The role of SIRT6 and FOXO3a acetylation in the development of drug resistance in breast cancer

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ABSTRACT

Drug resistance is one of most challenging problems that need to be addressed in order to improve treatment efficiency and overcome cancer. Apart from epigenetic changes, the regulation of transcription factors, such as p53 and E2F1, via deacetylation, independent of histone modification, have been documented to be involved in cancer progression and drug resistance. In this work, I demonstrated that sirtuins (deacetylase enzyme family) especially SIRT6, have a crucial role in the development of epirubicin and paclitaxel resistance in breast cancer. The levels of SIRT6 protein were elevated in MCF-7-epirubicin resistant- (MCF-7-Epi^{R}) and -paclitaxel resistant cells (MCF-7-Tax^{R}) compared to MCF-7 sensitive cells. The depletion of SIRT6 in both resistant cells sensitises these cells to epirubicin and paclitaxel treatments, whereas ectopic overexpression of SIRT6 in MCF-7 cells is sufficient to confer the resistance. I also found that the overexpression of SIRT6 prevents epirubicin induced cell death and cellular senescence by enhancing DNA damage repair. Moreover, its overexpression also deregulates G2/M cell cycle checkpoint induced by paclitaxel. FOXO3a and p53 are two main mediators for chemotherapeutic response and have been reported to be cellular targets of SIRT1 and SIRT2 via their deacetylase activities. Interestingly, the acetylation levels of these proteins are also regulated by SIRT6, as the levels of their acetylation are higher in *Sirt6*$^{-/-}$ MEFs compared to wild-type (WT) MEFs, especially in response to chemotherapy treatment. Additionally, the acetylation levels of FOXO3a are increased after SIRT6 depletion in MCF-7 cells. The depletion of SIRT1, -2, and -6 in MCF-7 as well as MCF-7-Epi^{R} and MCF-7-Tax^{R} cells increases the acetylation levels of FOXO3a compared to control cells. However, the acetylation levels of FOXO3a are noticeably higher in SIRT6 depleted cells compared to SIRT1 and SIRT2

depleted cells. These findings implicate SIRT6 as the main player in the regulation of FOXO3a acetylation in MCF-7 cells and SIRT6 reduces paclitaxel and epirubicin sensitivity, at least in part, through the modulation of FOXO3a acetylation and expression. Consistently, immunohistochemical analysis of breast cancer patient samples revealed that higher SIRT6 nuclear expression is significantly associated with poorer overall survival. Collectively, my study suggests that SIRT6 has a role in paclitaxel and epirubicin sensitivity via targeting the FOXO3a protein, and that SIRT6 could be a useful predictive biomarker and therapeutic target for paclitaxel and epirubicin resistant cancer.

PUBLICATIONS

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ABBREVATIONS

ABC	ATP-binding cassette
APS	Ammonium persulphate
Bcl-2	B cell lymphoma gene 2
BER	base excision repair
BSA	Bovine Serum Albumin
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
CBP	CREB binding protein
CLL	Chronic lymphocytic leukemia
CKI	Cyclin- dependent kinase inhibitor
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DR4	Death Receptor 4
DTT	Dithiothreitol
dNTP	Nucleotides
DSBs	Double strand breaks
ECL	Enhanced Chemi-Luminescence
EDTA	Ethylenediaminetetraacetic acid

EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase (MAPK)
FACS	Fluorescence activated cell sorting (flow cytometry)
FasR	Fas Receptor
FasL	Fas Ligand
FCS	Foetal calf serum
FGFR	Fibroblast growth factor receptor
FOX	Forkhead box
FOXM	Forkhead box, sub group M, transcription factor
FOXO	Forkhead box, sub group O, transcription factor
g	gravitational
GADD45α	DNA-damage inducible protein α of 45 kDa
GCN5	General control non-repressed protein5
GDH	Glutamate dehydrogenase
GNAT	Gen5 related N-acetyl transferase
h	hour
H2	Histone 2
H3	Histone 3
H3K9	Histone 3 lysine 9
H3K56	Histone 3 lysine 56
H4	Histone 4

H4K16	Histone 4 lysine 16
HAT	Histone aceyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HR	Homologous recombination
HRP	Horseradish peroxidase
IDH2	Isocitrate dehydrogenase 2
IGF-1	Insulin-like growth factor
IKK	I-kappa-B kinase
JNK1	c-Jun kinase
m	Milli
M	Molar
MAPK	Mitogen activated protein kinase
MBC	Metastatic breast cancer
MDM2	Murine double minute2
MDR1	Multidrug resistance protein 1
MEFs	Mouse embryonic fibroblasts
Min	minutes
MLL	Mixed lineage leukemia
MnSOD	Manganese superoxide dismutase
MST1	Mammalian Ste20-like kinase

n	nano
NAM	Nicotinamide
NAD+	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
Na3VO4	Sodium orthovanadate
NER	Nucleotide excision repair
NFY	Nuclear transcription factor Y
NHEJ	Non-homologous end joining
NSC	Non-specific control
PARP1	Poly ADP-ribose polymerase 1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PML	Promyelocytic leukemia
PP2A	Protein phosphatase 2A
PR	Progesterone receptor
PKB	Protein kinase B
PTEN	Phosphatase and tensin homologue on chromosome ten
PTM	Post-translation modification
Rb	Retinoblastoma
ROS	Reactive oxygen species
RPM	Revolutions per minute

RT	Room temperature
s	Seconds
SAHA	suberoylanilide hydroxamic acid
SDS	Sodium dodecyl sulphate
SERM	Selective ER modulators
Sir2	Silent Information Regulator 2
SIRTs	Sirtuins
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween 20
TCA	Tricholoroacetic acid
TEMED	N,N,N,'N-tetramethylethylenediamine
TGFB	Transforming growth factor B
TKI	Tyrosine kinase inhibitor
TNF	Tumour necrosis factors
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAIL	TNF-Related Apoptosis Inducing Ligand
Tris	Tris (hydroxymethyl)aminomethane
Tween 20	polyoxyethylene-sorbitan monolaurate
µ	Micro
v/v	volume:volume ratio
w/v	weight:volume ratio
XRCC4	X-ray repair cross complementing protein 4

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a disease related to genetic alterations in the cells resulting in the upregulation of oncogenes and/or the repression of tumour suppressive genes. Tumorigenesis involves multiple processes that transform healthy human cells into aggressive malignant cells. At least six key characteristics leading to this alteration have been identified and are denoted as the hallmarks of cancer. They include inappropriate sustention of growth signalling, a loss of limitation on the replicative potential of the cell, resistance to anti-growth signalling, evasion of programmed cell death, induction of angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000).

1.1.1 Breast cancer

Breast cancer is the most common malignancy in women and one of the major causes of death worldwide with over one million cases per year. Breast cancer is reported to account for 23% of all female cancers and 10% of all diagnosed cancers (Ferlay et al., 2010; Kamangar et al., 2006). It can also develop in males, albeit at a lower frequency, who account for 1% of all breast cancers (Gómez-Raposo et al., 2010).

There are different risk factors associated with breast cancer, such as age (premenopausal women are particularly susceptible), obesity, alcohol, as well as radiation exposure. Additionally, family history is another predisposing factor. Of all breast cancers, 10% are believed to develop due to the inheritance of mutated genetic predisposition genes (K. McPherson *et al,* 2000). Breast cancer type1 susceptibility protein (*BRCA1*) and breast cancer type 2 susceptibility protein

(*BRCA2*) are among the most common genes that have been identified to increase the risk of hereditary breast cancer (Miki et al., 1994; Wooster et al., 1995). BRCA1 and BRCA2 act as tumour suppressors due to their ability to control genomic stability via the regulation of DNA damage repair. The mutation of these two genes often results in the loss of their functions, which lead to an increased incidence of tumorigenesis (Scully and Livingston, 2000).

Breast cancer is a heterogeneous disease, and is clinically classified into different subtypes based on the cellular origin, and the expression profiles of estrogen receptor alpha (ERα), the progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2 or ErbB2) proteins (Anderson and Matsuno, 2006). These subtypes include luminal A and B, HER2-overexpressing, triple negative and basal-like (Berrada et al., 2010). The basal-like and "triple negative" breast cancers display similar genotypes, and denote the absence of ERα, PR and the HER2. Together these two subtypes account for 15% of all breast cancers (Cleator et al., 2007).
The expression of ERα is the primary and most robust marker for distinguishing between ERα positive (ERα+) and ERα negative (ERα-) cellular subtypes. It has been documented that 70% of all breast cancer cases exhibit an increase in ERα expression and subsequently ERα induced cell proliferation.

ERα is a member of the nuclear receptor superfamily of transcription factors that can bind to a number of hormone ligands. In this instance, ERα is activated via the binding of estrogen, typically 17β estradiol, leading to a change in its conformation, facilitating the subsequent phosphorylation of the receptor, engagement with DNA, and the consequent recruitment of its coactivators (Osborne and Schiff, 2005). The

activated ERα then acts as a transcription factor by binding to estrogen response elements (EREs) in the promoters of cognate target genes, many of which are involved in cell proliferation, differentiation and survival (Madureira et al., 2006). A wide range of therapeutic interventions against ERα are now available to inhibit hormone-induced tumour growth, and as a result, ERα expression is correlated with better survival in breast cancer patients (Ali and Coombes, 2000). PR is also a member of the nuclear receptor superfamily, and as such its mode of regulation and action is similar to that of ER (Rubel et al., 2010).

Human epidermal growth factor receptor 2 (HER2) is a cell surface receptor tyrosine kinase. HER2 overexpression occurs in 20-30% of all breast cancer cases (Moasser, 2007). This receptor is activated by the engagement of its ligand by the ligand binding domain, leading to stable self-dimerisation and *trans*autophosphorylation, and the subsequent recruitment of additional signalling factors, including Human epidermal growth factor 3 (HER3) and epidermal growth factor receptor (EGFR). HER2 activation promotes growth, invasion, and survival signalling pathways via the upregulation of phosphoinositide-3-kinase signalling (PI3K).

1.1.2 Breast Cancer treatment

Breast cancer is generally treated by surgery, in combination with endocrine therapy, chemotherapy and radiation.

1.1.2.1 Endocrine (or 'Hormone') treatment

Endocrine treatment is the first option for ERα+ patients, as this type of cancer is dependent on aberrant estrogen-induced cell proliferation. Thus, the inhibition of ER and/or estrogen activity results in a reduction of estrogen-dependent tumour. Selective ER modulators (SERMs) are a class of agents, including tamoxifen and

raloxifen, which function as ER agonists or antagonists in a dose and tissue dependent manner. The mechanism of action of tamoxifen is to bind to ER, leading to a change in ER conformation which blocks coactivator binding, with subsequent recruitment of corepressors and repressors of ER-responsive gene transcription (Dutertre and Smith, 2000). It has been reported that about 30% of patients will not respond to tamoxifen treatment due to *de novo* or intrinsic resistance. However, some of the patients that initially respond to the treatment may eventually develop resistance, *i.e.* .acquired' resistance (Schiff et al., 2003). PR positive breast cancer pateints are also considered as a hormone positive breast cancer, which is generally respond to taxomifen treatment.

For breast cancer patients with HER2 overexpression, small molecule inhibitors such as lapatinib and monoclonal antibodies such as trastuzumab have been developed to specifically target and inhibit this receptor; accordingly, this is referred to as 'targeted' therapy.

1.1.2.2 Chemotherapeutic treatment

Conventional chemotherapeutic drugs are also used to treat breast cancer patients. This category of agents includes non-target compounds that mainly induce cell cycle arrest and/or cell death.

Anthracyclines, including doxorubicin and epirubicin; and also taxanes, including paclitaxel (Taxol) and docetaxel (Taxotere) are the most effective and widely used chemotherapeutic agents. These agents are used for treating patients diagnosed with triple negative breast cancer and also those who have acquired resistance to endocrine treatment (Piccart, 2003; Wu et al., 2011). These agents are particularly

important for treating advanced or metastatic breast cancer (MBC), as they can represent the only option for treatment.

Typically, 30–70% of MBC patients will respond to anthracyclines and taxanes, however their efficacy is often transient due to the development of resistance to one or both sets of drugs (Cortes and Baselga, 2007; Moreno-Aspitia and Perez, 2009). Drug resistance, either intrinsic or acquired, is thought to cause more than 90% of chemotherapeutic treatment failure in cancer patients (Longley and Johnston, 2005). When resistance to anthracyclines and taxanes develops, few further treatment options exist. Treatment with chemotherapeutic agents, such as capecitabine, gemcitabine and vinorelbine will follow, however many reports indicate a low response and poor survival (Dean-Colomb and Esteva, 2008; Estevez et al., 2008). Therefore, understanding the molecular mechanisms underlying taxane and anthracycline resistance is critical for the development of more effective chemotherapeutic strategies.

Taxanes and anthracyclines act via very different mechanisms. The mechanism of taxanes, paclitaxel for example, is to bind to α-tubulin leading to the prevention of depolymerisation of microtubules (Manfredi and Horwitz, 1984). Consequently, normal mitotic spindle dynamics are inhibited, leading to a transient block at the G2 to M phase transition and subsequent cell death (Jordan et al., 1996). The structure of paclitaxel is present in Figure 1.1 (bottom panel).

It has been suggested that there are many different mechanisms by which cancer cells can develop taxane resistance in *in vitro* models. These include the exclusion of taxanes from the cell by ATP-binding cassette transporters, the expression of certain tubulin isoforms and microtubule-associated proteins, the mutation of the tubulin

gene, and alterations in mitotic checkpoint signalling such as the extracellular signal-regulated kinases (ERK1 and 2), a component of the mitogen-activated protein kinase (MAPK) family (Chien and Moasser, 2008; Orr et al., 2003).

Epirubicin is an analogue of doxorubicin which has a change in the configuration of 4-hydroxyl group on the sugar ring from α to β position (Minotti et al., 2004). This modification is believed to reduce the cardiotoxicity typically associated with doxorubicin, yet the cytotoxic effects of this agent remain (Figure1.1).

Anthracyclines exert their function through their ability to intercalate into DNA, and bind to DNA topoisomerase II, leading to the induction of DNA cleavage in an ATP-dependent manner (Wang et al., 2001). The formation of reactive oxygen species (ROS) is another mechanism triggered by anthracyclines, which contributes to the cytotoxic activity of these drugs (Jacobson, 1996). As a result, the cellular response to such DNA damage including the initiation of DNA damage repair, the activation of cell cycle-checkpoint and induction of apoptosis or senescence, is a main factor that determines the genotoxic efficacy of these drugs.

As the induction of DNA damage is the principle mechanism of action of anthracyclines, it has been suggested that topoisomerase modulation and alteration in DNA damage response genes may be involved in the development of anthracycline resistance (Chien and Moasser, 2008). Further mechanisms of resistance also include drug transport proteins, and an upregulated antioxidant stress response.

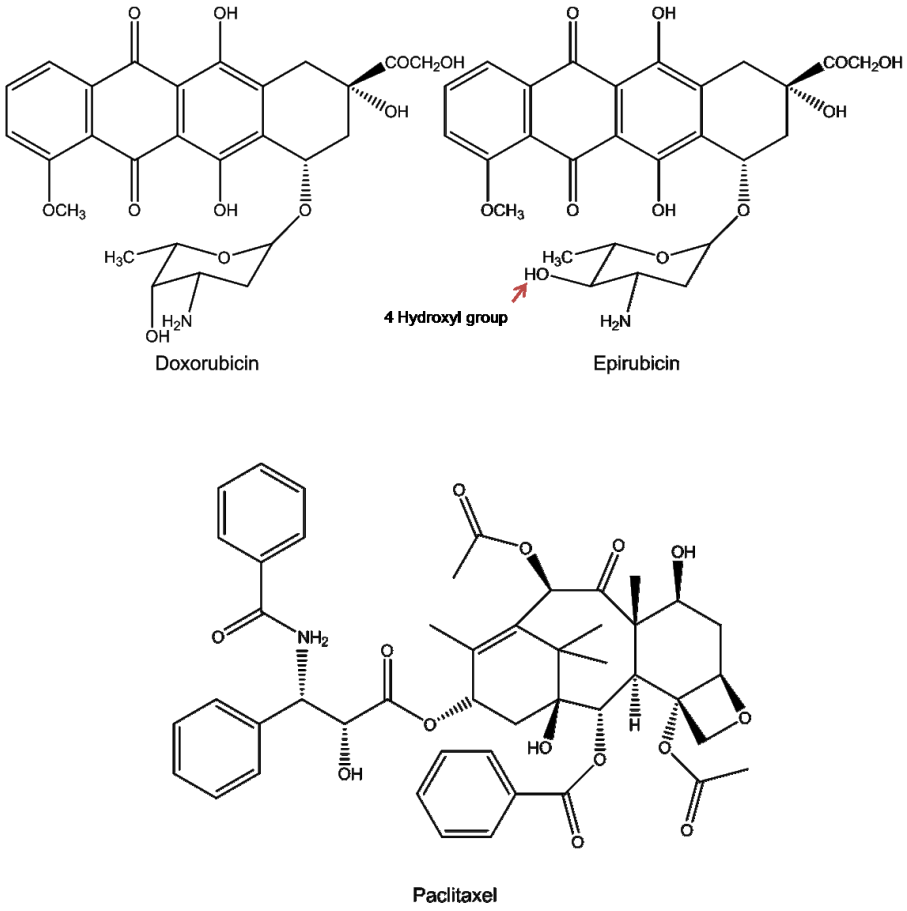


Figure 1.1 Schematic representation of chemotherapeutic structures Top: Structure of the anthracyclines, doxorubicin and epirubicin, showing that the 4-hydroxyl group on the sugar ring is changed in its position from α to β position. Bottom: structure of paclitaxel

1.2 DNA damage and repair

DNA damage can be caused by ROS, environmental genotoxins, and radiation, as well as chemotherapeutic drugs, which can result in single or double strand breaks (DSBs). Single strand break is typically rectified by the base excision repair (BER) or the nucleotide excision repair (NER). Single strand break leads to the activation of ATR-dependent Chk1 phosphorylation. This in turn triggers single-stranded DNA (ssDNA) repair (Smits et al., 2010). Moreover, single strand break repair requires the involvement of poly ADP-ribose polymerase 1 (PARP1) binding to sites of DNA damage, which leads to the recruitment of the other repair proteins, such as XRCC1, which initiate the repair process (Polo and Jackson, 2011).

DSBs are another type of “insult” to DNA which is extremely dangerous to the cell and can be caused by ROS and genotoxic agents such as chemotherapeutic drugs. There are two main pathways involved in DSB repair: non-homologous end joining (NHEJ) and homologous recombination (HR) (Houtgraaf et al., 2006).

NHEJ is “error prone” as only 25-50% of nuclear DSBs in yeast and mammalian cells are repaired by precise NHEJ, whereas HR is “error free” which provides 100% accuracy by employing sister chromatids as templates for repair (Shrivastav et al., 2008).

The initial step of NHEJ is the recruitment of the heterodimer of Ku70 and Ku80 at the break sites and the subsequent accumulation of other repair factors that will process DNA religation. The PI3K-like family member, DNA-dependent protein kinase, (DNA-PK), for instance, is one of the recruited factors following Ku70/Ku80 binding. The activation of this factor leads to its binding to DNA and subsequent an induction of its autophosphorylation at specific sites, including Thr2609 and Ser2056.

In turn, this results in the conformational change of the DNA-PK/Ku complex, and subsequently the recruitment of the other NHEJ repair factors, such as the DNA ligase IV/ X-ray repair cross complementing protein 4 (XRCC4) complex to process the ligation (Lieber, 2010).

DSB repair via HR additionally requires the MRN complex, consisting of MRE11, Rad50 and NBS1. The site of damage is recognised by the MRN complex which then recruits other repair factors, such as Rad51 and BRCA1/2 that help to resect the DNA ends. It has been shown that BRCA1/2 deficiency in cells results in the impairment of the HR pathway and increase the sensitivity of cancer cells in response to PARP inhibitors (Aly and Ganesan, 2011). This exemplifies that some deregulated proteins and pathways involved in DNA damage repair could be explored as a mechanism in the development of drug resistance.

1.3 Drug resistance

1.3.1 Possible mechanism of drug resistance

The common treatment for cancer is chemotherapy; however, most therapies eventually fail because of the development of drug resistance. There are two forms of drug resistance, which are acquired and intrinsic (*De novo*) resistance. Acquired resistance is the type of which the patients are initially response to the treatment but this treatment become inefficient over time, whereas intrinsic resistance is a type that patients do not respond to the first line of treatment (Florea and Büsselberg, 2013; Schiff et al., 2003). It has been proposed that at least three main mechanisms are involved in the development of cancer drug resistance (Cortes and Baselga, 2007; Moreno-Aspitia and Perez, 2009). Firstly, the impair of expression and activity of transporter proteins involved in the uptake of hydrophilic chemotherapeutic agents

can result in a reduction of drug uptake in cancer cells (Lee and Swain, 2005; Longley and Johnston, 2005). Secondly, the upregulation of transporters involved in the efflux of hydrophobic chemotherapeutic agents can also lead to the free diffusion of these substances through the plasma membrane (Dean-Colomb and Esteva, 2008). For example, the upregulation of membrane ATP-binding cassette (ABC) family transporter expression has been reported to promote the development of resistance to different agents, referred as multidrug resistance (Szakacs et al., 2006). Multidrug resistance protein 1 (MDR1), also known as P-glycoprotein or ABCB1 is a member of this transporter family commonly upregulated in multidrug resistance. The upregulation of P-glycoprotein causes an increase in the drug efflux of many agents, including anthracyclines, taxanes, etoposide and tyrosine kinase inhibitors (TKIs), such as imatinib (Fletcher, 2010). Thirdly, the hyperactivation of cellular survival and proliferative signalling can also culminate in an enhancement of DNA damage repair and the alteration of drug metabolism, and this will allow cancer cells to counteract the cytotoxic and cytostatic effects of chemotherapeutic substances (Estevez et al., 2008; Manfredi and Horwitz, 1984). Interestingly, the development of acquired drug resistance is mostly associated with a deregulation of transcription of genes involved in the cellular response to cytotoxic and cytostatic drugs (Olmos et al., 2011b).

1.4 Protein acetylation and deacetylation

The activity of proteins is tightly regulated by post-translational modifications, including acetylation, methylation, phosphorylation and ubiquitination. The term “acetylation” defines a reaction that introduces an acetyl functional group (-$COCH_3$) into a chemical compound. This reaction encompasses the substitution of the

hydrogen atom of a hydroxyl group with an acetyl group (-$COCH_3$). Proteins, including histones and transcriptional factors are targeted for acetylation, which occurs as a form of post-translational modification. Protein acetylation typically occurs on lysine residues (Lys) at the N-terminal tail of histones as part of the gene regulation process; however, other amino acid residues, like serines (Ser) and threonine (Thr), can also be acetylated. The acetylation status of proteins is tightly controlled by the opposing activities of “histone acetyltransferase” (HAT) and “histone deacetylase" (HDAC) enzymes.

Acetylation of histones leads to the alteration of chromatin structure, affecting gene expression (Gregory et al., 2001). Additionally, acetylation neutralizes the positive charges of histone tails, often causing a decrease in the interaction between histones and DNA and subsequently a relaxed form of chromatin. This relaxation then allows the access of transcription factors and other associated proteins to initiate gene transcription. In contrast, the chromatin structure remains tightly packed in non-acetylated proteins, leading to the limitation of binding and interaction between DNA and transcription factors. This results in the silencing of gene transcription (Marks et al., 2001; Struhl, 1998).

Moreover, HATs and HDACs can also mediate the acetylation status of non-histone proteins. The acetylation/deacetylation of transcriptional factors plays an important role in post-translational regulatory mechanisms by changing the structures of chromatin and protein functions (Hassig and Schreiber, 1997; Sadoul et al., 2008). These reactions are analogous to phosphorylation by the action of kinases or dephosphorylation by the action of phosphatases. Acetylation of several transcriptional factors has been suggested to have a crucial role in tumorigenesis, cancer progression as well as drug resistance. It has been demonstrated that the

activities and functions of tumour suppressors such as p53, p73 and FOXO3a, are partly regulated by their acetylation status (Brooks and Gu, 2003; Brunet et al., 2004; Glozak et al., 2005; Olmos et al., 2011b). Interestingly, not only can acetylation modify protein activity be modified by the status but it can also crosstalk with other post-translational modifications, such as phosphorylation, methylation, and ubiquitination, and involved in the dynamic control of cell signalling (Olmos et al., 2011b; Yang and Seto, 2008).

1.4.1 Histone Acetyltransferases (HATs)

Several different HAT protein families have been identified based on the highly conserved structures within each family. The most well established families are Gen5 related N-acetyl transferase (GNAT) and p300/CREB binding protein (CBP). HATs can regulate the acetylation status of both histone and non-histone proteins. For instance, p53 and FOXOs are acetylated by p300/CBP, resulting in the change of their cellular expression and activity (Gu and Roeder, 1997).

1.4.2 Histone deacetylases (HDACI, II,and IV)

There are two protein families that exhibit deacetylase activity, and they are the classical HDAC family and NAD+-dependent HDAC (known as sirtuins). Classical HDAC consists of 3 sub-classes, I, II, and IV, whereas sirtuins belong to class III HDACs. The main characteristic that distinguishes between classical HDACs and sirtuins are the cofactors that potentiate their enzymatic activity. Sirtuins use NAD+ as a cofactor, whereas the other HDACs require Zn^{2+} as a cofactor for their action. However, this thesis will mainly focus on the functions and activities of sirtuins.

1.4.3 Sirtuins (HDAC class III)

Mammalian sirtuins are defined by their homology with the catalytic domain of yeast histone deacetylase Silent Information Regulator 2 (*Sir2*). Yeast Sir2 was originally identified in *Saccharomyces cerevisiae* (Blander and Guarente, 2004). It has also been found to be highly conserved in nematode (*Caenorhabditis elegans*, *C. elegans*) as well as fruit flies (*Drosophila* melanogaster). *Sir2* is known to be involved in the epigenetic silencing of mating loci in yeast by deacetylating the N-terminal tails of H3 and H4 resulting in a more compact chromatin structure. Therefore, Sir2 is proposed to have a crucial function in longevity and genomic stability in response to metabolic and environmental stimuli (Gorospe and de Cabo, 2008; Guarente and Picard, 2005; Kennedy et al., 2005; Longo and Kennedy, 2006). Mammalian sirtuins consist of seven family members, which are named from SIRT1-7. Sirtuins are primary described as proteins processing histone deacetylase activity (SIRT1, 2, 3, 5, 6 and 7) as well as mono-ADP ribosyltransferase activity (SIRT4 and 6). Recently, SIRT5 has been shown to render demalonylase and desuccinylase activity, whereas SIRT6 also has deacylation function. As they require NAD^+ as a cofactor for their enzymatic activities, Sirtuins also act as cellular sensors for energy fluctuation. SIRT1, 6 and 7 are mainly localised in the nucleus, whereas SIRT2 and SIRT3 are predominantly cytoplasmic and mitochondrial, respectively. However, both of SIRT2 and SIRT3 can translocate into the nucleus under certain conditions. SIRT4 and 5 are localised in the mitochondria. The functions of sirtuins (SIRTs) depend on their cellular localisation and can be classified into 4 main processes, namely the regulation of chromatin, stress-induced cell survival, the regulation of homeostasis, and cell differentiation and development (Bosch-Presegue and Vaquero, 2011; Vaquero et al., 2004).

Because of their enzymatic activity and function in the regulation of homeostasis, SIRTs have been proposed to play a crucial role in the development of age-related diseases, including cancer (Olmos et al., 2011b).

The catalytic function of SIRTs is to hydrolyse the acetyl lysine moieties on histones and other proteins and break the glycosydic bond between nicotinamide (NAM) and ADP-ribose of NAD^+. This results in the acetylation of the ADP-ribose motif depicted in Figure 1.2. The deacetylase activity has been reported for SIRT1, 2, 3, 5, 6 and SIRT7.

For mono-(ADP)-ribosylation, the mechanism of this reaction is generally involved the transferring of ADP-ribose to conserved arginine side chain, which is next to two adjacent glutamine residues by breaking down of glycosidic bond between nicotinamide and ADP-ribose (Figure 1.2 continued). There are only SIRT4 and SIRT6 has been reported to display mono-(ADP)-ribosylation. SIRT6 has been shown to undergo intramolecular mono-ADP- ribosylation, which possibly lead to self-regulating SIRT6 function (Beauharnois et al., 2013).

The characteristic of mammalian SIRTs is defined by the highly conserved catalytic domain of Sir2 in yeast; however the relative length of individual SIRT is varied due to the length of their C and N-termini. This variation of the C- and N- termini contributes to the different roles and functions of individual SIRTs (Beauharnois et al., 2013).

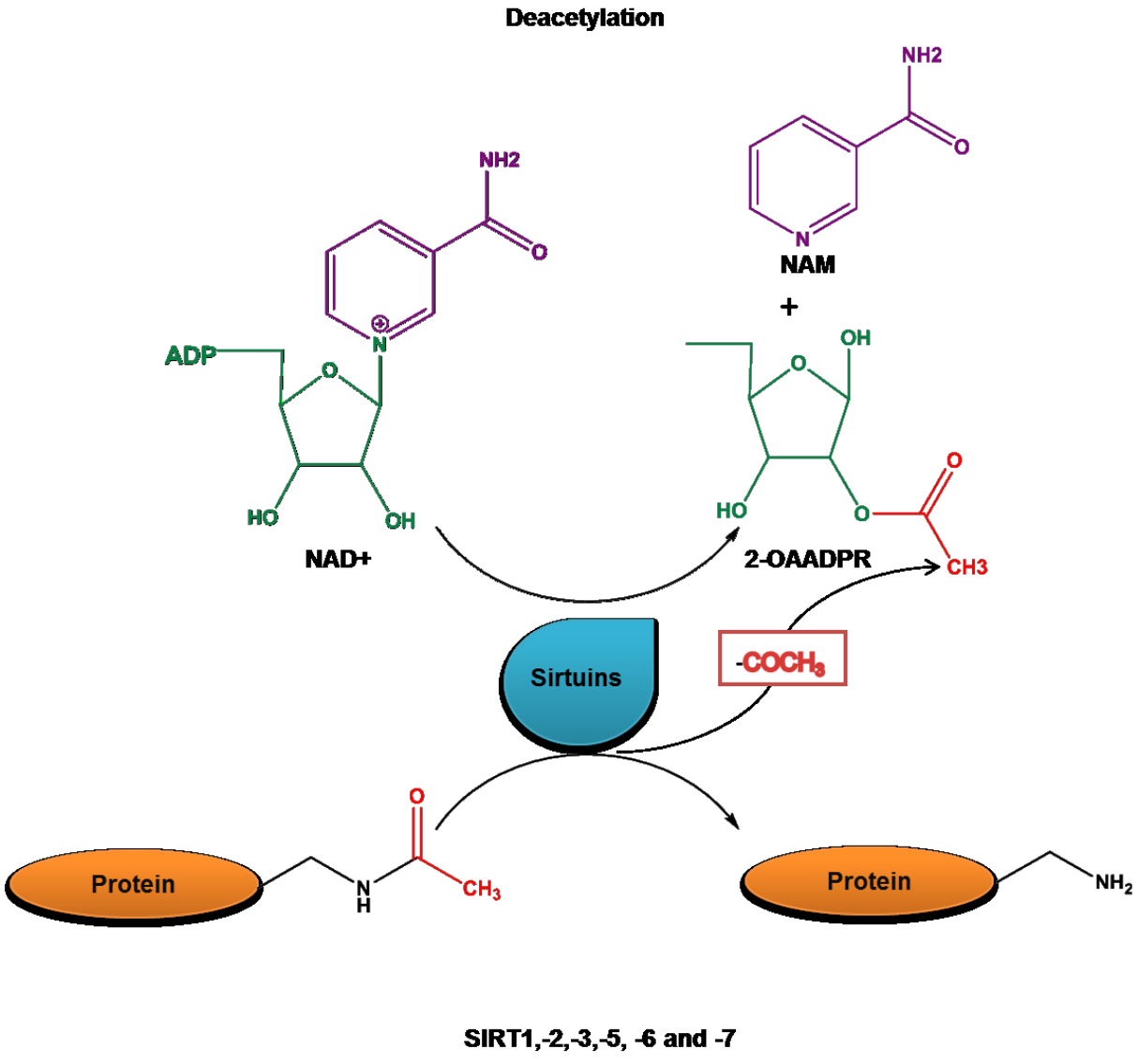


Figure 1.2 Schematic showing the enzymatic activity of Sirtuins in the regulation of deacetylation and mono-(ADP) ribosyltransferase.

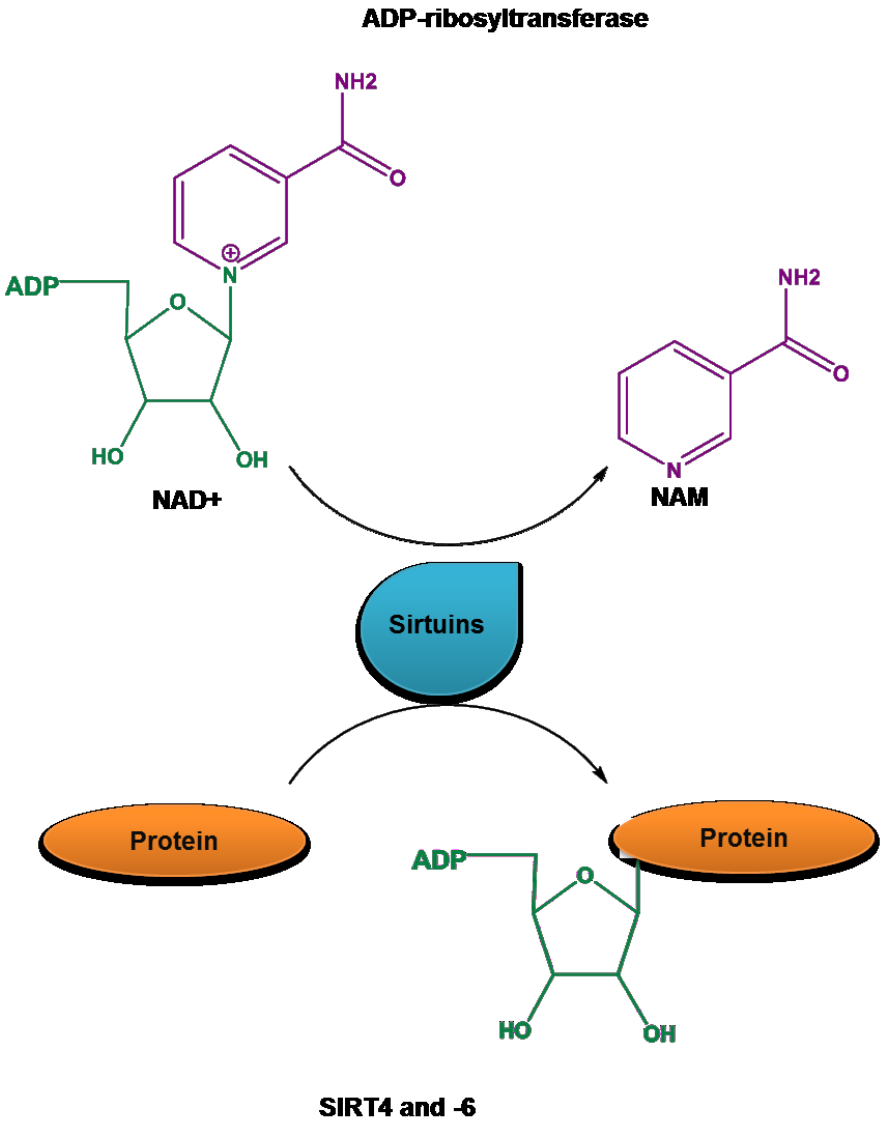


Figure 1.2 Schematic diagramme (Continued) showing the enzymatic activities of Sirtuins in the regulation of deacetylation and mono-(ADP) ribosyltransferase.

1.4.3.1 SIRT1

The most studied member of this family in mammals, SIRT1, is primarily described as a nuclear deacetylase for histones and non-histone proteins. The enzymatic activity of SIRT1 is to prevent the transfer of the acetyl moiety from the ε-amino group of lysine residues of its cellular targets leading to the regulation of its target gene expression and protein activity. SIRT1 has been shown to play a key role in many cellular processes, including cell longevity, differentiation, anti-apoptosis, cellular senescence, ageing, metabolism, DNA damage, oxidative stress and cell survival (Yuan et al., 2013).

1.4.3.2 SIRT2

SIRT2 is mainly present in the cytoplasm and regulates many of the cytoplasmic substrates, including α-tubulin which has a potential role in microtubule dynamics. However, it has also been reported that SIRT2 can shuttle into the nucleus and deacetylate histone proteins as well as transcription factors, such as p53 and FOXO proteins (Dryden et al., 2003; Peck et al., 2010; Wang et al., 2007a). SIRT2 is involved in many cellular functions, such as cell cycle progression, cell death and stress response. One function of SIRT2 in the regulation of cell cycle progression, especially during the G2/M phase is to deacetylate histone 4 on lysine 16 (H4K16), leading to a close conformation of chromatin and preventing the binding of transcriptional factors (Dryden et al., 2003; Vaquero et al., 2004). In agreement, the acetylation levels of H4K16 in *Sirt2*-deficient mouse embryonic fibroblasts (MEFs) remain high during mitosis, resulting in a delay in S phase.

1.4.3.3 SIRT3-5

SIRT3, SIRT4, and SIRT5 are mitrochondrial sirtuins that act as oxidative sensors. They are primarily involved in the regulation of energy metabolism, apoptosis as well as ageing by modulating protein acetylation (predominantly SIRT3 and SIRT5) and also mono-ADP-ribosylation (primarily SIRT4) (Olmos et al., 2011b).

Many mitochondrial proteins, including acetyl-coenzyme A synthetase, isocitrate dehydrogenase 2 (IDH2), glutamate dehydrogenase (GDH) and manganese superoxide dismutase (MnSOD) have been shown to be deacetylated by SIRT3, suggesting a crucial role of SIRT3 in the regulation of energy metabolism and redox activities (Yuan et al., 2013).

SIRT4 exhibits NAD^+ -dependent adenosine diphosphate (ADP)-ribosyltransferase activity to catalyse the transfer of ADP-ribosyl group onto its targets. The modulation of cellular metabolic function and fatty acid oxidation has been shown to be a function of SIRT4 (Haigis et al., 2006; Nasrin et al., 2010; Yuan et al., 2013). SIRT4 also functions together with SIRT3 to maintain the level of mitochondrial NAD^+ in response to genotoxic stress, resulting in the promotion of cell survival (Yang et al., 2007; Yuan et al., 2013).

SIRT5 initiates the urea cycle for ammonia detoxification and disposal by deacetylating carbonyl phosphate synthetase 1. It also displays NAD^+ dependent lysine demalonylase and desuccinylase activities to remove malonyl and succinyl groups from its targets, including GDH, carbonyl phosphatase synthetase 1, pyruvate dehydrogenase, succinate dehydrogenease, suggesting an important role of SIRT5 in the regulation of metabolic pathways (Du et al., 2011; Park et al., 2013; Peng et al., 2011; Yuan et al., 2013).

1.4.3.4 SIRT6

SIRT6 is a chromatin-associated nuclear protein and is found in the heterochromatic region of the nuclear fraction. SIRT6 protein has two isoforms in both human and mouse. One is full-length with an intact catalytic domain. The other is a shorter isoform that the amino acids (197-205) from the catalytic domain are missing. Therefore, the function of SIRT6 is generally based on the full-length form (Tennen et al., 2010).

SIRT6 displays both deacetylase and mono-ADP-ribosyltrasnferase activities (Liszt et al., 2005; Michishita et al., 2008). Recently, it has been shown to display the ability to remove long-chain fatty acyl group from lysine residue as a part of tumour necrosis factors (TNF)-α protein regulation to promote its secretion (Jiang et al., 2013; Yuan et al., 2013).

The high levels of SIRT6 expression have been found in the brain, muscle, liver, heart, and thymus gland, suggesting a role for SIRT6 in the transcriptional regulation in tissue-specific manner (Beauharnois et al., 2013; Pereira et al., 2012).

The functions of SIRT6 include the regulation of metabolism, inflammatory reaction, aging, transcription, apoptosis and stress resistance. It has been documented that *Sirt6* deficient mice display genomic instability, metabolic defects and degenerative pathologies associated with ageing. *Sirt6*$^{-/-}$ mice are also hypersensitive to DNA damaging agents (Mostoslavsky et al., 2006). These mice die around 4 weeks after birth due to lethal hypoglycaemia which is a direct consequence of the suppression of glycolytic genes via the deacetylation of H3K9 by Sirt6 (Kawahara et al., 2009; Michishita et al., 2008; Zhong et al., 2010). SIRT6 also has a crucial role in telomere maintenance and functions. It has been shown that SIRT6-depleted cells exhibits

pre-mature cellular senescence, telomere dysfunction and end to end fusion of chromosome (Beauharnois et al., 2013; Dimauro and David, 2009). Additionally, SIRT6 also contributes to the energy balance maintenance by regulating of metabolism. SIRT6 deacetylates the general control non-repressed protein5 (GCN5). This facilitates the acetylation of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α), resulting in the repression of glucose production in liver cells (Beauharnois et al., 2013; Dominy et al., 2012). In the immune response system, SIRT6 employs a similar set of cellular targets as SIRT1. This includes the negative regulation of the transcriptional activity of NF-κB by deacetylation of H3K56Ac. As a consequence, there is a reduction of pro-inflamatory cytokine production and a reduction in the expression of anti-apoptotic genes.

In human cells, SIRT6 is a key factor for maintaining a normal replicative lifespan and for preventing pre-mature ageing. Moreover, SIRT6 is shown to have a role in the regulation of DNA damage repair, as it has been found to be involved in BER (Mostoslavsky et al., 2006) and promotion of DSB repair through both NHEJ and HR (Kaidi et al., 2010; Mao et al., 2011; McCord et al., 2009). In order to enable DNA repair, SIRT6 is recruited to DSB sites where it interacts and stabilizes DNA-PK, another DSB repair factor (McCord et al., 2009). SIRT6 also promotes DNA end resection, a crucial step in DSB repair by HR, through C-terminal binding protein CtIP deacetylation (Kaidi et al., 2010) and mono-ADP-ribosylation of PARP1, enhancing DSB repair under oxidative stress (Mao et al., 2011).

1.4.3.5 SIRT7

SIRT7 is a nuclear protein that has been shown to be highly specific for H3K18 deacetylation and involved in the maintenance of transformed phenotypes of cancer

cells (Barber et al., 2012; Yuan et al., 2013). SIRT7 is also a positive regulator of RNA Pol I in the modulation of ribosomal gene (rDNA) transcription (Ford et al., 2006; Michishita et al., 2005). SIRT7 has been found to deacetylase p53 and is likely to be involved in cell survival and the development of resistance to genotoxic drugs (Vakhrusheva et al., 2008). Moreover, SIRT7 depletion leads to a marked reduction in tumorigenicity of human cancer cell xenografts in mice, suggesting a role of SIRT7 in the regulation of chromatin structure, cancer cell transformation and tumorigenesis *in vivo* (Barber et al., 2012; de Oliveira et al., 2013).

1.5 Sirtuins and their targets in cancer

As SIRTs exhibit a broad range of cellular functions, their imbalance could represent a potential cause for the development of many human diseases, including cancer, diabetes, cardiovascular and neurodegenerative diseases. However, contradictory roles of SIRTs in cancer have been reported. Some studies have shown that SIRTs act as a tumour suppressor by regulating DNA damage repair leading to genomic stability. Conversely, other studies argue that SIRTs enhance cell survival under stress conditions could favour tumorigenesis and cancer progression, thus implicating that sirtuins also have an oncogenic role.

1.5.1 SIRT1 and cancer

SIRT1 has been found to be upregulated in a board spectrum of cancers, such as lymphomas, leukaemia and soft-tissue sarcomas (Chang-Su, 2007; Fraga et al., 2007a; Fraga and Esteller, 2007). Overexpression of SIRT1 has been reported to be involved in the downregulation of tumour suppressor genes, as well as in the

development of drug resistance, including ionising radiation (Chu et al., 2005; Matsushita et al., 2005; Olmos et al., 2011b; Pruitt et al., 2006).

SIRT1 regulates a wide range of targets. The tumour suppressor p53, has been found to be one of the cellular substrates of SIRT1. p53 has a crucial role in cell cycle regulation and apoptosis. Many studies have reported that SIRT1 can bind to and deacetylase p53 (Brunet et al., 2004; Reagan-Shaw and Ahmad, 2007). The acetylation of p53 has been shown to increase its protein stability, nuclear localisation and transcriptionally activation independent of its phosphorylation status (de Oliveira et al., 2013). Thus, the deacetylation of p53 by SIRT1 leads to the inhibition of p53 activity and the subsequent initiation of cancer.

FOXO3a has also been shown to be one of the cellular targets of SIRT1. FOXO3a acetylation has been found to be increased under stress response conditions. Moreover, the deacetylation of FOXO3a via SIRT1 leads to the shift of its transcriptional activation from proapoptotic genes (*Fas ligand* and *BIM*) to genes that regulate cell cycle arrest ($p27^{Kip1}$) and DNA damage repair (*GADD45*) (Brunet et al., 2004).

The correlation of SIRT1 and the oncogene *c-Myc* has been reported in liver cancer. These two proteins positively regulate each other in a feedback loop that promotes cancer cell proliferation by increasing the expression of Ki67 (proliferative marker) and decreasing of p53 expression (Jang et al., 2012). Additionally, SIRT1 binds to retinoblastoma (pRb), a tumour suppressor which controls cell cycle regulation via its interaction with E2F1. The formation of SIRT1-Rb complexes leads to the inhibition of pRb activity, resulting in the promotion of cell cycle progression in cancer (Wong and Weber, 2007).

Ku70 is a well-known DNA repair factor for DSBs. The acetylation of Ku70 is controlled by CBP and PCAF and has been found to be increased in response to ionising radiation, oxidative stress and chemotherapeutic agents, and resulted in the transactivation of proapoptotic genes, such as *Bax* (Cohen et al., 2004). SIRT1 also mediates the deacetylation of Ku70 in response to stress, suggesting a role for SIRT1 in promoting cell survival and inhibiting apoptosis in mammalian cells.

In breast cancer, SIRT1 overexpression has been found in chemoresistant cells, compared with their drug sensitive counterparts (Chu et al., 2005; Matsushita et al., 2005). Additionally, it has been proposed that the ability of SIRT1 to activate stress response and DNA repair could allow cancer cells to counteract the oxidative and genotoxic stress caused by chemotherapeutic agents (Alcaín and Villalba, 2009; Olmos et al., 2011b). Interestingly, some studies have revealed that downregulation of SIRT1 using small interfering RNA–mediated molecule (siRNA) can partially reverse the resistant phenotype of cancer cells by repressing the expression of P-glycoprotein, a marker for multidrug resistance. In contrast, ectopic overexpression of SIRT1 can increase the expression of P-glycoprotein and confer resistance to doxorubicin treatment by cancer cells (Chu et al., 2005; Olmos et al., 2011b).

It has also been documented that the repression of SIRT1 expression is regulated via two p53 binding sites on its promoter (Chen et al., 2005; Olmos et al., 2011b). Therefore, loss of p53 function is predicted to enhance SIRT1 expression, leading to an increase in drug resistance in many cancer cells, including breast cancer (Figure 1.3).

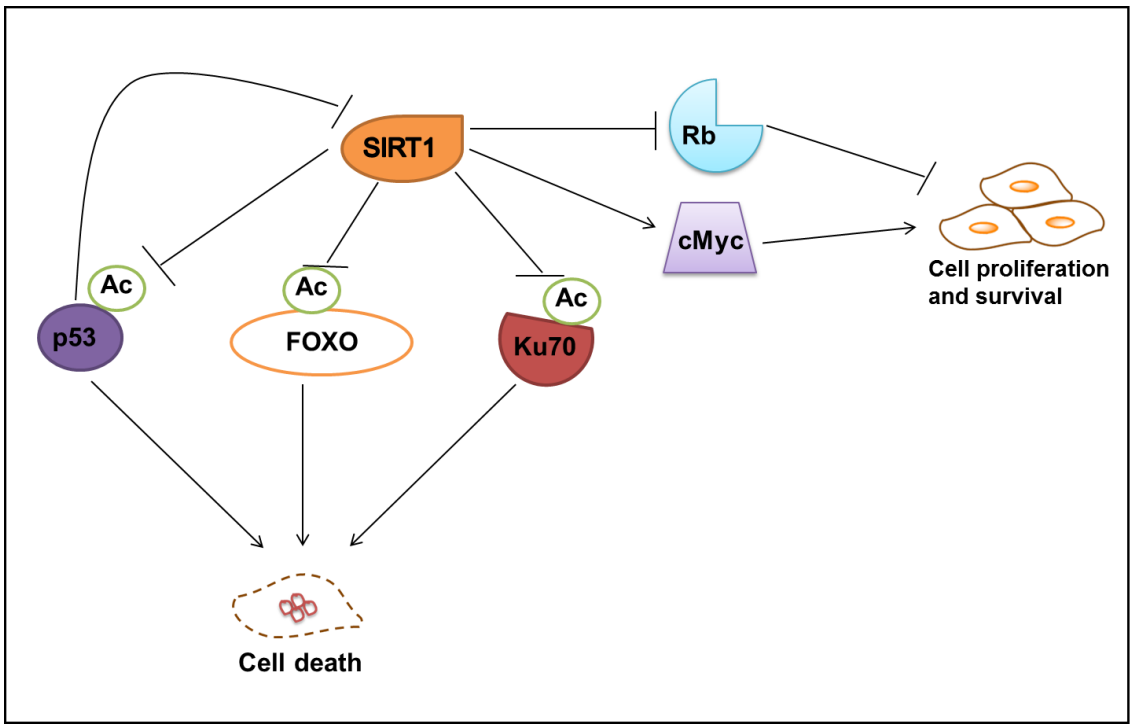


Figure 1.3 Schematic representation of the regulation of SIRT1 and the involvement of its downstream targets in cancer summary

1.5.2 SIRT2 and cancer

The overexpression of SIRT2 has been reported to prolong the M phase and cause delays to the mitotic exit of the cell cycle (Olmos et al., 2011b). Moreover, SIRT2 expression has been found to be downregulated in many cancers, including human glioma, breast cancer, head and neck squamous cell carcinoma (Cleveland et al., 2003; Hiratsuka et al., 2003; Olmos et al., 2011b). SIRT2 has been proposed to act as a G2/M mitotic check point regulator preventing chromosomal instability (Inoue et al., 2006), suggesting a role for SIRT2 as a tumour suppressor. Additionally, SIRT2 depletion results in an induction of mitotic regulators such as the Aurora kinase A and -B that drive mitotic catastrophes (Cha and Kim, 2013). Furthermore, it has also been reported that SIRT2 regulates NF-κB-dependent gene expression by deacetylating p65 at Lys310 in the cytoplasm (Rothgiesser et al., 2010). In fact, p65

is hyperacetylated at the same residue in *Sirt2*$^{-/-}$ cells upon TNF-α stimulation, which in turn enhances the expression of specific, acetylated p65-dependent target genes, such as *Mpa21*, *Ip10* and *Ccl20*.

SIRT2 also has a role in tumourigenesis and cancer progression. