The regulation of FOXM1 in breast cancer

Thesis submitted by

Mesayamas Kongsema

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 ORIGINALITY

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ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women worldwide. In the UK, there is 1 in 8 of women that have a risk of being diagnosed with breast cancer in a lifetime. Clinically, the common treatment using for breast cancer therapy are combinations of surgery, radiotherapy, endocrinal (hormonal) therapy and chemotherapy. The DNA damaging agent epirubicin has been shown to be an effective chemotherapeutic drug for breast cancer treatment. Nevertheless, there are more than 90% of patients with metastatic cancer that found to be resistant to the drug.

FOXM1 is a transcription factor that has been reported to be responsible for a resistance to various chemotherapeutics, including epirubicin. Accumulating evidence has revealed that FOXM1 is regulated by modifications at the post-translational levels. In this study, I show that FOXM1 can also be regulated by SUMOylation and ubiquitination. In response to epirubicin treatment, FOXM1 is modified primarily by SUMO1, and not SUMO2/3, in breast cancer cells. The SUMOylation of FOXM1 is targeted by RNF168 and leads to its ubiquitination, nuclear exportation and degradation through the proteasome degradation pathway. Unfortunately, the study about OTUB1 about its property in deubiquitinating ubiquitinated FOXM1 does not show the significant results. Collectively, this thesis identifies and characterises the role of SUMOylation, ubiquitination and RNF168 in modulating FOXM1 expression and activity, by promoting its degradation. My data suggest that these proteins and PTMs might be interesting targets for the development of novel therapeutic strategies for breast cancer treatment and for overcoming conventional chemotherapeutic drug resistance.
PUBLICATIONS


* The authors contributed equally and should be considered to joint first authors.
TO MY BELOVED MUM AND DAD
THANK YOU FOR EVERYTHING
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase-Promoting Complex, also called cyclosome</td>
</tr>
<tr>
<td>APLF</td>
<td>Aprataxin and PNK-like factor</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternative reading frame</td>
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<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
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<td>ATR</td>
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<td>Base excision repair</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast cancer gene 1/2</td>
</tr>
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<td>BSA</td>
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<tr>
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<td>CDKs</td>
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CKS1 - CDK subunit 1
CRL - Culling RNG ligase
CtIP - C-terminal binding protein interacting
DAPI - 4',6-diamidino-2-phenylindole
DCIS - Ductal carcinoma in situ
DDR - DNA damage response
DFS - Disease-free survival
DMEM - Dulbecco's Modified Eagle Medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DNA-PKcs - DNA-dependent protein kinase catalytic subunit
DNR - Daunorubicin
dNTPs - Deoxynucleotide triphosphates
DOX - Doxorubicin
DSB - DNA Double strand break
DTT - Dithiothreitol
DUB - Deubiquitylation enzyme
ECL - Enhanced chemiluminescence
EDTA - Ethylenediaminetetraacetic acid
ERα - Oestrogen Receptor α
ERE - Oestrogen response element
EXO1 - Exonuclease1
FACs - Fluorescence-activated cell sorting
FANC - Fanconi anaemia RING-type ligase
FCS - Foetal calf serum
FHA - Forkhead-associated domain
Fkh - Forkhead gene
FKHL16 - Forkhead drosophila homolog-like 16
FLIM - Fluorescence-lifetime imaging microscopy
FOX - Forkhead transcriptional factor box
FRET - Förster resonance energy transfer
GFP - Green fluorescence protein
GFR - Growth factor receptors
h - Hour
HECT - Homologous to the E6-AP (UBE3A) carboxyl terminus
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2 - Human epidermal growth factor 2
HFH11 - Hepatocyte nuclear factor3/ forkhead homolog11
HR - Homologous recombination
HRP - Horseradish peroxidase
IDA - Idarubicin
IDS - Invasive ductal carcinoma
IgG - Immunoglobulin G
JNK1 - c-Jun N-terminal kinase
KIS - Kinase interacting with Stathmin
LMA - Low melting point agarose
M - Molar (mole per litre)
MBC - Metastatic breast cancer
MCF-7 - Michigan Cancer Foundation-7 cells
MCF-7 EpiR - Michigan Cancer Foundation-7 epirubicin resistant cells
MDC1 - Mediator of DNA damage checkpoint
MDR1 - Multidrug resistance protein
MEF - Mouse embryonic fibroblasts
min - Minute
MMP - Matrix metalloproteinase
MMR - Mismatch repair
MPP2 - M-phase phosphoprotein
MRE11 - Meiotic recombination
MRN - MRE11-RAD50-NBS1 complex
M-phase - Mitosis phase
NRD - N-terminus repressing domain
Na$_3$VO$_4$ - Sodium ortho-vanadate
NaCl - Sodium chloride
NaF - Sodium fluoride
NBS1 - Nijmegen breakage syndrome
NER - Nucleotide excision repair
NHEJ - Non-homologous end joining
NMA - Normal melting point agarose
NSC - Non-specific control
OD - Optical density
OligoDT - Oligo deoxythymidine
OS - Overall survival
OTU - Ovarian tumor
OTUB1 - Otubain 1 or OTU domain, ubiquitin aldehyde binding 1
PARP - Poly ADP-ribose polymerase
PBS - Phosphate buffered saline
PFA - Paraformaldehyde
P-gp - P-glycoprotein
PLK - Polo-like kinase
PMSF - Phenylmethysulfonyl fluoride
PNK - Polynucleotide kinase
pRb - Phospho Retinoblastoma protein
PSG - Penicillin/Streptomycin and L-glutamine
PTM - Post-translational modification
RA - Retinoic acid
Rb - Retinoblastoma protein
RF - RING finger domain
RFC4 - Replication factor C4
RING - Really interesting new gene
RNA - Ribonucleic acid
RNFs - RING-type zinc finger proteins
ROS - Reactive oxygen species
RPA - Replication protein A
RPM - Round per minute
RT-qPCR - Reverse transcriptase quantitative polymerase chain reaction
<table>
<thead>
<tr>
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<tr>
<td>SAC</td>
<td>Spindle assembly checkpoint complex</td>
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<tr>
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</tr>
<tr>
<td>SCGE</td>
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<td>SSBR</td>
<td>Single strand break repair</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier or small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
</tbody>
</table>
TEMED - N,N,N',N'-Tetramethylethylenediamine

TOPIIα - Topoisomerase IIα

TSG - Tumour suppressor gene

Ub - Ubiquitin

UbL - Ubiquitin-like protein

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

WIN - Winged helix

XLF - XRCC4-like factor

XRCC4 - X-ray repair cross-complementing protein 4

53BP1 - p53-bonding protein1
CHAPTER 1

INTRODUCTION
1.1 Cancer

Cancer is a disease caused by uncontrolled cell growth. There are eight biological capabilities which have been declared associated with the multistep development of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). These include the ability for cells to replicate indefinitely, invade and metastasis to distance organs, maintain signalling for cell proliferation, escape from surveillance of growth suppressors, reprogramming of energy metabolism, angiogenesis inducible, evading immune destruction, and prevent cell death (Figure 1.1). In general, cancer can be differentiated into two types, defined by the cellular or tissue type: carcinoma is a cancer developed from epithelial cells and sarcoma is a cancer of the connective tissue.

Cancer is one of the leading causes of death. In 2010, it was estimated that there are over 12 million new cancer cases every year, with more than 7 million people dying from cancer (Ferlay et al., 2010). More recent data from WHO revealed that, in 2012, there were 14.1 million cancer cases diagnosed, with 8.2 million people dying from cancer (WHO, 2012). The most common malignancies world-wide are lung, breast and colorectal cancers (Ferlay et al., 2010).
Figure 1.1 Schematic diagrams of cancer hallmarks and enabling characteristics. 
A. The six out of eight essential properties required for cancer development. B. The eight cancer hallmarks (two emerging hallmarks were added later in 2011) with two enabling characteristics that have a role in driving cancer progression. (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).
1.2 Oncogenes and Tumour suppressor genes

The development of cancer can be attributed to genes being altered in a defective manner. The two types of genes that are related to cancer genetics are oncogenes and tumour suppressor genes. The first evidence that showed a genetic alteration could result in cancer was obtained in the study in Burkitt's lymphoma, where it was shown that the mutation of \textit{MYC} oncogene can induce cancer initiation (Croce, 2008).

1.2.1 Oncogenes

Proto-oncogenes are genes that have the potential of becoming oncogenes when they attain a gained-of-function or when their activity is increased inappropriately. This results in cancer development (Bishop, 1991). Proto-oncogenes are believed to have been mutated by point mutation, amplification or rearrangement (Todd and Wong, 1999). Oncogenes encode proteins called oncoproteins which are classified into 6 groups: apoptosis regulators, signal transducers, growth factors (GF), growth factor receptors (GFR), chromatin remodelers, and, transcription factors (TF) (Croce, 2008).

J Michael Bishop and Harold Vermus were the first to find a cellular oncogene (\textit{c-onc}) and were awarded a Nobel Prize for that (Vogt, 1997). There are three possible mechanisms for oncogene activation; these include gene amplification, mutation and chromosomal rearrangement. For example, HER2/neu (human epidermal growth receptor 2) is one of the important oncogenes, which was found to be overexpressed in around 25% of breast cancers. In breast cancer, the activation and amplification of the \textit{HER2/neu} oncogene leads to a poor prognosis. (Croce, 2008). Another example of an
oncogene is *RAS* oncogene, which is activated through a point mutation. The RAS proteins play a crucial role in many important pathways that help to sustain normal cell proliferation (Downward, 2003). There are 3 members of the RAS family, of which mutational activation is always found in human tumours: *KRAS*, *NRAS* and *HRAS* (Croce, 2008; Downward, 2003; Rodenhuis, 1992). These 3 members of the RAS family have approximately 85% amino acid sequence identity. Around 20% of all tumours have been found to contain an activating mutation in one of these *RAS* genes (Downward, 2003). Interestingly, each *RAS* gene mutation is common between different types of cancer. *KRAS* mutations are normally found in adeno-carcinomas of lung cancers, colorectal cancers, and pancreatic cancers. Alternatively, the *NRAS* mutation is commonly found in a subset of acute leukemias and myelodysplastic syndromes, whereas mutations of *HRAS* are very rare, presenting in less than 1% of a total *RAS* mutations in tumours (Downward, 2003; Rodenhuis, 1992). An example of a gene presenting the last type of oncogene activation, a chromosomal rearrangement, is the *c-MYC* gene. In general, *c-MYC* is expressed during embryogenesis and is also found in highly proliferative tissues like the skin epidermis and gut (Pelengaris et al., 2002). Translocation de-regulation of *c-MYC* is the most general activation mechanism in cancers of B and T cells, while *c-MYC* gene fusion is common in soft-tissue sarcomas (Croce, 2008).
1.2.2 Tumour Suppressor genes

Tumour suppressor genes (anti-oncogenes) are genes that encode proteins that prevent cells from becoming cancerous. It is believed that, in some cases, they can act by negatively regulating cell proliferation. Mutation or deletion of a tumour suppressor gene leads to a loss of its function, potentially resulting in cancer development (Lai et al., 2012; Osborne et al., 2004). The first study that revealed the existence of tumour suppressor genes was done in 1971 (Knudson, 1971). During the study on retinoblastoma, Knudson stated that the hereditary and the nonhereditary form of retinoblastoma are, somehow, linked together. He raised the “two hit hypothesis” to clarify the relationship between the two forms of retinoblastoma. In detail, his hypothesis was that retinoblastoma is a cancer that is caused by 2 mutational events. In the hereditary form, which is a common form of retinoblastoma, one mutation (the first hit) is inherited through the germinal cells in the dominant hereditary form. Subsequently, the second hit is developed in somatic cells, inducing the initiation of the disease. In contrast, the nonhereditary form is caused by a double mutation in somatic cells. These lead to the bilateral forms of retinoblastoma (Knudson, 1971; Payne and Kemp, 2005). In 1986, a study was performed to confirm that the mutation of the retinoblastoma gene, Rb, is a major course of retinoblastoma. This confirms Knudson’s theory about the tumour suppressor gene (Friend et al., 1986).

Another well-known tumour suppressor gene is TP53, which encodes a protein called p53. The p53 protein is a transcription factor that has an important role in many cellular processes, such as cell cycle control, apoptosis and stress signalling (Dai and Gu,
2010). It is also described as "the guardian of the genome" because it is involved with the conservation of the genome stability by preventing the insurgence of mutations (Dai and Gu, 2010; Matlashewski et al., 1984). Around 20% to 35% of breast cancers have shown to carry a mutation of p53 (Lacroix et al., 2006).

Other interesting tumour suppressor genes important for breast cancer and ovarian cancer are \textit{BRCA1} and \textit{BRCA2}. These genes encode proteins called breast cancer susceptibility protein 1 and 2, respectively. These proteins take part in two fundamental cellular processes: DNA damage repair and transcriptional regulation (Welcsh and King, 2001). Clinically, breast or ovarian cancer patients presenting a mutation in either \textit{BRCA1} or \textit{BRCA2} have a poor prognosis. In conclusion, a mutation in tumour suppressor genes together with the activation of oncogenes can promote the insurgence and progression of cancer, by promoting cell proliferation, cell cycle progression and the evasion of apoptosis (Todd and Wong, 1999).

\subsection*{1.3 Breast Cancer}

Breast cancer is the most common cancer in women. Over one million patients are diagnosed every year, and the rate is increasing in all regions worldwide (Ferlay et al., 2010; Kamangar et al., 2006). Breast cancer usually is derived from epithelial tissue of the duct or lobule (glands). The most common breast cancer is the ductal carcinoma, a cancer of the mammary duct in the breast. This type of breast cancer can also be classified in to 2 sub-types: the ductal carcinoma \textit{in situ} (DCIS), also known as a well-
defined border cancer with no invasiveness, and the invasive ductal carcinoma (IDS) (Anand, 2007). Breast cancer can also affect males, but this is a rare condition with only 1% of possibility (White et al., 2011). This study was mainly performed on MCF-7 cells, which are derived from a woman who suffered from breast cancer. Hence, the word “breast cancer” in this study will only be referring to the breast cancer in women.

The most common treatments for breast cancer are endocrine therapy, radiotherapy and chemotherapy. Radiotherapy is used locally to stop the cancer growth or to reduce the size of the tumour mass, while chemotherapy and endocrinal therapy are commonly used for a systemic treatment. Around 70% of patients with breast cancer are oestrogen receptor (ER) positive. In this type of breast cancer, the cell growth is controlled by ERα, a member of the Nuclear Receptor (NR) superfamily of transcription factors. ERα promotes FOXM1 transcription and leads to an upregulation of cell proliferation (Karadedou, 2006; Millour et al., 2010). Despite ER positive breast cancer can be treated with anti-oestrogen therapy (hormonal therapy), one-third of the patients will still not respond to the treatment (Karadedou, 2006). For the patients who have ER negative breast cancer or who have had a relapse from the endocrine therapy, the systemic chemotherapy is the only treatment option.
1.4 Clinical management of breast cancer

Chemotherapy resistance is the cause for 90% of treatment failure in breast cancer patients with metastasis (Longley and Johnston, 2005). In general, treatments for breast cancer patients are administered according to etiologic and pathological factors, such as patient age, histological grade and size of the tumour mass, nodal status and some receptors, like HER2 or ER/PR (Guarneri and Conte, 2004). Clinically, breast cancer is a heterogeneous disease. According to a previous study by Anderson and Matsuno, it can be differentiated according to an expression of hormonal receptors (positive or negative; +/-). Moreover, the ER/PR positive group has 2 subtypes which are luminal A and luminal B (Anderson and Matsuno, 2006).

The luminal subtype can be classified into 2 subtypes: luminal A (ER+, PR+ and HER2- with a low proliferative rate), and luminal B (ER+, PR+, and HER2+ with a high proliferative rate). Other types of breast cancer are the ER- with HER2+ (Anderson and Matsuno, 2006; Perou, 2011) and the triple negative breast cancer (ER-, PR- and HER2-). Triple negative tumours are classified as a basal-like breast cancer because they express cytokeratin 5, 6 and 17. The cytokeratin expression is commonly found in basal epithelial tissues, such as the skin and airways (Perou, 2011). The clinical management of breast cancers depends on a medical diagnostic. Treatment is normally a combination of various clinical procedures, such as surgery and radiotherapy. In metastatic breast cancer, the most important therapies are the systemic therapies, which include endocrinial (hormonal) therapy, molecular targeted therapy and
chemotherapy. Systemic therapies also help get rid of circulating tumour cells in the blood vessels and in the lymphatic system.

1.4.1 Endocrine (hormonal) therapy

Endocrine therapy or hormonal therapy is the treatment for patients with oestrogen receptor positive breast cancer. Oestrogen has an important role in various physiological processes. Oestrogen action is primarily mediated by two oestrogen receptors, ERα and ERβ (Ali and Coombes, 2002; Burns and Korach, 2012; Deroo and Korach, 2006). In normal breasts, about 15% - 25% of epithelial cells are ER+ (Ali and Coombes, 2002). Despite the fact that the role of oestrogen in promoting breast cancer is undefined, the majority of primary breast cancers (around 70% – 80%) have been found to express ERα at a high level. This evidence confirms that ERα has a crucial role in cancer development (Ali et al., 2011). Consequently, ERα has become one of the effective targets in breast cancer treatment. There are many ways of inhibiting the effect of oestrogen in breast cancer with ER+. Selective ER modulators (SERMs), including tamoxifen, are a common treatment for ER+ breast cancer. This type of treatment results in cancer regression, in approximately 30% of breast cancer cases (Ali and Coombes, 2002). Tamoxifen is a non-steroidal triphenylethylene agent that binds to ERα and antagonises its activity (Ali et al., 2011). Another anti-oestrogen drug that can be used for breast cancer treatment is raloxifene and faslodex (Ali et al., 2011; Chlebowski, 2014; Ingle et al., 2013). When comparing tamoxifen with raloxifene, tamoxifen has been found to have a higher efficiency than raloxifene and faslodex, but with a greater side effect of endometrial cancer risk than the other two (Ali et al., 2011;
Chlebowski, 2014; Howell et al., 2004). Faslodex is a selective oestrogen receptor down-regulator (SERD) that sometimes is called Fulvestrant according to its trade name.

1.4.2 Molecular targeted therapy

Clear understanding of the molecular events in the development of cancer aids the identification of new anti-cancer therapy targets. Molecular targeted therapy is a therapy that focuses on a specific molecule that is involved with a crucial mechanism in cancers, such as cell invasion and metastasis, cell death, cell cycle control or angiogenesis (Alvarez et al., 2010). In breast cancer, the first two examples of approved molecular targeted agents against the human epidermal growth factor receptor 2 (HER2) are lapatinib and trastuzumab (Alvarez et al., 2010; Sanchez-Munoz et al., 2009). Trastuzumab is commonly known as Herceptin, following its trade name. HER2 is a surface receptor tyrosine kinase (TK) that was found to be overexpressed in one third of breast cancers (Moasser, 2007). Another interesting molecular target for the treatment of breast cancer is the vascular endothelial growth factor (VEGF), which can be targeted by an agent called bevacizumab (Alvarez et al., 2010). In addition, poly ADP-ribose polymerase (PARP) inhibitors can be used as a combinational therapy together with other cancer therapy. This combination might promote the efficiency of the main therapy. PARP is a crucial mediator of base excision repair (BER) mechanism, necessary for a single strand break (SSB) repair in cells. The loss of PARP activity results in an accumulation of DNA SSB. These can be converted into DNA double strand break (DSB) by cellular replication and/or transcription machinery (Patel et al.,
2011). Taken together, increasing the cancer therapy options helps oncologists and doctors find and improve treatments for patients (Sanchez-Munoz et al., 2009).

1.4.3 Taxanes

Taxanes are one of the well-known chemotherapy agents for breast cancer treatment. Taxanes are products of plants of the Taxus genus. Clinically, there are two available taxanes (Markman, 2003) that are commonly used for cancer treatment: paclitaxel (Taxol) and docetaxel (Taxotere). These are microtubule-stabilising agents that have a role in interfering with spindle microtubule dynamics causing cell cycle arrest and subsequent cell death (McGrogan et al., 2008). The result from preclinical studies revealed that docetaxel binds stronger to β-tubulin than paclitaxel, as well as having longer intracellular retention time and more potential in inducing cell death (Bachegowda et al., 2014). Despite this, both drugs have become commonly used as chemotherapeutic agents for treating both early-stage and metastatic breast cancer with benefits, in terms of the overall survival (OS) and the disease-free survival (DFS) (Bachegowda et al., 2014; McGrogan et al., 2008).

1.4.4 Anthracyclines

Anthracyclines are anti-cancer chemotherapeutic drugs with a wide spectrum activity against various cancers, with the exception of some cancers, including colon cancer, which do not response to them. The main mechanism of action of anthracyclines is the DNA intercalation and the inhibition of the Topoisomerase IIα (TOPIIα) enzyme (Khasraw et al., 2012; Zunino and Capranico, 1990). Anthracyclines have been reported
to increase events of DNA cleavage by purified Topoisomerase II (Zunino and Capranico, 1990). The first two anthracyclines which were discovered in 1960s are daunorubicin and doxorubicin (Weiss, 1992). They were isolated from the pigment reducing Streptomyces peucetius (Minotti et al., 2004). Doxorubicin (DOX) differs from daunorubicin (DNR) only by a single hydroxyl group (red circles in Figure 1.2). This similarity has important effect on the spectrum activity of doxorubicin (hydroxyl-daunorubicin) and daunorubicin. Doxorubicin is normally used for the treatment of breast cancer, aggressive lymphomas, childhood solid tumours, soft tissue sarcomas, while daunorubicin is important for acute lymphoblastic or myeloblastic leukemias (Minotti et al., 2004).

Many analogues of doxorubicin and daunorubicin were marketed in various countries, one of them being idarubicin (IDA), an analogue of daunorubicin, which is available in the US. None of the analogues have stronger efficacy in term of cancer treatment than the original two anthracyclines, but there differ in toxicity (Weiss, 1992). Another interesting analogue of the original anthracycline is epirubicin, an analogue of doxorubicin.

In 1982, epirubicin was approved to be used for cancer treatment, and more than 2,000 publications have since characterised its efficacy and safety (Khasraw et al., 2012). Because of a small difference in its structure, epirubicin activity differs slightly from that of doxorubicin. Interestingly, this difference primarily affects its pharmacokinetics and metabolism, thus enhancing its total body clearance and making epirubicin have a shorter terminal half-life. Epirubicin has been shown to have less side effects in cancer
patients, including cardiotoxicity, when compared with doxorubicin (Minotti et al., 2004). Therefore, epirubicin has become a common choice in breast cancer treatment, and is commonly combines with some other treatments in adjuvant therapy.

Figure 1.2 Analogues of important anthracyclines: doxorubicin, daunorubicin, epirubicin and idarubicin. Red circles indicate the difference between DOX and DNR. Yellow stars indicate residues that are the side chain of DNR or IDA terminates with a methyl in place of a primary alcohol compared with DOX or EPI. Arrows indicate structural modifications in EPI compared with DOX, or in IDA compared with DNR (Minotti et al., 2004).
1.5 Chemotherapy resistance

Common therapies for breast cancer are endocrine therapy, molecular targeted therapy and chemotherapy (Section 1.3). For breast cancer patients who are oestrogen receptor negative (ER-) or HER2 negative (HER2-), such as patients presenting the basal-like triple negative breast cancer, or patients who have a relapse from endocrine or molecular targeted therapies, conventional chemotherapies remains the limited choice.

Cancer treatment failure in more than 90% of patients with metastatic cancer is caused by resistance to the chemotherapeutic drugs (Lippert et al., 2011; Rivera, 2010). Patients with metastatic breast cancer (MBC) are primarily treated with taxane or anthracycline containing regimens. However, in a lot of cases, patients are treated with these regimens as an adjuvant therapy, leading to an increasing number of patients becoming resistant to treatment (Rivera, 2010). Normally, breast cancer patients are treated with a combination of multiple drugs to avoid the insurgence of resistance to a single agent. The simultaneous resistance to different drugs with different targets and chemical structures is known as multidrug resistance (MDR) (Szakacs et al., 2006). An important mechanism of MDR is the overexpression of drug efflux proteins, including ATP-binding cassette (ABC) transporters (Szakacs et al., 2006; Zelnak, 2010). ABC transporters have a role in protecting cells or tissues from being damaged by extruding toxic agents, including chemotherapeutic drugs, out of the cells (Zelnak, 2010). The most studied ABC transporter is the multidrug resistance protein 1 (MDR-1), which is also known as permeability-glycoprotein (P-gp). It functions by promoting the efflux of chemotherapeutic drugs out of cells, and by reducing the intracellular drug levels.
Drug efflux is not the only cause of chemotherapy resistance. For instance, resistance to taxanes can arise from the loss of β-II-tubulin and the overexpression of β-III-tubulin. As taxanes work by binding to β-tubulin, resistance to their action can be caused by an altered expression of certain β-tubulin isotypes. Alternatively, altered expression of the topoisomerase llα (TOP2A) gene can lead to anthracycline resistance (Zelnak, 2010). Finally, an increase in the DNA repair mechanisms or the down regulation in apoptotic signalling can also promote the insurgence of chemotherapeutic drug resistance.

1.6 Cell cycle

The cell cycle is a strictly regulated process of cell replication and proliferation. It can be divided into four distinct phases as G1, S, G2 and M phases. While the cells are in S (synthesis) and M (mitosis) phases, the cellular genome is replicated, and separated into the two daughter cells, respectively. Both phases are followed by gap periods: G2 and G1 phases (Berckmans and De Veylder, 2009). In the presence of specific anti-mitotic signals or an absence of mitotic signals, cells will exit the cell cycle by entering into a quiescent state, which is called G0 (Park and Lee, 2003). The cell cycle is tightly regulated, because a small mistake can lead to serious consequences for the development of the whole organism, such as cancer initiation (Berckmans and De Veylder, 2009). Key proteins for the regulation of the cell cycle are the cyclins and the cyclin-dependent kinases (CDKs). CDKs belong to a family of serine/threonine protein kinase that require a specific cyclin for their activation at specific points of the cell cycle.
(Berckmans and De Veylder, 2009; Vermeulen et al., 2003). To date, nine CDKs have
been identified, but only five of them have been reported to be active during the cell
cycle: CDK1 (also known as CDC2), CDK2, CDK4, CDK6 and CDK7 (Vermeulen et al.,
2003). During the cell cycle, the levels of CDKs remain stable, while the protein levels of
the cyclins change. In this way, cyclins periodically activate CDKs. When CDKs are
activated, they induce their downstream targets by phosphorylating them at specific
sites (Park and Lee, 2003; Vermeulen et al., 2003).

Every phase of the cell cycle has at least one checkpoint, where regulatory proteins can
promote cell cycle arrest (Lukas et al., 2004). The most interesting inhibitory proteins
are CDK inhibitors (CKIs), which are inhibitors for the cyclin/CDK complexes. According
to their structures and their specificity, CKIs have been classified into two families: CDK
interacting protein/kinase inhibitor protein (Cip/Kip) family and the inhibitor of Cdk4 and
6 (Ink4) families. The Cip/Kip family members, which are p21^{Cip1}, p27^{Kip1} and p57^{Kip2},
have a wide range of substrate specificity and interfere with the activities of cyclin D-, E-
, A- and B-dependent kinase complexes. Conversely, the Ink4 family members, which
are p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} only target CDK4 and CDK6 by forming
stable complexes with them and prevent the binding of the cyclins (Lim and Kaldis,
2013; Vermeulen et al., 2003).

The cell cycle can be arrested at G1/S, S and G2/M, M checkpoints to evaluate
extracellular growth signals, DNA integrity and cell size (Park and Lee, 2003). In late G1
phase, there is a stage called the “restriction point”, which is controlled by the
retinoblastoma (Rb) protein, which is a tumour suppressor. This stage has a crucial role
in cancer prevention, as alterations in the regulation of the transition from G1 to S phase could lead to cells proliferating independently of mitogenic stimuli (Lapenna and Giordano, 2009). When the cells enter the cell cycle, CDK4 and CDK6 form an active complex with D-type cyclins (D1, D2 and D3) and initiate the phosphorylation of Rb to inactivate its function (pRB). This pRB inactivation leads to an increase in transcription of a few cell cycle genes, particularly of E-type cyclin (E1 and E2), by the E2F transcription factor. In late G1, the CDK2-cyclin E heterodimer reinforces the RB1 phosphorylation on additional sites, initiating the S-phase and the genes involved (Berckmans and De Veylder, 2009; Lapenna and Giordano, 2009; Park and Lee, 2003). The E2F transcription factor can also target genes that are involved in the DNA repair and chromatin dynamics (Berckmans and De Veylder, 2009). After the cells pass through the restriction point, pRb is still in a hyper-phosphorylated form due to the sequential kinase activity by CDK2-cyclin A and CDK1-cyclin A to ensure that the cell is entering into G2 phase.

The centrosome cycle starts during DNA replication. The centrosome duplicates in late S phase to early G2 phase and the duplicated chromosome makes poles for the mitotic spindle (Lapenna and Giordano, 2009). In late G2, CDK1 associates with cyclin A to initiate mitosis. However, if there is DNA damage during the G2 phase, the cell cycle will be arrested immediately at the G2/M checkpoint to prevent the damaged DNA from being carried on to the daughter cells. An important mediator of this checkpoint is the major mitosis-promoting of the CDK1-cyclin B kinase activity (Lukas et al., 2004). In case of DNA damage, combinations of factors come into account to prevent CDK1-cyclin B activation. These include ataxia telangiectasia mutated (ATM), ataxia
telangiectasia and their downstream effectors RAD3-related protein (ATR), and checkpoint kinases CHK1/CHK2. In this case, CDK1 is activated at the G2/M boundary to repair the damage lesion (Lapenna and Giordano, 2009; Lukas et al., 2004). Other key regulators of the G2/M checkpoint are the mitotic serine/threonine polo-like kinase (PLK) family members: PLK1 and PLK3. They promote the beginning of the M phase and can be down-regulated by an ATM-dependent mechanism. The overexpression or mutation of PLK1 has been found to be associated with tumorigenesis, and with poor prognosis (Schmitt et al., 2007). In the M phase, the spindle assembly checkpoint (SAC) complex ensures that chromatids are correctly attached to the mitotic spindles. The SAC complex is regulated by an anaphase-promoting complex/cyclosome (APC/C) and its cofactors. To complete the cell division process, an activated CDK1 needs to be switched off by the proteolysis of cyclin B via APC/C mediated pathway. SAC deficiency is a frequent characteristic of cancer cell lines (Lapenna and Giordano, 2009).

1.7 DNA damage response and repair

The DNA damage response (DDR) is highly complex. The mechanism includes “sensor” proteins that sense the damage lesion, “transducer” proteins that transmit the DNA damage signal, and “effector” proteins that receive the signal from transducer proteins (Schmitt et al., 2007). There are thousands of DNA damage events occurring in human cells every day. This is a consequence of both exogenous and endogenous factors. DNA damage can interfere with DNA replication and transcription as it can lead to mutations and chromosomal aberrations (Polo and Jackson, 2011). DNA damage
signalling and repair responses have a crucial role in the maintenance of the genome integrity, as it prevents the transmission of the DNA damage lesions and its consequences to daughter cells (Ciccia and Elledge, 2010; Hoeijmakers, 2001; Hoeijmakers, 2007). DNA damage can be both single-strand break (SSB) and double-strand break (DSB).

SSBs are less cytotoxic when compared with DSBs, but it is the most common type of DNA damage. This type of DNA damage has been found to be at a frequency of tens of thousands per cells a day. This is the consequence of spontaneous DNA decay and the direct attack by intracellular metabolites. Endogenous reactive oxygen species (ROS) are also a main cause of SSBs (Caldecott, 2008). In contrast, DSBs are the most deleterious forms of DNA damage as there is no intact complementary DNA strand that can act as a template during the repair. DSBs can occur in response to various factors, such as topoisomerase II inhibitor agents (such as epirubicin) which stop re-ligation of the double-stranded DNA cleavage (Polo and Jackson, 2011).

1.7.1 DNA double strand break repair

In order to neutralise the effect of the DNA damage on the cells, DNA damage repair is necessary (Figure 1.3). Normally, mismatched DNA bases are fixed by a process called mismatch repair (MMR). A minute alteration of DNA bases can be corrected by base excision repair (BER), which cuts out the damaged base. More complex lesions, such as intrastrand crosslinks and pyrimidine dimers, are repaired by nucleotide excision repair (NER), by the removal of the oligonucleotide, that contain the damaged bases (Ciccia and Elledge, 2010). SSBs are commonly repaired by a single-strand break
repair (SSBR), but DSBs are mainly repaired by either of two mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR) (Caldecott, 2008; Khanna and Jackson, 2001).

NHEJ is an effective mechanism that operates during all phases of the cell cycle and carries out the ligation of DNA ends with small processing at the end joining site. Conversely, HR repair is specific to late S and G2 phases of the cell cycle. HR works by using an undamaged homologous sequence as a template for the DNA repair, preferably the sister chromatid, and it is believed to be a highly precise DNA repair method for DSBs (Kass and Jasin, 2010). In HR, the DNA ends are first resected by a nuclease in a 5' to 3' direction. Subsequently, the 3' single-stranded tails, the product of the resection, enter the undamaged homologous DNA double helix, and are elongated by DNA polymerase. After branch-migration, the resulting DNA crossovers, also known as Holliday junctions, are resolved by many processes to produce two undamaged and repaired DNA molecules. As a result, HR is an accurate DNA repair mechanism with no loss or addition to the DNA sequence. Alternatively, NHEJ does not require an undamaged homologous partner and does not depend on extensive homologies between the two recombining ends. During NHEJ repair, sometimes after limited degradation at the termini, the two ends are joined together (Jackson, 2009; Khanna and Jackson, 2001). Error-prone repair can lead to genome instability through the accumulation of chromosomal aberrations and mutations. In addition, hyper-recombination also leads to genome instability phenotypes, such as gene deletion, amplification and loss of heterozygosity (Moynahan and Jasin, 2010).
Figure 1.3 DNA damage and repair mechanisms DNA can be damaged by various DNA damage agents. Most of the DNA repair processes, including BER, NER, HR, EJ and mismatch repair, are responsible for DNA damage lesion removal (Adapted from Hoeijmakers 2001).
The choice of DSB repair pathway is controlled by many agents, such as the nature of the damage lesion and the phase of the cell cycle. Accumulated evidence states that while NHEJ works throughout in every cell cycle phase, HR is restricted to late S and G2 phases. This is due to the presence of the sister chromatids: in mammalian cells, the sister chromatid, which is the template for HR repair, is present only during the S and G2 phases of the cell cycle (Rothkamm et al., 2003; Yata et al., 2012). A recent study demonstrates that even though NHEJ is modulated throughout the cell cycle, it only increase its activity from the G1 to the G2/M (G1 < S < G2/M) phases (Mao et al., 2008). For HR, it is almost absent during the G1 phase, to then become most active in the S phase, and descending in G2/M phase (Mao et al., 2008). In addition, an important regulatory step that drives to the choice for the DSB repair pathway is the process of DSB resection, which is needed for HR but not NHEJ (Polo and Jackson, 2011).

1.7.2 Signalling in DNA damage response

The DNA damage response (DDR) is a complex signal transduction pathway that can sense the presence of damaged lesions and transduce the signal to influence cellular responses (Ciccia and Elledge, 2010). DNA repair is closely correlated with the regulation of the cell cycle through the activation of orchestrated signalling pathways. One of the main aspects of the DNA damage signalling is the induction of cell cycle checkpoints which delay or stop cell cycle progression during the persistence of the DNA damage, thus preventing the replication of the damaged DNA or the segregation of abnormal chromosomes during mitosis. These signalling mechanisms can also lead
cells to triggering of programmed cell death (apoptosis) or long-term cell-cycle arrest (senescence), particularly when the damage persists (Jackson, 2009). DSB recognition and repair requires the action of various proteins that are involved with DNA damage response pathways: sensors, transducers, mediators and effectors. The DSB response pathway (Figure 1.4) allows the transduction of a signal from a sensor that recognises the DSB to its downstream effectors via a transduction cascade (Khanna and Jackson, 2001).

DNA damage by anthracyclines, such as epirubicin, causes double strand breaks. DSBs are recognised by the MRE11-RAD50-NBS1 (MRN) sensor complex. This complex promotes the activation of a transducer kinase ATM (Ataxia Telangiectasia Mutated) that phosphorylates members of the complex and prepares the DNA for HR (Ciccia and Elledge, 2010; Jackson, 2009). To organise the complex, MRE11 (meiotic recombination 11), an exonuclease enzyme, binds directly to NBS1, DNA and RAD50 (Williams et al., 2007). Specifically, RAD50, a member of a structural maintenance of chromosome (SMC) related protein family, contains an ATP-binding cassette (ABC) ATPase that interacts with MRE11 and associates with the DNA ends of DSBs. MRE11 has both endonuclease and exonuclease activities that help stabilising the DNA ends. These activities are important for the initial steps of DNA end resection, crucial for the initiation of HR mechanisms (Ciccia and Elledge, 2010; Williams et al., 2007). NBS1, the last sub unit of the MRN complex, interacts with MRE11 and contains supplementary protein-protein interaction domains that are crucial for DDR. In addition, NBS1 also interacts with ATM through its C-terminal region. This promotes the recruitment of ATM to the site of damage, where ATM is activated (Ciccia and Elledge,
The ATM kinase, as a signal transducer, activates the effector CHK1 and CHK2, with the help of mediator proteins, including mediators of the DNA damage checkpoint (MDC1), p53-binding protein1 (53BP1) and BRCA1. CHK proteins have a role in spreading the signal throughout the nucleus. Ultimate targets of these signalling cascades involve cell cycle regulators, DNA repair factors, programmed cell death machineries, and transcription factors (Harrison and Haber, 2006; Polo and Jackson, 2011).

For example, ATM kinase controls the resection of the DNA ends via C-terminal binding protein interacting protein (CtIP), that interacts with BRCA1 and MRN, in a BRCA1-C (BRCA1-BARD1 heterodimer, CtIP, MRN and damaged DNA) complex (Ciccia and Elledge, 2010). The most extensively studied among DDR proteins is the ssDNA-binding complex replication protein A (RPA). This protein accumulates at resected DSBs in an MRN and CtIP dependent manner. An important regulatory step in DSB repair is a resection by the MRN complex. The resulting single-stranded DNA (ssDNA) overhangs are then coated by RPA before being substituted by RAD51 (Polo and Jackson, 2011). An assembly of RAD51 filaments on RPA-coated ssDNA leads to HR repair (Ciccia and Elledge, 2010). The RAD51 nucleofilament and some other HR factors mediate the search for homology in the sister chromatid, followed by strand invasion into the homologous template (Polo and Jackson, 2011).
Figure 1.4 DSB damage and repair signalling. DNA can be damaged by either exogenous or endogenous agents, resulting in DNA double strand breaks (DSBs). DSBs are sensed by “sensors”, and then the signal is transmitted to the signal “transducers” to activate the downstream “effectors” via a transduction cascade. Then cells enter into the cell cycle arrest, apoptosis, or DNA repair pathways (Adapted from Khanna and Jackson, 2001)
DSBs can also be recognised by Ku heterodimers (Ku70 and Ku80 subunits). Ku heterodimers are involved with guarding genomic integrity via their ability to bind DSB and promoter DNA repair by NHEJ (Walker et al., 2001). The Ku protein binds to broken DNA ends, then Ku translocates inwards from the ends making the extreme termini accessible to the DNA-dependent protein kinase (DNA-PKcs), which is the catalytic subunit, so as to allow the NHEJ to be initiated (Mahaney et al., 2009; Yoo and Dynan, 1999). Moreover, binding of Ku to the damaged end of the DNA is also required for the recruitment of X-ray repair cross-complementing protein group 4 (XRCC4) (Mari et al., 2006) and the XRCC4-like factor (XLF) which are also known as Cernunnos (Yano et al., 2008). Briefly, after the DNA-PKcs is recruited, other factors, including polynucleotide kinase (PNK), nuclease Artemis, Aprataxin, and Aprataxin PNK-like factor (APLF), are also recruited to the DNA damage site. Accordingly, XRCC4, with the help of stimulatory factor XLF, promotes the ligation of the damaged end (Mahaney et al., 2009).

Poly (ADP-ribose) polymerase (PARP) 1/2 can also detect DSBs. PARP, probably with the help of DNA ligase III, works in an alternative pathway of NHEJ (alt-NHEJ) that functions as a backup of the classical pathway. Moreover, PARP1 directly competes with Ku to bind to the DNA ends to promote HR (Wang et al., 2006). During the DNA repair process, PARP1 is required for a rapid accumulation of MRE11 and NBS1 (MRN complex) at the DNA damage site, in an MDC1 independent manner (Haince et al., 2008).
1.8 Transcription factor Forkhead box M1 (FOXM1)

The transcription factors forkhead box (FOX) genes encode for a family of evolutionarily conserved transcriptional regulators that were named after the *Drosophila melanogaster* fork head gene (*fkh*) (Hannenhalli and Kaestner, 2009; Weigel et al., 1989). The *fkh* gene is expressed in the early stages of embryo development. A *fkh* mutation leads to defects to the embryo head during embryogenesis (Weigel et al., 1989). The FOX proteins are defined by a common DNA-binding domain (DBD), of about 100 amino acids in length, called the forkhead box or winged helix domain (Myatt and Lam, 2007). This domain consists of three α-helices, three β-sheets, and two large loops, also known as ‘wing’ regions that flank the third beta-sheet (Jackson et al., 2010). More than 50 FOX genes have been identified in humans, which can be further classified into 19 subfamilies. The DBD is very well conserved across families and species (Jackson et al., 2010). The FOX proteins play a central role in various cellular processes not only during embryogenesis and development, but also in adult organisms (Benayoun et al., 2011). Interestingly, various FOX families, including FOXA, FOXC, FOXM, FOXO, and FOXP, have been found to be linked to cancer genesis and progression (Myatt and Lam, 2007). In this study, I would mainly focus on the role of FOXM1 in breast cancer.

The FOXM1 (Forkhead box M1) protein is one of the transcription factors that have a crucial role in many biological processes, such as cell cycle progression, cell proliferation, angiogenesis, apoptosis, tissue homeostasis, and DNA damage repair (Figure 1.5). FOXM1 has been found to be overexpressed in various types of cancer, including breast cancer. FOXM1 was previously known as Trident, hepatocyte nuclear
factor 3/forkhead homolog 11 (HFH11), M-phase phosphoprotein 2 (MPP2) and Winged helix (WIN), forkhead drosophila homolog-like 16 (FKHL16) (Koo et al., 2012). The human and mouse FOXM1 share 79% homology. In humans, the FOXM1 gene is located on the chromosome 12 band p13, composing of 10 exons of which two are alternatively spliced (Korver et al., 1997a). Alternative splicing of exon A1 (Va) and A2 (Vlla) are present in the human FOXM1 gene (Figure 1.6) providing up to 3 distinct FOXM1 variants: FOXM1a, FOXM1b and FOXM1c (Koo et al., 2012; Yao et al., 1997).

FOXM1a contains both alternative exons, Va and Vlla. Presence of those inserts in the C-terminal transactivation domain (TAD) leads to an entirely inactive form of FOXM1. However, this TAD disruption may cause FOXM1a to be a dominant negative form because the TAD activity in DNA binding of this variant is still functional (Koo et al., 2012; Laoukili et al., 2007; Ye et al., 1997). In contrast, FOXM1b and FOXM1c are transcriptionally active. FOXM1c contains only exon Va at C-terminal of DBD, while FOXM1b contains none of the alternative exons. Both FOXM1b and FOXM1c can promote the expression of target genes in an isoform-specific manner (Koo et al., 2012). They act as transactivators through the binding to a conventional FOXM1 consensus site: TAAACA (Korver et al., 1997b).
Figure 1.5 FOXM1 plays crucial roles in various cellular processes. FOXM1 is a member of transcription factor families that have a crucial role in many biological processes, such as cell cycle progression, cell proliferation, angiogenesis, apoptosis, tissue homeostasis, DNA damage repair, and chemotherapeutic drug resistance (Adapted from Koo et al., 2012).
Figure 1.6 Schematic of known alternative splice variants and 3 protein regions of human FOXM1. A. Axon A1 and A2 are the two out of the ten exons. These two exons are alternatively spliced in FOXM1 gene. In C-terminal of DBD, there is exon A1 that encodes 15 amino acids. In C-terminal transactivation domain (TAD), there is exon A2 that encode 38 amino acids. Alternative splicing of these two exons resulted in three different isoforms of FOXM1: 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) (adapted from Koo et al., 2012; Laoukili et al., 2007).
1.8.1 FOXM1 is important for cell proliferation and cell cycle control

FOXM1 has been found to be expressed in all embryonic tissues and many of the rapidly proliferating cells. In adults, FOXM1 is expressed at high levels in the thymus and testis, and at moderate levels in the lungs and intestine. All of these tissues have a rapid proliferation rate (Laoukili et al., 2007). Interestingly, in a study of the \textit{Foxm1}\(^{-/-}\) mice, loss of the \textit{Foxm1} gene is embryonic lethality. \textit{Foxm1}\(^{-/-}\) embryos were found to be dead in the uterus due to pulmonary defects and liver development abnormalities (Korver et al., 1998; Krupczak-Hollis et al., 2004).

FOXM1 encourages cell proliferation by promoting cell cycle progression. FOXM1 expression is essential for G1/S and G2/M phases of the cell cycle and also for the mitotic spindle integrity. FOXM1 deficiency is involved with the decrease in Plk1, Aurora B kinase, cyclin A2 and B1 protein levels (Kim et al., 2006; Krupczak-Hollis et al., 2004). During the G1/S phase of the cell cycle, FOXM1 regulates CDK subunit 1 (CKS1), S phase kinase-associated protein 2 (SKP2), kinase interacting with Stathmin (KIS), and c-Jun N-terminal kinase 1 (JNK1), by acting as a transcriptional activator (Petrovic et al., 2008; Wang et al., 2005; Wang et al., 2008). FOXM1 is also required for the transcription of SKP2 and CKS1, which are specific subunits of the Skp1-Cullin 1-F-box (SCF) ubiquitin ligase complex. This complex targets cyclin-dependent kinase inhibitor (CDKI) proteins, p21\(^{Cip1}\) and p27\(^{Kip1}\), for degradation during the G1/S transition, resulting in cell cycle progression to the S phase and in mitosis (Wang et al., 2005).

FOXM1 activity can also be controlled by phosphorylation and dephosphorylation (Figure 1.7). FOXM1 phosphorylation is increased as cells progress from the G1 to S
and G2 phases, and then FOXM1 is dephosphorylated when the cells exit mitosis (Chen et al., 2009; Fu et al., 2008). Briefly, In the G1/S phase, FOXM1 phosphorylation is initiated by CDK2-cyclin E complex, while in the G2/M phase it is mediated by CDK1-cyclin B1 (Major et al., 2004). CDK2-cyclin E and CDK2-cyclin A complexes are also important for phosphorylation of FOXM1 in the G2/M phase (Laoukili et al., 2007).

Interestingly, a new conserved CDK1-dependent phosphorylation site (Ser-251) in FOXM1 has been shown to be important of its activation. Mutation of Ser-251 inhibits FOXM1 phosphorylation and results in a decrease in its transcriptional activity (Chen et al., 2009). It has already been confirmed that FOXM1 activation by phosphorylation can enhance the activity of many mitotic regulators, including Plk1 (Fu et al., 2008), Cyclin B1 (Leung et al., 2001), survivin, centromere protein A (CENPA), centromere protein B (CENPB), and Aurora B kinase (Wang et al., 2005). In order to exit cellular mitosis, FOXM1 is degraded through the proteasome degradation pathway by the Anaphase-Promoting Complex, also known as cyclosome, (APC/C). This pathway is regulated by Cdh1 (known co-factor for APC/C). The APC/C-Cdh1 complex binds directly to both D- and KEN-box motifs that are located on the N-terminus of FOXM1 (Laoukili et al., 2008).

In conclusion, all evidence confirms that FOXM1, and its post-translational modifications are necessary for cell cycle progression.
Figure 1.7 Schematic diagram of FOXM1 regulation during the cell cycle. Protein and mRNA levels of FOXM1 are upregulated in late G1 phase, FOXM1 activity is subjected to different levels of regulation. Increasing FOXM1 activity in G2/M phase is related to its hyperphosphorylation. This suggests that phosphorylation has a crucial role in activating FOXM1 (Kwok, 2010; Laoukili et al., 2007; Murakami et al., 2010).
1.8.2 FOXM1 in DNA repair and chromosome integrity maintenance

Various findings have revealed the importance of FOXM1 in maintaining genomic integrity. Loss of FOXM1 or FOXM1 overexpression can lead to chromosomal abnormalities (Laoukili et al., 2005; Wonsey and Follettie, 2005). As is widely known, FOXM1 is necessary for the timely mitotic entry. In addition, the centromere protein F (CENP-F, mitosin), a kinetochore binding protein, is also a direct target of FOXM1. CENP-F is part of the mitotic checkpoint (Laoukili et al., 2005). A study revealed that FOXM1 also has a crucial role in the regulation of various genes that are important for chromosome segregation and mitosis, including CENP-A, KIF20A and Nek2. FOXM1 defects have been confirmed to lead to mitotic spindle defects, delays in mitosis and mitotic catastrophe (Wonsey and Follettie, 2005).

There is evidence that the activity of FOXM1 is suppressed at the DNA damage checkpoint. FOXM1 has been shown to be repressed by p53, which is important in cell cycle delay and G2/M checkpoint, in response to DSBs. Induction of p53 by DNA-damage leads to the downregulation of FOXM1 (Barsotti and Prives, 2009; Pandit et al., 2009). In contrast, another report showed that FOXM1 remains active during the DNA damage response, in the G2 arrest. FOXM1 activates the expression of its downstream targets, including PLK1, Cyclin A and Cyclin B, which are crucial for cell cycle re-entry from G2 arrest and checkpoint recovery (Alvarez-Fernandez et al., 2010).

In a previous study from our lab, FOXM1 has been reported to be involved in DNA damage repair, following epirubicin treatment. FOXM1 is downregulated after epirubicin treatment in wild-type breast cancer cells but not in epirubicin resistant cells. The study
found that FOXM1, activated by ATM and E2F1, promotes cell survival in epirubicin resistant breast cancer cells (Millour et al., 2011). Consistently, FOXM1 interacts with NFκB in the presence of chemotherapeutic drugs, to protect the cells from DNA damage. Moreover, FOXM1 also regulates the expression of HR repair genes, including EXO1, PLK4 and RFC4 (Park et al., 2012).

1.8.3 FOXM1 and cancer

FOXM1 is a transcription factor that plays a crucial role in a wide spectrum of cellular processes, such as cell proliferation, DNA damage repair, apoptosis, and cell cycle progression. Moreover, overexpression of FOXM1 is commonly found in a multitude of cancers, including breast cancer (Koo et al., 2012; Wonsey and Follettie, 2005).

One of the most important processes for tumorigenesis and cancer progression is angiogenesis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The vascular endothelial growth factor (VEGF) family is important for tumour angiogenesis and the VEGF ligand binds to tyrosine kinase receptor (VEGFR) to promote its activation through dimerisation and phosphorylation (Shibuya and Claesson-Welsh, 2006). Indeed, VEGF is a direct transcriptional target of FOXM1 and this is mediated through two FOXM1 binding sites in the VEGF promoter. Mutation of these binding sites significantly reduces the ability of FOXM1 to induce activity of the VEGF promoter. In agreement, overexpression of FOXM1 leads to the promotion of angiogenesis in glioma cells (Zhang et al., 2008). A study in pancreatic cancer cells also showed that the inhibition of FOXM1 expression reduces the expression of matrix metalloproteinase-2 (MMP-2), MMP-9, and VEGF, resulting in the inhibition of cellular migration, invasion
and angiogenesis (Wang et al., 2007). A study in gastric cancer revealed that FOXM1 has a role in tumorigenesis by inhibiting cellular senescence. FOXM1 depletion leads to the down regulation of c-MYC and Skp2, together with the accumulation of CDK inhibitor p27\(^{kip1}\). Moreover, FOXM1 knock down can also inhibit telomerase, an enzyme that participates in cell immortality (Zeng et al., 2009). In addition, Foxm1\(^{-/-}\) MEFs revealed a high level of β-galactosidase, p19\(^{ARF}\), and p16\(^{INK4A}\) proteins, which are hallmarks of premature cellular senescence (Wang et al., 2005). FOXM1 has been proved to help preventing cancer cells from undergoing stress-induced premature senescence. Overexpression of FOXM1 suppresses cellular senescence and the expression of p53 and p21 (Li et al., 2008).

FOXM1 is widely known to be one of the key regulators of the G1/S and G2/M transition, and of the mitotic progression. Consistent with this, FOXM1 also regulates the expression level of various genes, including cyclin B, aurora kinases, PLK1, and survivin. (Koo et al., 2012). Foxm1\(^{-/-}\) MEFs and FOXM1 depleted human osteosarcoma cells show a mitotic blockage and accumulation of CDKI proteins, including p21\(^{Cip1}\) and p27\(^{Kip1}\) (Wang et al., 2005). Additionally, the protein and mRNA levels of FOXM1 and Bmi-1, its downstream target, are at the maximum levels at G2/M phase. It has been confirmed that FOXM1 regulates Bmi-1 expression through c-Myc. These three proteins are major regulators of tumourigenesis (Li et al., 2008).

Interestingly, some cancer cells and stem cells share identical properties, such as self-renewal ability, immortality and sustained proliferative capacity (Koo et al., 2012). Recently, FOXM1 has been revealed to have a crucial role in regulating cancer initiation.
by provoking stem cell-like self-renewal properties in cancer cells. This study, using a functional 3D organotypic epithelial tissue regeneration system, showed that FOXM1 overexpression disturbs epithelial differentiation, promotes hyperproliferative characters, leading the cells to acquire similar properties that can be observed in human epithelial hyperplasia (Gemenetzidis et al., 2010). A study of embryonic development revealed that FOXM1, induced by retinoic acid (RA), is suppressed during differentiation of pluripotent P19 embryonic carcinoma cells at the early stage. This downregulation of FOXM1 is consistent with a lower expression of stem cell pluripotency genes, including Oct4 and Nanog (Xie et al., 2010). In addition, a study in mouse embryos also showed that increasing Foxm1 activity induces the proliferation of respiratory epithelium and promotes the growth of lung tumours (Wang et al., 2010). Together these findings confirm that FOXM1 has an important role in cancer development.

1.8.4 FOXM1 and chemotherapy resistance

Conventional chemotherapy is a common systemic treatment for patients with cancer. Chemotherapy helps to prevent cancer cells from proliferation and undergoing metastasis. FOXM1 has been found to have an important role for development of chemotherapeutic drug resistance. In breast cancer, FOXM1 is related to the resistance to various therapies including endocrine therapy (Millour et al., 2010), cisplatin (Kwok, 2010), Trastuzumab (also known as Herceptin), paclitaxel (Carr et al., 2010), and also anthracyclines (Halasi and Gartel, 2012; Millour et al., 2011; Monteiro et al., 2013; Park et al., 2012).
Hormonal therapy, also known as endocrine therapy, is the preferred option for most of breast cancer patients with ERα positive tumours. Normally, these patients are treated with anti-oestrogen agents, such as tamoxifen, that promote G1/S cell cycle arrest and apoptosis (Koo et al., 2012). However, ERα positive breast cancer patients present inconsistent unstable clinical outcomes in response to endocrinal therapy. A considerable number of patients at a metastatic stage is found to be resistance to the treatment (Ali and Coombes, 2002). Expression of ERα in breast carcinoma cells is physiologically regulated by FOXM1 (Madureira et al., 2006). Conversely, ERα is also found to regulate FOXM1 expression via an oestrogen response element (ERE) located within the proximal promoter region of FOXM1. This feedback mechanism might be responsible for an association between the mRNA levels of FOXM1 and ERα, which has been observed in breast cancer patient samples (Millour et al., 2010). A genome view study, using a cDNA microarray, revealed that FOXM1 is one of the oestrogen responsive genes in breast cancer cells (Cicatiello et al., 2004). Notably, tamoxifen treatment has been shown to repress FOXM1 in sensitive breast cancer cell lines, but not in resistant cell lines (Millour et al., 2010). All together, these studies indicate that high expression levels of FOXM1 are correlated to an endocrine therapy resistance in breast cancer.

Cisplatin, also known as cis-platinum or cis-diaminedichloroplatinum (II)/CDDP, is a platinum-based compound that has a high potential for cancer treatment (Koo et al., 2012). Cisplatin functions by forming DNA adducts (can be called as platinum–DNA adducts), intrastrand and interstrand, and thereby activating various signal-transduction pathways, such as DNA-damage repair, cell cycle arrest, and apoptosis (Kelland, 2007).
Recent studies have also revealed that FOXM1 expression is involved with cisplatin resistance (Kwok, 2010; Wang et al., 2013). In breast cancer, cisplatin resistant cell lines exhibit elevated FOXM1 in both protein and mRNA levels, when compared with sensitive breast cancer cell lines. Furthermore, an increase in FOXM1 expression is also correlated with an elevated expression of its downstream targets in the DNA repair pathway, including BRCA2 and XRCC1 (Kwok, 2010).

Another common drug for molecular targeted therapy is gefitinib, a small molecule tyrosine kinase inhibitor (TKI) for EGFR (Carter et al., 2009; van Erp et al., 2009). These molecular targeted therapy agents, including trastuzumab and gefitinib, mediate cytotoxic function in the cells via Forkhead box O3 (FOXO3a) activation, which is directly linked to FOXM1 signalling pathways (Koo et al., 2012). Although the treatment with trastuzumab is effective, only 30% of patients have found to be responsive to the treatment, because of the rapid development of resistance (Burris, 2000; Wilken and Maihle, 2010). FOXM1 is a downstream target of HER2 and has a tight correlation between their expressions (Bektas et al., 2008). In addition, gefitinib has also been found to have an indirect effect on FOXM1 suppression via FOXO3a activation in sensitive breast cancer cell lines. Moreover, overexpression of exogenous FOXM1 or a constitutively active FOXM1 (NH$_2$-terminal truncated form: ΔN-FOXM1) can promote gefitinib resistance (McGovern et al., 2009).

Commonly used chemotherapy drugs are taxanes and anthracyclines. Taxanes polymerise tubulin and induce apoptosis by disturbing microtubule dynamics that are required for cell mitosis (McGrogan et al., 2008). In breast cancer, FOXM1
overexpression confers resistance to paclitaxel (sometimes known as taxol) by altering the dynamics of the microtubules to prevent cancer cell death. FOXM1 depletion by a small interfering RNA (siRNA) or an alternate reading frame (ARF) derived peptide inhibitor helps to resensitise the cancer cells to paclitaxel (Carr et al., 2010). In recent studies, FOXM1 expression has also been found to be related with the cellular response to anthracyclines, including doxorubicin and epirubicin (Halasi and Gartel, 2012; Millour et al., 2011; Park et al., 2012). Both taxanes and anthracyclines are important therapeutic agents for breast cancer patients with metastasis (Zelnak, 2010). While taxanes work as microtubule dynamic interrupter, anthracyclines have a role in DNA intercalation and induction of DSBs (Minotti et al., 2004). In breast cancer cell lines, FOXM1 is found to be overexpressed in epirubicin resistance breast cancer cells, compared with sensitive cells. Depletion of FOXM1 promotes the sensitivity of breast cancer cells to epirubicin treatment (Millour et al., 2011; Monteiro et al., 2013). The regulation of FOXM1 by epirubicin is mediated by the activation of the DNA damage-transducer kinase ATM that promotes the expression of E2F transcription factor. This factor regulates FOXM1 via E2F-sites, which are located within the proximal promoter region of FOXM1. Moreover, FOXM1 has been indicated to be responsible for the DNA damage repair mechanisms and cell survival in response to epirubicin treatment (Millour et al., 2011). Depletion of FOXM1 in MDA-MB-231 cells (triple negative breast cancer cells) known chemotherapy resistant cells, leads to a higher sensitivity to doxorubicin. Using the same model, FOXM1-dependent resistance is found to be mediated by exonuclease 1 (EXO1), replication factor C4 (RFC4), and PLK4 (Park et al., 2012). Despite the fact that FOXM1 is required for DNA repair by HR via BRIP1 regulation,
overexpression of FOXM1 can promote cells viability, DNA repair and abrogate DSBs after treatment with epirubicin (Monteiro et al., 2013). Collectively, it can be implied that FOXM1 might be an interesting target to resensitise therapeutic resistance breast cancer cells (Carr et al., 2010).

1.9 Ubiquitination and SUMOylation

One important feature of DDR proteins is their mobilisation and local accumulation at a damaged lesion, which eventually results in the formation of discrete foci. Protein assembly at DNA damage sites is tightly controlled and achieved by post-translational modifications (PTM) of proteins. These modifications encourage the assembly or disassembly of DDR factors or control their times of residence at DNA breaks (Polo and Jackson, 2011). Most of the PTMs by ubiquitination are normally involved with protein targeting for proteolytic degradation via proteasome pathways, but some ubiquitination can also be controlled by protein localisation or its activity. Moreover, there are several ubiquitin-like proteins (UbLs) that have the capacity to target other proteins and regulate their functions. One of the most important UbLs is a small ubiquitin-related modifier (SUMO) that has been shown to covalently bind to various targeted proteins and play a crucial part in a huge number of cellular mechanisms, including genome maintenance, signal transduction, and gene expression (Gill, 2004).

Ubiquitin is a globular 76-amino-acid protein. It is highly conserved and is found to be different in only three amino acids between humans and yeast (Weissman, 2001).
Ubiquitin normally forms a covalent bond with targeted proteins at the lysine side chains of those proteins (Muller et al., 2001). Ubiquitination is mediated by the concerted action and sequential activity of E1-E2-E3 enzymatic cascade (Figure 1.8), which are activating (E1), conjugating (E2), and ligating (E3) enzymes, respectively (Bergink and Jentsch, 2009; Gill, 2004; Grabbe et al., 2011). Ubiquitination varies according to the lysine residues where they form in the chain (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63) or the amino-terminal methionine (Met1) of the ubiquitin monomer (Grabbe et al., 2011). The most common ubiquitin chains in cellular responses are the Lys 48 (K48)-linked ubiquitin chains and the Lys 63 (K63)-linked ubiquitin chains. While K48 chains usually act as markers for proteasomal degradation of the targeted protein, K63 chains are normally a marker for non-degradation and often orchestrate assembly of a protein complex in cellular signalling pathways (Nakada et al., 2010). Despite the fact that ubiquitination has a role in many cellular processes, including cell cycle and DNA damage repair response, it can also play a part in regulating cancer progression (Hoeller et al., 2006).

SUMO (previously known as sentrin), a member of UBL proteins, can be found in all eukaryotes and, like ubiquitin, it is highly conserved from yeast to humans (Muller et al., 2001). In the ubiquitination enzymatic cascade, E3 ligase normally provides substrate specificity by specific substrate-binding sites or, sometimes, a combination of E3 and E2. In contrast, SUMOylation has a surprisingly simpler cascade than ubiquitination (Figure 1.8). There are many different E2 and even more E3 enzymes in ubiquitination, but SUMOylation only involves an E1, a single E2 (which is Ubc9) and only a few E3 enzymes (Bergink and Jentsch, 2009).
In budding yeasts \textit{(Saccharomyces cerevisiae)}, there is only one type of SUMO, which is called Smt3. In contrast, there are four isoforms of SUMO that can be found in vertebrates including human: SUMO1, SUMO2, SUMO3 and SUMO4 \cite{Martin et al., 2007}. SUMO2 and SUMO3 differ from each other merely by only three amino at N-terminal residues (sometimes called as SUMO2/3), and are found to be functionally redundant \cite{Jackson and Durocher, 2013}. SUMO1 is around 11.6 kDa, contains 101 amino acids. SUMO1 has been found to share \~{}18\% homology with ubiquitin. The 3D structures of SUMO1, SUMO2/3 and ubiquitin are very similar to each other, but not SUMO4. Expression of SUMO4 is limited to the kidney and spleen. Nevertheless, the understanding of SUMO4 has yet to be established. It remains unclear whether SUMO4 might be simply be a pseudogene or whether it might bind non-covalently, and not be a part of PTM \cite{Martin et al., 2007}. Unlike ubiquitination, SUMOylation does not target proteins for proteasome-mediated degradation, but it takes a crucial part in regulating function of a targeted protein, including protein-protein interactions and the DNA damage repair responses \cite{Bergink and Jentsch, 2009; Park-Sarge and Sarge, 2009}. 
Figure 1.8 Comparison of ubiquitin and SUMO1 in the structure and pathways. Ubiquitin (Ub) and SUMO (S) are small modifiers that form covalent bonds with targeted proteins. Both modifications are reversible. These ubiquitination and SUMOylation can be by a single modifier (monoubiquitination or monoSUMOylation) or multiple modifiers to form specific chains (polyubiquitylation or polySUMOylation). The amounts of enzymes in *Saccharomyces cerevisiae* and in human are respectively shown in brackets. UBPs are ubiquitin-specific proteases, and ULPs are ubiquitin-like protein (SUMO)-specific proteases (Adapted from Bergink and Jentsch, 2009; Gill, 2004).
PTMs by SUMOylation have been reported to be important regulators of transcription. SUMOylation has various effects over the targeted proteins, but most of the SUMOylation events have been found to act as transcriptional repressors (Ouyang et al., 2009). A study in Helicoverpa armigera (cotton bollworm) revealed that a modification by SUMOylation has an important role in the suppression of the transcriptional activity of the forkhead transcriptional factor box A (FOXA), which is associated with a downregulation of the transcriptional downstream target of FOXA, such as DH-PBAN gene (Bao et al., 2011). Furthermore, SUMOylation of FOXL2, a transcription factor essential for ovarian development and maintenance, also leads to the inhibition of its transactivation ability (Georges et al., 2011): this FOXL2 SUMOylation can be found in both humans and mice (Marongiu et al., 2010). Recently, FOXC1 and FOXC2, which are important transcription factors in development and physiology, have been found to be targeted by SUMO. This modification results in the downregulation of the transcriptional activity of FOXC1/C2. SUMOylation deficient mutants FOXC1/C2 have a higher transcriptional activity when compared with the wild-type forms (Danciu et al., 2012). These studies indicate that this novel PTM, SUMOylation, has become an important reversible regulator for controlling the transcriptional activity of various transcription factors.
1.10 RING-type ubiquitin E3 ligase

Ubiquitin E3 ligases are involved with ubiquitinating enzymatic cascades by recognising targeted proteins (ubiquitin substrates) and mediating the ubiquitin transfer from conjugating (E2) enzymes to the target proteins (Deshiaies and Joazeiro, 2009). There are two major classes of E3 ligases (Figure 1.9): HECT (homologous to the E6AP carboxyl terminus) and RING (Really interesting new gene) domain containing E3 ligases (Metzger et al., 2012). The key feature of the HECT domain which contains the ligase, also called HECT-type E3, is a conserved cysteine residue. This residue forms an intermediate covalent thio-ester bond with the C-terminus of ubiquitin, before catalysing its transfer (Di Fiore et al., 2003; Rotin and Kumar, 2009). In contrast, RING-type E3 does not form a thio-ester bond with the ubiquitin residue like HECT-type E3. Instead, it brings the ubiquitin-E2 complex and its substrates (targeted proteins) into close proximity to transfer the ubiquitin residue directly from the conjugating E2 enzyme to the substrate (Di Fiore et al., 2003; Metzger et al., 2012; Ozkan et al., 2005).

The role of the RING-type E3 ligases is commonly specified by the RING domain which interacts with the ubiquitin-E2 complex to discharge the ubiquitin cargo from an E2 enzyme (Deshiaies and Joazeiro, 2009). RING-type E3 family is composed of three distinct groups (Figure 1.10); singles subunit, dimeric and multisubunit. Single subunit E3s, such as, parkin and RNF168, have one RING finger (RF) subunit that is surrounded by a protein-interacting motif. The RF subunit of the dimeric group and/or its surrounding regions acts as dimerisation sites to enhance the activity of E3. The dimeric group can be divided into two sub-groups which are homodimers (such as RNF4 and
RNF8) and heterodimers (such as BRCA1). The most complex RING-type E3s are multiple subunit E3s, such as, the culling RNG ligase (CRL) superfamily complex. This complex consists of the S phase kinase-associated protein 1 (SKP1)–culin 1 (CUL1)–F-box protein (SCF) and CRL2 E3 families, the anaphase promoting complex/cyclosome (APC/C) and the Fanconi anaemia (FANC). RING-type E3 enzymes control the specificity of substrate recognition by PTMs, including phosphorylation, glycosylation and SUMOylation (Lipkowitz and Weissman, 2011).

One of the most well-known RING-type E3 is RNF4. It has been reported to be involved with the DNA damage repair pathway. It is also an important DDR protein that is involved with HR repair in response to genotoxic stress (Yin et al., 2012). RNF4 (also known as SLX5, SNURF or RES4-26) is a dimeric RING-type E3 ligase that normally targets SUMOylated proteins to be ubiquitinated. RNF4 can be recruited to DSB lesions with a RING domain, a SUMO interacting motif (SIM) and a combination of some DNA repair proteins, including MRN sensor complex, MDC1 (DNA damage checkpoint 1), RNF8 and BRCA1 (Galanty et al., 2012; Yin et al., 2012). In order to target SUMOylated proteins, RNF4 engages the substrate proteins through its SIMs while the RING domain acts as a dimerisation domain and has a role in catalysing the ubiquitin transfer to the substrate. The stability of the ubiquitin-E2 complex is regulated by this RING domain dimerisation of RNF4 (Liew et al., 2010). Depletion of RNF4 results in defects of DSB repair and cell cycle arrest, caused by the impairment of ubiquitin adduct formation at the DSB sites (Galanty et al., 2012). The role of RNF4 in DDR in mammals is poorly understood because Rnf4 null mice normally die in the early stages of embryogenesis (Vyas et al., 2013). In addition to RNF4, RNF168 is also found to play an important role
in response to DSBs (Bartocci and Denchi, 2013; Morris et al., 2009). In some studies, the RNF168 pathway has been reported to cross-talk with RNF8 in order to promote the formation of ubiquitin chains (Bartocci and Denchi, 2013). At the DSB lesion, RNF8 is chained to the damage site through its forkhead-associated (FHA) domain which targets the phosphorylated forms of MDC1. RNF8 recognises UBC13, a ubiquitin E2 conjugating enzyme, through a RING-dependent interaction. Ubiquitination of the substrate protein by RNF8 leads to the recruitment of RNF168 to the damaged site to further amplify ubiquitin signal (Zhang et al., 2013). RNF168 contains a motif which interacts with the ubiquitin (MIU) domain. It can interact directly with the ubiquitin chain that is initially formed by RNF8 (Ohta et al., 2011). In addition, Ubiquitin chains play a crucial role in the recruitment and accumulation of various repair proteins, including ATM, BRCA1 and 53BP1 (Doil et al., 2009; Zhang et al., 2013). RNF168 activities at the damage site are not restricted to the amplification of the signal, but also to help sustain BRCA1 at the damaged site to promote DNA repair (Morris et al., 2009).
Figure 1.9 Schematic of ubiquitin pathway and the different mechanism of ubiquitin transfer by E3 ligases. In the beginning of the ubiquitin pathway, ubiquitin residue (Ub) is activated by ATP at the carboxyl terminus and conjugated, with a thio-ester bone, to active sites of an activating enzyme (E1). Then Ub is transferred to an active site of a conjugating enzyme (E2). Finally, two main groups of E3 ligases (HECT and RING type) have different ways to transfer Ub to the substrate: HECT-type E3 has a conserved cysteine residue that can directly form a thio-ester bond to Ub and transfer it to the substrate, while RING-type E3 acts as scaffolds by facilitating the interaction of Ub-E2 complex and the substrate before transferring Ub to the substrate without forming a bond with Ub (Adapted from Di Fiore et al., 2003).
Figure 1.10 The diagram of three distinct groups of E3 ligase. RING-type E3s can be differentiated into 3 groups according to their RF subunit (shown in red colour with a square shape) which are single RF subunit, dimeric RF subunit and multi-subunit RF. RING-type E3 controls a specificity of substrate recognition by PTMs, including phosphorylation, glycosylation and SUMOylation (Adapted from Lipkowitz and Weissman, 2011).
1.11 OTUB1

It is widely known that ubiquitination plays a crucial part in the regulation of a broad spectrum of cellular responses, including proteasome degradation, transcription and DDR. DUB is an enzyme that is important for balancing these processes by modulating ubiquitin signalling (Wiener et al., 2013). OTUB1 is a product of the OTUB1 gene, a member of the OTU (ovarian tumour) superfamily of predicted cysteine proteases. Some well-known members of this superfamily have been established as important regulators in various signalling cascades including inflammation and cancer signalling pathways (Mevissen et al., 2013), such as ubiquitin-editing enzyme A20 (also known as Tumour Necrosis Factor Alpha-Induced Protein 3 or TNFAIP3) and OTUD family members that are known as a negative feedback regulation of NF-κB signalling and tumour suppressor in Hodgkin lymphomas and primary mediastinal lymphomas (Malynn and Ma, 2009; Mevissen et al., 2013; Vereecke et al., 2009), OTULIN that prevents inappropriate pro-inflammatory signal (Fiil et al., 2013; Keusekotten et al., 2013), and ZRANB1 (also known as TRABID) that was discovered to have a role as a regulator of cytoskeleton and cell migration (Bai et al., 2011).

