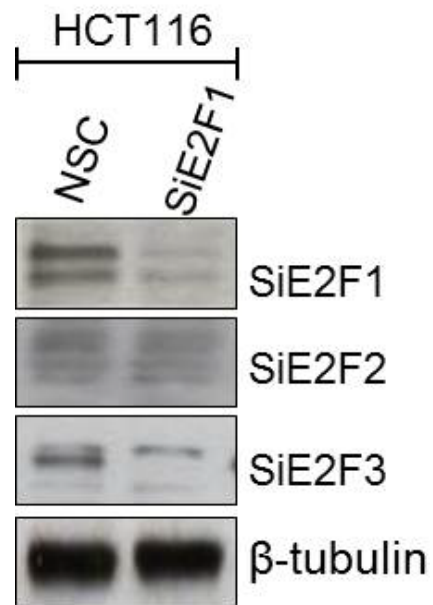
Previously it has been demonstrated that at least one member of the E2F family, E2F1, can induce apoptosis through a number of p53 dependent and independent mechanisms. I next performed SRB assays on HCT116 wt cells to investigate the role of E2F transcription activators in cell viability and 5-FU sensitivity (figure 3.46). The result shows that silencing of E2F1 increases cytotoxicity in p53 wt HCT116 cells (figure 3.46 B). In contrast, inhibition of E2F2 (figure 3.46 C) and E2F3 (figure 3.46D) didn't enhance 5-FU chemo-sensitivity. The role of E2F1 in cell cycle regulation and DNA synthesis has been studied comprehensively (dos Reis Vasques, Pujiz et al. 2010). My results could suggest that knock-down of E2F1 might decrease cell cycle progression in p53 wt HCT116 cells.

I next analysed the cell cycle phase distribution of HCT116 wt cells followed by knock-down of E2F1, E2F2 and E2F3 and exposure to 1µg/ml of 5-FU over a time course of 72h. Little effect on cell cycle progression was observed in untreated SiE2F1, SiE2F2 and SiE2F3 cells compared to untreated NSC (nonspecific control) cells, (left panel of figure 3. 44).

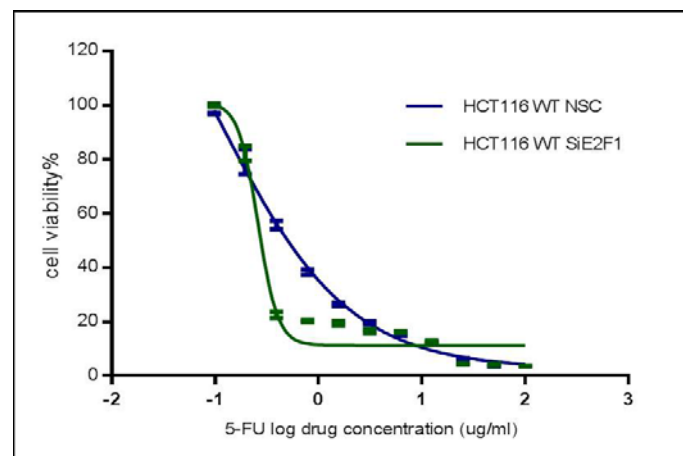
In 5-FU treated HCT116 NSC cells, G2/M arrest occurred after the 72 h exposure to 5-FU, evidenced by a larger fraction of cells in the G2/M phase (right panel figure 3.47), and in E2F1 deleted HCT116 wt cells decreases in S phase, G2/M phase and an increase in the population of cells in G0 (sub-G1), indicative of cell death and apoptosis (right panel figure 3. 47) were observed. As expected, no significant amount of cell death was observed in E2F2 and E2F3 deleted cells but an increased number of cells in S-phase and fewer number of cells in G2/M was observed compared to the control (right panel figure 3. 47). This could suggest that inhibition of E2F1 in HCT116 cells leads to release of arrested cells at the G1/S phase and

progression through S phase of the cell cycle and cell death and apoptosis in which is consistent with cell viability data showing a decrease in viability in E2F1 knock-down cells. Western blot analysis confirms the knock-down of E2F transcription activators (figure 3.47 A). As I mentioned earlier, E2F2 knock-down was unreliable at the protein level.

A



B



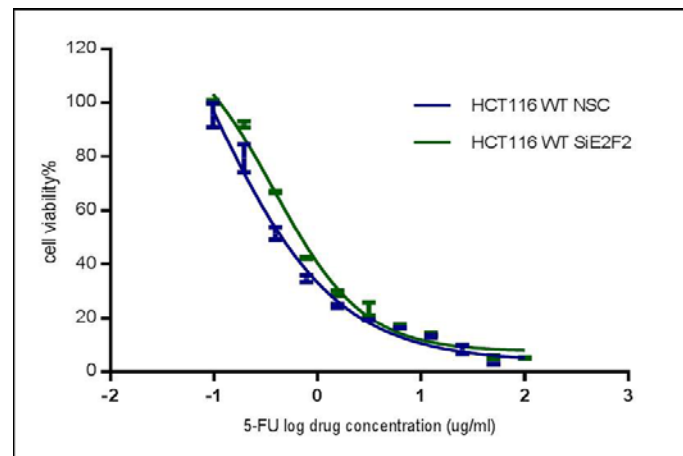
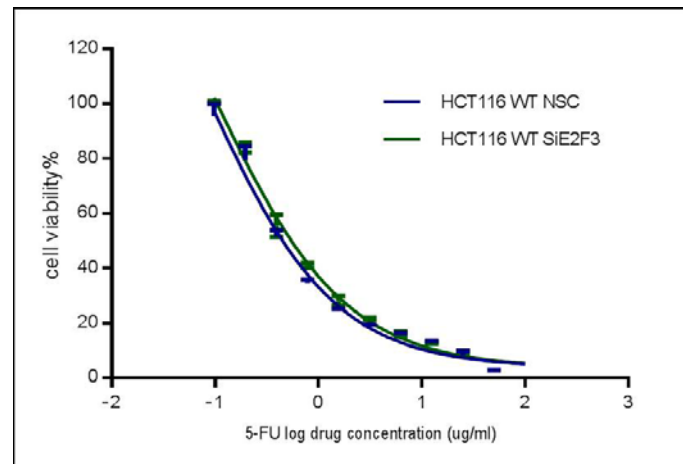
C**D**

Figure 3. 46 Effect of 5-FU in E2F transcription activators in HCT116 WT cells.

HCT116 cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with, NSC, SiE2F1, SiE2F2 and SiE2F3. Cells were seeded in to 96 well plates. 48h after the transfection, cells were harvested and seeded into 96 well plates and exposed to various concentrations of 5-FU for 72h for SRB assays. The rest of the cells were seeded into 6 well plates for FACS analysis. A) Western blot analysis confirming knock-down of E2F transcription activators. B) knock-down of E2F1 increases chemo-sensitivity to 5-FU (HCT116 SiE2F1 IC₅₀-0.27, HCT116 NSC IC₅₀-0.48 μ g/ml) but knock-down of E2F2 (C) and E2F3 transcription activator (D) did not make significant changes in IC₅₀ compared to control.

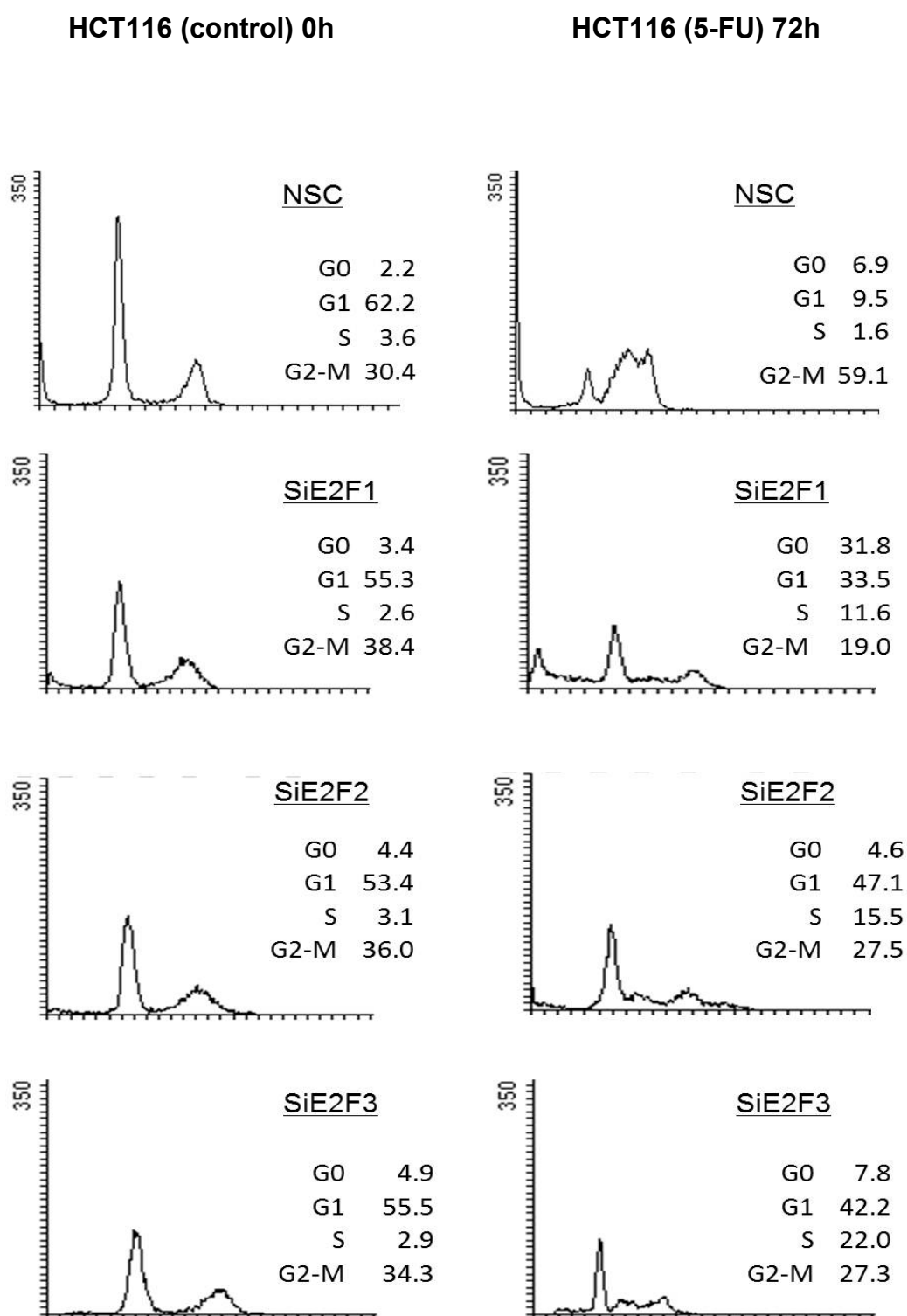


Figure 3. 47 Effect of 5-FU in E2F transcription activators in HCT116 WT cells. HCT116 cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with, NSC, SiE2F1, SiE2F2 and SiE2F3. Cells were seeded in to 6 well plates, followed by exposure to $1 \mu\text{g/ml}$ of 5-FU for 72h for FACS analysis. Cells were harvested and fixed in 70% ethanol and stained with PI. Significant amounts of cells in G0 are observed in HCT116 SiE2F1 cells treated with 5-FU cells compared to non-specific control or untreated control.

3.3.5 Silencing E2F1 in p53 mutant cells decreases chemo-sensitivity

In order to establish whether E2F transcription activators are required for 5-FU resistance in p53 mutant cells, I then continued to knock-down E2F transcription activators in HT29 p53 mutant cells and treated with 5-FU (figure 3.48). Surprisingly, SRB assays show that SiRNA mediated knock-down of E2F1 in HT29 cells are more resistant to 5-FU than HT29 NSC (figure 3.48 B). The IC₅₀ of HT29 control is 3.46µg/ml and HT29 SiE2F1 is 6.16 µg/ml, suggesting that E2F1 could be functioning as one of the main regulators of apoptosis in p53 mutant cells, and that inhibition of E2F1 reduces the amount of apoptosis in the face of DNA damage.

Similar to p53 wt cells, no effect was seen with either SiE2F2 or SiE2F3 knock-down in HT29 cells (figure 3. 48 C, D). Further to the SRB assays, FACS was performed to detect the cell cycle distribution in E2F1, E2F2 and E2F3 knock-down HT29 cells. No significant changes were observed in the SiE2F1, SiE2F2 and SiE2F3 untreated cells compared to untreated nonspecific control (left panel of figure 3. 49).

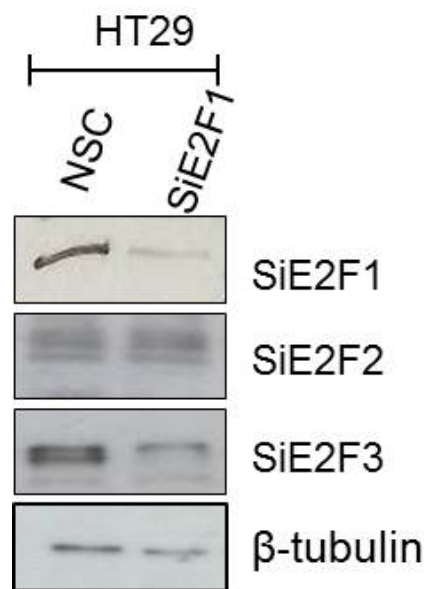
In HT29 NSC, (right panel of figure 3.49) cells in G1 phase increased from 51% (untreated) to 68% and G2-M decreased from 31.4 % (untreated) to 14.1% after 72h exposure to 5-FU.

Likewise, in HT29 SiE2F1 (right panel of figure 3.49) the proportion of cells in G1 phase increased from 55% (untreated) to 68% after 72h of exposure with 5-FU, 53% SiE2F2 untreated to 62% SiE2F2 5-FU treated and 53% SiE2F3 untreated to 62% SiE2F3 5-FU treated, were observed (right panel figure 3. 49). Small increases were observed in the number of cells in S-phase compared to the respective untreated control, and approximately a 50% reduction in the number of cells in G2/M

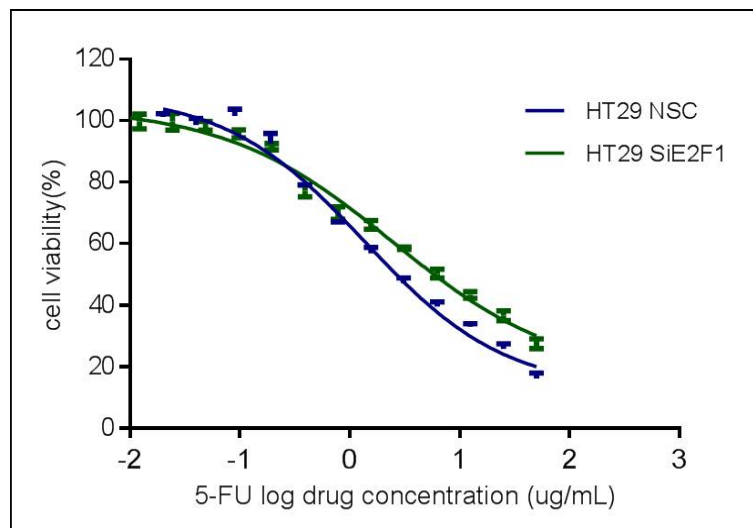
phase is was seen (right panel figure 3. 49), generally the number of cells in G0 phase (sub G1) increased following 5-FU treatment.

