

FOXM1 in breast cancer and drug resistance

Thesis submitted by

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DECLARATION OF ORIGINALITY

Unless otherwise stated in text, this thesis is the result of my own work.

ABSTRACT

Endocrine agents have become the primary adjuvant treatment for breast cancer. In addition to endocrine therapy, cytotoxic chemotherapeutic agents have also been frequently used in the neoadjuvant and adjuvant settings, to reduce tumour size prior to surgery or to reduce the chance of relapse or metastasis. However, patients can be resistant to endocrine and chemotherapeutic agents, or become resistant after long term treatment. In this study, I investigated the role and the regulation of FOXM1 in the sensitivity and resistance to the endocrine agent, tamoxifen, and the cytotoxic chemotherapeutic agent, epirubicin. Firstly, I demonstrated that tamoxifen repressed FOXM1 expression in sensitive but not in tamoxifen resistant breast cancer cell lines. In MCF-7 cells, FOXM1 protein and mRNA expression levels were regulated by ER-ligands, and depletion of ER α by RNA interference down-regulated FOXM1 expression. Importantly, ectopic expression of FOXM1 abrogated the cell cycle arrest mediated by the anti-oestrogen tamoxifen, and conferred tamoxifen resistance to MCF-7 cells. In contrast, silencing of FOXM1 in tamoxifen resistant cells abolished oestrogen-induced MCF-7 cell proliferation and overcame acquired tamoxifen resistance. Secondly, FOXM1 expression analysis in epirubicin resistant MCF-7 cells showed a higher level compared with MCF-7 cells. In addition, epirubicin treatment down-regulated FOXM1 expression in MCF-7, but FOXM1 protein level remained constant in epirubicin resistant MCF-7 cells. I established that p53 repressed FOXM1 expression in MCF-7 cells, while this protein is lost in the MCF-7 epirubicin resistant cells. I also found that ataxia-telangiectasia mutated (ATM) was overexpressed at protein and mRNA levels in epirubicin resistant MCF-7 compared with MCF-7 cells, and that ATM depletion strongly decreased FOXM1 expression. Epirubicin treatment increased DNA damage levels in MCF-7 cells while this remained constant in similarly treated epirubicin resistant MCF-7 cells, suggesting a higher level of DNA repair in these cells. Taken together, these results indicate that deregulation of FOXM1 may contribute to resistance to endocrine and cytotoxic agents through its involvement in cell proliferation and DNA repair.

PUBLICATIONS

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Kwok JM, Peck B, Monteiro LJ, Schwenen HDC, **Millour J**, Coombes RC, Myatt SS, Lam EW. *Mol Cancer Res* 2010; 8(1):24-34 FOXM1 confers acquired Cisplatin resistance in Breast Cancer cells.

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ABBREVIATIONS

5-FU	5-fluorouracil
ABC-transporters	ATP-binding cassette transporters
AF	Activation function
AI	Aromatase inhibitor
ARF	Alternative Reading Frame
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BRCA1	Breast cancer susceptibility gene 1
BSA	Bovine Serum Albumin
CDK	Cyclin dependent kinase
DNA	Deoxyribonucleic acid
cDNAs	Complementary deoxyribonucleic acid
CHK2	Checkpoint kinase 2
CKI	Cyclin dependent kinase inhibitors
DBD	DNA binding domain
DMEM	Dulbecco.'s Modified Eagle.'s Medium
DMSO	Dimethyl sulphoxide
DNA-PK	DNA-dependent protein kinase
dNTP	Di-nucleotide triphosphate
DSB	DNA double strand break
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
ERAP	ER-interacting proteins
ERE	Oestrogen response element
ERK	Extracellular signal-regulated kinase
FCS	Foetal Calf Serum
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor
HR	Homologous recombination
ICI or ICI182780	Fulvestrant
IGFR	Insulin-like growth factor
IR	Ionizing radiation
LBD	Ligand-binding domain
M	Mitotic phase
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MEF	Mouse embryonic fibroblasts
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MRP1	Multidrug resistance-associated protein 1
MUC4	Membrane-associated glycoprotein mucin-4
NBS1	Nijmegen breakage syndrome
NcoR	Nuclear receptor co-repressor

NHEJ	Non-homologous end-joining
NSC	Non-specific control
OHT	Tamoxifen
PARP1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate Buffered Saline
PGP	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphatidylinositol 3 kinases
PIK3CA	Phosphoinositol 3-kinase catalytic unit
PIP2	3,4,5-triphosphate
PIP3	Phosphatidylinositol 4,5-biphosphoate
PLK1	Polo-like kinase 1
PR	Progesterone receptor
pRB	Phosphorylated retinoblastoma protein
PTEN	Phosphatase and tensin homologue
RB	Retinoblastoma protein
RIP	Receptor interacting proteins
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
S	Synthesis phase
SERD	Selective ER α down-regulators
SERM	Selective ER α modulator
SMC1	Structural maintenance of chromosome protein 1
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
TBP	TATA-binding protein
TEMED	Tetramethylethylenediamine
TK	Tyrosine kinases
TKI	Tyrosine kinase inhibitor
uPA	Urokinase-type kinase plasminogen activator
uPAR	Urokinase-type kinase plasminogen activator receptor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XRCC4	X-ray repair cross-complementing protein 4

CHAPTER 1 INTRODUCTION

1.1 BREAST CANCER

The development of cancer or carcinogenesis is a process by which normal cells transform into cancer cells. It is characterised by a series of alterations occurring at genetic and cellular levels leading to uncontrolled cell division and consequently formation of a malignant mass. These alterations affect two types of genes: oncogenes and tumour suppressors. An oncogene is a gene involved in cell proliferation and differentiation signalling pathways, which has been modified by mutation or in a post-translational manner, and consequently has acquired potential to cause cancer. In contrast to oncogenes, tumour suppressors are anti-oncogenic and protect cells from deregulation by inhibiting cell growth and inducing cell death. Hanahan and Weinberg summarize all biological properties acquired by tumour cells as self-sufficiency in growth signals, loss of sensitivity to anti-growth signals, loss of apoptotic and senescence capacities, acquisition of sustained angiogenesis and invaded neighbouring tissues (Hanahan and Weinberg 2011). Cancer is now the leading cause of death in the UK (Cancer Research UK) and around 293,000 people are diagnosed every year in the UK. There are about 200 different types of cancer but four of them, breast, lung, colorectal and prostate, account for over half of all new cases.

1.1.1 Epidemiology

Breast cancer is the most common diagnosed cancer in the UK despite the fact that it is rare in men. In 2008, 48,034 new cases of breast cancer were diagnosed in the UK: over 99 % in women and less than 1 % in men. Moreover, in the last ten years female breast cancer incidence rates have increased by 6 % in the UK (Cancer Research UK. Breast Cancer. 2009). The UK breast cancer screening program was set up in 1988 and uses mammography to screen all woman aged between 50 and 70 every 3 years (Cancer Research UK. Breast Cancer. 2009). However, the majority of breast cancers are self-detected.

Survival rates for breast cancer vary greatly depending on the breast cancer stage. Overall, women's survival rate after five-year is 82 %, 72 % at ten years and

64 % at twenty years. Despite improvement of the survival rate in the past years, 12,116 deaths from breast cancer were reported in the UK in 2008 and breast cancer remains the most common cause of death in younger women aged 35-54 years (Fig. 1.1) (Cancer Research UK. Breast Cancer. 2009). Breast cancer accounts for around 16% of female deaths from cancer in the UK annually and causes of death from breast cancer vary greatly according to the age at diagnosis, node involvement, cardiovascular condition and osteoporosis. It was found that non-breast cancer-related deaths were more common than breast cancer-related deaths in a study involving 50 % of patients younger than 70 years and 50 % of patient over 70 years old. Two factors have different correlations with the type of death (Chapman, Meng et al. 2008). Cardiovascular disease was associated with significant increased risk of death from other causes than breast cancer, and osteoporosis was associated with significant risk of death from other malignancies. Breast cancer-related death was associated with lymph node involvement and metastases to other organs (Chapman, Meng et al. 2008). Between 1989 and 2008 the breast cancer mortality rate fell in each age range. The reduction in breast cancer mortality rates is likely to be due to improvements in screenings, specialization of care and the widespread adoption of tamoxifen treatment since 1992.

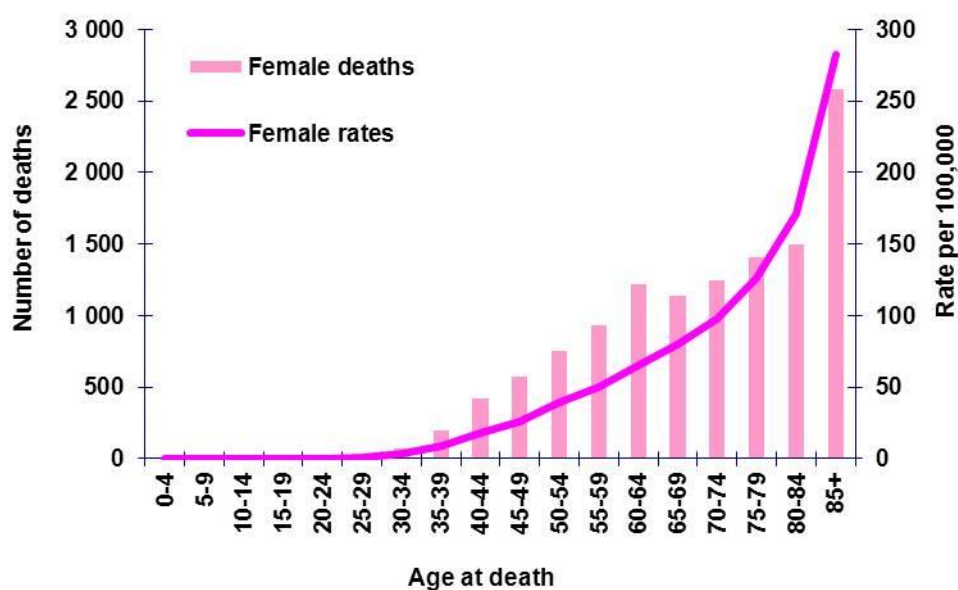


Figure 1.1 Number of deaths and ages-specific mortality rates of breast cancer, UK, 2008. Cancer Research UK.

1.1.2 Breast cancer development

Breast cancer development involves a progression through different mammary lesions and clinical stages starting from ductal hyperplasia, with subsequent evolution to atypical hyperplasia, *in situ* carcinomas progressing into invasive carcinomas (Fig. 1.2) (Allred, Mohsin et al. 2001). Among invasive carcinomas, 80 to 90 % are formed in breast ducts (referred as ductal carcinoma *in situ* or DCIS), while 10-20 % occur in breast lobules (referred as lobular carcinoma *in situ* or LCIS) (Fig. 1.2).

Mammography is the first examination undergone by women and is followed by a biopsy. These tests are used to define the breast cancer stage. Stage 1 is defined by a tumour no wider than 2 centimetres, but not spreading to the lymph nodes. In contrast, stage 2 refers to a tumour wider than 2 centimetres, which has no sign of spread to other organs. Stage 3 involves a tumour of 5 centimetres or less that has reached the lymph nodes, whereas stage 4 is a stage where the tumour is seen in lymph nodes and has spread to other parts of the body.

1.2 BREAST CANCER CLINICAL MANAGEMENT

For years, mastectomy has been the treatment of choice for all types of breast cancer. After surgery, radiation is the first therapy used to kill cells left over from surgery to reduce recurrence rates (Schoenfeld and Harris 2011). Chemotherapies were introduced in the 1940's to reduce tumour size before surgery, prevent recurrence, and treat cancers that have metastasized (Smith and Chua 2006). The discovery of the anti-oestrogen tamoxifen in 1966 and the identification of distinctive oestrogen receptor alpha (ER α) and progesterone receptor (PR) status in mammary tumour cells determined the significance of the hormone receptor status for breast cancer clinical management. Two types of tumours were established based on ER α /PR status: those with ER α (generally PR positive) benefit from anti-oestrogen or endocrine therapy, while those without ER α (generally negative for PR) do not respond to endocrine therapy. A transmembrane protein, human epidermal growth factor receptor 2 (HER2), was later found overexpressed on the surface of human breast cancer cells. Based on the poor prognosis of those patients ER α -/PR-/HER2+ receptor status, a monoclonal antibody specifically targeting the HER2, trastuzumab,

was developed and approved for the treatment of breast cancer in 1998 (Hynes and Stern 1994).

Current clinical management of breast cancer involves patients' first undergoing surgery to totally or partially remove the tumour depending on the breast cancer stage, followed by adjuvant radiotherapy for partially removed tumours. Then, chemotherapy and hormonal therapy is administrated according to the patient receptors status. Chemotherapy alone is administrated to ER α /PR/HER2 negative receptors. ER α /PR positive patients are administrated endocrine therapy in addition to chemotherapy, while HER2 positive patients will receive HER2-targeted therapy with chemotherapy (Fig. 1.2) (Smith and Isaacs).

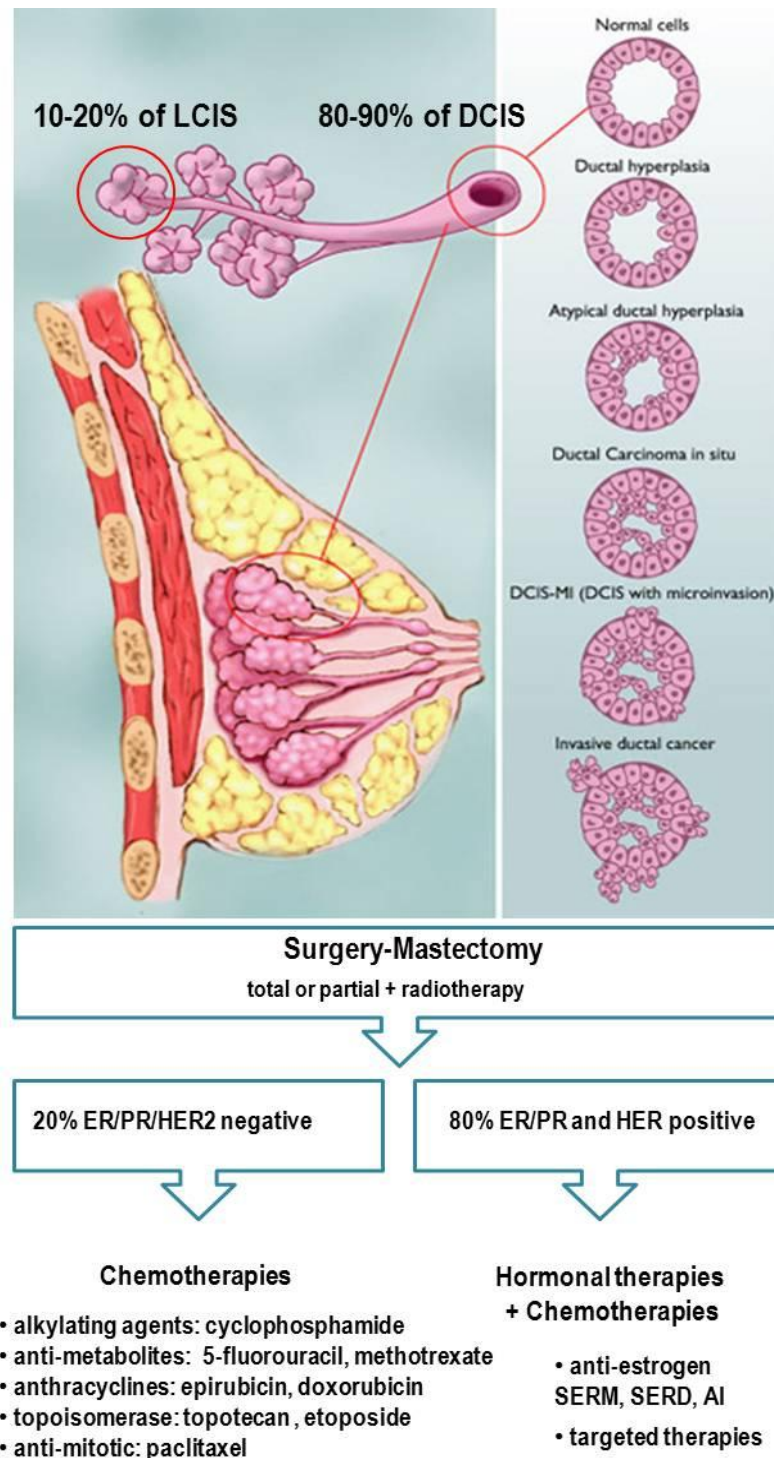


Figure 1.2 Two main types of breast cancer and therapies. After surgery, the hormone receptor status is determined. Patients ER/PR/HER2 negative receptors will be administrated chemotherapies, while ER/PR/HER2 positive receptors patients will be treated hormonal therapies in addition to chemotherapies.

1.2.1 Breast cancer chemotherapies

1.2.1.1 DNA damage agents

About 10 to 20 % of breast cancer patients are triple negative receptors for ER/PR/HER2. These patients have poorer prognosis than ER α positive breast cancer patients because they are only sensitive to chemotherapy agents. So far, no biomarker has been identified to allow for the development of targeted therapy for these patients. Chemotherapy agents for breast cancer can be divided into five categories according to their mechanisms of action: alkylating, anti-metabolite, topoisomerase inhibitor, anthracycline and anti-mitotic agents (Hortobagyi 1995, Rodler, Korde et al. 2010, Rodríguez-Lescure 2010).

Alkylating agents are one of the earliest used chemotherapy agents for the treatment of cancer, which alkylate many nucleophile functional groups in cells. Cisplatin and carboplatin, as well as oxaliplatin, are alkylating agents and act directly on DNA, causing cross-linking of DNA strands, abnormal base pairing, or DNA strand breaks, preventing cells from dividing (Euhus 2011). Alkylating chemotherapy drugs are effective during all phases of the cell cycle.

The structure of anti-metabolites is similar to those of vitamins, amino acids and precursors of DNA and RNA, and act by inhibiting cell division. Anti-metabolites block and prevent purines and pyrimidines from incorporating into DNA during the synthesis phase (S phase) of the cell cycle, stopping normal development and division. These anti-metabolites also effectively block RNA synthesis. Examples of anti-metabolites include 5-fluorouracil (5-FU), methotrexate and gemcitabine.

Anthracyclines are anti-tumour antibiotics that interfere with enzymes involved in DNA replication (Morris, Hudis et al. 2011). These antibiotics work in all phases of the cell cycle and are widely used for a variety of cancers. A major dose-limiting factor when giving anthracyclines is that they can permanently damage the heart if given in high doses. Examples of anthracyclines include daunorubicin, doxorubicin (Adriamycin®) and epirubicin.

Topoisomerase inhibitors interfere with two enzymes called topoisomerase I and II, which help separate the strands of DNA during transcription and cell division. Examples of topoisomerase I inhibitors include topotecan and irinotecan (CPT-11) while examples of topoisomerase II inhibitors include etoposide (VP-16) and teniposide.

Mitotic inhibitors are often plant alkaloids and other compounds derived from natural products. They act to stop mitosis or inhibit enzymes from producing proteins needed for cell division. They are effective during the mitotic phase (M phase) of the cell cycle, but can damage cells in all phases. These drugs are known for their potential to cause peripheral nerve damage, which can be a dose-limiting side effect. Examples of mitotic inhibitors include taxanes (paclitaxel (Taxol®) and docetaxel (Taxotere®) (Fig. 1.3).

Clinically, combinations of up to three chemotherapy drugs are administered together to achieve maximum efficiency against tumour growth. Some of the most common combinations used for breast cancer are CMF (cyclophosphamide, methotrexate and fluorouracil), FEC (epirubicin, cyclophosphamide and fluorouracil), E-CMF (epirubicin, followed by CMF), AC (doxorubicin (adriamycin) and cyclophosphamide) (Fig. 1.2) (Chu and Kiel 1982, Sledge, Neuberg et al. 2003, Blohmer, Schmid et al. 2010, Seidman, Brufsky et al. 2011). The oncologist may offer a choice of chemotherapy combinations as different combinations have different side effects.

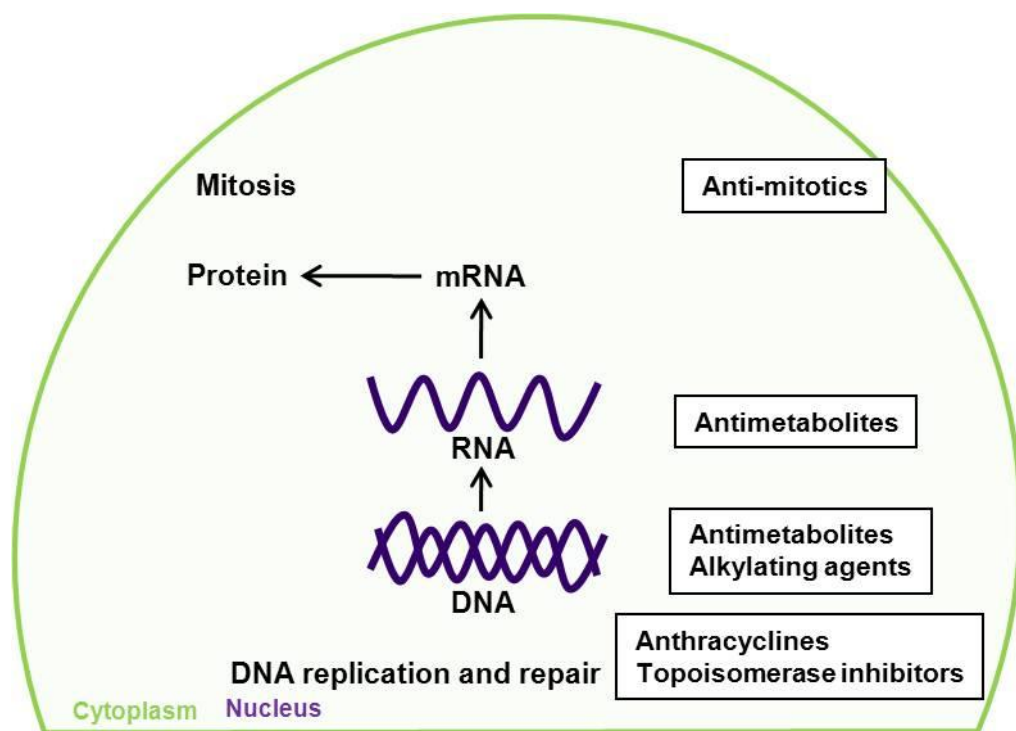


Figure 1.3 Targets for chemotherapies. Alkylating agents cross-link DNA strands and induce DNA strand breaks. Anti-metabolites prevent the incorporation of purines and pyrimidines into DNA in S phase. Anthracyclines inhibit multiple enzymes involved in DNA replication. Topoisomerase inhibitors inhibit topoisomerase enzymes that are required for DNA replication. Mitotic inhibitors inhibit enzymes required for mitotic execution.

1.2.1.2 DNA damage response pathways

Taken together, each category of chemotherapies affects DNA directly via cross-linking, synthesis inhibition, or indirectly via inhibition of enzymes required for DNA replication. Chemotherapy agents damage DNA and can further induce multiple DNA damage responses including cell cycle checkpoints, transcriptional activation and DNA repair.

Cell cycle checkpoints are regulatory pathways governing the order and timing of cell cycle transitions to ensure accurate completion of the cell cycle phases. The key regulators of the checkpoint pathways in the mammalian DNA damage response are ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) protein kinases. Both of these proteins belong to a structurally unique family of serine-threonine kinases. Although ATM and ATR share similar cellular substrates, they generally respond to distinct types of DNA damage (Traven and Heierhorst 2005). ATM is the primary mediator to DNA double strand breaks (DSBs) that can arise by exposure to ionizing radiation (IR), whereas ATR plays mainly a role in response to ultraviolet (UV) damage and stalls in DNA replication (single DNA breaks) (Abraham 2001). The key trigger in the G1 cell cycle checkpoint is the activation of p53, which is phosphorylated by checkpoint kinase 2 (CHK2), downstream target of ATM. Activated p53 upregulates a number of target genes involved in DNA damage response (MDM2, GADD45 α) and G1 cell cycle arrest (p21^{Cip1}) (Fig. 1.4) (Bartek and Lukas 2001). During the S phase checkpoint, ATM activates two parallel pathways. Firstly, ATM phosphorylates CHK2 to prevent cyclin-dependent-kinase 2 (cdk2)/cyclins activation and completion of DNA synthesis. Secondly, ATM phosphorylates a series of downstream substrates involved in DNA repair including Nijmegen breakage syndrome (NBS1), breast cancer susceptibility gene 1 (BRCA1) and structural maintenance of chromosome protein 1 (SMC1). The blockage of entry into mitosis is essential for the G2 checkpoint activation. ATM and ATR modulate the phosphorylation status of the cyclin-dependent CDC2 to keep it in its inactive form and prevent the entry into mitosis (Fig. 1.4) (Zhou and Elledge 2000).

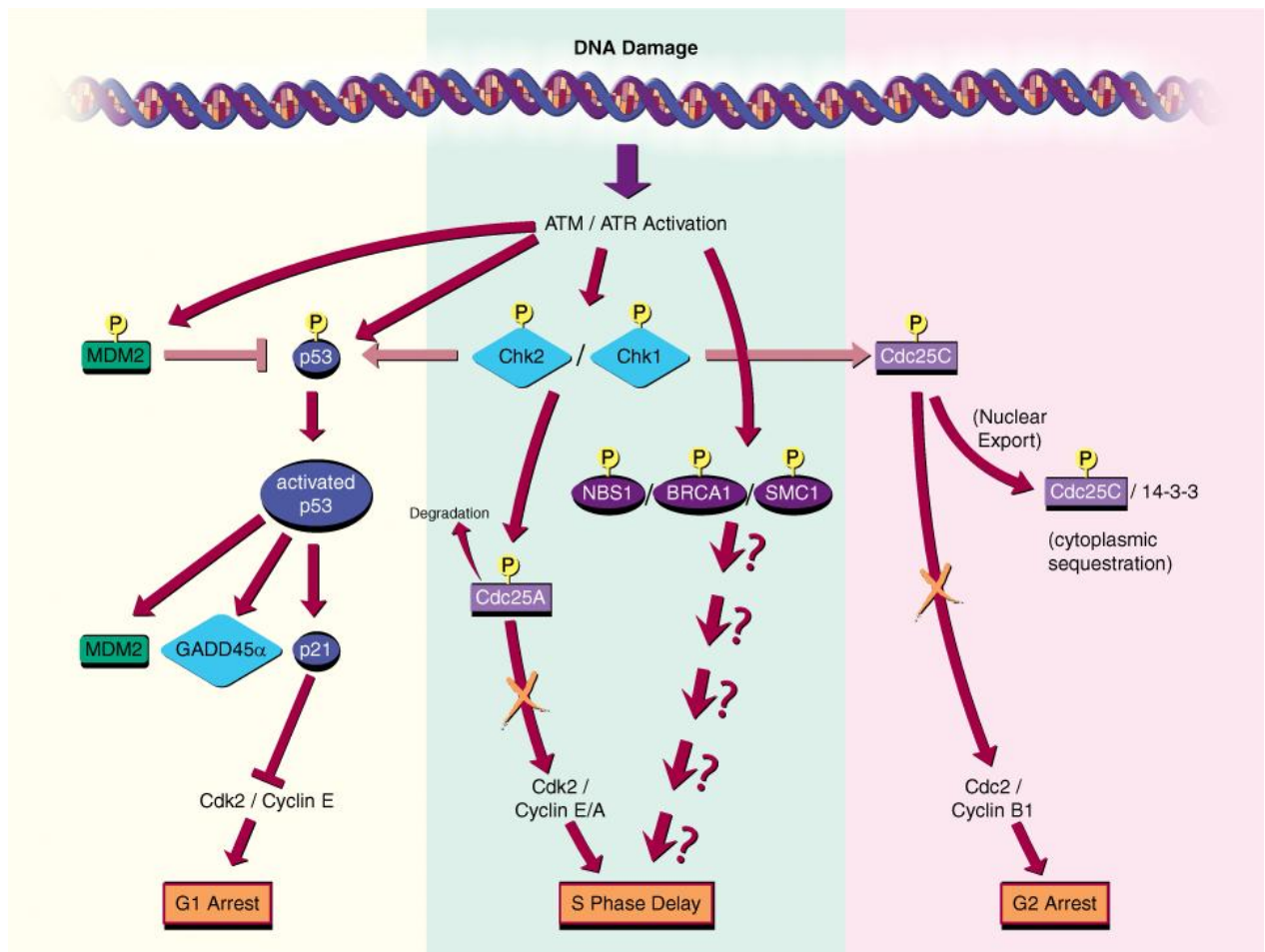


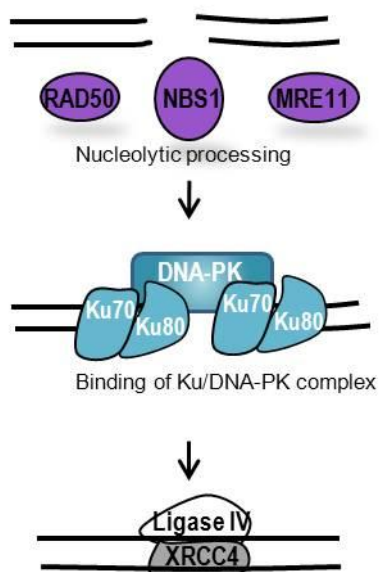
Figure 1.4 Cell cycle checkpoints following DNA damage. ATM/ATR activated by DNA damage trigger signalling cascades leading to cell cycle arrest and delay. ATM/ATR induce a series of phosphorylation affecting p53 and MDM2 leading to G1 arrest. ATM/ATR also phosphorylate and stabilize CHK1/2 and the complex NBS1/BRCA1/SMC1, which cause a delay in S phase. G2 arrest is induced by the phosphorylation of CDC25C, which prevent phosphorylation and activation of the CDC2/CYCLIN B1 complex (From R&D systems website).

Multiple DNA repair pathways can be activated following DNA alterations. Chemotherapy agents induce DSBs that are the worst form of DNA damage. The signal transduction activated by DSBs is strictly dependent on the ATM/ATR family of serine-threonine kinases that regulates two main DSB repair pathways: the homologous recombination (HR) and non-homologous end-joining (NHEJ). Emerging studies have demonstrated there is a cross-talk between the signal of ATM/ATR, and HR and NHEJ repair mechanisms (Kühne, Tjörnhammar et al. 2003). NHEJ involves the ligation of two DNA double strand break without sequence homology between the two DNA ends. Whereas in HR, the damaged DNA retrieves genetic information from an undamaged DNA that shares sequence homology (San Filippo, Sung et al. 2008).

NHEJ is the dominant repair mechanism in cells undergoing G0, G1 and early S phases. The central event involves the KU70/80 heterodimer binding to the two ends of the DSB enabling the recruitment of DNA-dependent protein kinase (DNA-PK). DNA-PK binds to a single strand of the DSBs, which triggers its kinase activity. One DNA-PK substrate is X-ray repair cross-complementing protein 4 (XRCC4) that forms a complex with the DNA ligase IV and stimulates DNA end-ligation (Fig. 1.5). Prior to the DNA end-ligation, the complex MRE11/RAD50/NBS1 exerts its exonuclease activity to clean up the DNA ends (Jackson 2002).

HR, particularly important during S and G2 phases, involves DNA ends resection by nucleases, homology DNA pairs allowing strand invasion, homologous recombination and DNA repair synthesis. This process can be divided into four steps: resection, strand invasion, branch migration and Holliday junction formation. The nucleolytic resection of the DNA in the 5' to 3' is an early event involving the complex MRE11/RAD50/NBS1 (Fig. 1.5). The resulting 3' single strand DNA ends are then bound by RAD51, which is a process involving the binding replication protein A (RPA), RAD52, RAD54 and BRCA1, BRCA2. The RAD51 nucleoprotein filament then interacts with the homologous undamaged DNA and catalyses strand exchanges, in which the damaged DNA invades the undamaged DNA. The 3' terminus of the damaged DNA is extended by a DNA polymerase, which copies genetic information from the undamaged DNA molecule. Finally, the ends are ligated using DNA ligase I and the DNA cross-overs, called Holliday junctions, are cleaved and ligated to two intact DNA molecules (Jackson 2002, Mao, Bozzella et al. 2008).

Non-Homologous End-joining



Homologous Recombination

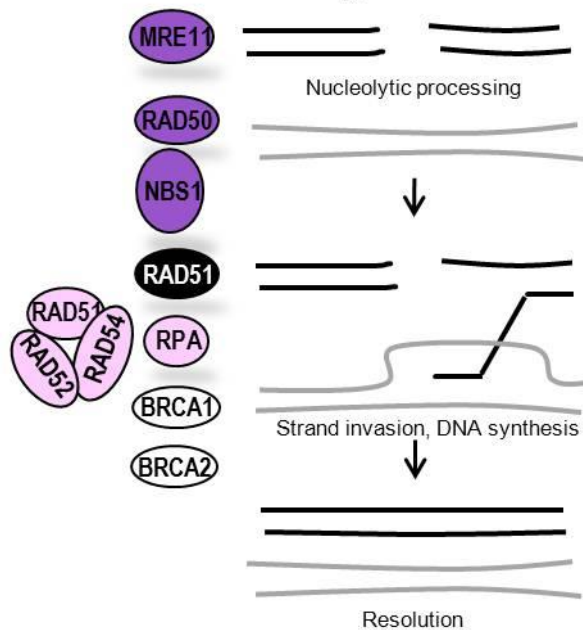


Figure 1.5 Double strand break repair. Homologous recombination process uses a homologous chromosome to repair DNA. When no homologue is present, breaks can be repaired with the non-homologous end-joining mechanism, in which two ends are ligated.

1.2.2 Breast cancer endocrine therapies

1.2.2.1 Anti-oestrogen therapies

About 70 to 80 % of breast cancer patients are ER α positive (in which more than half are also PR positive) and ER α positivity predicts for response to endocrine therapy (Kuukasjärvi, Kononen et al. 1996, Bauer, Brown et al. 2007). Therefore, anti-oestrogen therapies are usually given to patient diagnosed ER α positive when surgery and neoadjuvant chemotherapy were already administrated.

Tamoxifen (OHT) has been the standard adjuvant anti-oestrogen therapy for pre- and post-menopausal women with ER α and/or PR positive breast cancer for years. It is a selective ER α modulator (SERM) that functions as ER α antagonist in breast and as ER α agonist in the heart and bones (Smith and Chua 2006).

It blocks the binding of oestrogen and consequently its activity on ER α . Tamoxifen binding further prevents critical ER α conformational changes that are required for the association of co-factors and the transcriptional activity of ER α . However, the oestrogen-agonist effects of tamoxifen have been associated with serious life-threatening events including endometrial cancer, stroke and trombo-embolism. Selective ER α down-regulators (SERD) such as fulvestrant (ICI182,780) are used as alternatives to tamoxifen (Krell, Januszewski et al. 2011). In contrast to tamoxifen, SERDs are pure antagonists that bind with 100 fold greater affinity, inhibiting receptor dimerization and abrogating oestrogen signalling. Clinical studies have also shown that fulvestrant decreases ER α expression levels (Dauvois et al. 1992). Third-generation aromatase inhibitors (AIs) have also been introduced as an alternative to tamoxifen therapy for post-menopausal women which cancer has progressed following tamoxifen treatment. AIs prevent oestrogen synthesis by inhibiting the action of aromatase, an enzyme necessary for the conversion of androgens to oestrogens (Fig. 1.6A).

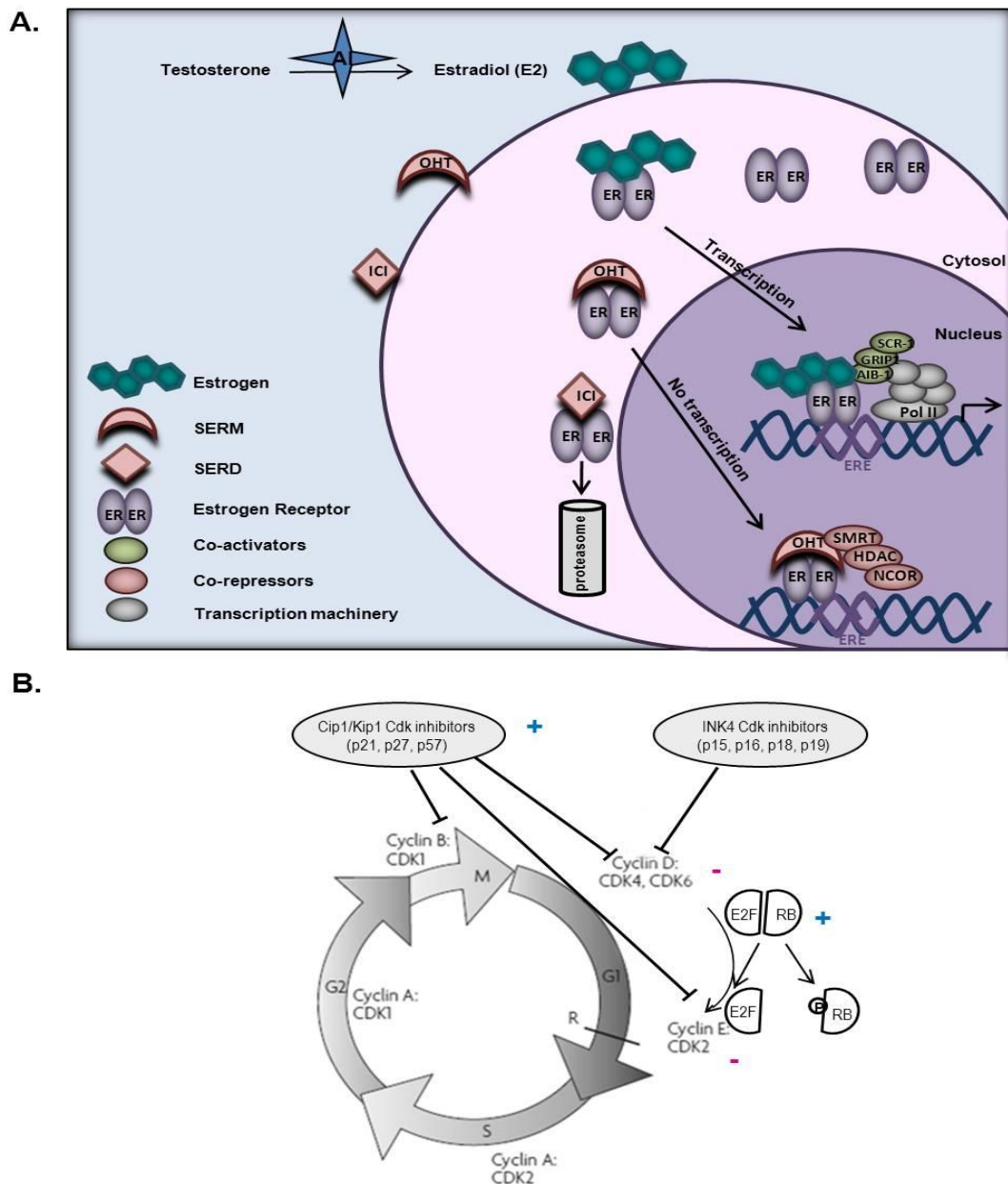


Figure 1.6 Effects of anti-oestrogens. A. ERs are free in the cytoplasm and can be bound by oestrogen to translocate to the nucleus, and recruit ER co-activators to activate ER-responsive genes transcription. When the anti-oestrogen OHT is added, it binds to ER, translocate to the nucleus and recruit ER co-repressors which inhibit gene transcription. In contrast, ICI binds ER and guides it to the proteasome for degradation. **B.** The cell cycle is divided in 4 phases which are regulated by different cyclin/cdk complexes. When cells are treated anti-oestrogens, cells arrest in G1 or G2 phases through the downregulation (-, in pink) of cyclin D1 and E and the upregulation (+, in blue) of cdk inhibitors and hypophosphorylated Rb. Adapted from Dehay and Kennedy, 2004.

The effect of anti-oestrogen therapies has been studied since 1975 and shown to induce cell cycle arrest in G1 resulting in a lower proportion of cells in S phase. Studies in MCF-7 breast carcinoma cells revealed a decrease of *CYCLIN D1* mRNA level and suggested that cyclins involved in G1 phase might be the target for anti-oestrogens to block cells entry in S phase (Lykkesfeldt, Madsen et al. 1994). Further studies using the pure ER α antagonist, ICI182,780, demonstrated a decrease in S phase cells combined with increased of hypophosphorylated Retinoblastoma protein (RB), which inhibits the transcriptional activity of E2F restricting the transcription of cyclins and cdk. Additionally, treatment of MCF-7 cells with the pure ER α antagonist decreased CYCLIN D1 at mRNA and protein levels. Studies of cyclin-dependent kinases revealed no change in their expression pattern. Anti-oestrogens treatment of breast cancer cell lines also showed an upregulation of two members of the cdk inhibitors Cip1/Kip1 family, p27^{Kip1} and p21^{Cip1}, which by consequent inhibit G1 and G2 cyclins (Fig. 1.6B). Tamoxifen can also induce apoptosis at a higher concentration of 5 μ mol/L in MCF-7 cells. Protein kinase C, C-MYC and p53 were identified as potential targets in triggering tamoxifen-induced apoptosis but the exact mechanism still remains elusive (Doisneau-Sixou, Sergio et al. 2003).

1.2.2.2 The oestrogen receptor pathway

Oestrogens, 17 β -estradiol (E2), estrone and estriol, are the most important regulators of breast cancer growth. Oestrogens are steroid hormones produced primarily by the developing follicles in the ovaries and the placenta, and act through the oestrogen receptors, ER α and ER β , products of different genes.

The oestrogen receptors belong to the nuclear receptor superfamily of transcription factors, which includes steroid hormones, thyroid hormones, vitamin D and retinoic acid. ER α and ER β are composed of three functional domains: the N-terminal, DNA binding domain (DBD), and the ligand-binding domain (LBD) (Fig. 1.7) (Ruff, Gangloff et al. 2000). The N-terminal domain of nuclear receptor encodes a ligand-independent activation function (AF-1) that is involved in protein-protein interactions, and transcriptional activation of target gene expression. The DNA binding domain contains a two zinc finger structure, which plays an important role in

receptor dimerization and in binding within the oestrogen response element (ERE). The LBD mediates ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene expression. The activation function 2 (AF-2) in the LBD is governed by the binding of ligands (Hall and McDonnell 2005).

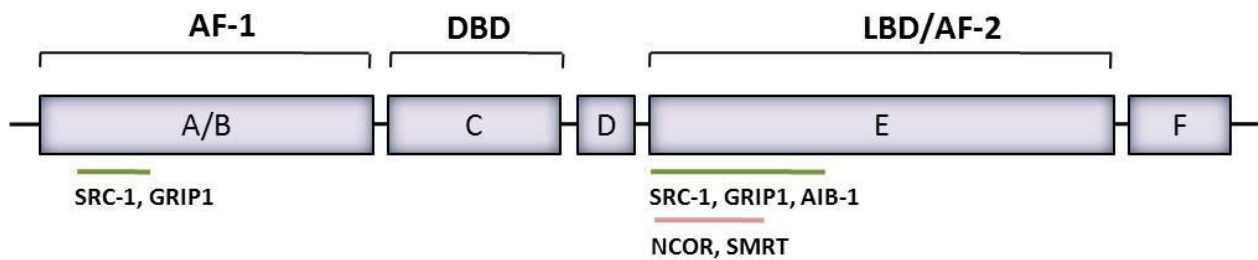


Figure 1.7 Direct interactions of oestrogen receptor with its cofactors. Oestrogen (E2)-bound oestrogen receptor (ER) directly interacts with co-activators SRC-1, GRIP1 or AIB-1 at the AF-1 or AF-2 domain (shown by green lines). Tamoxifen (OHT)-bound ER recruits co-repressors NCOR and SMRT to the AF-2 domain (shown by red line).

Oestrogen receptors act as transcription factors, either by activating or inhibiting the expression of a wide array of genes. ER α and ER β share a high degree of homology, 97 % in the DBD and 60 % in the LBD, and therefore interacts with the same oestrogen ligand (Bai and Gust 2009). However, the expression and characteristics of these receptors differ. ER α is widely expressed and is predominant in the breast, uterus and bone, while ER β is mostly expressed in ovary, prostate, testis, lung, thymus, spleen and areas of the brain. Animal models have shown that ER β can function as an inhibitor of ER α , and is often downregulated in breast tumours. While ER α activity induces breast cancer cell proliferation and tumourigenesis in mice under estradiol, ER β is anti-proliferative and prevents the formation of tumours (Paruthiyil, Parmar et al. 2004).

The ERs are sequestered in a multi-protein complex, including heat shock proteins, in the cytoplasm of cells until their activation by ligands (Fig. 1.7). The binding of the ligand on ER induces its conformational changes, promoting its dimerization, its binding to DNA and the recruitment of co-factors inducing transcription of target genes. Among ER co-factors, some ER modulators bind to the

AF-2, including ER-interacting proteins 140 and 160 (ERAP140 and ERAP160), receptor interacting proteins 140 and 160 (RIP140 and RIP160) and the p160 family (SRC-1, GRIP1, AIB1) (Halachmi, Marden et al. 1994, Oñate, Tsai et al. 1995). In addition to co-factors that modulate ER activity by binding to AF-2, AF-1 interacting co-factors have also been described, such as p68 RNA helicase, which enhances ER activity through AF-1 (Endoh, Maruyama et al. 1999). While co-activators enhance ER activity, co-repressors decrease the agonist effect of oestrogens. The first identified and most studied are the nuclear receptor co-repressor (NcoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT). Cloning of cDNAs stimulated by oestrogen resulted in the identification of numerous target genes including *pS2* and *CATHEPSIN D*, *CYCLIN D1*, *C-MYC* and many others (Elangovan and Moulton 1980, Brown, Jeltsch et al. 1984, Dubik and Shiu 1988, Altucci, Addeo et al. 1996). Promoter region analysis of these genes led to the discovery of two distinct mechanisms of ER binding, the “classical” and “non-classical” binding as genomic response (Fig. 1.7). The former involves the binding of ER within the ERE, while the latter involves the binding of DNA through a different motif including those for AP-1 and Sp-1 (Klinge 2001, Carroll, Meyer et al. 2006). Another mechanism, by which ER regulates transcription indirectly, referred to as the non-genomic response, identified about sixty years ago. Oestrogen binding sites were identified on the membrane of endothelial cells. Further studies showed that ER can mediate rapid signals originating from the membrane into the nucleus by the activation of growth factor receptors, tyrosine kinases, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinases (PI3K) (Fig. 1.7) (Migliaccio, Di Domenico et al. 1996, Migliaccio, Piccolo et al. 1998, Simoncini, Hafezi-Moghadam et al. 2000, Song, McPherson et al. 2002).

The success of targeted anti-oestrogen therapy motivated the search for genetic mutations occurring in breast cancer and the development of new targeted therapies.

1.3 GENETIC AND NEW THERAPIES

1.3.1 Genetic predispositions and mutations

Risk factors for breast cancer can be divided into environmental and genetic categories. A number of environmental risks related to reproductive events include age, menstruation age, age at first birth, menopause and exogenous uptake. In addition, a strong family history involving genetic alterations is recognized as the strongest risk factor.

Genetic predisposition to cancer occurs in a minority of patients and is conferred by inherited mutations in high penetrance genes. The *BRCA1* and *BRCA2* genes have been identified in the 1990's as "high risk" breast cancer susceptibility genes. Women carrying deleterious mutation of these genes have 45-65 % risks of developing breast cancer (Ahmed, Lalloo et al. 2009). Women with very strong family history can be tested for faulty *BRCA* genes, but account for just 3 %.

While some of these alterations might be inherited, most accumulate during a woman's lifetime. Gene alterations accumulated with time are known as somatic mutations that are permanent and transmissible in genetic material. A wide variety of genes is commonly mutated or incorrectly regulated in breast cancer cells and have been implicated in the development and progression of the disease. These genes encompass growth factors receptors and their ligands, intracellular signalling molecules, cell cycle regulators, apoptosis regulators and adhesion molecules leading to cell proliferation, inhibition of apoptosis and invasion (Cordon-Cardo 1995, She, Chandarlapaty et al. 2008). Studies of these altered molecules are promising as new potential targets for breast cancer therapy. The best example of such a therapy is trastuzumab, which has been shown to be effective in breast cancers overexpressing the growth factor HER2 (Bartsch, Wenzel et al. 2007).

1.3.2 Targeted therapies

The human epidermal growth factor receptor/HER family of transmembrane type I receptor tyrosine kinases are enzymes that are fundamental in cellular processes such as proliferation, differentiation and survival. The members of this family contain an extracellular ligand-binding domain, a single membrane-spanning region, a nuclear localization signal, and a cytoplasmic tyrosine kinase domain. HER1, HER3 and HER4 members interact with specific ligands, while no natural ligand has been identified for HER2. HER2 can be activated by hetero-dimerization with ligand-activated HER co-receptors, which modulate receptor internalization and prolong signal transduction (Prenzel, Fischer et al. 2001, Li and Hristova 2010). Conformational changes occurring of dimer receptors lead to auto-phosphorylation and initiation of several signal transduction cascades. These type I receptors transmit signals through the Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway stimulating cell division (Fig. 1.8). Cell lines studies suggest that these receptors modulate cell survival via the activation of AKT/PI3K pathway. *In vitro* and *in vivo* studies have established oncogenic properties (Hubalek, Brunner et al. 2010). Aberrant HER1 and HER2 signalling have been associated with cancer cell proliferation and survival. Activated HER2 was first identified by a point mutation in rat neuroblastomas (Perantoni, Rice et al. 1987). It was later found to be overexpressed in 25 % of some human breast cancers. The prognosis of those patients with HER2 expression on breast cancer cells surface is poor, which warranted the development of antibodies and small molecules specifically targeting this receptor.

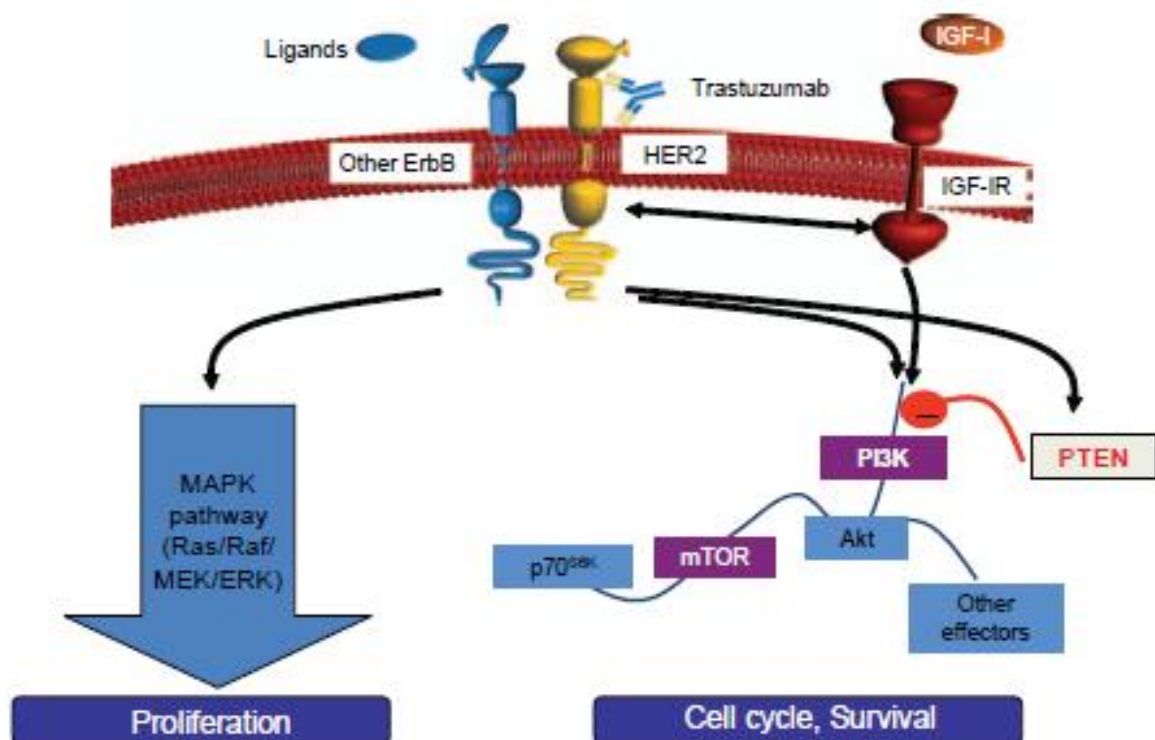


Figure 1.8 HER2 signalling and pathway targeted therapy. Hetero-dimerization of HER2, induced by the ligands of HER members, activates the phosphoinositol 3-kinase pathway including AKT leading to cell survival and the mitogen-activated protein kinases promoting cell proliferation (Hubalek et al 2010).

In the 1980's, the monoclonal antibody against HER2, trastuzumab, was developed and approved in 1998 for the treatment of metastatic breast cancer. In 2005, five adjuvant trials evaluated trastuzumab as a potent agent for early breast cancer with higher benefit than adjuvant chemotherapy and similar to that seen with adjuvant endocrine therapy (Harries and Smith 2002). Although trastuzumab's method of inhibiting HER2 activity is not fully understood, studies suggest that the drug promotes internalization and degradation of HER2, or disrupts the activation of the AKT/PI3K pathway. Trastuzumab administration leads to cell cycle arrest at the G1/S boundary, which often results from an increase of p27^{Kip1} level, and a subsequent decrease of CYCLIN D1 and cdk2 (Baselga, Albanell et al. 2001). Currently, trastuzumab is the only HER2-targeted therapy approved by the FDA for the treatment of early and metastatic breast cancer overexpressing HER2, therefore evaluating HER2 expression has become mandatory in early breast cancer (Fig. 1.8).

1.4 BREAST CANCER RECURRENCE

1.4.1 Recurrence of the disease

The recurrence rate among patients who did not receive adjuvant endocrine therapy is nearly 50 % throughout the first 10 years after diagnosis.

There are so many factors that account into the risk of recurrence. Some common indicators of recurrence include the involvements of lymph node and vasculature, the tumour size, the histological grade, the receptor status, proliferative capacity, oncogene expression and somatic mutation (Smith and Chua 2006).

Recurrent breast cancer can be categorized in three types: local recurrence appearing at the tumour site which may be considered as a failure of initial treatment, regional recurrence showing that cancer has spread is very common and distant recurrence or metastasis which is the most serious type and is associated with decreased patient survival. In 65-75 % of distant recurrences the breast cancer then spreads from the lymph nodes to the bones. In rare cases, breast cancer may also metastasize to other sites including the lungs, liver, brain or other organs.

1.4.2 Chemotherapy resistance

Resistance to chemotherapies is a major problem in the clinical management of breast cancer and most particularly for triple negative patients, who can only be administered chemotherapy drugs. Response rate for first line chemotherapies is between 30 and 70 %. However, these agents are efficient only for 6 to 10 months. Patients who initially respond become resistant to the initial agents and to multiple anti-cancer drugs, which have different structure and mechanism of action. This phenomenon is referred as multidrug resistance (MDR).

Chemotherapy resistance can be mediated by various mechanisms such as an increased activity of exporters or decreased activity of importers resulting in reduced intracellular drug concentrations or reduction of cellular uptake, respectively. Drug transport is mediated by the ATP-binding cassette transporters (ABC-transporters) (Coley 2008). An increased of the ABC-transporter P-glycoprotein (PGP) expression from 11 % to 30 % in patients, who had received doxorubicin and taxol, correlated with drug resistance (Leonessa and Clarke 2003). Furthermore, multidrug resistance-associated protein 1 (MRP1) is frequently found to be overexpressed in primary breast cancer and associated with relapse of node-positive and –negative patients who received cyclophosphamide, methotrexate and 5-fluorouracil (Riordan and Ling 1985, Tsuruo 1988). Additionally, the activation of detoxifying systems also allows cells to escape to the effect of chemotherapies. Upregulation of the cytochrome P450, aldehyde dehydrogenase and glutathione S-transferases particularly affect the toxicity of cyclophosphamide. Resistance can also be the consequence of defective apoptotic pathways or a change in cell cycle checkpoint. P53 is lost in cells where the cytotoxic effect of doxorubicin is delayed or lost. Mutations in p53 have been linked to doxorubicin resistance and to early relapse in breast cancer patients. Comparisons between mouse fibroblast cells containing wild-type p53 and p53 knock-out showed that the absence of p53 reduces apoptotic cell death and induces doxorubicin resistance. Mutated p53 loss of function was also associated with the abolition of p21^{Cip1} transcriptional activation resulting in cell cycle arrest defect. Expression of the anti-apoptotic family of protein BCL-2 has been detected in 80 % of breast cancers from women with primary tumours and having either node positivity or negativity. In contrast, the expression of the pro-apoptotic factor BAX is lost in some breast

tumours (Krajewski, Krajewska et al. 1999). Reduced BAX levels correlate with shorter overall survival, fast tumour progression and failure to respond to therapy (Coley 2008).

1.4.3 Anti-oestrogen therapy resistance

Currently, tamoxifen remains the treatment of choice for most women with ER α positive invasive breast carcinomas. Approximately 30 % of ER α positive breast cancer patients become resistant to tamoxifen. While anti-oestrogens have been available since the early 1970's, the mechanisms of action and development of resistance is still not fully understood. There are two forms of anti-oestrogen resistance: *de novo* resistance and acquired resistance. By definition, *de novo* resistance is present before drug exposure, while acquired resistance occurs after drug exposure. Additionally, endocrine resistance can be categorized into loss or mutation of ERs, specific resistance to anti-oestrogens, modified ER α interaction proteins and ligand independent ER α activation (Clarke, Liu et al. 2003).

Since the effects of anti-oestrogens are mediated through ERs, the degree of ER expression is a strong predictor of response. Loss of ER expression is the primary mechanism of *de novo* resistance to tamoxifen with ER/PR negative. However, the loss of ER occurs only in 15 % of cases (Ring and Dowsett 2004). In addition to genetic modifications, epigenetic changes such as CpG island hypermethylation causes transcriptional inactivation of ER gene (Issa, Ottaviano et al. 1994). In a study, the analysis of the methylation status of CpG dinucleotides in ER from patients with recurrent breast cancer following tamoxifen allowed the development of a predictive score that could be used to identify patients likely to respond to tamoxifen (Martens, Nimmrich et al. 2005). Further studies are undertaken to confirm the validity of this approach. A second ER was cloned from a rat prostate cDNA library and was named ER β (Kuiper, Enmark et al. 1996). ER β is highly homologous to ER α and binds to the same ligands but has different effects on gene transcription. There is divergent data concerning the expression of ER β , patient prognosis and anti-oestrogen responsiveness (Omoto, Inoue et al. 2001, Fuqua, Schiff et al. 2003, Iwase, Zhang et al. 2003). However, evidence suggest that ER β

protein expression is decreased in breast carcinoma compared to normal or benign lesions (Roger, Sahla et al. 2001).

The majority of patients that initially respond to tamoxifen acquire resistance after long-term exposure without losing ER α expression. Therefore, most of the published information on potential mechanisms of resistance has been documented by studies in ER α positive breast cancer cells selected by sustained exposure to anti-oestrogen.

A mechanism of drug resistance common to chemotherapies and endocrine therapies is the emergence of increased drug efflux or reduced drug influx. Intratumoural tamoxifen concentration was decreased in tamoxifen-resistant breast cancer compared to tamoxifen-responsive breast cancer. Although the mechanism responsible for altered tamoxifen accumulation is not understood, *in vitro* study shows that overexpression of MDR1 in MCF-7 cells reduces tamoxifen sensitivity (Clarke, Currier et al. 1992). In addition, lower concentration of tamoxifen active metabolite (4-hydroxy-N-desmethyltamoxifen) was found in patients carrying a variant of the CYP2D6 allele (Stearns, Johnson et al. 2003).

ER α is activated by binding of estradiol that induces recruitment of co-factors, conformational changes, phosphorylation of ER α , and its dimerization before binding of the ERE within the promoter of ER-responsive genes. This is referred to as the classical mode of action. The overexpression of AIB1, an ER co-activator, is observed in 50 % of breast tumours (Anzick, Kononen et al. 1997). AIB1 is also highly expressed in MCF-7 breast cancer cell line and in a mouse xenograft (List, Lauritsen et al. 2001). The overexpression and phosphorylation of AIB1 led to constitutive transcriptional activity of ER α conferring resistance *in vitro* and in xenograft models (Musgrove and Sutherland 2009). In addition, AIB1 overexpression is associated with reduced responsiveness to treatment in breast cancer patients and a worse disease-free survival (Osborne, Bardou et al. 2003, Alkner, Bendahl et al. 2010). *In vitro* studies suggest that overexpression of other co-activators such as SCR-1, may also be able to enhance ER activation by oestrogen and agonist activity of tamoxifen. However, no clinical data have verified SCR-1 role in tamoxifen resistant patients. While ER co-activators levels are found overexpressed in tumours that acquired tamoxifen resistance, NCoR levels, an ER co-repressors, are declined (Chan, Lykkesfeldt et al. 1999).

ER can also regulate gene expression by interacting with DNA directly via other transcription factors, such as FOS/JUN activating protein 1 (AP-1) and NF- κ B (non classical mode) (Nilsson, Mäkelä et al. 2001). The increase in transcriptional activity elicits an increase in ER α activity, which is associated with tamoxifen resistance.

Post-translational modifications, such as phosphorylation, acetylation and methylation, affecting ER α can also influence its interaction with factors and lead to anti-oestrogen insensitivity. Numerous studies showed cross-talk between ER α and growth factor receptor pathways, such as HER family and insulin-like growth factor (IGFR) family. ER α can be phosphorylated on several sites but the most reported is serine 118. ER can be phosphorylated at serine 118 by the MAPK ERK1/2, which is downstream of HER2 signalling pathway (Kato, Endoh et al. 1995). Phosphorylation enhances ER α ligand sensitivity and may lead to ligand-independent activation. Indeed, ERK1/2 expression and activity are increased in endocrine resistant breast cancer cell lines (Kronblad, Hedenfalk et al. 2005). Upstream RAS/MAPK pathway can be activated by IGF stimulation inducing phosphorylation of ER α serine 118 and resulting in enhanced activation (Kato, Endoh et al. 1995). A direct interaction between ER α and IGFR leads to activation of IGFR downstream targets, thereby leading to an increase in cell survival (Ring and Dowsett 2004). In MCF-7 breast cancer cell line, phosphorylation of ER α on serine 118 and serine 167 by the receptor tyrosine kinase RET and mTOR pathway leads to ligand-independent activation of ER-responsive genes and tamoxifen resistance (Morandi, Plaza-Menacho et al. 2011). The clinical relevance of serine 118 phosphorylation has not been yet established; some studies showed a bad prognosis correlated with phospho-serine 118, while other studies positively correlated phospho-serine 118 with response to endocrine treatments (Sarwar, Kim et al. 2006, Riggins, Schrecengost et al. 2007, Yamashita, Nishio et al. 2008).

1.4.4 Targeted therapy resistance

The anti-tumour effects exerted by the anti-HER2 antibody, trastuzumab, require modulation of key signalling pathways and cell cycle/apoptosis regulatory proteins that are not directly regulated by trastuzumab itself. Therefore, alterations in these pathways and regulatory proteins limit the therapeutic efficacy of trastuzumab

leading to resistance. Studies have shown that high expression of other HER family members or other growth receptors lead to trastuzumab resistance. For example, IGFR overexpression activates the AKT/PI3K pathway and renders trastuzumab-sensitive HER2-overexpressing SKBR3 cells resistant to treatment (Lu, Zi et al. 2001). The expression level of a receptor tyrosine kinase MET was increased in HER2-overexpressing breast cancer tumour samples with resistance to several targeted therapies (Shattuck, Miller et al. 2008). Aberrant regulation of the downstream signalling pathway of HER family, such as phosphoinositol 3-kinase pathway, also leads to resistance (Campbell, Russell et al. 2004). Furthermore, trastuzumab can induce the release of HER ligands conferring resistance to its anti-proliferative effect (Kong, Calleja et al. 2008). In addition to the high levels of ligands, the membrane-associated glycoprotein mucin-4 (MUC4), which is overexpressed in breast cancers, can mask HER2 interfering with trastuzumab binding (Nahta and Esteva 2006). Several studies also identified a truncated version of HER2 which lacks the extracellular domain (p95 HER2) and able to escape trastuzumab's binding. In a survey, Molina *et al.* found HER2 truncation more highly expressed in surgically excised node-positive breast cancer samples than node-negative ones (Molina, Sáez et al. 2002). Consistently, the comparison between MCF-7 transfected with HER2 and p95HER2 showed that only MCF-7/HER2 cells are sensitive to trastuzumab (Scaltriti, Rojo et al. 2007). The recruitment of phosphoinositol 3-kinase by a tyrosine kinase receptor catalyses the conversion of membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP₂) to 3,4,5-triphosphate (PIP₃). PTEN is the negative regulator of Class I PI3K converting PIP₃ back to PIP₂ and has been reported to be frequently lost (26 %) and mutated (6 %) in breast cancer. Furthermore, *in vitro* and *in vivo* studies demonstrated that knocking-down PTEN in HER2-overexpressing breast cancer cells induces trastuzumab resistance (Nahta and Esteva 2006). Additionally, somatic mutations in phosphoinositol 3-kinase catalytic unit (PIK3CA) were identified in 2004 in several malignancies including breast cancer (Campbell, Russell et al. 2004). *In vitro* studies showed that gain-of-function mutation of the *PIK3CA* gene lead to increased resistance to trastuzumab in breast cancer cells than breast cancer cell without *PIK3CA* mutation. Finally, p27^{Kip1} expression responsible for the G1/S blockage induced by trastuzumab is reduced in resistant cells derived from SKBR3 and sensitivity is restored by reintroduction of p27^{Kip1} (Chang 2007).

1.4.5 Potential strategies overcoming drug resistance

In breast cancer and leukaemia, expression of multidrug pump is observed in several tissues prior to exposure to chemotherapy and subsequently dramatically increased once resistance develops. Targeting of the pumps by small-molecular compounds is an attractive strategy to overcome MDR in cancer. Several inhibitors of pumps have been developed and are currently under clinical phased studies in different cancers. To increase the selectivity of MDR inhibition, gene silencing strategies were developed. Antisense oligonucleotide targeting *MDR1* mRNA partially resensitizes the human MDR xenograft in mice to doxorubicin (Yagüe, Higgins et al. 2004). Similarly, silencing of *MDR1* by RNAi reverses the resistance of doxorubicin-resistant leukemia cells to doxorubicin and taxol (Wu, Hait et al. 2003). The application of shRNA strategy showed a successful knock-down *in vivo* (Yagüe, Higgins et al. 2004).

Since chemotherapy agents exert their anti-tumour effect through production of DNA damage, inhibition of DNA repair machinery can be used. Poly (ADP-ribose) polymerase 1 (PARP1) belongs to a large family of nuclear enzyme that are activated by and recruited to the sites of DNA damages. PARP1 catalyses the transfer of NAD⁺ to acceptor proteins and induce the formation of poly (ADP-ribose) polymers important for the recruitment of the base excision repair machinery to the sites and repair of DNA. Based on the fact that PARP1 has a role on DNA repair, inhibition of PARP1 and consequently DNA repair on tumour resistant to chemotherapies effects may represent a good strategy. Two therapeutic strategies employ PARP inhibitors in the treatment of cancer. The first is the use of PARP inhibitors as sensitizers to DNA damaging chemotherapy agents, while the second aims to exert anti-tumour effect through production of DNA damage. Indeed, PARP inhibitors enhance cytotoxicity of DNA methylating agents (Veuger, Curtin et al. 2004). In 2005, pivotal evidence showed that the use of PARP inhibitors in cells *BRCA1* and *BRCA2* genes deficient resulted in selective cytotoxicity compared to wild-type or heterozygous (Bryant, Schultz et al. 2005, Farmer, McCabe et al. 2005). Based on this, a number of PARP inhibitors are currently in development for the treatment of cancers including breast cancer. The *BRCA1* and *BRCA2* proteins are described for their role in homologous

recombination, but BRCA proteins are also implicated in nucleotide excision and base excision repairs (Hartman and Ford 2002, Alli, Sharma et al. 2009). If PARP is inhibited, repair-associated breaks result in replication fork-mediated double strand break, which required BRCA1 and BRCA2-associated recombination (Arnaudeau, Lundin et al. 2001). Following successes in phase I and II, many pharmaceutical companies are now examining the efficacy of their PARP inhibitors in patients BRCA mutation-associated and triple negative breast cancers.

The use of signal transduction inhibitors represents a promising therapeutic approach as overactivated growth factor signalling pathways are involved in endocrine resistance of breast cancer. Drugs blocking the signalling pathways of HER1, IGFR1, MAPK and AKT/PI3K are advanced in clinical development (Baselga 2011). Results from pre-clinical studies suggest that these drugs can be effective in both tamoxifen sensitive and resistant breast cancer patients. Indeed, several reports showed that additive or synergistic effects are obtained in ER α positive breast cancer patients when treated with a combination of tamoxifen and signal transduction inhibitors such as tyrosine kinase and farnesyltransferase inhibitors (Johnston 2005, Baselga 2011). Similar results were obtained with the combination of mammalian target of rapamycin (mTOR) antagonists and an aromatase inhibitor letrozole in cell lines models (Baselga 2011). A synergistic effect has been observed when combining trastuzumab with tamoxifen in ER α positive and HER2 overexpressing BT-474 breast cancer cell lines (Chen, Wang et al. 2008). Moreover, treatment of MCF-7 cells with tamoxifen and gefitinib, a HER1 tyrosine kinase inhibitor (TKI), showed greater effects than tamoxifen alone by inhibition of cell growth and promotion of apoptosis (Gee, Harper et al. 2003). This pre-clinical data led to the development of clinical trials examining the combination of tamoxifen and signal transduction inhibitors in ER α positive breast cancer patients.

Because ER α co-activators are important in ER α function, their overexpression contributes to endocrine resistance. As mentioned, AIB1 is amplified in breast cancer and correlate with ER α and PR positive cells. Pre-clinical studies showed that overexpression of co-activator AIB1 increases tamoxifen agonist activity suggesting that histone acetyl transferase (HAT) activity is increased to allow gene transcription. Conversely, ER α co-repressor NCOR1 is

underexpressed in tamoxifen resistant mouse models (Lavinsky, Jepsen et al. 1998, Chan, Lykkesfeldt et al. 1999). Therefore, the treatment of breast cancer cells with histone deacetylase (HDAC) has been developed. TSA and SAHA treatments, two HDAC inhibitors, reduced breast cancer cell growth. In MCF-7 cells, TSA induces *CYCLIN D1* and *ERα* proteins degradation and derepression of *p21^{Cip1}* resulting in G1 arrest (Butler, Zhou et al. 2002, Alao, Stavropoulou et al. 2006, Kim, Bang et al. 2006, Munster, Thurn et al. 2011).

Currently, trastuzumab is the only HER2-targeted therapy approved by the FDA for the treatment of metastatic breast cancer overexpressing HER2. However, the HER2 overexpressing patients who originally responded to trastuzumab develop resistance. Therefore, the combination of trastuzumab with novel agents may increase the magnitude and duration of the response. Among novel biological agents, pertuzumab is a HER2 monoclonal antibody that blocks dimerization of HER2 with HER1 and HER3, and their signalling pathways. The combination of trastuzumab and pertuzumab showed synergistic effect inducing apoptosis in HER2 overexpressing breast cancer cells, but had no effect on trastuzumab resistant breast cancer cells (Nahta, Hung et al. 2004, Tanner, Kapanen et al. 2004). This result suggests a cross-resistance to HER2 antibodies that are not yet understood. An alternative is to produce antibody-toxin conjugates. However, the major limitation of this strategy is the activation of immune response.

In addition to anti-HER2 antibody strategy, TKIs that directly inhibit the cytoplasmic tyrosine kinase of the growth factor receptor are in development. Currently clinical trials are being conducted and showed a reduction in HER1 and HER2 phosphorylation after treatment. Lapatinib, TKIs inhibiting both HER1 and HER2, is currently being tested in clinical trials (Moy and Goss 2006, Sridhar, Hotte et al. 2010). Lapatinib has shown remarkable activity *in vitro* and *in vivo* including inhibition of MAPK and AKT activation and growth arrest and apoptosis in HER1- and HER2-dependent tumours (Xia, Mullin et al. 2002).

To date, much of the information on mechanisms of resistance has come from cell line studies and too few genes have been considered. The rate, at which breast cancer relapses, calls for new approaches to provide new target for the development of treatment capable of reversing drug resistance.

1.5 FORKHEAD BOX TRANSCRIPTION FACTORS

A forkhead box transcription factor was discovered in *Drosophila* in 1989 by Deftlef Weigel, who identified a novel structure and nuclear localization of a protein (Weigel, Jürgens et al. 1989, Weigel and Jäckle 1990). The structure described was three α -helical domains at the N-terminal region, three β -sheets and two large loop regions located at the C-terminal end, forming a structure which is similar to the wings of a butterfly, leading to the term winged-helix family. The comparison between the forkhead box factor and *HNF-3A*, a gene cloned from a rat hepatocyte, showed striking similarity (Lai, Prezioso et al. 1990). Fifty-five members grouped into 17 subfamilies (A-Q) (Myatt and Lam 2007) were then identified and described in vertebrates which have been given a wide range of names until Kaestner *et al* unified the nomenclature and the proteins name became FOX (Kaestner, Knochel et al. 2000). The FOX family is an extensive family in which members share greater than 90 % in their winged-helix forkhead DNA-binding domain (DBD) sequences (Clark, Halay et al. 1993, Kaestner, Knochel et al. 2000). Outside the DBD, FOX proteins differ significantly leading to differential function and regulation in many processes including metabolism, proliferation, development and differentiation, aging, angiogenesis, DNA repair and apoptosis.

1.6 FORKHEAD BOX M1 (FOX M1)

1.6.1 Structure

FOX M1 protein contains three regions: the N-terminal Repressor Domain (NRD) followed by a conserved DNA Binding Domain called Forkhead winged-helix domain (FKH). The C-terminal region harbours the transactivation domain (TAD) with several cyclin/cyclin-dependent-kinase-dependent phosphorylation sites (Fig. 1.9) (Yao, Sha et al. 1997). FOX M1 can transactivate target genes through two mechanisms. First, FOX M1 transactivates genes via the binding of its DBD to FOX M1 binding sites in the promoter of the gene (Wierstra and Alves 2006). Second, FOX M1 binds human promoters through the binding of FOX M1 DBD to TATA-boxes

and the binding of its central domain to TATA-binding protein (TBP) (Wierstra and Alves 2006). FOXM1 binds to the DNA binding sequence of genes it regulates and facilitates binding of RNA polymerase to transcribe genes.

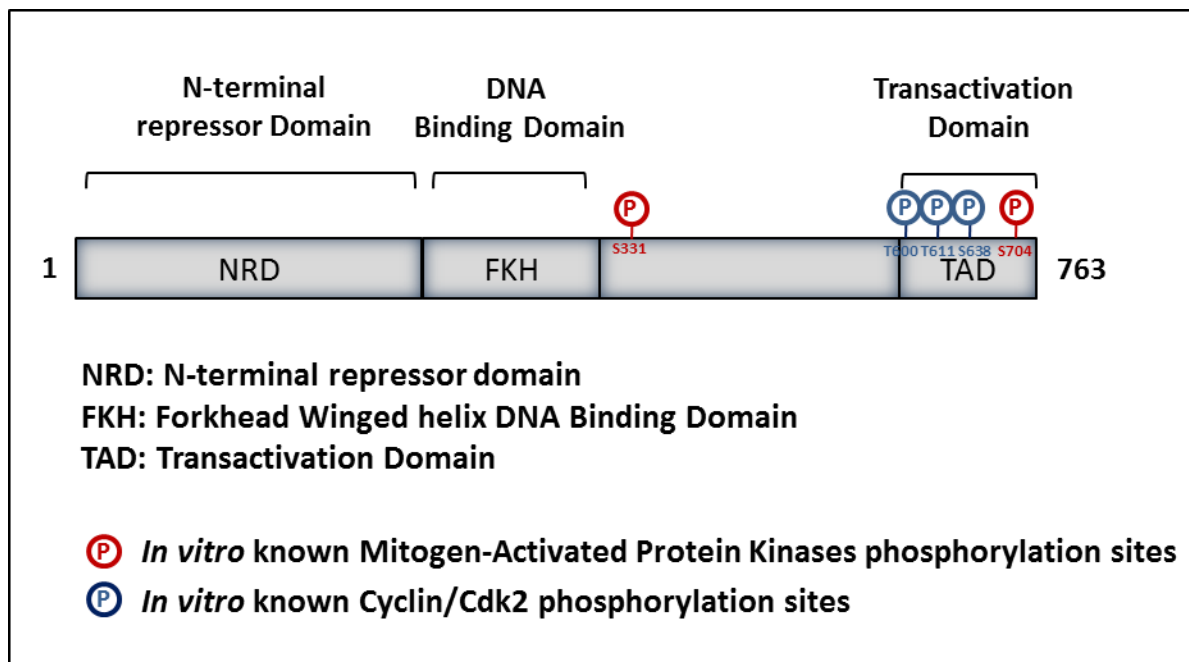


Figure 1.9 FOXM1 structure. FoxM1 protein contains 3 main regions: the N-terminal Repressor Domain (NRD). This region is followed by a conserved DNA Binding Domain called Forkhead winged-helix domain (FKH). The C-terminal region harbours the Transactivation Domain (TAD) with several activating Cyclin-Cdk-dependent phosphorylation sites. FoxM1 transcriptional activity also requires the presence of appropriate mitogenic signals involving the Raf/MEK/MAPK signalling pathway which phosphorylate FOXM1 in two sites.

1.6.2 Regulation

A partial fragment of FOXM1, named after WIN, was originally cloned from a rat insulinoma cell line and detected in human thymus, testis, lung and intestine (Yao et al. 1997). This fragment WIN was previously isolated in a screen for phospho-proteins in the mitotic phase, suggesting its regulation by phosphorylation (Westendorf, Rao et al. 1994, Yao, Sha et al. 1997). Study of FOXM1 in mouse

identified that its expression correlated with cycling cells (Korver, Roose et al. 1997). It is the only forkhead transcription factor known to be associated with proliferation and that displays a proliferation specific expression pattern (Costa, Kalinichenko et al. 2003, Laoukili, Stahl et al. 2007). It is expressed in embryos and proliferating tissues as well as in response to injury in adults (Yao, Sha et al. 1997, Ye, Holterman et al. 1999, Leung, Lin et al. 2001, Wang, Krupczak-Hollis et al. 2002, Wang, Chen et al. 2005, Tan, Raychaudhuri et al. 2007)

Detailed cell cycle analysis revealed that the expression of FOXM1 protein increases during G1 and S phases, reaching a maximal level in G2/M transition (Korver, Roose et al. 1997, Laoukili, Kooistra et al. 2005). Despite *FOXM1* mRNA and protein being expressed throughout the cell cycle, its transcriptional activity is tightly regulated in a cell cycle-dependent manner. Several important proliferation and anti-proliferation signals regulate FOXM1 transcriptional activity involving post-translational modifications and protein-protein interactions. The G1 phase regulator CYCLIN D1/CDK4 complex strongly and indirectly activates FOXM1 by releasing the TAD from RB repression and by the release of the TAD by the NRD repression. The complexes CYCLIN E/CDK2, CYCLIN A/CDK2 and CYCLIN A/CDK1 also phosphorylate and activate FOXM1 (Fig. 1.9), and the M phase associated CYCLIN B1/CDK1 complex may also phosphorylate and activate FOXM1. The Ras-Raf-MEK-ERK signalling pathway has been reported to regulate FOXM1 at multiple levels including nuclear localization, protein expression, and transcriptional activity. Antagonistically to these proliferation signals, the tumour suppressor ARF (Alternative Reading Frame) interacts with FOXM1 to prevent its transactivation and the tumour suppressor RB directly binds to the NRD and represses indirectly its TAD. The cyclin dependent kinases inhibitors p16^{INK4a}, p21^{Cip1} and p27^{Kip1} also repress FOXM1 through the inhibition of CYCLIN D1/CDK4, CYCLIN E/CDK2 and CYCLIN A/CDK2. Moreover, glycogen synthase kinase-3 α targets the TAD of FOXM1 and abolishes its transactivation (Wierstra and Alves 2006).

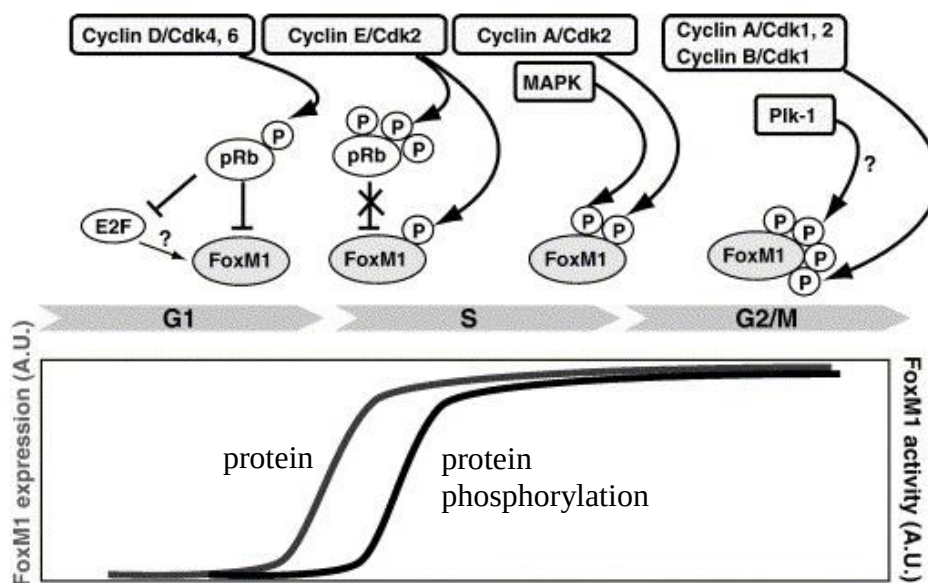


Figure 1.10 Cell cycle-dependent phosphorylation of FOXM1. Cell cycle-dependent regulation of FoxM1. FoxM1 protein expression (legend on the left) increases in late-G1. Upon mitogenic stimulation, Cyclin D/Cdk4, 6 and Cyclin E/Cdk2 inactivate pRb and relieve FOXM1 inhibition from pRb allowing the cells to progress into S phase. Increased FoxM1 transcriptional activity (legend on the right) in G2/M correlates with its hyperphosphorylation (Laoukili et al 2007).

1.6.3 FOXM1 function

1.6.3.1 Cell cycle

So far, the best described function of FOXM1 is its role in cell growth. It stimulates proliferation by promoting G1/S and G2/M transitions (Leung, Lin et al. 2001, Wang, Hung et al. 2001, Laoukili, Kooistra et al. 2005, Wierstra and Alves 2007). The use of MEF *foxm1*^{-/-} and siRNA interference demonstrated that FOXM1 is necessary for the expression of CDC25A, phosphatase required to activate CDK2 kinase activity during G1 and S phases. FOXM1 also facilitates CDK2 activation by induction of SKP2 and CKS1 expression, which induces cdk inhibitors degradation by the proteasome. In addition, CDC25A and cdk inhibitors regulate RB phosphorylation and E2F release, which in turn stimulate transcription and cell cycle progression. For the progression from G2 to M phase, FOXM1 controls the expression of CDC25B and CYCLIN B1 and upregulates PLK, SURVIVIN and AURORA B during the mitotic phase (Wang, Chen et al. 2005). Due to its role in mitosis, depletion of FOXM1 has dramatic consequences such as aneuploidy and polyploidy, failure of the prophase stage, misalignment of chromosomes at metaphase or mitotic spindle defect (Laoukili, Kooistra et al. 2005, Wang, Chen et al. 2005, Wonsey and Follettie 2005).

1.6.3.2 Regenerative cell proliferation

In addition to its role in cell growth, FOXM1 is important for tissue repair. It was shown that transgenic mice, with tissue-specific FOXM1 expression, display accelerated cell proliferation following partial hepatectomy, liver or lung injury (Ye, Holterman et al. 1999, Wang, Hung et al. 2001, Costa, Kalinichenko et al. 2003, Kalinichenko, Gusarova et al. 2003). In contrast, regenerative cell proliferation is reduced in mice with hepatocyte-specific and endothelial cell-specific FOXM1 knock-out (Wang, Kiyokawa et al. 2002, Zhao, Gao et al. 2006).

1.6.3.3 Senescence

In agreement with FOXM1 importance in cell proliferation, MEF *foxm1*^{-/-} and cells from mice with pancreas-specific knock-out display senescence (Wang, Chen et al. 2005, Zhang, Ackermann et al. 2006, Li, Smith et al. 2008, Park, Carr et al. 2009, Zeng, Wang et al. 2009). The role of FOXM1 in carcinogenesis has also been investigated and demonstrated that FOXM1 depletion sensitizes cells to oxidative stress and senescence (Park, Carr et al. 2009). Furthermore, it was recently reported that FOXM1 plays a role in senescence inhibition through transcriptional activation of Bmi1 and inhibition of p27^{Kip1} (Li, Smith et al. 2008, Zeng, Wang et al. 2009).

1.6.3.4 Apoptosis

FOXM1 has recently been demonstrated to regulate apoptosis. It was first reported that MEF FOXM1 knock-out and pancreas-specific FOXM1 depleted show an increase in apoptosis (Zhang, Ackermann et al. 2006, Tan, Raychaudhuri et al. 2007, Bhat, Halasi et al. 2009). In addition, FOXM1 inhibition by thiazole antibiotic, thiostrepton, siomycin A or ARF peptide inhibitor induce apoptosis in human cancer cells and SV-40 transformed human lung fibroblasts (Kalinichenko, Major et al. 2004, Radhakrishnan, Bhat et al. 2006, Kwok, Myatt et al. 2008, Bhat, Halasi et al. 2009). Although studies have demonstrated a correlation between FOXM1 inhibition and apoptosis, molecular mechanisms are not yet completely clarified.

1.6.3.5 DNA damage

The observation of aneuploidy and polyploidy in FOXM1 deficient cells indicates a requirement of FOXM1 in chromosome stability and integrity. Consistent with this, the percentage of DNA breaks increased when FOXM1 was depleted by knock-out and siRNA interference (Tan, Raychaudhuri et al. 2007). FOXM1 is likely to have a role in DNA damage but it is still elusive. A study has shown FOXM1 accumulation promoted by CHK2 following IR, UV and etoposide, and a role in regulation of DNA repair genes, including *XRCC1* and *BRCA2* (Tan, Raychaudhuri et al. 2007). In

addition, exogenous FOXM1 promotes cisplatin resistance in breast cancer cells (Kwok, Peck et al. 2010).

1.6.3.6 Angiogenesis

Histological studies showed strong FOXM1 staining in gastric tumours and lymph node metastases. The manipulation of FOXM1, by overexpression or silencing, demonstrated that FOXM1 promotes cell growth and angiogenesis via the modulation of genes involved in the degradation of the extracellular matrix and angiogenesis such as *uPA* (urokinase-type kinase plasminogen activator), *uPAR* (urokinase-type kinase plasminogen activator receptor), *MMP-2* (matrix metalloproteinase 2), *MMP-9* (matrix metalloproteinase 9) and *VEGF* (vascular endothelial growth factor) (Wang, Banerjee et al. 2007, Li, Zhang et al. 2009). This finding was confirmed by the discovery of FOXM1 binding sites in *VEGF* and *MMP2* promoters (Dai, Kang et al. 2007, Zhang, Zhang et al. 2008).

In summary, this tight antagonistic regulation of FOXM1 may require control to exclude aberrant regulation of FOXM1 downstream target genes and their functions that could result in tumourigenesis (Fig. 1.10).

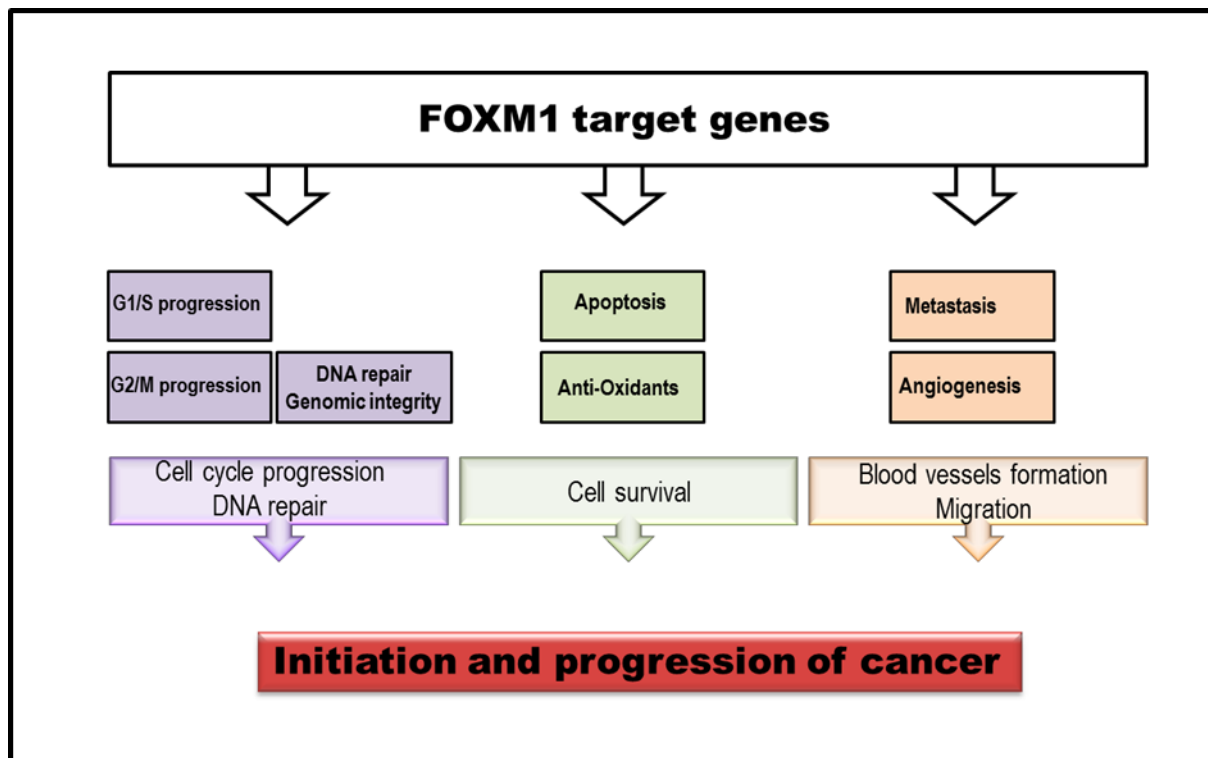


Figure 1.11 FOXM1 functions. FOXM1 regulates a wide range of genes involved in key biological processes. FOXM1 regulates genes involved in G1/S and G2/M transitions as well as in genomic integrity leading to cell cycle progression and DNA repair. FOXM1 also regulates genes involved in apoptosis, metastasis and blood vessels formation. Deregulation of FOXM1 and its downstream targets enhance cell cycle progression, DNA repair, survival and angiogenesis and participate to the initiation and development of cancers.

1.7 FOXM1 IN CANCER

FOXM1 has been shown to be overexpressed in an extensive number of human cancers, including gastric, cervical, breast, epidermal keratinocyte, lung, prostate, colon and hepatocellular carcinomas (Kalinichenko, Major et al. 2004, Pilarsky, Wenzig et al. 2004, Chandran, Ma et al. 2007, Yoshida, Wang et al. 2007, Gialmanidis, Bravou et al. 2009, Zeng, Wang et al. 2009, Teh, Gemenetzidis et al. 2010, Kretschmer, Sterner-Kock et al. 2011). Particularly, it has been reported that FOXM1 expression level increased with tumour grade and was inversely correlated with patient survival (Kalin, Wang et al. 2006, Liu, Dai et al. 2006). Furthermore, the chromosome band 12p13 where FOXM1 is located is frequently amplified in cervical, head and neck carcinomas (Willem and Mendelow 1997, Sato, Kobayashi et al.

2001). Mice models highlighted the functional role of FOXM1 in tumour initiation and progression. The knock-out of FOXM1 before liver cancer induction decreased liver tumour development, and the knock-out of FOXM1 before lung and colon cancers induction reduced the size and the number of adenocarcinomas (Kalinichenko, Major et al. 2004, Kim, Ackerson et al. 2006, Yoshida, Wang et al. 2007). Similarly, anchorage-independent growth on soft agar and tumour formation in nude mice were increased by FOXM1 overexpression indicating that FOXM1 enhances tumour development (Wang, Park et al. 2011). The overexpression of FOXM1 in mice model increased the invasion capacity of glioma cells indicating that FOXM1 has a critical role in metastasis (Dai, Kang et al. 2007, Raychaudhuri and Park 2011).

1.7.1 FOXM1 in breast cancer

A pioneer study in 2005 has revealed that *FOXM1* mRNA expression level is significantly overexpressed in 194 infiltrating ductal carcinomas, but not in untransformed breast epithelial tissues. Furthermore, RT-qPCR data showed a positive correlation between *FOXM1* transcript levels and the stage of breast cancer disease (Wonsey and Follettie 2005).

FOXM1 was identified among genes associated with high histological grade in ER α positive breast cancers. During a survey of a panel of 16 different breast cell lines, FOXM1 correlates with ER α at mRNA and protein levels (Madureira, Varshochi et al. 2006). *FOXM1* silencing results in a significant decrease of ER α expression levels in ER α positive breast cancer cells. Conversely, ectopic expression of FOXM1 led to an upregulation of ER α transcript and protein levels. Furthermore, chromatin immunoprecipitation assay identified FOXM1 binding site on *ER α* promoter region (Madureira, Varshochi et al. 2006). Taken together, these data indicate FOXM1 as a physiological regulator of ER α in breast carcinomas. Moreover, FOXM1 levels predict early metastatic relapse for endocrine dependent breast cancers (Yau, Wang et al. 2011).

In addition to ER α , FOXM1 is associated with HER2 receptor. *FOXM1* and *HER2* mRNA and protein levels expression correlate in breast cancer cell lines and patient samples. Consistently, investigations of mammary epithelium targeted HER2 mouse tumours resulted in an increase of FOXM1 expression (Francis, Myatt et al.

2009). Data from breast cancer cell lines demonstrated that HER2 directly regulates FOXM1 in HER2 overexpressing breast cancers.

While there is no biomarker identified for patients who are oestrogen and progesterone receptor negative and exhibit low HER2 expression breast cancer, these patients are treated with chemotherapy and have poorer prognostic than receptor positive cancer patients. However, a recent study has shown that FOXM1 is found elevated by DNA copy number alterations in triple negative breast cancer, suggesting that targeting FOXM1 would be beneficial regardless the receptor status (Han, Jung et al. 2008).

Taken together, FOXM1 inhibition could be a new strategy to treat breast cancer patients of any types.

1.7.2 Development of FOXM1 inhibitors

FOXM1 is an attractive target for targeted therapy because it has a key role in many biological processes and is overexpressed in a majority of cancers (Wang, Ahmad et al. 2010). This notion is supported by studies using RNA interference to knock-down FOXM1 expression. Depletion of FOXM1 in breast cancer cells lead to inhibition of cell growth, clonogenicity, migration and invasion (Wonsey and Follettie 2005). In addition, FOXM1 silencing reduced cell proliferation and anchorage dependent cell growth on soft agar in several prostate and lung cancer cell lines (Kalinichenko, Gusarova et al. 2003, Kalin, Wang et al. 2006).

Consistent with this, a study in 2004 revealed that FOXM1 is essential for initiation of carcinogen-induced liver tumours since liver cells with *FOXM1* conditional depletion fail to proliferate and are resistant to liver cancer development (Kalinichenko, Major et al. 2004). Further results reported that ARF26-44 peptide can bind and inhibit FOXM1 transcriptional activity resulting in inhibition of cell proliferation and induction of apoptosis (Gusarova, Wang et al. 2007).

Within the last ten years, an emerging class of naturally occurring thiostrepton group of antibiotics has shown a range of antibacterial, anti-parasitic and anti-cancer properties. In 2008, evidence showed that thiostrepton antibiotic selectively reduced FOXM1 expression resulting in breast cancer cell death. Furthermore, a study using a cell-based screening assay system identified another member of the family of

antibiotics, siomycin A, as a potent inhibitor FOXM1 transcriptional activity. The efficacy of siomycin A and thiostrepton was studied in neuroblastoma, liver, leukemia, melanoma and breast cancer and results showed that the antibiotics induced apoptosis (Bhat, Zipfel et al. 2008, Kwok, Myatt et al. 2008, Bhat, Halasi et al. 2009). Siomycin A has been shown to specifically reduce FOXM1 transcriptional activity, while the mechanism of action of thiostrepton still needs to be clarified.

Taken together, these data suggest that targeting FOXM1 is a promising strategy for treating breast cancer and many other cancers. Moreover, studies have shown FOXM1 involvement in resistance to targeted therapy and chemotherapy. It is possible that inhibiting FOXM1 in combination with anti-cancer therapies will improve the efficacy of currently available treatments.

1.8 HYPOTHESES AND OBJECTIVES: FOXM1 as a therapeutic strategy to overcome drug resistance

Several members of the forkhead family have been found to be involved in diverse mechanisms of drug resistance. Notably, *FOXO* and *FOXM1* members were associated with targeted therapies and chemotherapies resistance.

FOXO proteins play an important role in protection of cells against genotoxic and environmental stresses. Although FOXO3A activation by anti-cancer drugs induces cell cycle arrest and apoptosis, chronic activation by doxorubicin induces the transcriptional expression of MDR1 leading to increased drug efflux ability, which can render cancer cells resistant to drug therapy (Hui, Francis et al. 2008). Activated FOXO3A can also contribute to the development of resistance to HER2 targeted therapies by increasing AKT/PI3K activity through the induction of PIK3CA expression and promotion of cell survival (Chen, Gomes et al. 2010). Another FOXO member, FOXO1 protein serves as a protector against oxidative stress and its contribution to drug resistance was highlighted during human pregnancy, when the human endometrial stromal cells are exposed to high fluctuations in oxygen levels. Through the induction of the expression of manganese superoxide dismutase (MnSOD), FOXO1 confers resistance to oxidative stress-induced apoptosis (Kajihara, Jones et al. 2006). Furthermore, FOXO1 is highly expressed in paclitaxel-resistant ovarian cells and enhanced by paclitaxel exposure. FOXO1 overexpression was

frequently observed in tissue samples from paclitaxel-resistant patients compared to paclitaxel-sensitive patients. *FOXO1* silencing rendered chemoresistant cells sensitive to paclitaxel-induced apoptosis (Goto, Takano et al. 2008). In addition to taxane resistance, FOXO1 has been involved in breast cancer resistance to anthracyclines. A reporter assay showed that FOXO1 stimulates the transcription of *MDR1* gene expression in MCF-7 cells. MDR1 expression and doxorubicin resistance in MCF-7 resistant cells was reversed by *FOXO1* silencing indicating that FOXO1 expression is crucial for chemoresistance (Han, Cho et al. 2008).

In vitro studies showed that ectopic expression of FOXM1 confers breast cancer cells resistance to trastuzumab and paclitaxel. Resistance to the growth inhibitory effect of trastuzumab has been accounted by its ability to maintain low level of p27^{Kip1}, preventing its accumulation and cell cycle arrest. Moreover, FOXM1 transcriptionally activates the expression of *STATHMIN*, which inhibits the polymerization of the microtubules in response to the taxane agent paclitaxel (Carr, Park et al. 2010). An additional study demonstrated an increased FOXM1 protein expression in cisplatin resistant breast cancer cells (Kwok, Peck et al. 2010). FOXM1 contribution to cisplatin resistance is thought to be due to its role in DNA repair. However, the role and detailed mechanisms of FOXM1 involvement in DNA repair and resistance have not yet been elucidated. Moreover, it was recently reported that overexpression of FOXM1 partially protects osteocarcinoma cells from apoptosis induced by thiazole antibiotic (Bhat, Halasi et al. 2009). The emerging evidence from *in vitro* and *in vivo* studies demonstrate that FOXM1 plays an important role in initiation and progression of cancer by the regulation of many target genes and cross-talking with multiple signalling pathways (Kalin, Ustiyan et al. 2011). Therefore, FOXM1 signalling pathway is a promising therapeutic target and the development of agents targeting FOXM1 is likely to have a great impact for the treatment of drug resistant breast cancer.

1.8.1 FOXM1 regulation and role in tamoxifen sensitivity and resistance

Due to an increase of evidence in clinical resistance to a wide range of targeted therapeutic and chemotherapeutic agents, the development of novel drugs to overcome drug resistance is needed. Tamoxifen is the main endocrine treatment used for ER α positive breast cancer patients. However, 70 % of patients that initially respond to tamoxifen become resistant after long term treatment. ER α is a strong proliferative factor activating the expression of a wide range of genes encoding cytokines and factors associated with immune response, signal transduction, cell migration and cytoskeleton regulation. Data has showed that deregulation of ER α at transcriptional or posttranslational level can elicit anti-oestrogen resistance. Previous work in the laboratory reported that FOXM1 and ER α correlate at mRNA and protein levels in a panel of breast cancer cell lines. Further results showed that FOXM1 activates ER α transcriptional expression directly through the binding of forkhead site within *ER α* promoter in breast cancer cell lines (Madureira, Varshochi et al. 2006). Previous data also showed that FOXM1 is often regulated through a positive feedback loop with genes involved in proliferation such as *CYCLIN B1* and *PLK* (Leung, Lin et al. 2001, Laoukili, Kooistra et al. 2005, Fu, Malureanu et al. 2008). These co-regulations occur amongst genes involved in cell cycle, which could lead to uncontrolled proliferation and drug resistance of cancer cells. Therefore, it is important to investigate FOXM1 regulators. This thesis examines the regulation of FOXM1 by ER α , given that FOXM1 is frequently overexpressed in breast cancer, and ER α deregulation leads to the development of anti-oestrogen resistance. Furthermore, this thesis examines reversing anti-oestrogen resistance by downregulating FOXM1 as therapeutic approach.

1.8.2 FOXM1 regulation and role in chemotherapy sensitivity and resistance

Hormone receptor negative patients can only be administrated chemotherapeutic agents. Identification of potential biomarkers is urgently needed to elucidate novel therapies and improve the overall survival rate. FOXM1 has been

identified in a high resolution array to be amplified in hormone receptor negative breast cancer tissue patients suggesting that FOXM1 could be a potential biomarker in hormone receptor negative breast cancer (Han, Jung et al. 2008). FOXM1 target genes are involved in cell proliferation; oxidative stress and DNA repair processes. Recent data identified FOXM1 as a mediator of cisplatin resistance. FOXM1 involvement in cisplatin resistance is thought to be due to an increase in DNA repair but the detailed molecular events have not been clarified yet (Kwok, Peck et al. 2010). Further studies revealed that FOXM1 is stabilized by DNA damage agents through the phosphorylation by checkpoint kinase, CHK2, and leading to the regulation of genes involved in homologous recombination DNA repair mechanism (Tan, Raychaudhuri et al. 2007). Cisplatin is a strong alkylating agent given to treat a variety of cancers, but is limited by its toxicity profile. Nowadays, anthracyclines, doxorubicin or epirubicin, are the most widely prescribed chemotherapeutic agents. For nearly thirty years, the anthracyclines, doxorubicin and epirubicin, have been pivotal in the management of early stage breast cancer, particularly in hormone receptor negative cases (Boér 2010). As emerging evidence has shown that FOXM1 is involved in drug resistance and may in fact be a potential biomarker, this thesis investigates the involvement and regulation of FOXM1 in epirubicin-sensitive and – resistant breast cancer.

CHAPTER 2 MATERIAL AND METHODS

2.1 CELL CULTURE

2.1.1 Cell lines

The human breast carcinoma MCF-7, ZR-75-1, and MDA-MB-231, MDA-MB-453 cell lines originated from the American Type Culture Collection, were obtained from the Cancer Research UK Cell Line lab (CRUK, Clare Hall, UK), in which they were tested and authenticated, and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Foetal Calf Serum (FCS), 2 mM glutamine, and 100 units/ml penicillin/streptomycin at 37 °C in an atmosphere of 10 % CO₂.

The COS-1 cells were derived from kidney cells and grown in DMEM supplemented with 10 % Foetal Calf Serum (FCS), 2 mM glutamine, and 100 units/ml penicillin/streptomycin at 37 °C in an atmosphere of 10 % CO₂.

2.1.2 Stably transfected cell lines

Previously in our laboratory, parental MCF-7 cells were stably transfected with the N-terminal deleted FOXM1 fragment (Park, Wang et al. 2008) and the full-length FOXM1 in pcDNA3 expression vector and the transfection was maintained by DMEM supplemented with 1 µg/ml puromycin selection marker (Invitrogen, Paisley, UK).

2.1.3 Knock-out cells

The mouse embryonic fibroblasts (MEF) were derived from *wild-type*, *p53*^{-/-} and *p21*^{Cip1}^{-/-}. The MEF wild-type (*wt*) and *foxm1*^{-/-} were kindly provided by Pr René H. Medema (Department of Medical Oncology, University Medical Center Utrecht, The Netherlands). All cells were grown in humidified atmosphere 10 % CO₂ at 37 °C and in DMEM supplemented with 10 % FCS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin.

The wild-type 48BR (human fibroblast) primary skin fibroblasts were a generous gift by Dr Penny Jeggo (University of Sussex, UK). The human fibroblasts deficient in NBS1 (NBS1-LBI) were kindly given by Veronique Smiths (Unidad de investigacion, Hospital Universitario de Canarias, Spain). All cells were grown in humidified atmosphere 10 % CO₂ at 37 °C and in DMEM supplemented with 10 % FCS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin.

2.1.4 Drug resistant cell lines

The tamoxifen 4-OHT resistant MCF-7TAM^{R4} cells have been kindly given by Anne Lykkesfeldt (Institute of Cancer Biology, Denmark), and their growth conditions previously characterized and described [Lykkesfeldt, 1994 #229; Madsen, 1997 #230]. Briefly, a clone of MCF-7TAM^R cells were established by two series of one week treatment with 10⁻⁶ mol/L 4-OHT (OHT) and the resistant cells were continuously propagated with 10⁻⁶ mol/L OHT. The MCF-7 and derivatives cells were exposed to ER ligands: 10⁻⁸ mol/L estradiol (E2), 10⁻⁶ mol/L 4-OHT (OHT) or 10⁻⁷ mol/L ICI182780 (ICI) (prepared in ethanol), or only ethanol (vehicle control) for the indicated times prior to harvesting. For steroid starvation, these cells were cultured in phenol-free DMEM/F-12 containing 5 % double charcoal-stripped FCS.

The MCF-7EPI^R cell line is an epirubicin resistant cell line derived from parental MCF-7 cells. Previously in our laboratory, MCF-7 cells were subjected to a gradual concentration of epirubicin until cells acquire resistance up to 10 µmol/L of epirubicin (Pfizer, UK). MCF-7EPI^R cells were maintained in 1 µmol/L of epirubicin. Prior experiments, epirubicin was removed for 24 h and then cells were treated with epirubicin 1 µmol/L for the indicated times before harvesting. For RNA interference, the cells were treated with epirubicin 24 h after transfection. For ATM inhibition, cells were treated with Ku-55933 at 10 µmol/L for 24 h alone or in combination with 1 µmol/L epirubicin.

2.1.5 Cell line maintenance

Cells were grown and split at approximately 90 % twice a week. Media was aspirated and the cell monolayer was rinsed once with 1X PBS and then detached using 1X trypsin-EDTA mix. After centrifugation at 1200 rpm for 5 min, cells were resuspended in the appropriate media and seeded into an appropriate flask or dish.

For long term maintenance, cells were detached from the flask or dish by the addition of 1X trypsin-EDTA, spun 1200 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in FCS with 10 % dimethyl sulphoxide (DMSO) at 1 million cells/ml and 1 ml was transferred per cryotube and slowly frozen at -80 °C for 2 days before being transferred to storage in liquid nitrogen. For defrosting cells, cryotubes were placed for 1min in a waterbath at 37 °C and the defrosted solution was added to complete media and spun at 1200 rpm for 5 min. the DMSO containing supernatant was removed and cell pellets were resuspended in fresh supplemented medium in flask or dish.

2.1.6 Chemicals

Tamoxifen was maintained as a stock solution at 2 mM at -20 °C and diluted in fresh media prior to treatment (Pfizer, UK). **ICI182780** and **estradiol** were dissolved in ethanol and stored -20 °C at a concentration of 10^{-2} M (SigmaAldrich, UK). **Epirubicin** (2 mg/ml in 0.9 % sodium chloride) was obtained from Pfizer UK and stored at 4 °C. **Ku-55933** was dissolved in ethanol and stored at -20 °C at a concentration of 10 mmol/L (Tocris Bioscience, UK).

2.2 PROTEIN ANALYSIS

2.2.1 Preparation of total protein lysates and determination of protein concentration

Whole cell extracts was prepared by harvesting cells using 1X trypsin-EDTA and spinning at 1200 rpm for 5 min to obtained cell pellets. The supernatant was discarded and cell pellets were washed in 1X PBS (Phosphate Buffered Saline) and spun for an additional 5 min at 1200 rpm. The supernatant was discarded and cell pellets were frozen at -80 °C until lysis was performed. Frozen pellets were lysed in a lysis buffer containing 0.1 % Triton X100, 150 mM NaCl₂, 50 mM Tris-HCl (pH 7.8), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors ("Complete protease inhibitor mixture, as instructed by the manufacturer, Roche Applied Sciences, UK) on ice for 30 min (Table 2.1). Supernatant were collected after microcentrifugation at 13000 rpm at 4 °C for 10 min and protein concentration was measured using Bio-Rad D_c protein assay (Bio-Rad Laboratories, CA, USA) as instructed by the manufacturer. 20 µl of reagent S was added to 1 ml of reagent A. A standard curve was established by assaying 5 dilutions of a protein standard within the range of 0.2 mg/ml to 1.5 mg/ml protein. 25 µl of reagent A was added to 2 µl of each dilution and mixed with 200 µl of reagent B. After 15 min, absorbance was read at 700 nm. The protein concentration of the samples was determined by multiplying the absorbance of the sample by the standard curve's regression coefficient.

2.2.2 Western blotting or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein expressions were determined using SDS-PAGE gels made using a 37.5 % (w/v) acrylamide/bis stock solution, Tris-HCl and, 25 % of ammonium persulphate and tetramethylethylenediamine (TEMED). SDS-PAGE gels consist of a lower resolving gel and an upper stacking gel. The percentage of resolving gel used depends on the size of the protein of interest. The protein extracts were diluted at 20 µg in 2X SDS loading buffer (4 % (w/v) SDS, 62.5 mM Tris-HCL (pH 6.8), 1 % (v/v)

glycerol, 0.01 % (w/v) bromophenol blue, 10 % (v/v) β -mercaptoethanol) and boiled at 100 °C for 5 min. Samples were then run at a constant voltage of 100V in running buffer (Table 2.1). Proteins were separated alongside a Novex® Sharp Pre-Stained protein standard (Invitrogen, Paisley, UK) to identify the molecular weight of the separated proteins. Subsequently, proteins were transferred to nitrocellulose membranes (Whatman® Protran®) using a transfer buffer at 90 V for 1 h (Table 2.1). After the transfer, membranes were saturated with a 5 % blocking solution (Bovine Serum Albumin or Milk) diluted in TBST for 1 h at room temperature (RT) (Table 2.1). The primary antibody was diluted at 1:1000 in the blocking solution previously used and incubated overnight at 4 °C, the membranes were then washed three times with TBST (Table 2.1) for 15 min and incubated either with peroxidase-conjugated secondary antibody against mouse or rabbit IgG at 1:5000 dilution in TBST for 45 min at RT. After the TBST washing steps, membranes were incubated with Western Lightning® ECL (chemiluminescence peroxidase substrate) according to the manufacturer's instructions (Perkin Elmer, UK) and a signal was detected using Hyperfilm ECL (GE healthcare) on SRX-101A x-ray developer.

Reagent	Recipe
<u>Western Blotting</u>	
1X TG	25 mM Tris, 192 mM Glycine, pH 8.3
TBS 10X	24.23 g Tris HCl, 80.06 g NaCl add up to 1 L with ddH ₂ O and adjust pH to 7.6 with concentrated HCl
TBST	100 ml of TBS 10x, 890 ml ddH ₂ O, 10 ml Tween 10 % (v/v)
Lysis buffer	50 mM Tris pH 7.5, 150 mM NaCl, 0.10 % Triton x100 , 10 mM NaF, 1 tablet protease inhibitors (Roche)
Running buffer	1x TG, 0.1 % SDS
Transfer buffer	100 ml TG 10x, 800 ml ddH ₂ O, 100 ml Ethanol
Blocking solution	5 % (w/v) BSA, TBST, 0.02 % (w/v) sodium azide
<u>Chromatin Immunoprecipitation</u>	
TSE I buffer	0.1 % (w/v) SDS, 1 % (v/v) Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl
Buffer I	0.25 % (v/v) Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5
Buffer II	200 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5
Lysis buffer	1 % SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1 tablet protease inhibitors

Table 2.1 SDS-PAGE and ChIP buffers

Antibody	Company	Reference	Specie	kDa
<u>Transcription factors and coactivators</u>				
FOXM1 (WB, ChIP)	Santa Cruz	sc-502	Rabbit	100
FOXM1 (IF)	Santa Cruz	sc-500	Rabbit	100
ERα	Santa Cruz	sc-543	Rabbit	66
AIB-1	BD transduction laboratories	611105	Mouse	160
P-p53 (Ser15)	Cell Signaling	9284	Rabbit	53
p53	Santa Cruz	Pab1801	Mouse	53
<u>Histones and histones modifiers</u>				
HDACI	Santa Cruz	sc-6299	Goat	55
HDACII	Abcam	ab7029	Rabbit	60
Acetyl-H3 (ChIP)	Upstate	06-599	Rabbit	10
Acetyl-H4 (ChIP)	Upstate	06-866	Rabbit	17
<u>Cell cycle regulators</u>				
Cyclin A	Santa Cruz	sc-239	Mouse	54
Cyclin B1	Santa Cruz	sc-752	Rabbit	60
Cyclin D1	Santa Cruz	sc-246	Mouse	37
CDK2	Abcam	ab2363	Mouse	34
CDK4	Cell Signaling	DCS156	Mouse	30
p21	Santa Cruz	sc-6246	Mouse	21
Cdc25b	Abcam	ab2358	Mouse	90
PLK	Santa Cruz	sc-17783	Mouse	66
pS2	Santa Cruz	sc-28925	Rabbit	7-12
E2F1 (WB, ChIP)	Santa Cruz	sc-193	Rabbit	60
P-pRB (Ser807/811)	Cell Signaling	9308	Rabbit	110
pRB (ChIP)	BD Pharmigen	554136	Mouse	110
<u>DNA damage and checkpoint markers</u>				
P-ATM (Ser1981)	Upstate	MAB3806	Mouse	370
ATM	Calbiochem	Ab-3	Rabbit	370
P-Chk1 (Ser345)	Cell Signaling	133D3	Rabbit	59
Chk1	Santa Cruz	sc-8408	Mouse	56
P-Chk2 (Thr68)	Cell Signaling	C13C1	Rabbit	62
Chk2	Upstate	clone 7	Mouse	67
P-H2AX (Ser139)	Upstate	clone JBW301	Mouse	17
H2AX	Cell Signaling	2595	Rabbit	17
NBS1	Cell Signalling	3002	Rabbit	95
<u>Cell death markers</u>				
PARP	Cell Signaling	9542	Rabbit	89,116
<u>Ubiquitous proteins</u>				
Beta-Tubulin	Santa Cruz	(H-235)sc-9104	Rabbit	57

Table 2.2 Antibodies for western blotting and ChIP

2.3 PULL-DOWN using biotin-labelled oligonucleotides

Nuclear and cytoplasmic extracts were prepared using NE-PER® nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, USA). Briefly, cells were collected using trypsin and centrifuged to obtain dry pellet. CER I buffer (cytoplasmic extraction reagent) was added to the pellet, incubated for 5 min and centrifuge at maximal speed. The supernatant (cytoplasmic fraction) was transferred to a new eppendorf and stored at -80 °C until use. The insoluble pellet was then resuspended in NER buffer (nuclear extraction reagent) and incubated on ice for 40 min. After the same process of centrifugation, the nuclear fraction was transferred to a new eppendorf and store at -80 °C until use.

The biotinylated oligonucleotides, oestrogen response element (ERE) (ERE-wt 5'-GCCGATTGGCGACGTTCCGTACGTGACCTTAACGCTCCGCCGGCG-3', 5'-CGCCGGCGGAGCGTTAAGGTCACGTGACGGAACGTCGCCAATCGGC-3'), or (mERE3 5'-GCCGATTGGCGACGTTCCGTAAACGTTACGTTAACGCTCCGCCGGC-3', 5'-CGCCGGCGGAGCGTTAACGTAACGTTACGGAACGTCGCCAATCGGC-3'), were firstly annealed and bound to streptavidin beads on a rotator for 2 h at RT. 50 µg of protein extract were added with or without an excess of unlabelled competitor oligonucleotides and incubated for 1 h at 4 °C on the rotator. Beads were then washed with three times 1X PBS, loading buffer was added. Beads were boiled at 100 °C for 5 min and analysed by western blotting the supernatant.

2.4 IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Cells were harvested and lysed as described in the protein analysis section. While the cells pellets were being extracted, 20 µl of dynabeads per reaction (Invitrogen, Paisley, UK) were washed with 1X PBS and incubated for 1 h 4 °C with the appropriate antibody. After centrifugation at 14 000 rpm, protein lysates were incubated with the complexes beads/antibodies for 2 h at 4 °C. For immunoblot analysis, the immunoprecipitated samples were diluted in 2X SDS-loading buffer, boiled and run on SDS-PAGE gels. After the transfer, membranes were blocked, incubated with primary antibody overnight 4°C and then with the relevant peroxidase-

conjugated secondary antibody for 1 h RT and visualised using Western Lightning® ECL

2.5 CHROMATIN IMMUNOPRECIPITATION (ChIP)

2.5.1 Beads preparation

The day before chromatin immunoprecipitation was performed, 20 µl of dynabeads per condition (Invitrogen, Paisley, UK) were washed with TSE I buffer (Table 2.1) three times using a magnetic rack to discard the supernatant and were resuspended in 20 µl of TSE I buffer. One microgram of antibody and control immunoglobulin were incubated with the beads overnight on the rotator at 4 °C (Table 2.2).

2.5.2 Cells preparation

The cells were seeded in 10 cm dish to obtain 90 % confluency prior experiments. About 10 million of cells were cross-linked by adding 270 µl of 37 % formaldehyde to the 10 ml of cell medium (1 % formaldehyde) and were incubated at 37 °C for 10 min. Under the chemical hood, formaldehyde/medium mix was discarded and cells were gently washed twice with cold 1X PBS. One millilitre of the mix containing 100 mM Tris-HCl pH 9.4 and 10 mM DTT was added to each 10 cm dish to scrape the cells and transferred to an ice-cold sterile eppendorf. Cells were then centrifuged at 5 000 rpm for 5 min. Pellets were sequentially washed with 1 ml of 1X PBS, ChIP buffer I and ChIP buffer II (Table 2.1) to obtain chromatin.

2.5.3 Sonication

After these washing steps, cell pellets were resuspended in a ChIP lysis buffer (Table 2.1), incubated on ice for 10 min and sonicated for 12 min at 30 sec intervals. Optimisation of sonication times has been optimised with the different cell lines used. The sonication time was determined by running DNA (chromatin) on 1 % DNA gel electrophoresis and selecting time for which DNA size is between 100-500 bp.

2.5.4 DNA/beads-antibody incubation

After sequential washing steps, the lysates were microcentrifuged at 14000 rpm for 10 min at 4 °C. The chromatin pellets obtained were resuspended in the TSE I buffer, transferred to the beads-antibody complexes and incubated on the rotator for 2 h at 4 °C. Afterwards, the beads were washed with the TSE I buffer five times and the DNA was eluted with a mixture of 1 % (w/v) SDS and 0.1 M NaHCO₃ twice for 1 h on the rotator.

2.5.5 DNA elution, purification and Polymerase Chain Reaction (PCR)

The samples were decrosslinked at 65 °C overnight and DNA was purified using the QIAquick PCR Purification kit (Qiagen, Paisley, UK), as described in the manufacturer's instructions. After the purification, a Polymerase Chain Reaction (PCR) was set up with primers designed using the ABI Primer Express software (Table 2.3). 50 ng of total eluted DNA or 1% of eluted DNA (input) with 1 µM of a forward and reverse primer specifically designed for each target gene (at the DNA binding site tested and control site upstream), mixture of dNTP and mix reaction of the DNA polymerase kit containing 10X buffer, Q solution, MnCl₂ and Taq polymerase as described by the manufacturer (Qiagen, Paisley, UK) were incubated in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems).

The controls performed include:

- primers for a region where the protein of interest is absent (negative control) that were tested for each experiment (shown in the results).
- a non-template control that was included in each PCR reaction to spot contamination (not shown in the results).

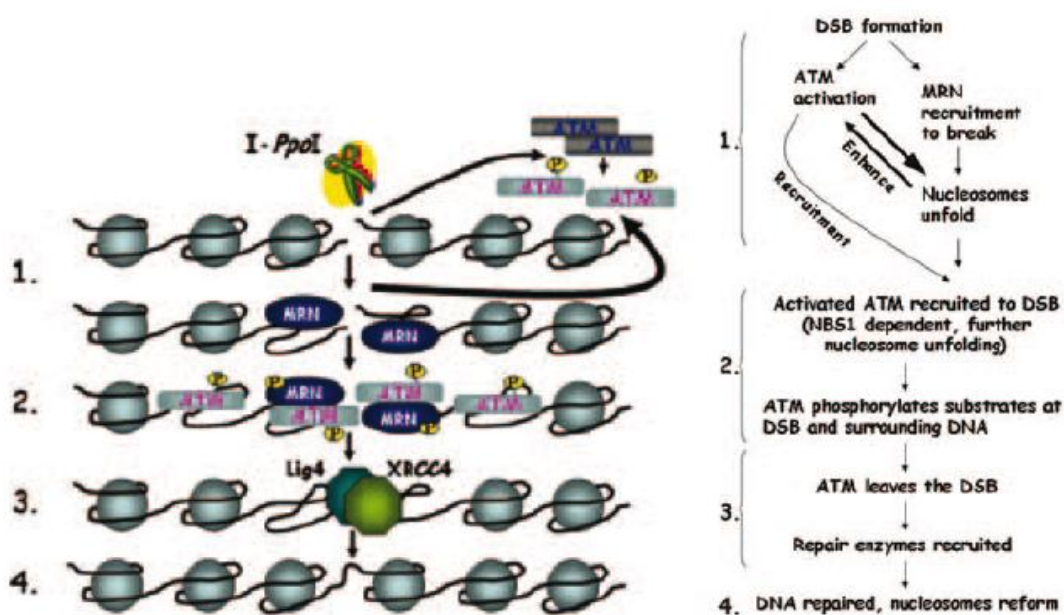


Figure 2.1 DSB detection and repair model. I-PpoI cuts DNA (1) inducing chromatin structural change that initiates ATM activation. Activated ATM is recruited to DSBs and phosphorylate MRN proteins (2). Repair proteins such as XRCC4 are recruited to the DSB (3) (from (Berkovich, Monnat et al. 2007)).

2.5.6 DNA gel electrophoresis

Agarose (SigmaAldrich, UK) was dissolved in the appropriate volume of 1X TAE (Table 2.4) at the concentration of 1 % (w/v) and the mixture heated until fully dissolved. Ethidium bromide was added once the mixture was allowed to cool. Samples diluted in DNA loading buffer (Table 2.4) and DNA ladder was resolved and DNA visualized under Ultra Violet light using UVIPro Platinum software.

Buffer	Recipe
Tris Acetate EDTA (TAE 50X)	2 M Tris, 57.1 ml acetic acid, 0.2 M EDTA pH 8.0, ddH ₂ O added until total volume 1 L
DNA Loading buffer	2.3 M sucrose and 100 mg Orange G made to 50 ml with ddH ₂ O

Table 2.3 DNA gel electrophoresis buffers

2.6 RNA ANALYSIS

2.6.1 Total RNA extraction

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Crawley, UK). The protocol was performed in line with the manufacturer's instructions.

Frozen cell pellets were resuspended in 350 µl of RLT buffer (containing 10% β-mercaptoethanol) and homogenised by pipetting. 350 µl of 70 % ethanol was added and the total mixture was transferred to the provided column, which was placed in a 2 ml collection tube, and spun in a benchtop centrifuge at 10000 rpm for 30 seconds (sec). The flow through was discarded and 700 µl of buffer RW1 was added to the column and spun at 10000 rpm for 30 sec. Then, 500 µl of RPE buffer was added to the column and spun at 10000 rpm for 30 sec. the low through was discarded and the column was spun for an additional 10000 rpm for 30 sec to remove any waste of the column. Next, the column was transferred to a clean sterile eppendorf tube. The

extracted RNA was eluted by the addition of 30 µl of RNase-free water to the centre of the column and spinning for 1 min at 10000 rpm. The purity and concentration of the RNA was determined using Nanodrop, which measures the spectrometric absorption at 260 nm and 280 nm. RNA samples were then stored at -80 °C or immediately used to make cDNA using first strand cDNA synthesis.

2.6.2 First strand cDNA synthesis

1 µg of total RNA was reverse transcribed into first strand cDNA using the Superscript III first strand cDNA synthesis system (Invitrogen, Paisley, UK). One microliter of random primers and 1 µl of 10 mM dNTPs mix (containing four bases adenine, cytosine, guanine and thymine) were added to 1 µg of total RNA to make a total volume of 14 µl using sterile RNase free water. The sample was heated for 5 min at 65 °C and placed immediately on ice for 1 min. Four microliters of 5X first strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT and 1 µl of the reverse transcriptase Superscript III were then added to make a total volume of 20 µl. The mixture was placed in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems) where they were incubated for 5 min at 25 °C for 5 min, then heated to 50 °C for 50 min. the reaction was terminated by heating the mixture at 70 °C for 15 min.

2.6.3 Primers

The following human and mouse gene-specific primer pairs were designed using the ABI Primer Express software:

	Forward sequence	Reverse sequence	Temp (° C)
ChIP primers (human):			
FOXM1 (ERE)	CCACTTCTTCCCCACAAG	CCGGAGCTTTCAGTTTGTTC	65
FOXM1 (E2F)	CCACTTCTTCCCCACAAG	CCGGAGCTTTCAGTTTGTTC	65
FOXM1 (cont)	CCACGCTTCCCCACAAG	CCGGAGCTTTCAGTTT	65
NBS1 (FHK)	AATTAAAAATTTTCCTTATGTTGCTTT	GGGCGCTTGCCCGCCACCTGGTGGTTGG	60
NBS1 (cont)	GCTAGAGTGCACTGGCATGA	AAGATCAGCATGGGCAACAT	60
DAB1	TGCTGCTTTTTTCTTCTCTCC	CTTCTTTCCCAAGTCTTC	64
B-actin	AATCCATCATGAAGTGTGACG	GATCCACATCTGCTGGAAGG	60
Gene expression primers (human):			
FOXM1	TGCAGCTAGGGATGTGAATCTTC	GGAGCCCAGTCCATCAGAACT	60
ERα	CAGATGGTCAGTGCCTTGTGG	CCAAGAGCAAGTTAGGAGCAAACAG	60
E2F1	CTGAGACAACCTTGAGGAAGAG	TTTGAACTGTACTAGCCAGTC	60
ATM	AATATCCATTACCGCAGCC	CACAATTTGCCGTAGGTAGTATC	60
ATR	AGTCCAGCCAGTCTCTACTCA	TGCCCATCCGGGACAA	60
NBS1	TTTTCAACCAGTTTTCCGTTACTTC	AACTGCGCGTATAAGCCAAT	60
MRE11	TGAGAACTGGCCTTCGATTCA	GGAGCCCAGACAAGCATGAT	60
RAD50	TCCAAATCTTGTGGAAGTGCAT	CTGCAAGCAGCCAGAACTTG	60
L19	TCTGGATGATGCTGTGCTACCT	GGCCACAGCTCAGACTGA	60
	Forward sequence	Reverse sequence	Temp (° C)
Gene expression primers (mouse):			
<i>Foxm1</i>	TGCAGCTAGGGATGTGAATCTTC	GGAGCCCAGTCCATCAGAACT	60
<i>Nbs1</i>	TGACAACCCGATAGAGGAGCAT	TCTTGCTCTCTGTCTGTCCAG	60
<i>Mre11</i>	TTCCCTCGGTGGGATTCAA	ACACCATCTGGCTGTCAGAA	60
<i>Rad50</i>	TAGCACACCAACACGTCGTA	CAGTGCCTTCCTCCTCTTGT	60
<i>Atm</i>	GCGCCACGCCTTGT	CAAACGTTGCCTGAAT	60
<i>L19</i>	TACACCTTCCCACTTACTGA	ATTCTCCGACTCTTCCTTT	60

Table 2.4 Human and mouse gene-specific primer pairs for RT-qPCR and ChIP

2.6.4 Real-time quantitative PCR (RT-qPCR)

The specificity of each primer was determined using NCBI BLAST module. Real time PCR was performed with ABI PRISM 7700 Sequence Detection System using SYBR Green Mastermix (Applied Biosystems, Brackley, UK).

Transcript levels were quantified using the standard curve method, where 1 µg of cDNA from each sample was mixed and diluted into serial dilutions (1/4, 1/16, 1/64, 1/256). L19, a non-regulated housekeeping gene, was used as an internal control to normalise the input cDNA.

The reaction mix contained 2 µl of sample and 23 µl of SYBR Green master mix, primers and RNase-free water were added to a final volume of 25 µl. All experiments were performed in triplicates.

2.7 DNA MANIPULATION

2.7.1 Plasmid amplification and extraction

The XL1-Blue competent cells were used to amplify plasmids. 50 µl of XL1-Blue (per vector) were thawed on ice and 2 µl of plasmid was added and incubated on ice for 30 min. The mixture competent cells and plasmid was heated at 42 °C in a waterbath for 45 sec and chilled on ice for 2 min prior incubation with 500 µl of SOC medium for 1 h at 37 °C. 100 µl was spread into warm LB-agar plates containing the selective antibiotic and incubated at 37 °C overnight.

Sixteen hours after incubation, individual colonies were grown overnight in 3 ml of LB-broth supplemented with the corresponding antibiotic. The following day, 1 ml of overnight culture was used for screening. Bacterial DNA was extracted using the miniprep protocol (Qiagen, Crawley, UK) following the manufacturer's instructions and DNA was digested using restriction enzymes to verify the presence of the insert. Positive clones were then grown overnight in 250 ml of LB-broth supplemented with the antibiotic and plasmid was extracted with a maxiprep protocol from Qiagen.

Reagent	Recipe
Luria-Bertani (LB) medium	1% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) NaCl
LB-agar medium	1% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) NaCl
SOC media	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 5 mM MgSO ₄ , 10 mM MgCl ₂ , 0.4% (w/v) glucose
Ampicillin	1 g Ampicillin in 10 ml ddH ₂ O (final concentration 100 mg/ml)
Ampicillin resistance selection media/agar	Ampicillin added to LB medium/agar at final concentration 100 µg/ml

Table 2.5 Bacterial culture reagents

2.7.2 DNA mutation and sequencing

Mutagenesis of plasmids were performed using the Stratagene Quickchange site-directed mutagenesis kit (Agilent Technology, Berkshire) as indicated by the manufacturer. Two complementary oligonucleotides containing the desired mutation and flanked by unmodified nucleotide sequence were designed prior mutagenesis. A PCR mix containing the DNA template, the forward and reverse primers, dNTP mixture, 10X reaction buffer and the *PfuTurbo* DNA polymerase was placed in a thermocycler for the amplification of mutated DNA. One microliter of *Dpn I* restriction enzyme was then added directly to the amplification reaction and incubated 1 h at 37 °C to digest the parental non mutated DNA. Next, the sample reaction was transformed into XL1-Blue competent cells, as described in the above section, and DNA was extracted using the miniprep kit to determine whether the DNA contains the

desired mutation. All plasmids DNA were sequenced at the Medical research Council DNA Core laboratory using an ABI 7500 PRISM automated sequencer.

2.7.3 Plasmid DNA transfection

Cells were seeded into 10 cm dish to reach confluency approximately 60%. Plasmids DNA (Table 2.6) were transfected using Fugene 6 (Roche Applied Sciences, UK). The ratio 3:1 (μ l of Fugene 6: μ g of DNA) was used as recommended by the manufacturer.

For one 10 cm dish, 988 μ l of DMEM (containing no supplement) was added to a sterile eppendorf tube and 12 μ l of Fugene 6 was added carefully. The mixture was shaken and left at RT for 5 min. 3 μ g of plasmid DNA (Table 2.6) was added, mixed and left at RT for 15 min. the mixture was then added gently to the dish containing cells and 10 ml medium. Overexpressions of desired proteins were confirmed 24 h after transfection after protein extraction and western blotting.

Vector	Selection	From/reference
pcDNA3-FOXM1	Ampicillin	Our laboratory
pcDNA3- Δ N-FOXM1	Ampicillin	Our laboratory
HEG0 (ER α)	Ampicillin	Tora et al, 1989, EMBO
pcDNA3-Flag- ER β	Ampicillin	J. Hartman
pcDNA3-Flag-p53	Ampicillin	N. Hadji
pCMV-E2F-1	Ampicillin	Helin et al, 1993
pFlag-Nbs1	Ampicillin	Kou-Juey Wu (Wu et al, 2007)

Table 2.6 Expression vectors

2.7.4 Luciferase assay

For promoter analysis, cells were treated with indicated drugs for Firefly/Renilla luciferase assays using the Steadyliteplus reporter assay system (Perkin Elmer, Cambridgeshire, UK) according to manufacturer's instructions. Briefly, the substrate (luciferin, ATP, magnesium and molecular oxygen) was added to the cells (seeded in 96-well plate) and the luminescence was measured after 15 min using a microplate reader (BMG Labtech, Offenburg, Germany). Subsequently, the substrate (coelenterate luciferin and molecular oxygen) for the Renilla was added to the previous mix and the cells, and luminescence was measured after 15 min. The ratio of luminescence of luciferase Reporter/Renilla reporter was calculated. All experiments were performed in triplicate.

Cells were transfected with the promoter constructs (Table 2.7) and 5 ng of Renilla (pRL-TK; Promega, Southampton, UK) as internal transfection control using Eugene-6 according to manufacturer's instructions (Roche Diagnostics Ltd, Burgess Hill, UK). For some experiments, cells were transfected with human *FOXM1* or *NBS1* promoters and 5 ng of Renilla alone, or in combination with 10 ng and 30 ng of expression vectors.

Promoter constructs	Length	From
<u><i>FOXM1</i> promoter</u>		
pGL3-Full length	2.4kb	René H. Medema
pGL3-Hind III	1.4kb	René H. Medema
pGL3-Apal	296bp	René H. Medema
pGL3-Apal-E2Fmut1	296bp	Made in the laboratory
pGL3-Apal-E2Fmut2	296bp	Made in the laboratory
pGL3-Apal-E2Fmut1/2	296bp	Made in the laboratory
<u><i>NBS1</i> promoter</u>		
pXP2-NBS1-FHK	1500bp	Kou-Juey Wu
pXP2-NBS1-FHKmut	1500bp	Made by myself

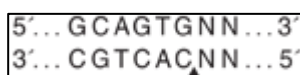
Table 2.7 Promoter constructs

2.7.5 Host cell reactivation assay (HCR)

The HCR assay is an assay measuring the DNA repair of single strand damaged DNA introduced into cells. This assay was performed using a damaged luciferase reporter vector, in which a CMV promoter drives the transcription of the luciferase gene. The plasmid harbouring luciferase was damaged prior transfection by a nicking endonuclease Nb.Bts1 (New England Biolabs) and was repaired by the cellular DNA repair machinery and only fully repaired plasmid will transcribe correctly to generate active luciferase.

Two micrograms of the cyclinB1 promoter luciferase reporter plasmid was damaged using 10,000 units/ml of a nicking endonuclease Nb.BtsI for 2 h at 37 °C (R0707S, New England Biolabs Ltd, Herts, UK) and 40 ng of plasmids were transfected along with 5 ng of Renilla plasmid (pRL-TK; Promega, Southampton, UK). One unit is defined as the amount of enzyme required to convert 1 µg of supercoiled plasmid to open circular form in 1 hour at 37°C in a total reaction volume of 50 µl. Each digestion was run on 1% agarose gel to confirm that the plasmid was linearized.

The endonuclease Nb.Bts1 cleaves DNA as a heterodimer of one large subunit (B subunit) and one small subunit (A subunit); and, in the absence of their small subunits, the large subunits behave as sequence-specific DNA nicking enzymes and only nick the bottom strand of the sequences at this position:



After the indicated time points, luciferase activity was measured and normalised against the internal control Renilla. Because of the variation in the transfection efficiency between undamaged and damaged plasmids, the percentage of luciferase recovery was determined comparing to the first time point at 0 h.

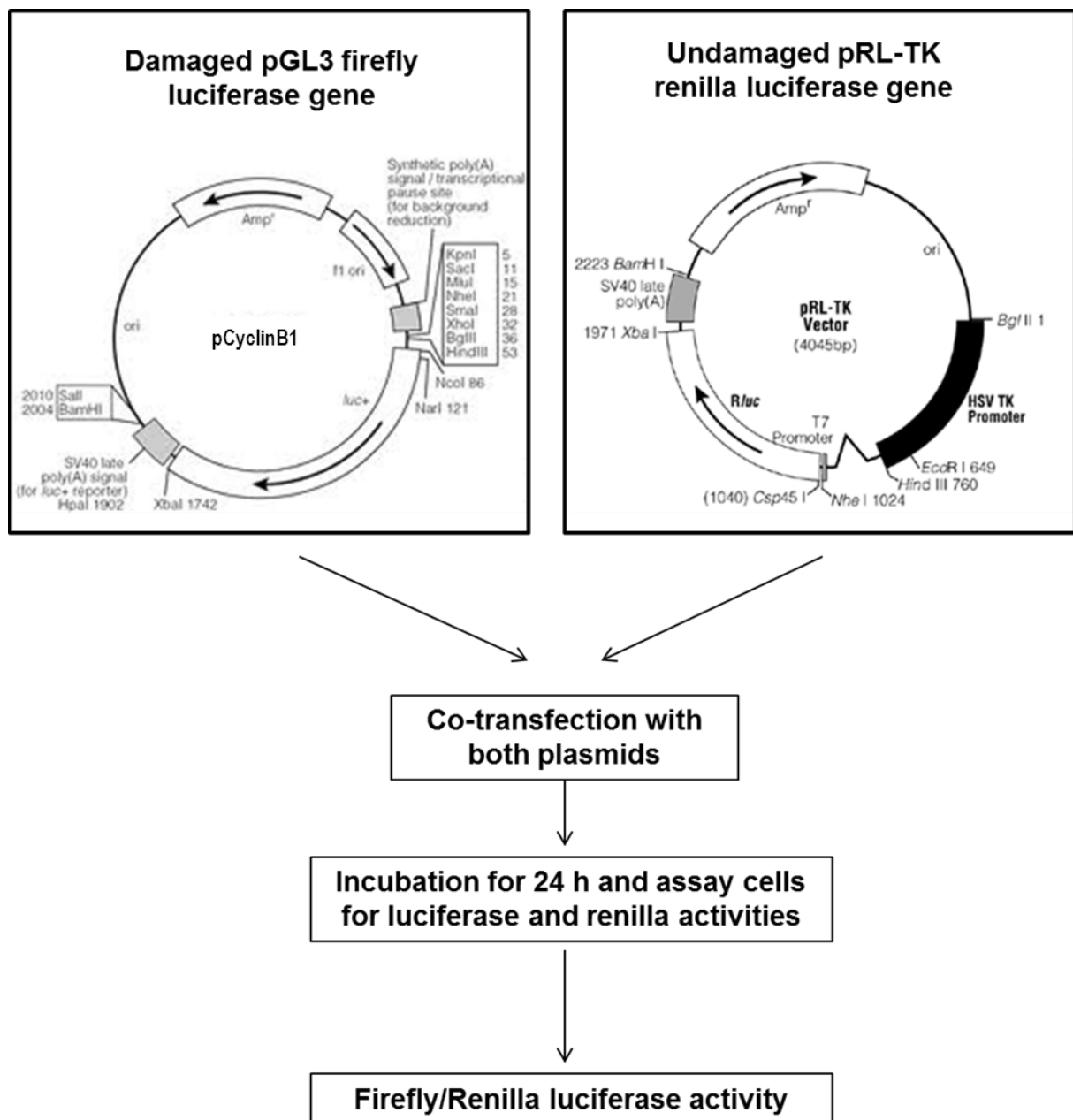


Figure 2.2 Host Cell Reactivation. Damaged pGL3-cyclinB1 luciferase reporter plasmid is transfected along with undamaged Renilla plasmid. Luciferase activity was measured and normalised against the internal control Renilla.

2.8 RNA INTERFERENCE

Transient RNA interference was used to specifically repress the expression of chosen genes and performed using small-interfering RNA (siRNA).

In this study, cells were transfected with the SMARTpool siRNAs purchased from Dharmacon (Lafayette, CO) using OligofectAMINE (Invitrogen, Paisley, UK) according to manufacturer's instructions. Briefly, cells were seeded in a 6-well plate 24 h prior transfection to reach a confluency of 50 % and the specific target gene was silenced using 50 nM of siRNA. For each well, 70 µl of Optimem was mixed with 5 µl of oligofectAMINE (Invitrogen, Paisley, UK) and incubated at RT for 10 min. This solution was then combined with 250 µl Optimem and 7,5 µl siRNA oligos for each gene. After 25 min incubation, 160 µl Optimem was added to the mixture reaching a final volume of 500 µl, which was added to the cells previously washed with 1X PBS. The 6-well plates were incubated at 37 °C for 4 h and 2 ml of culture medium containing 10 % FCS was subsequently added to cells to prevent toxic effects. Cells were harvested 24 h after transfection for protein and RNA analysis. SMARTpool FOXM1 siRNA (M-009762-00), ERα siRNA (L-003401-00), p53 siRNA (L-003329-00), p21^{Cip1} (L-003471-00), ATM (L-003201-00), NBS1 (L-009641-00), CHK1 (L-003255-00), CHK2 (L-003256-000) and siCONTROL non-targeting siRNA were used. All experiments were performed with a control mock condition. As control mock conditions showed the same results as the non/targeting siRNA, control mock condition was not shown in the data.

2.8 IMMUNOFLUORESCENCE MICROSCOPY

10000 cells per well were seeded into 8-well culture slide chambers (BD Falcon™, Oxford, UK) for confocal microscopy and 5000 cells per well into 96-well plate, black-walled with clear bottom (BD Falcon™, Oxford, UK) for foci/staining quantification with Image Xpress (Molecular Devices, Berkshire, UK). After 24 h, cells were treated with epirubicin 1 µmol/L for indicated times and then fixed and permeabilized with 100 % methanol for 10 min at RT. Wells were washed three times with 1X PBS and blocked with 5 % goat serum for 1 h at RT. Then, fixed cells were

incubated with a specific primary antibody for FOXM1 (Santa Cruz, sc-500) (1:250), P-ATM (Ser1981) (1:120) (Upstate, MAB3806), P-CHK2 (Thr68) (C13C1) or for P-H2AX (Ser139) (Upstate, JBW301) (dilution 1:250) overnight at 4 °C, followed by Alexa 488 (green)-conjugated anti-secondary antibody or Alexa 555 (red)-conjugated anti-secondary antibody (Invitrogen, Molecular Devices, UK) for 1 h at RT. Cells were counterstained with TO-PRO®-3 iodide or DAPI (Invitrogen, Molecular Devices, UK) to show the nuclei. Specific staining was visualised and images captured with Zeiss LSM 500 system confocal microscope. Foci quantification was performed with Image Xpress system microscopy and analysed with MetaMorph software (Molecular Devices, Berkshire, UK).

2.9 SRB assay

The cell survival was determined using the sulforhodamine B (SRB) colorimetric assay previously described (Skehan, 1990). The SRB dye binds proteins of the cell. It is an anionic aminoxanthene dye that forms an electrostatic complex with the basic amino acid residues of proteins under moderately acid conditions, which provides a sensitive linear response. Because the binding is stoichiometric, the quantity of dye dissolved from the stained and fixed cells is proportional to cell mass and representative of cell density. Therefore, the SRB assay detects the number of cells but not cell proliferation. Approximately 5000 cells per well seeded in 96-well plates were fixed with 100 µl of cold 40 % trichloroacetic acid for 1 h at 4 °C. After three washes with cold water, cells were stained with 0.4 % sulforhodamine B (that binds to protein basic amino acid residues) for 1 h at RT. Cells were then rinsed three times with 1 % acetic acid and left to dry overnight. The protein-bound dye was dissolved the next day in 10 mM Tris base solution for 30 min and measured at 495 nm using a Sunrise™ plate reader (Tecan Group Ltd). The results obtained were expressed as the mean of eight replicates relative to the results obtained for the vehicle control at 0 h providing the percentage of cell survival.

This assay is widely used for *in vitro* cytotoxicity screening. This assay has been used for high-throughput drug screening at the National Cancer Institute. Studies undertaken by groups showed that results from the SRB assay correlates well with

the MTT assay (tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Vichai and Kirtikara 2006).

Controls: all SRB assays with siRNA condition were performed with a mock control. No difference has been found compared with the non-targeting siRNA, therefore the mock data have not been included for clarity purpose.

2.10 CELL CYCLE ANALYSIS

Cell cycle analysis was performed using propidium iodide (PI) staining alone. Cells were trypsinized, collected by centrifugation, and washed in 1X PBS before fixing in 70 % ethanol. Fixed cells were washed twice in 90 % ethanol and then re-suspended in 1X PBS containing propidium iodide (1 mg/ml) supplemented with RNase (20 units/ml) for 15 min at 4 °C. The single cell suspensions were analysed on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) with CellQuest (BD Biosciences) acquisition software. For protein staining, cells were rinsed in PBS 1X before fixing in 70 % ethanol overnight at 4 °C. The fixed cells were then washed twice with 1X PBS and resuspended in 1X PBS containing 0.5 % of BSA. After centrifugation, the pellet was resuspended in 100 µl 1X PBS containing 1 % BSA and an antibody against P-ATM (Ser1981) (1:800), P-H2AX (Ser139) (1:400) or FOXM1-C20 (1:400) and incubated for 2 h at RT. After centrifugation, pellets were washed in 100 µl 1X PBS containing an anti-mouse or anti-rabbit Alexa 488 (green)-conjugated secondary antibody (1:400) (Invitrogen, Molecular Devices, UK) for 1 h at RT in the dark. After washing, pellets were counterstained with propidium iodide (1 mg/ml) supplemented with RNase (20 units/ml) for 15 min at RT. The samples were analysed by flow cytometry as described above. The experiments were performed in triplicate.

2.11 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism v5.0. The statistical significance of differences between the means of two groups was evaluated by

paired Student's *t* test and considered significant when n.s non significant, * $P \leq 0.1$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

CHAPTER 3 FOXM1 is a transcriptional target of ERalpha and has a critical role in breast cancer endocrine sensitivity and resistance

3.1 Introduction

Breast cancer is the most common cancer in women in the UK. The forkhead box family member FOXM1 was previously reported elevated in breast cancer adenocarcinoma as well as carcinoma from other origins (Pilarsky, Wenzig et al. 2004, Kalin, Wang et al. 2006, Kim, Ackerson et al. 2006, Yoshida, Wang et al. 2007). FOXM1 is highly expressed in embryo and proliferating adult tissues, while its expression is low in quiescent cells (Korver, Roose et al. 1997). FOXM1 regulates the expression of cell cycle regulatory genes involved in the G1/S and G2/M phase transitions (Laoukili, Kooistra et al. 2005). A cDNA microarray study previously identified FOXM1 as one of the 344 ER α responsive genes in breast cancer cells (Cicatiello, Scafoglio et al. 2004). Moreover, a recent study in our laboratory showed a positive correlation between ER α and FOXM1 protein expression in breast cancer cells (Madureira, Varshochi et al. 2006).

Oestrogens are the most important regulators of breast cancer growth and act through the oestrogen receptors, ER α and ER β . ER α activity induces breast cancer cell proliferation, while ER β is an anti-proliferative factor (Paruthiyil, Parmar et al. 2004). Oestrogens are heavily implicated in breast cancer because of their role in stimulating breast cell division, their activity during the critical periods of breast growth and development, and their effect on other hormones that stimulate breast cell division. Although ER α oncogenic potential, its expression in breast cancer patients is a good prognostic. Anti-oestrogen or endocrine therapies are effective only in patients with ER α positive breast cancer. Clinically, tamoxifen has been the most commonly used endocrine agent, and is suitable for both pre- and post-menopausal women. Tamoxifen is a selective ER α modulator that blocks the binding of oestrogen on ER α and induces the recruitment of co-repressors preventing ER α transcriptional activity.

Treatment of breast cancer with anti-oestrogens results in G1 cell cycle arrest and in some cases, cell death (Lykkesfeldt, Larsen et al. 1986). However, about half of the patients who initially respond to endocrine therapy become resistant despite continued expression of ER α (Ali and Coombes 2002, Goss, Muss et al. 2008,

Yamashita 2008). Indeed, loss of ER α expression occurs in a minority of resistant breast cancer while various mechanisms of endocrine resistance in ER α positive breast cancer have been reported. ER α is regulated through transcriptional, post-transcriptional and post-translational mechanisms, and deregulation of one of these processes can result in endocrine resistance (Musgrove and Sutherland 2009). Emerging data indicate that altered expression of several growth factors receptors and overexpression of ER α co-activators can constitutively phosphorylate and thereby activate ER α conferring breast cancer endocrine resistance.

The previous observation in our laboratory that ER α positive breast cancer cells express higher levels of FOXM1 led to hypothesise that FOXM1 may be regulated by ER α . Based on the recent evidence linking FOXM1 with drug resistance, I investigated the regulation of FOXM1 by ER α and its ligands in MCF-7 cells, and FOXM1 potential role in breast cancer endocrine resistance.

3.2 Results

3.2.1 Transcriptional regulation of FOXM1 by ER α in endocrine sensitive breast cancer cells

3.2.1.1 ER α ligands and ER α silencing modulate FOXM1 expression

To investigate whether FOXM1 is a target of ER α , the ER α positive MCF-7 breast cancer cell line and ER α negative MDA-MB-231 breast cancer cell line were treated with ER α ligands: estradiol (E2), tamoxifen (OHT) and fulvestrant (ICI). The efficacy of these treatments was verified by pS2 protein expression, a previously described oestrogen responsive gene (Soulez and Parker 2001). As expected, pS2 protein expression increased with the addition of E2 and decreased upon both anti-oestrogens OHT and ICI in MCF-7 cells (Fig. 3.1A). I further observed that E2 enhanced FOXM1 protein and mRNA expression within 24 h and remained high until 48 h in the ER α positive MCF-7 cells. By contrast, E2 did not change FOXM1 protein

and mRNA levels in the ER α negative MDA-MB-231 cells (Fig. 3.1B). The treatments with OHT and ICI reduced the expression of FOXM1 protein and mRNA in MCF-7 cells 24 h and 48 h after treatment, while FOXM1 expression was not affected by these treatments in MDA-MB-231 cells (Fig. 3.1B). Notably, E2 treatment decreased ER α protein expression in the ER α positive MCF-7 cells. Indeed, it has been reported that E2 increases ER α turnover (Nawaz, Lonard et al. 1999, Wijayaratne and McDonnell 2001). Moreover, ICI caused ER α degradation in the ER α positive MCF-7 cells (Long and Nephew 2006). These data have been confirmed in the ER α positive ZR-75-1 breast cancer cell line in our laboratory (data not shown; provided by Demetra Constantinidou).

In addition, the ability of OHT and ICI to antagonize E2-regulated FOXM1 upregulation was examined Figure 3.2. Therefore MCF-7 cells were stimulated by E2 for 4 h and then treated with OHT or ICI from 0 h to 48 h. The result showed that the effects of OHT and ICI following E2 stimulation were similar to those observed under normal growth conditions (Fig. 3.2). The ER α targets, pS2 and cyclin D1, and FOXM1 targets, PLK and CDC25B, were induced by E2 and repressed by the addition of OHT and ICI. Notably, FOXM1 expression pattern varied between Figures 3.1 and 3.2. Studies showed that if starvation is not perfect, some leakage might have occurred and cells slowly accumulate material leading to S phase initiation even during the period of incubation in low serum. Cells closer to initiation (later in S phase) can reach initiation mass sooner than cells earlier in the G1 phase. Cells later in the G1 phase at the time growth arrest are less likely to have a delayed cell division. Cells earlier in the cycle will not accrue enough leakage to initiate DNA synthesis and thus will exhibit a delayed cell division. FOXM1 expression pattern varies throughout the cell cycle. Therefore, it is possible that starvation was not reached Figure 3.2 leading to cells in different cell cycle phases with different FOXM1 protein expression levels (Cooper 2003, Cooper 2003).

In conclusion, the expression of FOXM1 protein and mRNA is upregulated by the ER α agonist and downregulated by ER α antagonists only in the ER α positive MCF-7 cells suggesting that ER α regulates FOXM1 expression. To provide further evidence of FOXM1 regulation by ER α , the effect of ER α silencing on FOXM1 protein

and mRNA levels was studied in MCF-7 cells. After 24 h transfection, ER α siRNA effectively silenced ER α at protein level and mRNA levels, and reduced the levels of FOXM1 protein and mRNA significantly (Fig. 3.3). In accordance with previous findings in the laboratory, FOXM1 siRNA also reduced ER α protein and mRNA levels (Madureira, Varshochi et al. 2006). Unfortunately, the quality of the western blot for this experiment is very poor and should be repeated.

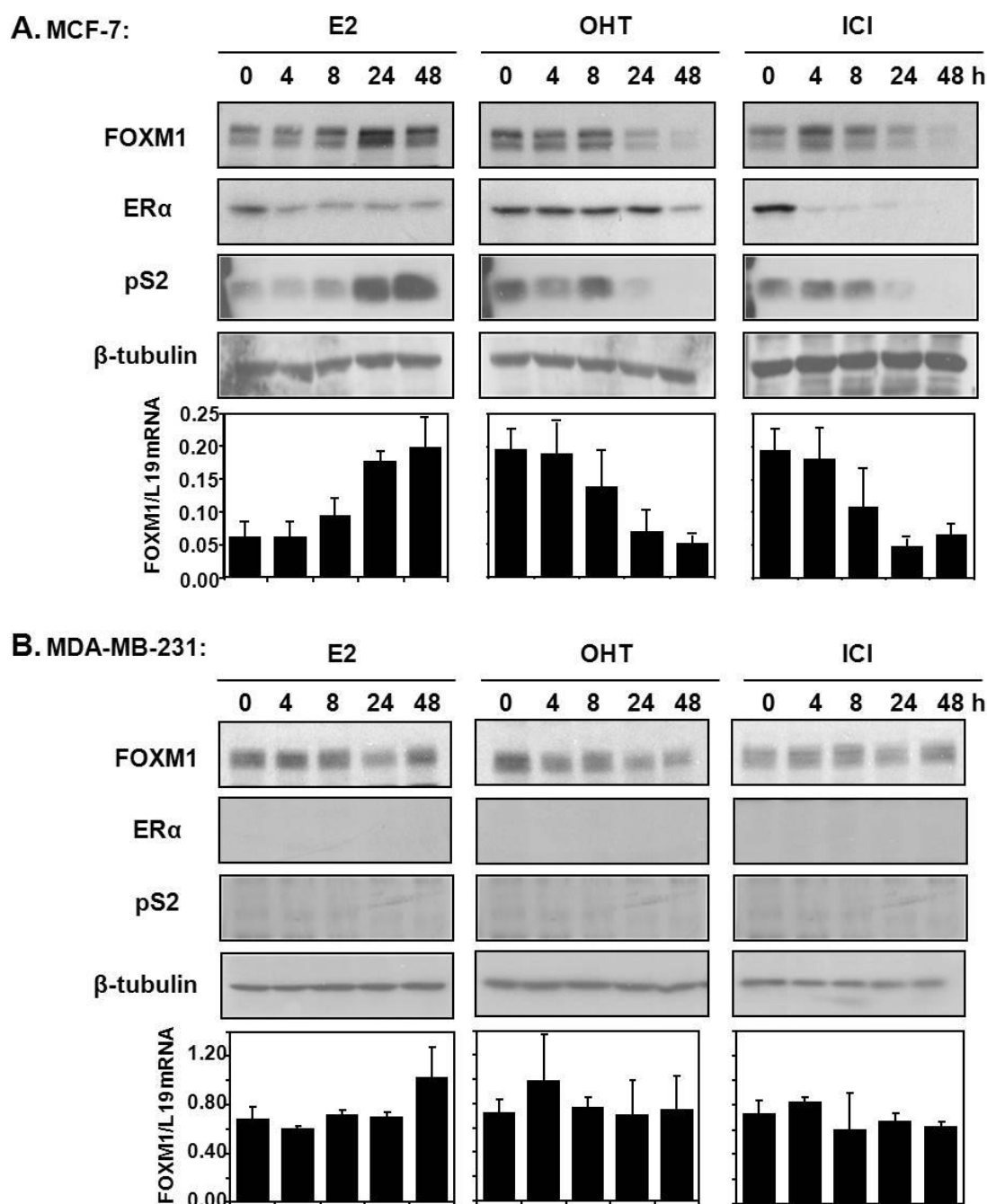


Figure 3.1 Expression of FOXM1 and ER α in response to E2, tamoxifen and ICI treatments in breast cancer cell lines. MCF-7 (A) and MDA-MB-231 (B) cells were cultured in 5 % double-charcoal striped FCS and phenol red free medium for 24 h before being stimulated with 10^{-8} mol/L of E2. Breast cancer cells cultured in 10 % FCS and phenol red medium were also treated with 10^{-6} mol/L of OHT or 10^{-7} mol/L of ICI. At times indicated, cells were collected and analysed for FOXM1, ER α , pS2 and β -tubulin expression by western blotting. FOXM1 mRNA levels of these cells were also analysed by RT-qPCR, and normalized with L19 RNA expression. All data shown represent the averages of data from three independent experiments, and the error bars show the standard deviations.

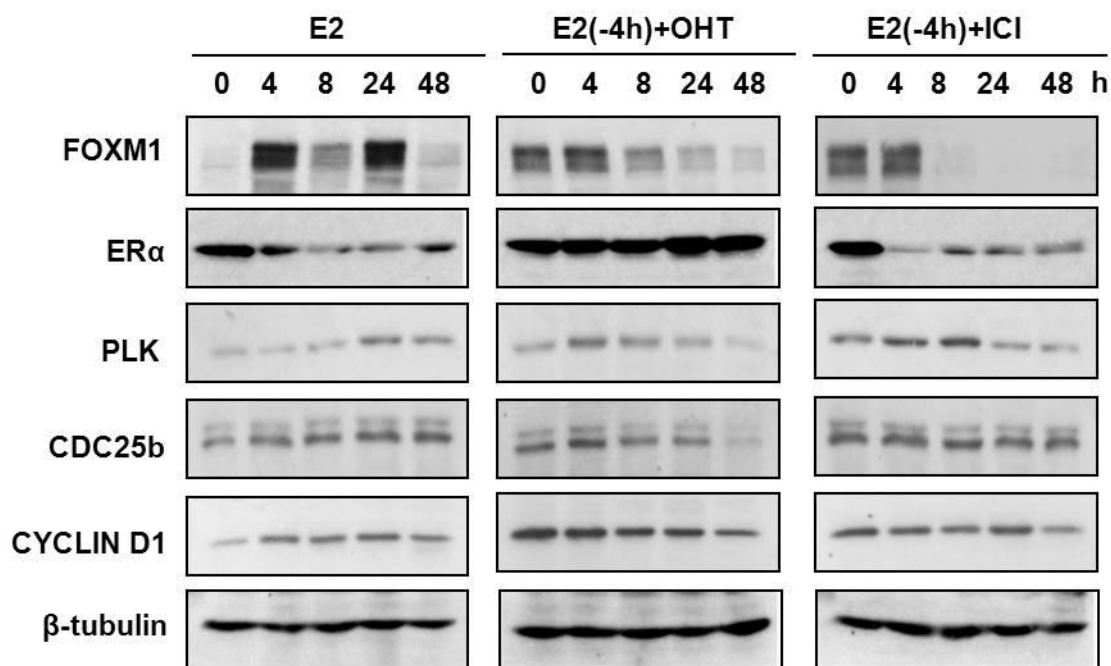


Figure 3.2 Induction of FOXM1 expression by E2 is antagonized by OHT and ICI in MCF-7 cells. MCF-7 cells were cultured in 5 % double-charcoal striped FCS and phenol red free medium for 24 h before stimulation with 10^{-8} mol/L of E2 (-4h). Four hours after E2 stimulation, the MCF-7 cells were treated with 10^{-6} mol/L of OHT or 10^{-7} mol/L of ICI for the indicated times. Cells were collected and analysed for FOXM1, ER α , PLK, CDC25b, CYCLIN D1 and β -tubulin protein expression using western blotting.

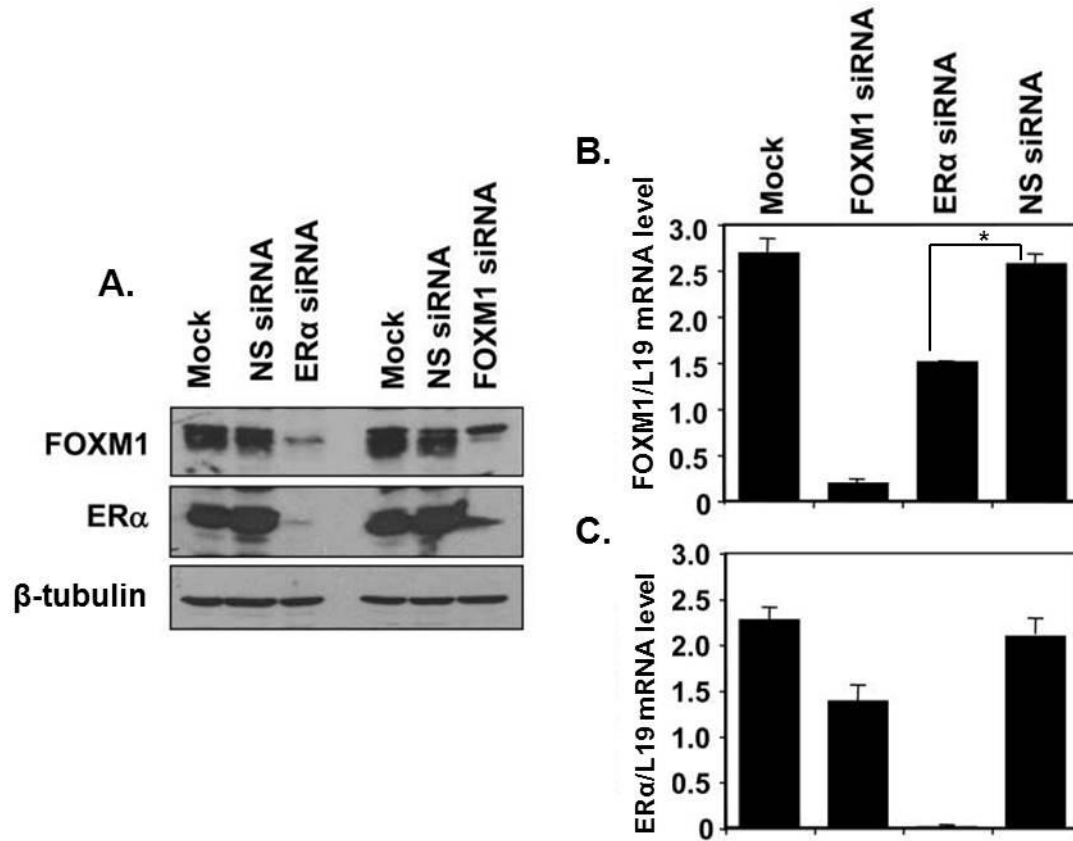


Figure 3.3 Effects of ERα silencing on the expression of FOXM1. MCF-7 cells were transiently transfected with ERα, FOXM1 or NS (non-specific) siRNA (100 nmol/L) with oligofectAMINE according to the manufacturer instructions. After 24 h transfection, cells were analysed for protein levels by western blot (**A.**) using specific antibodies as indicated and for mRNA levels of *FOXM1* (**B.**) and *ERα* (**C.**) by RT-qPCR. All data shown represent the averages of three independent experiments, and the error bars show the standard deviations. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$, **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

3.2.1.2 FOXM1 promoter responds to ER α ligands

The RT-qPCR data obtained in Figure 3.3 showed that FOXM1 is transcriptionally regulated by ER α . In order to elucidate whether FOXM1 is regulated at promoter level by ER α , Demetra Constantinidou and myself transiently co-transfected an ER α expression vector with three different FOXM1 promoter constructs in a luciferase reporter vector (Fig. 3.4A), previously made by Demetra Constantinidou in our laboratory, in COS-1 cells. We observed a 2-fold increase of luciferase activity of the 2 kb full length FOXM1 (pGL3-Full-length) and 1.5-fold increase of the luciferase activity of the 1.3 kb Hind III truncation constructs (pGL3-Hind III) 24 h after E2 treatment (Fig. 3.4B). The highest luciferase activity was obtained with the 300 pb Apa I truncation construct (pGL3-Apa I) that showed an enhancement of 2.5-fold upon 4 h E2 treatment, consistent with a previous study identifying a similar region of the FOXM1 promoter that responds to serum stimulation (Korver, Roose et al. 1997). As positive control, we performed this experiment with the *pS2* promoter (pGL3-ERE) under the luciferase reporter gene and observed an increase of 5-fold of the luciferase activity following E2 treatment. The addition of OHT for 24 h following E2 treatment resulted in a repression of all luciferase reporter gene activities previously induced by E2 (Fig. 3.4B).

These reporter assays were performed with the help of Demetra Constantinidou, who examined the proximal FOXM1 promoter sequence and identified potential ER α -responsive elements (EREs) by using the Transcription Element Search System (TESS website). These analyses revealed an ERE-like element located at -45 pb from the transcription start site that could explain the responsiveness to ER α ligands of the three FOXM1 promoters constructs used. Consequently, studies were focused on the characterization of the ERE-like element at -45 pb in the *Apa I* promoter fragment. By introducing mutations in both arms of the ERE-like palindrome of the *Apa I* truncation (Bourdeau, Deschenes et al. 2004) (Fig. S.D.7.1), Demetra Constantinidou observed by luciferase assay that the ERE3 mutant (mERE3) lost the majority of the responsiveness to E2 (Fig. S.D.7.2).

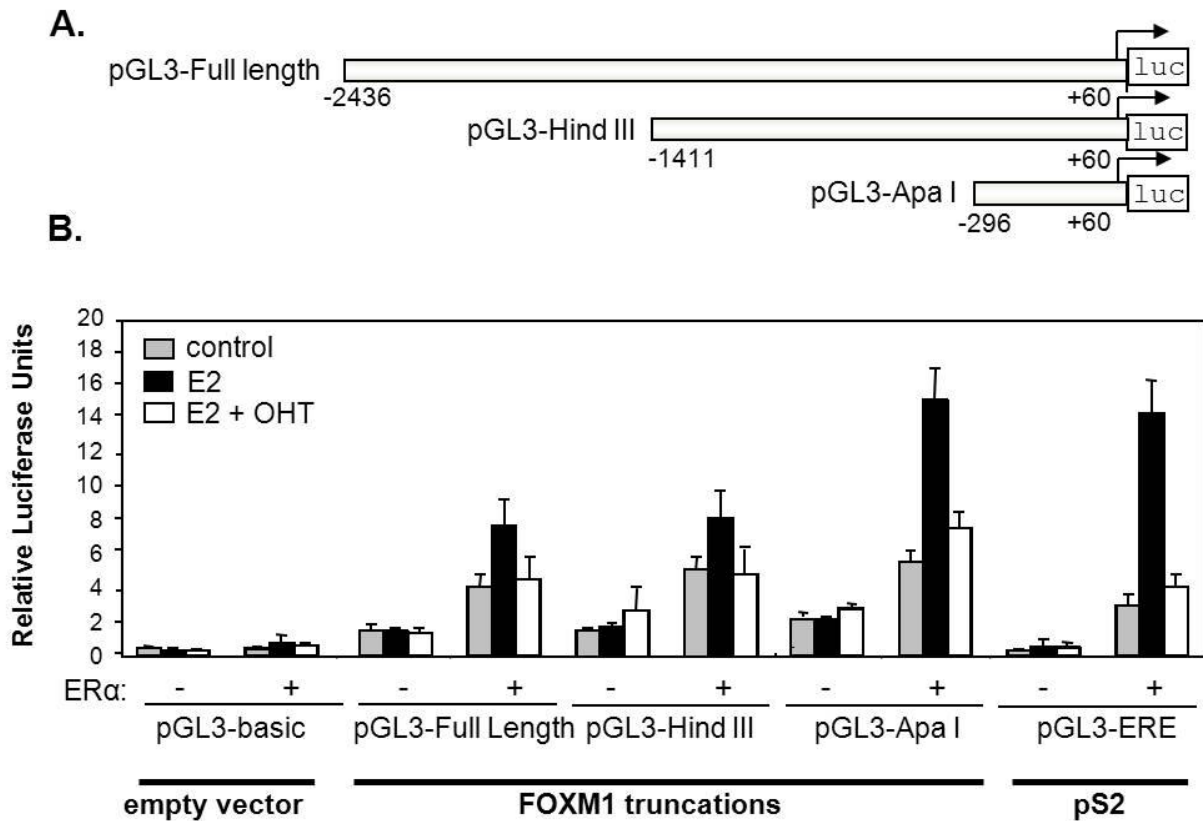


Figure 3.4 ER α induces the transcriptional activity of the human minimal *FOXM1* construction gene. Effect treatment with E2 alone or in combination with OHT and transient expression of ER α on *FOXM1* promoter activity. **A.** Schematic representation of the 2.4 kb full length, 1.4 kb Hind III and 0.3 kb Apa I *FOXM1*-luciferase reporter constructs previously made in the laboratory. **B.** COS-1 cells were cultured in 5 % double-charcoal striped FCS and phenol red free medium were transiently transfected with 20 ng of either the empty pGL3-basic, or *FOXM1* truncations: pGL3-Full length, pGL3-Hind III, pGL3-Apa I, or the control pGL3-ERE (pS2) promoter and 0 ng or 10 ng of ER α expression vector in the presence or absence of 10^{-8} mol/L of E2 alone for 4 h or 10^{-8} mol/L of E2 for 4 h followed by 10^{-6} mol/L of OHT for 24 h. Cells were treated 24 h after transfection and harvested for luciferase assay. All relative luciferase activity values were corrected by co-transfected Renilla activity. All data shown represent the averages of data from three independent experiments, and the error bars show the standard deviations.

3.2.1.3 ER α and HDAC2 bind on the ERE-like site of *FOXM1* promoter *in vitro*

To investigate whether ER α binds to the previously characterized ERE-like site *in vitro*, a pull-down assay with biotin-labeled oligonucleotides was performed using ER α positive MCF-7 and ZR-75-1 breast cancer cell lines. Streptavidin agarose beads were used to bind the wild-type oestrogen response element biotin-oligonucleotide (WT ERE-biotin) after incubation with nuclear protein extracts of breast cancer cells. Furthermore, a competition of the binding of the nuclear extract to the WT ERE biotin-oligonucleotide by addition of an excess of either wild-type (WT ERE-nonbiotin) or mutated (mut ERE3-nonbiotin) unlabelled-oligonucleotides was performed (Fig. 3.5). The binding of ER α to biotinylated oligonucleotides was analyzed by western blot and immunoblotted with an anti-ER α antibody. In Figure 3.5, I observed that ER α binding was completely abolished by the addition of unlabelled WT ERE oligonucleotide (WT ERE-nonbiotin, lane 2), while it was only slightly decreased with unlabeled mERE3 oligonucleotides (mERE3-nonbiotin, lane 3) under control vehicle condition in MCF-7 cells and ZR-75-1 cells. This result indicates that the WT ERE unlabeled-oligonucleotide has a higher affinity for ER α binding than mERE3. Moreover the effect of OHT, ICI and E2 on ER α binding on ERE sequence was verified in MCF-7 and ZR-75-1 cells (Fig. 3.5). The results show that ER α binding decreased in both cell lines upon ICI treatment (lane 12), whereas ER α binding seemed to be steady following OHT (lane 6) and E2 (lane 18) treatments. In agreement with previous studies and Figure 3.1, ICI decreased ER α protein level, while OHT stabilized it.

Collectively, these results demonstrated that ER α binds specifically to the ERE-like element found on *FOXM1* promoter *in vitro* under E2 and OHT treatments. However, these data did not explain the differential mechanism of *FOXM1* regulation by ER α upon OHT and E2, since ER α binds to the WT ERE sequence in both OHT and E2 conditions. To address this point, pull-down assays were performed in MCF-7 cells and immunoblotted with HDAC2 antibody, a histone deacetylase specifically overexpressed in breast cancer cells (Fig. 3.5). Interestingly, HDAC2 binds only to

the WT ERE biotin-oligonucleotides (WT ERE-biotin, lane 4 and 6) upon OHT but not under E2 (lane 16 and 18) conditions. This result suggests that OHT treatment induces ER α co-repressors binding including HDAC2 to repress FOXM1 expression. Surprisingly, HDAC2 was detected upon ICI treatment on the WT ERE biotin-oligonucleotides (WT ERE-biotin, lane 10). This result raised two issues for this experiment: the lack of a control input and the lack of protein extract quantification after incubation with the streptavidin-beads. These controls could add a quantitative value to the experiment and determine whether HDAC2 binds ERE under ICI treatment.

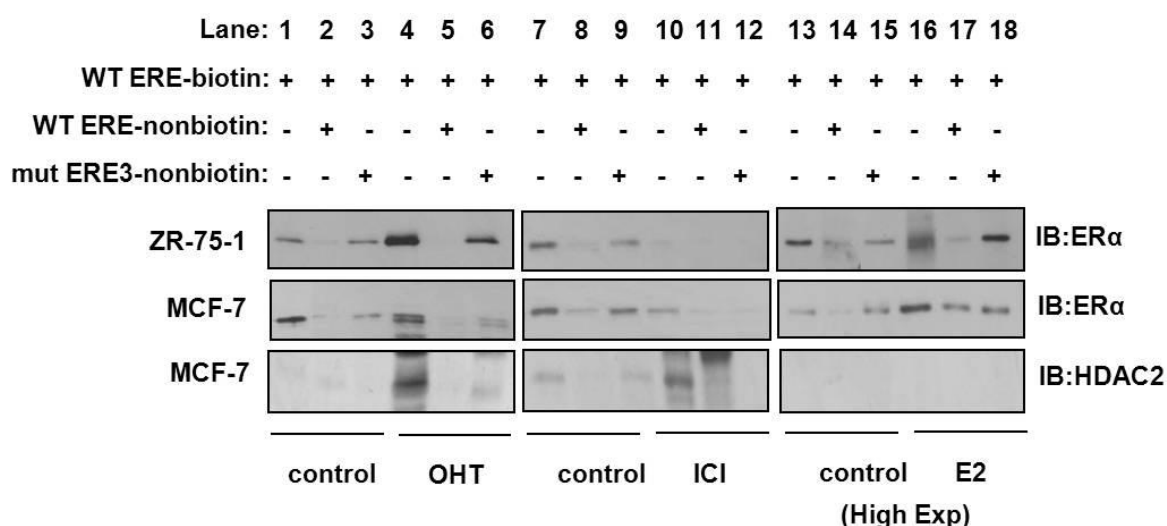


Figure 3.5 ER α binds directly to the ERE-like site on *FOXM1* promoter *in vitro*. MCF-7 and ZR-75-1 cells were cultured in 5 % double-charcoal striped FCS and phenol red free medium for 24 h before being stimulated with 10^{-8} mol/L of E2 for 24 h. Cells cultured in 10 % FCS and phenol red medium were treated with 10^{-6} mol/L of OHT, 10^{-7} mol/L of ICI or control vehicle (control) for 24 h. Nuclear extracts from MCF-7 and ZR-75-1 cells were incubated with biotin-oligonucleotides representing region of the *FOXM1* promoter containing the wild type ERE (WT ERE-biotin) in the absence or presence of molar excess of non-biotinylated ERE3 (mut ERE3-nonbiotin) or wild type ERE (WT ERE-nonbiotin) oligonucleotides. Proteins binding to the biotinylated oligonucleotides were pulled-down using streptavidine agarose beads and analysed by western blot using ER α antibody for ZR-75-1 cells, and with ER α and HDAC2 antibodies for MCF-7 cells. Nuclear extracts from MCF-7 cells were incubated with biotin-oligonucleotides representing region of the *FOXM1* promoter containing the wild type ERE or the mutated ERE3 site in the absence or presence of molar excess of non-biotinylated ERE3 or wild type ERE oligonucleotides. Proteins binding to the biotinylated oligonucleotides were pulled-down using streptavidine agarose beads and analysed by western blot using HDAC2 antibody.

3.2.1.4 ER α binds specifically to *FOXM1* promoter *in vivo*

I further studied the *in vivo* binding of ER α on *FOXM1* promoter in the MCF-7 and ZR-75-1 cell lines after OHT or ICI treatments using a semi-quantitative chromatin immunoprecipitation (Fig. 3.6A). The ER α -bound DNA was amplified with the *FOXM1* ERE site primers. In agreement with the pull-down studies, ER α occupied *FOXM1* promoter in control vehicle conditions and its occupancy decreased clearly after ICI treatment and was not affected by OHT treatment. For negative controls, samples were IP with non-specific IgG antibodies and PCR was performed on a region where ER α is absent. Semi-quantitative ChIP assays also showed an increase in the recruitment of HDAC1 and HDAC2 upon OHT treatment. Consistent with this finding, I showed a decrease of acetylated H3 and H4 on *FOXM1* promoter, indicating that OHT treatment caused the recruitment of HDACs that confer transcriptional repression to the ERE region of *FOXM1* promoter (Fig. 3.6B). Histones H3 and H4 are ubiquitously expressed and enriched on actively transcribed region. Therefore, this experiment should be repeated with appropriate negative controls including histone deacetylases such as HDACs or SIRTs.

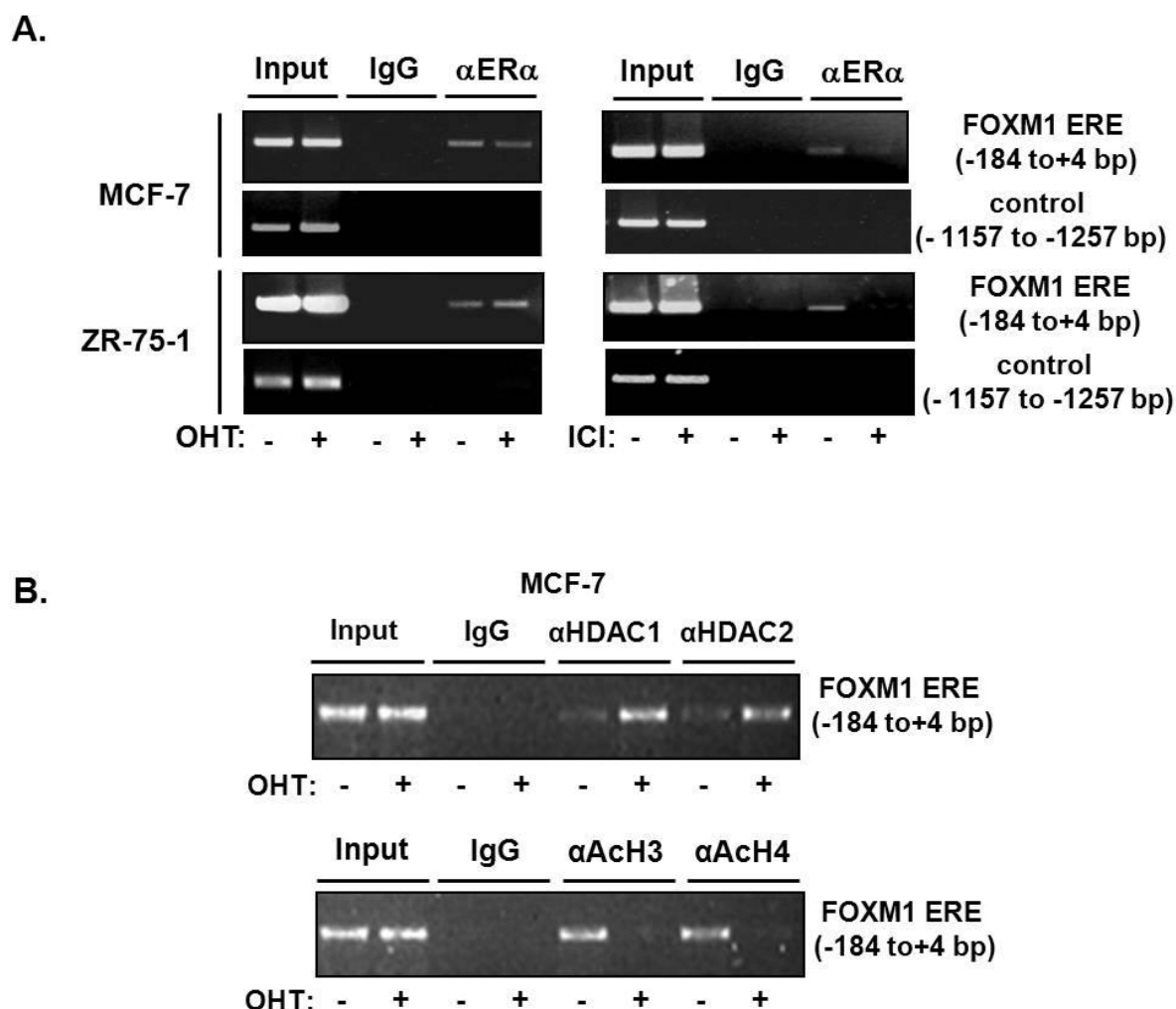


Figure 3.6 Chromatin immunoprecipitation (ChIP) analysis of the human *FOXM1* promoter. **A.** MCF-7 and ZR-75-1 cells untreated or treated with 10^{-6} mol/L of OHT or 10^{-7} mol/L of ICI for 24 h were used for ChIP assays using anti-IgG control, anti-ER α antibodies as indicated. **B.** MCF-7 untreated or treated with 10^{-6} mol/L of OHT for 24 h were used for ChIP assays using anti-IgG control and antibodies against acetylated H3 and H4, HDAC1 and HDAC2 as described above. After crosslink reversal, the co-immunoprecipitated DNA was amplified by PCR using primers amplifying the FOXM1 ERE containing region (-184/+4) and a control region (-1157/-1257), and resolved on 2 % agarose gel. Representative data from three independent experiments are shown.

3.2.1.5 FOXM1 silencing is cytotoxic for MCF-7 cells independent of the E2 mitogenic effect

Since the previous results showed FOXM1 as an ER α -responsive gene, I investigated the effects of FOXM1 knockdown on the survival of MCF-7 cells. Western blot analysis Figure 3.7A showed that FOXM1 was efficiently silenced by specific siRNA and that ER α expression was also decreased by FOXM1 siRNA. This is in line with previous findings in our laboratory that FOXM1 regulates ER α expression (Madureira, Varshochi et al. 2006). MCF-7 cells were oestrogen-starved for 48 h and then stimulated with E2 in the presence or absence of FOXM1 siRNA (Fig. 3.7). Notably, E2 treatment decreased ER α protein expression as already observed in Figure 3.1. The results of SRB assay showed that FOXM1 silencing induced the number of cells in the ER α positive MCF-7 cells following E2 or control vehicle (Fig. 3.7B). Taken together, the results indicate that FOXM1 has a role in the survival of ER α positive MCF-7 cells independent of E2. It would also be a good control to repeat this experiment with siRNA against ER α and FOXM1 combined to confirm that FOXM1 overcome the role of ER α in cell proliferation of MCF-7 cells. The effect of FOXM1 silencing on MCF-7 cell proliferation should be studied using different methods: vital staining or measure of DNA synthesis. Trypan blue selectively colour dead cells in blue, while the quantification of 3H-thymidine or bromodeoxyuridine incorporated into newly synthesized DNA indicate proliferation.

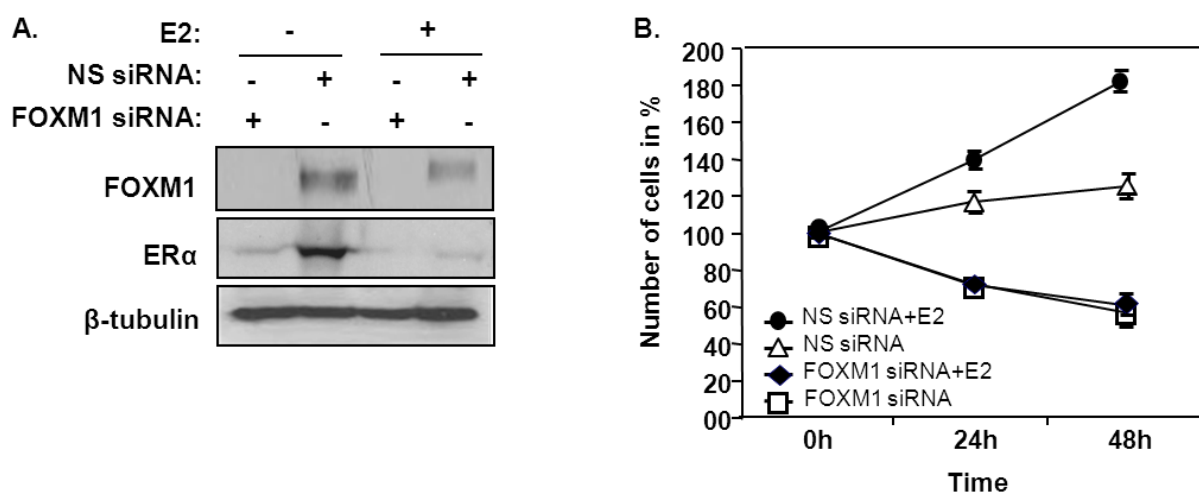


Figure 3.7 Effects of FOXM1 silencing on E2-induced proliferation of MCF-7 cells. MCF-7 cells were transiently transfected with non-specific (NS) and specific siRNA against FOXM1 (FOXM1) (100nmol/L) using oligofectAMINE according to the manufacturer instructions and, starved for 48 h and incubated with 10^{-8} mol/L of E2 for 24 h and analysed by western blotting with anti-FOXM1, ER α , β -tubulin (**A.**). Treatment with 10^{-8} mol/L of E2 for 0, 24, 48 h was performed before cells were harvested for SRB assays (**B.**). Representative data from three independent experiments are shown.

3.2.2 Deregulation of FOXM1 in tamoxifen resistant breast cancer cells

3.2.2.1 Deregulation of FOXM1 protein and mRNA expression in tamoxifen resistant cells

Since the majority of the patients who develop resistance to hormonal therapy are ER α positive, a dysfunction of ER α could result in FOXM1 deregulation and endocrine resistance in breast cancer cells. To address this question, I studied FOXM1 expression in tamoxifen sensitive MCF-7 cells (MCF-7) and tamoxifen resistant MCF-7 cells (MCF-7TAM^{R4}) after treatment with OHT. Consistent with Figure 3.1, OHT treatment caused a drastic reduction in FOXM1 protein and mRNA levels after 24 h until 72 h in MCF-7 cells, whereas no significant decrease was observed in MCF-7TAM^{R4} cells (Fig. 3.8A). Similar to FOXM1 expression pattern, FOXM1 target genes such as PLK, CDC25B, cyclin A and ER α showed a decrease in their protein expression in MCF-7 cells, but did not change in MCF-7TAM^{R4} cells, consistent with FOXM1 expression levels (Fig. 3.8A).

Additionally, to elucidate whether FOXM1 is involved in tamoxifen resistance, our group developed a stable MCF-7 cell line overexpressing a constitutive active form of FOXM1, the N-terminal deleted FOXM1 form (Δ N-FOXM1) which was previously described (Park, Wang et al. 2008). The protein and mRNA expression of FOXM1 under a constitutively active promoter (CMV) in MCF-7 cells led to a high and constant FOXM1 and Δ N-FOXM1 protein expression, and constant FOXM1 mRNA expression even following 72 h OHT treatment (Fig. 3.8B). As in MCF-7TAM^{R4} cells, FOXM1 targets protein expressions are consistent with FOXM1 expression pattern and remained unchanged. Moreover, to confirm whether FOXM1 has a pivotal role in tamoxifen resistance, different clones of MCF-7 cells (Pool of all clones, Clone 1, Clone 3, Clone 4) stably transfected with the full length wild-type FOXM1 (MCF-7-FOXM1) previously generated in the laboratory were used (Fig. 3.8C). Western blot analysis showed that the transfection of the full length FOXM1 abolished the downregulation of FOXM1 in clones 2 and 3, while FOXM1 was decreased in clone 1 and pool following OHT. This suggests that FOXM1 might be regulated at post-transcriptional levels by tamoxifen as FOXM1 expression is driven by CMV promoter in this experiment. Taken together, the full length FOXM1 overexpression partially

prevented the downregulation of FOXM1 targets including ER α , CDC25b, PLK and CYCLIN B1 in response to tamoxifen treatment (Fig. 3.8C).

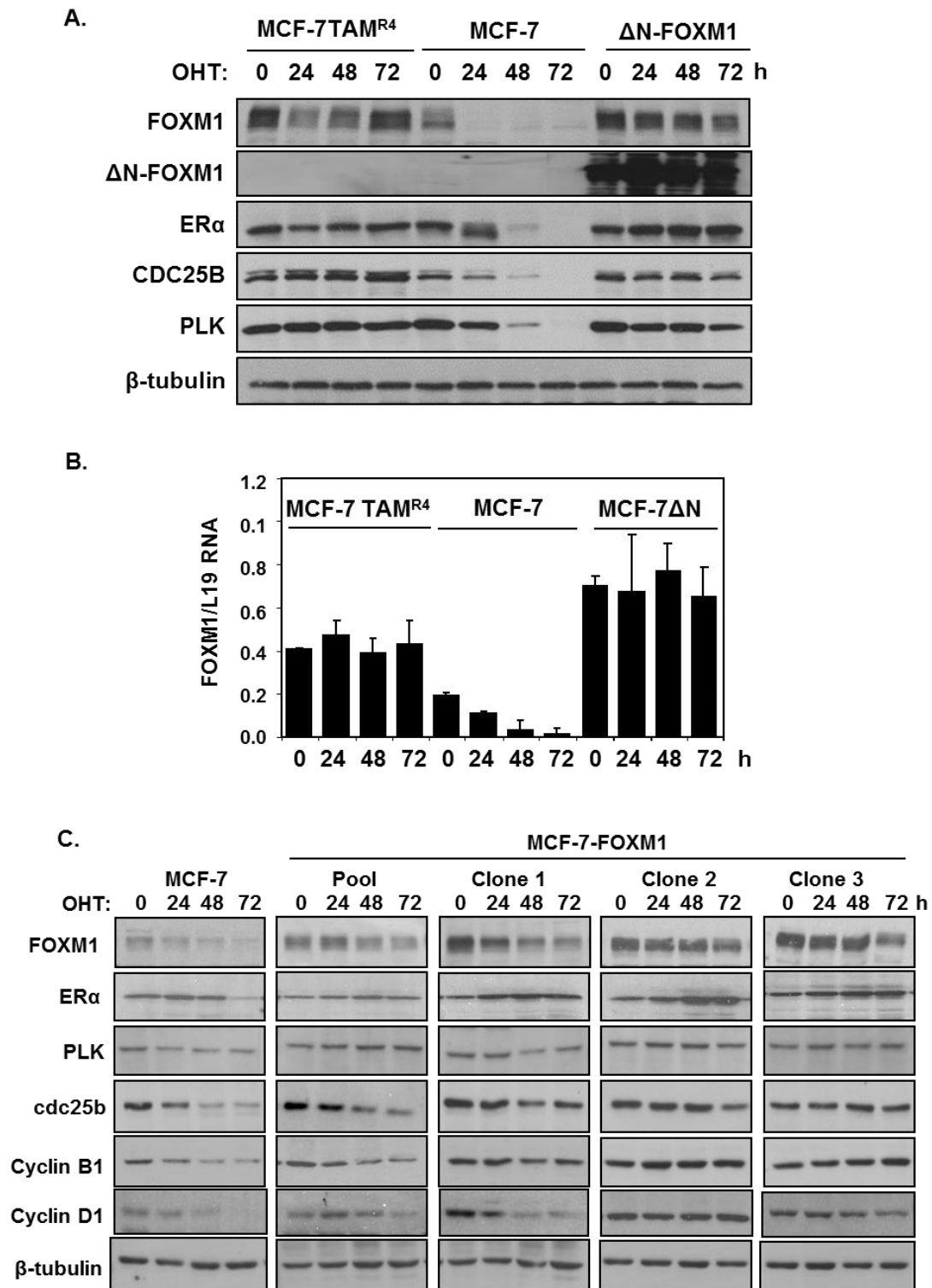


Figure 3.8 Full length and partial FOXM1 overexpression reduced the downregulation of FOXM1 and its target genes following tamoxifen treatment. MCF-7, MCF-7TAM^{R4} and MCF-7 ΔN-FOXM1 cells were treated with 10⁻⁶ mol/L of OHT in a time course of 72 h. **A.** Cell lysates were prepared at the times indicated, and the expression of FOXM1, ERα, CDC25B, PLK, and β-tubulin were analysed by Western blotting. **B.** Cells were harvested and FOXM1 mRNA levels were analysed by RT-qPCR, normalised with L19 housekeeping gene. **C.** Wild-type MCF-7 and overexpressing-FOXM1 MCF-7 (pool of clones, clone 1, 2 and 3) cell lysates were prepared at the times indicated, and the expression of FOXM1, ERα, PLK, CDC25B, CYCLIN B1, CYCLIN D1 and β-tubulin were analyzed by Western blotting.

3.2.2.2 Reduced G1 cell cycle arrest in tamoxifen resistant cells after OHT

I performed cell cycle analysis of the MCF-7, MCF-7TAM^{R4} and Δ N-FOXM1 MCF-7 cells following treatment with OHT (Fig. 3.9A). The results showed that OHT caused predominantly a G1 cell cycle arrest in the parental MCF-7 cells, but had comparatively lower effects on the cell cycle distribution of the MCF-7TAM^{R4} and Δ N-FOXM1 MCF-7 cells (Fig. 3.9B). The calculation of the percentage of increase relative to 0h in cells in G1 phase showed that OHT caused a G1 cell cycle arrest in MCF-7TAM^{R4} at a lower extent than in the wild-type MCF-7 cells, whereas OHT did not increase the number of cells in G1 phase in Δ N-FOXM1 MCF-7 cells (Fig.3.9B).

The cell cycle profile of the MCF-7 cells stably transfected with the wild-type FOXM1 (MCF-7-FOXM1) demonstrated that these cells underwent a cell cycle arrest at G1, concomitant with FOXM1 downregulation, but at a lower extent compared with MCF-7 cells transfected with the empty vector (Fig. 3.10). Similar to the western blot, pool and clone 1 MCF-7 cells did not show a strong difference in the number of cells in G1 phase compared with the wild-type MCF-7 cells, while clones 2 and 3 showed a lower increase in cells in G1 phase after 72h treatment. Collectively, these results indicate that FOXM1 has a role in mediating the G1 cell cycle arrest OHT-induced, and that its overexpression reverses MCF-7 sensitivity to G1 cell cycle arrest OHT-induced. Furthermore it would be interesting to overexpress a transcriptionally dead FOXM1 to demonstrate that FOXM1 activity is required for the inhibition of the G1 cell cycle arrest OHT-induced. At this point, it would also have been interesting to compare the proliferation of MCF-7, MCF-7TAM^{R4} and Δ N-FOXM1 MCF-7 cells following OHT.

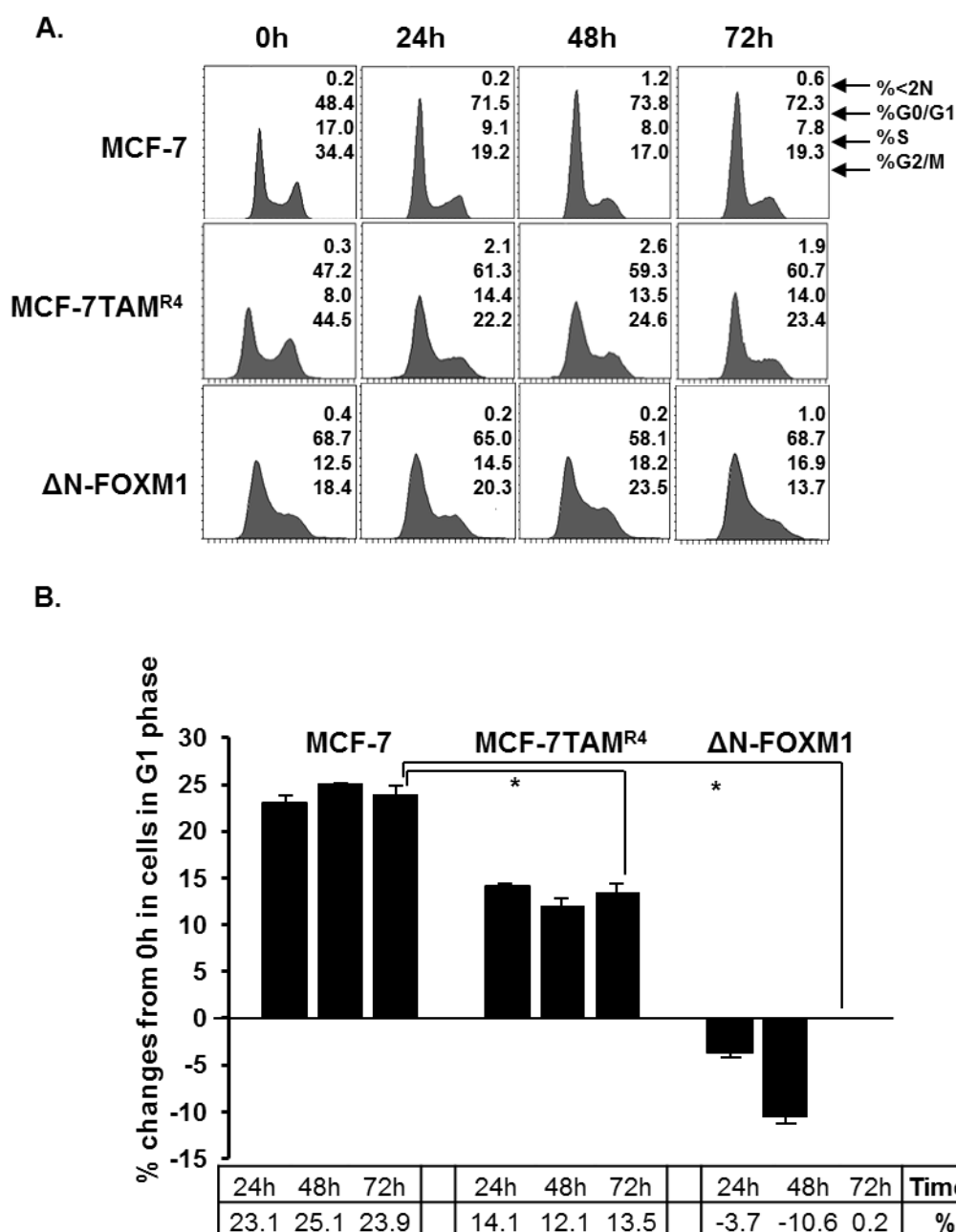


Figure 3.9 Cell cycle regulation in wild-type (MCF-7), tamoxifen resistant (MCF-7TAM^{R4}) and constitutively active ΔN-FOXM1 expressing MCF-7 cells in response to tamoxifen treatment. MCF-7, MCF-7 TAM^{R4} and MCF-7 ΔN-FOXM1 cells were treated with 10⁻⁶ mol/L of OHT in a time course of 72 h. **A.** Cell cycle phase distribution was analyzed by flow cytometry after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2/M) is indicated. Representative data from three independent experiments are shown. **B.** Percentage increase in cells in G1 phase relative to 0h. Statistical analyses were done using Student's *t* test. *, *P*≤0.1, **, *P*≤0.01 and ***, *P*≤0.001, significant and n.s, non significant.

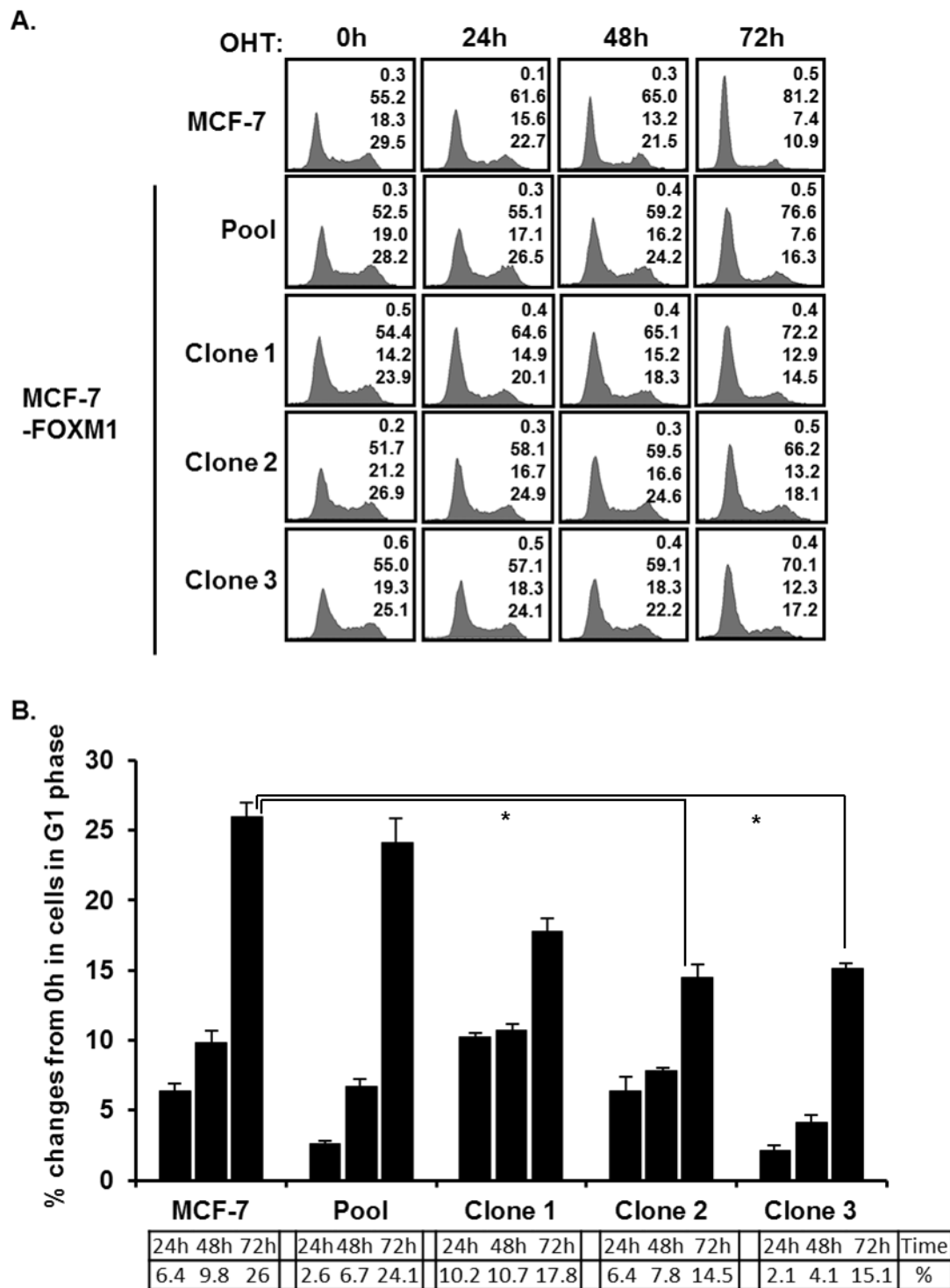


Figure 3.10 FOXM1 upregulation rescues OHT-induced cell growth arrest and decrease of endogenous FOXM1 in response to tamoxifen treatment. **A.** Wild-type MCF-7 and overexpressing-FOXM1 MCF-7 cells (pool of clones, clone1, 2 and 3) were fixed at 0, 24, 48, and 72 h after OHT treatment, and cell cycle phase distribution was analysed by flow cytometry after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2/M) is indicated. **B.** Percentage increase in cells in G1 phase relative to 0h. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$, **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

3.2.2.3 Combination of OHT and FOXM1 silencing has a cytostatic effect on MCF-7 tamoxifen resistant cells

Previous results showed that the constitutively active form of FOXM1 and the full length FOXM1 reduced the G1 cell cycle arrest induced by OHT. I next tested whether FOXM1 silencing would affect the survival of tamoxifen resistant cells to OHT treatment. The downregulation of FOXM1 by specific siRNA was verified by western blot (Fig. 3.11A). In the non-specific siRNA condition, the expression of FOXM1 was enhanced during the time course due to the fact that FOXM1 is a proliferative factor (Fig. 3.11A). Oppositely, FOXM1 protein expression remained low until 72 h in cells transfected with specific FOXM1 siRNA (Fig. 3.11A). Interestingly, western blot analysis also showed a downregulation of ER α protein expression upon FOXM1 silencing indicating that ER α is still under FOXM1 regulation in these cells. Additionally, I performed the SRB assay and observed that FOXM1 silencing decreased the survival of tamoxifen resistant cells (Fig. 3.11B). The combination of FOXM1 siRNA and OHT treatment had a cytostatic effect on these cells after OHT treatment (Fig. 3.11B). A recent publication showed that Thiostrepton directly interacts with FOXM1 protein to reduce FOXM1 transcriptional activity (Hegde, Sanders et al. 2011). It would be interesting to compare the effect of FOXM1 silencing to this inhibitor on the tamoxifen resistant cell proliferation and survival.

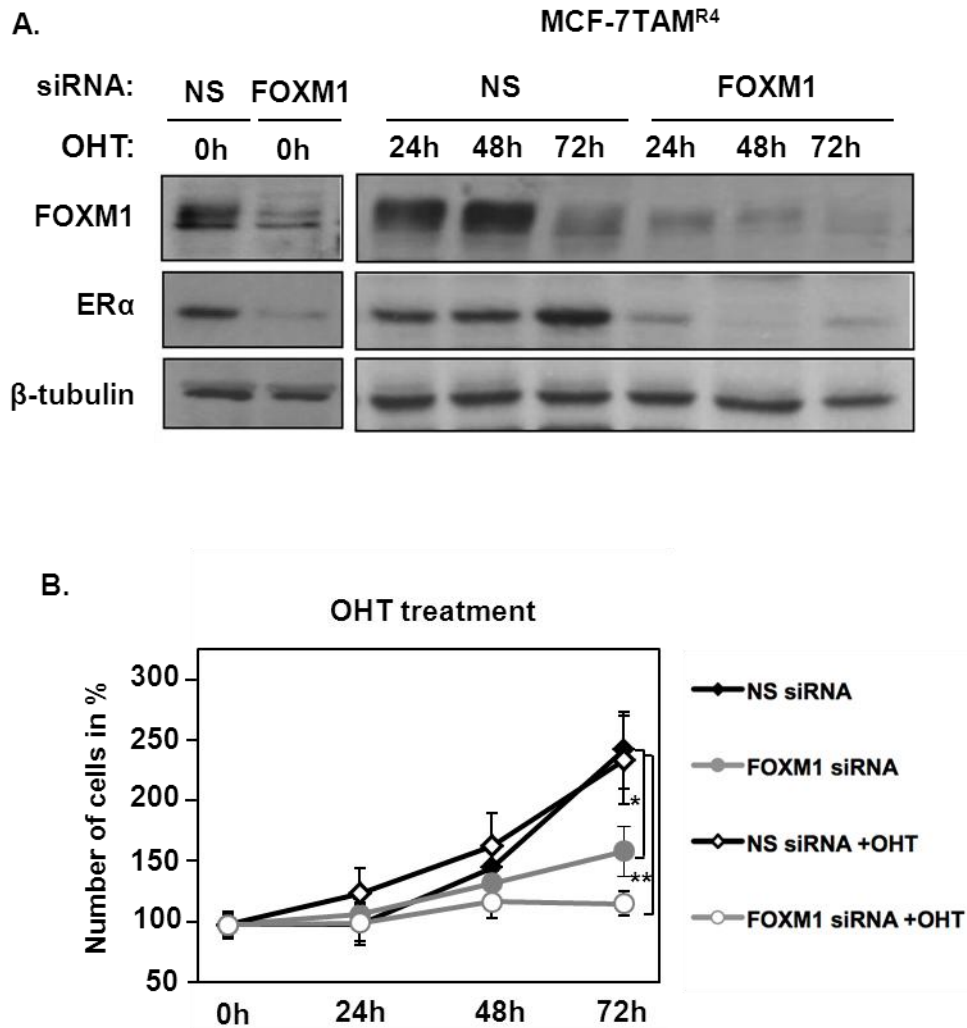


Figure 3.11 FOXM1 silencing rescues tamoxifen anti-growth effect in MCF-7TAM^{R4} cells. MCF-7TAM^{R4} cells were transiently transfected with non-specific or FOXM1 siRNA for 24 h (100nmol/L) using oligofectAMINE according to the manufacturer instructions, and were harvested at different time after transfection and analysed by western blot using FOXM1, ERα and β-tubulin antibodies (**A.**) and harvested for SRB assays (**B.**). Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$, **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

3.2.3 Potential mechanisms of tamoxifen resistance

3.2.3.1 FOXM1 phosphorylation and transcriptional activation

Western blot analysis Figure 3.12 showed that CYCLIN A, but not its catalytic partner CDK2, was downregulated after 24 h OHT treatment in MCF-7 cells and this decline in CYCLIN A level occurred in a slower kinetic in MCF-7TAM^{R4} and Δ N-FOXM1 cells. Consistent with this, the CDK2 activity revealed by the phosphorylation of pRB on Threonine 821 decreased drastically in MCF-7 cells compared with MCF-7TAM^{R4} and Δ N-FOXM1 cells. Given that the complex CYCLIN A/CDK2 phosphorylates FOXM1 and can activate its transcriptional activity (Wierstra and Alves 2006, Laoukili, Alvarez et al. 2008, Laoukili, Alvarez-Fernandez et al. 2008, Park, Wang et al. 2008), this finding suggests a potential mechanism by which FOXM1 can cause tamoxifen resistance in MCF-7TAM^{R4} and Δ N-FOXM1 cells.

Western blot analysis also showed that the level of CYCLIN D1 and its associated activity revealed by the CDK4 phospho-pRb (Ser807/811) antibody were overexpressed in the Δ N-FOXM1 cells and maintained in the MCF-7TAM^{R4} cells. In addition, the stable MCF-7-FOXM1 cell line also showed an increase in the expression levels of CYCLIN D1, particularly in clones 2 and 3 (Figure 3.8). Given that the complex CYCLIN D1/CDK4 phosphorylates FOXM1 in multiple sites and can activate its transcriptional activity (Anders, Ke et al. 2011), this finding suggests another potential mechanism by which FOXM1 can cause tamoxifen resistance in MCF-7TAM^{R4} and Δ N-FOXM1 cells.

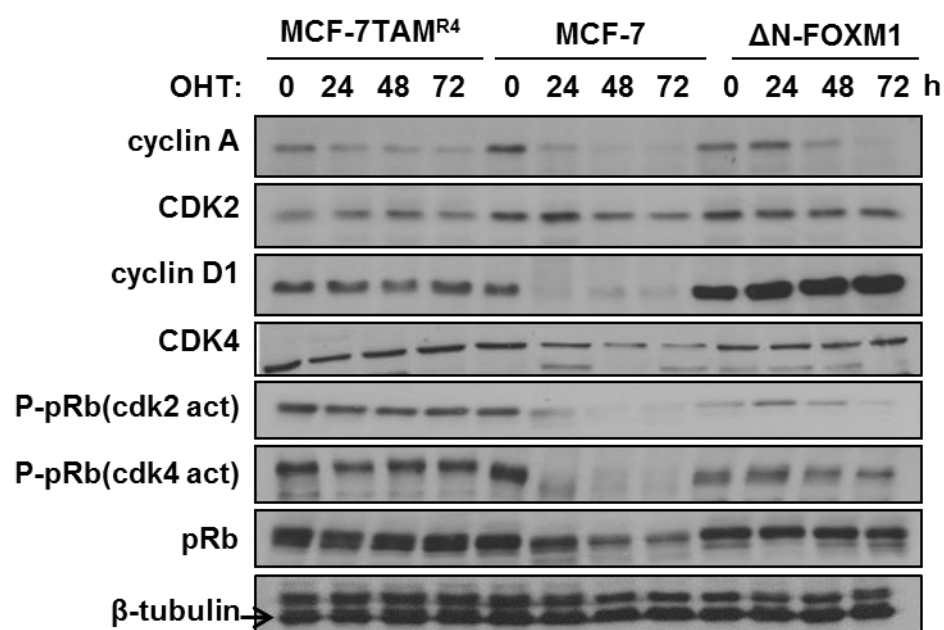


Figure 3.12 Constitutively active ΔN-FOXM1 expressing MCF-7 cells show the same protein expression pattern as MCF-7TAM^{R4} cells in response to tamoxifen treatment. MCF-7, MCF-7TAM^{R4} and MCF-7 ΔN-FOXM1 cells were treated with 10⁻⁶ mol/L of OHT in a time course of 72 h. Cell lysates were prepared at the times indicated, and the expression of CYCLIN A, CDK2, CYCLIN D1, CDK4, P-pRB(cdk2), P-pRB(cdk4), total pRB and β-tubulin were analysed by Western blotting.

3.2.3.2 ER α overexpression and silencing do not alter FOXM1 expression in tamoxifen resistant cells

Western blot analysis showed that ER α overexpression in MDA-MB-231 ER α -negative cells had no effect on FOXM1 protein expression (Fig. 3.13), while ER α -agonist increased FOXM1 protein expression in MCF-7 ER α positive cells (Fig. 3.1). Similarly, ER α silencing did not affect FOXM1 protein expression in MCF-7 tamoxifen resistant cells, while ER α silencing decreased FOXM1 protein level in MCF-7 cells (Fig. 3.3). These data suggest that FOXM1 regulation is controlled by other mitogenic factors in ER α -negative and tamoxifen resistant cells.

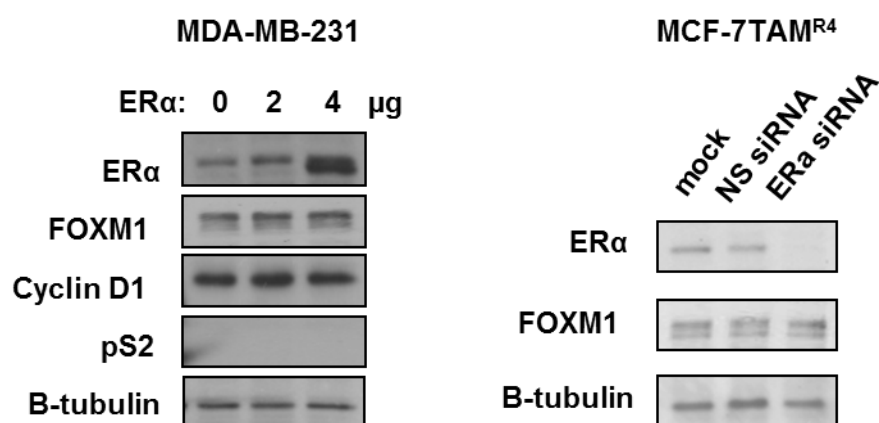


Figure 3.13 ER α ectopic expression and silencing in the tamoxifen resistant ER α -negative MDA-MB-231 and ER α -positive MCF-7TAM^{R4} breast cancer cells. MDA-MB-231 cells were transfected with increasing amounts of ER α . MCF-7TAM^{R4} cells were transiently transfected with non-specific siRNA and siRNA targeting ER α for 24 h (100nmol/L). The expression of FOXM1, ER α , CYCLIN D1, pS2 and β -tubulin was analysed by western blotting.

3.2.3.3 Protein deregulations in tamoxifen resistant cells

ER α is inhibited by the binding of tamoxifen which induces the recruitment of co-repressors, preventing ER α conformational changes and transcriptional activity. Semi-quantitative ChIP assays Figure 3.6B showed that tamoxifen treatment of MCF-7 cells induced the recruitment of HDACs (ER α co-repressor) decreasing the acetylation levels of histones 3 and 4 as observed for two ER α target genes, pS2 and C-MYC (Masiakowski, Breathnach et al. 1982, Carroll, Meyer et al. 2006). Thereby, it is possible that recruitment of ER α co-repressors and co-activators is deregulated in tamoxifen resistant cells leading to tamoxifen insensitivity. Protein analysis of AIB1 (co-activator) showed a high expression level in tamoxifen resistant MCF-7 cells even following tamoxifen, while AIB1 expression level decreased in MCF-7 cells treated (Figure 3.14).

A recent study showed C-MYB as an ER α target gene and determined the role of C-MYB in the proliferation of ER α -positive breast cancer cells, but not in ER α -negative cells (Drabsch, Hugo et al. 2007). While the role of C-MYB has been well studied in cell growth and transformation, little is known about B-MYB family member. Although a direct role of B-MYB in cancer has not been yet established, B-MYB is found amplified in breast, liver, ovarian carcinomas and cutaneous T-cells lymphomas (Forozan, Mahlamäki et al. 2000, Tanner, Grenman et al. 2000, Zondervan, Wink et al. 2000, Mao, Orchard et al. 2003). B-MYB expression is upregulated in metastasis compared to localised prostate tumours and its overexpression is associated with poor prognosis in breast cancer patients (Amatschek, Koenig et al. 2004). As FOXM1, B-MYB is a cell cycle-regulatory gene. Its expression is induced at G0/S and reached a maximum level in S phase (Lam, Bennett et al. 1995). A study showed that MYB mRNA and protein levels were not affected by FOXM1 silencing, whereas FOXM1 mRNA and protein levels were both reduced in a time-dependent manner after MYB silencing. These results suggest that MYB is a transcriptional activator of FOXM1 (Lefebvre, Rajbhandari et al. 2010). Therefore, altered B-MYB expression in breast cancer may affect FOXM1 expression and be involved in tamoxifen resistance. Preliminary data on tamoxifen resistant breast cancer cells confirmed that B-MYB is deregulated compared to wild-type MCF-7 cells (Fig.3.14).

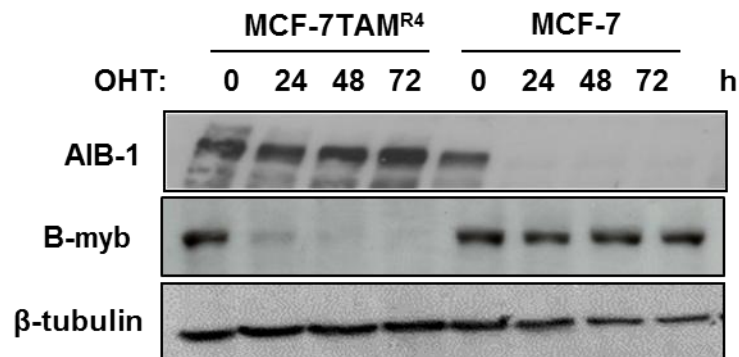


Figure 3.14 AIB-1 and B-myb expression pattern in tamoxifen sensitive and resistant MCF-7 cells. Both cell lines were treated with OHT over 72 h. The expression of AIB-1, B-MYB and β -tubulin were analysed by western blotting.

In addition to ER α , ER β is an oestrogen receptor encoded by a distinct gene than oestrogen receptor alpha, but both bind with equal affinity oestrogens. ER α promotes the proliferation of breast epithelium and cancer cells, while ER β has anti-proliferative and pro-apoptotic effects. A recent study in our laboratory has shown FOXM1 has a target of ER β 1, but only in ER α positive breast cancer cells. MCF-7 cells collected 24 hours after transfection with pcDNA3 as a control or pcDNA3-Flag-ER β 1 were subjected to semi-quantitative ChIP analysis with the use of an ER α antibody and an anti-Flag antibody, which recognized the transfected Flag-tagged ER β 1. The ChIP assays showed that there was an increase in ER β 1 recruitment to the ERE region on FOXM1 promoter when ER β 1 is transfected. Concomitantly, occupancy of ERE region by ER α was drastically reduced in MCF-7 cells with ER β 1 ectopic expression, indicating that ER β 1 expression caused the disassociation of ER α from ERE region of the FOXM1 promoter (Fig. 3.15). Given that ER β 1 is repressing FOXM1, it is possible that ER β 1 is downregulated in tamoxifen resistant cells leading to constant cell proliferation and tamoxifen resistance.

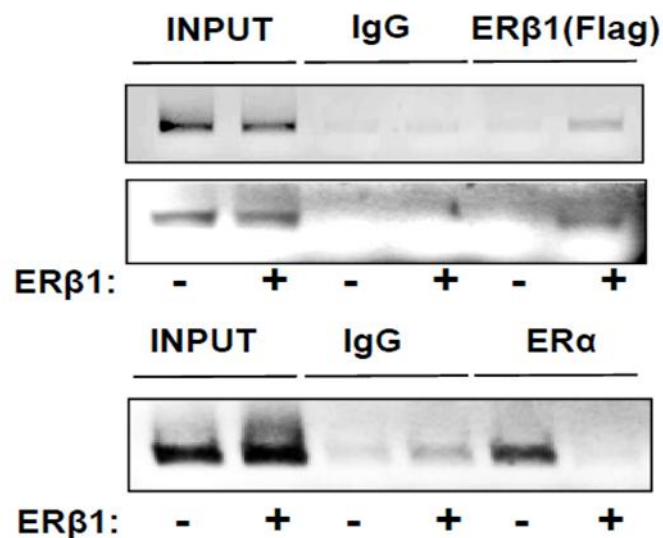


Figure 3.15 Chromatin immunoprecipitation of ERβ and ERα in MCF-7 cells. MCF-7 cells were transfected with ERβ and harvested for ChIP assays using anti-IgG, anti-Flag (recognized transfected ERβ) and anti-ERα antibodies as indicated. After crosslink reversal, the co-immunoprecipitated DNA was amplified by PCR using primers amplifying the FOXM1 ERE containing region (-184/+4) and resolved in 2% agarose gel.

3.3 Discussion

3.3.1 Regulation of ER and FOXM1 through a positive feedback loop in breast cancer cells

Colleagues in the laboratory previously studied *FOXM1* mRNA expression and its relationship to *ERα* mRNA level in breast cancer biopsy samples (Millour, Constantinidou et al. 2010). After exclusion of the data with high levels of FOXM1 expression (upper 25th percentile), a statistically significant correlation between *ERα* and *FOXM1* mRNA expression was found. This is in agreement with a recent breast cancer patient microarray dataset analysis indicating that high levels of FOXM1 mRNA expression (upper 25th percentile) are associated with poor prognosis in breast cancer (Martin, Patrick et al. 2008). The discordance between *ERα* and *FOXM1* mRNA expression in patient samples with high FOXM1 expression probably indicates that in these subjects FOXM1 expression is deregulated, with the control of

FOXM1 transcription by ER α overridden by other mitogenic signals or genetic changes.

A previous study in our laboratory also showed that the expression of ER α is regulated by FOXM1 in breast cancer cell lines (Madureira, Varshochi et al. 2006). In this report, I investigated the reciprocal regulation of FOXM1 expression by ER α . Using breast carcinoma cell lines, I showed that FOXM1 protein and mRNA expression are regulated by ER-ligands, including E2, OHT, and ICI (Fig. 3.1A). In addition, I also found that depletion of ER α by RNA interference in MCF-7 cells leads to the downregulation of FOXM1 expression (Fig. 3.3). Reporter gene assays demonstrated that ER α activates *FOXM1* transcription through an ERE located at -45 bp upstream of the transcriptional start site (Fig. 3.4) (Bourdeau, Deschenes et al. 2004). The direct binding of ER α to the *FOXM1* promoter was confirmed *in vitro* by DNA pull-down assays, and *in vivo* by semi-quantitative ChIP analysis (Fig. 3.5 and 3.6). Silencing of *FOXM1* by RNA interference had a cytostatic effect on tamoxifen resistant cells (Fig. 3.11). Conversely, ectopic expression of a constitutive active FOXM1 form can abrogate the G1 cell cycle arrest mediated by OHT (Fig. 3.9).

3.3.2 Uncoupled ER and FOXM1 feedback loop regulation in tamoxifen resistant breast cancer cells

The findings that tamoxifen represses FOXM1 expression in endocrine sensitive but not in resistant breast carcinoma cell lines, and that FOXM1 overexpression can abrogate G1 cell cycle arrest induced by tamoxifen, further suggested that deregulation of FOXM1 may contribute to anti-oestrogen insensitivity. *FOXM1* mRNA levels are higher in tamoxifen resistant MCF-7 cells relative to wild-type MCF-7 cells, suggesting that the main mechanism of FOXM1 regulation is transcriptional in these cells (Fig. 3.8B). Moreover, the constitutive active FOXM1 form overexpressing cells, where FOXM1 is under CMV promoter, showed a decrease in FOXM1 protein expression following tamoxifen indicating a post-translational mechanism of regulation too (Fig. 3.8C). The observation that there was no significant changes in ER α or endogenous FOXM1 levels in the MCF-7 cells expressing the active FOXM1 form highlighted an important positive feedback mechanism between ER α and

FOXM1, described here and previously (Madureira, Varshochi et al. 2006). This makes FOXM1 a particularly critical ER α target gene in breast cancer development and endocrine resistance, as the feedback loop will amplify the mitogenic action of oestrogens. Furthermore, it seems that this positive feedback transcriptional mechanism is uncoupled in ER α -negative breast cancer, as ER α overexpression did not induce FOXM1 expression (Fig. 3.13). In addition, the positive feedback transcriptional mechanism is also uncoupled in ER α -positive tamoxifen resistant breast cancer cells as ER α silencing did not affect FOXM1 expression while FOXM1 silencing reduced ER α expression in tamoxifen resistant cells (Fig. 3.13). This finding suggests that FOXM1 is a strong transcription activator in tamoxifen resistant cells.

3.3.3 Deregulated AIB1, an ER α co-factor, in tamoxifen resistant breast cancer cells

Over the last years, it has become evident that ER α activation is not only dependent on the binding of ligands, but also depends on interaction between co-factors and associated signaling pathways. Altered levels of ER α co-factors are observed in tamoxifen resistant patients. AIB1, an ER α co-activator, is found overexpressed in tamoxifen resistant breast cancer patients, and NCoR, an ER α co-repressor, is reduced in tumours that acquired tamoxifen resistance (Lavinsky, Jepsen et al. 1998, Osborne, Bardou et al. 2003). AIB1 protein analysis showed high expression in tamoxifen resistant MCF-7 cells even following tamoxifen, while AIB1 expression decreased in MCF-7 cells treated (Figure 3.14). Semi-quantitative ChIP assays demonstrated the mechanism by which tamoxifen represses FOXM1 expression in MCF-7 cells. Tamoxifen treatment of MCF-7 cells induced the recruitment of HDACs decreasing the acetylation levels of histones 3 and 4 as observed for two ER α target genes, pS2 and C-MYC (Masiakowski, Breathnach et al. 1982, Carroll, Meyer et al. 2006). Thereby, I speculate that recruitment of ER α co-activators is deregulated in tamoxifen resistant cells, which might affect the regulation of FOXM1 by ER α . However, ER α silencing did not affect FOXM1 expression in the tamoxifen resistant cells, which indicates that FOXM1 regulation is controlled by other mitogenic factors. For instance, the cell cycle regulator CYCLIN D1 regulates

positively FOXM1 and its overexpression is associated with tamoxifen resistance (Butt, McNeil et al. 2005, Wierstra and Alves 2006).

3.3.4 Deregulation of FOXM1 negative and positive regulators as potential mechanisms of tamoxifen resistance

The expression pattern of CYCLIN D1 showed a strong increase in its protein expression in MCF-7 cells overexpressing the active form of FOXM1 compared to wild-type and tamoxifen resistant cells. It is known that CYCLIN D1/CDK4 phosphorylates pRB releasing FOXM1 from pRB repression (Wierstra and Alves 2006), but CYCLIN D1/CDK4 also phosphorylates FOXM1 in multiple sites (Anders, Ke et al. 2011). Therefore, it is likely that a positive feedback loop between FOXM1 and CYCLIN D1 occurs in the FOXM1 overexpressing cells. Given the well-documented role of cyclin D1 in endocrine resistance (Lundgren, Holm et al. 2008, Wang, Dean et al. 2008, Finn, Dering et al. 2009, Yamashita, Takahashi et al. 2009, Zwart, Rondaij et al. 2009) and G1/S transition (Fung and Poon 2005, Myatt and Lam 2007, Tashiro, Tsuchiya et al. 2007), our data also support a role for FOXM1 in mediating breast cancer endocrine sensitivity and resistance at least in part through modulating CYCLIN D1 expression.

In addition to ER α , ER β is an oestrogen receptor encoded by a distinct gene than oestrogen receptor alpha, but both bind with equal affinity oestrogens. ER α promotes the proliferation of breast epithelium and cancer cells, while ER β has anti-proliferative and pro-apoptotic effects. ER β is expressed in several variants in normal and malignant tissues, but Roger and colleagues reported a decrease in ER β protein expression in pre-invasive mammary tumours compared to normal or benign lesions (Roger, Sahla et al. 2001). There is a conflicting data regarding the co-expression of both ERs and association with prognostic, endocrine responsiveness and survival. However, ER β has been in general shown to be associated with favourable prognostic for endocrine therapy in breast cancer. A recent study in our laboratory has shown FOXM1 has a target of ER β 1, but only in ER α -positive breast cancer cells. Indeed, I showed by semi-quantitative ChIP that ER β 1 competes with ER α on

the ERE of FOXM1 promoter to repress FOXM1 transcription (Fig. 3.15). Consequently, ER β 1 downregulation in tamoxifen resistant cells could lead to inhibition of cell cycle arrest tamoxifen-induced.

A recent study showed C-MYB as an ER α target gene and determined the role of C-MYB in the proliferation of ER α positive breast cancer cells, but not in ER α negative cells (Drabsch, Hugo et al. 2007). While the role of C-MYB has been well studied in cell growth and transformation, little is known about B-MYB family member. Although a direct role of B-MYB in cancer has not been yet established, B-MYB is found amplified in breast, liver, ovarian carcinomas and cutaneous T-cells lymphomas (Forozan, Mahlamäki et al. 2000, Tanner, Grenman et al. 2000, Zondervan, Wink et al. 2000, Mao, Orchard et al. 2003). B-MYB expression is upregulated in metastasis compared to localised prostate tumours and its overexpression is associated with poor prognosis in breast cancer patients (Amatschek, Koenig et al. 2004). As FOXM1, B-MYB is a cell cycle-regulatory gene. Its expression is induced at G0/S and reached a maximum level in S phase (Lam, Bennett et al. 1995). B-MYB activity is modulated by posttranslational modifications including phosphorylation during S phase by cyclin/cdk complexes (Sala, Kundu et al. 1997). It has been shown to promote DNA replication and maintenance of genomic integrity by regulating the transcription of genes essential for G2/M phase progression (García and Frampton 2006, Tarasov, Tarasova et al. 2008). Furthermore, B-MYB overexpressing cells were significantly enriched in genes involved in G2/M progression (Thorner, Hoadley et al. 2009). Based on the similarities of B-MYB and FOXM1 target genes, a cross-talk between B-MYB and FOXM1 has been investigated. Recent evidence demonstrated that B-MYB and FOXM1 co-regulate one another in a positive feedback loop (Lefebvre, Rajbhandari et al. 2010, Lorvellec, Dumon et al. 2010). ChIP analysis revealed that B-MYB and FOXM1 co-ordinate the transcriptional regulation of genes involved in proliferation in germinal centres of B-cell (Lefebvre, Rajbhandari et al. 2010). Therefore, I suggest that altered B-MYB expression in breast cancer may affect FOXM1 expression and be involved in tamoxifen resistance. The first western blot analysis on tamoxifen resistant breast cancer confirmed that B-MYB is deregulated compared to wild-type MCF-7 cells (Fig. 3.14). In addition, B-MYB has recently been associated with

tamoxifen resistance in a high-throughput screening for tamoxifen resistance (Gonzalez-Malerva, Park et al. 2011).

3.3.5 Conclusion

In this study, I showed a differential regulation of FOXM1 in endocrine sensitive and resistant breast cancer cell lines. These findings also provided potential insights in the mechanism of anti-oestrogen action and endocrine resistance, and showed that FOXM1 deregulation may involve deregulated AIB1, CYCLIN D1, ER β 1 or B-MYB proteins. Furthermore, this study raises a potential new strategy for the treatment of breast cancer endocrine resistant where FOXM1 is frequently overexpressed. Targeting FOXM1 with siRNA in this study had a cytostatic effect on tamoxifen resistant cells. Therefore, the inhibition of FOXM1 with small molecules, such as thiostrepton or siamycin A, in combination with tamoxifen might resensitise tamoxifen resistant cells to cell cycle arrest induced by tamoxifen as observed in this study.

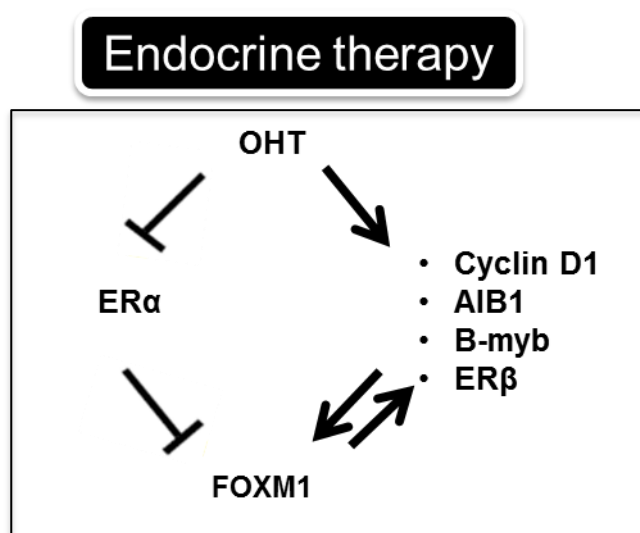


Figure 3.16 Potential pathways in endocrine therapy. Tamoxifen reduces ER α pathway and FOXM1 in endocrine sensitive cells leading to G1 cell cycle arrest. Whereas deregulated proteins including cyclin, proliferative factor, ER co-factor in tamoxifen resistant cells could prevent cell cycle arrest OHT-induced.

3.4 Future work

This report shows that targeting FOXM1 in tamoxifen resistant breast cancer cells is an attractive strategy considering that FOXM1 is overexpressed in tamoxifen resistant breast cancer cells and that FOXM1 deregulation is involved in tamoxifen resistance. I raised several potential mechanisms of FOXM1 deregulation in this study that need to be clarified. Treatment with tamoxifen induces the release of ER α co-activators and the recruitment of ER α co-repressors. However, I observed a high and constant expression of AIB1 in tamoxifen resistant breast cancer cells. Consequently, investigating the recruitment, expression and regulation of co-activators in tamoxifen resistant cells would reveal whether this process is deregulated and would provide new potential strategies to overcome resistance. For instance, histone deacetylase inhibitors are already in use as monotherapy or in combination with taxol and radiation for a wide range of cancers (Dowdy, Jiang et al. 2006, Lane and Chabner 2009, Mueller, Yang et al. 2011). Furthermore, the electrophile disulfite benzamide DIBA has given promising results in mice models. DIBA switched tamoxifen agonist to antagonist activity by facilitating the dissociation of co-activators and the association of co-repressor on the promoter of ER-responsive genes resulting in a decrease in xenograft tumor growth of tamoxifen resistant human cells in mice (Wang, Yang et al. 2006). This study also reveals a potential positive feedback loop between FOXM1 and CYCLIN D1. It was reported that CYCLIN D1 modulates the phosphorylation status of pRB leading to the release of FOXM1 by pRB (Gladden and Diehl 2003, Wierstra and Alves 2006). CYCLIN D1 is a validated anti-cancer target with several compounds in clinical trials. Therefore, further investigations of FOXM1 regulation by CYCLIN D1 and reciprocal would give new opportunities for treating tamoxifen resistant breast cancer patients. Finally, the study of ER β 1 in our laboratory identified ER β 1 as a negative FOXM1 regulator that could be downregulated in tamoxifen resistant cells. ER β 1 was observed decreased in pre-invasive mammary tumour compared to normal or benign lesions, but so far altered expression of ER β 1 in tamoxifen resistance has not been investigated (Roger, Sahla et al. 2001). Thereby, the study of ER β 1 expression would provide further molecular mechanism of tamoxifen resistance and potential strategy to overcome tamoxifen resistance. B-MYB has been recently identified as a positive regulator of FOXM1, which co-ordinates with FOXM1 to regulate genes involved in

G2/M phase transition. Our preliminary data suggest that B-MYB is also overexpressed in tamoxifen resistant ER α positive breast cancer cells and might participate to FOXM1 deregulation via a feedback loop. Therefore, it would be interesting to investigate B-myb role and regulation in tamoxifen resistance.

CHAPTER 4 ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance

4.1 Introduction

Cytotoxic chemotherapy agents are the most commonly drugs used in cancers. Taxanes and anthracyclines are cytotoxic chemotherapy drugs that have been frequently used in the neo-adjuvant and adjuvant settings to reduce tumour size prior to surgery and eliminate left over cells to prevent recurrence (Martin, Villar et al. 2003). These cytotoxic agents are used to treat patients with ER/PR/HER2 receptors in combination to anti-oestrogen or HER2-targeted therapies. Importantly, chemotherapy drugs are the only therapeutic option for triple receptor negative patients. Furthermore, cytotoxic chemotherapy agents are used to treat breast cancer patients that are resistant to endocrine and targeted therapies, and these agents are particularly important in the treatment of advanced or metastatic solid cancers (Alvarez 2010, Palmieri, Krell et al. 2010).

Anthracyclines, including doxorubicin (also called Daunorubicin) and epirubicin, are a group of *Streptomyces peucetius* bacteria-derived antibiotics commonly used in cancer chemotherapy. These compounds have been shown to be effective for the treatment of a broad spectrum of cancers such as breast, lung, and ovary carcinomas as well as leukaemia (Lown 1993, Nielsen, Maare et al. 1996). Despite being some of the most effective and widely used anti-cancer drugs in the clinic, patients relapse because of the development of acquired drug resistance (Gonzalez-Angulo, Morales-Vasquez et al. 2007, Broxterman, Gotink et al. 2009, Zelnak 2010). The exact mechanism of action of anthracyclines is still not completely clear, but likely to interfere with DNA replication and induce DNA intercalation triggering DNA damages (Gewirtz 1999, Rivera 2010). Resistance to these DNA targeting anti-cancer drugs is a major clinical obstacle for patients that initially respond to the treatment. It involves multiple mechanisms including enhancement of DNA repair. Consistently, DNA repair gene network signature has been found to be associated with anthracycline response in triple negative metastatic breast cancer (Rodriguez, Makris et al. 2010). A better understanding of the molecular mechanisms of anthracycline action and resistance is required for the development of novel strategies for the treatment of advanced or metastatic breast cancer and for overcoming resistance.

FOXM1 is required for normal G1/S and G2/M cell cycle phase transitions. Besides its involvement in cell cycle transitions, FOXM1 has a multifaceted role in biological processes. Notably, FOXM1 has been recently linked to DNA damage repair (Tan, Raychaudhuri et al. 2007, Kwok, Peck et al. 2010). In addition, FOXM1 dysregulation has been shown to be involved in the development of cisplatin resistance in breast cancer (Kwok, Peck et al. 2010). Accordingly, FOXM1 overexpression has been shown to confer resistance to the humanized anti-HER2 monoclonal antibody (trastuzumab) and microtubule-stabilizing drug (paclitaxel) (Carr, Park et al. 2010). Moreover, chapter 3 of this thesis shows that FOXM1 is a transcriptional target of ER α and play key role in breast cancer endocrine therapy resistance (Millour, Constantinidou et al. 2010). In this chapter, I investigated the expression and regulation of FOXM1 in epirubicin sensitive and resistant MCF-7 breast carcinoma cell lines and its involvement in epirubicin resistance.

4.2 Transcriptional regulation of FOXM1 by p53 in epirubicin sensitive MCF-7 cells

4.2.1 Activation of p53 transcriptionally represses FOXM1

The recent observation that p53 represses FOXM1 expression following daunorubicin treatment led to predict that epirubicin also activates p53 to repress FOXM1 expression in breast cancer cells (Barsotti and Prives 2009). To assess the role and mechanism by which p53 mediates the epirubicin response in breast cancer cells, I first examined the expression of FOXM1 in p53 positive MCF-7 and p53 negative MDA-MB-453 breast cancer cell lines. Western blot analysis of MCF-7 cells revealed that epirubicin treatment strongly induced the expression of the p53 protein and its target the cyclin-dependent kinase inhibitor p21^{Cip1} from 16 h post-treatment, and decreased FOXM1 protein level significantly from 24 h post-treatment (Fig. 4.1). Unsurprisingly, p53 and the inhibitor p21^{Cip1} were undetectable in MDA-MB-453 cell line before and after treatment. Consistently, RT-qPCR analysis revealed no significant decrease in *FOXM1* transcript level in MDA-MB-453 cells, while epirubicin induced a drastic reduction of *FOXM1* mRNA level in MCF-7 cells. Contrary to FOXM1 mRNA level, FOXM1 protein levels varied throughout the treatment. However, the variation in FOXM1 protein levels can be attributed to multiple levels of regulation and a shorter division time in these cell rather than treatment related.

Indeed, FOXM1 protein level varies throughout the cell cycle as well as cyclin A and B1 (Fig. 4.1) and is subject to post-translational modifications including phosphorylations. Collectively, these results show that FOXM1 is downregulated at mRNA and protein levels in response to epirubicin in the p53 positive MCF-7 cells, while FOXM1 expression remained relatively constant in the p53 negative MDA-MB-453 cells, suggesting that activated p53 plays a role in FOXM1 regulation.

To further confirm that p53 is responsible for the downregulation of FOXM1 expression in MCF-7 cells following epirubicin treatment, MCF-7 cells were transiently transfected with non-specific (NS siRNA) or p53-targeting siRNA (p53 siRNA), treated with epirubicin and FOXM1 expression examined. Western blot and RT-qPCR analysis showed that silencing of p53 attenuated FOXM1 downregulation at both protein and mRNA levels in response to epirubicin (Fig. 4.2A and 4.2C). The inability of p53 depletion to completely abolish the downregulation of FOXM1 also suggested that p53 might not be the sole regulator of FOXM1 expression in response to epirubicin (Fig. 4.2A). A previous study showed that p53 represses FOXM1 expression via pRB following daunorubicin treatment (Barsotti and Prives 2009). Thereby, one mechanism by which p53 can repress FOXM1 expression is through its ability to induce p21^{Cip1}, which can in turn repress cyclin-CDK-mediated pRB hyperphosphorylation, resulting in the repression of E2F transcriptional activity (Giacinti and Giordano 2006). Surprisingly, although p53 knock-down abrogated the induction of p21^{Cip1} and the downregulation of FOXM1 by epirubicin, silencing of p21^{Cip1} had little effect on the epirubicin-induced FOXM1 downregulation, suggesting that epirubicin can also repress FOXM1 expression via p21^{Cip1}-independent mechanisms (Fig. 4.2B and 4.2D). To further investigate the role of p53 and p21^{Cip1} in regulating FOXM1 expression in response to epirubicin, wild-type (*wt*), p53-deficient (*p53*^{-/-}), and p21-deficient (*p21*^{Cip1}^{-/-}) mouse embryo fibroblasts (MEFs) were subjected to epirubicin treatment and the expression of FOXM1 investigated (Fig. 4.3). Treatment of the *wt* and *p21*^{Cip1}^{-/-} MEFs with epirubicin resulted in a reduction of *FOXM1* mRNA expression within 16 h, further confirming that p21^{Cip1} is not essential for the repression of FOXM1 expression by epirubicin (Fig. 4.3). In contrast, epirubicin did not cause a downregulation of *FOXM1* mRNA expression in the p53-deficient MEFs (Fig. 4.3). Together these data support the idea that epirubicin represses FOXM1 expression at the transcriptional level through p53.

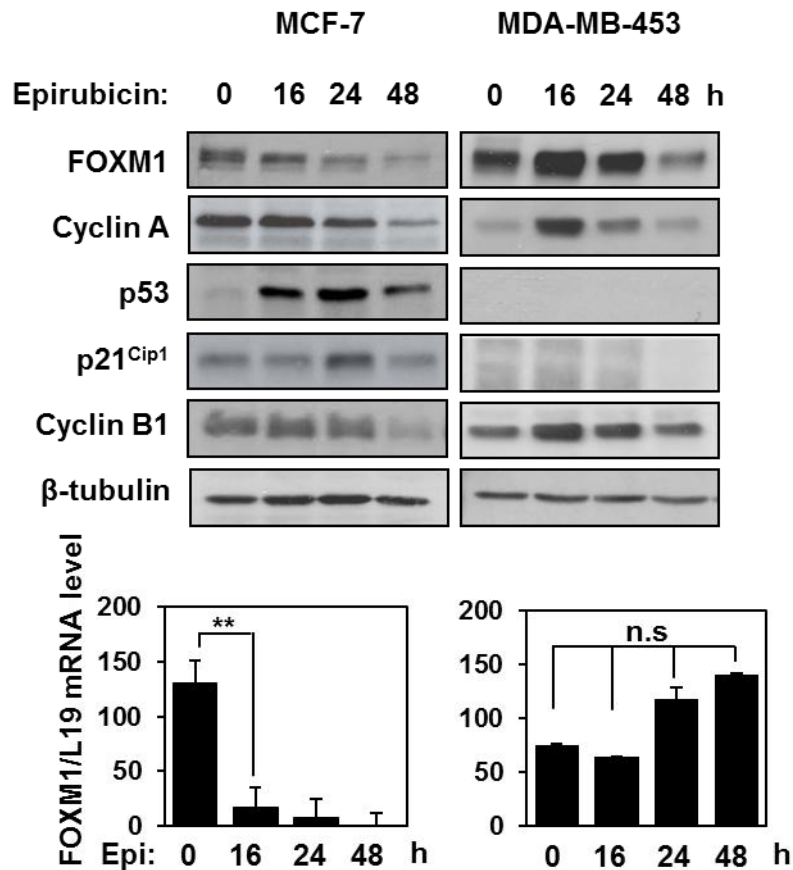


Figure 4.1 Expression of FOXM1 in response to epirubicin treatment in breast cancer cell lines. MCF-7 and MDA-MB-453 cells cultured in 10 % FCS and phenol red DMEM medium were treated with 1 μ mol/L of epirubicin. At times indicated, cells were collected and analysed for FOXM1, CYCLIN A, p53, p21^{Cip1}, CYCLIN B1 and β -tubulin expression by western blotting. *FOXM1* mRNA levels of these cells were also analysed by RT-qPCR, and normalized with *L19* RNA expression. All data shown represent the averages of data from three independent experiments, and the error bars show the standard deviations. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$, **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

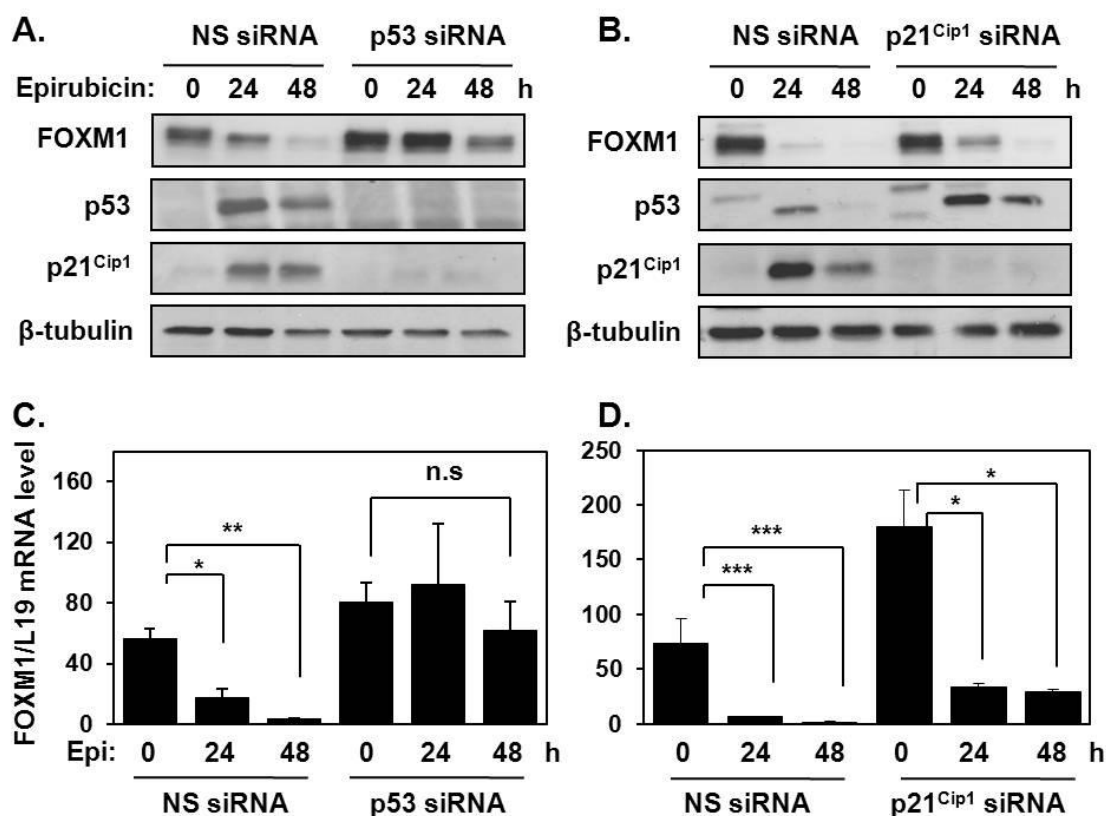


Figure 4.2 Activation of p53 in MCF-7 cells represses FOXM1 expression. MCF-7 cells cultured in 10 % FCS and phenol red DMEM medium were either transfected with non-specific siRNA (NS siRNA), siRNA smart pool against p53 (p53 siRNA) (**A. and C.**), or siRNA smart pool against p21^{Cip1} (p21^{Cip1} siRNA) (**B. and D.**) (100 nmol/L). Twenty-four hours after transfection, MCF-7 cells were treated with 1 μ mol/L of epirubicin and harvested for western blot and analysed using RT-qPCR at 0, 24 and 48 h. The protein expression levels were determined for FOXM1, p53, p21^{Cip1} and β -tubulin and the mRNA level was determined for FOXM1 and normalized with L19 RNA expression. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$, **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

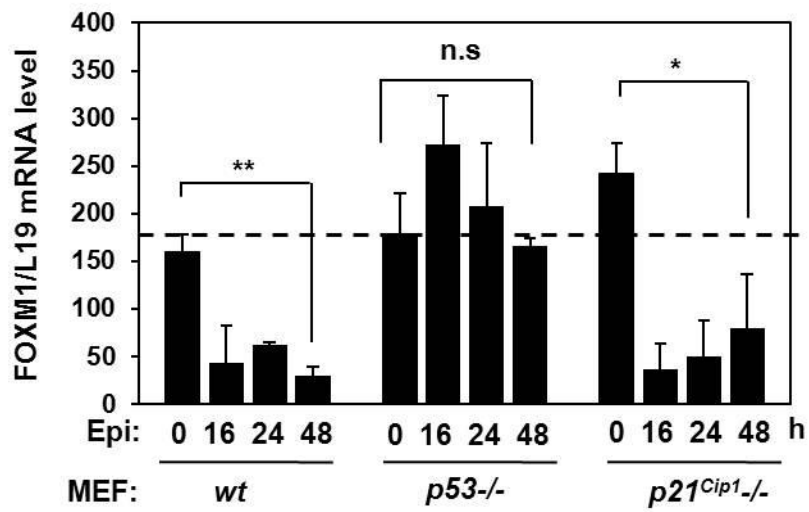


Figure 4.3 FOXM1 repression by p53 in a p21-independent manner. Wild-type, p53^{-/-} and p21^{Cip1}^{-/-} MEF cells cultured in 10 % FCS and phenol red DMEM medium were treated with 1 μ mol/L of epirubicin for 0, 16, 24 and 48 h, and RT-qPCR was performed to determine *FOXM1* mRNA transcript levels and normalize with L19 RNA expression. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$ and ** $P \leq 0.01$, significant.

4.2.2 p53 can regulate FOXM1 through an E2F site in its promoter

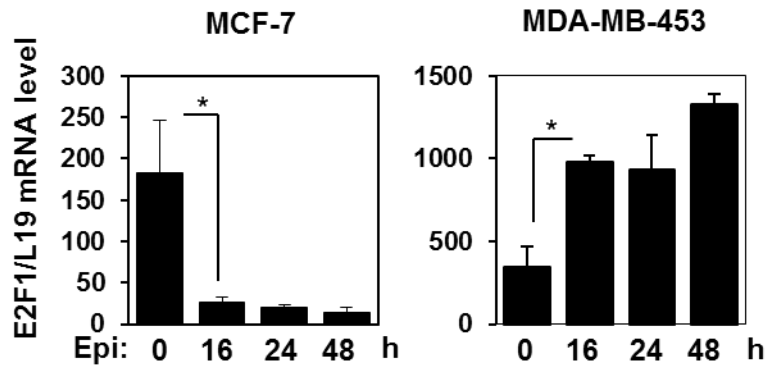
The pRB/E2F transcription factors are principal regulators of the cell cycle and function downstream of the p53 canonical pathway (Giacinti and Giordano 2006). To assess whether the E2F transcription factors are involved in the p53-dependent FOXM1 repression, we analysed the expression pattern of E2F1, a well-characterized E2F-responsive gene product as well as a subunit of the E2F transcription factor dimers (DeGregori and Johnson 2006). The other E2F family members are expressed in different tissues and contexts (Kusek, Greene et al. 2000). Treatment of MCF-7 cells with epirubicin markedly reduced *E2F1* mRNA levels within 16 h, whereas *E2F1* transcript level increased in response to epirubicin in the p53 negative MDA-MB-453 cells (Fig. 4.4A). Furthermore, the close correlation between the mRNA expression pattern of *E2F1* and *FOXM1* in MCF-7 cells suggests that p53 is likely to downregulate FOXM1 expression through the repression of E2F activity.

To provide further evidence that epirubicin represses FOXM1 expression through inhibition of E2F activity, MCF-7 cells were treated with epirubicin for 0 and 24 h, followed by ChIP analyses of E2F1 and its negative regulator pRB on *FOXM1* promoter (Fig. 4.4B). Our semi-quantitative ChIP assay showed that the *in vivo* occupancy of the proximal *FOXM1* promoter by E2F1 decreased and pRB increased after epirubicin treatment, indicating that epirubicin causes the depletion of the transactivator E2F and the accumulation of the transcriptionally repressive pRB protein on *FOXM1* promoter (Fig. 4.4B). Although, this experiment indicated a decreased in E1F and an increased in pRB occupancies on *FOXM1* promoter, a ChIP assay using a quantitative PCR method would provide the exact quantification of the recruitment of these proteins.

We next analysed the involvement of the putative E2F-binding sites in *FOXM1* promoter in FOXM1 repression upon epirubicin treatment. To this end, MCF-7 cells were transiently transfected with a luciferase reporter (pGL3) driven by either a 2.4 kbp (Full Length), a 1.4 kbp (HindIII), or a 296 bp (ApaI) *FOXM1* promoter, and the promoter activity was assayed at 0, 24 and 48 h after epirubicin treatment (Fig. 4.5).

The activity of all three *FOXM1* promoter constructs was markedly reduced following exposure to 1 μ mol/L epirubicin, consistent with the fact that the putative E2F-binding sites (site 1: -58 bp and site 2: -24 bp) locate inside all three *FOXM1* promoter constructs (Fig. 4.5). We next examined whether p53 exerts its repression on *FOXM1* promoter activity through these putative E2F-binding sites. To this end, we co-transfected into MCF-7 cells increasing amounts of pcDNA3-Flag-p53 together with either the wild-type Apal *FOXM1* promoter reporter (WT) or the Apal *FOXM1* promoter lacking one (E2Fmut1 or E2Fmut2) or both (E2Fmut1/2) putative E2F-binding sites. The results showed that p53 caused a drastic reduction (12.7 fold) in E2Fmut1 luciferase activity, comparable to that observed for WT (11.5 fold) (Fig. 4.6A). By contrast, the repression by p53 was considerably reduced in both the E2Fmut2 and the E2Fmut1/2, suggesting that the second putative E2F-binding site (site 2) mediates the repression of *FOXM1* promoter by p53. Next, activity of the wild-type Apal (WT) as well as mutated pGL3-Apal constructs (E2Fmut1, E2Fmut2, and E2Fmut1/2) was examined by co-transfection assays in MCF-7 cells with different amounts of pCMV-E2F1 expression vector. The results showed that the E2Fmut1 construct showed similar responsiveness to E2F1 as the WT (Fig. 4.6B). In contrast, both the E2Fmut2 and the E2Fmut1/2 mutants lost the majority of their responsiveness to E2F1 transfection. Together these co-transfection results provide strong evidence that the E2F-binding element located at -24 bp confers the responsiveness to p53 and E2F1. Taken together, these results indicate that epirubicin can induce p53 to repress *FOXM1* through modulating E2F activity on *FOXM1* promoter.

A.



B.

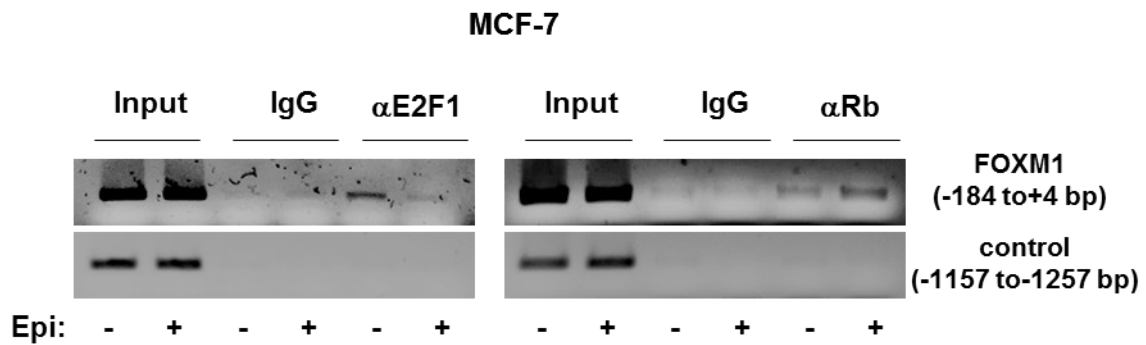


Figure 4.4 E2F1 is decreased in response to epirubicin in MCF-7 cells. **A.** MCF-7 and MDA-MB-453 cells cultured in 10 % FCS and phenol red DMEM medium were treated with 1 μ mol/L of epirubicin for 0, 16, 24 and 48 h and RT-qPCR was performed to determine *E2F1* transcript levels and normalize with *L19* RNA expression. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$ and ** $P \leq 0.01$, significant. **B.** MCF-7 cells untreated or treated with 1 μ mol/L of epirubicin for 24 h were used for ChIP assays using IgG negative control, anti-E2F1 and anti-pRB antibodies as indicated. After crosslink reversal, the co-immunoprecipitated DNA was amplified by PCR using primers amplifying the FOXM1 E2F-binding sites containing region (-184/+4 bp) and a control region (-1157/-1257 bp), and resolved in 1 % agarose gel.

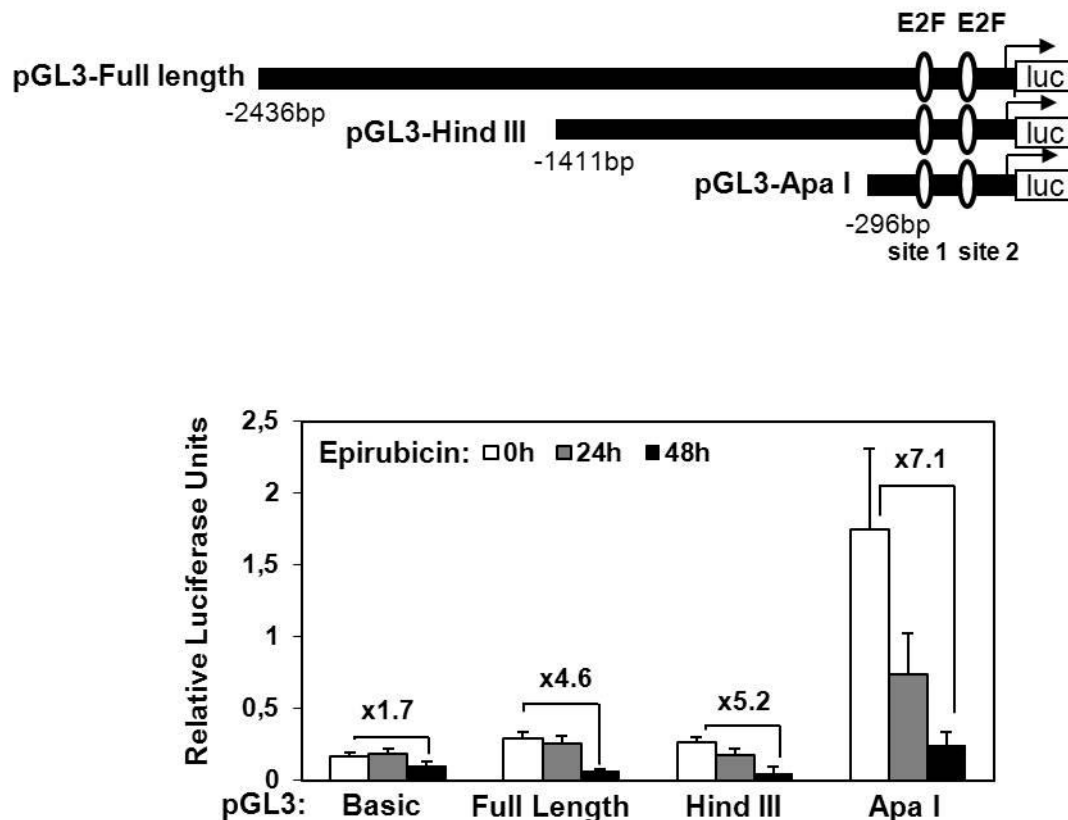


Figure 4.5 FOXM1 promoter activity in response to epirubicin. Schematic representation of the full length, HindIII and ApaI FOXM1-luciferase reporter constructs and the E2F-binding sites 1 (-58 bp) and 2 (-24 bp). MCF-7 cells were transiently transfected with 20 ng of either the empty pGL3-basic, pGL3-Full length, pGL3-HindIII or the pGL3-ApaI, and cells were treated with 1 μ mol/L of epirubicin. Cells were, as described in material and methods, harvested at 0, 24 and 48 h after treatment and assayed for luciferase activity. All relative luciferase activity values are corrected for co-transfected Renilla activity. The fold of repression were calculated between 0 h and 48 h of epirubicin treatment. Columns, means derived from three independent experiments; bars, SD.

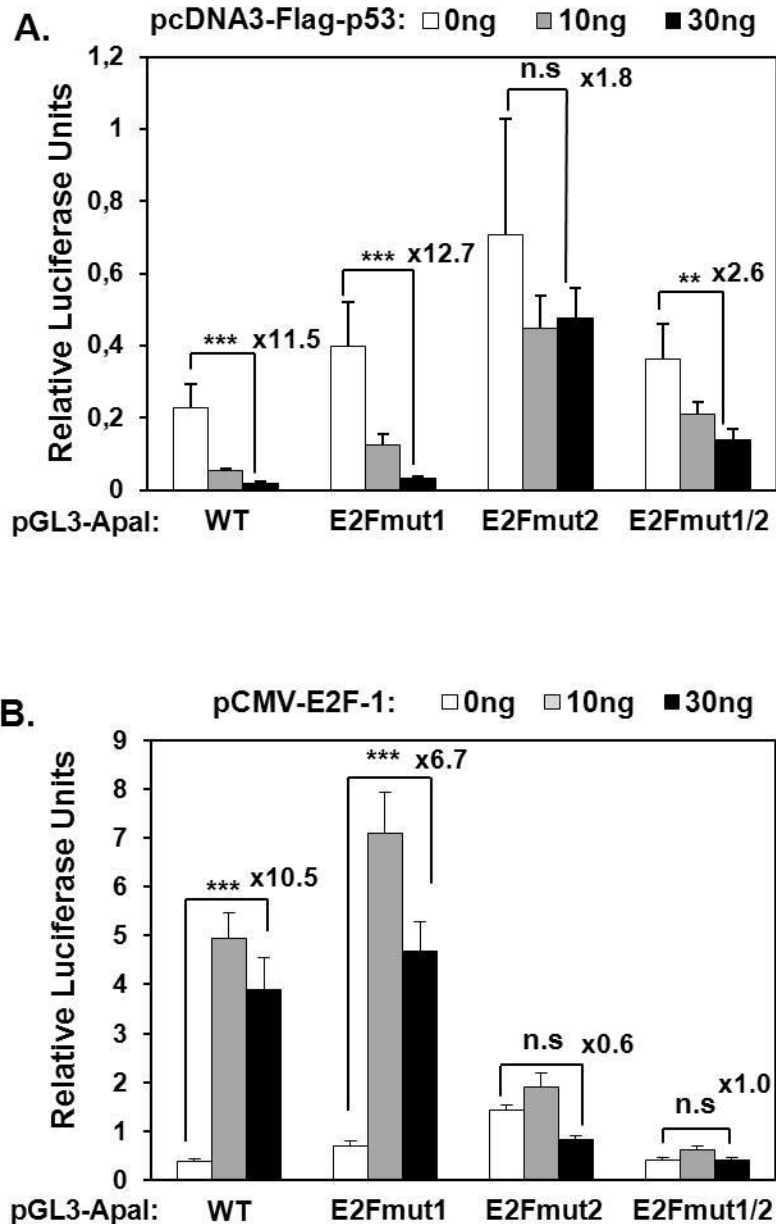


Figure 4.6 Modulation of FOXM1 promoter by p53 and E2F1 via E2F binding site. MCF-7 cells cultured in 10 % FCS and phenol red DMEM medium were transiently transfected with 20 ng of either the wild-type (WT), E2F-binding site 1 mutated (E2Fmut1), E2F-binding site 2 mutated (E2Fmut2), or E2F-binding site 1 and 2 mutated (E2Fmut1/2) pGL3-Apal constructs together with increasing amounts (0, 10 and 30 ng) of pcDNA3-Flag-p53 (**A.**) and pCMV-E2F1 (**B.**). Cells were harvested after 24 h transfection and assayed for luciferase activity as described in Material and Methods. All relative luciferase activity values are corrected for co-transfected Renilla activity. The fold of repression and activation were calculated and indicated between 0 h and 48 h of epirubicin treatment. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

4.3 Differential mechanism of FOXM1 regulation in epirubicin resistant MCF-7 cells

4.3.1 Deregulation of FOXM1 protein and mRNA levels in epirubicin resistant cells

The involvement of FOXM1 in DNA damage response and chemotherapy drug resistance led us to hypothesise that FOXM1 has a role in anthracycline sensitivity as well as resistance in breast cancer. In order to test this conjecture, an epirubicin resistant breast cell line MCF-7EPI^R was used for this study. MCF-7EPI^R cell line was established by a former PhD student by chronic exposure of the parental drug sensitive MCF-7 to stepwise increases in epirubicin concentration until a concentration of resistance up to 10 µmol/L. I confirmed by SRB assays that MCF-7EPI^R cells displayed strong resistance to cell death epirubicin-induced compared to the parental MCF-7 cells (Fig. 4.7A). I next examined the effect of epirubicin on the cell viability of MCF-7 and MCF-7EPI^R cells at 1 µmol/L, a concentration generally used in cancer therapy. The SRB assay revealed that survival of MCF-7 cells was significantly inhibited following epirubicin treatment, while survival of MCF-7EPI^R cells was relatively unaffected in the presence of epirubicin (Fig. 4.7B). There was also a significant difference in the survival rate between the epirubicin-treated MCF-7 and MCF-7EPI^R cells at both 24 h and 48 h. Cell cycle analysis showed that epirubicin exposure (1 µmol/L) induced an accumulation of MCF-7 cells at G2/M and sub-G1 phases, indicative of G2/M phase transition delay and cell death, whereas no significant changes in cell cycle profile are observed for MCF-7EPI^R cells (Fig. 4.7C).

Subsequent western blot analysis revealed no significant changes in the levels of FOXM1 and FOXM1 protein targets, CYCLIN B1 and PLK, following 48 h treatment with epirubicin (1 µmol/L) in MCF-7EPI^R cells in contrast to the downregulation observed in MCF-7 cells Figure 4.1 (Fig. 4.8A). Consistently, RT-qPCR analysis revealed no significant decrease in *FOXM1* transcript level in MCF-7EPI^R cells (Fig. 4.8B). Importantly, p53 and p21^{Cip1} protein levels were undetectable in MCF-7EPI^R cells by western blot analysis (Fig. 4.8A), the band detected in the p53 lane being an unspecific band. Although *p53* mRNA levels are not relevant for its functional activity, we further investigated whether MCF-7EPI^R cells could have lost *p53* mRNA expression. RT-qPCR analysis further showed that *p53* transcript level is reduced by 3.5 fold in MCF-7EPI^R cells compared to MCF-7 cells (Fig. 4.8C).

Collectively, these results show that FOXM1 expression is deregulated in epirubicin resistant MCF-7 cells likely due to the lost of repression by p53, suggesting that FOXM1 has a role in epirubicin sensitivity and resistance.

4.3.2 Increased DNA repair in epirubicin resistant cells

Next, I sought to determine the molecular mechanism that confers epirubicin resistance to MCF-7EPI^R cells. It has been previously shown that FOXM1 expression is associated with cisplatin-induced DNA damage response and drug resistance (Kwok, Myatt et al. 2008). I therefore examined the formation of DNA damage foci by P-H2AX staining in MCF-7 and MCF-7EPI^R cells following epirubicin treatment, including some enlargement of cells showed by the white arrows and represented in the white window. The results showed an increase in the mean number of P-H2AX foci/cell over time after epirubicin treatment in MCF-7 cells, while the level of P-H2AX foci/cell remained relatively constant in MCF-7EPI^R cells, suggesting higher DNA repair activities in these cells (Fig. 4.9). This result was also confirmed in a recent study (Monteiro, Khongkow et al. 2012). To investigate this further, we evaluated the expression level of the DNA repair protein ATM in MCF-7 and MCF-7EPI^R cells. Western blot and RT-qPCR analysis demonstrated that the levels of ATM protein and mRNA are strongly upregulated in MCF-7EPI^R cells compared to MCF-7 cells (Fig. 4.10A), thus suggesting a role of ATM in mediating an increase in DNA repair activity in resistant cells. The ATR mRNA expression level was investigated and showed an significantly elevated ATR mRNA level in MCF-7 cells compared with MCF-7EPI^R cells. This finding might suggest a difference in the activation of DNA repair proteins between these two cell lines (Fig. 4.10B).

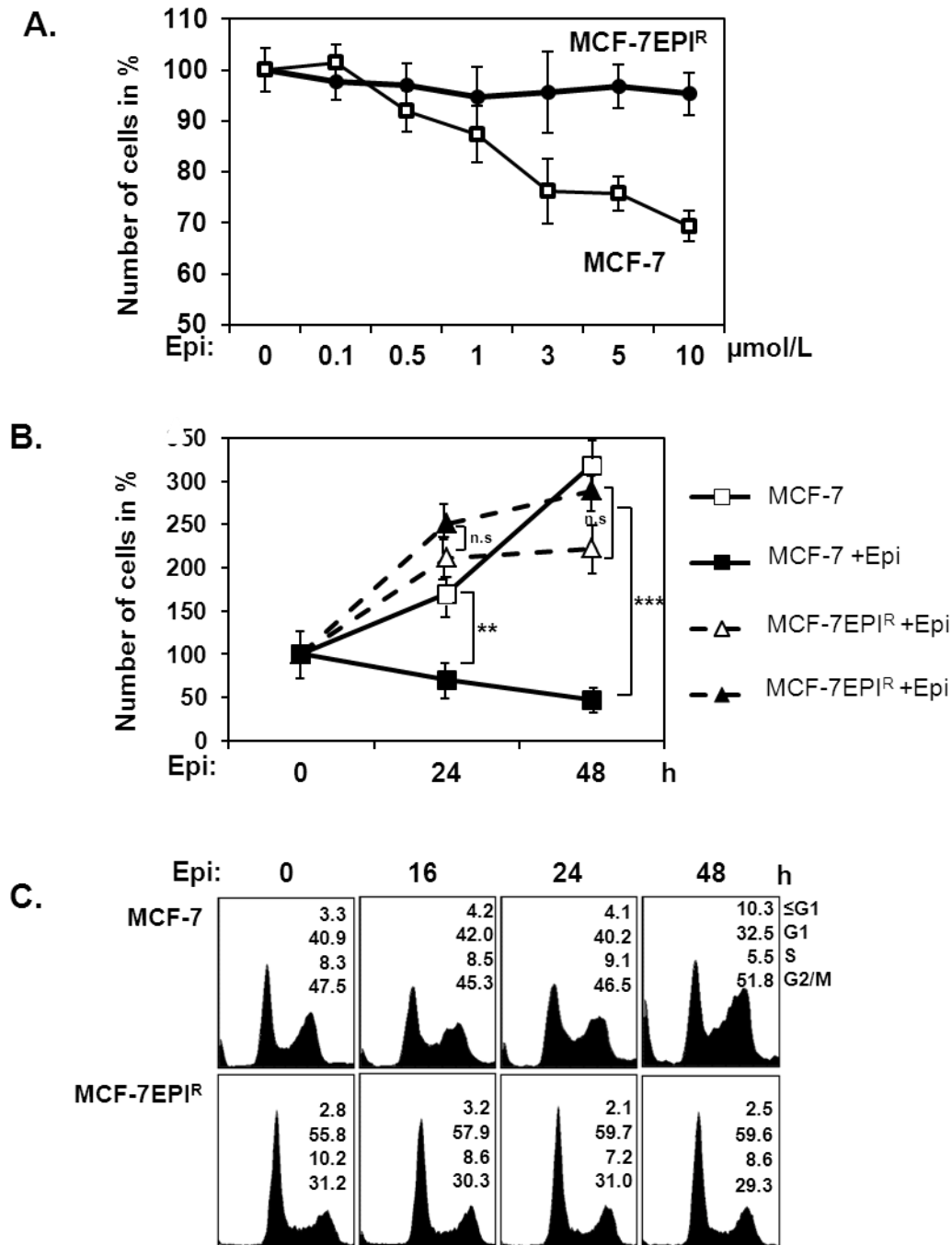


Figure 4.7 Characterisation of epirubicin resistant MCF-7EPI^R cells. **A.** MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red DMEM medium were treated with increasing concentrations of epirubicin for 24 h. Number of cells was measured using SRB assay as described in Material and Methods. **B.** MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red DMEM medium were treated with 1 $\mu\text{mol/L}$ of epirubicin for 0, 16, 24 and 48 h and SRB assay was performed. Statistical analyses were realized using Student t-test for untreated versus treated. **C.** MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red DMEM medium were treated with 1 $\mu\text{mol/L}$ of epirubicin for 0, 16, 24 and 48 h and cells were stained with propidium iodide and analysed FACS analysis carried out. Percentage of cells in each phase (sub-G1, G1, S, G2/M) is indicated. Representative data from three independent experiments are shown.

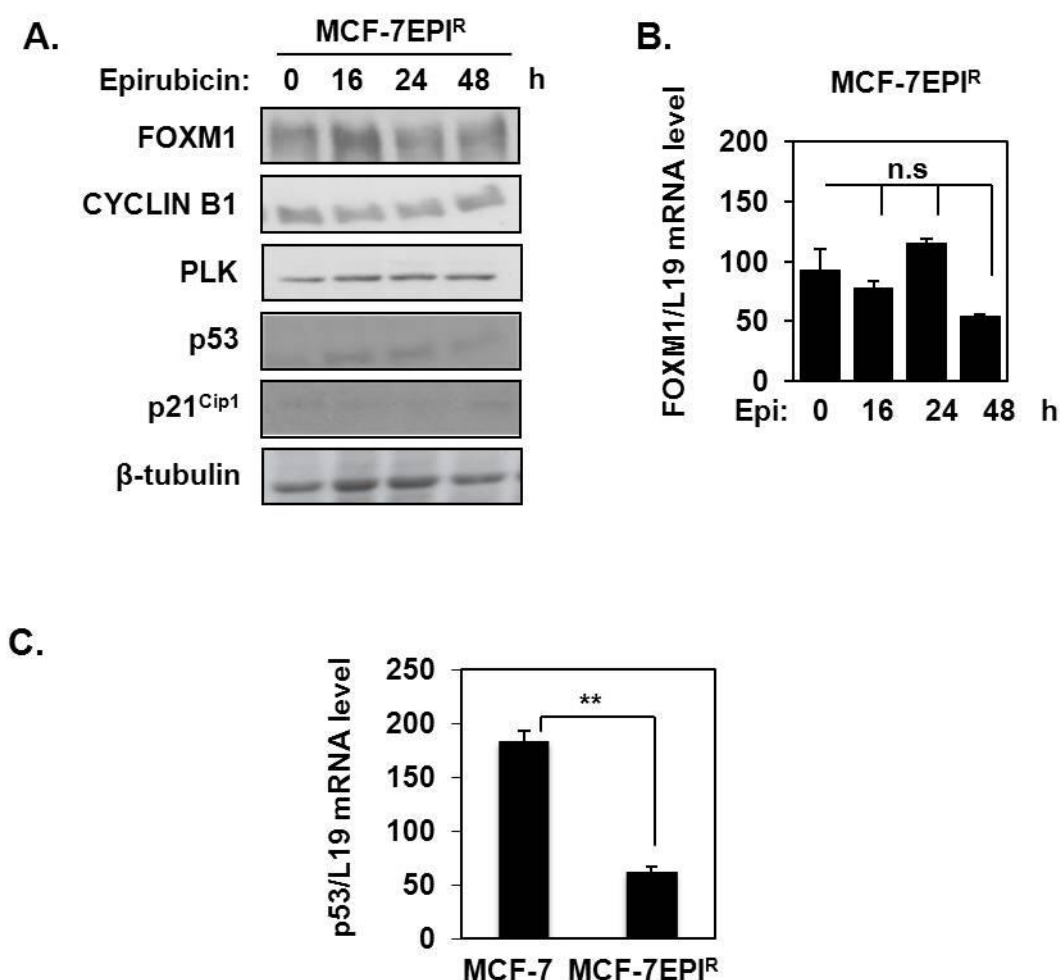


Figure 4.8 Inverse correlation between FOXM1 and p53 expression in MCF-7 and MCF-7EPI^R cell lines. **A.** and **B.** MCF-7EPI^R cells cultured in 10 % FCS and phenol red DMEM medium were treated with 1 μ mol/L of epirubicin for 0, 16, 24 and 48 h. At indicated time, cells were collected and analysed by western blotting to determine the protein expression levels of FOXM1, CYCLIN B1, PLK, p53, p21^{Cip1} and β -tubulin (**A.**), and by RT-qPCR (**B.**) to determine *FOXM1* mRNA transcript levels. Columns, means derived from three independent experiments; bars, SD. **C.** MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red DMEM medium were harvested to determine *p53* mRNA transcript level by RT-qPCR. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

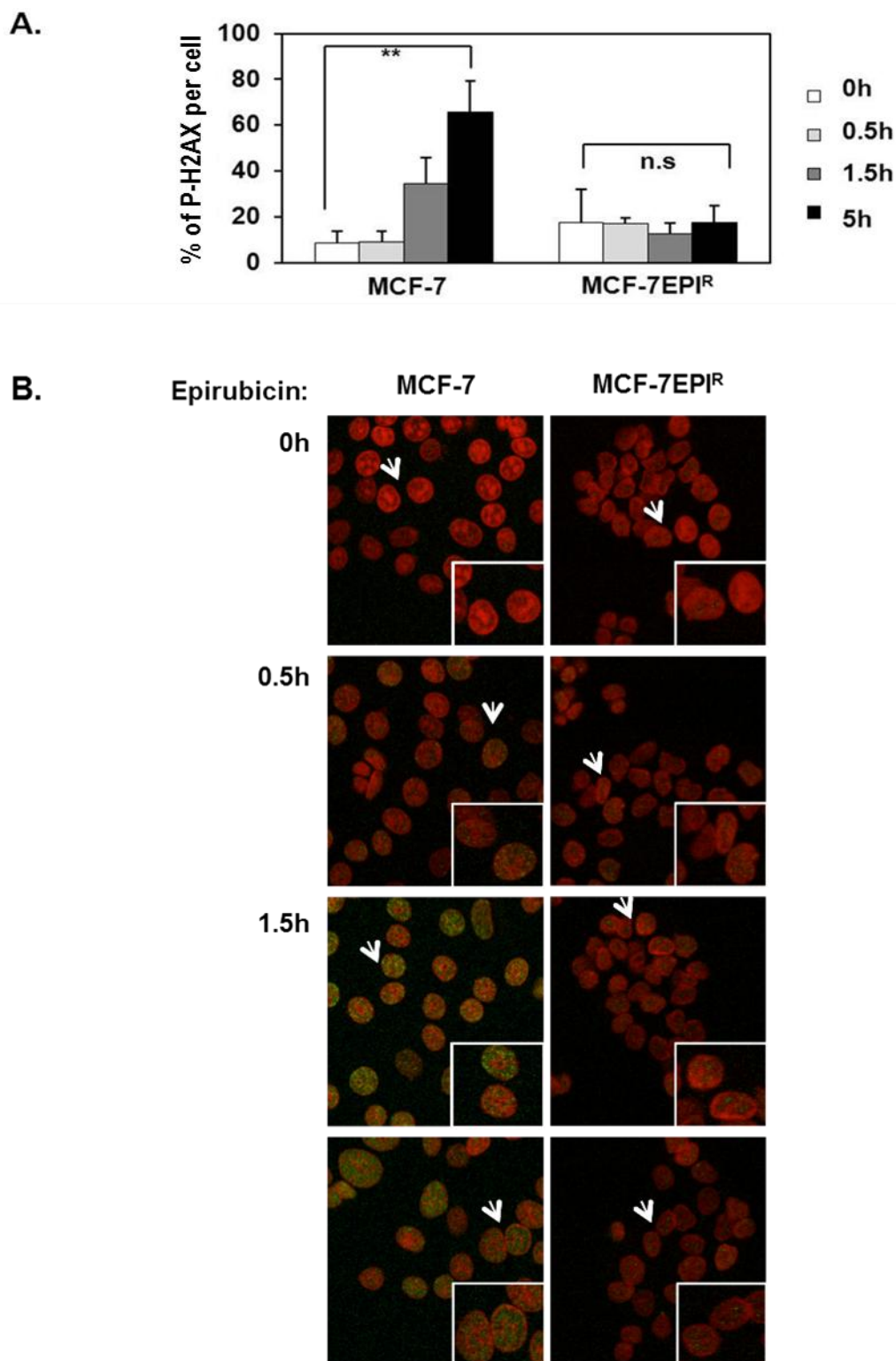


Figure 4.9 Epirubicin resistant MCF-7EPI^R cells show a reduction of DNA damage in response to epirubicin treatment. **A.** MCF-7 and MCF-7EPI^R cells treated with 1 μmol/L of epirubicin for 0, 0.5, 1.5 and 5 h were stained with P-H2AX antibody and DAPI. Images were visualized and scored by ImageXpress (Molecular Devices). The results are the average of three independent experiments. Mean ± SD. Statistical analyses were performed using Student's test. **, $P \leq 0.01$ significant; n.s non significant. **B.** MCF-7 and MCF-7EPI^R cells treated with 1 μmol/L of epirubicin were stained with P-H2AX antibody (green) and DAPI (red). Images visualized by confocal microscopy. Images: magnification: x 20; insets x 80.

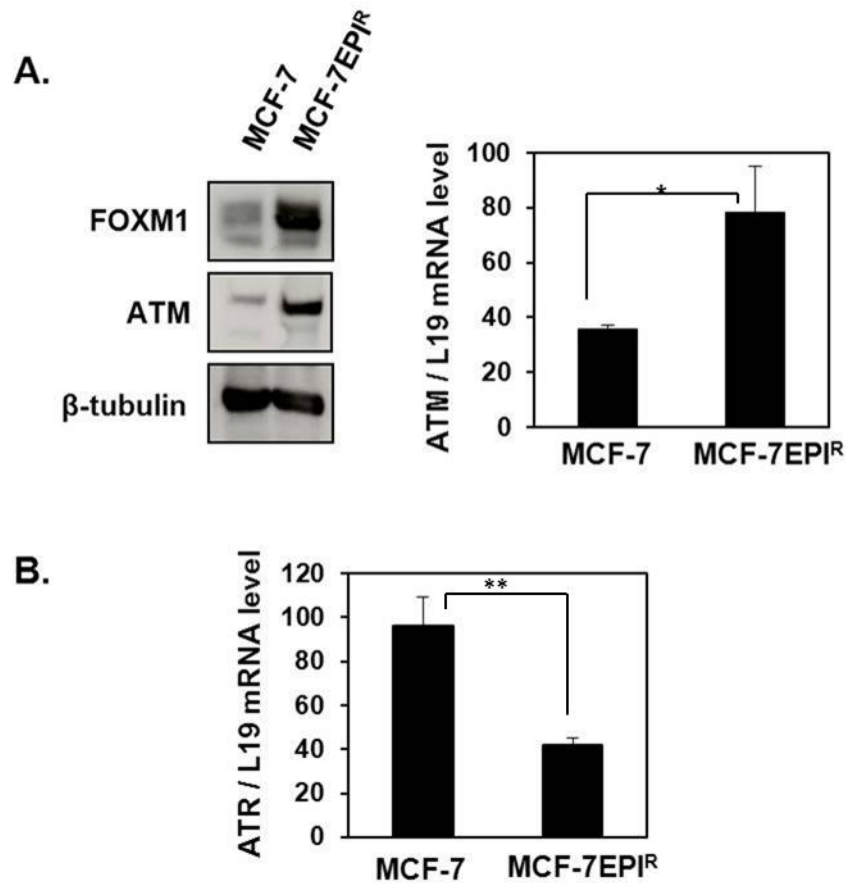


Figure 4.10 Increased expression of ATM in epirubicin resistant MCF-7EPI^R cells. MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red DMEM medium were analysed for FOXM1, ATM and β -tubulin by western blotting (A.). Cells were harvested to determine ATM (B.) and ATR (C.) mRNA levels using RT-qPCR. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

4.3.3 ATM is involved in FOXM1 regulation and epirubicin resistance

To determine whether the ATM signalling pathway is involved in FOXM1 regulation in response to epirubicin, we treated MCF-7 and MCF-7EPI^R cells with epirubicin in the absence or presence of Ku-55933, a known ATM inhibitor (Fig. 4.11). Western blot analysis demonstrated that epirubicin induced a shift of FOXM1 protein at a lower size in MCF-7 cells as already shown previously, while it did not affect FOXM1 protein level and/or size in MCF-7EPI^R cells (Fig. 4.11). However, the combination of epirubicin with Ku-55933 repressed E2F1 and FOXM1 protein expression in MCF-7 and MCF-7EPI^R cells, indicating that Ku-55933 re-sensitised the resistant MCF-7EPI^R cells to FOXM1 downregulation epirubicin-induced (Fig. 4.11). These results suggest that the ATM DNA damage response is involved in FOXM1 regulation in MCF-7EPI^R cells independent of p53 status. However, it has been shown that Ku-55933 alone has no effect on cell cycle while Ku-55933 combined with etoposide induces a G2 arrest (Hickson et al. 2004). Taking into account that FOXM1 expression is reduced when cell cycle is stopped, FOXM1 downregulation Figure 4.11 might be the consequence of Ku-55933 combined with epirubicin treatment.

Consequently, ATM expression and activity were investigated in MCF-7 and MCF-7EPI^R cells by western blot analysis (Fig. 4.12A). Treatment with epirubicin activated ATM phosphorylation (on serine 1981) and also induced ATM expression in MCF-7EPI^R within 24 h, while this induction was not detectable in MCF-7 cells (Fig. 4.12A). Phosphorylation of ATM downstream target CHK2 was strongly enhanced in MCF-7EPI^R cells and to a much lesser extent in MCF-7 cells. In addition, treatment with epirubicin strongly activated p53 reflected by the phosphorylation of p53 on serine 15. In contrast, phosphorylated p53 on serine 15 was undetectable in MCF-7EPI^R cells, demonstrating that p53 is not active in these cells.

To determine whether ATM is a key player of FOXM1 regulation, I silenced ATM expression using siRNA strategy (ATM siRNA) in both MCF-7 and MCF-7EPI^R cells, and studied FOXM1 expression in response to epirubicin (Fig. 4.12B). Western blot analysis showed that ATM knock-down had little effect on FOXM1 and E2F1 expression in MCF-7 cells. While the expression level of FOXM1 remained constant

in MCF-7EPI^R cells transfected with non-specific siRNA (NS siRNA) upon epirubicin treatment, epirubicin caused a decrease in FOXM1 protein expression in MCF-7EPI^R cells when ATM is silenced (Fig. 4.12B). Furthermore, E2F1 protein decreased in MCF-7EPI^R cells following ATM knock-down and epirubicin treatment, which correlates with FOXM1 protein expression. These findings confirmed a differential mechanism of regulation by ATM between MCF-7 and MCF-7EPI^R cells. These findings also suggest that the lack of active p53 and the induction of ATM in MCF-7EPI^R cells are responsible for FOXM1 expression in response to epirubicin.

As ATM is a protein kinase that phosphorylates proteins including p53, mdm2, h2ax, chk2 following DNA damage, I performed a time course with MCF-7 and MCF-7EPI^R cells treated with epirubicin (Zhou and Elledge 2000). I showed that FOXM1 protein level (higher band) increased in MCF-7EPI^R cells, while FOXM1 is downregulated in MCF-7 cells (Fig. 4.13A). Furthermore, I immunoprecipitated FOXM1 proteins and probed with anti-MPM2, an antibody recognising phosphorylated proteins, to investigate FOXM1 phosphorylation status in MCF-7 and MCF-7EPI^R cells epirubicin-treated. Immunoblotting with MPM2 antibody showed a decrease in phosphorylation of immunoprecipitated FOXM1 in MCF-7 cells, while FOXM1 phosphorylation remained high in MCF-7EPI^R cells following epirubicin (Fig. 4.13B). In addition, it has previously been shown that FOXM1 protein is phosphorylated by CHK2 after DNA damage. Given that CHK2 functions directly downstream of ATM in DNA damage response, it is predicted that the induction of FOXM1 expression by ATM may occur through post-translational mechanisms (Tan, Raychaudhuri et al. 2007). In contrast to Tan *et al.* study, silencing of both checkpoint kinases (CHK1 and CHK2) did not affect FOXM1 protein expression in untreated and treated MCF-7EPI^R cells (Fig. 4.13C). Notably, the loading of CHK1 siRNA protein samples was lower than non-specific and CHK2 siRNA, but FOXM1 levels still remained steady in these samples over the time course.

To further investigate FOXM1 regulation by ATM, I used the siRNA strategy to reduce *ATM* expression (ATM siRNA) and investigated *FOXM1* mRNA transcript levels in MCF-7EPI^R cells treated with epirubicin. ATM silencing significantly reduced *ATM* mRNA levels as well as *FOXM1* mRNA levels in MCF-7EPI^R cells treated with epirubicin, suggesting that ATM regulates FOXM1 at transcriptional level (Fig.

4.14A). Given that E2F1 protein expression followed FOXM1 protein expression pattern when ATM is silenced, I analysed *E2F1* mRNA levels in MCF-7EPI^R cells treated with epirubicin and performed ChIP assay. The results showed that *E2F1* mRNA expression and E2F1 occupancy of *FOXM1* promoter remained steady in MCF-7EPI^R cells treated with epirubicin (Fig. 4.14B and 4.14C).

These data suggested that ATM regulates FOXM1 transcriptionally via E2F1 and that FOXM1 might also be regulated by phosphorylations in MCF-7EPI^R cells. Given the role of ATM in DNA repair and the fact that ATM regulates FOXM1 in epirubicin resistant cells, it is likely that FOXM1 has a role in epirubicin resistance. In addition, the role of FOXM1 in epirubicin sensitivity and resistance is further supported by the observations that overexpression of FOXM1 in MCF-7 cells can decrease the sensitivity to epirubicin (supplementary data realised by Julia K. Langer Fig. S.D.7.3) and that FOXM1 knock-down in MCF-7EPI^R cells did mimic the cytotoxic effects of epirubicin on MCF-7 cells (Fig. 4.15). However, the preliminary SRB result did not show that ATM silencing has an effect on the survival of the epirubicin resistant cells compared to FOXM1 silencing (Fig. 4.15). These results could indicate that FOXM1 is regulated by multiple pathways including ATM, but remains the main regulator in epirubicin resistance.

Although the SRB assay showed that FOXM1 silencing reduced the survival of the epirubicin resistance cells, a clonogenic or colony formation assay would be a better method to test the effect of FOXM1 inhibition on epirubicin sensitivity and resistance. Clonogenic assay is the method of choice to determine cell reproductive death after treatment. Only a fraction of seeded cells retains the capacity to produce colonies (Franken, Rodermond et al. 2006). Testing FOXM1 silencing in epirubicin resistant cells using this assay would tell us whether FOXM1 is a key player for epirubicin resistance.

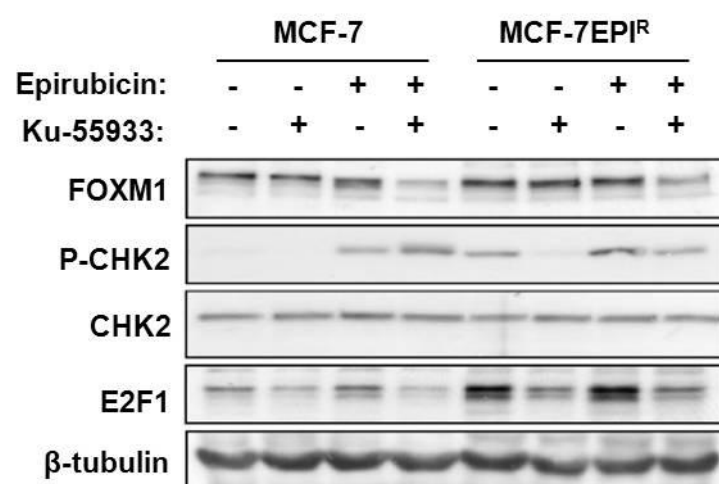


Figure 4.11 ATM inhibition and epirubicin downregulate FOXM1 in MCF-7EPI^R cells. MCF-7 and MCF-7EPI^R cells were treated with 1 μ mol/L of epirubicin alone or in combination with 10 μ mol/L of Ku-55933 for 24 h and the protein expression levels of FOXM1, P-Chk2, Chk2, E2F1 and β -tubulin were analysed by western blot analysis.

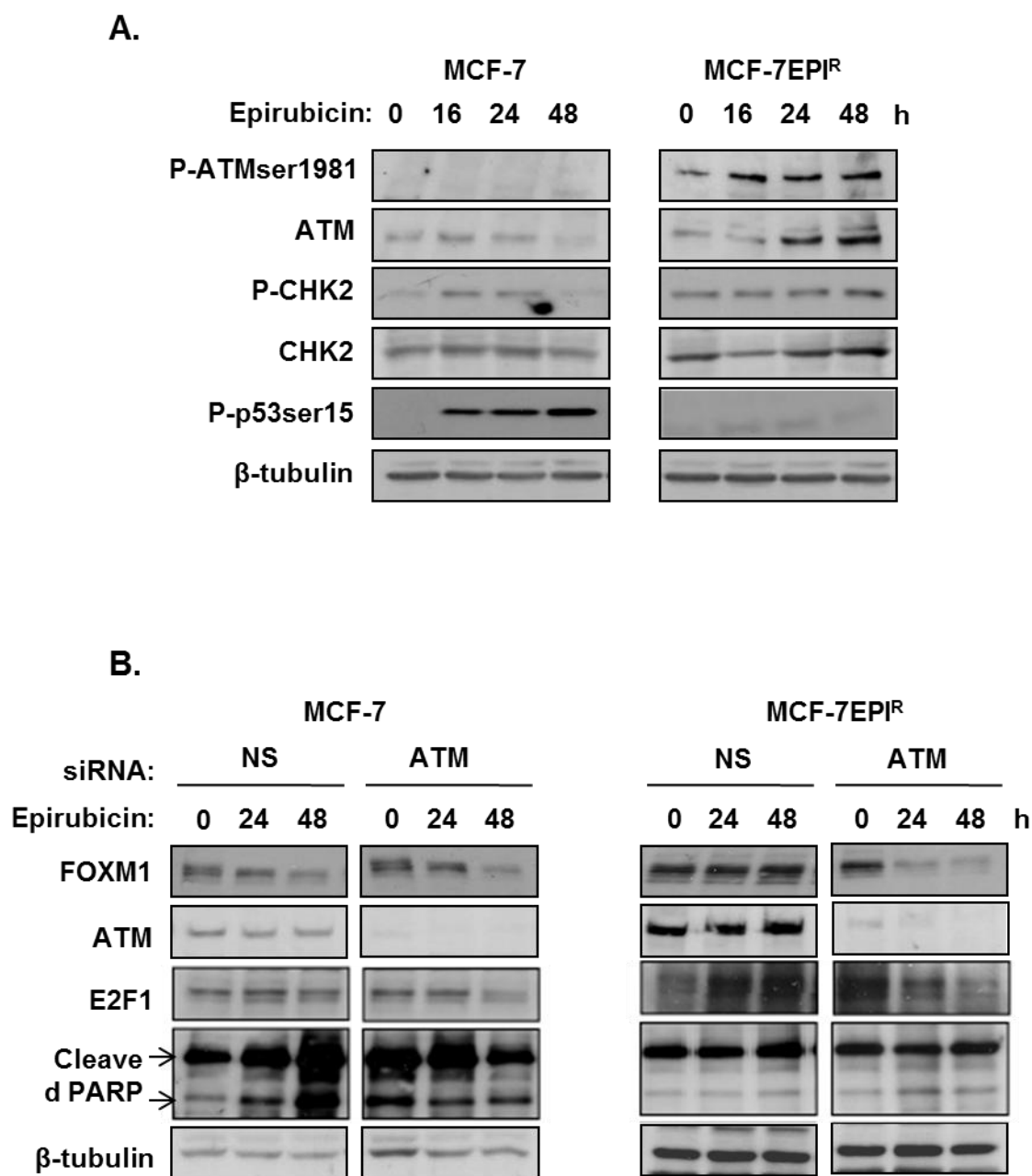


Figure 4.12 ATM is involved in FOXMI regulation in epirubicin resistant MCF-7EPI^R cells. **A.** MCF-7 and MCF-7EPI^R were treated with 1 μ mol/L of epirubicin and the protein expression levels of P-ATM, ATM, P-CBK2, CHK2, P-p53 (ser15) and β -tubulin were analysed by western blot analysis. **B.** MCF-7 and MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA (100 nmol/L) or siRNA smart pool against ATM (100 nmol/L). Twenty-four hours after transfection, cells were treated with 1 μ mol/L of epirubicin and harvested for western blot at 0, 24 and 48 h. The protein expression levels were determined for FOXMI, ATM, E2F1, cleaved PARP and β -tubulin.

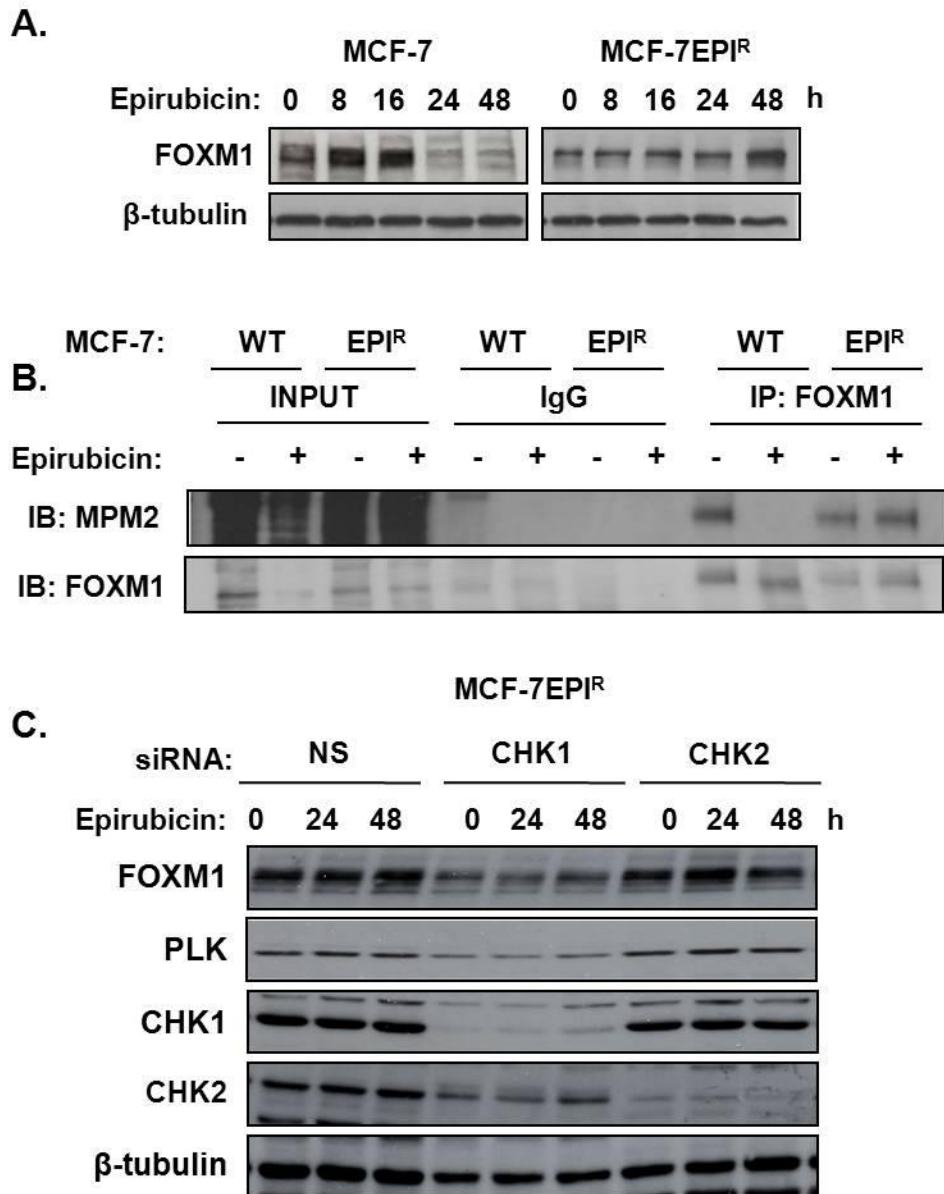


Figure 4.13 Phosphorylation of FOXM1 in MCF-7EPI^R cells. **A.** MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red medium were treated with 1 μ mol/L of epirubicin in a time course of 48 h. Cell lysates were prepared at indicated times, and the expression of FOXM1 and β -tubulin was analysed by western blotting. **B.** MCF-7 and MCF-7EPI^R cells cultured in 10 % FCS and phenol red medium were treated with 1 μ mol/L of epirubicin for 24 h. Cell lysates were subjected to immunoprecipitation using anti-FOXM1 antibody and immunoblotted with anti-MPM2 and FOXM1 antibodies. **C.** MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA, CHK1- or CHK2-targeting siRNA (100 nmol/L) and treated with 1 μ mol/L of epirubicin for 0, 24 and 48 h. The protein levels were analysed by western blotting using anti-FOXM1, anti-PLK, anti-CHK1, anti-CHK2 and anti- β -tubulin antibodies.

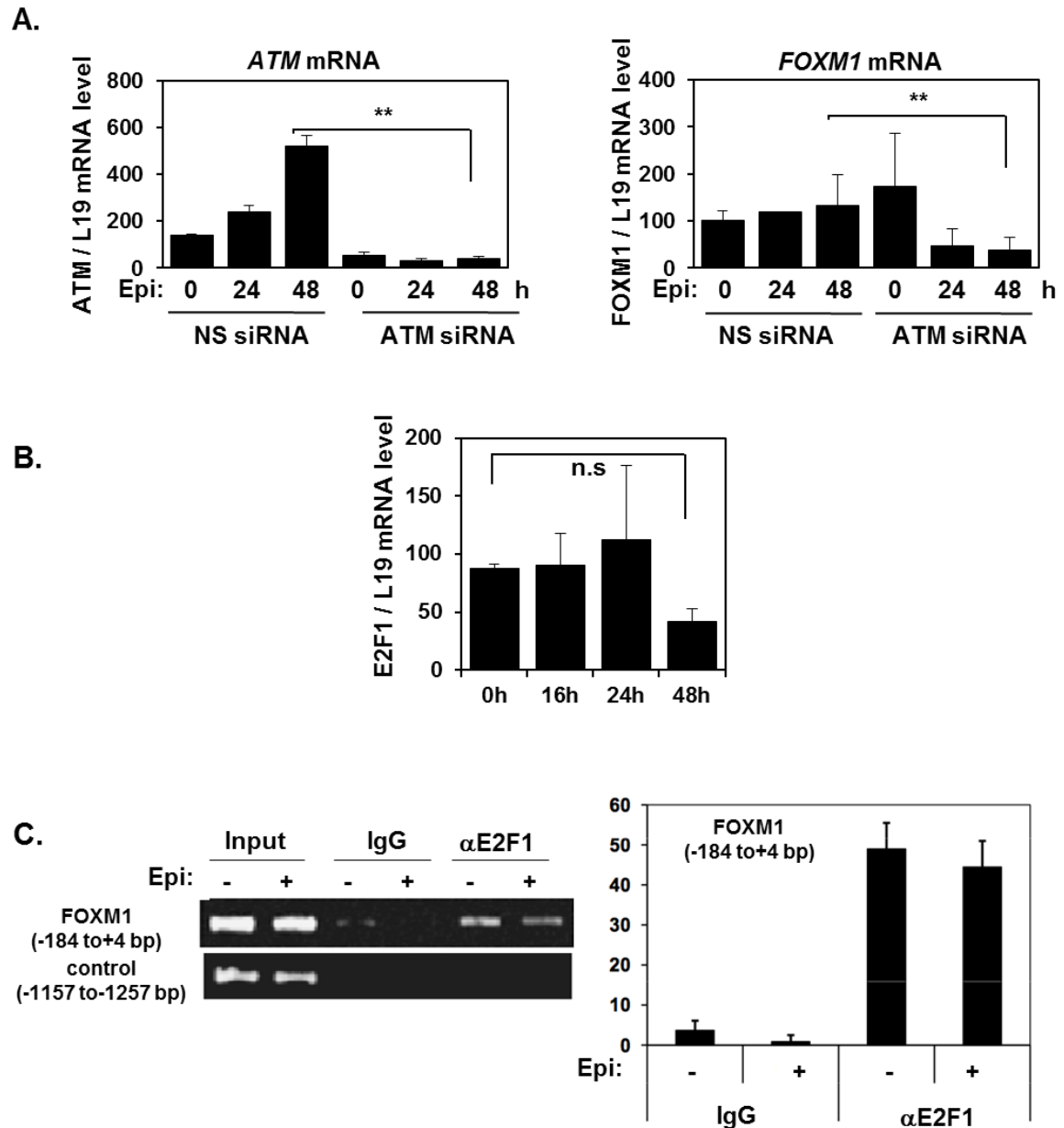


Figure 4.14 E2F1 occupancy on *FOXM1* promoter remains steady in MCF-7EPI^R cells.
A. Cells were transfected with non-specific and FOXM1-targeting siRNA (100 nmol/L) and were treated with 1 μ mol/L of epirubicin for 48 h and harvested for determination of *FOXM1* and *ATM* mRNA levels by RT-qPCR analysis. Statistical analyses were performed using Student's test. **, $P \leq 0.01$ significant; n.s non significant. **B.** MCF-7EPI^R cells were treated with 1 μ mol/L of epirubicin for 0, 16, 24 and 48 h and RT-qPCR was performed to determine E2F1 transcript levels. Columns, means derived from three independent experiments; bars, SD. **C.** After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR using primers amplifying the FOXM1 E2F1-binding sites containing region (-184/+4) and a control region (-1157/-1257), and resolved on 2 % agarose gel (left panel). Quantification by RT-qPCR gave similar results (right panel).

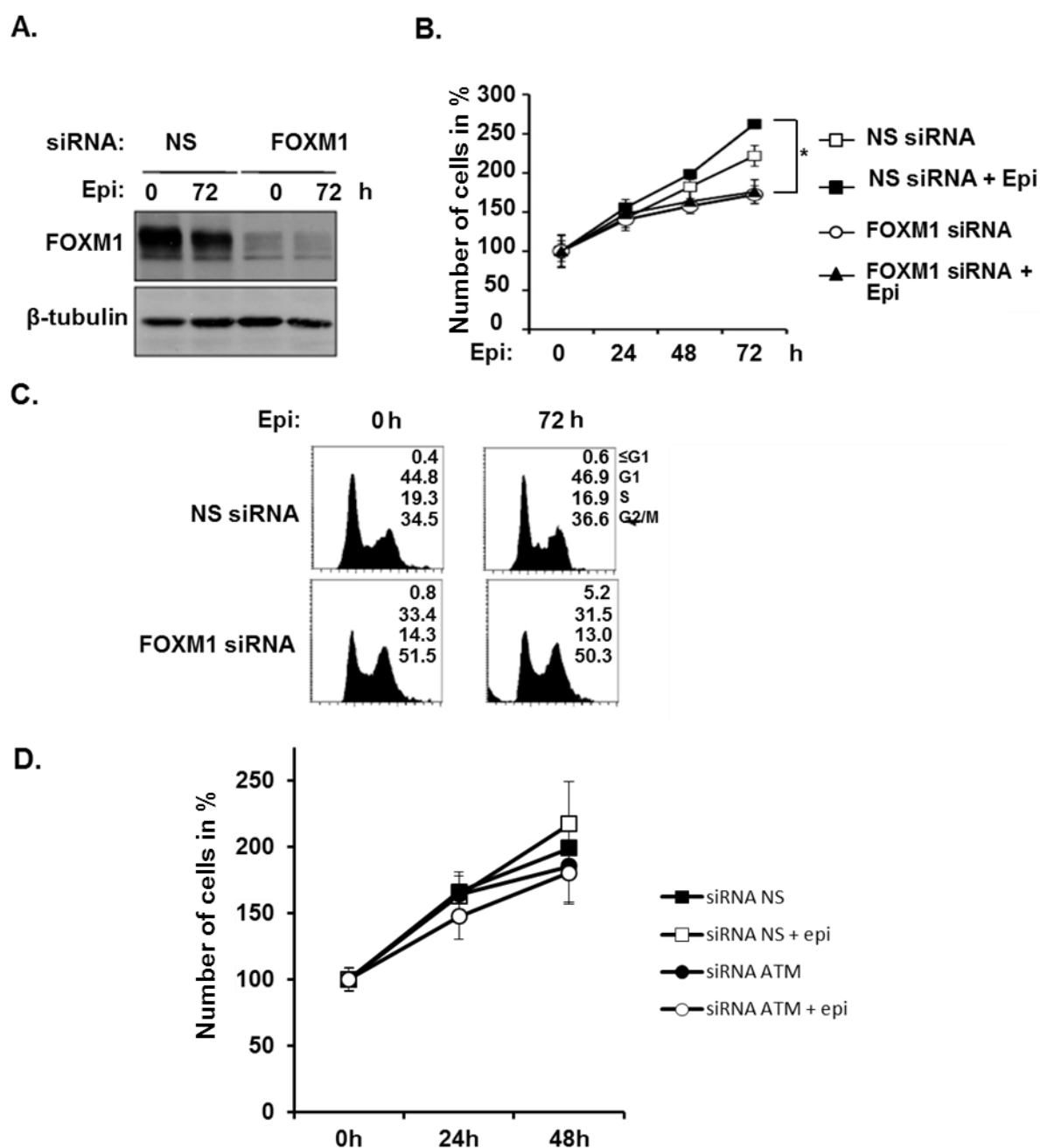


Figure 4.15 Silencing of FOXM1 combined with epirubicin treatment increases cell death in MCF-7EPI^R cells. **A.** MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA or FOXM1-targeting siRNA (100nmol/L) and treated with 1 μ mol/L of epirubicin for 72 h. Cells were harvested for western blot analysis to validate the silencing efficiency using anti-FOXM1 and anti- β -tubulin antibodies. **B.** The transfected MCF-7EPI^R cells treated with 1 μ mol/L of epirubicin were collected for SRB assay at 0, 24, 48, 72 h. **C.** The transfected MCF-7EPI^R cells treated with 1 μ mol/L of epirubicin were also collected for FACS analysis carried out after propidium iodide staining. Cell death was analysed using flow cytometry. The percentages of cells in sub-G1 were calculated. Columns, means derived from three independent experiments; bars, SD. **D.** MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA or ATM-targeting siRNA (100nmol/L) and treated with 1 μ mol/L of epirubicin for 72 h. The transfected MCF-7EPI^R cells treated with 1 μ mol/L of epirubicin were collected for SRB assay at 0, 24, 48 h. Experiment performed once in triplicates.

4.4 Discussion

4.4.1 FOXM1 is a crucial target of p53

FOXM1 has been found to be frequently upregulated in a large variety of human cancers (Wang, Kiyokawa et al. 2002, Kalinichenko, Major et al. 2004, Kalin, Wang et al. 2006, Kim, Ackerson et al. 2006, Liu, Dai et al. 2006, Laoukili, Stahl et al. 2007, Wang, Banerjee et al. 2007). In addition, emerging evidence revealed that FOXM1 also has a role in cancer drug resistance. Studies demonstrated that FOXM1 level is an important determinant of sensitivity to breast cancer chemotherapy drugs, such as trastuzumab, gefitinib, lapatinib, paclitaxel and cisplatin (Kwok, Peck et al. 2010). Consistent with these findings, this study established that FOXM1 is a crucial cellular target of the anthracycline epirubicin in breast cancer cells. FOXM1 expression is downregulated by epirubicin in the sensitive MCF-7 cells, but not in the resistant MCF-7EPI^R cells. Moreover, FOXM1 protein levels are higher in the epirubicin resistant MCF-7EPI^R cells relative to the sensitive MCF-7 cells. Taken together, these data suggest that FOXM1 also has a role in epirubicin resistance. In agreement, a recent study revealed that the anthracycline daunorubicin can repress FOXM1 expression through the sequential activation of p53, p21^{Cip1} and RB family of proteins (Barsotti and Prives 2009). Using *p53*^{-/-} and *wt* MEFs, we established that FOXM1 expression is negatively regulated by p53 (Fig. 4.3). However, epirubicin can effectively repress FOXM1 expression in the *p21*^{Cip1/-} MEFs (Fig. 4.3). This finding indicates that p53 can repress E2F activity and FOXM1 expression independent of the cyclin-dependent kinase inhibitor p21^{Cip1}, despite previous studies showing that the activation of pRB by the anthracycline daunorubicin is mediated at least partially through p21^{Cip1} (Barsotti and Prives 2009). Based on the fact that *E2F1* gene is an E2F-regulated gene, its expression reflects the cellular E2F activity. Transient reporter assays indicate that the effects of epirubicin, its cellular targets p53 and E2F1 are mediated through a proximal E2F-binding site within *FOXM1* promoter (Fig. 4.6). In agreement, a recent study revealed that a great majority of genes repressed by p53 and p73 contains E2F-binding sites, suggesting that p53 proteins repress gene expression through inhibiting E2F activity (Scian, Carchman et al. 2008). The direct binding of pRB and E2F1 on *FOXM1* promoter was confirmed *in vivo* by ChIP

analysis. ChIP assays also revealed that upon epirubicin treatment pRB level increased and E2F1 level decreased within *FOXM1* promoter region containing the E2F-binding site (Fig. 4.4). Collectively, these findings indicate that epirubicin can repress *FOXM1* expression through induction of p53, which in turn represses E2F activity through activating pRB and downregulating E2F1 expression.

4.4.2 p53 status is not a determinant of epirubicin response

Many chemotherapy agents in the treatment of cancer cause DNA damage that is sensed by the tumour suppressor protein p53, triggering DNA repair and inducing apoptosis (Liu and Kulesz-Martin 2001). In case of mutation or deletion of p53 gene, the efficiency of chemotherapy agents is compromised (Aas, Børresen et al. 1996, Reles, Wen et al. 2001). Mutations of p53 occur in more than half of all tumours and have been linked to drug resistance (Hollstein, Sidransky et al. 1991). Despite that the loss or mutation of p53 is associated with resistance to chemotherapy in many cancers including breast cancer (Aas, Børresen et al. 1996), this study shows evidence that DNA damage-sensing kinase ATM has also a role in regulating *FOXM1* expression and epirubicin resistance, independent of p53. For instance, epirubicin fails to activate p53 in MCF-7EPI^R cells, but reduces *FOXM1* protein expression in combination with ATM inhibitor treatment (Fig. 4.11). Similarly, the combination of epirubicin with ATM silencing completely abrogated *FOXM1* protein expression (Fig. 4.12). Furthermore, U2OS cells treated with epirubicin activates p53 but only reduces *FOXM1* levels in combination with caffeine (an ATM inhibitor) (Millour, de Olano et al. 2011). Taken together, this study shows that p53 is not the major component of epirubicin resistance. The role of p53 in drug sensitivity occurs through the activation of cell cycle arrest and apoptosis genes transcription. Based on its role, the transfection of p53 in MCF-7EPI^R was tested, but p53 was not sufficient to restore epirubicin sensitivity (data not shown). Similarly, ectopic expression of p53 in lung cancer cell lines failed to alter the sensitivity of the cell line to the chemotherapeutic agents adriamycin, taxol and carboplatin (Breen, Heenan et al. 2007).

4.4.3 FOXM1 is a target of ATM

Based on the fact that ATM controls DNA repair through protein phosphorylation leading to the activation of gene transcription, we analysed the regulation of FOXM1 by ATM. Downregulation of ATM using siRNA significantly reduced *FOXM1* mRNA levels, indicating that ATM regulates FOXM1 at transcriptional level (Fig. 4.14A). Consistently, ample evidence has demonstrated that ATM regulates E2F1 expression in response to DNA damage, although the mechanism involved is not completely understood (Blattner, Sparks et al. 1999, Carcagno, Ogara et al. 2009). For example, genotoxic stress has been reported to upregulate E2F1 expression at transcriptional level through the activation of ATM (Carcagno, Ogara et al. 2009). On the contrary, a previous study also showed that E2F1 expression is upregulated in response to DNA damage because of an increase in protein stability indicating a post-translational regulation mechanism (Blattner, Sparks et al. 1999). Current evidence indicates that E2F1 expression can be involved in proliferation and tumorigenesis as well as apoptosis and tumour suppression (Kusek, Greene et al. 2000, Polager and Ginsberg 2009). However, in the context of cancer chemotherapy, the current observations evidently suggest that E2F1 is linked to cell survival through promoting FOXM1 expression. In a previous microarray study, E2F1-3 proteins have been shown to promote the expression of genes involved in DNA replication, DNA repair and mitosis, and interestingly some of these E2F-regulated genes identified, such as *CYCLIN B1*, are also transcriptional target of FOXM1 (Polager, Kalma et al. 2002, Ren, Cam et al. 2002, Russo, Magro et al. 2006). Consistently, a number of recent studies have demonstrated that E2F1 expression is induced by a variety of DNA damaging agents and genotoxic chemotherapeutic drugs and mirrors that of p53. Based upon our current findings that ATM induces E2F activity and FOXM1 expression in response to DNA damage and that E2F can promote FOXM1 transcription, this study proposes that ATM enhances E2F1 expression and activates E2F-dependent *FOXM1* expression at transcriptional level in response to DNA damaging agents, such as epirubicin.

In addition, western blotting and immunoprecipitation experiments (Figure 4.13) in MCF-7 and MCF-7EPI^R cells suggested that FOXM1 phosphorylation is enhanced by epirubicin in MCF-7EPI^R cells. Taken together, our findings showed FOXM1

involvement in DNA damage response pathway and for the first time a relationship between FOXM1 and ATM. Numerous studies have shown that ATM regulates by phosphorylation numerous genes involved in cell cycle checkpoints, apoptosis and DNA repair. For instance, ATM phosphorylates the checkpoint kinase 2 (CHK2) on threonine 68 in response to DNA damage. When activated, CHK2 inhibits CDC25C phosphatase preventing entry into mitosis and allowing repair of the DNA. In addition, it has previously been shown that FOXM1 protein is phosphorylated by CHK2 on serine 361 in response to DNA damage. This phosphorylation event has also been proposed to increase the stability of the FOXM1 protein to promote expression of DNA repair genes (Tan, Raychaudhuri et al. 2007). Given that CHK2 functions directly downstream of ATM in DNA damage response, it is predicted that the induction of FOXM1 expression by ATM may therefore also occur through post-translational mechanisms in response to DNA damage (Tan, Raychaudhuri et al. 2007). In contrast, silencing experiments showed that neither CHK2 nor CHK1 is involved in FOXM1 regulation in MCF-7EPI^R cells (Figure 4.13C). Given that FOXM1 is stabilised after epirubicin in MCF-7EPI^R cells and that ATM knock-down completely abolished FOXM1 expression, we hypothesised that ATM could directly phosphorylate FOXM1. To investigate this hypothesis, I performed *in vitro* radioactive-labelled kinase assay using ATM, FOXM1 and CHK2 (positive control) recombinant proteins. While CHK2 protein did phosphorylate FOXM1 as already published (Tan, Raychaudhuri et al. 2007), I did not observe any phosphorylation events when ATM was added to FOXM1 protein (data not shown).

4.4.4 FOXM1 involvement in DNA repair and cell survival

Increased ATM expression in MCF-7EPI^R cells indicates that ATM may promote DNA repair to counteract the DNA damage-induced cell death triggered by genotoxic chemotherapy drugs. Consistent with this, the sustained levels of foci in the MCF-7EPI^R cells after epirubicin are significantly reduced when compared with the drug sensitive MCF-7 cells, as revealed by the P-H2AX staining (Fig.4.9). Moreover, this idea is further supported by our finding that depletion of ATM activity (by siRNA or Ku-55933 inhibitor) abolished the accumulation of FOXM1. Consequently, we

showed that FOXM1 silencing reduced cell survival (Fig. 4.15), and could sensitise the resistant MCF-7-EPI^R cells to epirubicin sensitivity. Conversely, FOXM1 overexpression delays apoptosis induced by epirubicin (Fig. S.D.7.3) and by cisplatin in MCF-7 cells as well as increased DNA repair (Kwok, Peck et al. 2010). Taken together, this study shows FOXM1 involvement in ATM DNA damage response.

4.4.5 Conclusion

In summary, our data suggest that the genotoxic chemotherapy agent, epirubicin, triggers the accumulation and activation of p53 and ATM, and it is the antagonistic signals of activated ATM and p53 that converge on E2F to control FOXM1 expression, DNA damage repair and cell survival. Specifically, p53 represses while ATM enhances E2F activity, FOXM1 expression, cell survival in response to epirubicin. In consequence, the development of epirubicin resistance can be due to the loss of p53 function and an increase in ATM expression and activity. The finding that ATM as well as p53 modulates FOXM1 expression may have important implications for the diagnosis and treatment of drug resistant cancers, particularly those lacking functional p53. For example, ATM and FOXM1 inhibitors can be important cancer therapeutics as they can cause cell death independent of p53 status. ATM and FOXM1 inhibitors can also be used in combination with conventional genotoxic therapeutic agents to enhance drug efficacy and overcome resistance. Furthermore, p53, ATM and FOXM1 could be useful biomarkers for the prediction of epirubicin sensitivity in cancer patients.

Chemotherapy

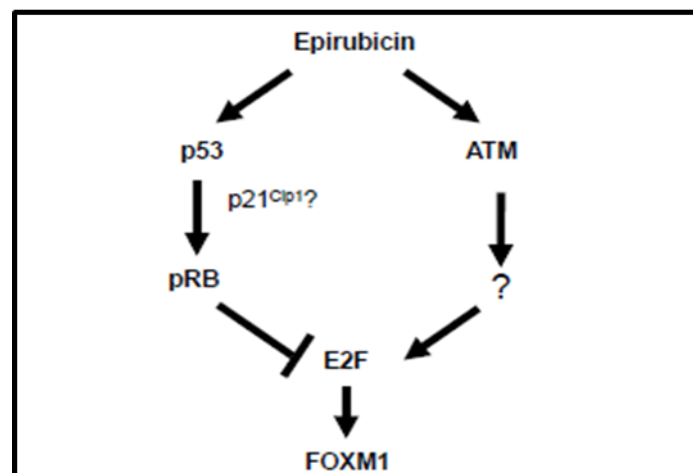


Figure 4.16 Antagonistic pathways in epirubicin treatment. Epirubicin triggers the accumulation and activation of p53 and ATM. It is antagonistic signals of activated ATM and p53 that converge on E2F directly or indirectly to control FOXM1 expression and might regulate DNA damage repair and cell survival.

4.5 Future work

Based on our results showing that epirubicin resistant MCF-7EPI^R cells have lost p53 activity and that DNA damage agents act through p53, the identification of new therapeutic agents is needed in combination with DNA agents to resensitise cells to cytotoxic drugs. DNA damage induces a cascade of protein kinases that repair DNA breaks through the regulation of DNA repair proteins. Emerging evidences show FOXM1 involvement in DNA repair response (Tan, Raychaudhuri et al. 2007, Kwok, Peck et al. 2010). It was first reported that CHK2 phosphorylates FOXM1 leading to its phosphorylation and regulation of DNA repair genes, XRCC1 and BRCA2 (Tan, Raychaudhuri et al. 2007). In our study, we showed a different mechanism of FOXM1 regulation in which ATM regulates FOXM1 at transcriptional level. Some evidence suggests that ATM also regulates FOXM1 at post-translational level, but our attempts to perform *in vitro* kinase assay failed. It would be beneficial to verify whether ATM can regulate FOXM1 phosphorylation and investigate the phosphorylation site involved. An alternative would be that FOXM1 is phosphorylated by another DNA damage kinase. Furthermore, investigation of the detailed role of FOXM1 in DNA repair would clarify what is FOXM1 primary role in the epirubicin resistant cells.

CHAPTER 5 FOXM1 regulates ATM phosphorylation and DNA damage response via transcriptional activation of NBS1

5.1 Introduction

Breast cancer is the most cancer in women and one of the most prevalent causes of death in women due to cancer relapse and metastasis to other organs (Jemal, Siegel et al. 2009). Cytotoxic chemotherapeutic drugs are largely used in the treatment of many cancers from different origins and to reduce the chance of metastasis (Martin, Villar et al. 2003, Smith and Chua 2006). Chemotherapy agents encompass alkylating agents, anti-metabolite, topoisomerase inhibitors, anthracyclines and anti-mitotic agents (Hortobagyi 1995, Rodler, Korde et al. 2010, Rodríguez-Lescure 2010). Each class of drug act differently, but all lead to DNA damages. Among cytotoxic chemotherapies, anthracyclines are anti-cancer antibiotics widely used and effective for the treatment of breast, lung, ovarian and leukaemia cancers (Lown 1993). Anthracyclines mechanism of action is thought to interfere with enzymes involved in DNA replication, but is also likely to be involved in DNA intercalation and DNA damage (Euhus 2011). ATM is a key factor activated following DNA damage that induces phosphorylation of its downstream target histone H2AX, leading to the recruitment of DNA repair proteins to the sites of damage (Fernandez-Capetillo, Chen et al. 2002, Celeste, Fernandez-Capetillo et al. 2003). Although ATM signalling pathway and downstream targets are known, further elucidations are necessary to understand all mechanisms activating ATM and DNA repair. It has been reported that changes in chromatin structure induced by the MRE11/RAD50/NBS1 (MRN) complex was essential for ATM auto-phosphorylation and activation (Lee and Paull 2004, Lee and Paull 2005).

The mammalian FOXM1 transcription factor belongs to the forkhead box superfamily and plays a critical role in cell proliferation, as it is required for G1/S and G2/M cell cycle transitions (Laoukili, Kooistra et al. 2005, Wierstra and Alves 2007). Besides its role in cell growth, FOXM1 also regulates organogenesis, angiogenesis, metastasis and DNA damage repair (Dai, Kang et al. 2007, Tan, Raychaudhuri et al. 2007, Raychaudhuri and Park 2011). Consistent with its roles, FOXM1 is found elevated in a broad spectrum of carcinomas (Kalinichenko, Major et al. 2004, Pilarsky, Wenzig et al. 2004, Chandran, Ma et al. 2007, Zeng, Wang et al. 2009). In addition to its involvement in tumorigenesis, FOXM1 dysregulation was implicated in tamoxifen resistant breast cancer cells through abrogation of tamoxifen anti-

proliferative effect (Millour, Constantinidou et al. 2010). Furthermore, FOXM1 dysregulation has been shown to play a role in the development of cisplatin and epirubicin resistance in breast cancer (Kwok, Peck et al. 2010, Millour, Constantinidou et al. 2010, Millour, de Olano et al. 2011). The role of FOXM1 in resistance to DNA damage agents is thought to be due to enhanced DNA repair. However, FOXM1 involvement in DNA damage signalling pathway has not been explored. In this report, the role of FOXM1 in ATM DNA damage response in epirubicin resistance was studied.

5.2 FOXM1 is involved in single and double stranded DNA repair in epirubicin resistant breast cancer cells

The previous study chapter 4 and published work from our laboratory showed FOXM1 involvement in resistance to DNA damage agents including epirubicin and cisplatin. The study chapter 4 showed that epirubicin enhances P-H2AX DNA damage foci in epirubicin sensitive breast cancer cells, while P-H2AX foci are sustained and low in epirubicin resistant breast cancer cells. This study suggests a higher DNA repair system in the epirubicin resistant cell line (Millour, de Olano et al. 2011). To investigate single stranded DNA repair mechanism of epirubicin resistant MCF-7EPI^R cells, the host-cell reactivation assay (HCR) was used. The plasmid harbouring firefly luciferase was damaged by a nicking endonuclease on a single strand of the DNA and repaired by the cellular DNA repair machinery. Only fully repaired plasmid transcribed correctly generate active firefly luciferase (Fig. 5.1) (Matijasevic, Precopio et al. 2001). According to differences in transfection efficiencies, the luciferase data were not normalised to the undamaged control plasmid, but normalised to Renilla and compared to the time 0 h (from the same transfection mix). Additionally, all controls with undamaged plasmid were performed (data not shown). The firefly luciferase activity of the damaged plasmid was recovered by 1174-fold 72 h post-transfection in epirubicin resistant MCF-7EPI^R cells, while luciferase activity was only recovered by 5.2-fold 72 h after transfection in epirubicin sensitive MCF-7 cells (Fig. 5.1A). This result indicates that MCF-7EPI^R cells have an enhanced mechanism of repair for single strand damage than MCF-7

cells. FOXM1 was examined for its role in DNA repair in MCF-7EPI^R cells using the HCR assay. The firefly luciferase activity of the damaged plasmid transfected combined with the non-specific siRNA (NS siRNA) in MCF-7EPI^R cells was recovered by 24.5-fold after 48 h transfection, which matches the result obtained Figure 5.1A (Fig. 5.1B). In contrast, FOXM1 silencing (FOXM1 siRNA) completely abrogated the luciferase recovery, suggesting that FOXM1 plays an important role in DNA repair in MCF-7EPI^R cells (Fig. 5.1B). Notably, these results remain preliminary and manual. It has also been reported that dysregulated FOXM1 is involved in cisplatin and epirubicin resistance and it is thought to be due to enhanced DNA repair mechanisms. Our lab has recently investigated FOXM1's role in double stranded DNA repair using HeLa cell lines harbouring an integrated direct repeat green fluorescent protein reporter for HR or NHEJ. These experiments showed that FOXM1 depletion reduced the HR DSB repair, but had no significant effects on NHEJ repair (Monteiro, Khongkow et al. 2012).

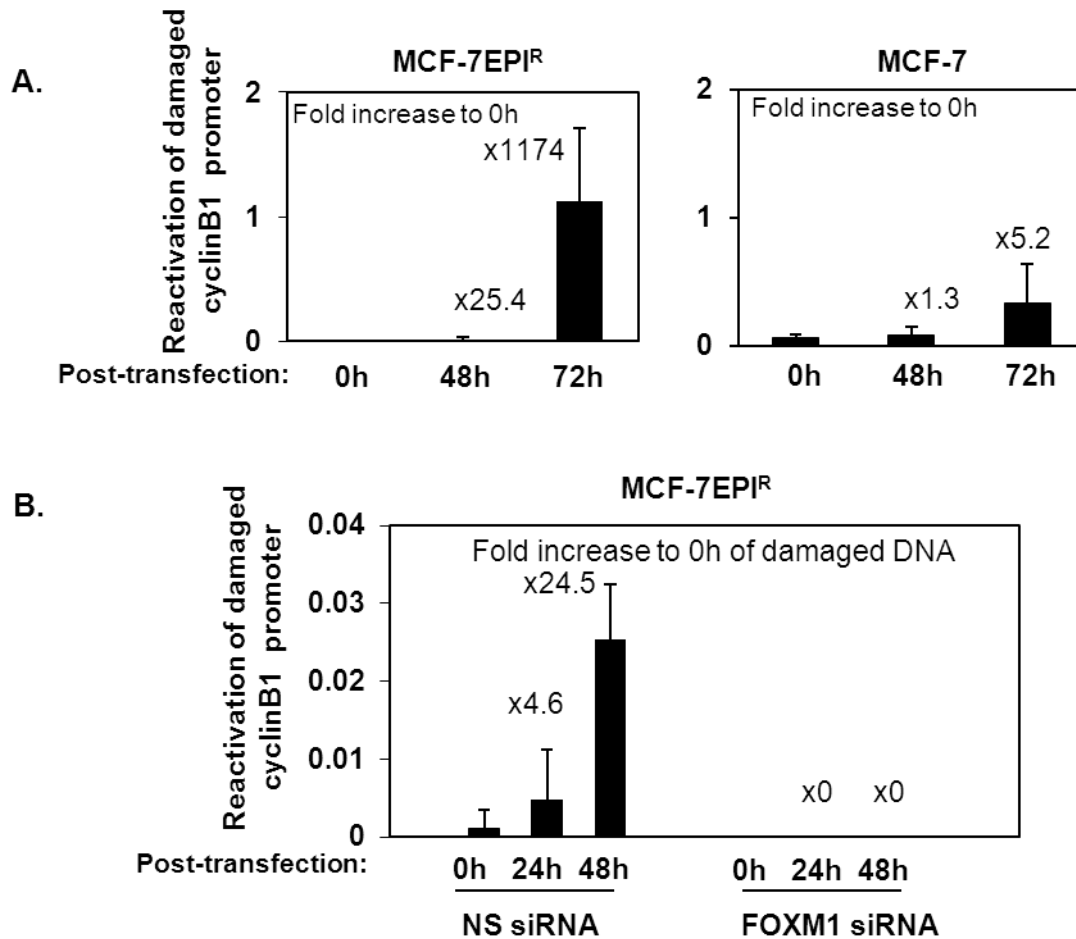


Figure 5.1 FOXM1 depletion alters single stranded DNA repair in MCF-7EPI^R cells. A. MCF-7EPI^R and MCF-7 cells were transiently co-transfected with damaged firefly luciferase and undamaged renilla luciferase plasmids. After 24 h transfection, luciferase activities were assayed at 0, 48 and 72 h and the ratios firefly/renilla were calculated. Folds increase relative to 0 h are shown. **B.** MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA or FOXM1-targeting siRNA (100 nmol/L). Twenty-four hours after transfection, cells were co-transfected with damaged firefly luciferase and undamaged renilla luciferase plasmids, and luciferase activities were assayed at 0, 24 and 48 h and the ratios firefly/renilla were calculated. Folds increase relative to 0 h are shown. Columns, means derived from three independent experiments; bars, SD.

5.3 Enhanced recruitment of FOXM1 and P-H2AX in MCF-7EPI^R cells following DNA breaks

As MCF-7EPI^R cells have high DNA repair in response to epirubicin (Fig. 4.9 and 4.10), the recruitment of DNA repair proteins at double strand breaks (DSB) sites was investigated. For this purpose, the eukaryotic homing endonuclease I-Ppol, which has a 15 base pair recognition sequence to cleave endogenous DNA sites in the human genome, was used to cleave specifically one site on the chromosome 1 on an intron of the *DAB1* gene. Expression of the I-Ppol leads to a cleavage of the I-Ppol target sites (*DAB1* gene), generating DSBs (equivalent to 0.8 Gy irradiation) and activating ATM-dependent signalling pathway (Berkovich, Monnat et al. 2007). Although it is inaccurate to compare DNA damage epirubicin-induced with DNA damage I-Ppol-induced because of the difference in DNA damage levels, the generation of DSBs by I-Ppol transfection was confirmed in MCF-7 and MCF-7EPI^R cells using the expression level of P-H2AX (Fig. 5.2). Western blotting of I-Ppol would have also been a good control in this case. The P-H2AX and FOXM1 were immunoprecipitated and the DNA sequence bound to these proteins was amplified using *DAB1* primers and normalised with β -actin housekeeping gene. DNA breaks induced by I-Ppol transfection induced a strong enhancement in the recruitment of P-H2AX and FOXM1 proteins on the sites of DNA breaks in MCF-7EPI^R cells relative to MCF-7 cells and to the respective IgG negative control (Fig. 5.2). These results suggest that the recruitment of DNA repair proteins to the DSB sites is enhanced in MCF-7EPI^R cells compared to MCF-7 cells epirubicin sensitive. In addition, these data might indicate that FOXM1 may be necessary either for H2AX phosphorylation or for the recruitment of P-H2AX at the DSBs. Impaired H2AX phosphorylation or recruitment would affect DNA repair proteins recruitment.

However, these results remain preliminary and imprecise. Indeed, Michael Kastan *et al.* improved this system by adding a mutant oestrogen receptor hormone-binding domain to I-Ppol to create a fusion protein that localized to the nucleus in response to 4-hydroxytamoxifen (4-OHT). Addition of 4-OHT to cells infected with an oestrogen receptor-I-Ppol retrovirus results in a time-dependent cleavage of I-Ppol site (Berkovich, Monnat et al. 2007). In Figure 5.2, the kinetic of transfection is likely

to be very different as MCF-7 cells are much easier and quicker to transfect than MCF-7EPI^R cells. Therefore, the level of H2AX recruitment in MCF-7 cells might be low because the transfection is rapid in these cells and 24 hrs after transfection the damages have already been repaired. Western blotting of I-Ppol in a time course could also provide us with extra information about the kinetic of transfection. However, the difference between the level of P-H2AX in the western blot and ChIP experiments for MCF-7 cells is striking. H2AX recruitment on DSB might not be on the amplified site. H2AX protein recruitment might also be low because these MCF-7 cells have a low level of DNA damage repair pathway activation. Indeed, in Figure 4.12 MCF-7 cells showed a very low level or no phospho-ATM after epirubicin treatment. The experiment has been repeated several times using western blot with high sensitivity detection methods, but the expression of phospho-ATM remained undetectable.

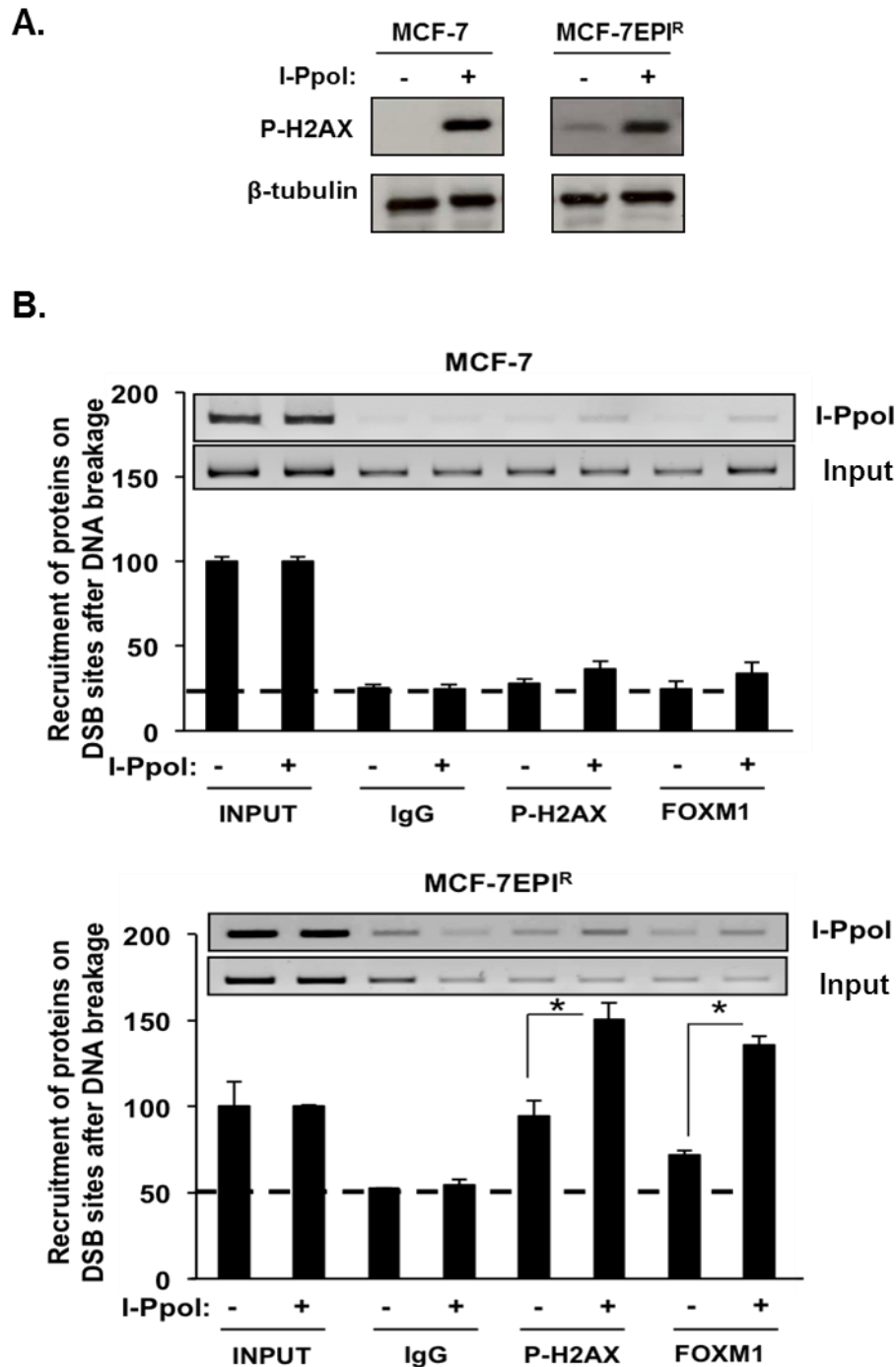


Figure 5.2 Increased in the recruitment of FOXM1 and repair factors at DNA breaks in MCF-7EPI^R cells. **A.** MCF-7 and MCF-7EPI^R cells were transfected with with 3 μ g of empty vector or vector encoding for I-Ppol and harvested for western blot analysis 48 h post-transfection. The protein expression levels were determined for P-H2AX and β -tubulin. **B.** Transient expression of the I-Ppol for 24 h leads to a cleavage of the I-Ppol target sites and generation of DSBs. After cross link reversal of epirubicin-treated MCF-7 and MCF-7EPI^R cells, the H2AXpSer139 and FOXM1 were immunoprecipitated and the DNA sequences bound to these proteins were amplified using DAB1 primers. Input DNA was used to normalise the amplified DNA. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

5.4 FOXM1 is required for the activation of ATM, H2AX and CHK2 DNA repair proteins

Since the HCR assay showed that FOXM1 is essential to repair DNA in MCF-7EPI^R cells and might be necessary for H2AX phosphorylation, the effect of FOXM1 silencing was examined on the phosphorylation of DNA repair proteins. For this purpose, MCF-7EPI^R cells were transiently transfected with non-specific siRNA and FOXM1-targeting siRNA and examined for their ability to phosphorylate ATM and its targets when treated with 1 μ mol/L of epirubicin. Western blot analysis confirmed that FOXM1 was effectively silenced at least over 48 h after treatment (Fig. 5.3A). The use of phosphospecific antibodies showed an increase in the phosphorylation of ATM, H2AX and CHK2 following epirubicin exposure in the non-specific siRNA condition (Fig. 5.3A). In contrast, the phosphorylation of ATM was completely abrogated, and phosphorylations of H2AX and CHK2 were reduced in treated MCF-7EPI^R cells when FOXM1 was silenced (Fig. 5.3A). To further confirm the reduction in ATM phosphorylation, the percentage of P-ATM positive cells were assessed by staining using flow cytometry in MCF-7EPI^R cells treated with non-specific and FOXM1-targeting siRNA. After 48 h treatment with 1 μ mol/L of epirubicin, the percentage of P-ATM positive cells increased compared to untreated condition in the non-specific siRNA condition, while it decreased in cells treated with siRNA targeting FOXM1, independent of epirubicin treatment (Fig. 5.3B). The validation of FOXM1 silencing was also confirmed by staining using flow cytometry, and for each antibody an IgG secondary antibody negative control was performed (Fig. 5.3B). Notably, FOXM1 enhancement at protein level Figure 5.3A was not observed using flow cytometry indicating that total FOXM1 protein expression remained the same and that FOXM1 induction observed by western blotting could be due to an increase in its phosphorylation levels. The reduction in H2AX phosphorylation observed Figure 5.3A was confirmed by immunofluorescence staining in MCF-7EPI^R cells treated with non-specific and FOXM1-targeting siRNA in absence and presence of epirubicin at 1 μ mol/L for 24 h (Fig. 5.4). Although our previous study chapter 4 showed low levels of P-H2AX foci in epirubicin resistant cells compared to MCF-7 cells, the foci were present and sustained over the treatment (Millour, de Olano et al. 2011). In this study, the quantification of P-H2AX foci in MCF-7EPI^R cells showed a decrease of

foci in cells treated with siRNA targeting FOXM1 compared to non-specific siRNA condition at 24 h epirubicin treatment (Fig. 5.4B). This result is in opposition to results from a recent study using the same cell lines (Monteiro, Khongkow et al. 2012). However, these results were confirmed in a human fibroblast cell line (Fig. 5.5). FOXM1 silencing in human fibroblast cells also abrogated ATM, H2AX and Chk2 phosphorylations, and by consequent their activation (Fig. 5.5 and 5.6). Figure 5.5 the use of the siRNA targeting ATM should be used to confirm the specificity of the phosphor-ATM antibody and total Chk2 and H2AX antibodies should be used Figure 5.6 as control. Taken together, these data show that FOXM1 silencing reduced phosphorylation events following DNA damage response in MCF-7EPI^R cells and suggest that it may reduce recruitment of these proteins at the DSBs sites.

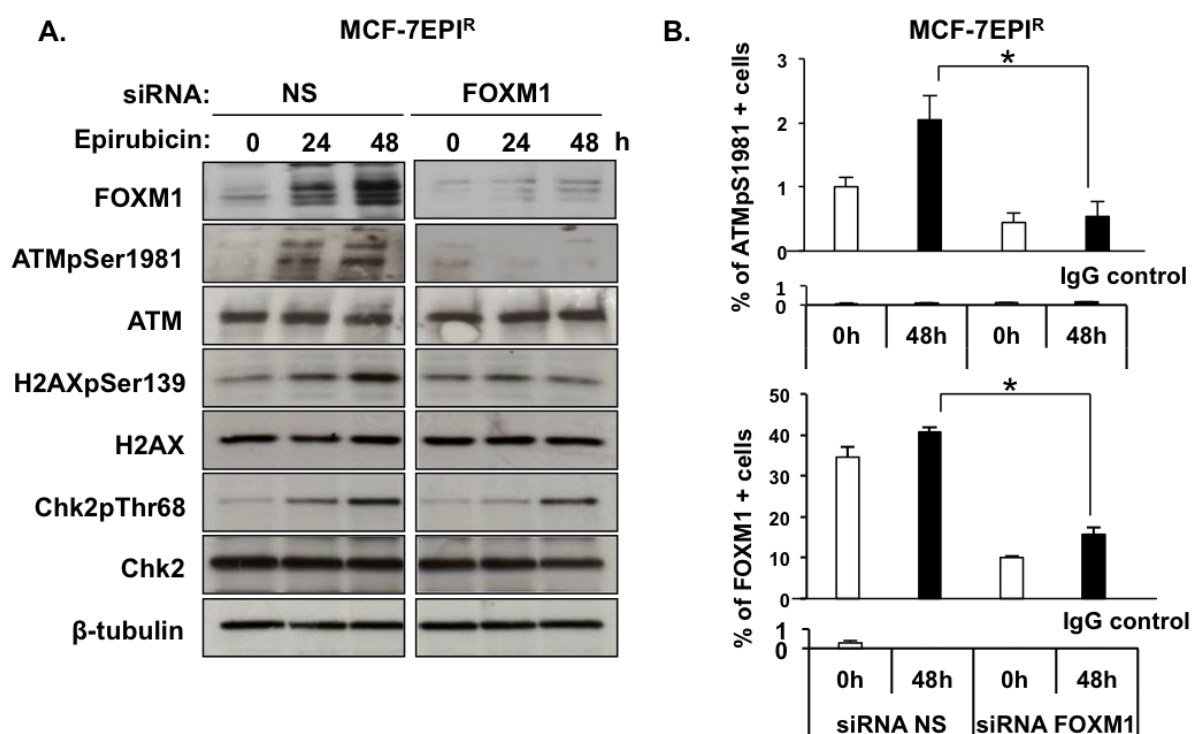
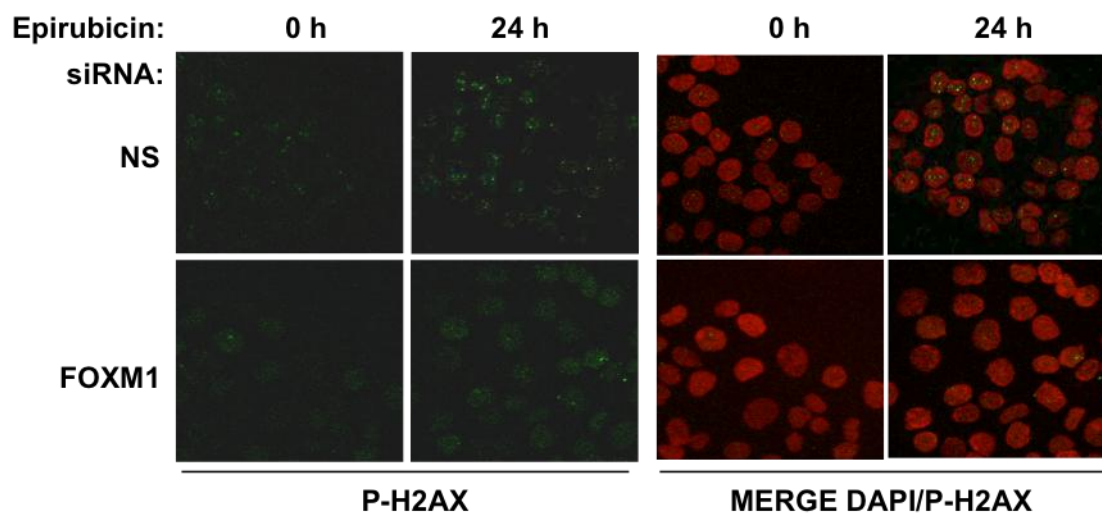


Figure 5.3 FOXM1 silencing reduces ATM phosphorylation on serine 1981 in MCF-7EPI^R cells. **A.** MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA or FOXM1-targeting siRNA (100 nmol/L). Twenty-four hours after transfection, MCF-7EPI^R cells were treated with 1 μ mol/L of epirubicin and harvested for western blot analysis at 0, 24 and 48 h. The protein expression levels were determined for FOXM1, ATMpSer1981, ATM, H2AXpSer139, H2AX, Chk2pThr68, Chk2 and β -tubulin. **B.** MCF-7EPI^R cells transfected with siRNA NS and siRNA FOXM1 untreated and treated with 1 μ mol/L of epirubicin 48 h were assessed by staining with an antibody against ATMpSer1981, or FOXM1, or an isotype IgG (negative control), followed by an Alexa 488-conjugated secondary antibody. The percentage of cells ATMpSer1981 or FOXM1 positive were determined by flow cytometry. Columns, means derived from three independent experiments; bars, SD. Statistical analyses

were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

A.



B.

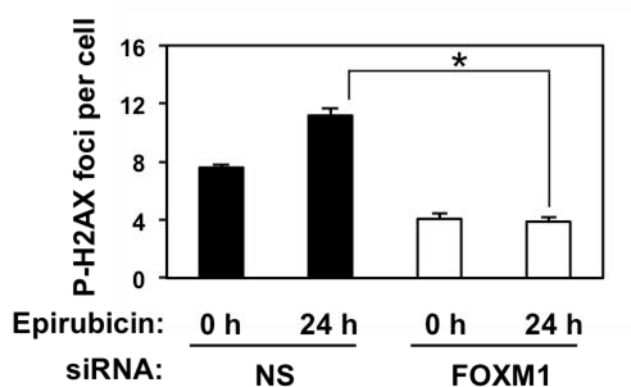


Figure 5.4 FOXM1 silencing decreases H2AX phosphorylation on serine 139 in MCF-7EPI^R cells. **A.** MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA or FOXM1-targeting siRNA (100 nmol/L). Twenty-four hours after transfection, MCF-7EPI^R cells were treated with 1 μ mol/L of epirubicin for 24 h and stained with H2AXpSer139 antibody (green) and DAPI (red). Images visualized by confocal microscopy. Images: magnification: x 20; insets x 80. **B.** The results were quantified using Image J and were the average of three independent experiments. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s, non significant.

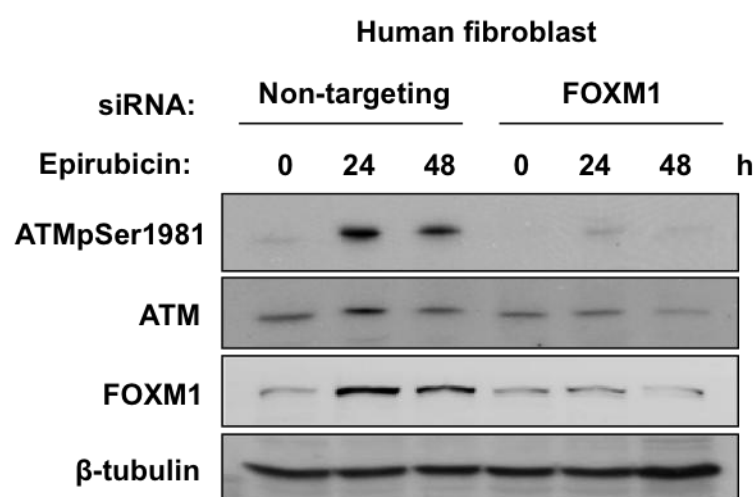


Figure 5.5 FOXM1 silencing in human fibroblast cells abrogates ATM phosphorylation.

Human fibroblast (48BRhtert) cells were either transfected with non-specific (NS) siRNA or FOXM1-targeting siRNA (100 nmol/L). Twenty-four hours after transfection, cells were treated with 1 μ mol/L of epirubicin and harvested for western blot analysis at 0, 24 and 48 h. The protein expression levels were determined for FOXM1, ATMpSer1981, ATM, FOXM1 and β -tubulin.

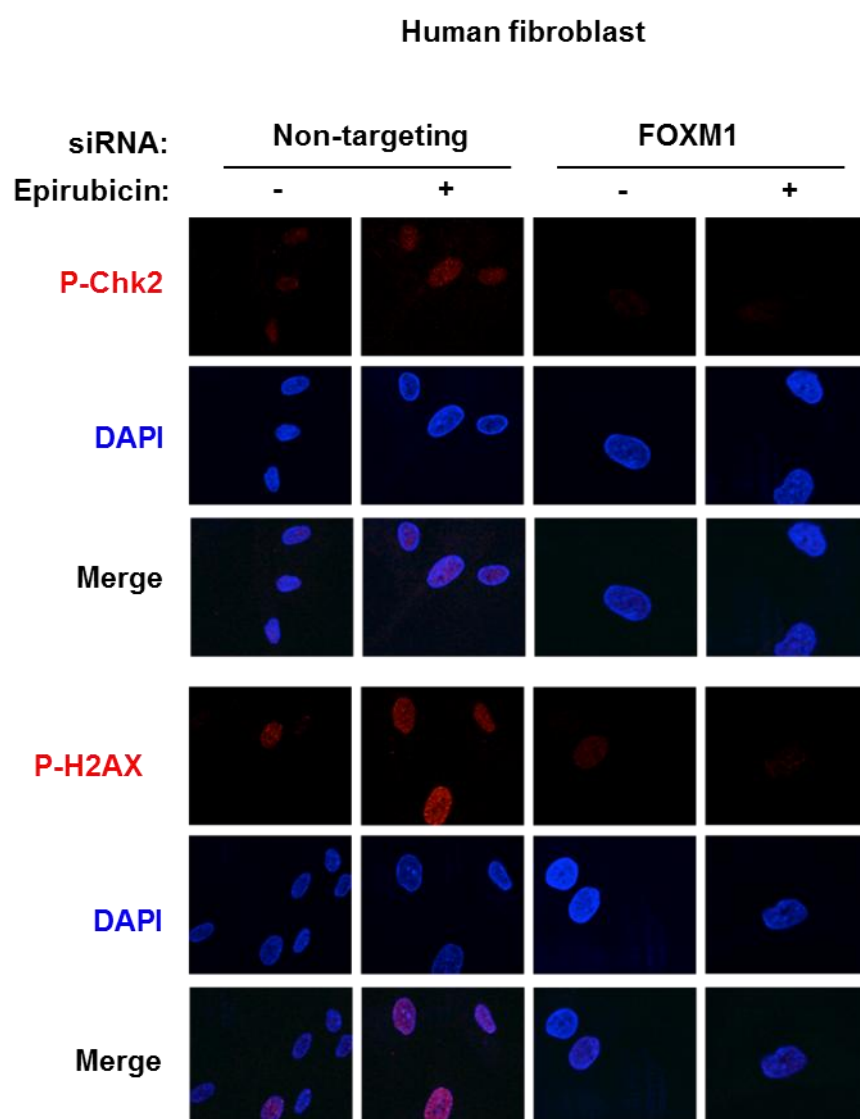


Figure 5.6 FOXM1 silencing in human fibroblast cells decreases Chk2 and H2AX phosphorylation. Human fibroblast (48BRhtert) cells were cultured, treated with 1 $\mu\text{mol/L}$ of epirubicin for 48 h and stained with Chk2pThr68 and H2AXpSer139 antibodies (red) and DAPI (blue). Images visualized by confocal microscopy. Images: magnification: x 20; insets x 80.

5.5 FOXM1 is required for ATM auto-phosphorylation upon epirubicin

It has been reported that ATM is regulated by the members of E2F family either at post-translational level or at promoter level (Berkovich and Ginsberg 2003, Hong, Paulson et al. 2008). To elucidate the mechanism by which FOXM1 regulates ATM, *ATM* mRNA level was examined in MCF-7EPI^R cells when FOXM1 was silenced in combination with 1 μ mol/L of epirubicin. RT-qPCR analysis showed that FOXM1 silencing did not affect *ATM* mRNA levels compared to the non-specific siRNA condition in MCF-7EPI^R cells (Fig. 5.7A). Furthermore, western blot analysis of wild-type (*wt*) and FOXM1 knock-out (*Foxm1*^{-/-}) MEFs cells treated with 1 μ mol/L epirubicin showed a reduction in P-ATM level, while ATM mRNA levels remained steady in *Foxm1*^{-/-} cells (Fig. 5.7B and C). Taken these results together, the reduction of FOXM1 levels by transient knock-down or genomic knock-out decreased the level of ATM phosphorylation, but did not affect its mRNA levels. The reverse was showed with FOXM1 overexpression in *wt* MEFs that increased ATM phosphorylation as well as Δ N-FOXM1 overexpression (Fig. 5.7D). Collectively, these data indicate that FOXM1 plays a role in regulating ATM auto-phosphorylation on serine 1981.

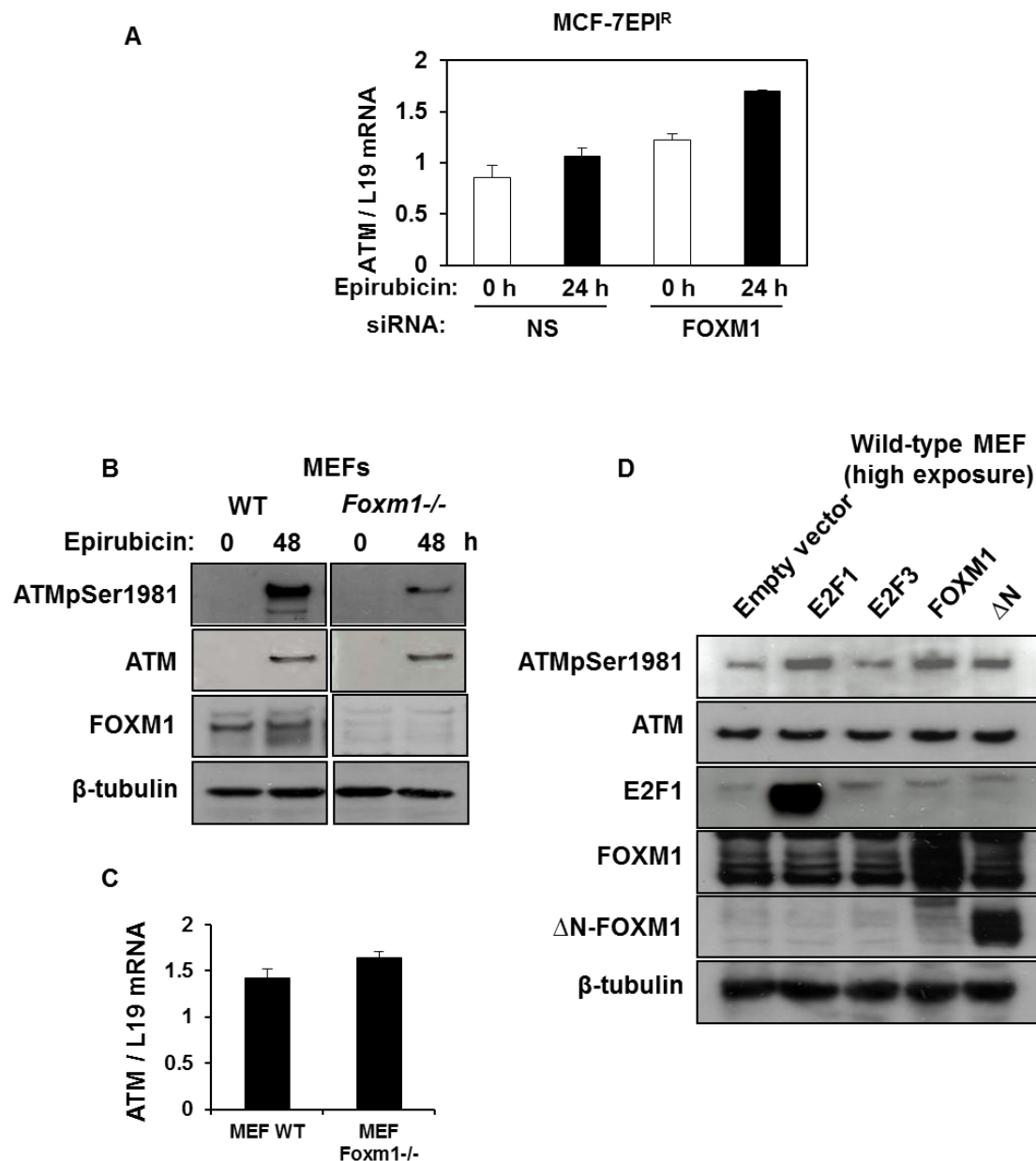


Figure 5.7 FOXM1 does not regulate ATM transcriptionally. A. MCF-7EPI^R cells were either transfected with non-specific (NS) siRNA or FOXM1-targeting siRNA (100 nmol/L). Twenty-four hours after transfection, MCF-7EPI^R cells were treated with 1 μ mol/L of epirubicin and harvested for RT-qPCR analysis at 0 and 24 h. ATM mRNA levels were determined and normalised to L19 gene. MEFs wild-type (WT) and knock-out for FOXM1 (*Foxm1*^{-/-}) were treated with 1 μ mol/L of epirubicin and harvested at indicated times for western blot (B.) and RT-qPCR analyses (C.). The protein expression of ATMpSer1981, ATM, FOXM1 and β -tubulin and the ATM mRNA were determined. Columns, means derived from three independent experiments; bars, SD. D. Wild-type MEFs were transiently transfected with empty vector and vectors encoding for FOXM1 and Δ N-FOXM1 and harvested after 24 h for western blot analysis. The protein expression of ATMpSer1981, ATM, FOXM1 and β -tubulin were examined.

5.6 Transcriptional regulation of NBS1 by FOXM1

It has been reported that ATM is recruited and fully activated at the DSB sites by the MRN complex (Lee and Paull 2004, Lee and Paull 2005). The modulation of ATM phosphorylation via the regulation of the MRN complex through FOXM1 was investigated in MCF-7EPI^R cells. To investigate whether FOXM1 regulates members of the MRN complex, the effect of FOXM1 silencing was examined on *MRE11*, *RAD50* and *NBS1* mRNA levels in MCF-7EPI^R cells. RT-qPCR data revealed that FOXM1 knock-down reduced significantly *NBS1* mRNA, but has no effect on *MRE11* and *RAD50* mRNA levels in MCF-7EPI^R cells (Fig. 5.8A). This finding was confirmed in the human fibroblast cell line (Fig. 5.8B). Indeed, FOXM1 silencing significantly reduced *NBS1* mRNA levels, but not *MRE11* or *RAD50* mRNA levels. This study was extended to breast cancer cell lines including MDA-MB-231, ZR-75-1 and MCF-7 cells. Each of these cell lines showed a significant reduction of *NBS1* mRNA after FOXM1 silencing, indicating *NBS1* as a potential transcriptional target of FOXM1 (Fig. 5.8C). Transient reporter assay was performed to study whether FOXM1 regulates *NBS1* at promoter level. Full length FOXM1 did not affect significantly *NBS1* promoter activity (data not shown), but ectopic expression of the active FOXM1 form, Δ N-FOXM1, induced a significant increase in the luciferase activity of *NBS1* promoter (WT FHK-luc) in MCF-7 cells in a dose-dependent manner (Fig. 5.9B). The study of *NBS1* promoter revealed a forkhead binding site located at -78 pb from the transcription start explaining the responsiveness of *NBS1* promoter to FOXM1 active form (Fig. 5.9A). Directed mutagenesis of the forkhead site (mFHK-luc) abrogated the transcriptional induction of *NBS1* promoter by Δ N-FOXM1 expression vector compared to the wild-type promoter WT FHK-luc (Fig. 5.9B). Chromatin immunoprecipitation assays showed that FOXM1 binds *NBS1* promoter after ectopic expression of FOXM1 in MCF-7 cells and after epirubicin treatment in MCF-7EPI^R cells (Fig. 5.9C). These results suggest that FOXM1 could affect ATM auto-phosphorylation via the transcriptional regulation of *NBS1*.

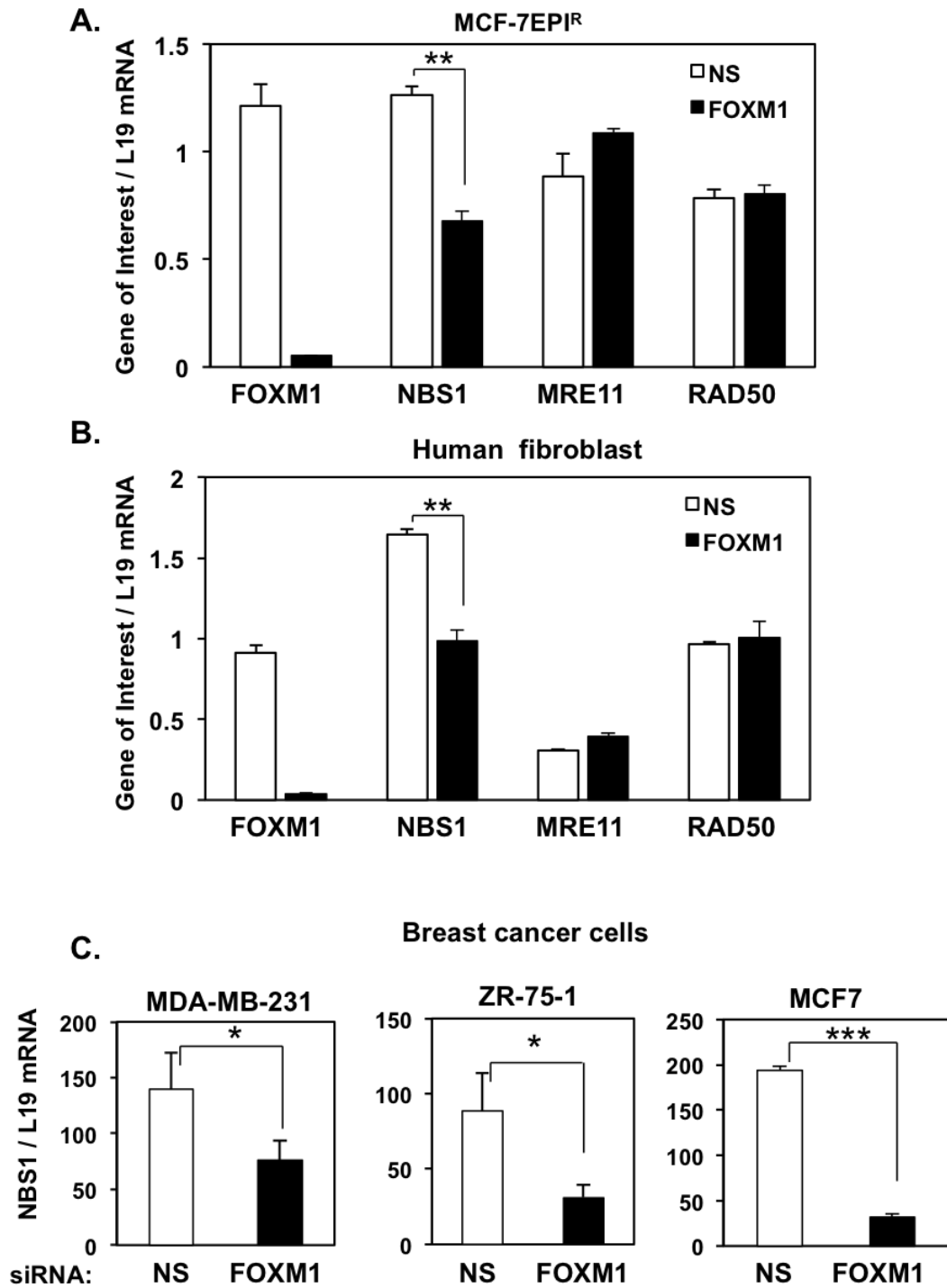


Figure 5.8 FOXM1 silencing significantly decreases NBS1 mRNA levels. A. MCF-7EPI^R cells, B. Human fibroblast cells, and C. MDA-MB-231, ZR-75-1 and MCF-7 cells were transiently transfected with non-targeting siRNA and siRNA against FOXM1, and harvested for RT-qPCR analysis. The mRNA levels of FOXM1, NBS1, MRE11 and RAD50 were examined. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. *, $P \leq 0.1$ **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant.

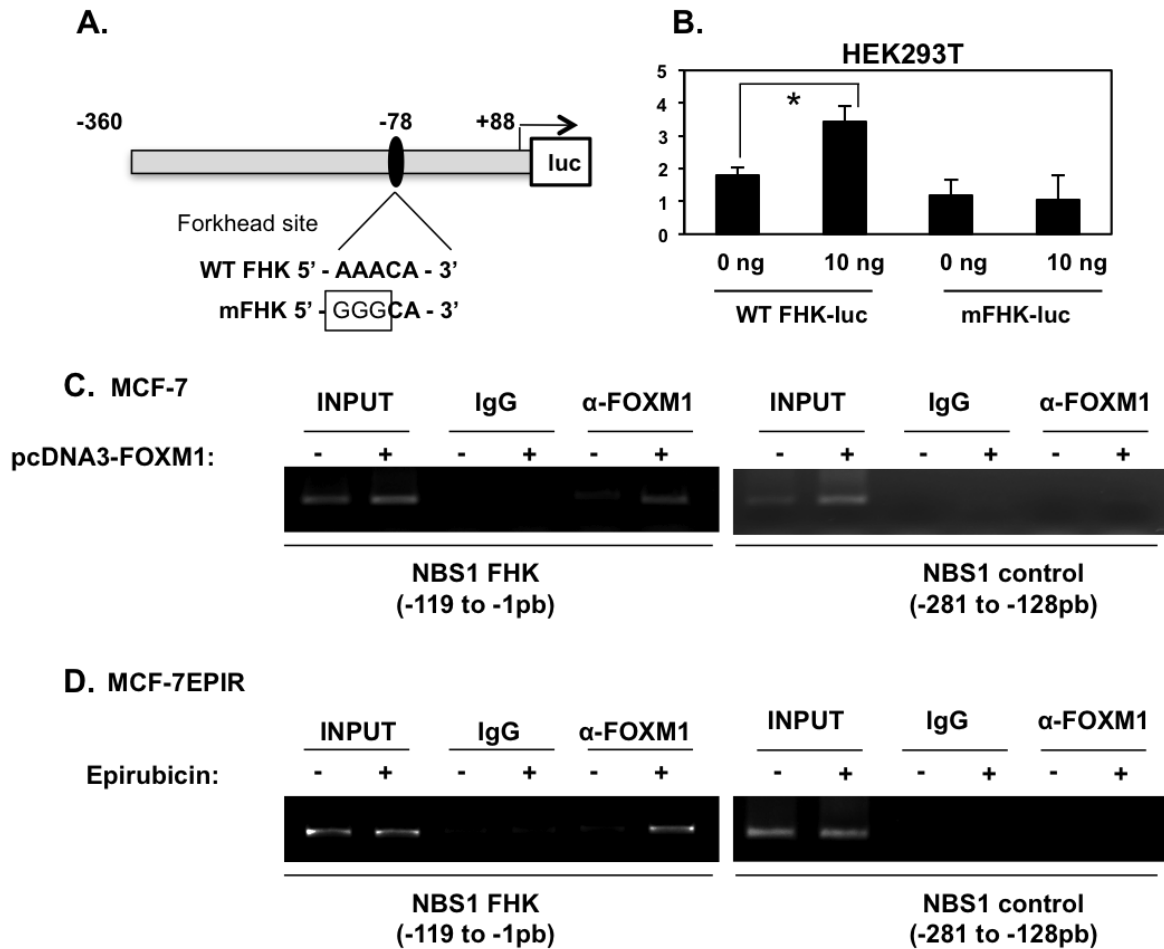


Figure 5.9 FOXM1 binds directly to NBS1 promoter through the Forkhead binding site (FHK). **A.** Schematic representation of *NBS1* luciferase promoter construction pXL2-Nbs1, wild-type Forkhead (WT FHK) and mutant Forkhead (mFHK) binding sites performed using site directed mutagenesis. **B.** HEK293T cells were cultured in 10% FCS DMEM medium and transiently transfected with pXL2-Nbs1 and increasing amount of deltaN-FOXM1 constructions (0 and 10 ng) and assessed for luciferase assay. All relative luciferase activity values are corrected for co-transfected Renilla activity. Columns, means derived from three independent experiments; bars, SD. Statistical analyses were done using Student's *t* test. **, $P \leq 0.01$ and ***, $P \leq 0.001$, significant and n.s., non significant. **C.** Chromatin immunoprecipitation (ChIP) analysis of *NBS1* promoter. MCF-7 cells were transfected with pcDNA3 (-) or pcDNA3/FOXM1 (+) and MCF-7EPI^R cells were treated with epirubicin. These cells were used for ChIP assay using anti-IgG and anti-FOXM1 antibodies as indicated. After cross-linking reversal, the co-immunoprecipitated DNA was amplified by PCR using primers for NBS1 FHK containing region (-119 to -1 pb) and a control region (-281 to -128 pb) and runned in 1% agarose gel.

5.7 NBS1 mediates ATM activation upon epirubicin

Since this study indicates that FOXM1 regulates *NBS1* transcriptionally and affects the phosphorylation of ATM, I hypothesised that FOXM1 regulates transcriptionally *NBS1*, which in turn regulates the auto-phosphorylation of ATM at the DNA break sites. To demonstrate that NBS1 is required for ATM activation, we used the *wt* and *Nbs1*^{-/-} MEFs cells transfected with empty control vector and vector encoding for NBS1. The full activation of ATM is only observed in MEFs cells treated with epirubicin and transfected with NBS1, but not in MEFs cells lacking NBS1 (Fig. 5.10). Taken together, these data indicate that ATM activation requires NBS1 and epirubicin treatment. This finding suggests that FOXM1 may acts upstream of ATM and NBS1 and could control ATM auto-phosphorylation through NBS1.

To determine whether FOXM1 can activate ATM auto-phosphorylation, western blot analysis could be performed on *Foxm1*^{-/-} cells transfected with empty vector or a plasmid encoding for NBS1 to circumvent the lack of FOXM1 and activate ATM.

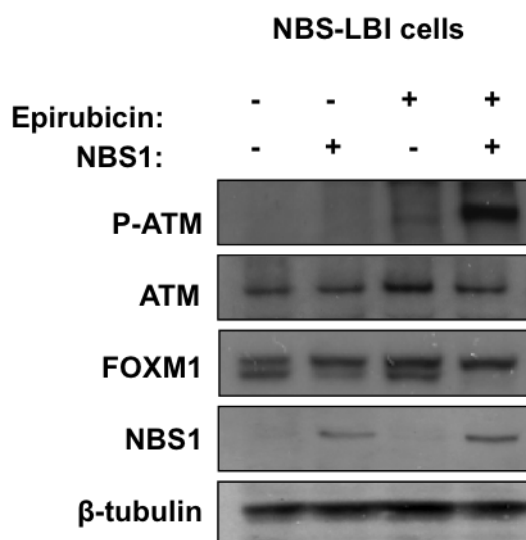


Figure 5.10 NBS1 is required for ATM activation upon epirubicin. NBS-LBI cells were either transfected with 3 µg of empty vector or vector encoding for NBS1 and treated with 1 µmol/L of epirubicin 24 h and harvested for western blot analysis at 0, 24 and 48 h. The protein expression levels were determined for ATMpSer1981, ATM, FOXM1, Chk2, NBS1 and β-tubulin.

5.8 Discussion

Chemotherapeutic drugs generate double strand breaks by free radical attack of deoxyribose, inhibition of re-ligation of DNA strand broken by topoisomerase II or DNA replication. DNA DSBs are the most deleterious form of DNA damage because they do not leave an intact complementary strand to be used as a template for DNA repair and they induce DNA damage signalling pathway leading to cell cycle arrest, apoptosis or repair. Deregulation in DNA damage signalling pathway can favour DNA repair and inhibit apoptosis to contribute to chemotherapeutic drug resistance. Cells rely on two major DNA DSBs repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR requires a homologous template, sister chromatid, and allows repair of DSBs in S and G2 phases of the cell cycle (San Filippo, Sung et al. 2008, Moynahan and Jasin 2010). In contrast, NHEJ can operate throughout the cell cycle without the need for DNA template.

5.8.1 FOXM1 is involved in ATM and its downstream substrates phosphorylations

In response to DSBs, DNA damage signalling pathway delays the cell cycle before and during DNA replication (G1/S and intra-S checkpoints) and before cell division (G2/M checkpoints) to prevent duplication and segregation of damaged DNA. DNA damage signalling cascades are complex events that require various proteins whose function can be categorised as DNA damage sensors, transducers, mediators and effectors. DNA damage signalling pathway involves two key serine/threonine kinases: ATM (ataxia telangiectasia mutated) and ATR (ATM and rad3-related). The MRE11/RAD50/NBS1 sensor complex detects DSBs and contributes to the recruitment and activation of the ATM transducer. Mediator proteins, such as MDC1 (mediator of DNA damage checkpoint), 53BP1 (p53-binding protein 1) and BRCA1, help activate effector kinases CHK1 and CHK2, which spread the signal throughout the nucleus (Lavin, Delia et al. 2006). In this study, FOXM1 silencing abrogates ATM phosphorylation in MCF-7EPI^R and fibroblast cells, which is required for phosphorylation events of the DNA damage cascade (Fig. 5.3A and 5.5). Similarly,

the level of P-ATM positive cells was reduced when cells were transfected with FOXM1-targeting siRNA (Fig. 5.3B). FOXM1 silencing also reduces phosphorylation of CHK2 and P-H2AX in MCF-7EPI^R cells (Fig. 5.3A) and human fibroblasts (Fig. 5.6), which inhibits activation of transcription factors, cell cycle and apoptosis regulators and repair proteins (Matsuoka, Rotman et al. 2000, Bartek and Lukas 2003, Iliakis, Wang et al. 2003). Although these results have been confirmed in different cell lines, it is in contradiction with results recently published. In this study I suggest that FOXM1 inhibition reduces P-H2AX and the activation of the DNA repair pathway, while the recent study shows that FOXM1 depletion increases P-H2AX and DNA damage. H2AX is a histone protein that is rapidly phosphorylated by ATM in response to DNA damage. Activated H2AX forms foci around the DSBs and helps to recruit the proteins responsible for DNA repair. In this study, I hoped to show that FOXM1 silencing reduced phosphorylations and recruitment of DNA repair proteins at the DSBs.

The mechanism of ATM regulation by FOXM1 was investigated by western blot and RT-qPCR. The results revealed that FOXM1 silencing inhibits ATM auto-phosphorylation, but does not affect total ATM protein and mRNA levels (Fig. 5.7). A recent study demonstrates that FOXO3A interacts with ATM to promote its auto-phosphorylation on serine 1981 (Tsai, Chung et al. 2008). However, ATM and FOXM1 direct interaction was not found (data not shown), suggesting that FOXM1 activates ATM auto-phosphorylation indirectly.

5.8.2 FOXM1 regulates NBS1

The role of the MRN complex in ATM activation has clearly been shown through analysis of ATM-dependent phosphorylation events in cells with MRN deficiencies (Uziel, Lerenthal et al. 2003). MRN complex stimulates ATM activation that induces p53, CHK2 and H2AX *in vitro* phosphorylations using recombinant proteins. The association between ATM and MRN is mediated through multiple protein-protein interactions, one between ATM and NBS1, and the other between ATM and MRE11/RAD50 (Lee and Paull 2004, You, Chahwan et al. 2005). As FOXM1 is a transcription factor, the transcriptional regulation of the MRN protein complex by

FOXM1 was investigated. RNA interference experiments demonstrated that FOXM1 knock-down significantly decreased *NBS1* mRNA in different human breast cancer cell lines and fibroblasts (Fig. 5.8). Furthermore, ectopic expression of the active form of FOXM1 induced *NBS1* promoter activity, while mutation of the forkhead binding site abrogated *NBS1* promoter activity induction following addition of FOXM1 active form (Fig. 5.9). Chromatin immunoprecipitation assays showed that FOXM1 directly binds *NBS1* promoter following FOXM1 overexpression and DNA damage treatment (Fig. 5.9C). Similarly, *NBS1* is a target of a transcription factor involved in cell growth control, C-MYC (Chiang, Teng et al. 2003). C-MYC function was known in promoting cell proliferation in normal and neoplastic cells, until its function was linked to the regulation of DNA DSB repair pathway.

5.8.3 NBS1 activates ATM auto-phosphorylation

Even though wild-type ATM is present, only reconstitution of MRN complex restores ATM auto-phosphorylation and phosphorylation of downstream substrates in MRN deficient cells (Uziel, Lerenthal et al. 2003, Lee and Paull 2004). In this study, rescue experiments in *Nbs1*^{-/-} fibroblast cells also showed that the combination of epirubicin treatment with *NBS1* fully induces ATM auto-phosphorylation (Fig. 5.10). Given that our promoter assay showed that FOXM1 regulates *NBS1*, the results could suggest that FOXM1 activates ATM through *NBS1* transcription (Fig. 5.11). Similarly, *NBS1* is required for E2F1 to induce p53 phosphorylation. In fibroblasts lacking *NBS1*, p53 and CHK2 phosphorylations were impaired, while E2F1 induced p53 and CHK2 phosphorylations in wild-type cells (Powers, Hong et al. 2004).

5.8.4 FOXM1 function in DNA repair

The role of FOXM1 in DNA repair was first showed in osteosarcoma cells with the direct regulation of XRCC1 and BRCA2, genes involved in DNA repair (Tan, Raychaudhuri et al. 2007). In this study, I showed that FOXM1 regulates *NSB1*, a sensor of DNA damage. A study found *NBS1* as a downstream target of C-MYC proliferation factor and showed the role of C-MYC in DNA repair for the first time

(Chiang, Teng et al. 2003). This study is also the first evidence of FOXM1 function in ATM activation and ATM-mediated DNA damage phosphorylation cascade. DNA damage signalling pathway activates DNA damage sensors, transducers, mediators and effectors required for cell cycle arrest, apoptosis and DNA repair. Cells were co-transfected with pre-damaged single strand DNA template and undamaged Renilla. The level of repair of damaged template was significantly enhanced in MCF7-EPI^R cells relative to MCF-7 cells, whereas DNA repair was prevented in MCF7-EPI^R cells treated with siRNA FOXM1 relative to non-specific siRNA (Fig. 5.1). These results indicate that FOXM1 is involved in HR. Furthermore, a recent study demonstrated the role of FOXM1 in HR, but not in NHEJ (Monteiro, Khongkow et al. 2012).

A growing body of evidence indicate the importance of chromatin organisation in the DNA damage response, with the most prominent modifications being the phosphorylation of histone H2AX at the site of DNA breaks. As H2AX is associated with chromatin at DNA break sites and plays a key role in recruiting DNA repair proteins to nuclear foci, the recruitment of FOXM1 to DNA break sites was assessed by chromatin immunoprecipitation upon local induction of DNA damage by specific endonucleases. The generation of sequence-specific DSBs by I-Ppol endonuclease (Monnat, Hackmann et al. 1999, Berkovich, Monnat et al. 2008) was verified by H2AX phosphorylation (Fig. 5.2A). Transient transfection of I-Ppol in MCF-7EPI^R cells increased the recruitment of phosphorylated-H2AX at DNA breaks sites (Fig. 5.2B). Importantly, DSBs enhance FOXM1 recruitment at DNA breaks in MCF-7EPI^R cells (Fig. 5.2B). In contrast, P-H2AX and FOXM1 recruitments at DNA breaks is not increased following I-Ppol transfection in MCF-7 cells (Fig. 5.2A). This result is in opposition with the result published by Berkovich *et al.* in which P-H2AX was recruited on DSBs in MCF-7 cells after 16 hrs transfection with I-Ppol (Berkovich, Monnat et al. 2007). Our MC-7 cell lines showed a low level of DNA repair as observed Figure 5.1A. Hence, this experiment should be repeated with low passage MCF-7 cell lines. This study did show a higher level of FOXM1 recruitment at DNA breaks I-Ppol-induced as well as P-H2AX in MCF-7EPI^R cells compared to MCF-7 cells (Fig. 5.2). H2AX interacts with MDC1, 53BP1, NBS1, RAD51 and BRCA1 at DNA break sites, but because FOXM1 silencing decreased H2AX phosphorylation, FOXM1 might affect protein assembly of sensors, mediators and effectors.

5.8.5 Conclusion

This study shows that FOXM1 activates the transcription of *NBS1*, member of the MRN sensor complex, inducing ATM auto-phosphorylation (on serine 1981) and the phosphorylation of ATM downstream targets including H2AX and CHK2 in MCF-7EPI^R cells (Fig. 5.3 and 5.4) and in human fibroblasts (Fig. 5.5 and 5.6). These results also showed that FOXM1 is required for the DNA damage phosphorylation events, suggesting that FOXM1 might affect DNA repair mechanisms in MCF-7EPI^R cells. FOXM1 silencing completely abrogated the elevated DNA repair mechanism in MCF-7EPI^R cells (Fig. 5.1B). The examination of protein recruitment at DNA breaks shows that FOXM1 is recruited after DNA breaks in MCF-7EPI^R cells and might have a role in protein assembly ATM-mediated (Fig. 5.2). Together these results indicate that FOXM1 is required for DNA damage signalling and DNA repair, and involved in protein assembly at DSB breaks. This study showed differences in DNA damage signalling induction, DNA repair factor recruitment and DNA repair between breast cancer cell sensitive and resistant to epirubicin (Fig. 5.11). Taken together, this study unravels that FOXM1 has a crucial role in promoting DNA repair response in MCF-7EPI^R cells and suggests that targeting FOXM1 could potentially resensitise epirubicin-resistant cells to DNA damage and cell death.

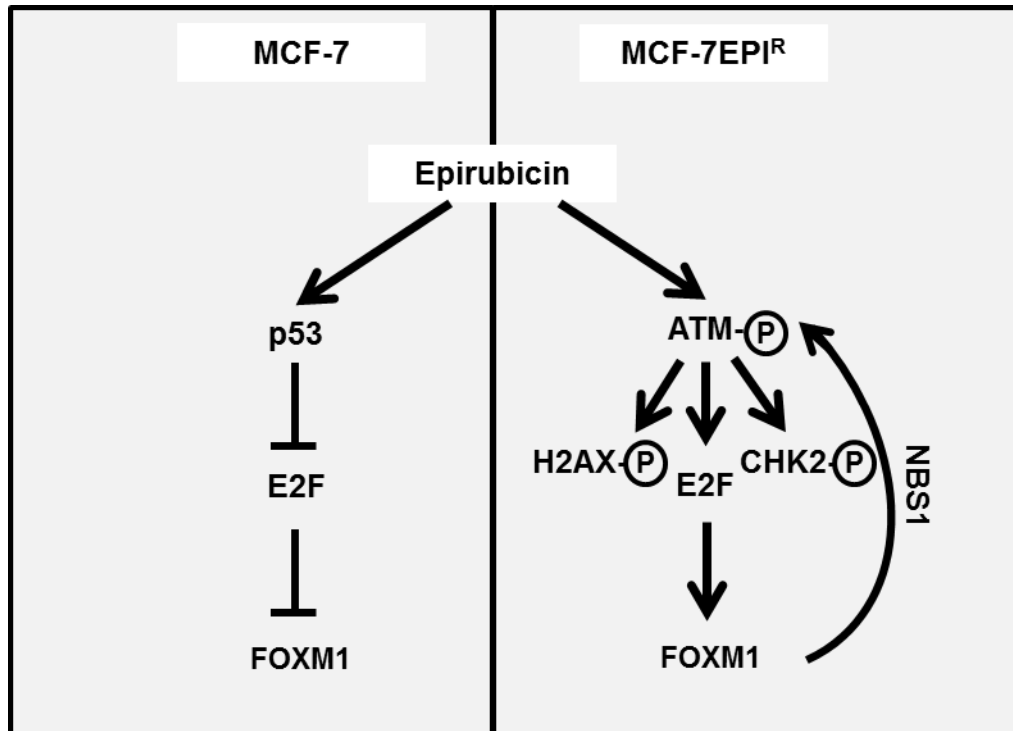


Figure 5.11 Differential pathways upon epirubicin in sensitive and resistant MCF-7 cells. In epirubicin sensitive MCF-7 cells, epirubicin activates p53 which downregulates E2F to repress FOXM1 expression and arrest the cell cycle. In epirubicin resistant MCF-7 cells, epirubicin activates ATM which in turn activates its downstream targets H2AX, CHK2 and E2F involved in DNA repair. E2F positively activates FOXM1 expression, which regulates NBS1 at promoter level. NBS1 is required to activate ATM auto-phosphorylation, which creates a feedback loop, controlling DNA damage repair and survival.

5.9 Future work

This report shows that targeting FOXM1 in epirubicin resistant breast cancer cells is an attractive strategy considering that FOXM1 is overexpressed in these cells and that FOXM1 inhibition results in DNA repair defect in these cells. Epirubicin resistant breast cancer cells have a high capacity to repair DNA, while FOXM1 silencing reduces ATM and its downstream targets activation. A previous study showed that FOXM1 regulates XRCC1 and BRCA2 DNA repair genes, consistent with FOXM1 inhibition resulting in defective DNA repair (Tan, Raychaudhuri et al. 2007). XRCC1 is important for two types of DNA repair, HR and NHEJ, while several lines of evidence indicate that BRCA genes are only critical for DSB repair by HR. In addition, this study showed that FOXM1 regulates NBS1 that is involved in both DSB repair mechanisms. Furthermore, a recent study showed that FOXM1 is involved in HR, but not in NHEJ (Monteiro, Khongkow et al. 2012). HR is particularly important during S and G2 phases, while NHEJ is dominant during G0, G1 and early S phases. It would be interesting to investigate whether a chemotherapeutic agent targeting G2 phase would be more effective than an agent arresting cells in G1 phase.

Recent work suggests that BRCA1 regulates the activity of MRN complex. Since FOXM1 regulates BRCA2 and a member of MRN complex, it could be possible that FOXM1 also regulates BRCA1. BRCA1 has been implicated in the transcription of several genes in response to DNA damage, such as p21Cip1 and GADD45 (Venkitaraman 2001). This could be another link between FOXM1 and DNA repair.

Protein assembly at sites of damage is important for the DNA damage response cascade and DNA repair. It would be interesting to investigate deeper whether FOXM1 affects protein assembly.

Previous studies showed that reduced FOXM1 expression significantly diminished DNA replication and mitosis in tumour cells. The current study suggests that inhibition of FOXM1 levels lead to defective DNA repair. Thereby, it would be beneficial to study FOXM1 inhibitor, thiostrepton, and investigate whether it inhibits ATM activation, reduces NBS1 levels as well as abolishes DNA repair and induces cell cycle arrest and cell death.

CHAPTER 6 FINAL DISCUSSION

Breast cancer is the most common cancer in the UK. Patients are treated according to their receptor status. ER/PR positive receptors breast cancer patients are treated with hormonal therapy and tamoxifen is the main agent given. However, 30% of patients that initially respond to tamoxifen become resistant. Hormone receptor (HR) negative breast cancer patients only respond to chemotherapy. A wide range of chemotherapeutic agents is used for solid tumours, but these agents are not specific to types of cancer. Based on the fact that there is no biomarker for HR negative patients, treatment administered are not specific to these breast cancers. In addition to the lack of specificity, patients treated with chemotherapeutic agents become resistant. Therefore, this information raises the urgent need to identify new targets involved in hormonal and chemotherapy drugs resistance, and to develop targeted agents.

FOXM1 is a master transcription factor involved in the regulation of cell proliferation, cell survival, angiogenesis and metastasis. FOXM1 is a proliferation specific factor largely expressed in developing embryos and observed at very low levels in adults. The upregulation of FOXM1 has been widely observed in several types of cancer, including breast cancer. The last past years, studies showed FOXM1 as an attractive target for anti-cancer drugs due to its specific expression in actively proliferating cells, especially cancer cells, and its role in cell cycle proteins regulation. Recent evidences raise FOXM1 as a novel target for prevention of drug resistance.

In this thesis, I have documented FOXM1 involvement in anti-estrogen resistance. A recent study demonstrated elevated FOXM1 mRNA and protein levels in cisplatin resistant breast cancer compared to cisplatin sensitive. I hypothesized that FOXM1 could have an important role conferring tamoxifen resistance through the upregulation of cell cycle target genes. In addition, FOXM1 has been recently linked with DNA repair mechanism through the regulation of BRCA2 and XRCC1 (Tan, Raychaudhuri et al. 2007). Therefore, I suggested that FOXM1 could also be involved in epirubicin resistance through the upregulation of DNA repair target genes.

If FOXM1 were found important in conferring both hormone receptor positive and negative drug resistances, FOXM1 inhibitors could be used to circumvent any types of breast cancer resistance. Indeed, I have detailed the mechanism of FOXM1 transcriptional regulation by ER α in tamoxifen sensitive breast cancer cells. Taken

together with a recent study, FOXM1 is regulated in a positive feedback loop with ER α that can be used to stop and perhaps kill breast cancer cells with the ER α antagonist, tamoxifen. I also unravelled that *FOXM1* mRNA is elevated in tamoxifen resistant cells related to tamoxifen sensitive cells, suggesting that FOXM1 is regulated at transcriptional level in tamoxifen resistant cells. My data indicate that FOXM1 is the major proliferation factor in tamoxifen resistant cells and that tamoxifen has no effect on the viability of these cells. I suggest that the lack of response to tamoxifen is likely to be due to deregulation of the ER-FOXM1 feedback loop and upregulation of the cyclin D1-FOXM1 loop. Furthermore, my studies showed that targeting FOXM1 in these cells can resensitise tamoxifen resistant cells to G1 growth arrest tamoxifen-induced. Indeed, FOXM1 regulates a large range of cell cycle regulators but this study hints that the targeting of FOXM1 results in a significant cell growth inhibition.

Pursuing the study, I found that FOXM1 is also upregulated in epirubicin resistant cells compared to epirubicin sensitive breast cancer cells. I unravelled a differential regulation of FOXM1 in sensitive and resistant cells. While FOXM1 is transcriptionally repressed by p53 via E2F1 in epirubicin sensitive cells, p53 is lost and FOXM1 is regulated by ATM through E2F1 in epirubicin resistant cells. A recent study has shown that FOXM1 phosphorylation and stabilization after DNA damage through the checkpoint kinase CHK2 promotes the transcriptional expression of DNA repair proteins, BRCA2 and XRCC1 (Anzick, Kononen et al. 1997, Tan, Raychaudhuri et al. 2007). This report identified NBS1 as a novel DNA repair FOXM1 target gene. In addition, this study showed that FOXM1 is a crucial component of the DNA damage response by activating ATM indirectly. In response to DNA damage, the MRE11/RAD50/NBS1 complex regulates the activation of ATM kinase. Here I showed that FOXM1 is required to regulate a member of the MRE11/RAD50/NBS1 complex, which is likely to promote ATM activation. ATM is the key activator of the double and single strand DNA repair pathways. Therefore, FOXM1 involvement in DNA repair was investigated and showed that FOXM1 knock-down completely abrogated DNA repair. FOXM1 is well-described to be required for cell survival but this study showed that FOXM1 is also essential for DNA repair and that targeting FOXM1 lead to DNA repair default and can induce cell death.

Drug resistance raises significant clinical challenges. The mechanisms by which cells acquire drug resistance are multiple. In the present study we have demonstrated for the first time that FOXM1 possesses a crucial role in tamoxifen and epirubicin resistance in breast cancer cells through enhancing cell proliferation and DNA-damage repair pathways. Several observations suggest that FOXM1 expression is an important determinant of tamoxifen and epirubicin sensitivity and resistance. Following drug treatment, FOXM1 was downregulated in the sensitive MCF-7 cells while the resistant MCF-7 cells showed an up-regulation of both FOXM1 mRNA and protein expression levels. Moreover, expression of the constitutively active Δ N-FOXM1 was sufficient to confer resistance to the cell cycle arrest OHT- and epirubicin-induced whereas the depletion of FOXM1 through siRNA knockdown reversed this effect.

These observations may have implications in the development of a treatment for tamoxifen and epirubicin resistant patients, suggesting it would be more efficient to target a key oncogene such as FOXM1, rather than targeting a proliferative gene or DNA repair machinery, where potential compensatory mechanisms could occur.

So far, the FOXM1 inhibitor, Thiostrepton, has only been approved by the FDA for the treatment of topical bacterial infection in cats and dogs. However, Thiostrepton has now been largely tested in different fibroblast, breast, colon; lung cancer cells (Bhat, Zipfel et al. 2008, Gartel 2008, Bhat, Halasi et al. 2009). Studies reveal that Thiostrepton inhibits FOXM1 expression, but not the expression of other members of the Forkhead box family. In addition, Thiostrepton inhibits the growth and induces apoptosis in human cancer cell lines of different origin (Bhat, Halasi et al. 2009). The anti-cancer properties of Thiostrepton in breast cancer were not only investigated *in vitro*, but also in xenograft mouse models of breast cancer *in vivo*. The encapsulation of Thiostrepton enhanced its solubility and accumulation into tumour sites. Micelle-thiostrepton nanoparticles reduces tumour growth rate with the suppression of FOXM1 protein and induction of cell death (Wang and Gartel 2011) .

Moreover, the inhibition of FOXM1 and co-treatment with anti-proliferative or DNA-damaging agents may be hypothesized to enhance therapeutic response. FOXM1 inactivation has already been tested for overcoming cisplatin resistance in breast cancer cells. SRB proliferative assays indicated that combination of cisplatin

and Thiostrepton showed synergistic effect on cell death rate in cells resistant to cisplatin, and that inhibition of FOXM1 is able to circumvent cisplatin resistance in breast cancer cells (Kwok, Myatt et al. 2008). The combination of Thiostrepton and Bortezomib (proteasome inhibitor) was also investigated and showed a similar synergistic effect on the induction of apoptosis in different cancer cells (Pandit and Gartel 2011).

The mechanisms by which FOXM1 activity and expression are upregulated in tamoxifen and epirubicin resistant cells require further investigation. However this study and recent publications suggest a positive feedback loop between FOXM1 with cyclin D1 and B-myb in tamoxifen resistant cells and with ATM in epirubicin resistant cells. These observations may also have implications in the development of new co-treatments. Cyclin D1 overexpression has been found in many cancer and correlate with the lack of response to tamoxifen in breast cancer. A study showed that inhibition of cyclin D1 expression by cyclin D1 shRNAs in human oral squamous cell carcinoma cells is associated with increased cisplatin chemosensitivity (Zhou, Zhang et al. 2009). DNA repair pathways can enable tumour cells to survive DNA damage that is induced by chemotherapeutic treatments; therefore, inhibitors of specific DNA repair pathways might be efficient when used in combination with DNA-damaging chemotherapeutic drugs. The combination of ATM inhibitor with IR and doxorubicin has been tested and showed a significant increase in the sensitivity of lung cancer cells to the cytotoxic effect of these treatments (Shaheen, Znojek et al. 2011). Patients with defect in one repair pathway could potentially be treated with the inhibitor of the other pathway and benefit from maximum treatment outcome with minimal toxicity. This thesis and a recent study suggest that FOXM1 plays a key role in homologous recombination (Monteiro, Khongkow et al. 2012). Therefore, targeting a critical protein in non-homologous end joining such as DNA-PK in combination with FOXM1 inhibitor might abrogate any possible repair and increase the sensitivity of resistant breast cancer cells to chemotherapies.

The novel thiazole antibiotic Thiostrepton is a potential pre-clinical candidate that should be further studied in co-treatment with key inhibitors for overcoming breast cancer drug resistance.

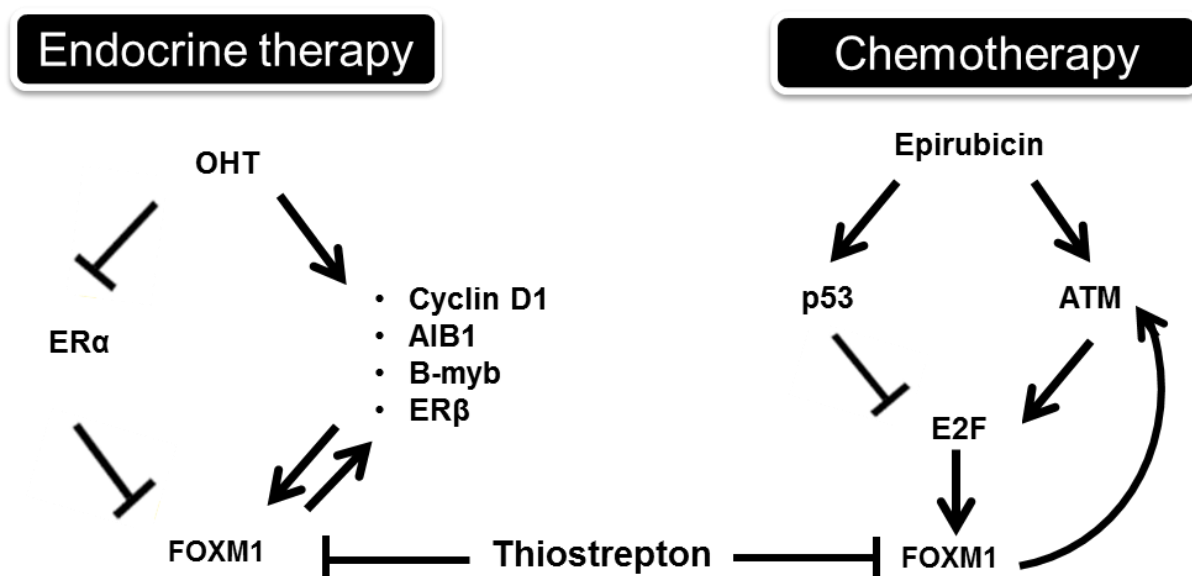


Figure 6.1 Thiostrepton as a potential candidate to overcome endocrine and chemotherapy resistance in breast cancer. Tamoxifen treatment (OHT) inhibits FOXM1 expression via ERα and FOXM1 functions in OHT-sensitive breast cancer cells, while OHT induces a range of key proteins regulated in a feedback loop with FOXM1 allowing cell survival and drug resistance in OHT-insensitive breast cancer cells. Epirubicin treatment represses FOXM1 expression via p53 upregulation in epirubicin sensitive cells, while FOXM1 is regulated in a positive feedback loop with ATM preventing DNA damage accumulation and cell death. The use of Thiostrepton in combination with these treatments could break the positive feedback loop regulating FOXM1 and its targets, and overcome Tamoxifen and Epirubicin resistance.

CHAPTER 7 SUPPLEMENTAL DATA

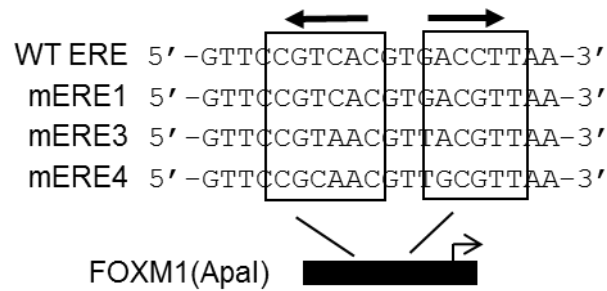


Figure S.D.7.1. Schematic representation of the Apa I FOXM1 construct, showing the wild-type ERE, and three mutants ERE (mERE) sequences (mutant analysed by Demetra Constantinidou).

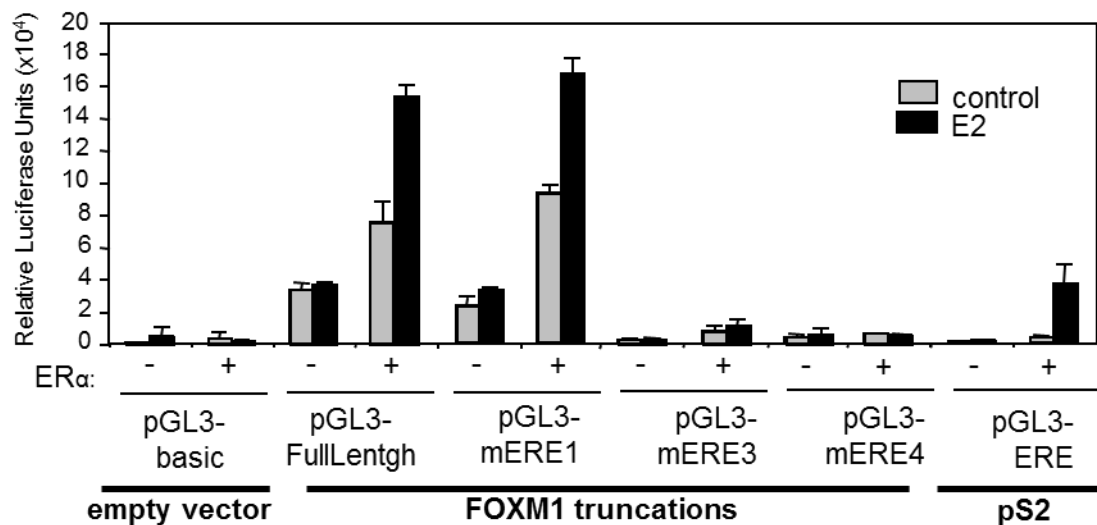


Figure S.D.7.2. ERα induces the transcriptional activity of the human FOXM1 gene through an ERE proximal to the transcription start site (experiment performed by Demetra Constantinidou). COS-1 cells were transfected with pGL3-F Length, pGL3-ApaI) or pGL3-ERE promoter constructs, together with increasing amounts (0, 0.1, 1, 10, and 20 ng) of ERα expression vector.

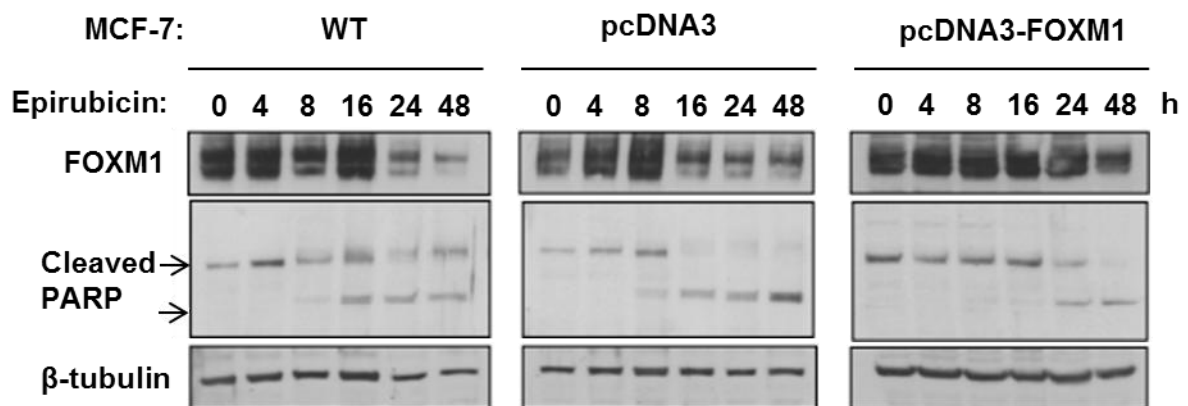


Figure S.D.7.3 Ectopic expression of FOXM1 reduces MCF-7 cells sensitivity to cell death (Experiment performed by Julia K. Langer). MCF-7 cells wild-type, stably transfected with pcDNA3 or with FOXM1 were treated with 1 μ mol/L of epirubicin for 0, 4, 8, 16 24 and 48 h. At indicated times, cells were harvested for western blot analysis to determine the protein expression of FOXM1, cleaved PARP, indicator of apoptosis, and β -tubulin.

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