Ubiquitin aldehyde binding 1 (OTUB1), or also known as OTU deubiquitinase is a deubiquitination enzyme (DUB) that has a role in a reverse process of ubiquitination, blocking the transfer of ubiquitin residues. The regulation of OTUB1 by PTMs remains unclear. Only one study has reported that OTUB1 is monoubiquitinated. This modification is mediated by UbcH5 E2 enzyme and is critical for the function of OTUB1 as a deubiquitinating enzyme (Li et al., 2014).
OTUB1 is believed to interfere with DNA repair by inhibiting ubiquitination which is one of the most important signals in DDR (Figure 1.11). In normal cells, OTUB1 separates UBC13, an E2 conjugating enzyme, from the ubiquitination process by binding to it. In the presence of DNA damage, OTUB1 separates from UBC13, and lets it participates in the ubiquitination, together with the help of an E3 ligase, in DNA damage signalling (Rose and Schlieker, 2010). OTUB1 has also been shown to be able to bind to the E2s of UBE2D and UBE2E families (Nakada et al., 2010; Sowa et al., 2009). OTUB1 can also suppress the RNF168-dependent ubiquitination pathway (Nakada et al., 2010). Interestingly, OTUB1 can inhibit the ubiquitination of both K48-linked (Wiener et al., 2013) and K63-linked ubiquitin chains (Nakada et al., 2010).

The function of OTUB1 in cancer development has not yet been thoroughly established. In fact, a recent study has revealed that OTUB1 might play a crucial role in colon cancer development and metastasis. OTUB1 expression levels in colon cancer tissues have been found to be 3.15-fold higher than in the corresponding non-cancerous tissues (Liu et al., 2014). However, the role of OTUB1 in breast cancer has never been studied. OTUB1 has been reported to regulate various DDR proteins that have a role in cancer progression and metastasis, including ERα (Stanisic et al., 2009), RNF8, BRCA1 (Nakada et al., 2010), and p53 (Li et al., 2014; Sun et al., 2012).
Figure 1.11 The blocking of ubiquitin transfer. Normally, OTUB1 isolates UBC13, an E2 conjugating enzyme, from ubiquitin loading, interfering with the formation of the ubiquitin chain. This is different from other deubiquitinating enzymes that commonly work by detaching ubiquitin residue from the substrate protein. In the presence of DNA damage, such as the damage from ionising radiation, OTUB1 disengages from UBC13, allowing it to form ubiquitin chains, in this case, a K63-linked chain, in the presence of an E3 enzyme (RNF168), to be a DNA damage signal (Adapted from Rose and Schlieker, 2010).
1.12 Thesis Aims

Chemotherapy resistance is the main obstacle in the treatment of breast cancer, especially for the patients who cannot be treated with hormonal therapy or other targeted treatments. Anthracyclines, including epirubicin, are effective first-line chemotherapeutic agents for breast cancer treatment. However, more than 90% of patients with metastatic cancer are found to be resistant to chemotherapeutic treatments (Lippert et al., 2011). FOXM1 is a transcription factor that is a key regulator of various cellular processes, including DNA damage repair response. FOXM1 overexpression has been reported to be involved with tumourigenesis and cancer progression, including in breast cancer (Koo et al., 2012). Moreover, FOXM1 is also involved with the resistance to various treatments, for example, endocrinial therapy (Millour et al., 2010), cisplatin (Kwok, 2010), Paclitaxel (Carr et al., 2010), and, most recently, epirubicin (Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013). Previous studies show that FOXM1 is primarily regulated by PTMs in response to treatment with chemotherapeutic drugs. This normal control is deregulated in drug resistant cancer cells. Crucial to the understanding of the regulation of FOXM1 is the post-translational regulation of FOXM1, which has yet to be clearly understood. FOXM1 has already been reported to be regulated by reversible phosphorylation throughout the cell cycle (Koo et al., 2012). In this thesis, I aim to study the role of SUMOylation and ubiquitination in terms of FOXM1 regulation, in response to epirubicin treatment.

In addition, E3 ligases are important for the ubiquitination of FOXM1, as ubiquitination is mediated by the concerted actions and sequential activity of E1-E2-E3 enzymatic
cascade (Bergink and Jentsch, 2009; Grabbe et al., 2011). The well-known E3 ligases that have been reported to be involved with DDR are RNF4, RNF8 and also RNF168 (Chapter 1.10). Understanding the interactions between these RNFs and FOXM1 might lead to a more thorough understanding of how FOXM1 can be regulated and how this control is deregulated in resistant breast cancer. This can be re-sensitised to chemotherapeutic agents. Moreover, OTUB1, a deubiquitination enzyme, has been reported to regulate various cancer related proteins, including, p53 (Li et al., 2014; Sun et al., 2012), and ERα (Stanisic et al., 2009). Interestingly, FOXM1 has previously been shown to be regulated by ER (Horimoto et al., 2011; Karadedou, 2006; Millour et al., 2011) and p53 (Qu et al., 2013). Taken together these data, I hypothesised that FOXM1 expression is regulated by PTMs in response to chemotherapeutic drug treatment. I also aimed to investigate the role of RNFs and OTUB1 in the regulation of FOXM1. If these proteins and their post-translation regulation were to have a strong impact over FOXM1 expression, they might become novel potential targets for treatment of breast cancer and for overcoming drug resistance.
CHAPTER 2

MATERIALS AND METHODS
2.1. Cell lines and cell culture

The human breast cancer cell line, MCF-7 (Michigan Cancer Foundation – 7), was acquired from the Cell culture service of Cancer Research UK (London, UK). This cell line originated from the American Type Culture Collection (ATCC).

MCF-7 Epi\textsuperscript{R} cells are breast carcinoma cells which are epirubicin resistant. MCF-7 Epi\textsuperscript{R} cells are non-clonal MCF-7 derivatives, generated in the laboratory. They are produced by culturing in medium supplemented with stepwise increases in amounts of epirubicin (Medac, Hamburg, Germany). The epirubicin concentration in the medium was increased until it reached a concentration of 10µM. The resistant cells were all maintained in medium supplemented with 10µM epirubicin.

MCF-7 Epi\textsuperscript{R} OTUB1 stable cell line was generated from MCF-7 Epi\textsuperscript{R} cells transfected with pcDNA3-OTUB1 plasmid. Transfected cells were selected by using 1mg/ml of neomycin (G418) (Sigma-Aldrich, UK). Single cells were isolated using 96-well plate serial dilution before screening by Western Blotting and qPCR.

Mouse embryonic fibroblasts (MEFs) wild type (\textit{Foxm1}\textsuperscript{+/+}) and Foxm1 knock-out (\textit{Foxm1}\textsuperscript{-/-}) cells were isolated from wild-type and Foxm1 nullizygous mouse embryos, as previously described (Laoukili et al., 2005). MEFs were used for investigating the role of FOXM1 as a model for FOXM1 null cells, compared to wt MEFs cells. Both types of MEFs were a gift from Professor René Medema, The Netherlands Cancer Institute, The Netherlands.
All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, UK) supplemented with 1% penicillin/streptomycin and 2 mmol/L L-glutamine (PSG, Sigma-Aldrich, UK), and 10% foetal calf serum (FCS, First (UK) Link Ltd., UK). Before being added to the media, PSG were filtered using a sterile 0.20 µm filter (Sartorius Stedim Biotech, UK). Cell lines were cultured at 37°C with 10% CO₂. For MCF7-EpiR cells, 10µM epirubicin was added to the medium (DMEM with FCS and PSG) maintaining the cells epirubicin resistance.

2.1.1. Sub-culturing and long-term maintenance

Cells were split when at ~60 - 70% confluence. After washing with phosphate buffered saline (PBS) twice, cells were then trypsinised using 1x trypsin in EDTA solution (Sigma-Aldrich, UK). Trypsinisation was stopped by adding twice the volume of complete medium, before the cells were spun down at 300x g for 4 min to remove the trypsin. Cells were then resuspended in fresh complete medium and seeded as required in new flasks or dishes.

For long-term maintenance, cells were trypsinised and washed with PBS. Cells were spun down at 300x g for 4 min to remove supernatant, and then resuspended in 1 ml of 90% (v/v) FCS and 10% (v/v) dimethyl sulphoxide (DMSO, Sigma-Aldrich, UK). The cell suspension was added to cryogenic vials (Corning, UK) and placed into a freezing container (Mr. Frosty, VWR International, Lutterworth, UK) and frozen in -80°C freezer.

To defrost cells, cryogenic vials were taken from the freezer and placed in a 37°C water bath until defrosted. The cell suspension was then mixed with complete medium and
spun down at 200x g for 2 min to collect the cells. The supernatant was discarded, and the cell pellet resuspended with complete medium in flasks or dishes.

2.1.2. Drug and treatment

Epirubicin Hydrochloride [2 mg/ml (3400 μM) in 0.9% Sodium chloride, Medac, Germany] is an anthracycline drug used for cancer chemotherapy (hereafter referred to as epirubicin). Epirubicin, obtained from Imperial College Healthcare (UK), was used at 1 μM for treating MCF-7 cells and MEFs.

Cycloheximide (CHX, Sigma-Aldrich, UK) is a compound that inhibits protein synthesis. Proteins degraded by an ubiquitin-proteasome pathway are found to be decreased after CHX treatment. CHX powder is soluble in 100% ethanol. The stock solution was reconstituted at a concentration of 100 mg/ml. The cells were treated at the final concentration of 100 μg/ml.

2.1.3. Single cells clone by serial dilution method

To generate a stable cell line, cells were transfected with an expression vector with resistance to antibiotics. Selective antibiotics were used for selecting transfected cells from cells without DNA inserted. Single clones were isolated using a 96-well plate serial dilution (Corning, UK). During the process of cloning, cells were always cultured with the appropriate selective agent. The transfection efficiency was checked by using a western blotting and qPCR.
2.2. Molecular plasmids and transformation

The pcDNA3-FOXM1 plasmid was cloned by Dr. Jimmy Kwok (previous PhD student in the laboratory). The pmCherry-FOXM1 plasmid was a gift from Dr. Cornelia Man (The Hong Kong Polytechnic University, Hong Kong). The pcDNA3-BRCA1 plasmid was a gift from Dr. Jenny Quinn (Queen’s University, Belfast). The pBOS-RNF4, pBOS-RNF4 CS1 mutation and pcDNA3-RNF4 plasmids were from Professor Jan Brosens (Reproductive Health, University of Warwick, UK). The pcDNA3-OTUB1, pcDNA3-RNF168 plasmids were kind gifts from Dr. Daniel Durocher (The Lunenfeld-Tanenbaum Research Institute Mount Sinai Hospital Joseph & Wolf Lebovic Health Complex, Canada). The HA-ubiquitin, FOXM1-Ubc9 and FOXM1 (5XK>R)-Ubc9 fusion, PGL2-CyclinB1 promoter and PGL2-Cyclin B1 mutant promoter, eGFP-FOXM1 wt and eGFP-FOXM1 5xK>R mutant plasmids were constructed by Dr. Stephen Myatt (previous postdoc in the laboratory). The tRFP-SUMO1 plasmid was a kind gift from Dr Simon Ameer-Beg (Guy’s hospital campus, King’s College London, UK).

All plasmids were amplified in One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, UK) and plasmid DNA extracted using a QIAGEN plasmid MAXI kit (QIAGEN, UK) according to the manufacturer protocol.
2.3. Transfections

2.3.1. FuGENE 6 transfection

FuGENE 6 transfection reagent (Promega UK) was used for transfecting plasmid DNA to MCF-7 cells. First, cells were seeded to approximately 60% confluency on the next day. The ratio for Transfection of FuGENE 6: plasmid DNA was 3:1 according to manufacturer recommendation and the previous titration. Briefly, according to the manufacturer protocol, FuGENE 6 was diluted by DMEM serum free medium and incubated for 5 min. Plasmid DNA was then added to the FuGENE-DMEM dilution and left for another 15 min to incubate before gently adding to the cells. At least 24 h incubation at 37ºC with 10% CO2 was required prior to the drug treatment or other experiment procedures.

2.3.2. XtremeGENE HP transfection

In order to increase the efficiency of the Transfection, XtremeGENE HP reagent (Roche Diagnostics, UK) was used to transfect the eGFP-FOXM1 (wt and 5X K>R) plasmids to MCF-7 cells for using in confocal microscopy experiments. Cells were seeded to be approximately 60% confluent on the following day. The manufacturer protocol was followed; the ratio of XtremeGENE HP to plasmid DNA at 3:1 was used. Certain amount of plasmid DNA was diluted in DMEM serum free or Opti-MEM I Reduced Serum Media (Life technologies, UK) before XtremeGENE HP was added. The complex mixture was left for incubation for 15 min, and then added directly dropwise to the cells. Before
further experiment was carried out, the cells were incubated at 37°C with 10% CO₂ for no less than 24 h.

2.3.3. Oligofectamine transfection

In order to knock down genes for functional analysis, small interfering RNAs (siRNAs, Dhharmacon, Epsom, UK) specific to the gene of interest were transfected into the cells using Oligofectamine reagent (Invitrogen, Life technologies, UK). At the first day, cells were seeded to be 70% confluent on the following day. The Oligofectamine mixture was prepared by adding oligofectamine to Opti-MEM and being incubated for 10 min. Then, the dilution of siRNA (50nM final concentration) in Opti-MEM and 1x siRNA buffer (Thermo scientific, UK) was added to the first mixture and left to incubate for 25 min. Following incubation, Opti-MEM was added until the manufacturer recommended volume was reached. The cells were washed with PBS after complete medium was removed. The mixture was then added to the cells dropwise and returned to the incubator at 37°C with 10% CO₂ for 4 h to 6 h before the addition of the complete media. The cells were placed back for further incubation for at least 24 h prior to experiments being carried out.
2.4. Protein preparation

2.4.1. Protein lysate extraction

Cells were harvested using trypsinisation and spun down at 300x g for 4 min to collect the cell pellets. The cell pellets were washed with PBS and transferred to 1.5 ml eppendorf tubes before being spun down for 4 min at 300x g. Cleaned cell pellets were then lysed with lysis buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA), 1mM Dithiothreitol (DTT), 1% (v/v) Nonidet P-40, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM Sodium fluoride (NaF), 1 mM Sodium ortho-vanadate (Na$_3$VO$_4$) and 100 mM N-Ethylmaleimide (NEM), and a “complete” protease inhibitor cocktail (Roche Diagnostics, UK). Once the pellets were lysed, they were placed on ice for 20 min and placed on vortex every 10 min. The whole lysate (supernatant) was collected by centrifugation at 14000x g at 4°C for 10 min.

The concentrations of the protein lysates were determined using the Bio-Rad D$_c$ Protein Assay kit (Bio-Rad, UK). Following the manufacturer protocol, the protein concentration was determined by microtitre plate reader (Sunrise, Tecan, Reading, UK) at 700 nm wavelength. Protein lysates were kept in a -80°C freezer until further require.
2.4.2. Nuclear and cytoplasmic protein fractionation

Sub-cellular protein fractionation is a method for studying nuclear-cytoplasmic localisation of proteins. In this study, fractionation was performed according to the manufacture protocol using NER-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, UK). Cells were harvested by trypsinisation. Cell pellets were resuspended in 200 µl of chilled CER I solution, containing complete protease inhibitor then vortexed at a high speed for 15 sec and incubated for a further 10 min on ice. After that, 11 µl of chilled CER II solution was added before vortexing for another 5 s and incubation on ice for 1 min. The supernatant (cytoplasmic fraction) was then collected by centrifugation at 13,000 x g 4°C for 5 min. The supernatant was transferred to a new pre-chilled eppendorf. The insoluble fraction was washed once with PBS (optional) before being lysed by chilled NER solution with complete protease inhibitor. The samples were then vortexed for 15 sec and incubated for 45 min (vortex every 10 min). Supernatant (nuclear fraction) was collected by centrifugation at 13,000 x g 4°C for 10 min. Both cytoplasmic and nuclear fractions were stored at -80°C until further use.
2.5. Western blotting

Protein expression was investigated using western blotting. Proteins were separated according to their molecular weights by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes for immunoblotting and visualisation.

2.5.1. SDS-PAGE and nitrocellulose membrane transferring

SDS-PAGE consists of two parts: a stacking gel (upper part) and a resolving gel (lower part). The percentages of resolving gel (varying an amount of bis-acrylamide solution, Table 2.1) 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).

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. Samples were loaded into the gel, Novex charp protein standard (Invitrogen, UK) served as a marked for determining molecular sizes.

Proteins were separated by molecular weight in running buffer [0.1% (w/v) sodium dodecyl sulphate, 25 mM Tris, 190 mM glycine] at 90 V using a Bio-Rad Mini-PROTEAN system (Bio-Rad, Hempstead, UK). 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.

**Table 2.1 Ingredients of the different percentages of SDS-PAGE gels.**

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<th></th>
<th>Resolving gel 5%</th>
<th>Resolving gel 7%</th>
<th>Resolving gel 10%</th>
<th>Resolving gel 12%</th>
<th>Resolving gel 14%</th>
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<td>2.5</td>
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<td>-</td>
</tr>
<tr>
<td>1.5M Tris pH 6.8 (ml)</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
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<tr>
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<td>100</td>
<td>100</td>
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<td>50</td>
</tr>
<tr>
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<td>5</td>
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2.5.2. Antibodies

The antibodies that were used in this study are following;

Table 2.2 Lists of antibodies that were used in western blot

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<th>species</th>
<th>Catalog No.</th>
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<td>Santa cruz</td>
<td>Rabbit</td>
<td>sc-752</td>
</tr>
<tr>
<td>ERK</td>
<td>Santa cruz</td>
<td>Rabbit</td>
<td>sc-292838</td>
</tr>
<tr>
<td>Flag M2</td>
<td>Sigma-Aldrich</td>
<td>Mouse</td>
<td>F1804</td>
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<tr>
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<td>Santa cruz</td>
<td>Rabbit</td>
<td>sc-17783</td>
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<tr>
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<td>Santa cruz</td>
<td>Mouse</td>
<td>sc-9996</td>
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<tr>
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<td>Abcam</td>
<td>Mouse</td>
<td>119D5</td>
</tr>
<tr>
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<td>Sigma-Aldrich</td>
<td>Rabbit</td>
<td>O9889-200UL</td>
</tr>
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<td>Cell signallng</td>
<td>Rabbit</td>
<td>#9542</td>
</tr>
<tr>
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<td>Abcam</td>
<td>Rabbit</td>
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<td>Sheep</td>
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</tr>
<tr>
<td>RNF168</td>
<td>Home made</td>
<td>Rabbit</td>
<td>-</td>
</tr>
<tr>
<td>RNF4</td>
<td>Novus Biologicals</td>
<td>Mouse</td>
<td>H00006047-A01</td>
</tr>
<tr>
<td>RNF8</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>ab4183</td>
</tr>
<tr>
<td>SUMO1</td>
<td>Santa cruz</td>
<td>Rabbit</td>
<td>sc-5308</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Santa cruz</td>
<td>Rabbit</td>
<td>sc-9104</td>
</tr>
<tr>
<td>γH2Ax</td>
<td>Cell signallng</td>
<td>Rabbit</td>
<td>#9718</td>
</tr>
<tr>
<td>Rabbit HRP</td>
<td>Dako</td>
<td>Goat</td>
<td>P0448</td>
</tr>
<tr>
<td>Mouse HRP</td>
<td>Dako</td>
<td>Goat</td>
<td>P0447</td>
</tr>
<tr>
<td>Sheep HRP</td>
<td>Dako</td>
<td>Rabbit</td>
<td>P0163</td>
</tr>
</tbody>
</table>

NOTE: RNF168 (Home-made) was a generous gift from Dr. Raimundo Freire, Hospital Universitario de Canarias, Spain.
2.5.3. Immunoblotting

After removing nitrocellulose membranes from the transfer tank, 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 (TBS-T, 20 mM Tris pH 7.6, 136 mM NaCl, 0.01% (v/v) tween). Having removed the stain, the membranes were blocked with 5% (w/v) BSA (BSA, Sigma) in TBS-T for 30 – 45 min. Membranes were incubated with primary antibodies in 5% BSA-TBS-T and 0.02% (v/v) azide overnight, shaking at 4°C.

On the following day, the membranes were washed with TBS-T for 15 min 4 times. The membranes were then incubated with secondary antibody conjugated with horseradish peroxidase (HRP) (Dako, Ely, UK) in TBS-T (1:5000 dilution) for 30 min, following this, the membranes were again washed 4 times (15 min per each wash). Once washed, Enhanced chemiluminescence detection system (ECL, Perkin Elmer, UK) was added to enable visualisation. Luminescence signal was detected using Amersham Hyperfilm ECL (GE Healthcare, UK) before being developed with a SRX-101A X-ray developer (Konica Minolta, Tokyo, Japan) or an Optimax 2010 X-Ray film processor (IGP, Essex, UK).
2.6. Co-immunoprecipitation assay (co-IP)

Cells were seeded and treated as usual, and then harvested using trypsinisation. Total protein lysate was prepared with lysis buffer as previously described. The protein concentrations were measured. The lysate was then pre-cleaned using Dynabeads (Life Technologies, UK) according to primary antibodies to be used for the IP experiments (protein A for rabbit antibody and protein G for mouse antibody). The mixes were rotated for 2 h at 4°C. After the pre-clean process, the Dynabeads were removed from the lysate using DynaMag magnetic stand (Life Technologies, UK). The lysates were then separated into 3 equal volumes (input, IgG control and pull-down; ratio of each condition was 1:20:20). The input was mixed with 2x SDS loading dye, like the normal western blot process, before boiling at 100°C for 5 min and kept at -20°C until further use.

For other conditions, lysate samples were incubated with a rabbit or mouse IgG negative control (Dako, UK) or specific primary antibodies on a rotator for 2 h at 4°C. After that, lysate was removed and the Dynabeads were washed 3 times with PBS. At the third wash, the Dynabeads were changed into a new eppendorf to avoid non-specific adhesion to the old eppendorf. Proteins were eluted from the Dynabeads using 2x SDS loading dye and boiled at 100°C for 5 min. Proteins were kept at -20°C until required.
2.7. Real-time reverse transcription quantitative PCR (RT-qPCR)

2.7.1. Total RNA isolation and quantification

Total RNA was extracted from cell pellets by using RNeasy mini kit (Qiagen, UK) according to the manufacturer protocol. Cells were briefly resuspended in Buffer RLT as recommended in the table 2.3:

Table 2.3 Volumes of Buffer RLT for lysing cell pellets

<table>
<thead>
<tr>
<th>Number of cell pellets</th>
<th>Volume of Buffer RLT (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5 x 10^6</td>
<td>350</td>
</tr>
<tr>
<td>5 x 10^6 – 1 x 10^7</td>
<td>600</td>
</tr>
</tbody>
</table>

Cell suspension was then directly transferred to a QIAshredder spin column which was placed into a 2 ml collection tube and centrifuged at 13,000 xg for 2 min to collect the flow through. The same volume of 70% ethanol was added to the lysate and mixed well. Samples were then transferred to an RNeasy spin column in a 2 ml collection tube and spun at ≥8000xg for 15 s, with the flow through discarded. The column membrane was then washed with 700 μl of Buffer RW1 and centrifuged for 15 s at ≥8000xg before washed for another 2 times with Buffer RPE. To ensure that no ethanol (from Buffer RPE) was left, the empty tube was spun down at 13,000 xg for a further minute (optional). For elution, the column was placed into a new 1.5 ml collection tube and eluted with 30–50 μl of RNasefree water. The eluted RNA concentration was measured using a NanoDrop ND-1000 (Thermo Scientific, UK) and the sample stored at -80°C until needed for further experiments.
2.7.2. cDNA synthesis

Complementary DNA (cDNA) was synthesised from 1 μg of total RNA. Reverse transcription was performed using Superscript III first strand cDNA synthesis (Life technologies, UK). 1μl of 50μM oligo(dT)20 Primer (Life technologies, UK) and 1μl of 10mM dNTPs (Life technologies, UK) were added to RNA. The volume of the mix was adjusted with Ambion DEPC-treated water (RNase-free water, Life technologies, UK) up to 13 μl. The mixture was incubated at 65ºC for 5 min before being cooled down at 4ºC for 4 min. One μl of reverse transcriptase Superscript III (200U/μl), 1μl of 0.1M DTT, 1μl of RNsaeOUT recombinant RNase inhibitor and 1x first-stand buffer (All are from Life technologies, UK ) were added to the first mixture. The mixture was incubated at 25°C for 5 min, then at 50°C for 60 min, and 70°C for 15 min (in the thermo cycler). The cDNA-containing mixture was diluted at the ratio of 1:4 with Ambion DEPC-treated water and kept at -20°C until qPCR analysis.

2.7.3. Primers design and optimisation

In this study, all primers were designed using Primer Express 3.0 software (Applied Biosystems, UK). This software produced an amplicon between 100 – 200 bp, according to the gene sequences from Pubmed (National Center for Biotechnology Information: NCBI, USA). Newly designed primers were optimised to find the concentration ratio of the primer. The standard curve from each concentration ratio was checked for an equation slope (theoretically between -3.3 to -3.8) and R² (should not be lower than 0.98). A list of primers is shown in Table 2.4 (Primers are written 5' to 3')
<table>
<thead>
<tr>
<th>Gene</th>
<th>species</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>L19</td>
<td>Human</td>
<td>GCGGAAGGGTACAGCCAAT</td>
<td>GCAGCGGGCGCAGAAN</td>
</tr>
<tr>
<td>L19</td>
<td>Mouse</td>
<td>CCCGTCAGCAGATCAGGAA</td>
<td>GTCACAGGCTTGCGGATGA</td>
</tr>
<tr>
<td>FOXM1</td>
<td>Human</td>
<td>TGCAGCTAGGGATGTAACTTC</td>
<td>GGAGCCCACTCACAAGACT</td>
</tr>
<tr>
<td>FOXM1</td>
<td>Mouse</td>
<td>GCAGAATCGGGTTAGGTAGGAG</td>
<td>GACACAGAGTCCTGCGCAAGATGT</td>
</tr>
<tr>
<td>OTUB1</td>
<td>Human</td>
<td>CAGGCCTGACGGCAAACGTG</td>
<td>AGTGCCTCAAAGTGGAGAA</td>
</tr>
<tr>
<td>OTUB1</td>
<td>Mouse</td>
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<td>TGCCCTCAAGGGAGAA</td>
</tr>
<tr>
<td>RNF4</td>
<td>Human</td>
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<tr>
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<td>CCCAAAAGAATGACCAAATGATA</td>
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</tr>
<tr>
<td>RNF168</td>
<td>Human</td>
<td>AAGAGCAAATGCTGCGCAAAC</td>
<td>GTGTGGAGATGGTTTGATGT</td>
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<tr>
<td>CCNB1</td>
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<td>TGTGGTAGAGTGCTGATGTTCATG</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Human</td>
<td>GCGGACTCAGCACAGA</td>
<td>GCTTTTCATTTTTTCTCTACACACA</td>
</tr>
</tbody>
</table>
2.7.4. Quantitative Polymerase chain reaction (qPCR)

The quantitative polymerase chain reaction (qPCR) was performed using a SYBR-green master mix (Applied Biosystem, UK) and run in a 7900HT fast real-time PCR system (Applied Biosystem, UK). Per one reaction, 2 μl of cDNA were mixed with 12.5 μl of SYBR-green master mix and optimised volumes of primers and made up to 25 μl with Ambion DEPC-treated water. The standard curve was produced from the mixture of 5 μl cDNA from every sample, and then diluted 1:4 for 4 serial dilutions. DEPC-treated water was used as no 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 30 s (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. A dissociation curve was used to confirm an absence of non-specific amplicon.
2.8. Sulphorhodamine-B assay (SRB)

Cells were seeded in 96-well plates (~5,000 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. To harvest, cells were fixed by adding 100 µl 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 3 times with slow running water before being stained with 100 µl 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. Following incubation, the plates were washed with 1% (v/v) acetic acid and left to be dry overnight. For measurement, 100 µl of 10 mM Tris-base (VWR International, UK) was used to dissolve SRB from cells in a shaker for 30 min. Optical density (OD) was read at 492 nm using a microtitre plate reader (Sunrise, Tecan, Reading, UK). Results were normalised to untreated or control wells. This assay was used for measuring protein content, the difference between each condition was assumed to be equivalent to a difference of the number of cells.
2.9. Luciferase reporter assay

Cells were grown in 96-well plates (approximately 5,000 cells/well) and left in an incubator at 37°C 10% CO2 overnight. On the following day, using FuGENE 6 transfection reagent, cells were co-transfected with a Luciferase reporter plasmid, Renilla plasmid (pRL-CMV, Promega, UK), transfection efficiency control, and the expression plasmid of interest.

To harvest, after 24 h of transfection, cells were washed with PBS before being lysed with 100 µl/well of 1x Steady lite plus reagent (Perkin Elmer, UK) and incubated for 15 min in a dark environment. Lysates were transferred to a luciferase plate (Perkin Elmer, UK) where the emission of Luciferase-derived light can be measured using a PHERAstar Plus microplate reader (BMG Labtech, Aylesbury, UK). Then, 25 µl of the renilla substrate mix (1% (w/v) coelenterazine (Lux Biotechnology, UK), 0.04 M EDTA, 0.5 M HEPES pH 7.8) was added to each well and incubated 20 min in the dark. The presence of EDTA inhibited the Luciferase enzyme. The luminescence light from Renilla was measured using the PHERAstar Plus microplate reader.

The Luciferase reading was normalised to the renilla reading, well by well. All measurements were performed in six replicates.
2.10. Immunofluorescent staining (IF)

Cells were grown on 4-chamber culture slides (BD Biosciences, UK) and transfected or treated as usual. Before staining, cells were washed twice with PBS and fixed with 4% (v/v) paraformaldehyde (PFA, Sigma-Aldrich, UK) in PBS for 15 min. The fixed cells were washed 3 times with PBS and then permeabilised with 0.2% (v/v) Triton X-100 (Sigma-Aldrich, UK) in PBS for 10 min. Cells were then washed another 3 times before 5% (v/v) goat serum (Sigma-Aldrich, UK) in PBS was added for 30 min to block the non-specific binding. Next, the cells were incubated in primary antibody diluted in 0.2% (v/v) goat serum at 4°C overnight. The excess antibodies were removed by washing with PBS 3 times. Once the cells were washed, Secondary antibodies (table 2.5) in PBS were added to the cells and the mixtures were left to incubate in the dark for 45 min.

Stained cells were then washed with PBS 3 times before the chambers were removed from the slides. The nucleus of the cells was stained and slides were mounted using Vectashield Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA) and covered by cover-slips. Slides were sealed using a clear nail vanish and kept at 4°C until visualised. Images were taken using a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) with 63x objective and LAS-AF software.
Table 2.5 List of antibodies which were used for IF staining

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Colour</th>
<th>species</th>
<th>company</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO1 (primary)</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>#4930</td>
<td></td>
</tr>
<tr>
<td>FOXM1 (primary)</td>
<td>mouse</td>
<td>MyBiosource</td>
<td>MBS120349</td>
<td></td>
</tr>
<tr>
<td>RNF168 (primary)</td>
<td>Rabbit</td>
<td>Home Made</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor 488 anti Rabbit</td>
<td>Green</td>
<td>goat</td>
<td>Lift Technologies</td>
<td>A-21070</td>
</tr>
<tr>
<td>Alexa Fluor 555 anti Rabbit</td>
<td>Green</td>
<td>goat</td>
<td>Lift Technologies</td>
<td>A-21428</td>
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<tr>
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<td>Red</td>
<td>goat</td>
<td>Lift Technologies</td>
<td>A-10680</td>
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<tr>
<td>Alexa Fluor 555 anti mouse</td>
<td>Red</td>
<td>goat</td>
<td>Lift Technologies</td>
<td>A-21422</td>
</tr>
</tbody>
</table>

2.11. Fluorescence lifetime imaging microscopy and Fluorescence Resonance Energy Transfer (FLIM-FRET)

Cells were seeded in 96-well plate overnight. The next day, cells were co-transfected with FRET donor and acceptor using XtremeGENE HP transfection (Roche Diagnostics, UK). After 24 h of transfection, cells were treated with epirubicin at various time points. To harvest, cells were washed and immersed in Hanks' Balanced Salt solution supplied with glucose and L-glutamine. FLIM-FRET measurement was done by Douglas Kelly, a PhD student in a Photonics Group, Department of Physics, Imperial College London, South Kensington Campus, London, UK, using an automated 96-well plate reading FLIM microscope Olympus IX81-ZDC (Olympus, UK) as previously described (Alibhai et al., 2013). Images were acquired with 40x long working distance air objective with an NA of 0.6 using Olympus LUCPLFLN 40X objective lens (Olympus, UK). Data were
analysed using FLIMfit software which was developed by the photonics group (Imperial College London, UK)

2.12. COMET Assay (Single cell gel electrophoresis, SCGE)

Microscopy slides with double frosted 45° ground edges (Thermo Scientific, UK) were emerged in 1% (w/v) normal melting point agarose (NMA, Promega, UK) in H₂O to prepare the pre-coated first layer. These slides were kept in 4°C until further use.

Cell pellets were re-suspended in 0.7% (w/v) of low melting point agarose (LMA, Promega, UK) in PBS at 37°C and spread homogenously on pre-coated glass slides. Slide with cells were then coated with 0.7% (w/v) LMA and immersed into ice-cold lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 10% (v/v) DMSO, 1% (v/v) Triton X-100, 1% (v/v) sodium sarcosinate, 250mM NaOH pH10, and N-lauroylsarcosine) for 1 h. The coating was done for one more layer. After being lysed, slides were placed in an electrophoresis tank filled with alkaline electrophoresis buffer (1 mM Na₂EDTA, 150 mM NaOH pH 12.8). The electric current was adjusted between 250 and 300 mA. Electrophoresis was performed on ice at 25 V for 15 min in the dark. Slides were immediately subjected to neutralised buffer (400 mM Tris pH 7.5) twice for 5 min. The nuclei were stained with DAPI. The slides were covered with cover slips. Individual cells was visualised using a Nikon Eclipse E400 Upright Microscope (Nikon, Japan) connected with a digital CCD camera ORCA-03G C8484-03G02 (Hamamatsu, Japan). DNA double strand breaks were analysed using the COMET assay IV software (Perceptive Instruments, UK).
CHAPTER 3

FOXM1 AND ITS MODIFICATION BY SUMOYLATION IN RESPONSE TO CHEMOTHERAPY IN BREAST CANCER CELLS
3.1 Introduction and Objectives

Anthracyclines, such as doxorubicin and epirubicin, are typical chemotherapeutic drugs for breast cancer therapy. They are members of the *Streptomyces peucetius*–derived antibiotics. The main mechanism of anthracyclines is inhibiting the nuclear topoisomerase II (TOPO II) enzyme (Nielsen et al., 1996) by restraining its catalytic activity (Stearns et al., 2004). Anthracyclines form a complex with DNA by intercalating between base pairs. Anthracyclines is the preferred treatment for advanced stage breast cancer, especially for hormone receptor (ER/PR) negative counterparts (Palmieri et al., 2010). For patients who relapse from other treatments, such as endocrine therapy, chemotherapy is the only option.

Forkhead box protein M1 (FOXM1) is a transcription factor that plays a key role in various cell regulation mechanisms such as cell cycle control, cell proliferation, apoptosis, DNA damage and repair (Koo et al., 2012). In response to genotoxic drugs, FOXM1 mediates drug resistance and enhances DNA repair by controlling DNA repair genes including BRIP1 (Monteiro et al., 2013), NBS1 (Khongkow et al., 2013) and Rad51 (Zhang et al., 2012). FOXM1 has been found to be upregulated in various cancers. In breast cancer, FOXM1 is involved in resistance to various cancer treatments, for example, endocrine therapy (Millour et al., 2010), cisplatin (Kwok, 2010), paclitaxel (Carr et al., 2010), and, especially, epirubicin (Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013).

FOXM1 has been reported to be controlled by different PTMs. One of these modifications is phosphorylation. In an inactive stage, transcriptional and
transactivational activity of FOXM1 is suppressed by its own N-terminal autorepressor domain (NRD) in G1 and early S phases (Koo et al., 2012). This NRD binds directly to its C-terminal transactivation domain (TAD) (Park et al., 2008). Phosphorylation of FOXM1 is increased throughout cell cycle progression from G1 to S and G2 phases. During G2, FOXM1 is phosphorylated by cyclin A-CDK complexes. This allows FOXM1 to be free from its autorepression (Laoukili et al., 2008). Additionally, phosphorylation of FOXM1 also culminates with its nuclear translocation during G2/M phase (Koo et al., 2012; Laoukili et al., 2008). At the mitosis exit, FOXM1 is dephosphorylated (Chen et al., 2009; Fu et al., 2008). Although many studies report about the phosphorylation of FOXM1, other PTMs including SUMOylation and ubiquitination remain poorly understood.

SUMOylation is a reversible PTM by the small ubiquitin-related modifier (SUMO) proteins. Although the structure of SUMO is similar to that of ubiquitin, the cellular outcomes of SUMOylation can be distinct from ubiquitination. While ubiquitination targets a protein for proteolytic breakdown or sub-cellular redistribution, SUMOylation takes part in various pathways, including sub-cellular localisation of SUMO-target protein such as RanGAP1. Besides regulating its nuclear translocation, SUMOylation of RanGAP1 is also required for its recruitment to the mitotic spindle during the mitosis (Joseph et al., 2002). In a study with Dictyostelium, SUMOylation is required for the nuclear export of MEK1, under starvation conditions (Sobko et al., 2002). SUMOylation has also been found to have a role in cell cycle progression. In a study of yeast, mutation of SUMO-activating enzyme (E1) and SUMO-conjugating enzyme (E2) results in cell cycle arrest at the G2/M phase. In addition, SUMO modifications are necessary
for anaphase-promoting complex/cyclosome (APC/C) activity in order to control cell cycle progression through SUMOylation-ubiquitination crosstalk (Hay, 2005). Moreover, SUMOylation has also been reported to involve in DNA damage response. For example, SUMOylation is required for an assembly of promyelocytic leukaemia nuclear (PML) bodies to participate in the DNA damage repair (Kerscher et al., 2006). SUMOylation of proliferating cell nuclear antigen (PCNA) is also important for RAD6-dependent DNA repair (Watts, 2006).

SUMOylation has been reported to have a fundamental role in regulating other forkhead proteins such as FOXA1 (Bao et al., 2011; Belaguli et al., 2012) and FOXC1/2 (Danciu et al., 2012). Interestingly, SUMOylation of these forkhead proteins leads to an inhibition of their function. In this chapter, I hypothesised that FOXM1 might be modified by SUMOylation. Therefore, I investigated the effect of SUMOylation on the role of FOXM1 in response to epirubicin. This should lead to a better understanding of how FOXM1 is regulated by post-translational modification, especially SUMOylation and ubiquitination.
3.2 Results

3.2.1 FOXM1 is involved in epirubicin resistance

FOXM1 is widely known to promote cellular migration and invasion, DNA damage repair, and chemotherapeutic drug resistance including epirubicin, an anthracycline chemotherapeutic drug (Millour et al., 2011). In order to confirm the importance of FOXM1 in epirubicin resistance (Monteiro et al., 2013), previously reported by our previous PhD student in the lab (Dr. Lara Monteiro), MCF-7 and MCF-7 Epi^R^ cells were treated with 1 μM epirubicin and harvested at different time points (0, 4, 8, 16, 24, and 48 h) for western blotting analysis. The expression levels of FOXM1, PARP, cyclin B1, γH2Ax and β-tubulin were determined. The results showed a decrease in FOXM1 protein levels after treatment with epirubicin in MCF-7 cells. In contrast, FOXM1 protein levels in MCF-7 Epi^R^ cells were stably expressed throughout the time course of epirubicin treatment (Figure 3.1A). Cyclin B1 was used as a positive control for the activity of FOXM1 as this is a well-established downstream target of FOXM1 (Leung et al., 2001). Accordingly, the down-regulation of FOXM1 activity observed in MCF-7 cells was also confirmed by a reduction in the expression levels of cyclin B1. Cleaved poly-ADP ribose polymerase (PARP, arrow) and γH2Ax are known markers of apoptosis and DNA damage responses, respectively. MCF-7 cells exhibited an increase in cleaved PARP after 24 h of epirubicin treatment and also displayed higher γH2Ax expression compared with MCF-7 Epi^R^ cells.
FOXM1 mRNA levels were also analysed using qPCR. The mRNA levels correlated with the results from western blotting; the expression of FOXM1 decreased after treatment with epirubicin in MCF-7 cells. In MCF-7 EpiR cells, FOXM1 mRNA levels were also shown to be highly expressed both before and after epirubicin treatment (Figure 3.1B). The resistant phenotype of MCF-7 EpiR cells was confirmed using SRB assay. The result showed that MCF-7 cells were significantly more sensitive to epirubicin compared with MCF-7 EpiR cells especially after treatment with concentrations higher than 0.2 μM (Figure 3.2).
Figure 3.1 Time course experiment in MCF-7 and MCF-7 EpiR cells. MCF-7 and MCF-7 EpiR cells were treated with 1 μM epirubicin and harvested at different indicated time points (0, 4, 8, 16, 24 and 48 h). A. Protein lysates were extracted and analysed by western blotting. B. The mRNA levels of FOXM1 of each time point was analysed by qPCR. Statistical analysis was performed using Student’s t-test. (**, p ≤ 0.001; ***, ≤ 0.0001 significant; ns, non-significant)
Figure 3.2 MCF-7 EpiR cells are more resistant to epirubicin than MCF-7 cells. MCF-7 and MCF-7 EpiR cells were treated with various indicated concentrations of epirubicin for 24 h. Cell proliferation was measured by SRB assay. The result was normalised to the untreated control (0 μM concentration). Results represent the average of three independent experiments including 6 replicates ± SD. Statistical analysis was performed using Student’s t-test. (**, p ≤ 0.001; ***, ≤ 0.0001 significant; ns, non-significant)
3.2.2 *Foxm1*⁻/⁻ MEFs are significantly more sensitive to epirubicin than wt MEFs

To confirm the role of FOXM1 in epirubicin resistance, I used SRB assay to compare wt and *Foxm1*⁻/⁻ MEFs. The cells were treated with epirubicin at various concentrations for 24 h. The result revealed that *Foxm1*⁻/⁻ MEFs were more sensitive to epirubicin compared with wt MEFs, especially at high epirubicin concentrations, 0.5 μM to 1 μM (Figure 3.3). To further analyse the importance of FOXM1 in DNA damage, COMET assay was performed under an alkaline condition using wt and *Foxm1*⁻/⁻ MEFs. Cells were treated with 1 μM epirubicin and harvested at 0, 6 and 24 h after treatment. The cell pellets were resuspended in low-melting point agarose (LMA) before being used for electrophoresis. The cellular DNA was stained with DAPI. Images were captured using 40x magnification objective. The percentage of the DNA in tail was quantified using COMET IV software. The results showed that at 6 h *Foxm1*⁻/⁻ MEFs had a significantly higher percentage of DNA in tails (damaged DNA) compared with wt MEFs (Figure 3.4A). At 24 h, the percentage of the DNA in tails of wt and *Foxm1*⁻/⁻ MEFs showed no significant difference (Figure 3.4A), but there were fewer *Foxm1*⁻/⁻ MEFs, compared with wt MEFs (Figure 3.4B). This showed that *Foxm1*⁻/⁻ MEFs have fewer surviving cells after 24 h of epirubicin treatment compared with wt MEFs. In conclusion, the cells with lower FOXM1 expression were more sensitive to epirubicin and had more DNA damage than the cells with higher FOXM1 expression levels. This data confirms that FOXM1 has an important role in mediating epirubicin resistance.
Figure 3.3 *Foxm1*–/– MEFs are more sensitive to epirubicin than wt MEFs. *Foxm1*–/– MEFs and wt MEFs were treated with various indicated concentrations of epirubicin for 24 h. Cell proliferation was measured by SRB assay. The result was normalised to the untreated control (0 μM concentration). Results represent the average of three independent experiments including 6 replicates ± SD. Statistical analysis was performed using Student’s *t*-test. (**, *p* ≤ 0.001; ***, *p* ≤ 0.0001 significant; ns, non-significant)
Figure 3.4 Foxm1<sup>−/−</sup> MEFs show more DNA damage than wt MEFs after epirubicin treatment. Foxm1<sup>−/−</sup> and wt MEFs were treated with 1 μM epirubicin and harvested after 0, 6 and 24 h of the treatment. The cells were used to perform the COMET assay to examine the DNA damage. **A.** Images were acquired with a Nikon Eclipse E400 Upright microscope (40x magnification objective). **B.** DNA damage was quantified using COMET Assay IV software and measured for percentages of DNA in tail. Represented data are the average of two independent experiments (**, p ≤ 0.001; ***, ≤ 0.0001 significant; ns, non-significant).
Figure 3.4 (continued)
3.2.3 FOXM1 can be modified by Small ubiquitin-related modifier1 (SUMO1)

Since our previous data shown that FOXM1 plays a critical role in epirubicin resistance, investigation of FOXM1 regulation might beneficial in order to overcome chemotherapeutic drug resistance. As SUMOylation is also important in DNA damage response (Galanty et al., 2009; Morris et al., 2009), I hypothesised that FOXM1 might be modified by SUMOylation and this could have a role in the epirubicin-mediated DNA damage response.

In order to test this hypothesis, MCF-7 cells were transfected with SUMO1/SUMO2/SUMO3 or empty vector control, together with the wt FOXM1 expression vector before the total protein lysates were used for western blotting analysis. The result showed multiple higher molecular weight bands (poly-SUMOylation) in FOXM1-SUMO1 co-expression condition, but this is not the case for the SUMO2, SUMO3 (SUMO2/3) or empty vector control (pcDNA3) transfected conditions (Figure 3.5). This result suggests that FOXM1 is predominantly targeted by SUMO1.

To confirm the presence of an interaction, MCF-7 cells were transfected with pcDNA3-Flag-FOXM1 (FOXM1) or pcDNA3-Flag empty vector control (Flag), together with tRFP-SUMO1. After 24 h of transfection, cells were treated with 1 μM epirubicin and harvested at 0, 6, 24 and 48 h after treatment with epirubicin. Immunoprecipitation was performed using ANTI-FLAG M2 affinity gel beads to pull down the Flag-tagged protein. Input and eluent were analysed by western blotting and probed with various specific antibodies (FOXM1, SUMO1, and Flag). Higher molecular weight bands were only found in the pcDNA3-Flag-FOXM1 transfected cells and were absent in the pcDNA3-
Flag empty vector transfected control (Figure 3.6). After 24 and 48 h of epirubicin treatment, the bands that correspond to the FOXM1-SUMO1 decreased in response to epirubicin treatment. This result further confirms the interaction between FOXM1 and SUMO1 and also that epirubicin has an effect on it.

A covalent interaction between SUMO1 and FOXM1 was identified using Ni$^{2+}$ pull down from His-tagged SUMO1 (His-SUMO1) expressing cells. The experiment was performed in collaboration with Dr. Stephen Myatt from our lab. MCF-7 cells were co-transfected with FOXM1, Ubc9 and His-SUMO1. First, the His-tagged protein was pulled down using Ni$^{2+}$-column under denaturing conditions (8M urea) to prevent de-SUMOylation and proteolysis. The expression of FOXM1 was analysed by western blotting. The higher molecular weight bands corresponded to SUMOylation of FOXM1 (Figure 3.7A).

In addition, an in vitro SUMOylation assay (Enzo Life Sciences, Exeter, UK) was also used to confirm the interaction between SUMO1 and FOXM1. From the preliminary experiment done by Dr. Stephen Myatt, in vitro SUMOylation assays exhibited FOXM1 SUMOylation in an ATP-dependent manner (Figure 3.7B).
Figure 3.5 FOXM1 interacts with SUMO1 but not SUMO2/3. MCF-7 cells were transfected with FOXM1 together with or without SUMO1 or SUMO2 or SUMO3. The cells were harvested 24 h after transfected and used for western blotting. The immunoblots were probed using antibodies against FOXM1, SUMO1, SUMO2/3 and β-tubulin (used as a loading control). Multiple higher molecular weight bands were observed (higher than FOXM1: ≥105 KDa). This experiment was performed in collaboration with Dr. Stephen Myatt.
Transfection:  pcDNA3/SUMO1  pcDNA3-FOXM1/SUMO1

1 μM epirubicin (h)  Input  IP:Flag  Input  IP:Flag

WB:Flag  260 kDa  0  6  24  48  0  6  24  48
160 kDa  110 kDa

WB:FOXM1  260 kDa  0  6  24  48  0  6  24  48
160 kDa  110 kDa

WB:SUMO1  260 kDa  0  6  24  48  0  6  24  48
160 kDa  110 kDa

Figure 3.6 Confirmation of the interaction between FOXM1 and SUMO1 and its degradation after epirubicin treatment. MCF-7 cells were transfected with pcDNA3-Flag-FOXM1 or pcDNA3-Flag empty vector control, and tRFP-SUMO1. The transfected cells were treated 1 μM epirubicin and harvested at different indicating time points (0, 6, 24 and 48 h). Co-IP was performed using ANTI-FLAG M2 Affinity Gel to pull down the Flag-tagged protein. Input represents 1:10 dilution of the pull down. Eluent was analysed by western blotting and the expression of FOXM1, SUMO1, and Flag were determined.
Figure 3.7 FOXM1 interacts with SUMO1 via covalent bond. A. FOXM1, Ubc9 and His-tagged SUMO1 were co-expressed. SUMOylated proteins were purified under denaturing conditions (8 M urea) using Ni$^{2+}$ column affinities pull down. Input and His-tagged protein were analysed by western blotting and probed with antibodies against FOXM1. B. His-tagged FOXM1 was induced to be expressed in *E. coli* using IPTG and isolated with Ni$^{2+}$ column affinity pull down. The extracted lysates from *E. coli* in the presence or absence of IPTG were incubated with Ubc9 and SUMO1 with and without Mg$^{2+}$-ATP in reaction buffer. SUMOylation of FOXM1 was examined using western blotting. These two experiments were performed in collaboration with Dr. Stephen Myatt.
3.2.4 The interaction of FOXM1 and SUMO1 increases after 6 h of epirubicin treatment before decreasing after 24 h of the treatment

Co-immunoprecipitation was used to study the interaction between FOXM1 and SUMO1. Input and eluent were analysed by western blotting. Higher molecular weight bands (160kDa) were found confirming that FOXM1 forms higher order complexes with SUMO1. The results from MCF-7 cells (Figure 3.8A and 3.8C) showed a stronger interaction of FOXM1-SUMO1 compared to MCF7 EpiR cells (Figure 3.8B and 3.8D). Interestingly, the endogenous FOXM1 levels in MCF-7 cells decreased after epirubicin treatment for 24 h (Figure 3.8C). However, the higher molecular weight band for FOXM1-SUMO1 complex remained constant. This suggests that there is an increase on FOXM1 SUMOylation upon epirubicin treatment in MCF-7 cells.

To confirm the increase in the interaction between FOXM1 and SUMO1 after epirubicin treatment, immunofluorescent staining was performed. MCF-7 and MCF-7 EpiR cells were treated with 1 µM epirubicin and harvested at different time points (0, 6, 24 and 48 h). Cells were then fixed with 4% paraformaldehyde (PFA) and stained with FOXM1 (Alexa488, shown as green), SUMO1 (Alexa555, shown as red) and DAPI (blue). The merged (yellow colour) showed the co-localisation of FOXM1 and SUMO1 (Figure 3.9). In accordance to the co-IP results, there was an increase in the FOXM1-SUMO1 interaction in MCF-7 especially after 6 h of epirubicin treatment, but this was not observed in MCF-7 EpiR cells. In contrast, there was a weaker FOXM1-SUMO1 interaction in MCF-7 EpiR cells compared to MCF-7 cells. After 24 h and 48 h of 1 µM
epirubicin treatment, the levels of co-localisation of FOXM1-SUMO1 were decreased in MCF-7 cells, but not in MCF-7 Epi$^R$ cells.

In collaboration with the Photonic group (Imperial College London, South Kensington Campus), Douglas Kelly (a PhD student) kindly helped me to perform the Fluorescence Lifetime Imaging and Förster resonance energy transfer (FLIM-FRET) experiment to confirm the interaction between FOXM1 and SUMO1, after epirubicin treatment. MCF-7 cells were co-transfected with eGFP-FOXM1 (donor) and tRFP-SUMO1 (acceptor). FLIM-FRET is used for measuring the exponential decay rate of fluorescent proteins. When two proteins are close enough (approximately less than 10 nm), there is energy transfer between the two fluorescent proteins. The energy transfer from the donor protein (in this case eGFP) to the acceptor protein (in this case tRFP) results in decreasing the lifetime of the donor fluorescence (Ishikawa-Ankerhold et al., 2012).

The FLIM-FRET result exhibited an increase in decay of the eGFP donor signal after treatment with epirubicin at 6 and 8 h (Figure 3.10). This result indicates that the FOXM1-SUMO1 interaction increased after epirubicin treatment. In contrast, the control condition (eGFP-FOXM1 with tRFP empty vector) showed little or no decay of eGFP-FOXM1 donor. This indicates that the decay of eGFP signals in FOXM1-SUMO1 co-transfected conditions was due to the FRET from the FOXM1-SUMO1 interaction.
Figure 3.8 Co-immunoprecipitation (co-IP) shows an interaction between FOXM1 and SUMO1. MCF-7 and MCF-7 EpiR cells were treated with 1 μM epirubicin and harvested after 0, 6 and 24 h of the treatment. Antibodies against SUMO1 were used to perform co-immunoprecipitation and probed for FOXM1, and vice versa. Input represents 1:10 dilution of pull down and IgG was used as a negative control. Protein eluent from beads and input were analysed using western blotting. Higher molecular weight bands confirmed the interaction between FOXM1 and SUMO1 (shown in asterisk, *)
Figure 3.9 Immunofluorescent staining shows co-localisation of FOXM1 and SUMO1. A. MCF-7 and B. MCF-7 Epi\textsuperscript{R} cells were treated with 1 µM epirubicin and harvested at 0, 6, 24 and 48 h after the treatment. The treated cells were then fixed with 4% PFA before being stained with antibodies against FOXM1 (Alexa488, shown as green), SUMO1 (Alexa555, shown as red) and DAPI (blue). Images were taken using a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) with 63x objective and LAS-AF software. Images are the representative of 3 independent experiments, results obtained from at least 10 different fields.
**Figure 3.9 (continued)**

B

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Figure 3.10 FLIM-FRET confirms an increase in the interaction of FOXM1-SUMO1 following epirubicin treatment. FLIM-FRET experiment was performed by Douglas Kelly, a collaborator from the photonic group (Imperial College London, South Kensington campus, London, UK). MCF-7 cells were co-transfected with eGFP-FOXM1 and tRFP-SUMO1. The transfected cells were used for performing the FLIM-FRET experiment after epirubicin treatment at indicated time points. Fluorescent life time was analysed and the graph was plotted using the mean lifetime ± SEM (standard error of the means). Statistical analysis was obtained with Tukey’s HSD test compared with 0h (*, p < 0.05 significant; ns, non-significant)
3.2.5 FOXM1 is SUMOylated at multiple sites

In order to get better insights into which FOXM1 sites could be potentially SUMOylated, I used SUMOplot (http://www.abgent.com/tools, Abgent, UK) and SUMOsp (http://sumosp.biocuckoo.org, CUCKOO Workgroup, University of Science & Technology of China, China) to predict FOXM1 SUMOylation sites (Figure 3.11A, B). The predicted sites were then mutated using the site-direct mutagenesis kit to create the FOXM1 5x(K>R) mutant plasmid. This plasmid carried 5 point mutations that created SUMO modification sites, by changing the lysine residues into arginine. This plasmid was generated by Dr. Stephen Myatt.

MCF-7 cells were transfected with FOXM1 wt or FOXM1 5x(K>R) plasmids, with or without SUMO1/Ubc9. As expected, cells transfected with FOXM1 wt revealed not only a band corresponding to SUMOylated FOXM1 in the presence of SUMO1 and Ubc9. In contrast, cells transfected with FOXM1 5x(K>R) mutation did not express the band corresponding to the SUMOylated FOXM1 (Figure 3.11C). This confirms that FOXM1 was indeed modified by SUMO1 at multiple sites (K201, K218, K460, K478 and K495).
Figure 3.11 FOXM1 is SUMOylated at multiple sites. A. The table shows 5 consensus sequences (Ψ is a hydrophobic residue; K is a lysine that has conjugated with SUMO; x is any amino acid; E is an acidic residue) of predicted SUMO modification site from SUMOplot (Abgent, UK) and SUMOsp (CUCKOO Workgroup, University of Science & Technology of China, China). B. The schematic shows positions of predicted SUMO modification sites from A. (NRD is an N-terminal regulatory domain; FKH is a forkhead domain; TAD is a trans-activation domain). C. MCF-7 cells were transfected with FOXM1 wt or 5x(K>R) mutant together with Ubc9 and SUMO1, or empty vector control. SUMOylation of FOXM1 was determined at higher molecular weight by western blotting.
3.2.6 SUMOylated FOXM1 is mainly cytoplasmic rather than nuclear

After confirming that FOXM1 is SUMOylated, I decided to investigate the impact of SUMOylation on the sub-cellular localisation of FOXM1. MCF-7 cells were transfected with FOXM1 in the presence or absence of SUMO1 and Ubc9. Multi-SUMOylation of FOXM1 was found at higher molecular weight band in the cytoplasmic fraction of these cells when co-transfected with SUMO1 and Ubc9 (Figure 3.12A). To further confirm this, MCF-7 cells were transfected with FOXM1 (wt)-Ubc9 or FOXM1 5x(K>R)-Ubc9. Ubc9-fusion directed SUMOylation system (UFDS) was used in this experiment in order to bypass the requirement of SUMO E3 ligase enzymes. It is widely known that the SUMOylation pathway requires E1-E2-E3 enzymatic cascade. Ubc9 is exclusively E2 SUMO-conjugating enzyme, but there are few E3 ligases in this cascade. The SUMO-E3 ligase for this pathway remains unknown. The UFDS is the best way to study the SUMOylation of FOXM1 excluding the interference of the SUMO-E3 ligases (Jakobs et al., 2007). As expected, cells with an overexpression of FOXM1 (wt)-Ubc9 showed an auto-SUMOylation in the cytoplasmic fraction but not in the FOXM1 5x(K>R)-Ubc9 transfected cells.

In addition, there was less FOXM1 in the cytoplasmic fraction of the cells transfected with FOXM1 5x(K>R)-Ubc9, compared with the FOXM1 wt-Ubc9 transfected cells (Figure 3.12B). Furthermore, confocal microscopy also confirmed that SUMOylation is important for cytoplasmic translocation of FOXM1. The GFP tagged-FOXM1 (wt)-Ubc9 was found in both cytoplasm and nucleus, while eGFP-tagged FOXM1 5x(K>R)-Ubc9 was present only in the nucleus fraction (Figure 3.12C).
Figure 3.12 SUMOylated FOXM1 is predominantly found in the cytoplasm of MCF-7 cells. 

A. MCF-7 cells were transfected with FOXM1 together with or without SUMO1 and Ubc9. Sub-cellular fractionation extraction was performed and the lysates were analysed by western blot. Lamin B and β-tubulin were used as loading controls for nuclear and cytoplasmic fractions, respectively.

B. FOXM1 (wt)-Ubc9 or FOXM1 (5x(K>R))-Ubc9 were transfected into MCF-7 cells.

C. MCF-7 cells were transfected with eGFP-FOXM1 (wt)-Ubc9 or eGFP-FOXM1 (5x(K>R))-Ubc9 and stained with DAPI (nucleus) and β-tubulin (cytoplasm). Images were taken with a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) with 63x objective and LAS-AF software.
To prove that there is a nuclear-cytoplasmic translocation of FOXM1, immunofluorescent staining was performed to examine the endogenous levels of FOXM1 protein levels in cells treated with leptomycin B (LMB), a nuclear export inhibitor, or a vehicle (ethanol). The result confirmed that FOXM1 can only be found in the nuclear fraction after LMB treatment. In contrast, FOXM1 was found in both nuclear and cytoplasmic fractions in the cells treated with vehicle alone (Figure 3.13). These results suggested that there is a nuclear export mechanism for FOXM1 which is SUMOylation-dependent.
Figure 3.13 Leptomycin B inhibits nuclear export of endogenous FOXM1. MCF-7 cells were treated with 6 h of 10 ng/ml leptomycin B (LMB) or vehicle (ethanol). After 24 h of treatment, cells were stained with antibodies against FOXM1 (green), DAPI (blue) and β-tubulin (red). Images were taken with a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) with 63x objective and LAS-AF software.
3.2.7 SUMOylation promotes the degradation of FOXM1 and this is mediated by Cdh1

Previously, FOXM1 was found to be degraded in a Cdh1 dependent manner during cellular mitotic exit (Laoukili et al., 2008). To determine whether this Cdh1-mediated effect could be affected by the SUMOylation status of FOXM1, I firstly confirmed the effect of overexpression and depletion of Cdh1. To this end, MCF-7 cells were transfected with Cdh1 expression vector or pcDNA3 empty vector control. The transfected cells were treated with CHX to inhibit protein synthesis. The protein expression was examined by western blotting. The results showed that FOXM1 was degraded faster in cells with Cdh1 overexpression when compared to the empty vector control (Figure 3.14). In order to silence Cdh1, MCF-7 cells were transfected with siCdh1 or NSC siRNA. The result showed that in Cdh1 depleted cells, FOXM1 protein degrades slower than the cells transfected with the NSC control (Figure 3.15). In conclusion, Cdh1 is found to promote the degradation of FOXM1.

Since SUMOylation was shown to be important for the nuclear export of FOXM1 (Figure 3.12 and 3.13), I next investigated the role of SUMO1 in the regulation of FOXM1 ubiquitination and degradation in Cdh1 dependent manner. MCF-7 cells were transfected with FOXM1 with and without Ubc9 and SUMO1 for 24 h before the transfected cells were treated with CHX. When SUMO1 and Ubc9 were overexpressed, FOXM1 protein was found to degrade faster than in the control cells in the absence of SUMO1/Ubc9 (Figure 3.16A). This result suggests that SUMOylation might have an important role in accelerating the ubiquitination and degradation of FOXM1.
Consequently, I hypothesised that SUMOylation is involved in the degradation of FOXM1 through the APC/C-Cdh1 complex. To test this, MCF-7 cells were transfected with V5-tagged-FOXM1 wt or V5-tagged-5x(K>R), with increasing levels of a constitutively active Cdh1 expression vector (Cdh1(4a)). This experiment was done in collaboration with Dr. Stephen Myatt. The results showed that the increasing levels of Cdh1 resulted in the presence of the higher molecular weight bands (arrow) in the position where the ubiquitination form of FOXM1 was expected. This higher molecular weight band can only be found in MCF-7 cells transfected with FOXM1 wt expression and not in those transfected with FOXM1 5x(K>R) expression (Figure 3.16B). This suggests that the SUMOylation status of FOXM1 might be important for the ubiquitination of FOXM1 and this is in a Cdh1 dependent manner.

To further investigate the effect of SUMOylation on the Cdh1-mediated degradation of FOXM1, cells expressing either FOXM1 wt or 5x(K>R) were treated with CHX in the presence or absence of Cdh1 overexpression. The cells overexpressing FOXM1 wt and Cdh1 revealed a significant higher degradation rate than in the cells lacking Cdh1 overexpression, or in the 5x(K>R) transfected cells (Figure 3.17). In contrast, the cells transfected with FOXM1 5x(K>R) showed no difference in their degradation rate between cells with or without the overexpression of Cdh1.
Figure 3.14 Cdh1 overexpression increases the degradation rate of the FOXM1 protein. A. MCF-7 cells were transfected with Cdh1 or empty vector control. Then, cells were treated with 100 µM CHX and harvested at several time points (0, 2, 4, 6 and 8 h). Densitometry was used to quantify the FOXM1 levels from western blots using ImageJ. β-tubulin was used for normalisation. The asterisk indicates pcDNA3-flag-Cdh1 band. B. Quantitative FOXM1 levels were normalised to 0 h CHX treatment. C. Quantitative FOXM1 levels were normalised to 2 h CHX treatment.
Figure 3.15 Cdh1 knockdown stabilises the FOXM1 protein. A. MCF-7 cells were transfected with siCdh1 or NSC. Then, cells were treated with 100 µM CHX before being harvested at several time points (0, 2, 4, 6 and 8 h). Densitometry was used to quantify the FOXM1 levels from western blot using ImageJ. β-tubulin was used for normalisation. B. Quantitative FOXM1 levels were normalised to 0 h CHX treatment. C. Quantitative FOXM1 levels were normalised to 2 h CHX treatment.
Figure 3.16 SUMOylation of FOXM1 accelerates the degradation of FOXM1 which is mediated by Cdh1. A. FOXM1 was overexpressed in MCF-7 cells with or without Ubc9/SUMO1. Transfected cells were treated with 100 µM CHX before being harvested at several time points (0, 4, 6 and 8 h). FOXM1 expression was analysed by western blot. Densitometry of FOXM1 levels was quantified with Image J and normalised to β-tubulin. Densitometry was the means ± SEM. Statistical analysis was analysed by Student’s t-test. (**, p ≤ 0.001; ***, ≤ 0.0001 significant; ns, non-significant) B. MCF-7 cells were transfected with FOXM1 wt or 5x(K>R) mutant, and increasing levels of Cdh1. FOXM1 bands were examined by western blotting. The higher molecular weight bands were indicated by the arrow. This experiment was performed in collaboration with Dr. Stephen Myatt.
Figure 3.17 Cdh1 overexpression affects the degradation of FOXM1 wt but not FOXM1 5x(K>R) mutant. MCF-7 Cells were transfected with V5-tagged FOXM1 wt or 5x(K>R) mutant before being treated with 100 µM CHX before being harvested at several time points (0, 2, 4, 6 and 8 h). Western blot was used for protein analysis. FOXM1 levels were quantified using Image J and normalised to β-tubulin. Densitometry represents the means ± SEM. Statistical analysis was performed using Student’s t-test. (**, p ≤ 0.001; ***, ≤ 0.0001 significant; ns, non-significant)
3.2.8 SUMOylation affects the ubiquitination of FOXM1

It is widely known that most of the SUMOylation events that occur within a cell leads to the crosstalk with the ubiquitination pathway (Bergink and Jentsch, 2009; Hunter and Sun, 2008; Praefcke et al., 2012). Therefore, I decided to investigate the role of SUMOylation of FOXM1 in its ubiquitination and degradation. Therefore, MCF-7 cells were transfected with FOXM1 wt or 5x(K>R) together with Flag-Cdh1(4a). The transfected cells were then used for a co-immunoprecipitation experiment. As expected, the ubiquitinated form of FOXM1 - the higher molecular weight - was found only in cells transfected with FOXM1 wt, but not in those transfected with the FOXM1 5x(K>R) mutant (Figure 3.18A). Interestingly, the study showed that Cdh1 was able to bind to both FOXM1 wt and 5x(K>R). This suggests that SUMOylation has no effect on the interaction between FOXM1 and APC/Cdh1 complex but affects its subsequent ubiquitination.

In addition, co-expression of HA-ubiquitin (Ub) with V5 tagged-FOXM1 wt or 5x(K>R) was performed before the transfected cells were used for co-immunoprecipitation. In agreement with the previous data, FOXM1 wt showed a stronger interaction with HA-ubiquitin, compared with FOXM1 5x(K>R) (Figure 3.18B). Collectively, these data shows that ubiquitination of FOXM1 cannot occur without SUMOylation.
Figure 3.18 SUMOylation affects FOXM1 ubiquitination. A. MCF-7 cells were co-transfected with FOXM1 wt or 5x(K>R), and together with Flag-Cdh1. Transfected cells were used for co-IP with a FOXM1 antibody after 24 h of transfection. Precipitated protein and total protein lysate (input) were analysed by western blotting. The membrane was probed with Flag, FOXM1 and β-tubulin antibodies.

B. MCF-7 cells were co-transfected with FOXM1 wt or 5x(K>R), with or without HA-ubiquitin. Protein lysates were then used for co-IP with V5 antibodies. Ubiquitination of FOXM1 was detected using HA antibodies.
3.2.9 SUMOylation-deficient form of FOXM1 shows an increased transcriptional activity of its downstream targets

Based on my results, I then proposed that the SUMOylation-deficient FOXM1 (5x(K>R)) mutant should have a higher transactivation activity over its downstream targets when compared with the wt form of FOXM1. To test this, the transactivation activity of FOXM1 5x(K>R) was compared with FOXM1 wt, by performing luciferase reporter assay using various FOXM1-responsive promoters. The result showed that SUMOylation-deficient FOXM1 (5x(K>R)) had a significantly higher transactivation activity over cyclinB1 (Figure 3.19A), 6x-DNA-binding element (6XDBE) (Figure 3.19C) and GADD45 (Figure 3.19D) promoters. In contrast, the result from the cyclin B1 mut3 promoter (containing mutations at 3 consensus forkhead binding sites) showed no difference between FOXM1 wt and 5x(K>R) (Figure 3.19B). This suggests that FOXM1 regulates its downstream targets, such as cyclin B1, through forkhead response elements (FHREs). When FOXM1 cannot be SUMOylated, it is more stable and unable to be degraded through the proteasome-degradation pathway. This leads to its increasing transactivational activity over its downstream targets.

To confirm the previous results, the luciferase assays were repeated using cyclin B1 (Figure 3.20A) and 6XDBE (Figure 3.20B) promoters with increasing amounts of FOXM1 wt or 5x(K>R) plasmids (0-100ng). This result also confirmed that the SUMOylation-deficient FOXM1 has a higher transactivation activity over its downstream targets compared to FOXM1 wt.
Figure 3.19 SUMOylation of FOXM1 suppresses cyclin B1 transcription. MCF-7 cells were co-transfected with a gradient of (0 - 25 ng) wt FOXM1-Ubc9 or 5x(K>R) FOXM1-Ubc9 (DNA concentration was normalised with empty vector control), and luciferase reporters driven by (A) wild-type cyclin B1 promoter, (B) cyclin B1 mut3 promoter contained three mutant consensus forkhead binding sites, (C) the FOXM1 6x-DNA-binding element (6XDBE), or (D) the GADD45 promoter. Luciferase assays were then performed following 24 h of transfection. The Luciferase activity was measured using PHERAstar Plus microplate reader. Reporter gene activity was normalised to the Renilla control activity. Graphs represent the mean of 3 independent experiments ±SEM. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.05 significant; ns, non-significant)
Figure 3.20 FOXM1 with 5x(K>R) mutation has higher transactivation activity over its downstream target than wild-type. MCF-7 cells were co transfected with gradient of (0 - 100 ng) wt FOXM1-Ubc9 or 5x(K>R) FOXM1-Ubc9 (DNA concentration was normalised with empty vector control), and luciferase reporters driven by (A) wild-type cyclin B1 promoter, (B) the FOXM1 6x-DNA-binding element (6XDBE) promoter. The Luciferase activity was measured using PHERAsstar Plus microplate reader. Reporter gene activity was normalised to the Renilla control activity. Graphs represent the means of 3 independent experiments ±SEM. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.05 significant; ns, non-significant)
3.2.10 SUMOylation-deficient FOXM1 induces higher epirubicin resistance when compared to FOXM1 wt

FOXM1 has already been well established that it is involved in the promotion of epirubicin resistance (Gomes et al., 2013; Halasi and Gartel, 2012; Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013). Therefore, I next investigated the effect of SUMOylation of FOXM1 on epirubicin resistance. MCF-7 cells were transfected with FOXM1 wt, FOXM1 5x(K>R) or an empty vector control. The transfected cells were treated with various concentrations of epirubicin (0 – 20 µM). Cell proliferation was then measured using SRB assay. The result showed that the SUMOylation-deficient FOXM1 transfected cells showed a significantly higher percentage of cell survival (Figure 3.21A). The overexpression levels of FOXM1 wt and 5x(K>R) were confirmed by western blotting (Figure 3.21B).
Figure 3.21 MCF-7 cells overexpressing FOXM1 5x(K>R) mutant are more resistant to epirubicin treatment than cells overexpressing FOXM1 wt. MCF-7 cells were transfected with FOXM1 wt, 5x(K>R) mutant, or pcDNA3 empty vector control. A. 24 h after transfection, cells were reseeded to 96-well plates overnight before being treated with various concentrations of epirubicin. Cell proliferation was indicated using SRB assay. Data represented 6 replicates (mean±SD). Statistical analysis was performed using a Student’s t-test (*, p ≤ 0.05 significant; ns, non-significant). The statistic compared between FOXM1 wt and FOXM1 5x(K>R) transfected cells. B. The protein expression was studied using western blotting. The membrane was probed with antibodies against FOXM1, GFP and β-tubulin. The asterisk indicates eGFP-FOXM1 specific bands.
3.3 Discussion and conclusion

FOXM1 is upregulated in various types of cancer, including primary breast cancer (Ahmad et al., 2010; Francis et al., 2009; Wonsey and Follettie, 2005). In many studies, FOXM1 has been shown to have a crucial role in promoting cancer metastasis (Koo et al., 2012; Xue et al., 2014), cancer invasion and angiogenesis (Wang et al., 2007), relapse from endocrine therapy (Millour et al., 2011), as well as resistance to chemotherapy (Carr et al., 2010; Khongkow et al., 2013; Kwok, 2010; Monteiro et al., 2013). In this chapter, I confirm that FOXM1 plays a crucial role in epirubicin resistance.