This suggests that release of the arrested cells at the G1/S phase and that cells are progressing slower through the S phase of the cell cycle, and reaffirms the notion that the inhibition of E2F1 activity could not sensitise p53 mutant HT29 cells to 5-FU treatment as no significant amount of cell death was noted in 5-FU treated E2F1, E2F2 and E2F3 deleted cells. Western blot analysis confirms that the knock-down of E2F transcription activators was successful apart from E2F2 knock-down. (Figure 3. 48 A), which was explained previously.

A



B



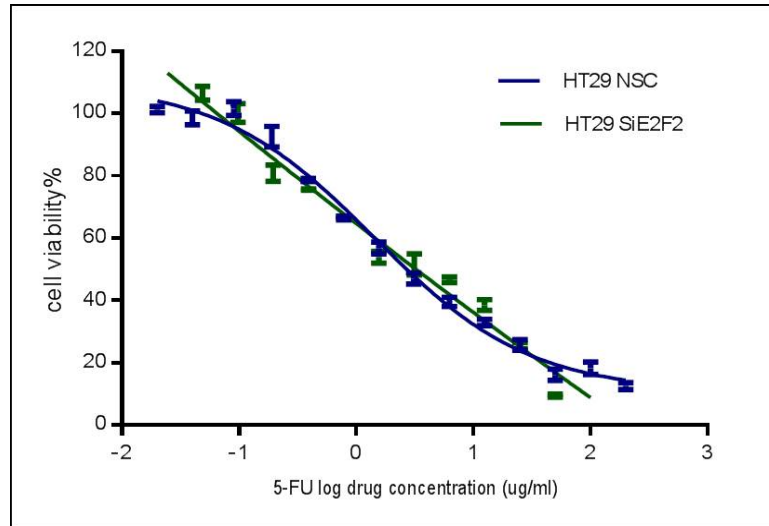
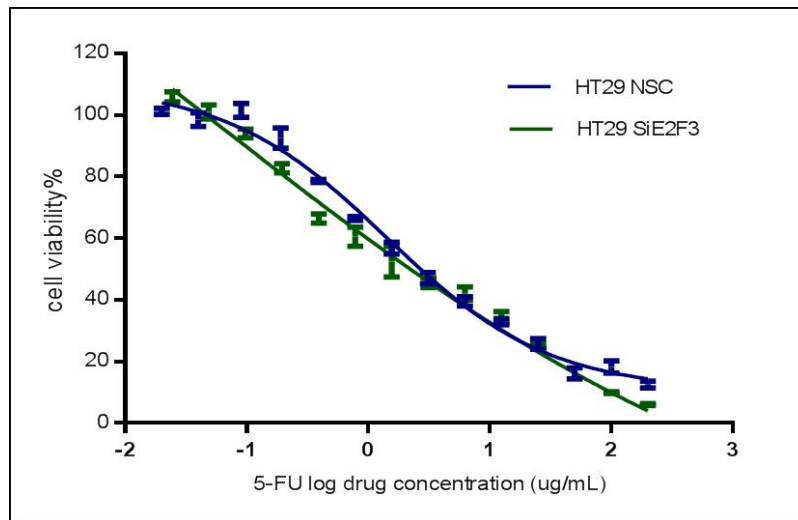
C**D**

Figure 3. 48 Effect of 5-FU on E2F transcription activators in HT29 cells.

HT29 cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with NSC, SiE2F1, SiE2F2 and SiE2F3. Cells were seeded in to 96 well plates. 48h after transfection, cells were harvested and seeded in to 96 well plates and exposed to various concentrations of 5-FU for 72 hours prior to SRB assays. The rest of the cells were seeded into 6 well plates for FACS analysis. Figure A. Western blot analysis confirms knock-down of E2F transcription activators. Knock-down of E2F1 transcription activators did not decrease cell viability to 5-FU treatment compared to NSC control (B), and no significant changes were seen following knock-down of E2F2 (C) and E2F3 (D) compared to control.

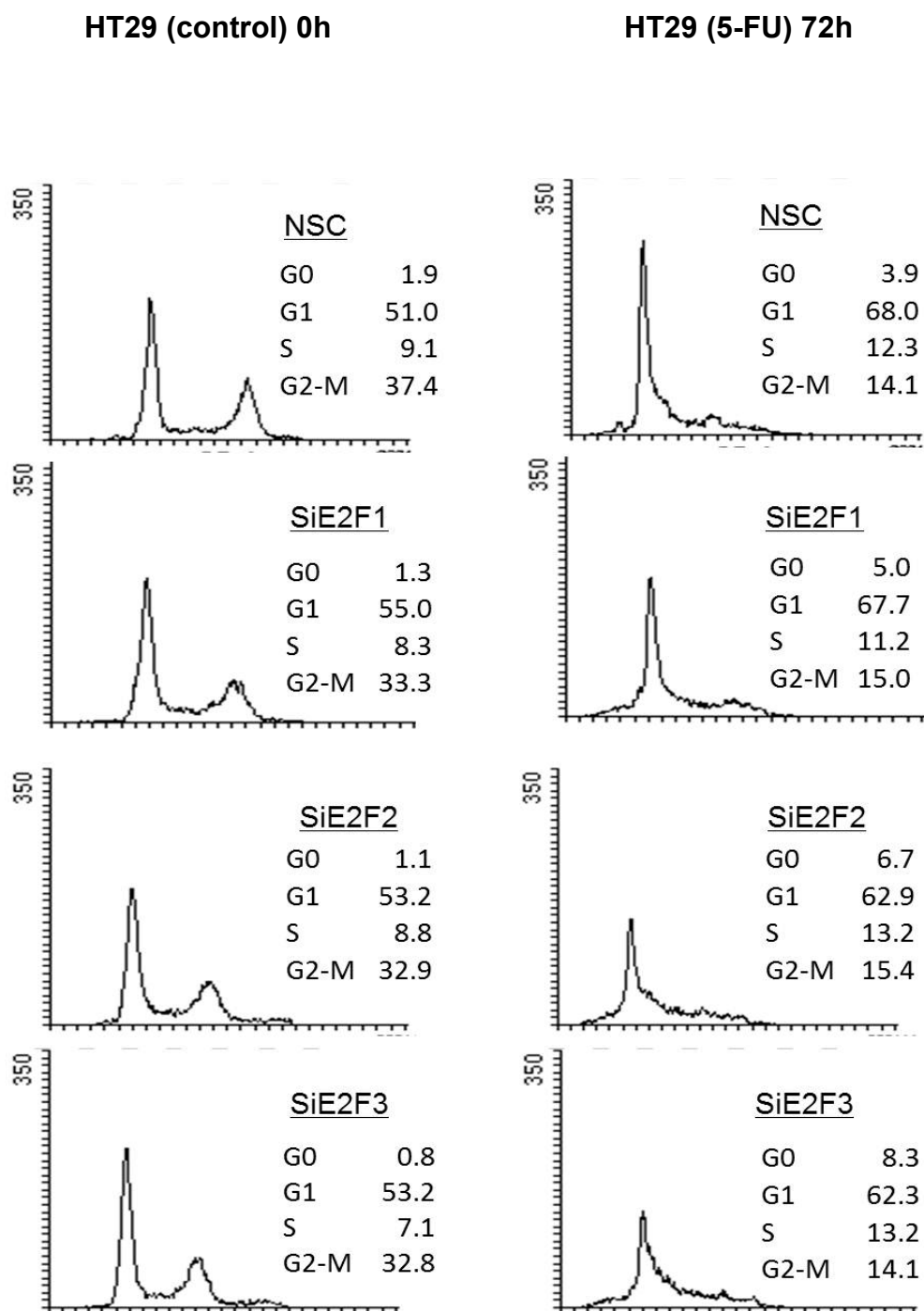


Figure 3. 49 Effect of 5-FU on E2F transcription activators in HT29 cells. HT29 cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with, NSC, SiE2F1, SiE2F2 and SiE2F3. Cells were seeded in to 6 well plate, followed by the exposure to $1 \mu\text{g/ml}$ of 5-FU for 72h for FACS analysis. Cells were harvested and fixed in 70% ethanol and stained with PI.

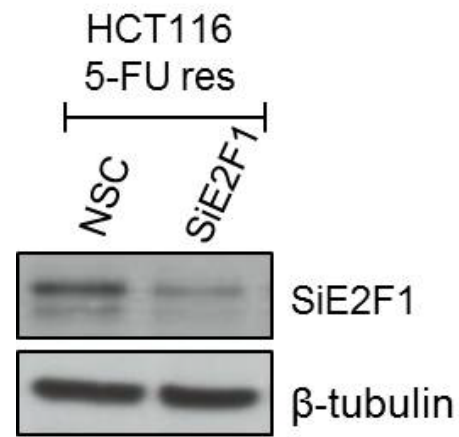
3.3.6 Silencing E2F1 in 5-FU resistant and FOXM1 over expressed cells decreases chemo-sensitivity

I next sought to detect the effect of E2F1 in acquired 5-FU resistant HCT116 cells (which have high basal expression of FOXM1) and MCF7 FOXM1 over-expressed cells. For this I decided to knock-down E2F1 in HCT116 5-FU Res cells and MCF7 FOXM1 over-expressed cell lines.

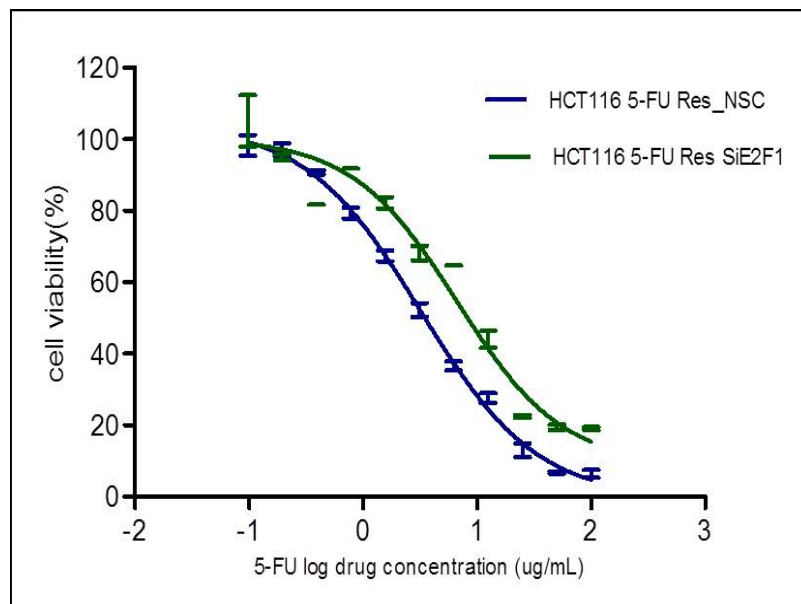
SRB assays were conducted and I observed that transient knock-down of E2F1 in HCT116 5-FU Res cells resulted in more resistance to 5-FU than in HCT116 5-FU Res NSC cells (figure 3.50 B). The IC₅₀ of HCT116 5-FU Res control was 3.23µg/ml and HCT116 5-FU Res SiE2F1 is 8.12µg/ml. In contrast, silencing E2F2 and E2F3 did not show any effect on 5-FU sensitivity in HCT116 5-FU Res cells (Figure 3.50 C, D). Unfortunately, I couldn't include the protein expression of E2F2 and E2F3 knock-down due to the loading error in the western blot gel.

Next, FACS analysis was performed to determine the effect of the E2F transcription activators on cell cycle distribution. No significant effects on the cell cycle were observed between control and 5-FU exposed E2F1, E2F2, E2F3 deleted cells (figure 3.51). This could be due to the concentration of 5-FU used for FACS (1µg/ml) was insufficient to cause any changes in cell cycle distribution in these resistant cells, due to their high FOXM1 expression.

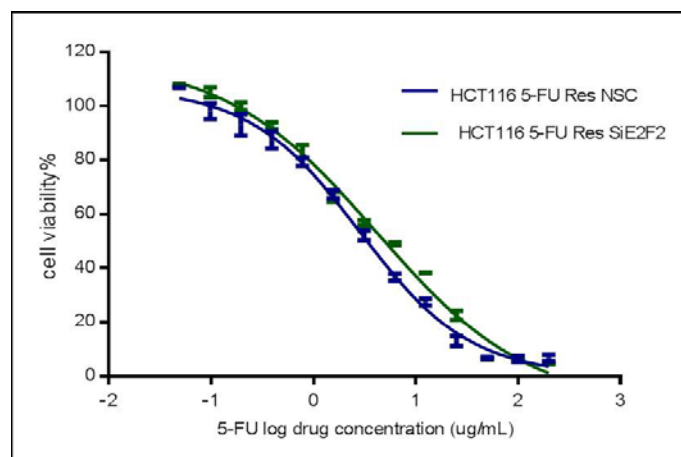
A



B



C



D

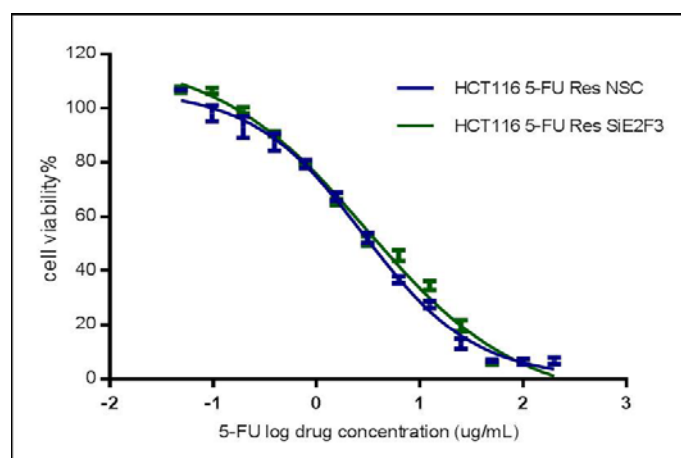


Figure 3. 50 Effect of 5-FU in HCT116 5-FU Res cells following E2F1 knock-down. Cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with NSC, SiE2F1, SiE2F2 and SiE2F3. Cells were seed in to 96 well plates. 48 hours after the transfection, cell were harvested and seed in to 96 well plates and exposed to various concentrations of 5-FU for 72 hours for SRB. The rest of the cells were seeded in to 6 well plate for FACS analysis. Figure 3.54 A. Western blotting confirming the knock-down of E2F1. B. Knock-down of E2F1 increases resistance to 5-FU. Knock-down of E2F2 (C) and E2F3 transcription activator (D) does not make any significant changes compared to control in acquired 5-FU Res cells.

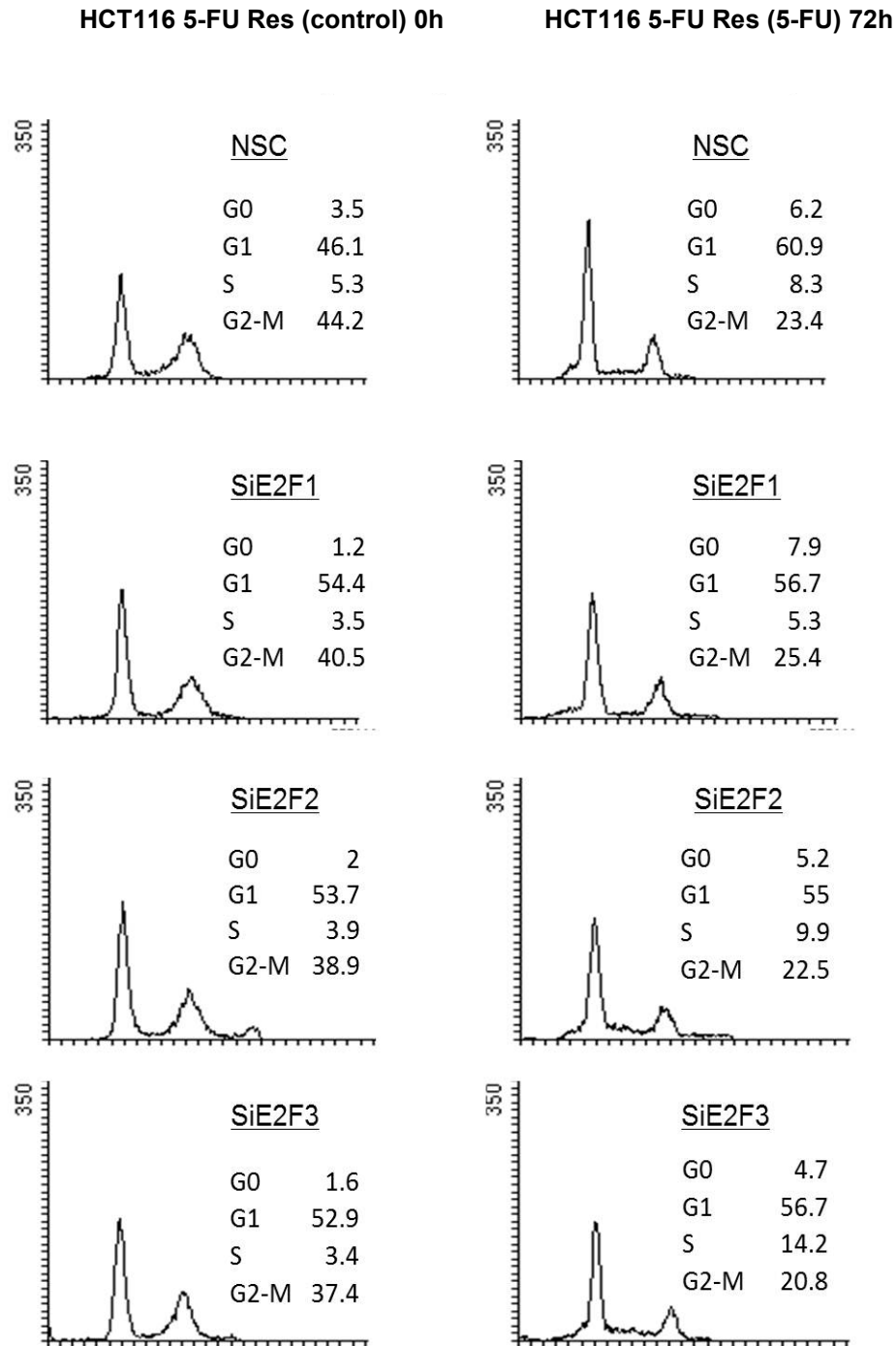
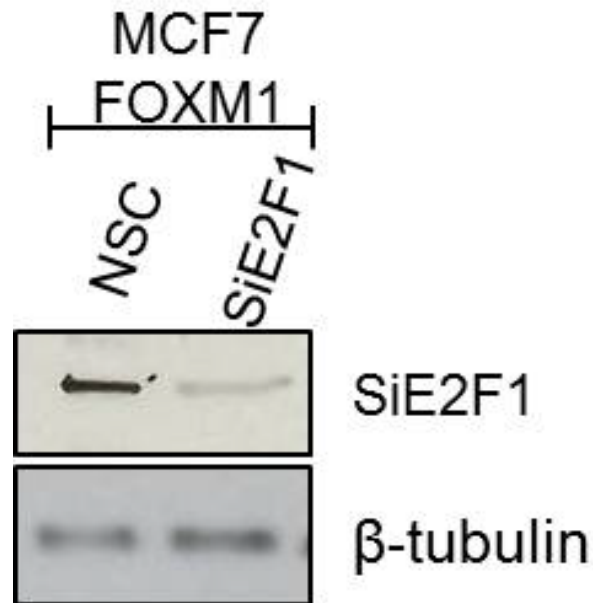


Figure 3. 51 Effect of 5-FU in E2F transcription activators in HCT116 5-FU Res cells. HCT116 5-FU Res cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with, NSC, SiE2F1, SiE2F2 and SiE2F3. Cells were seeded in to 6 well plate, followed by the exposure to $1 \mu\text{g/ml}$ of 5-FU for 72h for FACS analysis. Cells were harvested and fixed in 70% ethanol and stained with PI.

Similar to HCT116 5-FU Res cells, transient knock-down of E2F1 in MCF7 FOXM1 over-expressed cells shows increased resistance towards 5-FU compared to NSC (figure 3.52). (IC₅₀ of MCF7 FOXM1 NSC 3.07µg/ml, and MCF7 FOXM1 SiE2F1 10.96µg/ml). This supports the crucial role of E2F1 activity in mediating apoptosis in the response to 5-FU induced DNA damage in both FOXM1 high and low expressing CRC cells.

A



B

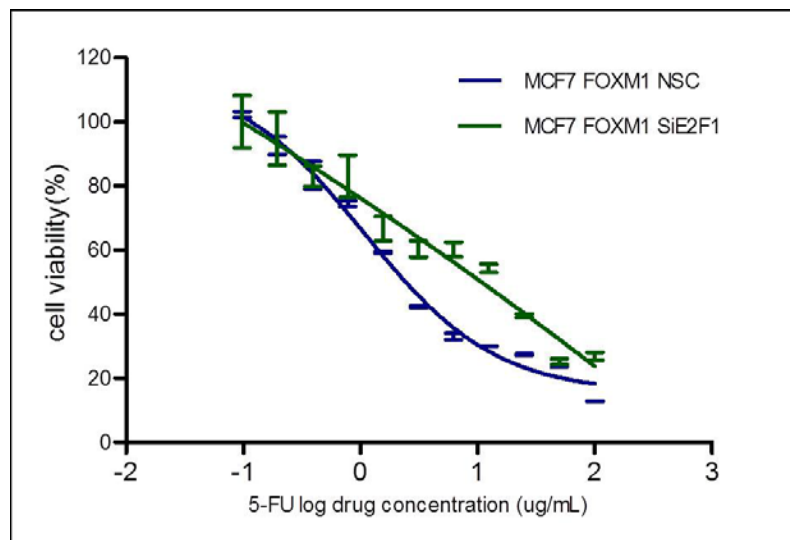


Figure 3. 52 Effect of 5-FU in E2F1 knock-down in MCF7 FOXM1 over-expressed cells. MCF7 FOXM1 over expressed cells were seeded in 60mm plates at a density of 30×10^4 cells/well and transfected with, NSC, SiE2F1. Cells were seeded in to 96 well plates 48 hours after the transfection and the day after, cells were exposed to various concentrations of 5-FU for 72 hours. Drug sensitivity was determined using SRB assays. A) Western blot confirming successful knock-down of E2F1. B) Knock-down of E2F1 transcription activator increases resistance to 5-FU. This experiment was done in triplicate. IC₅₀ was determined by fitting a sigmoidal dose-response curve to the data.

3.3.7 Discussion

Numerous studies have reported that E2F1 induces DNA synthesis and G1-S regulatory genes, including TYMS (Kasahara, Takahashi et al. 2000, Huang, Liu et al. 2007) and FOXM1 (Millour, de Olano et al. 2011). The E2F1 transcription factor is a cell cycle regulatory protein and also has a role in apoptosis (DeGregori, Leone et al. 1997, Hsieh, Fredersdorf et al. 1997, Ren, Cam et al. 2002, Wu, Zheng et al. 2009). The dual proliferative and apoptotic roles of the E2F1 transcription activator have made it difficult to define the precise significance in carcinogenesis and drug resistance.

Consistent with these studies, in this section I have studied the role of E2F1 in respect of FOXM1 and TYMS regulation and 5-FU resistance. In addition, I studied the involvement of other E2F transcription activators, E2F2 and E2F3, as it has been reported that loss of one E2F1 activator can be functionally compensated by another (Wu, Timmers et al. 2001).

I confirmed the role of E2F1 in TYMS regulation (DeGregori, Kowalik et al. 1995) through SiRNA mediated transient knock-down in MCF7 Dox^R cells. Further to this, transient knock-down and over-expression of E2F1, E2F2, and E2F3 was carried out in HCT116 cells. The results support a previous study showing the role of E2F1 in the regulation of TYMS and TK-1 genes (Helin 1998, Rahman, Voeller et al. 2004). In addition, my results also support another study which showed that E2F1 regulates FOXM1 in breast cancer (Millour, de Olano et al. 2011) and are also consistent with a further study in MEFs cells showing that loss of a single E2F family transcription activators can be functionally compensated by other related members (Wu, Timmers et al. 2001).

Unfortunately knock-down of E2F2 did not produce an efficient knock-down of E2F2 at the protein level, or other potential downstream targets but significant decreases were observed at the mRNA level. This could be due to the high stability E2F2 protein or the efficiency of the E2F2 antibody. Among the three E2F transcription activators, E2F1 knock-down led to slight decreases in TYMS and TK-1 protein levels and more significant decreases in mRNA levels. Notably, for the first time, both TYMS and TK-1 mRNA expressions were down-regulated in E2F2 and E2F3 deleted cells. In addition to the knock-down experiments, an overexpression study also confirms the direct regulation of TYMS and TK-1 mRNA expression by E2F2 and E2F3 transcriptional activators, (figure 3.40). Furthermore, E2F1 and E2F3 knock-down were associated with a significant decrease in both FOXM1 protein and mRNA (figure 3.39). E2F2 knock-down had no effect on FOXM1 protein but significant decreases were observed in FOXM1 mRNA. However there were problems with the E2F2 knock-down experiments as documented earlier. Overexpression of E2F1, E2F2 and E2F3 transcription activators led to increases in FOXM1 protein and mRNA levels, indicating that E2F2 and E2F3 could also regulate FOXM1 along with E2F1.

Over-expression of E2F transcription activators, especially E2F1 overexpression, leads to an increase in Rb phosphorylation (figure 3.41). The Rb tumour suppressor gene is a known regulator of E2Fs, and critical for balanced proliferation (Wenzel, Chong et al. 2011). Phosphorylation of Rb leads to dissociation of E2Fs and S-phase entry. Cell cycle regulatory protein p21^{Cip1} was also found to be decreased at the protein level of E2F1, E2F2 and E2F3 over-expressed cells, this confirms another study indicating that p21^{Cip1} protein levels were markedly elevated in (TKO cells) E2F1-3 deleted cells (Wu, Timmers et al. 2001). This could be due to over

expression of E2F transcription activators promoting cell cycle progression by positive feedback, through inhibition of p21^{Cip} that would promote Rb phosphorylation and then causing the release of E2F from Rb driving S-phase entry.

As E2F1 transcription factor known as key regulator in cellular proliferation, some studies show that inhibition of E2F1 increases chemo-sensitivity in CRC cells (de la Cueva, Ramírez de Molina et al. 2013). One property of E2F1 that distinguishes it from other E2F proteins is that E2F1 is a very effective inducer of apoptosis (DeGregori, Kowalik et al. 1995). E2F1 protein levels are induced following DNA damage and E2F1 activity is thought to contribute to the apoptosis.

In this context, I performed knock-down of E2F transcription activators to detect cell viability and 5-FU sensitivity in colorectal cancer cell lines. The results show that silencing of E2F1 increases cytotoxicity in p53 wt HCT116 cells. My result is in contrast with a previous study showing that E2F1 signals p53 dependent apoptosis, and E2F1 deficiency decreased p53-dependent apoptosis in TgT₁₂₁ E2F1^{-/-} mice (Pan, Yin et al. 1998), however this difference could be explained by the fact that these mice were not treated with DNA damaging agents. In my cell viability study, knock-down of E2F1 in HCT116 wt cells significantly decreased cell proliferation during 5-FU treatment, potentially loss of E2F1 decreases the ability of the cells to undergo cell cycle progression, as Pan et al described reduced proliferation in the setting of E2F1 deficiency in their report (Pan, Yin et al. 1998). In contrast, knock-down of the other two transcription activators, E2F2 and E2F3, didn't enhance chemo sensitivity. As expected, cell cycle analysis shows that 5-FU treatment in E2F1 deleted HCT116 wt cells led to changes indicative of cell death and apoptosis. Similar to the cell viability assays, no significant cell death was observed in E2F2 and E2F3 deleted cells.

However, down-regulation of E2F1 renders p53 mutant HT29 cells even more resistant to 5-FU, which was confirmed by both SRB and FACS data. These results would further support the role of E2F1 as a tumour suppressor. A previous study shows that in p53 mutant cells, E2F1 targets NOXA, PUMA, p73, p21^{Cip} and SIP are up-regulated. Knock-down of E2F1 in U937 cells blocked apoptosis in p53 mutant cells (Kojima, Burks et al. 2010). Based on this study my results might indicate that for p53 mutant cells, E2F1 is required for apoptosis. My results are also in accordance with another study which indicating that, in p53 mutant cells, E2F1 and its downstream target, p73 function as a backup to support apoptosis (Urist, Tanaka et al. 2004, Polager and Ginsberg 2009). Another study on U2OS (p53 wt) cells treated with doxorubicin and cisplatin indicates that activation of E2F1 can sensitise U2OS cells to chemotherapy. The authors also identified the tumour suppressor gene maspin as a novel E2F1 regulated gene which helps to promote apoptosis in p53 deficient cells (Ben Shachar, Feldstein et al. 2010). No changes were observed in HT29 cells transfected with SiE2F2 and SiE2F3, which could suggest that E2F2 and E2F3 might not have any role in apoptosis, however definitive conclusions cannot be made from the E2F2 knock-down experiments as we were unable to demonstrate reduced E2F2 protein on Western blot.

5-FU resistant p53 wt HCT116 5-FU Res cells were found to have high FOXM1 levels and (Result 3.3.6) also shows increased resistance towards 5-FU in SiE2F1 cells compared to control. Cell cycle analysis also indicates no changes in cell cycle distribution. Following E2F2 and E2F3 knock-down cell cycle analysis and SRB assay shows, control and SiE2F2 and SiE2F3 were similar, possibly indicating that E2F1 is the main regulator among E2F transcription activators, and also supporting another study showing that among E2F transcription activators, E2F1 is crucial for E2F dependent apoptosis (Lazzerini Denchi and Helin 2005). Likewise, transient

knock-down of E2F1 in MCF7 FOXM1 over expressed cells also resulted in increased resistance to 5-FU compared to control. This could indicate that E2F1 might act as a tumour suppressor in p53 mutant or FOXM1 overexpressed cells. Over-expression of FOXM1 is reported to be linked to loss of p53 function in thyroid cancer (Bellelli, Castellone et al. 2012).

The roles of FOXM1, E2F1, and p53 in cell cycle mediated progression and apoptosis are depicted in below in figure 3.53.

3.3.8 Future work

From these results, the roles of E2F1 in apoptosis in p53 mutant and 5-FU resistant cells were identified. To confirm the role of E2F1, more apoptosis assays (such as Annexin V, γ H2AX and caspase-Glo) should be performed on p53wt, p53 mutant and 5-FU resistant cell lines. I would also like to over-express E2F transcription activators and see if the results are the same as I observed in this study.

Finally, I would like to repeat E2F2 knock-down and over-expression experiments with an alternative E2F2 antibody as I could not observe any changes in the protein level during the transient transfection experiments.

CHAPTER 4

FINAL DISCUSSION AND CONCLUSION

5-Fluorouracil (5-FU) is an anti-metabolite. For many years it has been an important chemotherapeutic drug used in first-line treatment for a range of human cancers, particularly colorectal carcinoma and breast cancer (Longley, Harkin et al. 2003). 5-FU drug resistance is a major challenge in 5-FU based therapy, which ultimately leads to treatment failure and disease progression. Studies which investigate the mechanisms underpinning drug resistance could provide an opportunity for the development of novel agents, and allow the development of novel biomarkers that could predict resistance at an early stage.

FOXM1, TYMS, E2F1 and DDR gene expression in CRC cells treated with 5-FU

TYMS and TK-1 are essential factors for DNA synthesis and critical targets for 5-FU (Longley and Johnston 2005). High levels of TYMS expression have been observed in many cancers such as, colorectal, breast and gastric (Lenz, Leichman et al. 1996, Pestalozzi, Peterson et al. 1997, Aschele, Debernardis et al. 1999). Several studies indicate that TYMS expression is the key determinant of 5-FU sensitivity (Copur, Aiba et al. 1995, Johnston, Lenz et al. 1995, Lee, Park et al. 2006) and expression of TYMS and p53 have both been implicated as prognostic factors in CRC (van Triest, Pinedo et al. 2000). It has been reported that 5-FU is more efficient in cell lines carrying wild type p53. 5-FU induced apoptosis is dependent on a functional p53 pathway and loss of p53 function reduces cellular sensitivity to 5-FU (Longley, Harkin et al. 2003) (Osaki, Tatebe et al. 1997, Bykov, Issaeva et al. 2002). High TYMS expression and or lack of functional p53 could increase 5-FU resistance (Bunz, Hwang et al. 1999, Longley, Boyer et al. 2002). In my study, after 5-FU

treatment, mRNA and protein expression of TYMS was initially induced and then decreased in p53 wt cells but remained persistently up-regulated in p53 deficient cells. Furthermore, elevated persistent high expression of both FOXM1 protein and mRNA was observed in p53 mutant colon cancer cells following 5-FU treatment. Whilst in p53 wt cell lines, similar to TYMS, expression of FOXM1 decreased at 48h after an initial induction. This is in accordance with other studies showing that p53 is also an essential transcription factor that is required for the down-regulation of FOXM1, and cells with reduced p53 expression, FOXM1 is up-regulated after DNA damage (Barsotti and Prives 2009, Millour, de Olano et al. 2011). These studies support the idea that 5-FU activates through p53 to repress FOXM1 expression in colon cancer cells (Ju, Schmitz et al. 2007).

During DNA damage, DNA damage response genes (DDR) such as ATM, and ATR get activated, and phosphorylate down-stream targets such as γ H2AX, Chk2, and Chk1, which in turn directly or indirectly phosphorylate and stabilise other molecules such as E2F1, p53 (Oka, Tanaka et al. 2010). Once stabilised, p53 is responsible for the transcriptional activation of a series of proteins involved in cell cycle control, apoptosis, and senescence (Lavin and Gueven 2006). E2F1 levels increase following DNA damage and this is mediated by ATM dependent phosphorylation (Lin, Lin et al. 2001). Consequently, DNA damage response genes were analysed in colon cancer cell lines by western blot. Treatment with 5-FU induced ATM and Chk2 mainly in p53 mutant cells compared to p53 wt HCT116 cells. Increased levels of E2F1, another ATM target, were also found in p53 mutant cell lines. This could suggest that over expression of ATM, Chk2, and FOXM1 may promote DNA repair to overcome apoptosis or cell cycle arrest due to ineffectual tumour suppressor gene. This result is also in an agreement with another study which shows high levels of ATM and FOXM1 levels in drug resistant MCF7 cells compared with MCF7 wt cells

(Millour, de Olano et al. 2011). Furthermore, this result supports a previous study in colon cancer where low levels of pATM, γ H2AX and pChk2 were observed in normal and adenoma tissues, gradually increasing in cancer tissues, and reaching maximal levels in advanced carcinoma (Oka, Tanaka et al. 2010).

Role of FOXM1 in 5-FU resistance

Increased levels of FOXM1 are found in many types of cancers, including colon and breast (Chan, Yu et al. 2008, Gemenetzidis, Elena-Costea et al. 2010, Wang, Li et al. 2011, Koo, Muir et al. 2012, Park, Jung et al. 2012, Halasi, Pandit et al. 2013). The role of FOXM1 in CRC progression is reported in an in vivo study by Yoshida et al, where an increase in both the number and size of tumours was observed in Rosa26 FOXM1b transgenic mice compared to controls (Yoshida, Wang et al.) Over-expression of FOXM1 in primary colon tumours is an independent adverse prognostic factor in terms of both overall survival (45.6% % in FOXM1 high versus 85.9% in FOXM1 negative at 5 years) and development of metastasis, with FOXM1 high cases carrying a 4-fold risk of metastatic spread (Li, Wei et al. 2013).

Many studies show the key role of FOXM1 in chemotherapy drug resistance. Knock-down of FOXM1 in breast cancer cells improved sensitivity towards doxorubicin, and another study on FOXM1 overexpressed MCF7 cells demonstrated increased resistance to epirubicin compared with the parental MCF-7 cells (Carr, Park et al. 2010, Millour, de Olano et al. 2011, Park, Jung et al. 2012, Park, Jung et al. 2012, Bergamaschi, Madak-Erdogan et al. 2014). Consistent with these observations, in my study I have confirmed for the first time that, the critical role of FOXM1 in 5-FU drug resistance in colon cancer cells. Knock-down of FOXM1 in HCT116 cells

significantly increases sensitivity towards 5-FU while transient over-expression of FOXM1 increased 5-FU resistance in HCT116 cells.

HCT116 cells are p53 wt and MMR deficient due to mutated MLH1 on chromosome 3 (MSI-high), were more sensitive than DLD1 and HT29 (p53 mutant, MMR proficient) cells to 5-FU. Our results suggest that p53 status is more important than MMR in determining treatment efficacy as the MMR deficient cells were more sensitive than the other cell lines.

Transient transfections of FOXM1 in HCT116 cells revealed the role of FOXM1 in regulation of TYMS, TK-1 and interaction with E2F transcription activators, E2F1, E2F2 and E2F3. The development of acquired resistance is crucial for treatment failure. In my study acquired 5-FU Res cells show significantly higher levels of FOXM1 and TYMS at basal levels compared to parental HCT116 cells. In contrast, after 5-FU treatment, down-regulation of FOXM1 and TYMS was observed in HCT116 wt cells. But in the 5-FU resistant HCT116 cells, and p53 mutant DLD1 and HT29 cells persistent up-regulation of FOXM1 and TYMS protein expression was observed. This result suggested that TYMS is associated with FOXM1 and 5-FU drug resistance, and demonstrated the crucial role of FOXM1 in 5-FU drug resistance in CRC.

Following 5-FU treatment, increased cell death was detected in HCT116 wt cells, whereas no significant cell death or γ H2AX staining was observed in HCT116 5-FU Res cells. This is indicative of DNA fragmentation and cell death in (FOXM1-low) HCT116 wt cells and an increased DNA repair activity in (FOXM1-high) HCT116 5-FU Res cells. This observation is in accordance with a previous study demonstrating that increased DNA breaks are seen in FOXM1 over-expressed cells (Tan, Raychaudhuri et al. 2007, Kwok, Peck et al. 2010, Zhang, Wu et al. 2012) and that

knock-down of FOXM1 sensitizes human cancer cells to DNA damaging agents (Halasi and Gartel 2012). Furthermore, FOXM1 deficient mouse embryonic fibroblasts (MEFs) and SiFOXM1 osteosarcoma U2OS cells exhibit increased DNA breaks (Tan, Raychaudhuri et al. 2007). In these examples, FOXM1 could be acting to increase DNA repair, as FOXM1 is reported to directly regulating genes involved in various DNA damage repair pathways (Zona, Bella et al. 2014). A study on breast cancer shows that HR DNA repair genes are directly regulated by FOXM1 and that deletion of HR genes increased sensitivity to Doxorubicin (Park, Jung et al. 2012). Another study shows that Chk2 phosphorylates and increased the stability of the FOXM1 protein in response to DNA damage and increased transcription of XRCC1 and BRCA2 genes, both required for repair of DNA damage repair (Tan, Raychaudhuri et al. 2007).

Subsequently, I used ChIP assays to confirm whether FOXM1 is bound to the endogenous human TYMS promoter region. Strong enrichment of FOXM1 in the TYMS promoter region in both HCT116 and DLD1 cells was observed. Interestingly, significantly higher FOXM1 binding was observed in the TYMS promoter of HCT116 5-FU Res cells, confirming the significance of FOXM1 mediated 5-FU resistance through TYMS.

The ChIP-sequence data also confirmed direct binding of FOXM1 onto other 5-FU targets such as thymidine phosphorylase (TYMP) and TK-1. In addition, E2F transcription activators, E2F1 and E2F2 were found to be regulated by FOXM1, as were: Cyclin B1, Cyclins D1, and CDKN1A (p21^{Cip}), MMP2, MLH1, and DNA damage repair genes. Staining on human colorectal cancer tissues also confirmed the clinical association between FOXM1, TYMS, and TK-1.

In summary, from these data, I have demonstrated for the first time that FOXM1 possesses a crucial role in 5-FU drug resistance in colon cancer cells through the

direct regulation of 5-FU targets TYMS, TK-1, FOXM1 also regulates TYMP, and cell cycle regulatory and DNA repair genes.

My study provides new insights into the molecular mechanisms underlying the regulation of TYMS in colon cancer. All these data imply that cancer cells depends upon FOXM1 activity and therefore targeting FOXM1, could provide a useful therapeutic strategy.

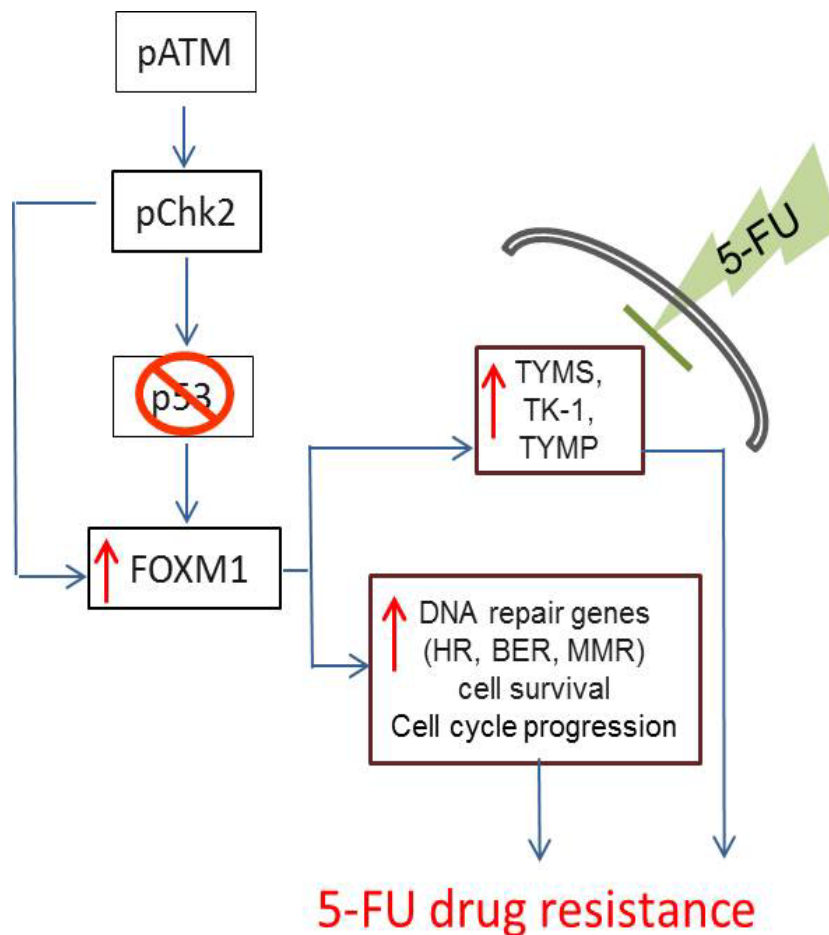


Figure 4. 1 Schematic representation of the role of FOXM1 in 5-FU drug resistance. During DNA damage ATM /Chk2 phosphorylate and stabilize p53 and FOXM1. In the absence of active p53, FOXM1 expression is up-regulated. Up-regulation of FOXM1 increased the expression of FOXM1 targets such as DNA repair genes, cell cycle progression genes and therapeutic targets of 5-FU, thymidylate metabolites. Over expression of DNA repair gene and thymidylate metabolites leads to 5-FU drug resistance.

E2F transcription activators in FOXM1, TYMS and TK-1 regulation

E2F1 is the most important member of the E2F family of transcription activators that functions in cell cycle progression and apoptosis induction in response to DNA damage (Wu, Zheng et al. 2009) and is known to regulate TYMS (Kasahara, Takahashi et al. 2000) (Huang, Liu et al. 2007) and FOXM1 (Millour, de Olano et al. 2011) independently. Thus it was important to clarify the function of E2F1 in TYMS and FOXM1 regulation and 5-FU resistance.

The dual roles, (cell cycle proliferation and apoptotic) of the E2F1 transcription activator have meant that its precise function in cancer development and drug resistance are not easily determined. It has been extensionally reported that E2F1 induces DNA synthesis and G1-S regulatory genes, including TYMS (Rahman, Voeller et al. 2004, Huang, Liu et al. 2007, Wu, Zheng et al. 2009) and overexpression of E2F1 increases the levels of TYMS and leads to 5-FU resistance (Banerjee, Schnieders et al. 1998). A study on epirubicin drug resistance in breast cancer cells revealed the significance of E2F1 in the regulation of FOXM1, as SiRNA of E2F1 attenuated FOXM1 induction via p38 (de Olano, Koo et al. 2012).

To this extent, I studied the role of E2F1 in FOXM1 regulation and 5-FU drug resistance (chapter 3.3) and also looked at the E2F2 and E2F3 transcription activators Knock-down and over expression of E2F1, E2F2 and E2F3 in HCT116 cells confirmed their role in TYMS and TK-1 regulation (Dyson 1998, Giangrande, Zhu et al. 2004, de la Cueva, Ramírez de Molina et al. 2013). In addition to this, results showed that E2F transcription activators can regulate each other, which is consistent with a previous study shows that loss of one of the E2F family transcription activators can be functionally compensated by other related members

(Wu, Timmers et al. 2001). My results also confirmed the role of E2F1 in FOXM1 regulation (Millour, de Olano et al. 2011). The transient transfection experiments showed that E2F2 (only at the mRNA level) and E2F3 could also regulate FOXM1.

E2F transcription activators in 5-FU mediated apoptosis

As I mentioned earlier, during DNA damage ATM/ATR become activated, stabilised and phosphorylates down-stream targets such as chk1/Chk2, p53, E2F1. Phosphorylation of p53 by ATM/Chk2 induces or represses several genes that regulate cell cycle arrest, DNA repair or apoptosis, including p21^{Cip} WAF1, MDM2 and GADD45. (Saito, Goodarzi et al. 2002). Along with cell cycle regulation, E2F1 is reported to be involved in apoptosis in both in vivo and in vitro. A variety of experiments have revealed the role of E2F1 in apoptosis in cells (Kowalik, DeGregori et al. 1995) and deregulated E2F1 activity in mouse contributes to tumour development (Guy, Zhou et al. 1996, Yamasaki, Jacks et al. 1996, Pierce, Fisher et al. 1998). Another study shows that activated E2F and wild-type p53 co-operate to mediate apoptosis (Bellelli, Castellone et al. 2012) similarly E2F1 deficiency decreases p53 and other p53 target genes and causes a reduction in apoptosis (Pan, Yin et al. 1998). My results show that knock-down of E2F1 in p53 wt HCT116 cells decreased cell viability, decrease in S phase, G2/M phases and an increase in population of cells in G0 (sub-G1), indicative of cell death and apoptosis. It could be that loss of E2F1 decreases the ability of the cells to undergo cell cycle progression, and this effect is possibly associated with p53 status. This might support other study showing that E2F1 has an inhibitory effect on p53 and upon Adriamycin (ADR) treatment. E2F1 forms a physical complex with p53 and this complex is recruited to

p53 target genes where E2F1 then attenuates p53 dependent apoptosis (Yoshihara, Wu et al. 2012). The response of p53 target genes to DNA damage and apoptosis has been observed in the absence of E2F1. In p53 mutant cells, E2F1 and p73 bind together and activate TP53 and target genes (Yoshihara, Wu et al. 2012, Pützer and Engelmann 2013). A different study shows that p53 can stabilize and activate directly through the activation of ATM or indirectly by many other ATM targets (Shiloh and Ziv 2013) (Zhu, Alvarez et al. 2004).

According to a previous study, E2F1 can induce apoptosis in the presence of p53 (Pan, Yin et al. 1998). My studies demonstrated that knock-down of E2F1 in p53 mutant HT29 cells increased resistance to 5-FU and increased the proportion of cells observed in S-phase compared to respective untreated control, and the proportion of cells in G2/M phase was reduced and the amount of cells in G0 was not significantly altered compared to control (as opposed to HCT116 cells where a substantial increase in G0 fraction after E2F1 knock-down. This could suggest that there is release of arrested cells at G1/S phase, and cells are then progressing more slowly through S-phase. My results also confirm the concept that inhibition of E2F1 activity could not sensitise p53 mutant HT29 cells to 5-FU treatment (Yoshihara, Wu et al. 2012, Pützer and Engelmann 2013), and my data could also support another study showing that E2F1 can induce apoptosis in the absence of p53 (Hsieh, Fredersdorf et al. 1997). Activation of p73 by E2F1 functions as backup in p53 deficient cells to ensure that damaged cells can undergo apoptosis (Pützer and Engelmann 2013). E2F1 induced apoptosis could inhibit through an additional C-terminal domain of pRb, which binds to the E2F1. This secondary binding site within pRb inhibits E2F1 induced apoptosis. After DNA damage this domain becomes acetylated, resulting in E2F1 release without affecting the binding of pRb to other E2Fs, released E2F1

induced its apoptotic targets (Dick and Dyson 2003) (Hsieh, Fredersdorf et al. 1997, Markham, Munro et al. 2006). In contrast, other studies show the direct role of Rb in enhancing E2F1 apoptosis after DNA damage. Rb knock-down experiments reduced transcription and apoptosis upon chemotherapy in vitro and in vivo (Ianari, Natale et al. 2009).

It is interesting that knock-down of E2F1 in FOXM1 high expressing 5-FU resistant HCT116 cells led to increased resistance towards 5-FU. Cell cycle analysis also indicates no changes in cell cycle distribution. Transient knock-down of E2F1 in MCF7 FOXM1 over expressed cells also confirms knock-down of E2F1 in FOXM1 over expressed cells causes increased resistance to 5-FU. These results indicate that E2F1 might act as a tumour suppressor in p53 mutant and FOXM1 high expressing cells, supporting a study showing that loss of p53 function is linked to over-expression of FOXM1, (Bellelli, Castellone et al. 2012) and in the absence of p53, E2F1 is important for apoptosis (Pützer and Engelmann 2013).

Regarding the other E2F family transcriptional activators, E2F2 and E2F3, their modulation did not result in any changes significant changes following 5-FU treatment. However it should be noted that conclusions regarding E2F2 are limited due to the lack of efficacy of E2F2 knock-down in my data. A previous study showed that E2F1 is important for apoptosis induction by Adriamycin, but not E2F2 or E2F3 (Wang, Liu et al. 2004). A further report showed that E2F3 can also induce apoptosis but is dependent on E2F1, whereas E2F1 did not require E2F3 for the induction of cell death (Lazzerini Denchi and Helin 2005).

Targeting FOXM1 to promote 5-FU sensitivity

The thiazole antibiotic, thiostrepton was recently identified as a proteasome inhibitor known to specifically inhibit the transcriptional activity of FOXM1 (Walter, Hunter et al. 2011). Consistent with this idea p53 wt HCT116, p53 mutant DLD1, and HT29 cell lines were treated with thiostrepton. The treatment significantly decreased protein and mRNA expression of FOXM1, and its downstream targets TYMS, and TK-1. In HT29 and HCT116 cells, down-regulation of both E2F1 protein and mRNA was observed, consistent with previous studies showing that thiostrepton treatment can inhibit FOXM1 and its downstream targets. Previous reports have shown that p53 is necessary for FOXM1 regulation and p53 deletion increases FOXM1 expression (Barsotti and Prives 2009, Millour, de Olano et al. 2011). My data demonstrated that thiostrepton can inhibit FOXM1, and its downstream targets in both p53 wt and p53 mutant cells. Furthermore, thiostrepton treatment in 5-FU drug resistant HCT116 cells produced similar IC50 as the parental HCT116 cells cell lines, indicating that thiostrepton mediated inhibition of FOXM1 can help to reverse acquired 5-FU resistance.

ChIP assay on thiostrepton treated HCT116, DLD1 and HCT116 5-FU Res cell lines showed significant decreases in FOXM1 binding on the TYMS promoter region. In addition, decreases in FOXM1 binding to related targets were observed in TK-1 TYMP, E2F transcription activators E2F1 and E2F2, MMP1, MMP2 and MLH1. These results demonstrated that thiostrepton can modulate the expression of FOXM1 targets through direct binding on the transcription factor FOXM1.

During chemotherapy for patients, anticancer drugs are often used in combination, to

increase the response rates and long-term survival (Bezerra, Castro et al. 2008). Colorectal cancer treatment is based on 5-FU in combination with other cytotoxic drugs such as oxaliplatin or the topoisomerase I inhibitor CPT-11 (Irinotecan) (de la Cueva, Ramírez de Molina et al. 2013). Therefore, I tested the combination of FOXM1 inhibitor thiostrepton and 5-FU in colon cancer cell lines and a synergistic relationship between thiostrepton and 5-FU was observed. This supports previous reports showing that thiostrepton enhances chemo-sensitivity to drugs like bortezomib (Pandit and Gartel 2011, Wang, Halasi et al. 2012) and docetaxel (Li, Yao et al. 2014).

Furthermore, I have demonstrated that combination treatment can significantly increase γ H2AX foci in p53 wt, p53 mutant, and acquired 5-FU resistant colorectal cancer cell lines. This could indicate that thiostrepton enhances 5-FU mediated DNA damage by inhibiting FOXM1 and its genomic targets involved in DNA repair pathways, supporting previous studies showing that deletion of FOXM1 increases γ H2AX staining (Tan, Raychaudhuri et al. 2007, Monteiro, Khongkow et al. 2013). Likewise, FACS data demonstrate that combination treatment significantly increases the G0 (sub G1) population, indicating cell death and apoptosis in colon cancer cells, compared with either drug alone. This could suggest that inhibiting FOXM1 by thiostrepton down-regulate FOXM1 and its regulatory genes involved in cell cycle progression, which in turns enhances cell cycle arrest and cell death, this is in accordance with a previous study demonstrating that the role of FOXM1 in cell cycle regulatory genes and the percentage of G0-G1 phase cells was significantly elevated in FOXM1 siRNA transfected cells compared to control (Wu, Liu et al. 2010).

I subsequently found that combination treatment significantly increased caspase-dependent apoptosis in p53 wt and p53 mutant colorectal cancer cells and HCT116

5-FU Res cells, supporting other studies shows proteasome inhibitors like thiostrepton can induce p53 independent apoptosis in human cancer cells (Pandit and Gartel 2011, Zhang, Cheng et al. 2014). Colony formation assays, showed that combination treatment strongly suppressed the ability of colon cancer cells to form colonies compared to either drug alone in HT29 and DLD1, but in HCT116 and HCT116 5-FU Res cell line similar effects were observed with thiostrepton and combination treatment.

The combination of 5-FU and thiostrepton significantly reduces migration of colon cancer cells; suggesting that thiostrepton inhibited FOXM1 and down-regulated FOXM1 targets, metastatic regulator, MMP2 and MMP9, which are crucial in the process of tumour invasion and metastasis (Ahmed, Uddin et al. 2012). This is also in accordance with the ChIP-seq data. This is also in accordance with a previous study showing FOXM1 silencing inhibited the expression and activity of MMP-2, MMP-9 and VEGF (Xue, Xiao et al. 2012).

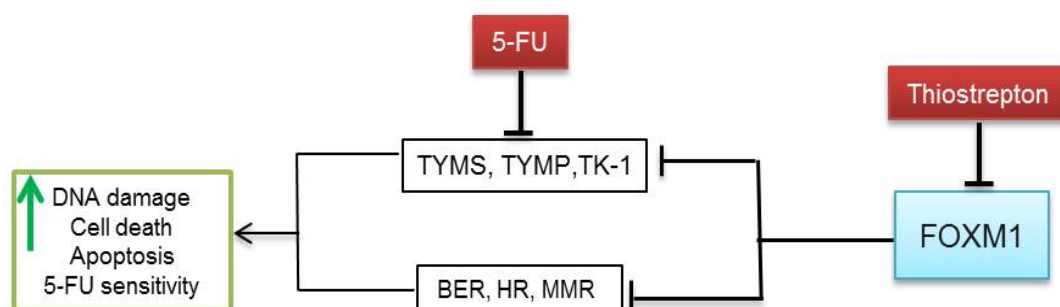


Figure 4. 2 Schematic representation of the combination of FOXM1 and 5-FU in drug sensitivity. The combination of 5-FU and thiostrepton inhibits FOXM1 and its downstream targets, leading to enhanced DNA damage, apoptosis and cell death.

In conclusion, I have identified the role of FOXM1 in 5-FU drug resistance in colorectal cancer and detected that FOXM1 has a direct role in the regulation of thymidylate metabolism in colon cancer, and hence 5-FU drug resistance. A strong correlation between FOXM1 and TYMS was identified in human colorectal tissue specimens.

E2F transcription activators were identified to regulate FOXM1, and my ChIP data shows FOXM1 has significant binding site on E2F transcription activators E2F1 and E2F2. Knock-down of E2F1 transcription activators increases resistance towards 5-FU in p53 mutant and FOXM1 over expressed cells.

Targeting DNA damage and cell cycle checkpoint signalling pathways are important for 5-FU therapy (Wyatt and Wilson 2009, Matuo, Sousa et al. 2010, Fujinaka, Matsuoka et al. 2012). In my study, I have demonstrated that FOXM1 regulates thymidylate metabolites. Other studies enlightened the regulation of FOXM1 in many homologous recombination repair genes, mismatch repair gene and base excision repair genes (Tan, Raychaudhuri et al. 2007, Somyajit, Subramanya et al. 2010, Monteiro, Khongkow et al. 2013, Zhou, Wang et al. 2014). Consequently, targeting FOXM1 by thiostrepton and 5-FU could be a potential strategy to treat colon cancer. Novel thiostrepton analogues are now being developed which may have even more potency even in p53 mutant cells, which should benefit disease outcomes in the future.

CHAPTER 5

SUPPLEMENTAL DATA

Table 5.1 Details of cases in the tissue microarray obtained from U.S. Biomax (ref. BC051110).

Pos	No.	Sex	Age	Organ	Pathology diagnosis	Grade	Stage	TNM	Type †
A1	1	M	51	Colon	Adenocarcinoma	1	III	T4N1M0	Malignant
A2	2	F	42	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
A3	3	M	64	Colon	Adenocarcinoma	1-2	II	T4N0M0	Malignant
A4	4	F	33	Colon	Mucinous adenocarcinoma	1	II	T4N0M0	Malignant
A5	5	F	63	Colon	Adenocarcinoma (smooth muscle)	-	II	T4N0M0	Malignant
A6	6	F	42	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
A7	7	M	32	Colon	Mucinous adenocarcinoma	1	II	T3N0M0	Malignant
A8	8	F	86	Colon	Adenocarcinoma	1-2	II	T4N0M0	Malignant
A9	9	M	71	Colon	Adenocarcinoma	1	III	T4N2M0	Malignant
A10	10	M	55	Colon	Adenocarcinoma	1-2	III	T3N2M0	Malignant
A11	11	F	40	Colon	Adenocarcinoma	1	III	T3N1M0	Malignant
A12	12	M	61	Colon	Adenocarcinoma	1	II	T3N0M0	Malignant
B1	13	M	70	Colon	Adenocarcinoma	1	II	T3N0M0	Malignant
B2	14	F	71	Colon	Adenocarcinoma (sparse)	-	II	T3N0M0	Malignant
B3	15	M	71	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
B4	16	F	58	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
B5	17	F	47	Colon	Mucinous adenocarcinoma	2	IV	T4N0M1	Malignant
B6	18	M	28	Colon	Mucinous adenocarcinoma	1-2	II	T4N0M0	Malignant
B7	19	F	50	Colon	Adenocarcinoma	1	III	T4N2M0	Malignant
B8	20	M	77	Colon	Adenocarcinoma	1-2	II	T3N0M0	Malignant
B9	21	M	47	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
B10	22	M	55	Colon	Adenocarcinoma with necrosis	2	III	T3N2M0	Malignant
B11	23	M	70	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
B12	24	F	40	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
C1	25	F	53	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
C2	26	M	34	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
C3	27	F	63	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
C4	28	M	74	Colon	Adenocarcinoma	2	I	T2N0M0	Malignant
C5	29	M	74	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
C6	30	M	72	Colon	Adenocarcinoma	2	I	T2N0M0	Malignant
C7	31	M	51	Colon	Adenocarcinoma (chronic inflammation of smooth muscle)	-	III	T4N2M0	Malignant
C8	32	F	34	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
C9	33	M	69	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
C10	34	M	55	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
C11	35	M	46	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
C12	36	F	70	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
D1	37	F	56	Colon	Adenocarcinoma (smooth muscle and necrotic tissue)	-	III	T3N1M0	Malignant
D2	38	M	70	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant

Pos	No.	Sex	Age	Organ	Pathology diagnosis	Grade	Stage	TNM	Type †
D3	39	M	72	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
D4	40	M	79	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
D5	41	F	51	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
D6	42	M	67	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
D7	43	F	60	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
D8	44	F	67	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
D9	45	M	66	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
D10	46	M	73	Colon	Adenocarcinoma (smooth muscle, fibrous tissue and blood vessel)	–	III	T4N1M0	Malignant
D11	47	M	75	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
D12	48	F	55	Colon	Mucinous adenocarcinoma	2	III	T4N1M0	Malignant
E1	49	F	44	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
E2	50	M	35	Colon	Adenocarcinoma	2	III	T4N1M0	Malignant
E3	51	F	53	Colon	Adenocarcinoma with necrosis	2	II	T3N0M0	Malignant
E4	52	M	70	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
E5	53	M	68	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
E6	54	M	61	Colon	Adenocarcinoma	2	III	T4N1M0	Malignant
E7	55	F	53	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
E8	56	F	71	Colon	Adenocarcinoma	1–2	II	T3N0M0	Malignant
E9	57	M	58	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
E10	58	M	71	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
E11	59	F	51	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
E12	60	M	60	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
F1	61	M	64	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
F2	62	F	49	Colon	Adenocarcinoma	2	III	T4N1M0	Malignant
F3	63	M	50	Colon	Adenocarcinoma	2	I	T2N0M0	Malignant
F4	64	F	50	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
F5	65	F	60	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
F6	66	F	49	Colon	Mucinous adenocarcinoma	1–2	IV	T4N1M1	Malignant
F7	67	F	30	Colon	Adenocarcinoma	2	III	T3N2M0	Malignant
F8	68	F	80	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
F9	69	F	70	Colon	Adenocarcinoma	2	III	T3N2M0	Malignant
F10	70	M	30	Colon	Adenocarcinoma	2	III	T2N1M0	Malignant
F11	71	M	43	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
F12	72	M	50	Colon	Adenocarcinoma	2	IV	T2N1M1	Malignant
G1	73	M	41	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
G2	74	M	81	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
G3	75	M	79	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
G4	76	M	49	Colon	Adenocarcinoma	1	II	T4N0M0	Malignant
G5	77	F	56	Colon	Adenocarcinoma (chronic inflammation of mucosa)	–	II	T3N0M0	Malignant
G6	78	F	73	Colon	Adenocarcinoma	2	III	T4N2M0	Malignant
G7	79	M	77	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
G8	80	M	43	Colon	Adenocarcinoma	3	III	T4N1M0	Malignant
G9	81	F	60	Colon	Adenocarcinoma	3	II	T3N0M0	Malignant
G10	82	F	67	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
G11	83	F	50	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
G12	84	M	82	Colon	Adenocarcinoma	2	II	T3N0M0	Malignant
H1	85	M	65	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
H2	86	M	58	Colon	Adenocarcinoma	2	III	T3N1M0	Malignant
H3	87	F	58	Colon	Mucinous adenocarcinoma	3	II	T4N0M0	Malignant
H4	88	F	30	Colon	Mucinous adenocarcinoma	3	II	T4N0M0	Malignant
H5	89	M	62	Colon	Adenocarcinoma	2	IV	T4N0M1	Malignant
H6	90	M	38	Colon	Adenocarcinoma	2	III	T4N1M0	Malignant
H7	91	F	22	Colon	Adenocarcinoma	3	III	T4N2M0	Malignant
H8	92	F	61	Colon	Adenocarcinoma	3	II	T4N0M0	Malignant
H9	93	M	28	Colon	Mucinous adenocarcinoma	3	II	T3N0M0	Malignant
H10	94	F	59	Colon	Adenocarcinoma (colonic mucosa tissue)	–	III	T3N1M0	Malignant
H11	95	M	33	Colon	Adenocarcinoma	3	II	T3N0M0	Malignant
H12	96	M	75	Colon	Adenocarcinoma	3	II	T4N0M0	Malignant
I1	97	M	35	Colon	Adenocarcinoma	3	III	T4N1M0	Malignant
I2	98	M	68	Colon	Adenocarcinoma	3	IV	T4N0M1	Malignant
I3	99	F	44	Colon	Adenocarcinoma	2	II	T4N0M0	Malignant
I4	100	M	56	Colon	Adenocarcinoma	3	III	T4N1M0	Malignant

Pos	No.	Sex	Age	Organ	Pathology diagnosis	Grade	Stage	TNM	Type †
I5	101	F	72	Colon	Adenocarcinoma	3	II	T4N0M0	Malignant
I6	102	M	36	Colon	Adenocarcinoma	3	II	T4N0M0	Malignant
I7	103	F	74	Colon	Adenocarcinoma	3	III	T4N1M0	Malignant
I8	104	M	72	Colon	Adenocarcinoma	3	II	T4N0M0	Malignant
I9	105	M	47	Colon	Adenocarcinoma	3	III	T4N2M0	Malignant
I10	106	F	48	Colon	Mucinous adenocarcinoma	3	IV	T4N0M1	Malignant
I11	107	M	49	Colon	Mucinous adenocarcinoma	3	III	T4N1M0	Malignant
I12	108	M	71	Colon	Adenocarcinoma	3	III	T4N1M0	Malignant
J1	109	M	49	Colon	Adenocarcinoma	3	II	T4N0M0	Malignant
J2	110	F	31	Colon	Adenocarcinoma	3	IV	T4N1M1	Malignant
J3	111	M	35	Colon	Normal colonic tissue	-	-	-	Normal
J4	112	M	30	Colon	Normal colonic mucosa tissue (sparse)	-	-	-	Normal
J5	113	M	30	Colon	Normal colonic tissue	-	-	-	Normal
J6	114	M	25	Colon	Normal colonic tissue	-	-	-	Normal
J7	115	M	30	Colon	Normal colonic mucosa tissue	-	-	-	Normal
J8	116	M	28	Colon	Normal colonic tissue	-	-	-	Normal
J9	117	F	21	Colon	Normal colonic tissue with cataplasia	-	-	-	Normal
J10	118	F	21	Colon	Normal colonic tissue	-	-	-	Normal
J11	119	M	35	Colon	Normal colonic tissue	-	-	-	Normal
J12	120	M	33	Colon	Normal colonic tissue	-	-	-	Normal

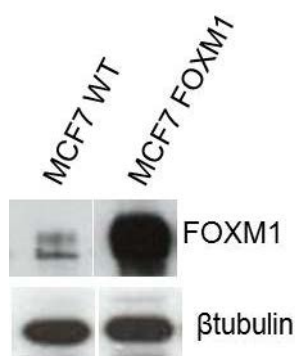
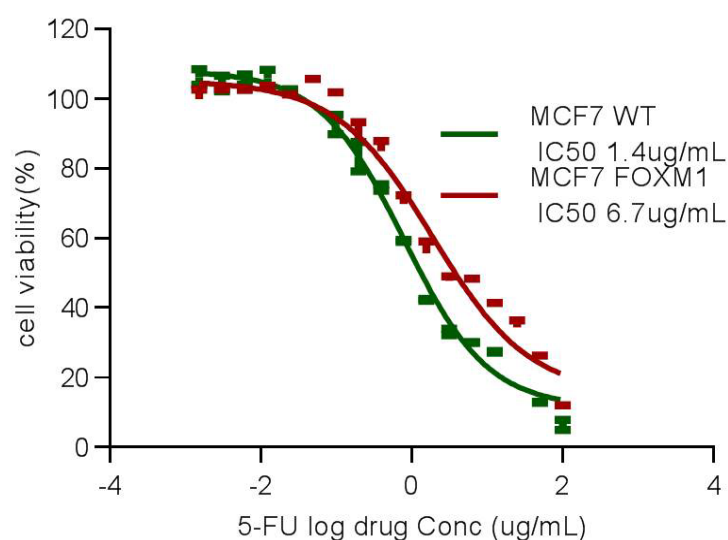
A**B**

Figure 5.1 Overexpression of FOXM1 increases resistance to 5-FU.

MCF7 cells were stably transfected with FOXM1 pCMV vector (here after MCF7 pCMV FOXM1 will be referred to MCF7 FOXM1 cells) and MCF7 wt cell lines were treated with 5-FU. Western blots were performed to see the expression of FOXM1 in MCF7 FOXM1 and MCF7 wt cells, the result confirms the expression of FOXM1 is high in MCF7 FOXM1 cells. SRB assay also revealed that FOXM1 over expressed cells are 4 fold more resistant to 5-FU than MCF7 wt cells (IC₅₀-1.8±0.31 μ g/ml) and MCF7 FOXM1 (IC₅₀-6.7±0.42 μ g/ml).

Lane 1- NSC (nonspecific control)

Lane 2- SiE2F1

Lane 3-SiE2F2

Lane 4- SiEF3

Lane 5- SiE2F1+ SiE2F2+ SiE2F3

Lane 6- SiFOXM1

Lane 7- pCDNA3 FOXM1

Lane 8-pCDNA3 (empty vector)

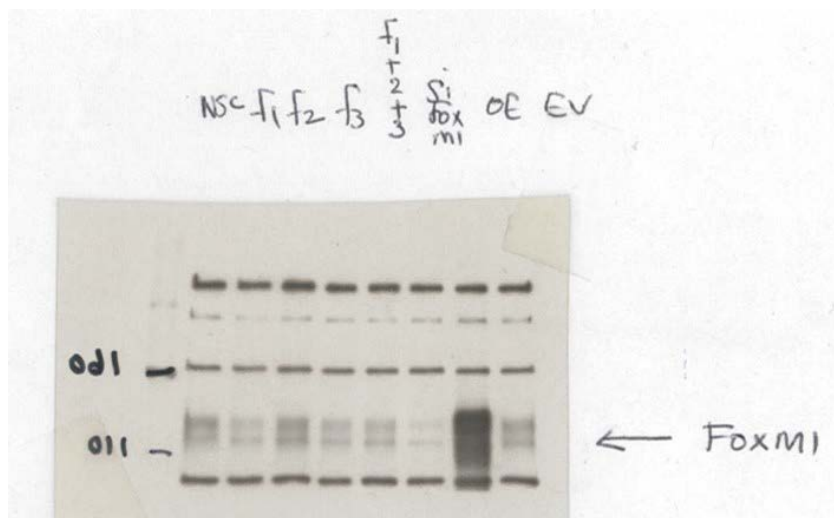


Figure 5. 2:

Over-expression and knock-down of FOXM1 affect 5-FU sensitivity in CRC (The original un-spliced film of figure 3.2A). HCT116 cells were transiently transfected with SiNSC/ SiE2F1/ SiE2F2/ SiE2F3/ SiFOXM1/ FOXM1 pcDNA3 plasmid/empty vector pcDNA3 and transfection efficiency was confirmed by western blot.

Table 5.2 Below table shows raw mRNA values (Quantity and CT values - I use quantity to calculate the gene expression) of L19, FOXM1, TYMS and E2F1 in HCT116 cells treated with 5-FU. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. Statistically significant decreases were observed in FOXM1 (P value = 0.0068), TYMS (P value = 0.0172) and E2F1 (P value=0.0008) mRNA levels at 48h.

L19					
Quantity	0 H	6H	18H	24H	48H
	1020.529	1012.4943	1027.9905	1123.3021	1081.246
	1046.423	865.5684	1055.3093	1041.2466	997.5622
	1047.885	927.2828	968.9427	866.9337	1095.6309
Average	1038.279	935.11515	1017.4142	1010.49413	1058.14637

FOXM1					
Quantity	0 H	6H	18H	24H	48H
	823.0709	408.54996	670.1711	799.82043	450.49835
	719.2044	395.22415	684.8401	828.8462	524.3738
	722.619	433.67258	699.7437	854.96375	555.3596

TYMS					
Quantity	0 H	6H	18H	24H	48H
	1142.835	829.17163	1001.8947	1143.6951	854.5208
	1133.498	769.72925	1011.8524	1202.459	954.4486
	1098.369	910.5921	1094.5554	1230.9286	998.0015

E2F1					
Quantity	0 H	6H	18H	24H	48H
	604.7258	556.6107	553.3173	585.5806	401.92303
	587.3936	507.29102	582.5266	611.70825	398.1012
	587.2163	535.2444	597.87103	623.005	442.60892

L19 CT Value				
0H	6H	18H	24H	48H
16.03177	16.72691	16.00956	15.73919	16.15147
15.95537	16.53396	16.23315	15.97049	16.10118
15.95111	16.32395	16.18994	16.52915	15.81525

FOXMI CT Value				
0H	6H	18H	24H	48H
21.35151	23.68106	22.03501	21.44682	23.35599
21.80016	23.79135	21.963	21.32826	22.85095
21.78441	23.48258	21.8914	21.22507	22.66

TYMS CT Value				
0H	6H	18H	24H	48H
18.39397	19.38881	18.80208	18.39163	19.29544
18.41941	19.61947	18.77142	18.23628	18.95252
18.51702	19.09837	18.52781	18.16372	18.81416

E2F1 CT value				
0H	6H	18H	24H	48H
24.81172	25.11164	25.1331	24.9281	26.28948
24.91692	25.44726	24.94701	24.77019	26.32404
24.91801	25.25323	24.85296	24.704	25.94067

Table 5.3 Below table shows raw mRNA values (Quantity and CT values - I use quantity to calculate the gene expression) of L19, FOXM1, TYMS and E2F1 in HT29 cells treated with 5-FU. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. Not any decreases were observed in FOXM1, E2F1 and TYMS after 5-FU treatment.

L19 Quantity	0H	6H	18H	24H	48H
	1071.548	1009.808	1138.774	1372.595	1185.832
	991.9625	950.0814	1221.044	1268.529	1184.636
	1056.768	957.8305	1138.646	1325.831	1141.885
Average	1040.093	972.5731	1166.155	1322.318	1170.784

FOXM1 Quantity	0H	6H	18H	24H	48H
	429.9657	234.7936	1302.417	1239.498	1399.561
	385.071	235.2346	1159.368	1303.294	1323.61
	428.1476	250.7164	1315.345	1469.413	1336.857

TYMS Quantity	0H	6H	18H	24H	48H
	764.3713	676.176	993.6406	1081.628	1094.603
	755.453	527.1229	1021.659	1062.546	991.4692
	784.1384	684.3752	1036.268	1091.375	1239.982

E2F1 Quantity	0H	6H	18H	24H	48H
	929.7045	994.0477	1067.751	1129.905	1314.844
	952.7765	857.2672	1138.488	1174.258	1338.078
	908.8566	874.1755	1146.641	1127.7	1439.73

L19 CT Value				
0H	6H	18H	24H	48H
15.03768	14.617	14.69596	14.35021	14.7614
15.04493	15.24924	14.59371	14.46088	14.63621
15.33727	15.0261	15.34848	14.70452	14.88275

FOXMI CT Value				
0H	6H	18H	24H	48H
26.00606	27.85778	22.61396	22.76551	22.39378
26.01903	27.85204	22.61396	22.6119	22.53408
26.01903	27.85204	22.61396	22.6119	22.53408

TYMS CT Value				
0H	6H	18H	24H	48H
22.79362	23.17072	21.98676	21.72579	21.68911
22.82972	23.93666	21.90123	21.78054	21.99349
22.71509	23.13364	21.85756	21.6982	21.30554

E2F1 CT Value				
0H	6H	18H	24H	48H
22.01002	21.76323	21.49944	21.29078	20.73173
21.91962	22.30918	21.26287	21.14878	20.66713
22.09366	22.23715	21.23655	21.29798	20.39709

Table 5.4 Below table shows raw mRNA values (Quantity and CT values - I use quantity to calculate the gene expression) of L19, FOXM1, TYMS and E2F1 in DLD1 cells treated with 5-FU. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. Not any decreases were observed in FOXM1, E2F1 and TYMS after 5-FU treatment.

L19					
Quantity	0H	6H	18H	24H	48H
	1003.421	1043.642	1198.83	1178.549	1132.758
	1010.879	1126.287	1248.063	1162.946	1169.315
	955.1263	1061.216	1205.992	1120.026	1152.962
AVERAGE	989.8088	1077.048	1217.628	1153.84	1151.678

FOXM1					
Quantity	0H	6H	18H	24H	48H
	1073.516	1188.96	1296.227	1280.573	1440.814
	1021.545	1003.946	1409.489	1434.144	1455.643
	1010.947	1097.549	1389.414	1404.014	1578.83

TYMS					
Quantity	0H	6H	18H	24H	48H
	1058.329	1236.792	1459.77	1368.457	1464.093
	1069.849	1269.57	1428.532	1451.127	1512.565
	1077.594	1348.345	1481.964	1494.249	1561.139

E2F1					
Quantity	0H	6H	18H	24H	48H
	793.2664	922.6923	934.3073	825.6658	1003.239
	784.5936	846.9683	1005.275	900.9139	1069.201
	800.5096	821.1838	1028.426	991.9516	1101.546

L19 CT Value				
0H	6H	18H	24H	48H
17.79829	17.68653	17.29235	17.34086	17.45354
17.77723	17.46984	17.1779	17.37876	17.36323
17.93855	17.63905	17.27541	17.48569	17.40327

FOXMI CT Value				
0H	6H	18H	24H	48H
19.98601	19.64643	19.35925	19.39964	19.00766
20.15099	20.20877	19.08074	19.02309	18.97362
20.18566	19.9124	19.12843	19.09368	18.70353

TYMS CT Value				
0H	6H	18H	24H	48H
21.9468	21.48441	20.99257	21.18424	20.98379
21.91467	21.40679	21.05675	21.01019	20.88715
21.89327	21.22817	20.94779	20.9233	20.79335

Lane1 – Empty vector
 Lane2 - pCMVHA E2F1
 Lane3 - pCMVHA E2F2
 Lane4 - pCMVHA E2F3
 Lane5 - pcDNA3 FOXM1

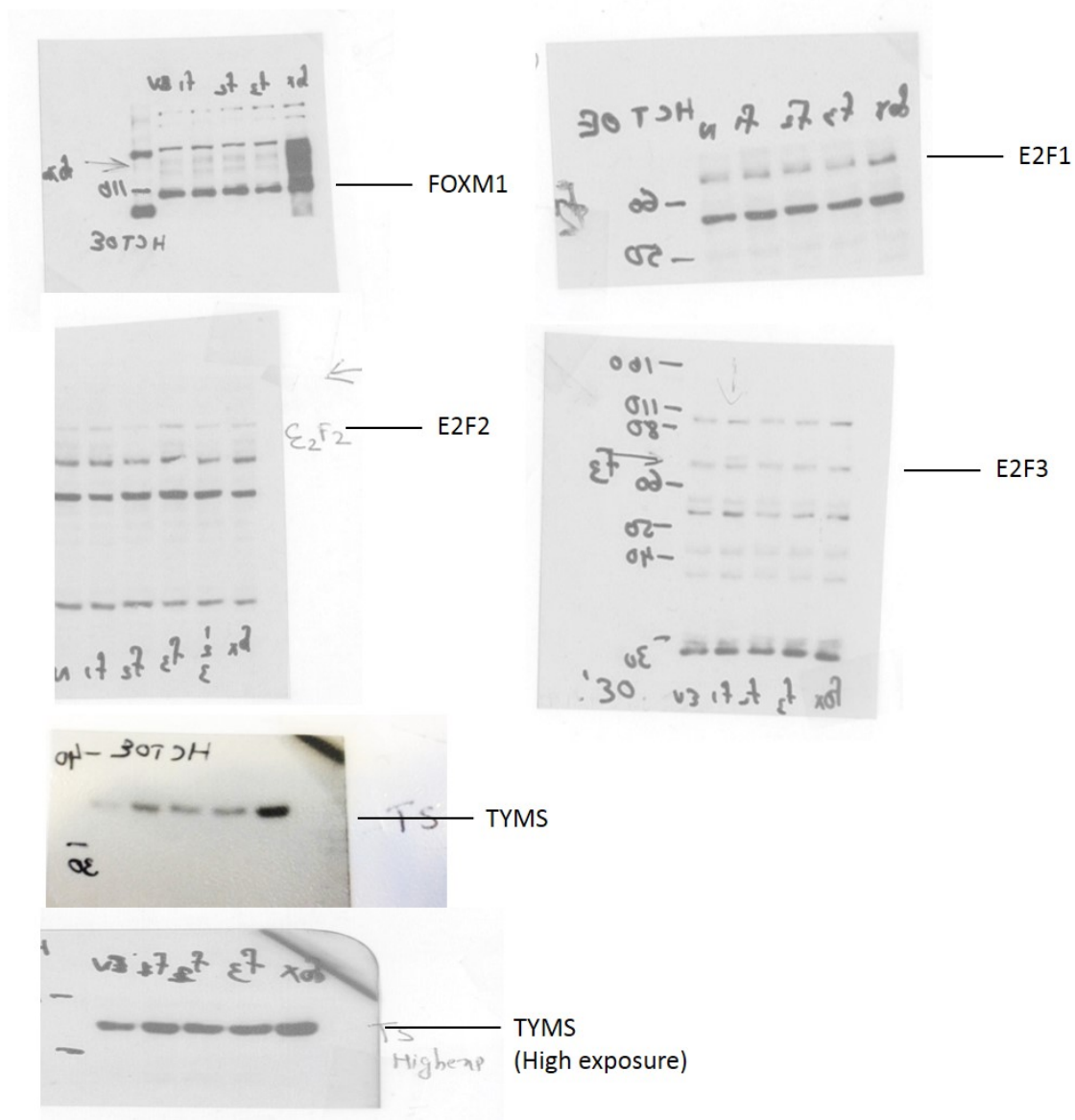


Figure 5.3:

HCT116 cells transfected with pCDNA3 empty vector, E2F1, E2F2, E2F3 and FOXM1 plasmids. (The original un-spliced film). All transfections were optimised and mediated by X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol. After 48h of transfection, cells were harvested and analysed by western blot.

Table 5.5: Below table shows raw mRNA values (Quantity and CT values- I use quantity to calculate the gene expression) of TYMS in pCDNA3 FOXM1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. TYMS mRNA expression found higher compared to control pCDNA3 (P value equals 0.0122). Statistical significance was determined by a student's T-test.

L19		
Quantity	pCDNA3	pCDNA3 FOXM1
	1092.899	1009.01624
	1342.624	1011.68524
	1019.12	1006.6471
	1151.547	1009.116193

TYMS		
Quantity	pCDNA3	pCDNA3 FOXM1
	6459.963	6796.4824
	5691.461	6502.621
	6502.635	6412.71

L19 CT Value	
pCDNA3	pCDNA3 FOXM1
13.25851	13.502951
12.62858	13.494865
13.78859	13.510146

TYMS CT Value	
pCDNA3	pCDNA3 FOXM1
21.92358	21.832457
22.15084	21.967821
21.91176	21.967821

Table 5.6: Below table shows raw mRNA values (Quantity and CT values) of TK-1 in pCDNA3 FOXM1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. TK-1 mRNA expression found slightly higher compared to control pCDNA3 but no statistical significance was observed.

L19		
Quantity	pCDNA3	pCDNA3 FOXM1
	1298.478	1154.0475
	1298.391	1258.4221
	1301.405	1172.8525
Average	1299.425	1195.107367

TK-1		
Quantity	pCDNA3	pCDNA3 FOXM1
	967.9623	897.938
	895.7839	919.5465
	1040.99	970.59094

L19 CT Value	
NSC	SiFOXM1
19.01058	17.05768
17.59481	17.06117
17.69718	17.45059

TK-1 CT Value	
NSC	SiFOXM1
21.8805	22.10833
22.11561	22.03618
21.64818	21.87227

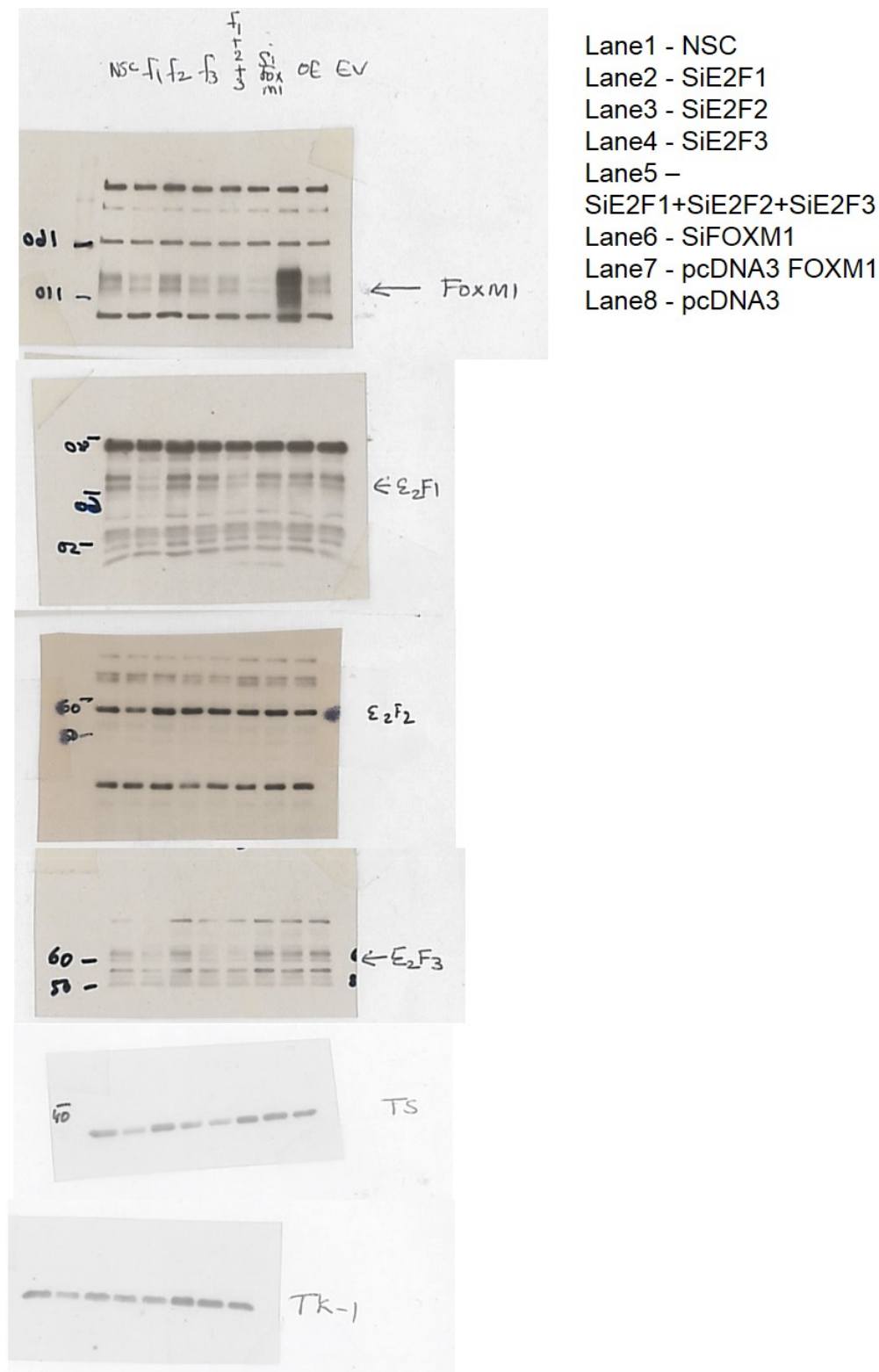


Figure 5.4:

HCT116 cells transfected with NSC, SiE2F1, SiE2F2, SiE2F3 and SiFOXM1. (The original un-spliced film). All transfections were optimised and mediated by oligofectamine transfection reagent following the manufacturer's protocol. After 48h of transfection, cells were harvested and analysed by western blot.

Table 5.7 Below table shows raw mRNA values (Quantity and CT values) of TYMS in SiFOXm1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. Significant change observed in TYMS mRNA expression compared to control. The two-tailed P value equals 0.0239

L19		
Quantity	NSC	SiFOXm1
	626.8763	1260.76
	732.0924	1078.03
	882.1256	1772.15
Average	747.0314	1370.314

TYMS		
Quantity	NSC	SiFOXm1
	4123.25	6612.805
	4841.391	6949.95
	4868.609	6918.447

L19 CT Value	
NSC	SiFOXm1
14.48499	12.82115
14.69537	13.30044
13.91434	13.47677

TYMS CT Value	
NSC	SiFOXm1
22.7292	21.88162
22.4411	21.79239
22.22251	21.74941

Table 5.8 Below table shows raw mRNA values (Quantity and CT values) of TK-1 in SiFOXm1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. Significant change observed in TK-1 mRNA expression compared to control. The two-tailed P value equals 0.0045

L19		
Quantity	NSC	SiFOXm1
	917.3549	1282.659
	999.2098	1280.577
	952.7639	1268.506
Average	956.4429	1277.247

TK-1		
Quantity	NSC	SiFOXm1
	828.5285	928.8051
	871.0966	942.7855
	935.3701	926.8192

L19 CT Value	
NSC	SiFOXm1
17.01058	17.05768
17.59481	17.061174
17.69718	17.450588

TK-1 CT Value	
NSC	SiFOXm1
22.74267	21.005783
22.2004	21.960455
21.98441	22.012278

Table 5.9 Below table shows raw mRNA values (Quantity and CT values) of E2F3 in SiFOX M1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. Significant change observed in E2F3 mRNA expression compared to control. The two-tailed P value equals 0.0028

L19		
Quantity	NSC	SiFOX M1
	883.256	1058.356
	911.3938	1078.836
	896.7839	1077.667
Average	897.1446	1071.62

E2F3		
Quantity	NSC	SiFOX M1
	641.3204	1015.46
	634.9728	1191.026
	638.3204	1087.026

L19 CT Value		
	NSC	SiFOX M1
	18.94413	16.55596
	16.90745	16.5109
	18.47888	16.64678

E2F3 CT Value		
	NSC	SiFOX M1
	27.48644	26.01166
	27.51837	25.49774
	27.49517	25.79311

Table 5.10 Below table shows raw mRNA values (Quantity and CT values) of E2F1 in SiFOXm1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. No significant changes observed in E2F1 mRNA expression compared to control.

L19		
Quantity	NSC	SiFOXm1
	946.3655	1162.271
	964.1655	1071.78
	982.4426	1104.799
Average	964.3245	1112.95

E2F1		
Quantity	NSC	SiFOXm1
	924.3223	1048.774
	969.586	1046.1
	1045.973	1077.58

L19 CT Value		
	NSC	SiFOXm1
	17.98626	16.06896
	16.63382	16.31397
	16.57705	16.22225

E2F1 CT Value		
	NSC	SiFOXm1
	25.35904	24.92483
	25.1947	24.9336
	24.93402	24.83169

Table 5.11 Below table shows raw mRNA values (Quantity and CT values) of E2F2 in SiFOX M1 transfected HCT116 cells. Gene expression was quantified using a standard curve and normalised to the housekeeping gene L19. No significant changes observed in E2F2 mRNA expression compared to control.

L19		
Quantity	NSC	SiFOX M1
	951.0515	1137.515
	949.5703	1133.427
	1041.665	1083.197
Average	980.7623	1118.046

E2F2		
Quantity	NSC	SiFOX M1
	897.2018	1111.737
	938.6639	996.9693
	1020.786	1032.486

L19 CT		
Value	NSC	SiFOX M1
	18.99713	17.03105
	17.57337	17.04186
	17.2954	17.17799

E2F2 CT		
Value	NSC	SiFOX M1
	26.00421	25.10737
	25.81523	25.56315
	25.46439	25.41672

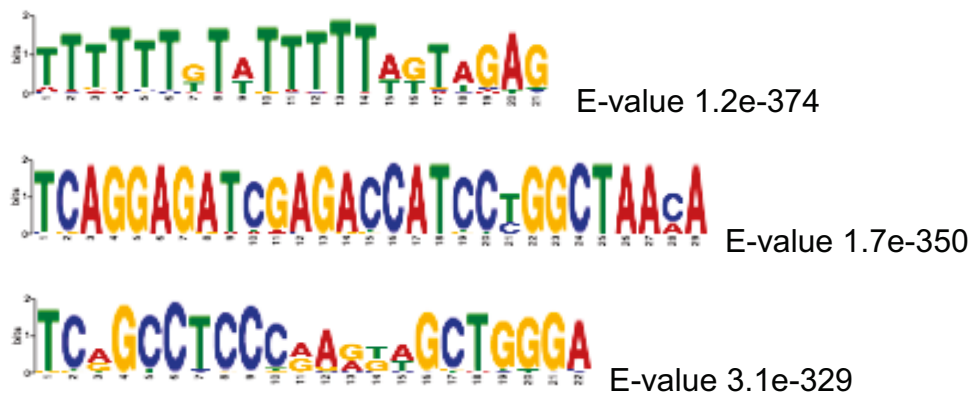


Figure 5.5 significantly enriched 3 top motifs logo were identified by MEME software (200bp regions around peak centre). From each motif more than 50 significant matches identified.

Table 5.12 Below table shows immunohistochemistry scoring of TYMS, TK1 and FOXM1 in 111 colon cancer tissue samples. The scoring was calculated based on the percentage and intensity of the staining in TYMS Cytoplasm, TK-1 cytoplasm and nucleus, FOXM1 cytoplasm and nucleus.

catalog number	position	TS cytoplasm			TK-1						FOXM1					
					CYTOPASM			NUCLEUS			CYTOPASM			NUCLEUS		
		%	intensity	Score	%	intensity	Score	%	intensity	Score	%	intensity	Score	%	intensity	Score
BC051110 A1		90.00	3	270.00	0	-		0	-		90	1	90.00	40	1	40.00
BC051110 A2		90	1	90.00	0	-		0	-		100	1	100.00	20	1	20.00
BC051110 A3		0			0			0			80	1		60	2	120.00
BC051110 A4		0			0			0			0			0	-	
BC051110 A5		50	2	100.00	0	-		0	-		90	1	90.00	50	1	50.00
BC051110 A6		90	2	180.00	0	-		10	1	10.00	100	3	300.00	20	2	240.00
BC051110 A7		80	2	160.00	0	-		0	-		100	2	200.00	30	2	260.00
BC051110 A8		90	2	180.00	80	1	80.00	0	-		100	1	100.00	70	2	2140.00
BC051110 A9		100	3	300.00	80	1	80.00	0	-		100	2	200.00	30	1	130.00
BC051110 A10		0			0			0	-		90	1	90.00	0	-	
BC051110 A11		100	3	300.00	90	2	180.00	20	1	20.00	90	3	270.00	30	1	130.00
BC051110 A12		0			0			0			0			0		
BC051110 B1		60	1	60.00	0	-		0	-		90	1	90.00	60	2	120.00
BC051110 B2		0			0			0	-		100	1	100.00	20	1	20.00
BC051110 B3		0			0			0			100	1	100.00	30	1	130.00
BC051110 B4		50	1	50.00	0	-		0	-		100	2	200.00	70	3	210.00
BC051110 B5		0	-		40	1	40.00	0	-		90	3	270.00	50	3	150.00
BC051110 B6		70	2	140.00	60	1	60.00	10	1	10.00	100	2	200.00	70	1	170.00
BC051110 B7		70	3	210.00	90	1	90.00	0	-		100	2	200.00	20	1	120.00
BC051110 B8		0	-		0	-		0	-		100	1	100.00	10	1	110.00
BC051110 B9		80	1	80.00	0	-		0	-		100	2	200.00	30	1	130.00
BC051110 B10		80	3	240.00	80	1	80.00	0	-		100	3	300.00	0	-	
BC051110 B11		40	1	40.00	90	1	90.00	0	-		100	2	200.00	20	1	120.00
BC051110 B12		100	1	100.00	90	1	90.00	0	-		100	2	200.00	20	1	120.00
BC051110 C1		0	-		0	-		0	-		50	1	50.00	0	-	
BC051110 C2		80	1	80.00	90	1	90.00	0	-		100	3	300.00	10	2	220.00
BC051110 C3		50	1	50.00	0	-		0	-		90	1	90.00	20	1	120.00
BC051110 C4		90	2	180.00	0	-		0	-		100	2	200.00	60	1	160.00
BC051110 C5		90	2	180.00	0	-		0	-		100	2	200.00	20	1	120.00
BC051110 C6		80	3	240.00	80	1	80.00	10	1	10.00	100	3	300.00	70	2	2140.00
BC051110 C7		90	1	90.00	40	1	40.00	10	1	10.00	100	3	300.00	70	2	2140.00
BC051110 C8		90	3	270.00	70	1	70.00	0	-		100	2	200.00	60	2	120.00
BC051110 C9		70	2	140.00	60	1	60.00	0	-		100	2	200.00	70	2	2140.00
BC051110 C10		90	2	180.00	80	1	80.00	0	-		100	2	200.00	20	1	120.00
BC051110 C11		90	2	180.00	80	1	80.00	0	-		100	2	200.00	40	1	140.00
BC051110 C12		70	1	70.00	90	1	90.00	0	-		100	2	200.00	90	3	270.00

BC051110 D1	0	-	0	-	0	-	90	2 180.00	10	1 10.00
BC051110 D2	80	2 160.00	0	-	0	-	90	2 180.00	20	1 20.00
BC051110 D3	80	2 160.00	0	-	0	-	50	1 50.00	20	1 20.00
BC051110 D4	80	2 160.00	0	-	0	-	90	2 180.00	20	1 20.00
BC051110 D5	70	2 140.00	80	1 80.00	50	1 50.00	90	3 270.00	20	1 20.00
BC051110 D6	80	1 80.00	0	-	0	-	90	1 90.00	0	-
BC051110 D7	90	2 180.00	80	1 80.00	0	-	90	3 270.00	40	3 120.00
BC051110 D8	90	2 180.00	70	140.0 20	40	1 40.00	90	3 270.00	50	3 150.00
BC051110 D9	90	2 180.00	0	0-	0	-	90	1 90.00	70	1 70.00
BC051110 D10	60	1 60.00	50	1 50.00	0	-	90	3 270.00	60	2 120.00
BC051110 D11	0	-	0	1-	70	1 70.00	0	-	0	-
BC051110 D12	70	1 70.00	80	1 80.00	0	-	100	3 300.00	80	2 160.00
BC051110 E1	60	1 60.00	0	-	0	-	90	1 90.00	20	1 20.00
BC051110 E2	60	1 60.00	0	-	0	-	80	1 80.00	60	1 60.00
BC051110 E3	0	-	0	-	0	-	90	2 180.00	20	1 20.00
BC051110 E4	40	1 40.00	0	-	0	-	90	2 180.00	10	1 10.00
BC051110 E5	0	-	0	-	0	-	0	-	0	-
BC051110 E6	0	-	0	-	0	-	90	2 180.00	20	1 20.00
BC051110 E7	80	3 240.00	90	1 90.00	0	-	90	2 180.00	10	1 10.00
BC051110 E8	50	1 50.00	90	1 90.00	0	-	90	2 180.00	10	1 10.00
BC051110 E9	30	2 60.00	0	-	0	-	90	1 90.00	30	1 30.00
BC051110 E10	0	-	70	1 70.00	0	-	90	2 180.00	30	3 90.00
BC051110 E11	80	3 240.00	90	1 90.00	0	-	90	2 180.00	10	1 10.00
BC051110 E12	80	1 80.00	80	1 80.00	0	-	90	3 270.00	10	1 10.00
BC051110 F1	0	-	0	-	0	-	90	2 180.00	20	2 40.00
BC051110 F2	50	3 150.00	0	-	0	-	90	1 90.00	10	1 10.00
BC051110 F3	80	3 240.00	0	-	0	-	90	1 90.00	10	1 10.00
BC051110 F4	0	-	0	-	0	-	90	2 180.00	20	2 40.00
BC051110 F5	80	1 80.00	0	-	0	-	90	2 180.00	20	1 20.00
BC051110 F6	0	-	0	-	50	1 50.00	80	3 240.00	20	3 60.00
BC051110 F7	100	3 300.00	90	180.0 20	0	-	90	3 270.00	10	1 10.00
BC051110 F8	80	1 80.00	90	1 90.00	0	-	90	2 180.00	10	1 10.00
BC051110 F9	30	1 30.00	0	-	0	-	90	1 90.00	20	1 20.00
BC051110 F10	90	1 90.00	0	-	20	1 20.00	90	2 180.00	80	1 80.00
BC051110 F11	70	2 140.00	0	-	0	-	90	2 180.00	20	1 20.00
BC051110 F12	90	1 90.00	0	-	0	-	90	2 180.00	60	1 60.00

BC051110 G1	0	-	0	-	0	-	90	190.00	10	110.00
BC051110 G2	0	-	0	-	0	-	0	-	0	-
BC051110 G3	50	2 100.00	0	-	0	-	90	2 180.00	40	140.00
BC051110 G4	100	3 300.00	90	190.00	0	-	90	3 270.00	20	120.00
BC051110 G5	90	2 180.00	0	-	0	-	90	2 180.00	40	140.00
BC051110 G6	80	180.00	80	180.00	0	-	90	2 180.00	20	120.00
BC051110 G7	100	3 300.00	90	180.0 20	0	-	90	3 270.00	80	3 240.00
BC051110 G8	0	-	90	180.0 20	0	-	90	3 270.00	0	-
BC051110 G9	0	-	0	-	0	-	0	-	0	-
BC051110 G10	70	170.00	0	-	0	-	90	3 270.00	20	240.00
BC051110 G11	0	-	0	-	0	-	90	3 270.00	30	130.00
BC051110 G12	0	-	60	160.00	50	2 100.00	90	190.00	60	2 120.00
BC051110 H1	0	-	0	-	0	-	60	160.00	0	-
BC051110 H2	60	160.00	0	-	0	-	90	190.00	10	110.00
BC051110 H3	0	-	0	-	0	-	20	120.00	0	-
BC051110 H4	0	-	0	-	0	-	0	-	0	0-
BC051110 H5	0	-	0	-	0	-	90	190.00	60	2 120.00
BC051110 H6	70	2 140.00	0	-	0	-	90	3 270.00	60	3 180.00
BC051110 H7	90	190.00	0	-	0	-	90	190.00	80	180.00
BC051110 H8	0	-	0	-	0	-	90	2 180.00	60	2 120.00
BC051110 H9	0	-	90	180.0 20	0	-	90	3 270.00	0	-
BC051110 H10	0	-	0	-	0	-	90	3 270.00	0	-
BC051110 H11	80	2 160.00	0	-	0	-	0	-	0	-
BC051110 H12	0	-	90	180.0 20	0	-	90	2 180.00	80	2 160.00
BC051110 I1	60	160.00	0	-	0	-	70	3 210.00	50	2 100.00
BC051110 I2	0	-	0	-	0	-	90	190.00	20	120.00
BC051110 I3	90	2 180.00	0	-	0	-	50	2 100.00	0	-
BC051110 I4	0	-	0	-	0	-	90	1	70	2 140.00
BC051110 I5	0	-	0	-	50	150.00	0	-	40	280.00
BC051110 I6	90	3 270.00	0	-	0	-	90	3 270.00	0	-
BC051110 I7	0	-	80	180.00	0	-	90	190.00	80	3 240.00
BC051110 I8	80	180.00	0	-	0	-	90	190.00	20	120.00
BC051110 I9	0	-	0	-	0	-	90	3 270.00	10	110.00
BC051110 I10	0	-	90	180.0 20	0	-	90	3 270.00	0	-
BC051110 I11	100	2 200.00	0	-	0	-	100	2 200.00	0	-
BC051110 I12	0	-	80	180.00	0	-	0	-	30	130.00

BC051110	J1	30	130.00	0	-	0	-	80	180.00	0	-
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BC051110	J3	100	1100.00	0	-	0	-	CANT DETER	#VALUE !	0	-
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BC051110	J5	100	2200.00	0	-	0	-	CANT DETER	#VALUE !	0	-
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BC051110	J11	100	3300.00	0	-	0	-	80	2160.00	80	180.00
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Figure 5.6 TYMS flanking region. FOXM1 consensus sites (TAAACA) is highlighted.

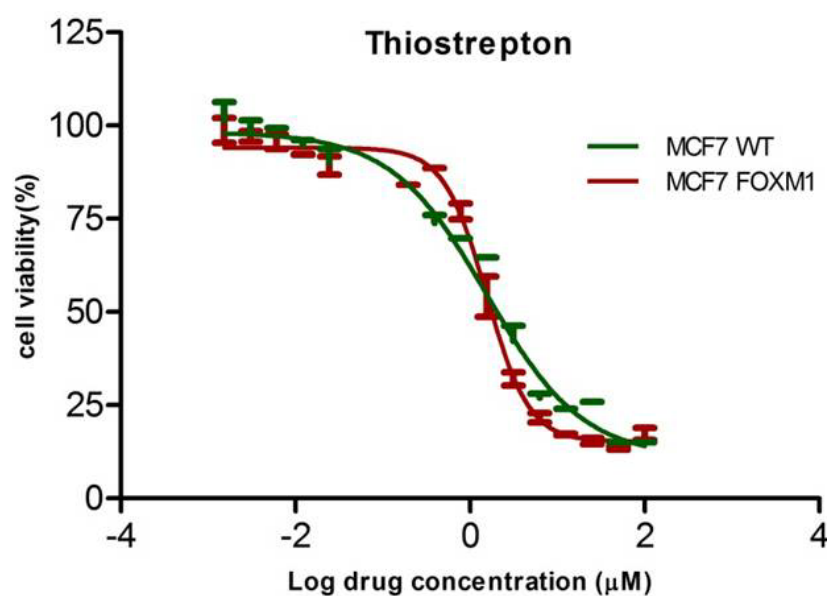


Figure 5.7 MCF7 wt and MCF7 pCMVFOXM1, expressing a CMV promoter drive. FOXM1 (MCF7 FOXM1 over expressed cells) were treated with thiostrepton ranging from 0-200 μM for 72h. SRB assay indicates MCF7 wt and MCF7 FOXM1 over expressed cells shows similar IC₅₀ after the thiostrepton treatment. Results were normalized to untreated. Results shown are mean and SD of three independent experiments, each with three replicates. IC₅₀ was determined by fitting a sigmoidal dose-response curve to the data.

CHAPTER 6

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