When comparing MCF-7 and MCF-7 EpiR cells, FOXM1 is shown to be down-regulated by epirubicin treatment in MCF-7, at both protein and mRNA levels. However in MCF-7 EpiR cells, FOXM1 expression levels are higher. This FOXM1 down-regulation is accompanied by a decrease on its downstream target, cyclin B1. Consistent with previous studies from our lab, after epirubicin treatment, MCF-7 cells displays higher levels of γH2Ax (a marker for DNA double stranded breaks and genomic instability) and cleaved PARP (a cell death marker) when compared to MCF-7 EpiR cells (Monteiro et al., 2013). This suggests that FOXM1 is responsible for the promotion of epirubicin resistance in breast cancer. In order to confirm the involvement of FOXM1 in epirubicin resistance, the role of FOXM1 in DNA damage repair and cell survival was studied. Foxm1 depleted mouse embryonic fibroblasts (Foxm1−/− MEFs), (were found to have a highly significant lower percentage of cell survival compared to wt MEFs, especially when treated with a high epirubicin concentration of epirubicin (≥ 0.05µM). In regards to DNA damage, alkaline COMET assay confirmed that the absence of FOXM1 leads to an increase in the DNA damage levels. After 6 h of epirubicin treatment, Foxm1−/− MEFs
showed higher percentages of DNA in tails (damaged DNA) compared to wt MEFs. Although quantitative data analysis showed no difference after 24h of epirubicin treatment, the figures obtained using confocal microscopy exhibited dramatically lower number of cells in Foxm1−/− MEFs compared with wt MEFs. This shows that Foxm1−/− MEFs have lower cell survival rates that the wt MEFs. At 24 h of epirubicin treatment, the cells capable of repairing the damaged DNA, those with higher FOXM1 levels, have already done it while cells unable to repair DNA have probably died at this time point. In summary, these data strongly support the previous findings from our lab that FOXM1 enhances DNA damage repair and plays a key role in increasing epirubicin resistance (de Olano et al., 2012; Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013).

Understanding the regulation of FOXM1, especially at the post-translational level, is crucial to improve current cancer therapy. For example, phosphorylation has been reported to regulate FOXM1 (de Olano et al., 2012; Joshi et al., 2013; Millour et al., 2011). FOXM1 phosphorylation inhibits its own autorepression in the cytoplasm (Laoukili et al., 2008) and initiates mitosis by entering the nucleus (Koo et al., 2012; Laoukili et al., 2008). Once mitosis is finished, FOXM1 is dephosphorylated (Chen et al., 2009; Fu et al., 2008). However, PTMs of FOXM1 by ubiquitination and SUMOylation remain unclear. Additionally, it has been reported in many studies that SUMOylation plays a role in the inactivation of a variety of transcription factors, including the Forkhead related family members FOXA1 (Bao et al., 2011; Belaguli et al., 2012) and FOXC1/2 (Danciu et al., 2012). Therefore, I hypothesised that SUMOylation might have an impact in the regulation of FOXM1 post-translationally and this might have a
functional outcome. To determine whether this was the case, MCF-7 cells were transfected with FOXM1, Ubc9 and SUMO1/SUMO2/SUMO3, or empty vector control. Protein lysis was performed in the presence of N-ethylmaleimide (NEM). When studying SUMO modifications, NEM works as a SUMO conjugated stabiliser by covalently changing the sulfhydryl group of SUMO-specific proteases at the catalytic cysteine (Babic et al., 2006; Li and Hochstrasser, 1999). The results showed that FOXM1 can form an interaction with SUMO1. Furthermore, this interaction was shown to be specific between FOXM1 and SUMO1 and not SUMO2/3. This interaction was confirmed by immunoprecipitation experiments using ANTI-FLAG M2 affinity gel. SUMO1-FOXM1 complexes were found at higher molecular weight than the endogenous FOXM1. As expected, co-IP results from endogenous proteins also displayed bands similar to the band of SUMO1-FOXM1 complexes. Collectively, these results confirm that FOXM1 forms a complex with SUMO1. In addition, FOXM1 SUMOylation was further confirmed using an in vitro SUMOylation assay. The covalent bond between both proteins was then identified in order to prove that FOXM1 is modified specifically by SUMO1 and not just by a random interaction. His-tagged SUMO1 was transfected into cells together with FOXM1 and Ubc9, before the His-tagged proteins were pulled down using Ni²⁺-column affinity pull down under denaturing conditions (Lamsoul et al., 2005; Li et al., 2012; Zilio et al., 2013). The bands corresponding to the complex of His-tagged SUMO1 and FOXM1 were found at a higher molecular weight (160 kDa). This demonstrates that the interaction between SUMO1 and FOXM1 occurs via a covalent linkage.

Although the endogenous band for FOXM1, observed in input conditions, was decreased after 24 h of epirubicin treatment, the SUMO1-FOXM1 complex showed the
same expression levels when compared with the untreated control. This suggests that epirubicin treatment has an effect on FOXM1 by increasing its SUMOylation status after a short time of exposure to epirubicin. The decreasing protein levels of FOXM1 after 24 h in the input conditions indicate that there might be a degradation of FOXM1 after the treatment. In agreement, the results from the immunofluorescent staining revealed an increase in the co-localisation between FOXM1 and SUMO1 in MCF-7 cells after epirubicin treatment. In contrast, the co-localisation in MCF-7 EpiR cells remained stable and at low levels both before and after epirubicin treatment. In MCF-7 EpiR cells the co-localisation fluorescence intense was lower than in MCF-7 cells. This suggests that SUMOylation of FOXM1 increases in response to epirubicin treatment only in sensitive breast cancer cells. In epirubicin resistance MCF-7 cells, there is lower level of FOXM1 when compared with the MCF-7 cells. This in agreement with the stable level of FOXM1 in MCF-7 EpiR cells after epirubicin treatment. Correspondingly, FLIM-FRET results further confirm that in MCF-7 cells SUMOylation levels increase after epirubicin treatment. Collectively, the experiment shows that FOXM1 is SUMOylated between 4 to 6 h of epirubicin treatment, before it starts to be degraded between 16 to 24 h of epirubicin treatment. Importantly, this shows that SUMOylation precedes the degradation of FOXM1.

To further examine the role of SUMOylation in the regulation of FOXM1 a mutant FOXM1 plasmid was generated harbouring 5 point-mutations at predicted SUMO modification sites (5x (K>R)). This plasmid was generated by Dr. Stephen Myatt in our lab. The SUMOylated sites were predicted using SUMOplot and SUMOsp. Western blot results of MCF-7 cells transfected with the SUMO-deficient FOXM1 plasmid confirmed
that this mutant is in fact unable to be SUMOylated. As it is widely known, FOXM1 is active in the nucleus where it regulates the transcription of its downstream targets, while the inactive form of FOXM1 is normally located in the cytoplasm. The regulation of FOXM1 by SUMOylation was then investigated by transfection MCF-7 cells with different forms of FOXM1 to determine their sub-cellular localisation. The nuclear-cytoplasmic fractionation was performed using Ubc9-fused FOXM1 wt or 5x(K>R) transfected cells. The Ubc9-fusion directed SUMOylation system (UFDS) was used in order to bypass the requirement of SUMO E3 ligase enzymes (Jakobs et al., 2007). Only Ubc9-fused FOXM1 wt transfected cells showed SUMOylation in the cytoplasmic fraction, but not in the Ubc9-fused FOXM1 5x(K>R) transfected cells. The results from the protein fractionation showed that the SUMOylation of FOXM1 is most likely to occur in the cytoplasmic fraction rather than in the nuclear fraction. The results from confocal microscopy also confirmed that FOXM1 wt was found both in the nucleus and cytoplasm; however, FOXM1 5x(K>R) was only found in the nucleus. This suggested that SUMOylation might play a role in the nuclear export mechanism for FOXM1. To confirm the nuclear export of FOXM1, LMB was used to inhibit protein export in the cells (Jang et al., 2003; Wolff et al., 1997). MCF-7 cells were treated with LMB before the immunofluorescent staining has been performed to investigate the sub-cellular localisation of the endogenous FOXM1 protein. The immunofluorescent study showed that LMB was able to inhibit the nuclear export of FOXM1 to the cytoplasm (Jang et al., 2003; Wolff et al., 1997). All together, these data confirms that the SUMOylation of FOXM1 results in its nuclear exclusion. As mentioned before, cytoplasmic FOXM1 is normally associated with an inactive form of the protein that is unable to regulate gene
transcription. Therefore, I decided to investigate the effect of SUMOylation on the transactivational activity of FOXM1.

My observations are in agreement with various studies that SUMOylation targets various transcription factors to inhibit their functions. Some of these factors include, FOXA1 (Bao et al., 2011) and FOXC1/FOXC2 (Danciu et al., 2012). Previous reports have already shown that Cdh1 is required for the degradation of FOXM1 (Laoukili et al., 2008; Park et al., 2008). Accordingly, I also found that the degradation rate of FOXM1 is enhanced in cells overexpressing Cdh1, and when the transcription of FOXM1 is inhibited by CHX. On the other hand, in the absence of Cdh1, FOXM1 protein is found to be more stable. Cdh1 not only works as an adaptor for the interaction between FOXM1 and the APC/C complex, but is also reported to be essential for the ubiquitin-mediated proteolysis of FOXM1 (Li et al., 2012; Park et al., 2008). The FLIM-FRET results together with the co-IP data, confirm that SUMOylation occurs in MCF-7 cells in response to epirubicin treatment, between 4 to 6 h preceding the degradation of FOXM1.

Therefore, I hypothesised that SUMOylation is required for the Cdh1-mediated degradation of FOXM1. The results from the CHX treatment show that the FOXM1 degraded faster in the presence of SUMO1/Ubc9 overexpression and epirubicin. Moreover, when MCF-7 cells were transfected with FOXM1 wt or 5x(K>R) mutant with increasing amounts of Chd1, the higher molecular weight band associated with the ubiquitinated form of FOXM1 was only found in the FOXM1 wt transfected cells. This
indicates that ubiquitination-mediated degradation of FOXM1 cannot occur without SUMOylation.

This is consistent with other studies showing that Cdh1 has a crucial role in APC/C-dependent degradation via the ubiquitin-proteasome pathway (Cataldo et al., 2013; Hyun et al., 2013; Li et al., 2012). This further supports the idea that SUMOylation of FOXM1 leads to the degradation of FOXM1 via the ubiquitin-proteasome. To confirm this, MCF-7 cells were transfected with FOXM1 wt or 5x(K>R) before being treated with CHX. In cells overexpressing Cdh1, there was a markedly increase in the degradation rate of FOXM1 wt in comparison with empty vector transfected cells. Interestingly, the degradation rate of SUMOylation-deficient FOXM1 was unaffected by overexpression of Cdh1, indicating that SUMOylation is required for the Cdh1-mediated degradation of FOXM1.

Furthermore, the co-IP results of ubiquitin also confirm that there is an interaction with FOXM1 wt but not with the SUMOylation-deficient FOXM1. Surprisingly, Cdh1 can form a complex with FOXM1 5x(K>R). This suggests that SUMOylation is not required for the interaction between FOXM1 and Cdh1, but it is crucial for the ubiquitination of FOXM1.

Additionally, SUMOylation is also found to induce a decrease on the transactivation activity of FOXM1 since SUMOylation-deficient FOXM1 has a higher transactivation activity on the downstream targets of FOXM1, including cyclin B1 (Leung et al., 2001) and 6xForkhead response element (FHRE) promoters (6xDNA binding element: 6XDBE) (Furuyama et al., 2000; Laoukili et al., 2005). However, this transactivation activity is dependent on the FHREs since the cyclin B1 mutant3 (Cyclin B1 with
mutation of FHREs) promoter shows no difference between FOXM1 wt and 5x(K>R). Moreover, I also found that FOXM1 5x(K>R) overexpression has a positive effect on the cell survival rate when compared to FOXM1 wt. This evidence indicates that SUMOylation-deficient FOXM1 enables the cells to overcome the anti-proliferative effect of epirubicin more than the FOXM1 wt.

Taken together, my results confirm the importance of FOXM1 in epirubicin resistance and DNA damage response. In addition, I have shown that FOXM1 can be modified predominantly by SUMO1 in response to epirubicin treatment. However, it does not exclude the involvement of SUMO2/3 in the modification of FOXM1 and its activity. Moreover, SUMOylation plays an important role in the FOXM1 Cdh1-mediated degradation via the ubiquitin-proteasome pathway. I have also shown that the SUMOylation-deficient FOXM1 has a higher transactivation activity on the down-stream targets of FOXM1 (cyclin B1 and 6XDBE) and it enhances epirubicin resistance in MCF-7 cells. Therefore, this study shows that SUMOylation has a crucial role in the regulation of FOXM1 in terms of its degradation and inhibition of its function. According to my study, SUMOylation is an interesting candidate that drives the molecular switch that controls the role of FOXM1 in epirubicin-induced DNA damage response and could potentially have future clinical applications. Understanding more about the regulation of FOXM1 by SUMOylation might lead to the development of an inhibitor that promotes the degradation of FOXM1. Such inhibitor could have a therapeutic use in combination with epirubicin in order to disable the resistant-downstream target gene network that is under the control of FOXM1.
3.4 Future work

Although SUMOylation is linked with ubiquitination and degradation of FOXM1 by an APC-C/Cdh1 complex, SUMOylation does not mediate the interaction of FOXM1 with the complex. This suggests that, there might be another protein that acts as a mediator between them. According to various studies, SUMO-targeting ubiquitin ligases (STUbLs), such as RNF4 and RNF8, have a role in mediating the crosstalk between SUMOylation and ubiquitination. To further investigate the regulation of FOXM1, I would first study the role of E3 ubiquitin ligases such as RNF4, RNF8 and RNF168. These RING-type E3 ligases have been reported to mediate the DNA damage response (DDR) (Galanty et al., 2012; Mallette et al., 2012; Munoz et al., 2012; Nakada et al., 2010; Yin et al., 2012). Then, the regulation of FOXM1 by E3 ligases would be investigated. By overexpression and silencing of E3 ligases including RNF4, RNF8, and RNF168, FOXM1 expression and degradation would be assessed. Subsequently, I would determine the presence of an interaction between FOXM1 and the E3 ligases using co-IP. Once the interaction has been confirmed, one can use luciferase reporter assays to determine the effect of the E3 ligases on the transactivation activity of FOXM1. Finally, I will also investigate the importance of SUMOylation in the regulation of FOXM1 by E3 ligases. Using western blotting analysis, the degradation of FOXM1 wt and 5x(K>R) can be determined in the presence of E3 ligases and the interaction between E3 ligases and FOXM1 wt and 5x(K>R) would be defined.
CHAPTER 4

THE REGULATION OF FOXM1 BY RING-TYPE E3 LIGASES: RNF4 AND RNF168
4.1 Introduction and Objectives

Ubiquitin E3 ligase is widely known to be important for recognising ubiquitin substrates, in targeted proteins, and mediating ubiquitin transfer from a conjugating (E2) enzyme to these proteins (Deshaies and Joazeiro, 2009). There are two major classes of E3 ligase: HECT (homologous to the E6AP carboxyl terminus) and RING (Really interesting new gene) domain containing E3 ligases. HECT-type E3 works by first forming a covalent bond with ubiquitin residues which are transferred from E2 to the substrate protein (Metzger et al., 2012). In contrast, RING-type E3 ligases help to bring the ubiquitin-E2 complex and the substrate protein into close proximity to discharge the ubiquitin residue directly from the E2 enzyme to the substrate protein (Metzger et al., 2012; Ozkan et al., 2005; Rotin and Kumar, 2009).

FOXM1 plays a key role in DNA damage response (DDR) and epirubicin resistance in breast cancer (Halasi and Gartel, 2012; Koo et al., 2012; Millour et al., 2011; Monteiro et al., 2013; Park et al., 2012; Wonsey and Follettie, 2005). In the previous chapter, I have found that SUMOylation of FOXM1 leads to its degradation via an APC/Cdh1 complex (Chapter 3). However, the APC/Cdh1 complex does not mediate this process directly. This suggests that there are other E3 ligases that might be involved in this proteasome degradation pathway. Previous studies reported that RING-type E3 ligases, including RNF4, RNF8, RNA168 and BRCA1, are involved in DDR (Galanty et al., 2012; Mallette et al., 2012; Oestergaard et al., 2012; Wu et al., 2010; Yin et al., 2012). The role of RING-type E3 ligase is commonly defined by a RING domain that interacts with the ubiquitin-E2 complex (Deshaies and Joazeiro, 2009). Mutations on this RING
domain of RING-type E3 ligases (by point mutations from a zinc-coordinating cysteine residue to a serine residue - CS mutation) lead to a catalytically inactive form of this ligase (Ahner et al., 2013; Luo et al., 2012; Tatham et al., 2008). This mutation results in the lack of ability of E3 ligase to ubiquitinate its substrate proteins.

In this study, I intended to investigate one of the potential candidate E3 ligases, RNF4, RNF8 and RNF168, that might be involved in the regulation of FOXM1. In my previous chapter, I have concluded that it is possible that following SUMOylation, FOXM1 is ubiquitinated. This ubiquitination could be mediated by one or more of these E3 ligases before the degradation of FOXM1. Therefore, I decided to identify which E3 ligase is involved in the degradation of FOXM1. The identification of this E3 ligase could prove useful to develop new and improved cancer therapeutics targeting FOXM1 and its role in promoting drug resistance.
4.2 Results

4.2.1 Evaluation of the expression levels of RNF4, RNF8 and RNF168 in MCF-7 and MCF-7 EpiR in response to epirubicin treatment

E3 ligases including RNF4, RNF8 and RNF168 have been reported to be involved in promoting ubiquitination of targeted proteins, leading to their degradation in response to genotoxic stresses (Galanty et al., 2012; Mallette et al., 2012; Oestergaard et al., 2012; Yin et al., 2012). In order to assess the expression of RNFs in response to epirubicin treatment, MCF-7 and MCF-7 EpiR cells were treated with epirubicin at different times (0, 4, 8, 16, 24, and 48 h). The protein lysates and mRNA were then extracted from these cells. Western blotting and qPCR were used to study the expression levels of protein (Figure 4.1) and mRNA (Figure 4.2), respectively. The RNF4 expression levels in MCF-7 and MCF-7 EpiR cells were found to correlate with FOXM1. In MCF-7 cells, RNF4 and FOXM1 levels decreased after epirubicin treatment but this was not the case in MCF-7 EpiR cells. For RNF8, the result showed no significant change in both MCF-7 and EpiR cells, with and without epirubicin treatment. Interestingly, in MCF-7 cells the protein expression levels of RNF168 were found to be more highly expressed than in MCF-7 EpiR cells. Moreover, the mRNA levels of RNF 168 in MCF-7 cells were significantly increased after epirubicin treatment, especially after 16 h.
Figure 4.1 Western blot analysis of FOXM1, RNF4, RNF8 and RNF168 in MCF-7 and MCF-7 Epi\textsuperscript{R} cells following epirubicin treatment. MCF-7 and MCF-7 Epi\textsuperscript{R} cells were treated with 1µM epirubicin for 0, 4, 8, 16, 24 and 48 h. Protein lysate was extracted and analysed by western blotting. The membrane was probed with antibodies against FOXM1, RNF4, RNF8 and RNF168. β-tubulin served as a loading control.
Figure 4.2 RNF4, RNF8 and RNF168 mRNA expression levels in MCF-7 and MCF-7 EpiR in response to epirubicin treatment. The transcript levels of RNF4, RNF8 and RNF168 from MCF-7 and MCF-7 EpiR cells were analysed by qPCR. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.005; **, ≤ 0.001 significant; ns, non-significant)
4.2.2 The expression levels of RNF4, RNF8 and RNF168 affect FOXM1 protein levels

Next, I examined the relationship between the different RNFs and FOXM1. In order to further examine the potential regulation of FOXM1 by RNFs, exogenous RNFs expression plasmids were transfected into MCF-7 cells. After 24 h of transfection, the cells were harvested and analysed for western blotting. The overexpression results showed that FOXM1 protein levels were dramatically upregulated when MCF-7 cells were overexpressed with RNF4 (pcDNA3-flag-RNF4), when compared with the empty vector control (pcDNA3). In contrast, MCF-7 cells with RNF168 overexpression (pcDNA3-flag-RNF168) showed a decrease in the protein levels of FOXM1 when compared with the control (pcDNA3). Notably, in the cells transfected with RNF8 (pcDNA3-HA-RNF8) there was a discrete decrease in the FOXM1 protein levels when compared with the control (Figure 4.3). These data might indicate that RNF4 has a role in enhancing the expression of FOXM1, while RNF168 has a role in suppressing its expression. Unfortunately, RNF8 was not found to have an effect on FOXM1 protein expression, displaying a similar trend as RNF168.
Figure 4.3 Effects of RNF4, RNF8 and RNF168 overexpression on FOXM1 in MCF-7 cells. A. MCF-7 cells were transfected with RNF4, RNF8, RNF168 or pcDNA3 empty vector control. Whole protein lysates were extracted and used for western blotting analysis. The immunoblot membranes were probed with antibodies against FOXM1, RNF4, RNF8, RNF168, and β-tubulin. Asterisks indicate flag-RNF4, HA-RNF8 and flag-RNF168, respectively. B. Densitometry of FOXM1 levels were quantified by Image J and normalised to β-tubulin.
Conversely, I also performed the knock-down experiment to confirm the above results. To this end, MCF-7 cells were transfected with specific siRNA against RNFs (siRNF4, siRNF8, siRNF168) or the non specific control (NSC). The following day, the cells were harvested and analysed by western blotting. In the RNF4 silenced cells, there was a down-regulation of FOXM1 protein expression levels, while in the RNF168 knock-down cells a slight upregulation of FOXM1 expression was observed when compared with the NSC control. Unfortunately, the knock-down of RNF8 did not show to increase the levels of FOXM1 (Figure 4.4). These results also confirm the overexpression data in which FOXM1 protein levels can be upregulated by RNF4 and suppressed by RNF168. However, the effect of RNF8 on FOXM1 protein levels remains unclear.
Figure 4.4 Effects of RNF4, RNF8 and RNF168 knock-down on FOXM1 expression in MCF-7 cells. A. MCF-7 cells were transfected with siRNF4, siRNF8, siRNF168, or NSC. The immunoblot membranes were probed with antibodies against FOXM1, RNF4, RNF8, RNF168, and β-tubulin B. Densitometry of FOXM1 levels were quantified by Image J and normalised to β-tubulin
4.2.3 Expression levels of RNF4 and RNF168 affect cyclin B1 transcription

I next examined the effects of the three potential RING-type E3 ligases over the transactivation activity of FOXM1. MCF-7 cells were transiently co-transfected with increasing amounts of different RING-type E3 expression vectors and a luciferase reporter containing the wild-type cyclin B1 promoter. The result showed that RNF4 promoted the transcription activity of cyclin B1, a downstream transcriptional target of FOXM1, while RNF168 repressed its activity. By contrast, RNF8 and BRCA1 expression had no significant effects over the activity of cyclin B1 promoter (Figure 4.5). Collectively, these data suggests that RNF4 and RNF168 might work as upstream regulators of FOXM1, while the role of RNF8 remains unclear.
Figure 4.5 RNF4 overexpression promotes the transcription of cyclin B1 promoter while RNF168 overexpression suppresses its transcription activity. A. MCF-7 cells were co-transfected with increasing amounts (0-30ng) of different RING-type E3s, including RNF4, RNF8, RNF168 or BRCA1 (DNA concentrations were normalised to empty vector control), and luciferase reporters containing the wild-type cyclin B1 promoter. The Luciferase activity was measured by PHERAstar Plus microplate reader. Reporter gene activity was normalised to the Renilla control activity. Graphs represent the mean of 3 independent experiments ±SEM Statistical analysis was performed using Student’s t-test compared with 0ng of RING-type E3 transfection. (**, p ≤ 0.01 significant; ***, p ≤ 0.001 significant; ns, non-significant)
4.2.4 Overexpression of RNF4 promotes FOXM1 expression while a knock-down of RNF4 suppresses FOXM1 expression

According to the previous results (Section 4.2.2 and 4.2.3), RNF4 has been shown to be able to promote FOXM1 expression and its transactivation activity. To further investigate the influence of RNF4 expression over FOXM1, MCF-7 cells were transfected with pcDNA3-flag empty vector or pcDNA3-flag-RNF4 for 24 h before treatment with 1 µM epirubicin. After 24 h of treatment, the cells were harvested for western blotting and qPCR analyses. The results, for both protein and mRNA, showed an upregulation of FOXM1 in RNF4-overexpressing MCF-7 cells when compared with the control, in both the absence and the presence of epirubicin. Interestingly, FOXM1 expression levels remained elevated even after 24 h of epirubicin treatment (Figure 4.6). Conversely, MCF-7 cells with a depletion of RNF4 (siRNF4) exhibited a downregulation of FOXM1 expression both with and without epirubicin treatment (Figure 4.7). Taken together, these data indicate that RNF4 might have a role in promoting the expression of FOXM1.
Figure 4.6 Effects of RNF4 overexpression on FOXM1 in MCF-7 cells in a response to epirubicin treatment. MCF-7 cells were transfected with siRNF4, or the NSC control. The cells were harvested after 0, or 24 h of epirubicin treatment. A. The cell lysates were analysed by western blotting. The asterisk indicates flag-RNF4. The arrow indicates the endogenous levels of RNF4. B. The mRNA from the cells was collected and the expression levels of RNF4 and FOXM1 were analysed using qPCR. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05; **, ≤ 0.01 significant; ns, non-significant).
Figure 4.7 Effects of RNF4 knock-down on FOXM1 expression in MCF-7 cells in response to epirubicin treatment. MCF-7 cells were transfected with siRNF4, or the NSC control. The cells were harvested after 0, or 24 h of epirubicin treatment. A. The cell lysates were analysed by western blotting. B. The mRNA from the cells was collected and the expression levels of RNF4 and FOXM1 were analysed using qPCR. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05; **, ≤ 0.01 significant).
4.2.5 RNF4 promotes the growth of MCF-7 cells, but fails to re-sensitise these cells to epirubicin treatment

Various studies reported that FOXM1 promotes epirubicin resistance (Khongkow et al., 2013; Monteiro et al., 2013). In previous experiment, I found that overexpression of RNF4 has a role in increasing the expression levels of FOXM1 (Figure 4.6 and 4.7). I therefore hypothesised that RNF4 might also affect the sensitivity of the cells to epirubicin. To this end, MCF-7 cells were transfected with pcDNA3-flag-RNF4 (RNF4) or pcDNA3-flag empty vector control (pcDNA3) for 24 h before SRB assays were performed in the presence of various indicated concentrations of epirubicin (0 µM – 20 µM). The percentages of the cell survival and cell growth were quantified. Surprisingly, the result showed no differences in the percentages of cell survival between RNF4 overexpressing cells and the control, after 24 h of epirubicin treatment (Figure 4.8A). Conversely, I knocked down RNF4 in MCF-7 cells and performed similar SRB assays. The results showed that in RNF4 depleted cells there was no difference in the percentages of cell survival compared to the NSC (Figure 4.9A).

Interestingly, although the expression of RNF4 was unable to change the sensitivity of MCF-7 cells to epirubicin, it affected the ability of the cells to proliferate. The SRB result revealed that, in the absence of epirubicin, the overexpression of RNF4 leads to an increase in the growth rate, while the depletion of RNF4 leads to a slower growth rate when compared with the corresponding controls (Figure 4.8B and 4.9B). In summary, the expression of RNF4 did not affect the sensitivity of MCF-7 cells to epirubicin treatment instead it only promoted their cell growth.
Figure 4.8 RNF4 overexpression does not affect the sensitivity of MCF-7 cells to epirubicin nor cell growth. MCF-7 cells were transfected with pcDNA3-RNF4 or pcDNA3 empty vector control. The cells were then treated with various concentrations of epirubicin 24 h before the cell proliferation was analysed by SRB assays. The results were normalised to the untreated conditions (0μM concentration). A. The percentages of survival and B. the percentages of cell growth were plotted. Results represent the average of 6 replicates ± SD. Statistical analysis was performed using Student’s t-test (***, ≤ 0.001 significant; ns, non-significant).
Figure 4.9 RNF4 knock-down does not affect the sensitivity of MCF-7 cells to epirubicin but reduces cell growth. MCF-7 cells were transfected with siRNF4, or NSC. The cells were treated with various concentrations of epirubicin 24 h before the cell proliferation was identified by an SRB assay. A. The percentages of cell growth and B. the percentages of cell growth were plotted. The result was normalised to an untreated condition (0 μM concentration). Results represent the average of 6 replicates ± SD. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05; **, ≤ 0.001 significant; ns, non-significant).
4.2.6 RNF4 promotes long-term cell proliferation in MCF-7 cells

To further study the role of RNF4 on long-term cell proliferation, RNF4 was overexpressed or knocked down in MCF-7 cells. The transfected cells were then reseeded into 6-well plates with a very low cell number (500 cells/well) for colony formation assays. The ability of cells to proliferate was gauged by crystal violet staining to define the role of RNF4 in long-term growth control. The result showed that overexpression of RNF4 significantly promoted long-term cell proliferation (Figure 4.10). Conversely, the RNF4 depleted cells revealed significantly lower rates of the cell proliferation when compared with the NSC control (Figure 4.11). Interestingly, both for short and long-term growth rates were extremely low when RNF4 was depleted from the cells. The SRB assay revealed no growth from RNF4-knocked down MCF-7 cells (Figure 4.9B) and less than 1% proliferation in 15 days of a long-term assay. This suggests that RNF4 has a crucial role in promoting long-term cell proliferation in MCF-7 cells.
Figure 4.10 RNF4 overexpression showed no effect over long-term cell growth. MCF-7 cells were transfected with pcDNA3-RNF4 or pcDNA3 empty vector control. A. The transfected cells were reseeded in 6-well-plate at a very low concentration (500 cells per well) for clonogenic assay. The cells were harvested every 2 days after seeding. B. The percentage of cell proliferation was measured using crystal violet staining. Results represent the average of triplicates ± SD. Statistical analysis was performed using Student’s t-test (***, ≤ 0.001 significant; ns, non-significant).
Figure 4.11 RNF4 knock-down showed a reduction in long-term cell growth. MCF-7 cells were transfected with siRNF4, or NSC. A. The transfected cells were reseeded in 6-well-plates at a very low concentration (500 cells per well) for clonogenic assay. The cells were harvested every 2 days after seeding. B. The percentages of cell proliferation were measured using crystal violet staining. Results represent the average of triplicates ± SD. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ***, p ≤ 0.001 significant; ns, non-significant).
4.2.7 The regulation of cyclin B1 transcription activity by RNF4 partially depends on the RING-domain of RNF4

Since RNF4 had an effect on the expression of FOXM1, I hypothesised that it might also have a role in regulating the transcription of cyclin B1 by modulating the transactivation activity of FOXM1. The result from my previous experiment confirmed that RNF4 has a role in promoting cyclin B1 promoter, a downstream transcriptional target of FOXM1 (Figure 4.5). Therefore, I investigated whether the regulation of cyclin B1 transcription activity depends on the forkhead response elements (FHREs). To address this, MCF-7 cells were co-transfected with increasing amounts (0 – 30 ng) of Flag-RNF4 wt and a luciferase reporters containing a wild-type cyclin B1 promoter or a mutant cyclin B1 mut3 promoter containing both the forkhead binding sites mutated. DNA concentration were normalised to the pcDNA3 flag empty vector control. The reporter gene activity was normalised to the activity of a Renilla control. Surprisingly, the result showed an upregulation of a cyclin B1 promoter activity in both wt and mutant3 transfection (Figure 4.12). This result indicates that the same upregulation of the transcriptional activity of cyclin B1 promoter partially relies on the presence of FHREs. This also indicates that the induction of cyclin B1 promoter by RNF4 does not require FHREs as well as other transcription factor-binding sites.

I also studied the importance of the RING-domain of RNF4 in promoting the transcriptional activity of cyclin B1 promoter. To test that, RNF4 CS mutation was used. This is a mutation from cysteine into serine residues in the RING finger domain. It destroys the RING domain of RNF4 and abolishes the ability of RNF4 to function
properly (Ahner et al., 2013; Luo et al., 2012; Pero et al., 2001). Firstly, MCF-7 cells were co-transfected with increasing amounts (0 – 30 ng) of RNF4 wt or RNF4 with CS mutations, and the wt cyclin B1 promoter reporter. The following day, luciferase assay was performed and the result was normalised to the activity of the Renilla control. Remarkably, increasing levels of RNF4 CS mutation transfection showed the same levels of cyclin B1 promoter transcriptional activity (Figure 4.13). This indicates that the RING-domain of RNF4 might have a crucial role in regulating cyclin B1 transcriptional activity as the mutation of RNF4 could not promote the transcriptional activity of cyclin B1 promoter.
Figure 4.12 The effect of RNF4 over the transcription of cyclin B1 partially depends on forkhead response elements (FHREs). MCF-7 cells were co-transfected with increasing amount (0 - 30 ng) of flag-RNF4 wt (DNA concentration was normalised by empty vector control), and luciferase reporters containing the wild-type cyclin B1 promoter or cyclin B1 mut3 promoter. The Luciferase activity was measured by PHERAstar Plus microplate reader. Reporter gene activity was normalised to the Renilla control activity. Graphs represent the mean of 3 independent experiments ±SEM. Statistical analysis was performed using Student’s t-test (ns, non-significant).
Figure 4.13 The RING domain of RNF4 is important for promoting a transcription of cyclin B1. MCF-7 cells were co-transfected with increasing amount (0 - 30 ng) of RNF4 wt or RNF4 with (CS1) mutation of RING domain (DNA concentration was normalised by empty vector control), and luciferase reporters containing wild-type cyclin B1 promoter. The Luciferase activity was measured by PHERAstar Plus microplate reader. Reporter gene activity was normalised to the Renilla control activity. Graphs represent the mean of 3 independent experiments ±SEM. Statistical analysis was performed using Student’s t-test compared between wt and CS1 mutation at the same amount of DNA transfection. (***, p ≤ 0.001 significant; ns, non-significant)
4.2.8 RNF4 does not delay the degradation rate of FOXM1 in response to epirubicin treatment

Previously, RNF4 was found to promote the expression of FOXM1. Moreover, MCF-7 cells overexpressing RNF4 showed higher FOXM1 protein levels after 24 h epirubicin treatment compared to the pcDNA3-flag empty vector control transfected cells (Figure 4.3). I then hypothesised that the overexpression of RNF4 might also delay the degradation of FOXM1. In order to test this hypothesis, the degradation rate of FOXM1 was determined after cycloheximide (CHX) treatment in combination with epirubicin. MCF-7 cells were transfected with flag-RNF4 or an empty vector control. The following day, the cells were treated with 1 μM epirubicin for 2 h prior to 100 μM cycloheximide treatment at indicated time points (0, 1, 2, 4, 6 and 8 h). The cells were harvested for the protein extraction. The FOXM1 protein expression was examined by western blotting. Unexpectedly, the result revealed no significant difference between the degradation rate of FOXM1 in cells with and without overexpression of RNF4 (Figure 4.14).

In addition, a similar experiment was performed using MCF-7 cells transfected with siRNA smart pool targeting RNF4 (siRNF4) or non-target siRNA control (NSC). This result also showed no noticeable differences in the degradation rate of FOXM1 between RNF4 depleted cells and the NSC control (Figure 4.15). These data show that RNF4 does not play a direct role in the degradation of FOXM1.
Figure 4.14 Overexpression of RNF4 has no significant effect on the degradation of FOXM1. A. MCF-7 cells were transfected with RNF4 or empty vector control (pcDNA3). Then, cells were treated with 1 μM epirubicin 2 h prior to 100 μM cycloheximide (CHX) treatment at several time points (0, 1, 2, 4, 6 and 8 h). Densitometry was used to quantify the FOXM1 level from western blot using imageJ. β-tubulin was used for normalising. B. Quantitative FOXM1 level was normalised relative to 0 h CHX treatment. Graphs represent the mean of 3 independent experiments ±SD. Statistical analysis was performed using Student’s t-test compared between pcDNA3 empty vector and RNF4 overexpression at the same time point (ns, non-significant). The asterisk indicates an endogenous RNF4.
Figure 4.15 depletion of RNF4 has no significant effect over the degradation of FOXM1. A. MCF-7 cells were transfected with siRNF4 NSC. Then, cells were treated with 1 μM epirubicin 2 h prior to 100 μM cycloheximide (CHX) treatment at several time points (0, 1, 2, 4, 6 and 8 h). Densitometry was used to quantify the FOXM1 level from western blot using ImageJ. β-tubulin was used for normalisation. B. Quantitative FOXM1 level was normalised relative to 0 h CHX treatment. Graphs represent the mean of 3 independent experiments ±SD. Statistical analysis was performed using Student's t-test compared between pcDNA3 empty vector and RNF4 overexpression at the same time point. (ns, non-significant)
4.2.9 RNF168 suppresses FOXM1 expression

In contrast to RNF4, RNF168 was found to down regulate the expression of FOXM1 (Figure 4.3 and 4.4). To further investigate the regulation of FOXM1 by RNF168, MCF-7 cells were transfected with pcDNA3-flag-RNF168 (RNF168) or pcDNA3-flag empty vector control (pcDNA3) before treatment with 1 μM epirubicin and 24 h. The following day, cells were harvested for western blotting and qPCR analyses. The western blot confirmed that RNF168 overexpression was successful. The result showed the down regulation of FOXM1 protein both before and after epirubicin treatment in RNF168 overexpressed cells, compared with the pcDNA3 transfected control (Figure 4.16A). The qPCR result showed a small reduction on the FOXM1 mRNA levels in the absence of epirubicin while it showed no differences after 24 h of epirubicin treatment (Figure 4.16B). This indicates that RNF168 might down-regulate FOXM1 at post translational levels.

Next, I performed the siRNA experiment to confirm the overexpression results. MCF-7 cells were transfected with siRNF168 and NSC to investigate the effects at protein and mRNA levels of FOXM1 in an RNF168 depleted environment. After 24 h of transfection, the cells were harvested and analysed by western blotting and qPCR. RNF168 depleted MCF-7 cells show a significant higher FOXM1 protein levels in both before and after 24 h of epirubicin treatment. However FOXM1 mRNA levels were found to be more stable in RNF168 silenced cells before and after 24 h of epirubicin treatment (Figure 4.17). This indicates that RNF168 depletion might stabilise FOXM1 protein levels, especially in the presence of epirubicin.
Figure 4.16 Effect of RNF168 overexpression over FOXM1 in MCF-7 cells in a response to epirubicin treatment. MCF-7 cells were transfected with pcDNA3-flag-RNF168 (RNF168), or the pcDNA3-flag empty vector control (pcDNA3). The asterisk indicates pcDNA3-flag-RNF168. The arrow indicates endogenous RNF168. The cells were harvested after 0, or 24 h of epirubicin treatment. A. The cell lysates were analysed by western blotting. B. The mRNA from the cells was collected and the expression levels of RNF168 and FOXM1 were analysed using qPCR. Statistical analysis was performed using Student’s t-test. (*,p ≤ 0.05; ns, non-significant).
Figure 4.17 Effect of RNF168 knock-down on FOXM1 in MCF-7 cells in a response to epirubicin treatment. A. MCF-7 cells were transfected with siRNF168, or the NSC control. The cells were harvested after 0, or 24 h of epirubicin treatment. A. The cell lysates were analysed by western blotting. B. The mRNA from the cells was collected and the expression levels of RNF168 and FOXM1 were analysed using qPCR. Statistical analysis was performed using Student’s t-test. (ns, non-significant).
4.2.10 RNF168 sensitises MCF-7 cells to epirubicin treatment

My previous experiment indicated that RNF168 represses FOXM1 protein expression levels. Hence, RNF168 was hypothesised to increase the sensitivity of MCF-7 cells to epirubicin treatment. To test this, MCF-7 cells were transfected with pcDNA3-flag-RNF168 (RNF168) and pcDNA3-flag empty vector control (pcDNA3). The transfected cells were subjected to an SRB assay with a range of concentrations of epirubicin treatment (0 – 20 µM). The percentages of cell survival were measured and quantified. As expected, the result from the RNF168-overexpressed cells exhibited extremely significant low percentages of cell survival after treatment with epirubicin for 24 h (Figure 4.18A). Conversely, I also performed the siRNA experiment to confirm the effect of RNF168 on epirubicin resistance and sensitivity. MCF-7 cells were transfected with siRNF168 and NSC before performing an SRB assay with a various concentrations of epirubicin (0 – 20 µM). The depletion of RNF168 increased the percentages of cell survival in MCF-7 cells (Figure 4.19A). Additionally the result indicated that RNF168 does not change the growth rate of MCF-7 in the absence of epirubicin (Figure 4.18B and 4.19B). All together, the results indicate that RNF168 has a role in increasing the sensitivity of MCF-7 cells to epirubicin, but not their cell proliferation capacity.
Figure 4.18 RNF168 overexpression promotes sensitivity of MCF-7 cells to epirubicin but does not affect cell growth. MCF-7 cells were transfected with pcDNA3-RNF168 or pcDNA3 empty vector control. The cells were treated with various concentrations of epirubicin 24 h before the cell proliferation was identified by an SRB assay. The result was normalised to the untreated condition (0 μM concentration). A. The percentage of cell survival and B. the percentages of cell growth were plotted. Results represent the average of 6 replicates ± SD. Statistical analysis was performed using Student's t-test (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ***, p ≤ 0.001 significant; ns, non-significant).
Figure 4.19 RNF168 depletion reduces the sensitivity of MCF-7 cells to epirubicin but does not affect cell growth. MCF-7 cells were transfected with siRNF168, or NSC. The cells were treated with various concentrations of epirubicin for 48 h before the cell proliferation was analysed by SRB assay. A. The percentage of cell growth and B. the percentages of cell growth were plotted. The result was normalised to the untreated condition (0 μM concentration). Results represent the average of 6 replicates ± SD. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ***, p ≤ 0.001 significant; ns, non-significant).
4.2.11 RNF168 does not play a crucial role in controlling long-term cell proliferation in MCF-7 cells

To emphasise that RNF168 is not responsible for cell proliferation ability, MCF-7 cells expressing flag-RNF168 or pcDNA3 empty vector were used for clonogenic experiments. The transfected cells were seeded into 6-well plates with a very low number of cells. Cell proliferation was determined using crystal violet staining. Consistently, the results showed no significant difference in the percentage of long-term cell proliferation (Figure 4.20). Similarly, the result from RNF168 depleted cells also showed the same growth rate as the NSC conditions (Figure 4.21). These data are consistent with the SRB assay result (Figure 4.18B and 4.19B). It can be assumed that RNF168 has no distinguishable effects on both short-term and long-term cell proliferation of MCF-7 cells.
Figure 4.20 RNF168 overexpression showed no effects over cell growth in long-term experiment. MCF-7 cells were transfected with pcDNA3-RNF168 or pcDNA3 empty vector control. The transfected cells were seeded in 6-well-plate at very low concentrations (i.e. 500 cells per well) for clonogenic assays. The cells were harvested every 2 days after being seeded. A. The cell proliferation was measured using crystal violet staining. B. The percentages of cell proliferation curve were plotted. The result represents the average of triplicates ± SD. Statistical analysis was performed using Student’s t-test (ns, non-significant).
Figure 4.21 RNF168 knock-down showed no effects over cell growth in long-term experiment. MCF-7 cells were transfected with siRNF168, or NSC. The transfected cells were re-seeded in 6-well-plate at a very low concentration (500 cells per well) for clonogenic assays. The cells were harvested every 2 days after being seeded. A. The cell proliferation was measured using crystal violet staining. B. The percentages of cell proliferation curve were plotted. The result represents the average of triplicates ± SD. Statistical analysis was performed using Student’s t-test (ns, non-significant).
4.2.12 The Forkhead binding sites of cyclin B1 are important for the transcription activity regulated by RNF168

It is widely known that cyclin B1 is a downstream target of FOXM1 (Leung et al., 2001). Since I have shown that RNF168 has a role in suppressing cyclin B1 transcriptional activity, it was necessary to examine whether RNF168 regulates the transactivation activity of FOXM1 over cyclin B1 promoter. To this end, I used the cyclin B1 mut3 promoter (containing mutations in the three consensus forkhead binding sites) for luciferase assays. First, MCF-7 cells were co-transfected with increasing amount (0-50ng) of pcDNA3-flag-RNF4 wt, and luciferase reporters, wt cyclin B1 promoter or cyclin B1 mut3 promoter. The activity of the reporter gene was normalised to the activity of the Renilla control. The result showed significantly lower transactivation activity of cyclin B1 wt promoter compared with that of the cyclin B1 mut3 promoter (Figure 4.22). This result provides evidence that the regulation of cyclin B1 transcription by RNF168 requires the consensus forkhead binding sites. In the case of the wild type forkhead binding sites transfection, RNF168 might regulate the cyclin B1 promoter though FOXM1 that binds to it. By mutating the forkhead binding sites, FOXM1 could no longer bind to the cyclin B1 promoter and this affected the transcription of cyclin B1 promoter by RNF168.
Figure 4.22 RNF168 overexpression promotes the transactivation activity of the cyclin B1 wt promoter but not the cyclin B1 mutant promoter (cyclin B1 mut3). MCF-7 cells were co-transfected with increasing amounts (0-50ng) of RNF168 (DNA concentration was normalised by empty vector control), and luciferase reporters containing wild-type cyclin B1 promoter, or cyclin B1 mut3 promoter containing the three mutant consensus forkhead binding sites. Luciferase assay was then performed after 24 h following transfection. The luciferase activity was measured by PHERAstar Plus microplate reader. Reporter gene activity was normalised to the Renilla control activity. Graphs represent the mean of 3 independent experiments ±SEM. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ***, p ≤ 0.001 significant)
4.2.13 RNF168 promotes the degradation of FOXM1 in response to epirubicin treatment

The previous result showed the importance of RNF168 in suppressing FOXM1 protein expression levels, especially after epirubicin treatment (Figure 4.16 and 4.17). Therefore, an experiment was then performed to determine whether the expression of RNF168 promotes the degradation of FOXM1 in the presence of epirubicin. First, MCF-7 cells were overexpressed with pcDNA3-flag-RNF168 (RNF168) or pcDNA3-flag empty vector control (pcDNA3). The cells were treated with 1 μM epirubicin for 2 h before being treated with 100 μM CHX. The cells were then harvested at various time points indicated (0, 1, 2, 4, 6, 8 h) for western blotting analysis. The degradation rate of FOXM1 was determined by densitometry from the western blot. The result displayed a dramatically faster degradation rate of FOXM1 in MCF-7 transfected with flag-RNF168, when compared with the control (Figure 4.23). A similar experiment was also performed in MCF-7 cells transfected with siRNA smart pool targeting RNF168 (siRNF168). The result was similar: the FOXM1 protein in RNF168 depleted cells was found to have a slower degradation rate than the NSC control (Figure 4.24).
Figure 4.23 Overexpression of RNF168 accelerates FOXM1 degradation in the presence of epirubicin. A. MCF-7 cells were transfected with RNF168 or empty vector control. Then, cells were treated with 2 h 1 μM epirubicin prior to 100 μM cycloheximide (CHX) treatment for several time points indicated (0, 2, 4, 6, and 8 h). Densitometry was used to quantify the FOXM1 level from western blot using imageJ. β-tubulin was used for normalising. B. Quantitative FOXM1 levels were normalised relative to 0 h CHX treatment. Graphs represent the mean of 3 independent experiments ±SD. Statistical analysis was performed using Student’s t-test compared between pcDNA3 empty vector and RNF168 overexpression at the same time point. (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ns, non-significant)
Figure 4.24 RNF168 knock-down has a slight but significant effect on FOXM1 degradation in the presence of epirubicin. A. MCF-7 cells were transfected with siRNF168, or NSC. The cells were then treated with 2 h of 1 μM epirubicin before adding 100 μM of cycloheximide (CHX). Cells were collected at several time points indicated (0, 2, 4, 6, and 8 h). Densitometry was used to quantify the FOXM1 levels from western blot using imageJ. β-tubulin was used for normalisation. B. Quantitative FOXM1 level was normalised relative to 0 h CHX treatment. The graphs represent the mean of 3 independent experiments ±SD. Statistical analysis was carried out using Student’s t-test comparing pcDNA3 empty vector and RNF168 overexpressed cells at the same time point. (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ns, non-significant)
Next, the effect of epirubicin in the regulation of FOXM1 degradation by RNF168 was investigated. To test that, I transfected MCF-7 cells with the flag-RNF168 (RNF168) or pcDNA3-flag empty vector control (pcDNA3), then treated them with 100 µM CHX, without the presence of epirubicin. The cells were then collected at various time points (0, 1, 2, 4, 6 and 8 h), without epirubicin for western blot analysis. Consistently, the FOXM1 protein from the flag-RNF168 transfected MCF-7 cells degraded faster when compared with the pcDNA3 control transfected cells (Figure 4.25). However, the difference was not as obvious as for the experiment performed in the presence of epirubicin (Figure 4.23 and 4.34). This might indicate that RNF168 promotes the degradation of FOXM1 specifically in the presence of epirubicin.
Figure 4.25 Overexpression of RNF168 slightly accelerates FOXM1 degradation in the absence of epirubicin. A. MCF-7 cells were transfected with RNF168 or empty vector control. Then, cells were treated with 2 h 1 μM epirubicin prior to adding 100 μM of CHX. Cells were collected at several time points (0, 2, 4, 6 and 8 h). Densitometry was used to quantify the FOXM1 levels from western blot using imageJ. β-tubulin was used for normalisation. The arrow indicates the endogenous levels of RNF168. The asterisk indicates the pcDNA3-flag-RNF168. B. Quantitative FOXM1 levels were normalised to the 0 h time point of CHX treatment.
4.2.14 FOXM1 interacts with RNF168 but not with RNF4

To confirm the interactions between RNF4, RNF168 and FOXM1, I performed co-immunoprecipitation (co-IP) experiments using MCF-7 cells treated with 1 μM epirubicin for 0, 6 and 24 h. Initially, antibodies against FOXM1 were used to perform the pull-down experiment of the FOXM1-containing complexes. Input and eluent were analysed by western blotting. The interaction between FOXM1 and RNF4 or RNF168 was assessed using antibodies against RNF4, RNF168 and FOXM1 (for affinity checking). Only the band corresponding to RNF168, but not RNF4, was detected both before and after epirubicin treatment. FOXM1 was found to be degraded after 24 h of epirubicin treatment (Figure 4.26). Interestingly, the interaction between FOXM1 and RNF168 was found to increase at 24 h of epirubicin treatment as the RNF168 band was stronger at this time point (see the long exposure of RNF168 in Figure 4.26). This indicates that FOXM1 interacts with RNF168. The interaction is also increased after a longer time of treatment with epirubicin and this interaction might culminate with the initiation of the degradation of FOXM1.

The reverse co-IP was also performed using antibodies against RNF168 to pull-down the RNF168 complexes after the MCF-7 cells were treated with epirubicin at indicated time points (0, 6, and 24 h). Input and eluent were analysed by western blotting. Similarly, the result revealed a stronger intensity for the FOXM1 band at the conditions of 0 h and 6 h of 1 μM epirubicin treatment, and a faint band at 24 h (Figure 4.27). At 24 h treatment, there was a decrease in the intensity of the FOXM1 band. This might be due to cells that start undergoing cell death mediated by the epirubicin treatment.
leading to the global protein degradation. In addition, no RNF4 band was found. This indicates that RNF4 does not interact with RNF168. This evidence emphasised a direct interaction between RNF168 and FOXM1 and also that FOXM1 is degraded after 24h of epirubicin treatment.

Additionally, I carried out the co-IP experiments using RNF4 antibodies after the MCF-7 cells were treated with epirubicin at indicated time points (0, 6, and 24 h). Input and eluent were analysed by western blotting. Consistently, neither FOXM1 nor RNF168 were detected on the subsequent western blot analysis (Figure 4.28). This result further confirmed that RNF4 does not form complexes with neither FOXM1 nor RNF168.
Figure 4.26 Co-immunoprecipitation (co-IP) experiments using FOXM1 antibodies show an interaction of FOXM1 with RNF168 but not RNF4. MCF-7 cells were treated with 1 μM epirubicin for 0, 6 and 24 h. Antibodies against FOXM1 were used to perform co-immunoprecipitation. Proteins precipitated and inputs were analysed by western blotting and probed for RNF168, RNF4 and FOXM1. The asterisk indicates IgG bands. Arrows indicate RNF168 or RNF4. Input represents 1:10 dilution of pull down and IgG was used as a negative control.
Figure 4.27 Co-immunoprecipitation (co-IP) experiments using RNF168 antibodies demonstrate an interaction of RNF168 with FOXM1 but not RNF4. MCF-7 cells were treated with 1 μM epirubicin for 0, 6 and 24 h. Antibodies against FOXM1 were used to perform co-immunoprecipitation. Protein precipitated and inputs were analysed by western blotting and probed for RNF4, FOXM1 and RNF168 expression. The asterisk indicates IgG bands. Arrows indicate RNF168 or RNF4. Input represents 1:10 dilution of pull down and IgG was used as a negative control.
Figure 4.28 Co-immunoprecipitation (co-IP) using RNF4 antibodies exhibits an interaction of RNF4 with neither RNF168 nor FOXM1. MCF-7 cells were treated with 1 μM epirubicin for 0, 6 and 24 h. Antibodies against FOXM1 were used to perform co-IP. Proteins pulled down with beads and inputs were analysed by western blotting for RNF168, FOXM1 and RNF4. The asterisk indicates IgG bands. Arrows indicate FOXM1 or RNF168 or RNF4 specific bands. Input represents 1:10 dilution of pull down and IgG was used as a negative control.
To confirm the interaction between RNF168 and FOXM1, MCF-7 cells were transfected with pcDNA3-Flag-RNF168 (RNF168) or pcDNA3-Flag empty vector control (pcDNA3). After 24h of transfection, cells were treated with 1 μM epirubicin and harvested at 0, 6, and 24 h after the treatment. Immunoprecipitation was performed using ANTI-FLAG M2 affinity gel beads to pull down the Flag-tagged protein. Input and eluent were analysed by western blotting and probed with various specific antibodies against FOXM1, RNF4, and RNF168 (for precipitation control). The result showed that only in cells overexpressing flag-RNF168 precipitated with FOXM1 and this was not observed in cells transfected with empty vector control (Figure 4.29). This result confirmed the interaction between FOXM1 and RNF168.

To further validate the previous result (Figure 4.28), I also transfected MCF-7 cells with flag-RNF4 (RNF4) or the pcDNA3-flag empty vector control (pcDNA3). The following day, the transfected cells were treated with 1 μM epirubicin and harvested at 0, 6, and 24 h after the treatment with epirubicin. Immunoprecipitation was performed using ANTI-FLAG M2 affinity gel beads to pull down the Flag-tagged protein. Input and eluent were analysed by western blotting. The western blot membrane was probed with antibodies against FOXM1, RNF4, and RNF168 (for precipitation control). Consistently, the western blot result failed to show both FOXM1 and RNF168 bands (Figure 4.30). The result indicates that there is no interaction between RNF4 and RNF168 or FOXM1.
Taken together, these data confirms that RNF168 might regulate FOXM1 by directly binding to FOXM1, while RNF4 does not bind to FOXM1. It can be concluded that RNF4 does not directly regulate FOXM1 but it can affect other factors that have an impact on the expression and function of FOXM1. This can also lead to the changes on the transcription and translation levels of FOXM1.

**Figure 4.29 Confirmation of the interaction between FOXM1 and RNF168.** MCF-7 cells were transfected with pcDNA3-flag-RNF168 or the pcDNA3-flag empty vector control. The transfected cells were treated with 1 μM epirubicin for 0, 6, 24 and 48 h before immunoprecipitation was performed using ANTI-FLAG M2 Affinity Gel to pull-down the flag-tagged protein. Input and precipitates were analysed by western blotting and probed for FOXM1, RNF4, and RNF168. The asterisk indicates pcDNA3-flag-RNF168. Arrows indicate endogenous RNF168 or RNF4. Input represents 1:10 dilution of pull down.
Figure 4.30 Immunoprecipitation confirms that there is no interaction between RNF4 and FOXM1 or RNF168. MCF-7 cells were transfected with pcDNA3-flag-RNF168 or the pcDNA3-flag empty vector control. The transfected cells were treated with 1 μM epirubicin for 0, 6, 24 and 48 h before immunoprecipitation was performed using ANTI-FLAG M2 Affinity Gel to pull-down the flag-tagged protein. Input and precipitates were analysed by western blotting and probed for FOXM1, RNF168, and RNF4. Asterisks indicate pcDNA3-flag-RNF4. Arrows indicate endogenous RNF4. Input represents 1:10 dilution of pull down.
4.2.15 RNF168 promotes the degradation of FOXM1 wt but not the SUMOylation-deficient FOXM1

In previous experiments (Chapter 3), SUMOylation-deficient FOXM1 5x(K>R) plasmid was found to be more stable than the FOXM1 wt as SUMOylation of FOXM1 promotes its degradation (Myatt et al., 2014). I therefore hypothesised that RNF168 could affect the degradation FOXM1 wt more than the mutant FOXM1 5x(K>R). To test this, I performed two parallel experiments using firstly eGFP-FOXM1 wt (Figure 4.31) followed by eGFP-FOXM1 5x(K>R) (Figure 4.32), to study the degradation of FOXM1 after inhibition of protein synthesis (CHX treatment) together with overexpression of RNF168.

Firstly, I transfected MCF-7 cells with eGFP-FOXM1 wt together with flag-RNF168 or empty vector control (pcDNA3). The transfected cells were treated for 2 h with epirubicin prior to the CHX treatment. The cells were harvested at indicated time points (0, 2, 4, 6 and 8 h). The protein expression levels were examined by western blotting. As expected, eGFP-FOXM1 wt transfected cells, overexpressing flag-RNF168, revealed a significantly faster degradation rate (Figure 4.31) than the cells without RNF168 (pcDNA3 empty vector control). I also quantified the degradation rate of the endogenous FOXM1 which might be affected by the expression of RNF168. Western blot result showed that, in cells overexpressing RNF168, the endogenous FOXM1 levels were found to be more stable than the pcDNA3 transfected control (Figure 4.31C). This confirms the previous result that RNF168 promotes the degradation of FOXM1 (Figure 4.23 and 4.24).
Next, to see the effect of the SUMOylation-deficiency FOXM1 mutant on the RNF168-mediated FOXM degradation, I transfected MCF-7 cells with eGFP-FOXM1 5x(K>R) together with flag-RNF168 or empty vector control (pcDNA3). The transfected cells were treated with 2 h epirubicin prior to the CHX treatment. The cells were harvested at indicated time points (0, 2, 4, 6 and 8 h) for western blotting analysis. In contrast to eGFP-FOXM1 wt transfected cells, eGFP-FOXM1 5x(K>R) showed no significant difference in the degradation rate of FOXM1 in the presence of RNF168 overexpression compared to the pcDNA3 transfected control (Figure 4.32). This result was compared to the difference in the degradation rate of endogenous FOXM1, which after overexpression of RNF168, is faster than the pcDNA3 transfected control (Figure 4.32C). This confirms that the overexpression of RNF168 is able to affect only the degradation of the SUMOylated FOXM1 protein and not the endogenous.
Figure 4.31 RNF168 overexpression promotes the degradation of the eGFP-FOXM1 wt in the presence of epirubicin. A. MCF-7 cells were co-transfected with eGFP-FOXM1 wt and, flag-RNF168 or pcDNA3-flag empty vector control. The following day, the cells were treated with 2 h 1 μM epirubicin before the addition of 100 μM of CHX. Cells were then collected at indicated time points (0, 2, 4, 6 and 8 h) for western blot analysis. Densitometry was used to quantify the FOXM1 levels from western blots using imageJ. β-tubulin was used for normalisation. Quantitation analysis B. eGFP-FOXM1 and C. endogenous FOXM1 levels were normalised to the 1h of CHX treatment. Graphs represent the mean of 3 independent experiments ±SD. Statistical analysis was performed using Student’s t-test comparing pcDNA3 empty vector and RNF168 overexpressed cells at the same time point. (*, p ≤ 0.05 significant; **, p ≤ 0.01 significant; ***, p ≤ 0.001 significant; ns, non-significant)
Figure 4.32 RNF168 overexpression does not significantly affect eGFP-FOXM1 5x(K>R) degradation rate in the presence of epirubicin. A. MCF-7 cells were co-transfected with eGFP-FOXM1 5x(K>R) and, flag-RNF168 or pcDNA3-flag empty vector control. The following day, the cells were treated with 1 μM epirubicin for 2 h before the addition of 100 μM of CHX. Cells were then harvested at several time points indicated (0, 2, 4, 6 and 8 h) for western blot analysis. Densitometry was used to quantify the FOXM1 levels from western blots using ImageJ. β-tubulin was used for normalisation. Quantitative B. eGFP-FOXM1 5x(K>R) and C. endogenous FOXM1 levels were normalised relative to 1 h CHX treatment. Graphs represent the mean of 3 independent experiments ±SD. Statistical analysis was analysed using Student’s t-test compared between pcDNA3 empty vector and RNF168 overexpressed cells, at the same time point.
I also repeated the CHX experiment to compare the degradation rate of eGFP-FOXM1 wt or eGFP-FOXM1 5x(K>R) in the presence of RNF168. According to the previous data (Figure 4.31, 4.32 and Chapter 3), I hypothesised that FOXM1 wt should also degrade faster in the presence of RNF168. To prove this, MCF-7 cells were co-transfected with the pcDNA3-flag-RNF168 and eGFP-FOXM1 wt or eGFP-FOXM1 5x(K>R) expression plasmids. The transfected cells were treated with epirubicin for 2 h prior to adding CHX at various indicated time points (0, 1, 2, 4, 6, and 8 h) and the cell pellets were then harvested for western blotting analysis. Predictably, eGFP-FOXM1 wt was found to degrade significantly faster in the presence of RNF168 and epirubicin, when compared with eGFP-FOXM1 5x(K>R) (Figure 4.33A and 4.33B). In contrast, the degradation rate of the endogenous form of FOXM1 in both conditions showed a similar trend to each other, comparing between eGFP-FOXM1 wt and eGFP-FOXM1 5x(K>R) transfected cells (Figure 4.33A and 4.33C). As the transcription process was inhibited by CHX treatment, the endogenous FOXM1 expression was not affected by the ectopic expression of FOXM1, eGFP-FOXM1 (wt) and eGFP-FOXM1 5x(K>R). As a result, the endogenous level of FOXM1 in the both conditions should be affected by only the overexpression of RNF168. Collectively, this result suggests that SUMOylation of FOXM1 is important for RNF168-mediated degradation of FOXM1.
Figure 4.33 RNF168 overexpression promotes eGFP-FOXM1 wt degradation in the presence of epirubicin. A. MCF-7 cells were co-transfected with eGFP-FOXM1 wt or 5x(K>R), and flag-RNF168. The following day, the cells were treated with 2 h of 1 μM epirubicin prior to the addition of 100 μM CHX. Cells were harvested at several time points (0, 2, 4, 6 and 8 h). Densitometry was used to quantify the FOXM1 level from western blot using imageJ. β-tubulin was used for normalisation. Quantitative B. eGFP-FOXM1 and C. endogenous FOXM1 levels were normalised relative to the 1 h treatment of CHX.
In addition, I examined the effect of RNF168 overexpression in the absence of epirubicin. To address this, MCF-7 cells were co-transfected with pcDNA3-flag-RNF168 and eGFP-FOXM1-wt or 5x(K>R) expression plasmids. The following day, the transfected cells were treated with CHX. Western blotting was used to examine the degradation rate of FOXM1. The result showed a faster degradation rate of exogenous eGFP-FOXM1 wt transfected cells compared with the eGFP-FOXM1 5x(K>R) (Figure 4.34). Interestingly, the difference in the degradation rate between eGFP-FOXM1 wt and eGFP-FOXM 5x(K>R) was smaller than in the experiment with epirubicin treatment (Figure 4.33).

In conclusion, this data indicates that the SUMOylation of FOXM1 is important for its RNF168-mediated degradation mediated RNF168, especially in the presence of epirubicin treatment.
**Figure 4.34** RNF168 overexpression has a mild effect on eGFP-FOXM1 wt degradation in the absence of epirubicin. A. MCF-7 cells were co-transfected with eGFP-FOXM1 wt or eGFP-FOXM1 5x(K>R), and flag-RNF168. The following day, the cells were treated with 100 μM (CHX). Cells were collected at several indicated time points (0, 2, 4, 6 and 8 h) of the treatment, without epirubicin. Densitometry was used to quantify the FOXM1 level from western blot using imageJ. β-tubulin was used for normalisation. Quantitative B. eGFP-FOXM1 and C. endogenous FOXM1 levels were normalised relative to the 1 h CHX treatment.
4.2.16 RNF168 interacts with FOXM1 wt but not with the SUMOylation-deficient FOXM1

I next investigated the role of SUMOylation in the interaction between RNF168 and FOXM1 in response to epirubicin treatment. MCF-7 cells were transfected with either eGFP-FOXM1 wt or eGFP-FOXM1 5x(K>R) before being treated with 1 µM epirubicin at 0, 6, and 24 h. The co-IP experiment was performed using antibodies against RNF168. The precipitates from co-IP experiment were used for western blot analysis. The western blot membrane was probed with antibodies against FOXM1 and RNF168 (for affinity testing). The band corresponding to the eGFP-FOXM1 was found in the cells transfected with eGFP-FOXM1 wt at 0 h and 6 h but not at 24 h. The endogenous FOXM1 band was also found in this experiment, but at 24 h of treatment with epirubicin, the endogenous FOXM1 band appeared to be degraded (Figure 4.35A, the top panel). In contrast, the cells transfected with eGFP-FOXM1 5x(K>R) only showed the band corresponding to the endogenous FOXM1 (indicated by asterisk,* ) whereas the eGFP-FOXM1 was not present (Figure 4.35A, the bottom panel). Antibodies against RNF168 were used to probe the membrane to confirm its ability to pull-down RNF168 (Figure 4.35B). The IgG band was found in the IgG control and the IP conditions. This confirmed the importance of SUMOylation in terms of mediating the interaction between RNF168 and FOXM1. Moreover, this also indicates that FOXM1 can only interact with RNF168 when it is modified by SUMOylation.
Figure 4.35 Co-immunoprecipitation (IP) by RNF168 antibodies shows an interaction between RNF168 and the eGFP-FOXM1 wt. MCF-7 cells were transfected with eGFP-FOXM1 wt or eGFP-FOXM1 5x(K>R) before treatment with 1 μM epirubicin for 0, 6 and 24 h. Antibodies against RNF168 were used for performing co-immunoprecipitation and the membranes were probed with antibodies targeting FOXM1 and RNF168. Precipitates from beads and inputs were analysed using western blotting. Asterisks indicate eGFP-FOXM1 wt or 5x(K>R). Arrows indicate the endogenous levels of FOXM1. Input represents 1:10 dilution of pull down and IgG was used as a negative control.
4.3 Discussion and conclusion

It is clear from my results that FOXM1 is modified by SUMOylation, which leads to its ubiquitination and degradation (Chapter 3). Although this degradation can occur via the ubiquitin-proteasome pathway through the APC/Cdh1 complex, the complex itself does not mediate the ubiquitination process (Myatt et al., 2014). This suggests that there should be another protein complex that works as a mediator between SUMOylated FOXM1 and leads to its ubiquitination. A number of studies have shown that various RING-type ubiquitin ligases play a key role in the crosstalk between SUMOylation and ubiquitination (Galanty et al., 2012; Galanty et al., 2009; Guzzo et al., 2012; Morris et al., 2009). These ubiquitin ligases have also been reported to be involved in the DNA damage repair pathway and chemotherapy resistance (Galanty et al., 2012; Mallette et al., 2012; Oestergaard et al., 2012; Wu et al., 2010; Yin et al., 2012). Moreover, some of the RING-type ubiquitin ligases, such as BRCA1, RNF4 (Galanty et al., 2012; Morris et al., 2009; Rojas-Fernandez et al., 2014) as well as RNF8 (Danielsen (Bartocci and Denchi, 2013; Danielsen et al., 2012; Galanty et al., 2009) have been reported to target SUMOylated proteins via their SUMO interacting motifs (SIMs) for ubiquitination. Although RNF168 has never been reported to have a SIM consensus, various studies reported that it works together with RNF8 in order to target SUMOylated proteins (Bartocci and Denchi, 2013; Danielsen et al., 2012; Galanty et al., 2009; Wrighton, 2010). As a consequence, in this chapter, I focused mainly on studying the role of RNF4, RNF8 and RNF168 in the regulation of FOXM1.
First, I examined the protein expression levels of RNF4, RNF8 and RNF168 in response to epirubicin treatment. Interestingly, I observed that there is a correlation between the expression levels of RNF4 and FOXM1. In MCF-7 cells, both were found to be downregulated after epirubicin treatment. Similar to what is observed for the FOXM1 protein levels in MCF-7 EpiR cells (Chapter 3), RNF4 appeared to be expressed at higher levels in comparison to MCF-7 cells. In contrast to RNF4 and FOXM1, the protein and mRNA levels of RNF168 were found to be significantly up-regulated after epirubicin treatment in MCF-7 cells but not in MCF-7 EpiR cells, although the expression was very low for the untreated cells. This suggests that RNF168 might have an inverse correlation with FOXM1 and might be modulated by DNA damage under the chemotherapy-induced genotoxic stress. For RNF8, the expression levels of both protein and mRNA did not change after the treatment and there were no differences between the two cell lines. However, it does not preclude RNF8 from having a role in FOXM1 regulation. RNF8 can still modulate FOXM1 expression in directly through an intermediate FOXM1 regulator or in another cell type. For example, RNF 8 has been shown to collaborate with RNF168 to modulate target protein expression (Danielsen et al., 2012; Galanty et al., 2009; Mallette et al., 2012).

Based on these results, I reasoned that RNF4 might promote FOXM1 expression and its activity, while RNF168 might have the reverse effect on FOXM1. To address this, I performed an overexpression and a silencing study of the RNF proteins in MCF-7 cells to determine their effects on the expression of FOXM1. Consistently with my hypothesis, the result showed that FOXM1 was up-regulated by RNF4, but down-regulated by RNF168. Interestingly, RNF8 expression was found to have a discrete
effect on the expression of FOXM1. Overexpression of RNF8, down-regulated the expression of FOXM1 while RNF8 knock-down resulted in an up-regulation on the expression of FOXM1. Although to a smaller extent, RNF8 had a similar effect on FOXM1 to RNF168. These data showed the positive regulation of FOXM1 expression by RNF4 and the negative regulation of FOXM1 by RNF168.

Additionally, RNF4 and RNF168 were also found to have an effect on the transactivation activity of FOXM1 over its known downstream target, cyclin B1 (Leung (Laoukili et al., 2005; Leung et al., 2001). The RNF4 significantly increases the transactivation activity of cyclin B1 promoter while RNF168 noticeably suppresses it. In addition, the transcription activity of cyclin B1 did not show a significant change upon the transfection with increasing levels of RNF8 or BRCA1. Again, RNF8 may modulate RNF168 activity rather than expression. The result from luciferase reporter assay from RNF8 shows a similar yet less strong trend compared to RNF168. This emphasises the outstanding positive effect of RNF4 and the negative effect of RNF168 on the FOXM1 downstream transcriptional target, cyclin B1. It is possible that RNF4 and RNF168 regulate transcriptional activity of cyclin B1 promoter via the regulation of FOXM1. To further understand the apparent opposite regulation of FOXM1 by RNF4 and RNF168, FOXM1 expression levels were then examined by overexpression of RNF4 and RNF168.

Initially, I observed the changes in FOXM1 protein levels by epirubicin under the condition of overexpression and depletion of RNF4, in MCF-7 cells. The levels of RNF4 were found to affect the mRNA and protein expression levels of FOXM1 in MCF-7 cells,
both before and after epirubicin treatment. I also found that RNF4 affects the cell growth rate of MCF-7 cells in the same way as FOXM1 does, in both short-term and long-term cell proliferation. These data correlates with various studies showing that FOXM1 promotes cell proliferation and tumour cell growth (Nakamura et al., 2010; Yang et al., 2013). Unfortunately, the changes in FOXM1 levels from the effect of RNF4 expression did not affect the sensitivity of MCF-7 cells to epirubicin treatment. This result is in contrast with previous studies showing that the regulation of FOXM1 leads to chemotherapy resistance while the depletion of FOXM1 promotes cell sensitivity to epirubicin (Halasi and Gartel, 2012; Millour et al., 2011; Monteiro et al., 2013; Park et al., 2012). It is possible that the expression of RNF4 affects the FOXM1 expression level but it does not affect the FOXM1 level and posttranslational modification of FOXM1 under epirubicin-induced genotoxic stress condition. This presumption is in agreement with the result from CHX experiment. The result from the CHX study showed that RNF4 expression does not have a direct effect on the stability and degradation of FOXM1. The inhibition of protein translation by cycloheximide treatment revealed no difference between the overexpression or knock-down of RNF4 compared with pcDNA3 empty vector and non-silencing control, respectively. This indicates that RNF4 might affect only FOXM1 transcription or translational levels indirectly but not directly through the proteasome degradation pathway. This might clarify that the fact that the expression of RNF4 affect the FOXM1 expression levels and the cell proliferation but not the epirubicin sensitivity of MCF-7 cells.

As mentioned above, RNF4 is able to regulate transcriptional activity of cyclin B1 promoter. In this Chapter, I also confirm the importance of the RING-domain of RNF4 in
regulating the transactivation functions of FOXM1 by the regulation of its known downstream target, *cyclin B1*. The function of RING-type E3 ligase is commonly specified by its RING domain that interacts with ubiquitin-E2 complex (Deshaises and Joazeiro, 2009). Mutations at the RING domain of RING-type E3 ligases (by point mutations from a zinc-coordinating cysteine residue to a serine residue - CS mutation) is resulted in catalytically inactive form of the ligase. The RING-type E3 ligase with this CS mutation is known to be lack of ability of E3 ligase to ubiquitinate its substrate proteins (Ahner et al., 2013; Luo et al., 2012; Tatham et al., 2008). Here, I show that a point mutation of zinc-coordinating cysteine residues into serine residues (CS mutation), leads to the loss of the function of RNF4 in promoting the transcriptional activity of *cyclin B1* promoter. However, RNF4 only partially regulates the transcription activity of *cyclin B1* via FOXM1. My result showed that RNF4 can transactivate both wt and mut3 *cyclin B1* promoters, but it is more effective in activating the *cyclin B1* promoter with the wild-type forkhead binding domains. It seems therefore that RNF4 only partially regulates *cyclin B1* transcription via FOXM1 and it also affects other transcription factor binding sites on the promoter.

I also determined the regulation of FOXM1 by RNF168. The overexpression and knock-down experiments reveal that RNF168 negatively regulates FOXM1 both in the absence and in the presence of epirubicin treatment. This down-regulation of FOXM1 appears to result in the increased sensitivity of MCF-7 cells to epirubicin. Accordingly, overexpression of RNF168 results in a significant increase of epirubicin sensitivity, while the knock-down shows the reverse effect. These data are consistent with previous studies demonstrating that FOXM1 promotes epirubicin resistance (Halasi and Gartel,
2012; Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013). I also found that RNF168 is a negative repressor of *cyclin B1* transcriptional activity, a downstream target of FOXM1, and this repressive property is forkhead binding domain dependent. This confirms that RNF168 promote *cyclin B1* promoter activity via the regulation of FOXM1 that is bound to the forkhead binding domain of *cyclin B1* promoter. Surprisingly, the down regulation of FOXM1 by RNF168 does not affect short-term cell growth and the long-term cell proliferation, without the presence of epirubicin. This suggests that RNF168 is activated by genotoxic stress. These data indicate that RNF168 is not only involved in the ubiquitination of histone H2A (Morris et al., 2009; Nakada et al., 2010) in order to drive the DNA damage signalling (Mattiroli et al., 2012; Pinato et al., 2011) but also involved in chemotherapeutic sensitivity.

As shown in my previous study (Chapter 3), SUMOylation of FOXM1 leads to its ubiquitination and subsequent degradation (Myatt et al., 2014). Through my studies, I found that RNF168 might be the modulator of FOXM1 degradation in this context. First, I discovered that overexpression of RNF168 accelerates the degradation rate of FOXM1 while depletion of RNF168 promotes its stability. Furthermore, this effect is enhanced in the presence of epirubicin. This is supported by a study in U2OS cells which showed that RNF168 is capable of ubiquitinating (K48-linked chain) the transcriptional cofactor JMJD2A. This in turn leads to the proteasomal degradation of the targeted protein in an RNF8 dependent manner in response to doxorubicin (Mallette et al., 2012). This suggests that RNF168 might be the mediated molecule that plays a necessary role in the SUMOylation and ubiquitination crosstalk of FOXM1. Here, the direct interaction between FOXM1 and RNF168, but not RNF4, has also been confirmed using co-
immunoprecipitation. Together these results strongly suggest that RNF168 directly binds to FOXM1 to promote its degradation.

I also confirm the impact of SUMOylation in the subsequent degradation of FOXM1 mediated by RNF168. In accordance to various studies, SUMOylation modifies and promotes the degradation of various Forkhead proteins, including FOXA1 (Bao et al., 2011) and FOXC1/FOXC2 (Danciu et al., 2012). The result from the CHX treatment with the overexpression of RNF168 revealed a faster degradation rate of eGFP-FOXM1 wt compared to the degradation of eGFP-FOXM1 5x(K>R), especially in the presence of epirubicin. Moreover, these data highlighted that RNF168 is important for promoting the degradation of SUMOylated FOXM1, especially under the epirubicin-induced genotoxic stress. This regulation can take place by direct binding of RNF168 to SUMOylated FOXM1 but not to the SUMOylation-deficient FOXM1. These data strengthen the findings that SUMOylation of FOXM1 is important for its ubiquitination and subsequent degradation (Myatt et al., 2014), and this can be promoted by an overexpression of RNF168.

In conclusion, my data suggest that RNF4 indirectly promotes the expression of FOXM1 but it is not directly involve with the degradation of FOXM1 through the proteasomal pathway. This upregulation leads to an increase in some of the functions of FOXM1 including promotion of cell proliferation and increasing the transcription of cyclin B1, a FOXM1 downstream target. However, this upregulation does not significantly alter the sensitivity of MCF-7 cells to epirubicin. Furthermore, this study indicates that the regulation by RNF4 of the transactivation activity of FOXM1 over a cyclin B1 promoter
occurs in RING-domain dependent manners. In contrast, RNF168 directly binds to SUMOylated FOXM1 to down-regulate its expression and promotes its degradation. Although the influence of RNF168 over FOXM1 expression and degradation does not affect the basal proliferation state of MCF-7 cells, this negative regulation by RNF168 on FOXM1 significantly affects the sensitivity of MCF-7 cells to epirubicin and transactivation activity of cyclin B1 promoter in a forkhead binding domain-dependent manner. Without a forkhead binding domain RNF168 cannot repress the transcriptional activity of cyclin B1. This finding also emphasises the importance of SUMOylation in terms of the formation of the RNF168-FOXM1 complexes.
4.4 Future work

According to my previous results, the mechanisms of SUMOylation and ubiquitination have been clarified but the deubiquitination mechanism remain unknown. Since, OTUB1, a Lys48-specific deubiquitinating enzyme (Wiener et al., 2013), has been shown to inhibit RNF168-dependent poly-ubiquitination pathway (Nakada et al., 2010) in future studies, I would examine the expression patterns of OTUB1 in MCF-7 and MCF-7 EpiR cells and its role in FOXM1 expression. Then, I would study the role of OTUB1 in DNA damage and epirubicin sensitivity in both sensitive and resistant breast cancer cell lines. In addition, I would attempt to identify a potential interaction between OTUB1 and FOXM1 using co-immunoprecipitation experiments.

Furthermore, I would determine whether RNF8 has a role in RNF168 and FOXM1 complex formation. To date, the SUMO interacting motifs (SIMs) of RNF168 have never been identified. Various studies have shown that RNF168 always works together with RNF8 in the DNA damage response pathways (Danielsen et al., 2012; Galanty et al., 2009; Mallette et al., 2012). It is also possible that the SIMs in RNF8, a SUMO-Targeted Ubiquitin Ligase (STUbL), have a role in targeting SUMO residues on FOXM1 and recruiting RNF168 to form a complex with FOXM1.
CHAPTER 5

THE INTERACTION BETWEEN OTUB1, A DEUBIQUITINATING ENZYME, AND FOXM1 IN RESPONSE TO EPIRUBICIN
5.1 Introduction and Objectives

OTUB1 (ovarian tumour domain-containing Ub aldehyde-binding protein 1) is a deubiquitinating enzyme (DUB) that belongs to the OTU (ovarian tumour) superfamily and has a role in inhibiting the formation of ubiquitin chains on target proteins (Nakada et al., 2010; Rose and Schlieker, 2010). The OTU-superfamily of proteases is homologous to the ovarian tumour gene product of *Drosophila* which was reported to have a role in oocyte morphogenesis (Makarova et al., 2000). OTUB1 contains an OTU domain, which has approximately 130 amino acids. This domain is highly conserved from yeast to humans (Messick et al., 2008). Some well-known members of this superfamily have been established as important regulators in various signalling cascades crucial for inflammation and cancer, such as ubiquitin-editing enzyme A20, OTUD family proteins, OTULIN, VCP/IP and ZRANB1 (Malynn and Ma, 2009; Mevissen et al., 2013; Vereecke et al., 2009). OTUB1 is expressed ubiquitously in human tissues. It is the first protein in this superfamily to be confirmed with deubiquitinating properties (Zhang et al., 2012). OTUB1 is in a heterogeneous group of cysteine proteases as it contains an active cysteine thiol catalytic site. Cysteine proteases commonly work by cleaving proteins at the bond between the ubiquitin residue and the modified protein (Balakirev et al., 2003). In the proteasome degradation pathway, de-ubiquitination of substrate proteins by DUBs in OTU superfamily family, such as YOD1 and OTUB1, play a critical part in the process by allowing the unfolded protein to be transported to the narrow proteolytic chamber of the proteasome (Ernst et al., 2009; Pickart and Fushman, 2004).
OTUB1 has been reported to disrupt RNF168-dependent ubiquitination pathway by binding to UBC13, a well known E2 conjugating enzyme, by impeding its binding to RNF168. This binding interferes with the ubiquitinating event and blocks the transfer of ubiquitin residues from UBC13 to the targeted protein (Nakada et al., 2010). OTUB1 has also been shown to bind to the E2s of UBE2D and UBE2E families (Nakada et al., 2010; Sowa et al., 2009). In cancer, OTUB1 has been found to be upregulated and involved in colon cancer development and metastasis (Liu et al., 2014). Nevertheless, the role of OTUB1 in breast cancer has not yet been established.

FOXM1 has been reported to plays an essential part in DNA damage repair and chemotherapeutic resistance (de Olano et al., 2012; Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013; Myatt et al., 2014). It is upregulated in the chemotherapeutic resistant breast cancer (including epirubicin resistance) when compared to the sensitive counterpart (Millour et al., 2011; Monteiro et al., 2013). In a study of MDA-MB-231, triple negative breast cancer, the knock-down of FOXM1 leads to an increase of cell sensitivity to epirubicin treatment (Park et al., 2012).

In chapter 3, my study revealed that FOXM1 is ubiquitinated prior to degradation (Laoukili et al., 2008; Myatt et al., 2014). OTUB1 is also reported to interrupt the DNA repair pathway by inhibiting the ubiquitination (Rose and Schlieker, 2010). Interestingly, OTUB1 is also able to inhibit the ubiquitination of both K48-linked (Wiener et al., 2013) and K63-linked ubiquitin chains (Nakada et al., 2010). Therefore, I hypothesised that OTUB1 might stabilise FOXM1 by removing its ubiquitin residues and prevent its degradation.
OTUB1 has been reported to regulate various proteins, such as p53 (Li et al., 2014; Sun et al., 2012), oestrogen receptor alpha (ERα) (Stanisic et al., 2009), and RNF168 (Nakada et al., 2010). Interestingly, these proteins have been shown to play a crucial role in tumorigenesis. For this reason, I decided to study the role of OTUB1 in the regulation of FOXM1. In previous studies, FOXM1 has also been showed to be regulated by ERα (Horimoto et al., 2011; Karadedou, 2006; Millour et al., 2011) and p53 (Qu et al., 2013). In Chapter 4, I showed that RNF168 regulates FOXM1 by promoting its degradation. Together these data suggest that OTUB1 could either regulate FOXM1 directly or indirectly via other proteins such as RNF168, p53 or ERα.

Investigating the role of OTUB1 in the regulation of FOXM1 could prove useful for understanding breast cancer progression and epirubicin resistance and may lead to the development of novel targets for improving breast cancer therapy.
5.2 Results

5.2.1 Sensitive cells express higher levels of OTUB1 compared with resistant cells

In order to study the protein expression pattern of OTUB1 in sensitive and resistant breast cancer cell lines, MCF-7 and MCF-7 EpiR cells were treated with 1 µM epirubicin. The cells were then harvested at different time points (0, 4, 8, 16, 24 and 48 h) for western blotting analysis. Interestingly, western blot results revealed that there were 2 bands of OTUB1 in MCF-7 cells but in MCF-7 EpiR cells only the bottom band was present (figure 5.1A). In MCF-7 cells, the top band was found to be degraded after 24 h of epirubicin treatment. In addition, qPCR results revealed that there was more OTUB1 mRNA expression in MCF-7 cells than in MCF-7 EpiR cells. Moreover, the expression of the OTUB1 mRNA in MCF-7 cells was increased after the treatment with 1 µM epirubicin, especially after 24 h treatment when it was significantly increased compared with the untreated condition. Similarly to the western blot results, at 48 h of epirubicin treatment OTUB1 transcript levels were dramatically decreased in MCF-7 cells. In contrast, OTUB1 mRNA expression in MCF-7 EpiR cells was constant after epirubicin treatment (Figure 5.1B).
Figure 5.1 Expression of OTUB1 following epirubicin treatment in MCF-7 and MCF-7 EpiR cells. MCF-7 and MCF-7 EpiR cells were treated with 1 μM epirubicin and harvested at time points indicated (0, 4, 8, 16, 24 and 48 h). A. Protein lysates were extracted and analysed by western blotting. B. The mRNA expression levels of OTUB1 for each time point was analysed by qPCR. Statistical analysis was performed using Student’s t-test. (*) p ≤ 0.005; (**) ≤ 0.001 significant; ns, non-significant)
According to the previous results, Foxm1−/− MEFs are sensitive to epirubicin (Chapter 3.2.2) and display more epirubicin-induced DNA damage when compared with wt MEFs. In order to study the effect of FOXM1 on OTUB1 expression, I then tested whether the expression of OTUB1 in Foxm1−/− MEFs was different from wt MEFs. Both MEFs (wt and Foxm1−/−) were treated with 1 μM epirubicin and harvested after 0, 6 and 24 h of treatment. Protein and mRNA were extracted for western blotting analysis. The result revealed a thicker slower migrating band of OTUB1 in Foxm1−/− MEFs, that was decreased in wt MEFs (Figure 5.2A). As the FOXM1 band from western blotting in Foxm1−/− MEFs was unclear, qPCR analysis was used to confirm the depletion of Foxm1 in Foxm1−/− MEFs. The result confirmed that the FOXM1 mRNA levels in Foxm1−/− MEFs could not to be determined (Figure 5.2B). I then investigated the OTUB1 mRNA expression levels. Consistently, the results showed higher OTUB1 mRNA expression in Foxm1−/− MEFs compared with wt MEFs (Figure 5.2C). In summary, this evidence indicates that OTUB1 might be involved in epirubicin sensitivity. In addition, the expression of FOXM1 might also be inversely correlated with that of OTUB1.
Figure 5.2 Protein levels of OTUB1 are higher in Foxm1⁻/⁻ MEFs compared with wt MEFs. Foxm1⁻/⁻ MEFs and wt MEFs were treated with 1 μM epirubicin and the cells were harvested after 0, 6, and 24 h of treatment. A. Protein lysates were extracted and analysed by western blotting. The mRNA expression levels of B. FOXM1 and C. OTUB1 for each time point were analysed by qPCR. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.005; **, ≤ 0.001 significant; ns, non-significant)
5.2.2 OTUB1 locates in both nuclear and cytoplasmic fractions

In order to study the sub-cellular localisation of OTUB1, nuclear-cytoplasmic fractionation was performed. MCF-7 and MCF-7 EpiR cells were treated with 1μM epirubicin for 0, 6 and 24 h. Cytoplasmic and nuclear fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit. The protein lysates were subjected to western blot analysis. The result showed that although OTUB1 can be found both in the nucleus and cytoplasm, with the faster migrating species localised primarily in the cytoplasm of both MCF-7 and MCF-7 EpiR cells, according to the bottom band pattern. It is possible that OTUB1 functions mainly in the cytoplasm of these cells. Interestingly, the higher molecular weight band of OTUB1, which is normally only found in MCF-7 cells, was not as strong as it was previously shown in whole-cell lysates of these cells. This could be the result of its degradation during the experimental procedure. As previously shown in Chapter 3, the study of some post-translational modification forms of FOXM1 requires the presence of specific protease inhibitors. The loss of the top band could be attributed to the different composition of the lysis buffer used in this experiment. For whole-cell lysates, RIPA lysis buffer was used (see Materials and Methods). In contrast, the nuclear-cytoplasmic fractionation kit uses a lysis buffer with an undisclosed recipe (Thermo Scientific, UK). Similarly, FOXM1 protein was also found in both cytoplasm and nucleus (Figure 5.3). As mentioned in Chapter 3, FOXM1 can be imported-exported from the cells. OTUB1 as a deubiquitinating enzyme might deubiquitinate the FOXM1 protein and lead to its nuclear re-entry of FOXM1. Lamin B and β-tubulin were used as loading controls for nucleus and cytoplasm, respectively.
Figure 5.3 OTUB1 locates in both cytoplasmic and nuclear fractions. MCF-7 and MCF-7 EpiR cells were treated with 1μM epirubicin for 0, 6, and 24 h. Sub-cellular fractionation was performed and lysates subjected to western blot analysis. Lamin B and β-tubulin were used as loading controls for nuclear and cytoplasmic fractions, respectively.
5.2.3 OTUB1 expression increases the sensitivity of MCF-7 cells to epirubicin

To determine the importance of OTUB1 in epirubicin resistance and sensitivity in breast cancer cell lines, I performed SRB assays using MCF-7 cells transfected with a mammalian expression vector encoding OTUB1. The transfected cells were seeded in 96-well plates before being treated with various concentrations of epirubicin (0 – 20 µM) 24 h after transfection. Cell proliferation was measured by SRB assays in terms of percentage of cell survival. The result indicates that the overexpression of OTUB1 increased the sensitivity of MCF-7 cells to epirubicin for some of the drug concentrations tested (Figure 5.4). The biggest significant differences can be found at higher concentrations (more than 0.3 µM) after 24h of epirubicin (Figure 5.4A). Unfortunately, at 5 µM there was no statistical significance. At 48h of epirubicin treatment, there was a difference for concentrations higher than 1 µM (Figure 5.4B), which is the optimal concentration previously determined by a PhD student in the lab (Millour et al., 2010; Monteiro et al., 2013). Western blot analysis confirmed that the overexpression of pcDNA3-flag-OTUB1 (OTUB1) was achieved (Figure 5.4C).
Next, the effect of OTUB1 silencing on epirubicin-sensitivity was also studied. To this end, MCF-7 cells were transiently transfected with siRNA pool against OTUB1 (siOTUB1) or the non-silencing siRNA control (NSC). Following transfection, cell proliferation was determined by SRB assay (Figure 5.5A and B) and efficient knock-down assessed by western blotting (Figure 5.5C). Consistently, cells with OTUB1 silencing exhibited a significantly higher percentage of viable cells following treatment with epirubicin (Figure 5.5), compared with the NSC-transfected controls, with the exception for 2 time-points (the 1.25 µM concentration of 24h epirubicin treatment and the 0.3 µM concentration of 48h epirubicin treatment). The non-significant results might due to the high standard deviation. Overall, these data indicate that OTUB1 could have a role in increasing the sensitivity of MCF-7 cells to epirubicin.
Figure 5.4 Overexpression of OTUB1 decreases the cell viability of MCF-7 cells after epirubicin treatment. MCF-7 cells were transfected with pcDNA3-OTUB1 or pcDNA3 empty vector control. The cells were treated with various concentrations of epirubicin for A. 24 h and B. 48 h. Cell proliferation was measured by SRB assay. The result was normalised to the untreated control (0 μM concentration). Results represent the average of three independent experiments including 6 replicates ± SD. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.05; **, p ≤ 0.01; ns, non-significant) C. The expression levels of OTUB1 and β-tubulin were analysed by western blotting. Arrows indicate the specific OTUB1 bands, asterisk indicates pcDNA3-flag-OTUB1 transfected band.
Figure 5.5 OTUB1 silencing in MCF-7 cells increases the rates of cell survival after epirubicin treatment. MCF-7 cells were transfected with siOTUB1 or NSC siRNA. The cells were treated with various concentrations of epirubicin for A. 24 h and B. 48 h. Cell proliferation was measured by SRB assay. The result was normalised to the untreated control (0 μM concentration). Results represent the average of three independent experiments including 6 replicates ± SD. Statistical analysis was performed using Student’s t-test. (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 significant; ns, non-significant) C. The protein expression levels of OTUB1 and β-tubulin were analysed using western blotting. Arrows indicate the specific OTUB1 bands.
The previous SRB results suggested that OTUB1 might have a role in increasing cell sensitivity to epirubicin. Therefore, I decided to investigate the role of OTUB1 in DNA damage in response to epirubicin treatment by using COMET assay. MCF-7 cells were transfected with siOTUB1, NSC or transfection reagent only (Mock). The cells were then treated with 1 µM epirubicin for 0, 6, and 24 h, prior to harvesting. The cell pellets were then resuspended in low-melting point agarose (LMA) before being subjected to electrophoresis. The cellular DNA was stained with DAPI, and images captured using 40x magnification objective. The percentages of DNA in tail were quantified using COMET IV software and the quantified percentages of DNA in tail of each condition were plotted. Percentages of DNA in tails represent the length of COMET tail and the fraction DNA in the tail, thus the data from percentages of DNA in tail s represent the DNA damage amount in the cells. Analysis of the images revealed that there was a decrease in the percentages of DNA in tail in the OTUB1 knock-down MCF-7 cells treated with 1 µM epirubicin for 24 h (Figure 5.6). However, there were no significant differences after 6 h of treatment. These results show that OTUB1 might have a role in increasing the amount of DNA damage hence decreasing the cell survival rate of MCF-7 cells.
Figure 5.6 OTUB1 silencing results in less DNA damage after epirubicin treatment. MCF-7 cells were transfected with siOTUB1 or NSC or Mock. The cells were then treated with 1 µM epirubicin before the COMET assay was performed to examine the DNA damage. **A.** Images were acquired with a Nikon Eclipse E400 Upright microscope (40x magnification objective). **B.** DNA damage was quantified using open COMET plug-in software to measure for the percentages of DNA in tail. Data represent the average of two independent experiments. (**, p ≤ 0.001; ns, non-significant)
5.2.4 Overexpression of OTUB1 decreases the growth rate of MCF-7 EpiR cells when treated with epirubicin

Since my previous results indicated that OTUB1 was involved in sensitivity to epirubicin, I hypothesised that OTUB1 overexpression could re-sensitise MCF-7 EpiR cells to epirubicin treatment. To this end, MCF-7 EpiR cells were transfected with pcDNA3-flag-OTUB1 (OTUB1) or pcDNA3 empty vector control (pcDNA3), before being re-seeded in 96-well plates for SRB assays. The result showed that overexpression of pcDNA3-flag-OTUB1 in MCF-7 EpiR cells reduced the percentage of cell growth following epirubicin treatment (Figure 5.7).

In addition, the pcDNA3-flag-OTUB1 transfected MCF-7 EpiR cells were used for COMET assays in order to corroborate the hypothesis that OTUB1 can re-sensitise the epirubicin resistant cells. Surprisingly, there was no DNA damage found in neither MCF-7 EpiR cells transfected with pcDNA3-flag-OTUB1 nor pcDNA3 empty vector (Figure 5.8). This indicates that the transfected pcDNA3-flag-OTUB1 cannot re-sensitise MCF-7 EpiR cells to epirubicin treatment.

In conclusion, although SRB assays showed a significant difference in the percentage of the cell survival between OTUB1 overexpressed cells and the empty vector control, the result from COMET assay revealed no difference. This might be caused by the low transfection efficiencies of MCF-7 EpiR cells.
Figure 5.7 Overexpression of OTUB1 in MCF-7 EpiR cells reduces the growth rate in higher concentration of epirubicin. MCF-7 EpiR cells were transfected with pcDNA3-OTUB1 or pcDNA3 empty vector control. The cells were treated with various concentrations of epirubicin for A. 24 h and B. 48 h. Cell proliferation was measured by SRB assay. The result was normalised to the untreated control (0 μM concentration). Results represent the average of three independent experiments including 6 replicates ± SD. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 significant; ns, non-significant). C. The protein expression levels of OTUB1 and β-tubulin were analysed using western blotting.
Figure 5.8 Overexpression of OTUB1 fails to resensitise MCF-7 Epi\textsuperscript{R} cells to epirubicin-mediated cell death. MCF-7 Epi\textsuperscript{R} cells were transfected with pcDNA3 OTUB1, or pcDNA3 empty vector. The cells were then treated with 1 µM epirubicin before the COMET assay was carried out to examine the amount of DNA damage. Images were acquired with Nikon Eclipse E400 Upright microscope (40x magnification objective). B. DNA damage was quantified using open COMET software to measure for the percentages of DNA in tail. Data represents the average of two independent experiments. (**, p ≤ 0.001; ns, non-significant)
5.2.5 An interaction of FOXM1 and OTUB1 has been found in MCF-7 cells

OTUB1 has been reported to regulate various proteins, such as p53 (Li et al., 2014; Sun et al., 2012) and ERα (Stanisic et al., 2009). Additionally, FOXM1 has also been reported to be regulated by ERα (Horimoto et al., 2011; Karadedou, 2006; Millour et al., 2011) and p53 (Qu et al., 2013). Thus, OTUB1 might regulate FOXM1 indirectly through these proteins. Moreover, as is shown in Chapter3, FOXM1 can also be regulated by ubiquitination (Laoukili et al., 2008; Myatt et al., 2014). Consequently, OTUB1 might have a role in the regulation of FOXM1 through the deubiquitination pathway. To test this hypothesis, an interaction between FOXM1 and OTUB1 was investigated.

Co-immunoprecipitation (co-IP) was used to test the interaction between FOXM1 and OTUB1. Co-IP was performed using antibodies against FOXM1 and OTUB1. In untreated MCF-7 cells, the protein interaction between FOXM1 and OTUB1 was confirmed. OTUB1 was co-immunoprecipitated with FOXM1 and the reverse co-IP was also validated (Figure 5.9A). This interaction was also found in MCF-7 EpiR cells (Figure 5.9B). The result indicates that there is a direct interaction between FOXM1 and OTUB1 in both MCF-7 and MCF-7 EpiR cells.
Figure 5.9 Co-immunoprecipitation showed an interaction between FOXM1 and OTUB1 in MCF-7 and MCF-7 EpiR cells. MCF-7 and MCF-7 EpiR cells lysates were extracted and co-IP was performed using FOXM1 and OTUB1 antibodies. Protein eluent from beads and input were analysed using Western blotting. Membrane was probed with FOXM1 and OTUB1 antibodies. Input represents 1:10 dilution of pull down and IgG was used as a negative control.
Next I investigated the effect of epirubicin treatment on the interaction between FOXM1 and OTUB1. To this end, MCF-7 cells were treated with 1 μM epirubicin. The cells were harvested after 0, 6 and 24 h of treatment and co-IP assays performed. The study revealed no changes in the interaction of FOXM1 and OTUB1 in MCF-7 cells (Figure 5.10A). The interaction of FOXM1 was also found in MCF-7 EpiR cells both before and after epirubicin treatment (Figure 5.10B). Again, the 31kDa-band OTUB1 in MCF-7 cells was not found in this co-IP experiment.
Figure 5.10 The interaction between FOXM1 and OTUB1 does not change after epirubicin treatment. MCF-7 and MCF-7 EpiR cells were treated with 1μM epirubicin before being harvest at 0, 6, and 24 h after the treatment. The cells lysates were extracted and co-IP was performed using FOXM1 and OTUB1 antibodies. Protein eluent from beads and input were analysed using Western blotting. Membrane was probed with A. FOXM1, and B. OTUB1 antibodies. Input represents 1:10 dilution of pull down and IgG was used as a negative control.
5.2.6 The bidirectional regulation effect of OTUB1 and FOXM1

To determine whether OTUB1 regulates FOXM1 expression and *vice versa*, specific siRNAs were used to perform silencing experiments. Initially, MCF-7 cells were transfected with siRNA pool targeting OTUB1, FOXM1, NSC, or Mock. The effect of FOXM1 or OTUB1 silencing in the absence of epirubicin treatment was examined using western blotting and qPCR. The result showed that OTUB1 silencing was achieved both at protein and at mRNA levels. However, the depletion of OTUB1 did not have an effect on FOXM1 protein and mRNA expression levels (Figure 5.11). Similarly, the depletion of FOXM1 did not change the OTUB1 protein and mRNA expression levels, even though the FOXM1 silencing was successful (Figure 5.12).

To further study the effect of drug treatment following the silencing of both OTUB1 and FOXM1, I depleted FOXM1 and OTUB1 in MCF-7 cells. Following the silencing, cells were treated with 1 µM epirubicin and harvested at 0, 6 and 24 h after the treatment. Western blotting was performed to analyse the protein expression and ImageJ was used to quantify both FOXM1 and the double bands corresponding to OTUB1 (Figure 5.13). The densitometry revealed that when FOXM1 was silenced, OTUB1 showed a moderate decrease throughout the time course in the FOXM1 silenced cells compared with the NSC controls (Figure 5.13B). In contrast, in the OTUB1 silenced cells there was an increase in FOXM1 expression levels that was more obvious at 6 h. However, after 24 h of epirubicin treatment there was a decrease in FOXM1 in the OTUB1 silenced cells (Figure 5.13C). This result suggests that the silencing of FOXM1 does not have a
significant effect over OTUB1 expression levels, while OTUB1 might promote the
decrease of FOXM1 protein levels after 24 h of epirubicin treatment.

Additionally, MCF-7 cells were transfected with pcDNA3 empty vector control (pcDNA3),
pcDNA3-flag-FOXM1 (FOXM1) or pcDNA3-flag-OTUB1 (OTUB1). The transfected cells
were then treated with 1µM epirubicin and harvested at 0, 6 and 24 h after the treatment
(Figure 5.14). Unfortunately, the overexpression of FOXM1 revealed only small
differences between the overexpressing cells and the pcDNA3 transfected control ones.
The overexpression of OTUB1 also showed a small effect on FOXM1 protein
expression levels. This result suggests that FOXM1 expression does not affect the
OTUB1 expression levels and vice versa.

In summary, the results from overexpression and silencing experiments were
inconclusive. This indicates that FOXM1 might not be an upstream regulator of OTUB1
and it might not control OTUB1 expression. However, OTUB1 might have an effect on
FOXM1 protein expression, especially after epirubicin treatment.
Figure 5.11 OTUB1 silencing does not affect FOXM1 expression at basal levels. A. MCF-7 cells were transfected with siOTUB1, NSC or Mock. The mRNA expressions of B. OTUB1 and C. FOXM1 were analysed by qPCR. The levels of OTUB1 and FOXM1 mRNA were normalised to L19 mRNA. Statistical analysis was performed using Student’s t-test. (**, p ≤ 0.01; ns, non-significant)
Figure 5.12 FOXM1 silencing does not affect OTUB1 expression at basal level. A. MCF-7 cells were transfected with siFOXM1, NSC or Mock. The mRNA expressions of B. FOXM1 and C. OTUB1 were extracted and analysed using qPCR. The levels of OTUB1 and FOXM1 mRNA were normalised with L19 mRNA. Statistical analysis was performed using Student's t-test. (**, p ≤ 0.01; ns, non-significant)
Figure 5.13 FOXM1 silencing has no significant impact on OTUB1 expression and vice versa. **A.** MCF-7 cells were transfected with siOTUB1, siFOXM1 or NSC, before being subjected to 1 µM epirubicin. The cells were harvested after 0, 6 or 24 h of the treatment. The cell lysates were analysed by western blotting. Densitometry of **B.** OTUB1 and **C.** FOXM1 levels were quantified by ImageJ and normalised with β-tubulin.
Figure 5.14 FOXM1 overexpression has no significant impact on OTUB1 expression and vice versa. A. MCF-7 cells were transfected with pcDNA3-FOXM1, pcDNA3-OTUB1 or pcDNA3 empty vector control, before being subjected to epirubicin treatment for 0, 6 or 24 h. Whole cell lysates were analysed by western blotting. Arrows indicate endogenous OTUB1. Asterisk indicates the pcDNA3-flag-OTUB1 band. Densitometry of B. OTUB1 and C. FOXM1 levels were quantified by Image J and normalised with β-tubulin.
5.2.7 Stably overexpression of flag-OTUB1 was generated in MCF-7 Epi\textsuperscript{R} cells

In order to overcome the low transfection efficiency of MCF-7 Epi\textsuperscript{R} cells, I generated MCF-7 Epi\textsuperscript{R} cells stably transfected with pcDNA3-flag-OTUB1 expression vector (pcDNA3-flag-OTUB1). Firstly, MCF-7 Epi\textsuperscript{R} cells were transfected with pcDNA3-flag-OTUB1 vector containing a geneticin (G418) resistance gene. The next day, the transfected cells were treated with 200 µg/ml geneticin and the medium changed until no cell death was observed. This would mean that only the positive pcDNA3-flag-OTUB1 transfected cells had been selected. The amount of geneticin ideal for selection was initially optimised using SRB assay and a killing curve obtained for MCF-7 Epi\textsuperscript{R} cells. From this result, 200 µg/ml of geneticin was chosen as the optimal concentration since it was the lowest amount of drug that could kill the maximum number of cells (Figure 5.15). When growth was achieved, single cell clones were seeded and expanded for 3 - 4 weeks in 96-well plates through serial dilutions (Figure 5.16A). Afterwards, to confirm the successful stable transfection of pcDNA3-flag-OTUB1, cells were harvested and analysed by western blot (Figure 5.16B). From the eight clones assessed, only clone number 8 was found to overexpress pcDNA3-flag-OTUB1.
Figure 5.15 Geneticin killing curve of MCF-7 Epi<sup>R</sup> cells MCF-7 Epi<sup>R</sup> cells were seeded in 96-well plates and treated with various concentrations of geneticin (0-1000 µg/ml). The percentage of cell survival was determined using SRB assay. According to the graph, 200 µg/ml of geneticin was the optimal concentration for cell selection as it is the lowest concentration that can kill the highest number of cells.
Figure 5.16 Screening of single cell clones of the pcDNA3-flag-OTUB1 stably transfected MCF-7 EpiR cell line A. The diagram shows the seeding and the serial dilutions performed (Adapted from “Cell cloning by serial dilution in 96 well plates protocol” (corning) by John A Ryan). B. Single cell clones were selected and grown in the presence of 200 μg/ml of geneticin. Western blot was used to screen for the overexpression of pcDNA3-flag-OTUB1. Total ERK was used as a loading control. Arrow indicates endogenous OTUB1. Asterisk indicates the transfected pcDNA3-flag-OTUB1 band.
5.2.8 Characterisation of OTUB1 single cell clones stably transfected MCF-7 EpiR cells

In order to study the effect of overexpressing flag-OTUB1 on FOXM1 expression and epirubicin sensitivity in MCF-7 EpiR cells, the pcDNA3-flag-OTUB1 stably transfected MCF-7 EpiR cells (clone8) were analysed by western blotting, qPCR and SRB assays. Clone number 1 was included as a negative control.

Clone1 and clone 8 were treated with 1 μM epirubicin and harvested after 0, 6 and 24 h of treatment. The levels of FOXM1 and OTUB1 were determined by western blotting (Figure 5.17A). The comparison of clone1 and clone 8 by densitometry quantification revealed no significant differences in the protein levels of FOXM1; however, OTUB1 overexpression was again confirmed for clone number 8 (Figure 5.17B). Because of this result, clone 8 cells were then treated with 1 μM of epirubicin and harvested at 0, 4, 8, 16, 24, and 48 h of a time course for western blotting analysis.

Since I hypothesised that OTUB1 would contribute to the degradation of FOXM1, I would expect the FOXM1 levels to decline over the time course with epirubicin treatment. Instead, the expression of FOXM1 was relatively constant and was even increased after 48 h of epirubicin treatment (Figure 5.18A). The densitometry quantification also showed no significant changes on the FOXM1 levels after the treatment (Figure 5.18B). The mRNA expression levels of FOXM1 and OTUB1 were also examined by qPCR. Surprisingly, there were no significant differences for the transcript levels of OTUB1 and FOXM1 mRNA levels after 8 h of epirubicin treatment (Figure 5.19A). However, at 0 h and 4 h of epirubicin treatment, the mRNA expression
levels of FOXM1 and OTUB1 in clone number 8 was unexpectedly higher compared to clone number 1 (Figure 5.19B).

After examining the transcript and protein levels of FOXM1 and OTUB1, the sensitivity of both clones number 1 and 8 to epirubicin was assessed by SRB assay. Cells were treated with increasing amounts of epirubicin for 24 h. The result showed no differences in the growth rate between clone 1 and clone 8, even though the concentration of epirubicin was increased to 100 μM. This result indicates that the pcDNA3-flag-OTUB1 stable transfection failed to re-sensitise MCF-7 EpiR cells to epirubicin.
Figure 5.17 Protein analysis of MCF-7 Epi$^R$ stably transfected with pcDNA3-flag-OTUB1. A. Single cell clones number 1 (Flag-OTUB1 negative) and number 8 (flag-OTUB1 positive) were treated with 1 μM epirubicin and harvested after 0, 6 and 24 h of epirubicin treatment. Total protein lysates were analysed by western blotting. Arrow indicates endogenous OTUB1. Asterisk indicates the transfected pcDNA3-flag-OTUB1 band. B. Densitometry analysis of FOXM1 levels were quantified by Image J and normalised with the bottom band of total ERK.
Figure 5.18 Time course treatment analysis of MCF-7 EpiR clone number 8 stably transfected with pcDNA3-flag-OTUB1

A. Single cell clone number 8 (flag-OTUB1 positive) was treated with 1 μM epirubicin and harvested at different time points (0, 4, 8, 16, 24 and 48 h). Total protein lysates were analysed using western blotting. Arrow indicates endogenous OTUB1. Asterisk indicates the transfected pcDNA3-flag-OTUB1 band.

B. Densitometry of FOXM1 levels were quantified by ImageJ and normalised to the bottom band of total ERK.
Figure 5.19 Transcript levels of MCF-7 Epi\textsuperscript{R} cells stably transfected with pcDNA3-flag-OTUB1. The mRNA expression levels of A. OTUB1 and B. FOXM1 from clones number 1 and 8 after treatment with 1 µM of epirubicin. The cells were harvest at various time points (0, 4, 8, 16, and 24 h) and were analysed using qPCR. The mRNA expression levels of OTUB1 and FOXM1 were normalised with L19. Statistical analysis was performed using Student’s t-test (*, p ≤ 0.05; **, ≤ 0.01 significant; ns, non-significant).
Figure 5.20 Percentage of cell growth of OTUB1 transfected clones number 1 and 8 in MCF-7 EpiR cells. Single cell clones number 1 and 8 were treated with various concentrations of epirubicin for 24 h. Cell proliferation was measured by SRB assay. The result was normalised to the untreated control. Results represent the average of three independent experiments including 6 replicates ± SD. Statistical analysis was performed using Student’s t-test (ns, non-significant).
5.3 Discussion and conclusion

Many studies have shown that FOXM1 plays a crucial role in DNA damage repair and has a role in chemotherapeutic agent resistance (de Olano et al., 2012; Khongkow et al., 2013; Millour et al., 2011; Monteiro et al., 2013; Myatt et al., 2014). Previous work done in our lab, has shown that ectopic expression of FOXM1 in MCF-7 cells results in increased cell viability, enhancement of DNA damage repair mechanisms and decreased DNA damage γH2Ax foci in response to genotoxic stress (Monteiro et al., 2013). FOXM1 has also been found to be overexpressed in chemotherapy resistant cell lines, further supporting a role for FOXM1 in DNA damage-induced resistance (Millour et al., 2011; Monteiro et al., 2013). Moreover, depletion of FOXM1 in chemotherapy resistant cells, such as MDA-MB-231 cells (triple negative breast cancer), was able to re-sensitise these cells to epirubicin-mediated cell death (Park et al., 2012). It is well established that FOXM1 can be regulated by PTMs, specifically via phosphorylation (Koo et al., 2012). However, the role of FOXM1 SUMOylation and ubiquitination remains unclear.

In Chapters 3 and 4, I have shown that FOXM1 is regulated by SUMOylation and ubiquitination. It is also well known that OTUB1 inhibits the ubiquitin pathway by separating E2 ubiquitin conjugating enzyme from the ubiquitin residues (Rose and Schlieker, 2010). Moreover, OTUB1 has been found to suppress RNF168-dependent ubiquitination pathway by impeding the bound of UBC13 (E2) to RNF168 and preventing it to work as an ubiquitin ligase (Nakada et al., 2010). In the context of cancer development and progression, OTUB1 has been reported to be involved in the
development of metastasis in colon cancer (Liu et al., 2014). OTUB1 has also been found to de-ubiquitinate ERα leading to its stabilisation (Stanisic et al., 2009). However, the role of OTUB1 in breast cancer and epirubicin resistance remains unclear. In fact, OTUB1 is a recently newly discovered protein found to be involved in DNA damage (Nakada et al., 2010). I have previously demonstrated in Chapter 4, that RNF168 has a role in promoting the degradation of FOXM1. Since OTUB1 has been shown to regulate RNF168, I hypothesised that OTUB1 could also play a role in the regulation of FOXM1. Moreover, as a deubiquitinating enzyme, it is possible that OTUB1 is involved in the deubiquitination of FOXM1. The regulation of FOXM1 by OTUB1 will provide a clearer understanding of how FOXM1 is regulated. In this chapter, I have studied the relationship between OTUB1 and FOXM1 and the role of OTUB1 in epirubicin resistance in breast cancer cell lines.

I found that the protein and mRNA levels of OTUB1 were deregulated in MCF-7 cells and MCF-7 EpiR cells. Accordingly, OTUB1 mRNA levels in MCF-7 EpiR cells were down-regulated in comparison with MCF-7 cells. Interestingly, western blot analysis showed a double band pattern for OTUB1 protein (31 kDa and 35 kDa) in MCF-7 cells (Figure 5.1). In contrast, the higher molecular weight band (35 kDa) was absent in the MCF-7 EpiR cells. This higher molecular band of OTUB1 could represent a modified form of OTUB1 in MCF-7 cells. In fact, to date, there are not many known PTMs targeting OTUB1. Yet, one study in U2OS cells showed that OTUB1 can be modified by mono-ubiquitination. The ubiquitinated-deficient OTUB1 mutant leads to the loss of its function as a deubiquitinating enzyme (Li et al., 2014).
Similarly to MCF-7 EpiR cells, Foxm1<sup>−/−</sup> MEFs also expressed lower levels of OTUB1 than wt MEFs. A previous study in our lab showed Foxm<sup>−/−</sup> MEFs were found to be more sensitive to epirubicin than wt MEFs (Monteiro et al., 2013). Similarly to what was observed for MCF-7 cells, Foxm1<sup>−/−</sup> MEFs also showed increased levels of the higher molecular band of OTUB1 (35kDa) than the wt MEFs. The fact that putative bands of similar pattern as FOXM1 are found in Foxm1<sup>−/−</sup> MEFs was surprising. However, the absence of FOXM1 was confirmed by qPCR results in Foxm1<sup>−/−</sup> MEFs. In addition, nuclear-cytoplasmic fractionation was performed to determine the sub-cellular localisation of OTUB1 and FOXM1. The western blot revealed that despite the fact that OTUB1 was found in both the cytoplasmic and nuclear fractions, it was preferentially located in the cytoplasm. In agreement, a previous study in U2OS cells shows that OTUB1 regulates p53 stability in the cytoplasm. This study also claims that OTUB1 is a cytoplasmic deubiquitinating enzyme (Sun et al., 2012; Sun and Dai, 2014). It is possible that OTUB1 might work in the cytoplasm to de-ubiquitinate FOXM1, which was also found in the cytoplasm after being SUMOylated and ubiquitinated (Chapter 3). The loss of the top band of OTUB1 in the sub-cellular fractionation experiment might due to the difference in composition of the lysis buffer used in this experiment, as mentioned above.

It is widely known that ubiquitination is associated with DNA double strand break (DSB) repair (Bohgaki et al., 2013; Li et al., 2011; Nakada et al., 2010; Zhao et al., 2014) and this repair is widely known to have a key role in chemotherapy resistance. As a result, deubiquitinating enzymes such as OTUB1 might be involved in chemotherapy resistance. In order to examine the role of OTUB1 in epirubicin resistance, MCF-7 cells
were transfected with pcDNA3-flag-OTUB1 or pcDNA3 empty vector control and cell survival determined by SRB assays. The transfected cells were treated with various concentrations of epirubicin. The results showed that OTUB1 overexpressing cells were more sensitive to epirubicin, especially at high concentrations compared to the empty vector control cells. In addition, I examined the effect of OTUB1 silencing on epirubicin sensitivity in MCF-7 cells. The MCF-7 cells were then transfected with siRNA against OTUB1 (siOTUB1) or NSC, or mock transfected, before the cell viability determined by SRB assays. The cell survival results showed that OTUB1 depleted cells were more resistance to epirubicin compared with the NSC siRNA-transfected cells. This suggests that OTUB1 might have an important role in mediating epirubicin resistance in MCF-7 cells. In accordance, OTUB1 has been found to play a part in the DNA damage response pathway (Nakada et al., 2010). In this chapter, I studied the role of OTUB1 in DNA damage and epirubicin resistance. COMET assay was used to determine the amount of DNA damage in OTUB1 silenced MCF-7 cells. According to the SRB results, the OTUB1 knock-down cells showed significant differences at lower concentrations (0.1 µM) of epirubicin. As a result, the COMET assay was performed using the OTUB1 silenced cells. The result showed that in OTUB1 depleted cells treated with epirubicin for 24 h, there was less DNA damage than in the NSC control. Collectively, these data suggest that silencing of OTUB1 promotes epirubicin resistance in MCF-7 cells, confirming that OTUB1 has a role in DNA damage response pathway. This is further supported by a previous study which shows that the ubiquitin-deficient mutant OTUB1 leads to an inhibition of DNA-damage induced apoptosis (Li et al., 2014).
So far these results show that OTUB1 has a role in promoting sensitivity of MCF-7 cells to epirubicin. Next, in order to prove that OTUB1 plays a part in sensitising cells to epirubicin-mediated cell death, I overexpressed OTUB1 in MCF-7 EpiR cells. To this end, MCF-7 EpiR cells were transiently transfected with pcDNA3-flag-OTUB1. SRB and COMET assays were then performed to assess the effects on cell survival and DNA damage. The SRB assay result shows that the pcDNA3-flag-OTUB1 transfected MCF-7 EpiR cells have a lower cell growth compared to the control pcDNA3 empty vector transfected cells. Although the percentage of cell growth diminishes, the pcDNA3-flag-OTUB1 transfected MCF-7 EpiR cells do not die following the treatment with epirubicin. Similarly, COMET assays show no differences in the amount of DNA damage after treatment with epirubicin. These results suggest that OTUB1 is unable to re-sensitise MCF-7 EpiR cells to epirubicin, but maintains the growth rate of these cells constant. In addition, it is possible that the MCF-7 EpiR cells overexpressing OTUB1 represent a selectively enriched MCF-7 EpiR cell population that can withstand and adapt to the expression of OTUB1. One possibility is that these cells can deregulate or alter existing signalling pathways crucial to maintaining the epirubicin resistant phenotype.

Although the pcDNA3-flag-OTUB1 overexpression cannot re-sensitise MCF-7 EpiR cells to epirubicin, it promotes DNA damage and epirubicin sensitivity in MCF-7 cells. In addition, the OTUB1 expression levels show a negative correlation with FOXM1 expression levels (i.e. MCF-7 cells expressed higher levels of OTUB1 and lower levels of FOXM1) in comparison with MCF-7 EpiR cells. This led me to investigate whether FOXM1 and OTUB1 can regulate each other. Based on previous studies it has been shown that FOXM1 is regulated by ERα (Millour et al., 2010) and that ERα is regulated
by OTUB1 (Stanisic et al., 2009). It is therefore possible that OTUB1 regulates FOXM1 indirectly via the regulation of other upstream FOXM1 regulators, such as ERα or RNF168. To test whether OTUB1 directly regulates FOXM1, the interaction between FOXM1 and OTUB1 needs to be identified. Using co-immunoprecipitation, I found an interaction of FOXM1 and OTUB1 in both MCF-7 and MCF-7 EpiR cells. In addition, the interaction increased after 6 h of epirubicin treatment in both cell lines. Interestingly, in MCF-7 cells this interaction decreased after 24 h treatment while the interaction in MCF-7 EpiR cells remained through the 24 h of treatment. As I mentioned above, the OTUB1 35kDa band was found only in MCF-7 but not in MCF-7 EpiR cells. This higher molecular weight band might be a modified form of OTUB1 that impairs disassociation of OTUB1 from FOXM1. To further elucidate the regulation of FOXM1 by OTUB1 and vice versa, I then knocked down and overexpressed OTUB1 to investigate whether there was an effect on FOXM1 expression. Both in the absence or presence of epirubicin treatment, there were no differences in either protein or mRNA levels. Conversely, I also depleted and overexpressed FOXM1 to study the effect on OTUB1 levels. Similarly, there were no differences in either the protein or mRNA levels of OTUB1. This data showed that OTUB1 has no effect on FOXM1 expression, and FOXM1 does not regulate OTUB1 expression. However, this does not exclude the hypothesis that OTUB1 regulates FOXM1 indirectly. It is already known that upon DNA damage FOXM1 might be recruited to damage sites to initiate repair together with other cofactor proteins (Monteiro et al., 2013; Tan et al., 2007). One could hypothesise that OTUB1 de-ubiquitinates one of these FOXM1-repair-cofactors that will affect the function of FOXM1. This is supported by the study of Nakada et al. (2010), in which
OTUB1 is shown to act as an inhibitor of DSB-induced chromatin ubiquitination (Nakada et al., 2010). The ubiquitination of chromatin serves as a signal for recruiting other repair proteins (Doil et al., 2009) to the damaged site. In this way, it is possible that OTUB1 removes these ubiquitin signals to mark the end of the DNA damage repair process. In addition, it is also possible that OTUB1 de-ubiquitinates a FOXM1 regulator protein, such as E2F1. In fact, E2F1 has been reported to be able to be modified by ubiquitination (Putzer and Engelmann, 2013). E2F1 has been found to function together with FOXM1 as co-factors (Sullivan et al., 2012) as well as to be a key regulator of FOXM1 (Millour et al., 2011).

Finally, in order to understand the role of OTUB1 in epirubicin resistance, I generated a MCF-7 EpiR single-cell clone stably overexpressing OTUB1 to ensure that the entire cell population contained pcDNA3-flag-OTUB1. Unfortunately, the stable OTUB1 transfected cells show no differences in FOXM1 levels, neither before nor after epirubicin treatment. When the cells were assayed for SRB, the result also showed that the stable OTUB1 transfection could not re-sensitise MCF-7 EpiR cells to epirubicin. However, this can be due to the fact that the OTUB1 expressing cells have adapted to the tumour suppressing function of OTUB1. In summary, although the levels of OTUB1 in sensitive and resistant cells appear to be differentially regulated, OTUB1 expression levels increase the sensitivity of MCF-7 cells. However, OTUB1 fails to re-sensitise MCF-7 EpiR cells to epirubicin.
5.4 Future work

From the results obtained in this chapter, the role of OTUB1 in terms of FOXM1 regulation and epirubicin resistance remains unclear. To further explore the role of OTUB1 in DNA damage and repair, more experiments should be done. For example, to clarify the role of OTUB1 in DNA damage, one could use repair assays specific for homologous recombination (HR) and non-homologous end joining (NHEJ) (Khongkow et al., 2013). Although my results did not show a direct regulation of FOXM1 by OTUB1, it is still possible that there could be an indirect regulation of FOXM1 by OTUB1. As a deubiquitination enzyme, OTUB1 might have a role in deubiquitinating FOXM1 or its upstream regulators. In order to study that, the ubiquitination of FOXM1 should be examined in cells in which OTUB1 is overexpressed and silenced. In addition, the OTUB1 35kDa-band that was found in MCF-7 but not in MCF-7 EpiR cells, could represent a modified form of OTUB1 (Chapter 5). This putative modified form should be identified and characterised. Immunofluorescent and FILM-FRET experiments (Myatt et al., 2014) using confocal microscopy could be useful techniques to confirm the subcellular co-localisation of FOXM1 and OTUB1 in response to epirubicin treatment. This will provide a better understanding of how these two proteins interact and are regulated, and what is the role of OTUB1 de-ubiquitination activity in regards to the FOXM1-mediated DNA damage response. In fact, if OTUB1 has a role in the regulation of FOXM1 and epirubicin resistance, it could represent a potential interesting target for overcoming chemotherapy resistance.
CHAPTER 6

FINAL DISCUSSION

AND CONCLUSION
Data from Cancer Research UK and World Health Organization (WHO) have shown that cancer is the major cause of death worldwide. In 2012, there were 14.1 million patients diagnosed with cancer and more than 8 million people died from the disease (WHO, 2012). Breast cancer is the most common cancer in women, with over 1 million people getting diagnosed every year and the rate increasing annually (Ferlay et al., 2010; Kamangar et al., 2006). Conventional chemotherapy is the most common treatment strategy for breast cancer. Chemotherapy resistance is the main cause of treatment failure (>90%) in patients with metastasis (Longley and Johnston, 2005).

The Forkhead box protein M1 (FOXM1) has been found to be upregulated in many types of cancer, including breast cancer (Koo et al., 2012). In addition, FOXM1 upregulation has been found to be related to resistance to various cancer treatments, including endocrine therapy (Millour et al., 2010), cisplatin (Kwok, 2010), Trastuzumab (also known as Herceptin), paclitaxel (Carr et al., 2010), and also anthracyclines (Halasi and Gartel, 2012; Millour et al., 2011; Monteiro et al., 2013; Park et al., 2012). I have confirmed in Chapter 3 that FOXM1 plays an important role in preventing DNA damage and promoting epirubicin resistance. As shown in a previous study, the lack of FOXM1 leads to increase level of DNA damage and loss of the DNA repair ability (Monteiro et al., 2013). In agreement, depletion of FOXM1 can re-sensitise the epirubicin resistant triple negative MDA-MB-231 breast cancer cells to epirubicin (Park et al., 2012) and the highly resistant MCF-7 EpiR cells to undergo cellular senescence (Khongkow et al., 2013). Moreover, FOXM1 has been shown to promote the DSB repair (Millour et al., 2011; Monteiro et al., 2013) and target some genes, including EXO1, PLK4 and RFC4, that are necessary for the DSB repair mechanism (Park et al., 2012). In accordance,
FOXM1 has become an attractive target for breast cancer therapy and for reversing of the chemotherapy resistance. For example, a small molecule FOXM1 inhibitor, thiostrepton, can specifically induce cell death in breast cancer cells. This drug has also been shown to have a minimal toxicity against non-cancerous cells (Kwok et al., 2008). There is accumulating evidence that FOXM1 is regulated at the transcriptional and post-translational levels in response to conventional chemotherapeutic drug treatments (Lam et al., 2013). Thus, an understanding of the PTMs of FOXM1 might help to reveal how FOXM1 is regulated and lead to the development of drugs, which can improve the breast cancer therapy and overcome the FOXM1-mediated chemotherapy resistance.

SUMOylation and ubiquitination has been reported to be important for regulating the transcription and degradation of targeted proteins. Although SUMOylation has various effects on target proteins, most of the SUMOylation events have been found to result in transcriptional repression (Ouyang et al., 2009). Here, I establish that FOXM1 can be modified by SUMO1 in response to cytotoxic anticancer agents, including epirubicin and paclitaxel treatment (Myatt et al., 2014). In my study, interaction of FOXM1 and SUMO2/3 could not be found in these breast cancer cell lines. However, it is still possible the FOXM1 can interact with SUMO2/3 in response to other stress stimuli or in other cell types.

It has previously been shown that SUMOylation of FOXM1 leads to its ubiquitination and subsequent nuclear export. Once FOXM1 has been exported out of the nucleus, FOXM1 is targeted by the proteasome system and degraded (Figure 6.1). This is in agreement with studies on other Forkhead transcription proteins, such as FOXA (Bao et
al., 2011), FOXL2 (Georges et al., 2011; Marongiu et al., 2010), and FOXC1/C2 (Danciu et al., 2012), showing that SUMOylation leads to their degradation. I have also shown in Chapter 3 that the SUMOylation-deficient FOXM1 (FOXM1 5x(K>R)) cannot be ubiquitinated and accumulates in the nucleus, as confirmed by Leptomycin B treatment (Jang et al., 2003; Wolff et al., 1997). Interestingly, this finding is in agreement with a study performed by Dr. Stephen Myatt, in which mutations in SUMO1 specific-sites on FOXM1 leads to an upregulation of the function of FOXM1 and delays its mitotic exit (Myatt et al., 2014). These data emphasise that SUMOylation could be a novel switchable regulator that controls the transactivation activity of many important transcription factors, including FOXA, FOXL2, FOXC1/2 and FOXM1.
Figure 6.1 Model summarising the impact of SUMOylation on FOXM1 function and degradation. In normal cells, FOXM1 is SUMOylated at the mitotic exit or after undergoing genotoxic stress. This leads to the ubiquitination and nuclear export of FOXM1. Subsequently, FOXM1 degradation is mediated via the APC/C-Cdh1 complex. FOXM1 degradation is required for the initiation of the mitotic exit from the cell cycle and the down regulation of its downstream target, such as Cyclin B1 and Aurora B kinase. In contrast, FOXM1 5x(K>R) which contains 5 point mutations of SUMO specific-motifs cannot be SUMOylated. This SUMOylation-deficient FOXM1 also leads to a defect in the ubiquitination and, thus this results in the accumulation of FOXM1 in the nucleus. Consequently, the cells with SUMOylation-deficient FOXM1 are delayed in the mitotic exit.
In addition, I also show in Chapter 3 that the APC/C-Cdh1, a complex responsible for targeting cell cycle proteins for ubiquitination and subsequent degradation, is associated with the regulation of FOXM1 degradation. This association has been shown to target FOXM1 to degradation and therefore resulting in mitotic exit (Laoukili et al., 2008; Park et al., 2008). This APC/C-Cdh1 complex has been reported to bind directly to D- and KEN-box motifs at the N-terminus of FOXM1 (Laoukili et al., 2008). I also found that increasing concentrations of Cdh1 protein could promote and target exclusively the wild-type, but not the SUMOylation-deficient, FOXM1 for degradation. This can explain why the mutations to SUMO specific-motifs on FOXM1 can lead to an increase in the ability of FOXM1 to transactivate the cyclin B1 promoter, a transcriptional downstream target of FOXM1 (Laoukili et al., 2005; Leung et al., 2001), in a forkhead binding element-dependent manner. Accordingly, this SUMOylation-deficient FOXM1 has also been found to effectively promote epirubicin resistance (Halasi and Gartel, 2012; Millour et al., 2011; Monteiro et al., 2013; Park et al., 2012).

Although the study in Chapter 3 shows that Cdh1 is important for the binding of the APC/C complex to FOXM1 (Laoukili et al., 2008; Park et al., 2008), its degradation is not entirely dependent on the APC/C complex as ubiquitination of SUMOylated FOXM1 will depend on other SUMOylation and/or substrate-specific ubiquitination. This suggests that other ubiquitin ligases such as RNF4, RNF8 and RNF168, could act as the mediators of the crosstalk between SUMOylation and ubiquitination of FOXM1. Importantly, these ligases have been reported to be involved with the DNA damage response (Munoz et al., 2012; Nakada et al., 2010). I investigated this by exploring the possibility that some of these potential RING-type E3 ligases (eg. RNF4, RNF168 and
RNF8) can act as upstream regulators of FOXM1 and prime its ubiquitination. I found that the expression of FOXM1 correlates with RNF4 expression, which is inversely related to RNF168, while RNF8 does not show much difference after epirubicin treatment. The result from RNF4 indicates that it has a role in promoting the FOXM1 expression at both mRNA and protein levels. As RNF4 is known to be one of the STUbLs, SUMO-targeted ubiquitin E3 ligases (Fryrear et al., 2012; Hu et al., 2010; Yin et al., 2012), I then hypothesised that RNF4 might target FOXM1 to regulate its expression. At odds with my prediction, RNF4 did not form a direct interaction with FOXM1 or RNF168. Furthermore, RNF4 did not have an effect on the degradation of FOXM1 via the ubiquitin-proteasome pathway. A possible explanation could be that RNF4 normally targets the poly-SUMO2/3 chains (Hu et al., 2010; Tatham et al., 2008; Yin et al., 2012), and my results from chapter 1 show that FOXM1 is not modified by SUMO2/3. In addition, unlike SUMO2/3, SUMO1 cannot form poly-SUMOylation chains, which are crucial for binding by RING-finger proteins, such as RNF4 and RNF8. (Bergink and Jentsch, 2009). This could explain why RNF4 does not target FOXM1 and regulate its expression in MCF-7 cells. Nevertheless, the modification of FOXM1 by SUMO1 happens at 5 sites which are closed located (Myatt et al., 2014), and this might function in a similar manner as the SUMO2/3 chains. The fact that that RNF4 does not regulate the degradation of FOXM1 in a response to epirubicin treatment might clarify the reason why RNF4 only affects the FOXM1 protein and mRNA expression levels and the cell proliferation, but not the epirubicin sensitivity of MCF-7 cells.

However, I have confirmed the importance of the RING-domain of RNF4 by showing that it indirectly regulates the transcriptional activity of cyclin B1. The CS RNF4 mutant
contains a mutation in the RING finger domain, resulting in a change from cysteine to serine (Ahner et al., 2013; Luo et al., 2012; Tatham et al., 2008), which abolishes the ability of RNF4 to ubiquitinate targeted proteins (Ahner et al., 2013; Luo et al., 2012; Pero et al., 2001). This mutation resulted in the loss of the regulation of the transcriptional activity of cyclin B1 by RNF4, indicating that ability of RNF4 to promote ubiquitination is required for it to regulate the activity of FOX proteins, including FOXM1.

In contrast to RNF4, RNF168 suppresses the expression of FOXM1, especially after epirubicin treatment. Although RNF168 expression did not affect the growth of MCF-7 cells, it sensitised the cells to epirubicin treatment. This might be because the regulation of FOXM1 expression by RNF168 is modulated by the epirubicin-induced genotoxic stress signals. This is consistent with previous reports, which suggest a role for FOXM1 in the promotion of epirubicin resistance (Halasi and Gartel, 2012; Millour et al., 2011; Monteiro et al., 2013; Park et al., 2012). By repressing FOXM1 expression, RNF168 promotes MCF-7 cells sensitivity to epirubicin. Moreover, RNF168 has also been reported to be involved in the ubiquitination of histone H2A and DDR signalling (Mattiroli et al., 2012; Pinato et al., 2011). Interestingly, RNF168 can also promote the transcriptional activity of the cyclin B1 promoter in a forkhead binding element-dependent manner, further suggesting RNF168 regulates FOXM1 expression and activity. Importantly, my data show that RNF168 forms complexes with FOXM1 and therefore suggest that RNF168 may bind FOXM1 directly to promote its degradation. Notably, the result from the co-immunoprecipitation experiment showed that RNF168 can interact only with the SUMOylated form of FOXM1, but not with the SUMOylation-deficient FOXM1. Moreover, in the presence of epirubicin, RNF168 can only promote
the degradation of the wild type FOXM1, but not of the SUMOylation-deficient FOXM1. Together these results suggest that RNF168 mediates the degradation of FOXM1 and this process requires FOXM1 SUMOylation. Interestingly, RNF168 does not have an obvious SUMO-interacting motif (SIM) yet it has been clearly shown to target SUMOylated FOXM1. SIMs are motifs that other RING-domain E3 ligases use to interact with their SUMOylated substrates. However, it is possible that RNF168 targets SUMOylated FOXM1 via a mediator protein such as RNF8 or some other STUbLs. RNF8 has also been reported to have SIMs that recognise SUMOylated proteins (Bartocci and Denchi, 2013; Danielsen et al., 2012; Galanty et al., 2012) and it has also been found to function together with RNF168 (Danielsen et al., 2012; Galanty et al., 2009; Mallette et al., 2012). In line with this, a study performed in U2OS cells showed the interaction between RNF168 and RNF8. This interaction was capable of generating k48 linked ubiquitin chains to JMJD2A, a transcriptional co-factor, thus helping in its degradation (Mallette et al., 2012). Consistently, the expression of RNF8 also affected FOXM1 in a similar way as RNF168 but its impact was less noticeable. Taken together, I demonstrate that SUMOylation leads to the ubiquitin-proteasome degradation of FOXM1 and this is at least in part mediated by RNF168, which might occur with the help of Ring-domain proteins, such as RNF8 (Figure 6.2). The fact that FOXM1 expression is modulated by SUMOylation and ubiquitination led me to ask if deubiquitinating enzymes might also have a role in FOXM1 regulation. In the last chapter of this thesis, I then investigated the importance of a deubiquitinating enzyme, OTUB1, in regulating FOXM1 expression.
OTUB1 (Ovarian Tumour domain-containing Ubiquitin aldehyde-Binding protein 1) is a deubiquitination enzyme (DUB) that has been reported to inhibit DSB-induced ubiquitination (Nakada et al., 2010; Rose and Schlieker, 2010). In cancer, OTUB1 was found to be upregulated and involved in colon cancer development and metastasis (Liu et al., 2014). In addition, OTUB1 has also been found to be a repressor for RNF168-dependent poly-ubiquitination (Nakada et al., 2010). Furthermore, OTUB1 has also been reported to regulate various proteins, such as p53 (Li 2014, Sun 2012), oestrogen receptor alpha (ERα) (Stanisic 2009). Interestingly, these proteins have been shown to play a crucial role in carcinogenesis and epirubicin resistance (Horimoto et al., 2011; Millour et al., 2010; Millour et al., 2011). In line with this, I hypothesised that OTUB1 might have a role in stabilising FOXM1, by inhibiting its ubiquitination by RNF168. Thus, in chapter 5, I intended to explore the importance of OTUB1 in regulating FOXM1 and epirubicin sensitivity. However, my results suggested that OTUB1 does not promote FOXM1 stability. I also found an inverse correlation between OTUB1 and FOXM1 expression. This can be seen in both MCF-7 cells and MEFs. MCF-7 cells were observed to have higher levels of OTUB1 compared with MCF-7 EpiR cells. In agreement, Foxm1−/− MEFs also showed a higher OTUB1 expression than wt MEFs. It is also noticeable that OTUB1 is upregulated in sensitive cells (MCF-7 cells), but not in their resistant counterparts. In fact, OTUB1 has been reported to promote apoptosis and inhibit cell proliferation in a p53-dependent manner (Sun et al., 2012). In agreement, my experiments also indicated that an overexpression of OTUB1 in sensitive cells might lead to an increase in the sensitivity of the cells to epirubicin-induced DNA damage and cell death.
**Figure 6.2 Model summarising the regulation of FOXM1 at post-translational levels.** During transcriptional activation, FOXM1 is phosphorylated by some cell cycle specific cyclins and cdks, complexes and some other kinases such as ATM. Once phosphorylated, FOXM1 is translocated to the nucleus initiating the transactivation of downstream target genes. Then, when FOXM1 becomes inactive, at the mitotic exit, FOXM1 is removed from its binding on target genes, and becomes SUMOylated in a UBC9-dependent manner. This SUMOylation leads to its ubiquitination, which results in the nuclear export of FOXM1 and RNF168-mediated degradation, via the APC/C-Cdh1 complex.
The OTUB1 protein in MCF-7 cells exists as a doublet (31kDa and 35kDa). Interestingly, the higher molecular weight band (35kDa) is absent in MCF-7 EpiR cells. This higher molecular band of OTUB1 could represent a modified form of OTUB1 in MCF-7 cells. In fact, to date, not many known PTMs have been shown to be targeting OTUB1. Yet, one study in U2OS cells showed that OTUB1 can be modified by mono-ubiquitination. The ubiquitinated-deficient OTUB1 mutant loses its function as a deubiquitinating enzyme (Li et al., 2014).

Previous work showed that FOXM1 is regulated by ERα (Cicatiello et al., 2004; Horimoto et al., 2011; Karadedou, 2006; Madureira et al., 2006; Millour et al., 2010). A study in breast cancer cell lines by our lab also demonstrated that ERα activates FOXM1 expression by directly binding to the FOXM1 promoter through an oestrogen-response element (ERE) (Horimoto et al., 2011; Millour et al., 2010). At the same time, ERα has also been found to be regulated by OTUB1 (Stanisic et al., 2009). OTUB1 can de-ubiquitinate ERα, leading to its stabilisation (Stanisic et al., 2009). In addition, OTUB1 has also been shown to promote the stability and enhance the activity of p53 (Sun et al., 2012). Interestingly, p53 has also been shown to downregulate FOXM1 and promote cell sensitivity to DNA damage (Millour et al., 2011). Hence, I firstly examined the effect of OTUB1 on epirubicin sensitivity, and found that OTUB1 overexpression increases the sensitivity of MCF-7 cells to epirubicin while a knockdown reduces the sensitivity of these cells. Moreover, in OTUB1 depleted cells there was a lower amount of DNA damage compared with the control, as revealed by COMET assays. These data suggest the possibility that OTUB1 might downregulate FOXM1 through an intermediate protein, such as ERα (Stanisic et al., 2009) or p53 (Sun et al., 2012). They also indicate
that OTUB1 overexpression in the resistant MCF-7 EpiR cells should re-sensitise these cells to epirubicin. However, the results showed otherwise, and there was a slight reduction in their growth rates. These results are consistent with a recent study showing that OTUB1 suppresses cell growth through stabilising p53 (Li et al., 2014). However, my results from the COMET assay showed that the overexpression of OTUB1 could not re-sensitise the MCF-7 EpiR cells from their resistant phenotype.

All together, these data led me to hypothesise that OTUB1 might have a subtle role in suppressing FOXM1 expression.

Although the co-IP in Chapter 5 showed a direct binding between OTUB1 and FOXM1 in both MCF-7 and EpiR cells, OTUB1 expression did not have an effect over FOXM1 expression, at both mRNA and protein levels. Conversely, FOXM1 expression also did not affect the expression of OTUB1. It is possible that FOXM1 and OTUB1 might form a complex, as OTUB1 can also bind to RNF168 (Nakada et al., 2010) and p53 (Sun et al., 2012), but this does not appear to be regulating the expression of FOXM1. This complex might, however, play a role in other pathways, such as the ubiquitination of histone-H2AxA (Nakada et al., 2010) in response to DNA damage (Wiener et al., 2012). Moreover, in MCF-7 cells OTUB1 affected their cell growth and epirubicin sensitivity yet it fail to re-sensitise MCF-7 EpiR cells to the drug. This also suggests that there might be other DUBs playing a role in the stabilisation of FOXM1. In future, deubiquitinase profiling assays need to be performed (Ye et al., 2011) in order to unravel potential new DUBs. It is possible that OTUB2, another deubiquitinating enzyme, might compensate for the loss of OTUB1 in FOXM1 regulation. As a consequence, further work has to be
done to define the role of OTUB1/2 in the de-ubiquitination of FOXM1 and drug sensitivity.

Although OTUB1 has been found to form a complex with FOXM1, it might not regulate FOXM1 expression. It is possible that OTUB1 de-ubiquitinates a FOXM1 regulator protein, such as E2F1. In fact, E2F1 has been reported to be able to be modified by ubiquitination (Putzer and Engelmann, 2013). E2F1 has previously been found to cooperate with FOXM1 to regulate target gene expression (Millour et al., 2011; Sullivan et al., 2012).

The role of FOXM1 in tumorigenesis and in promoting drug resistance renders it a target for therapeutic intervention. Indeed, thiazole antibiotics, including thiostrepton and Sianomycin A, have been shown to inhibit FOXM1 expression specifically at the mRNA and protein levels. Thiostrepton has been shown to bind directly to the DNA-binding domain of FOXM1 and thereby preventing it from activating the transcription of target genes and hastening it degradation. Interestingly, thiostrepton appears to target cancer cells specifically and has minimal effects on the proliferation of non-cancerous cells (Kwok et al., 2008). The reason for this is unclear but it could be due to the fact that cancer cells express high levels of FOXM1 and have become addicted to the elevated levels of FOXM1 for its continuous survival. Indeed, while silencing FOXM1 in breast cancer cells can sensitisise them to DNA damaging agents (Khongkow et al., 2013), FOXM1 depletion efficiently induces drug resistant breast cancer cells, which express high levels of FOXM1, to undergo senescence. The finding that FOXM1 expression is modulated by SUMOylation and ubiquitination also suggests the idea that
small molecules or natural compounds that enhance SUMOylation and ubiquitination may enhance the efficacy of conventional chemotherapeutic drugs. In the same way, it is also important to unveil the mechanism that regulates FOXM1 SUMOylation and ubiquitination, as this may open up further avenues for pharmacological intervention to inhibit FOXM1 activity and expression.

In summary, my work has established that FOXM1 is regulated by post-translational modifications (PTMs), such as SUMOylation and ubiquitination. This suggests that SUMOylation of FOXM1 by SUMO1 leads to its ubiquitination and subsequently RNF168-mediated degradation, via the APC/C-Cdh1 dependent proteasome pathway. I have also confirmed the importance of FOXM1 in DNA damage signalling and in promoting epirubicin resistance (Monteiro et al., 2013). Through SUMOylation FOXM1 can be down regulated. This reduction in FOXM1 expression promotes the sensitivity to epirubicin in MCF-7 cells (Khasraw et al., 2012; Minotti et al., 2004). This study provides valuable information about the regulation of FOXM1 protein, an oncoprotein that plays an important role in breast cancer chemotherapy resistance. Crucially, this could represent the start-point for the development of targeted therapies that will promote the degradation of FOXM1 in order to reduce its activity in breast cancer and help prolong breast cancer patient life.
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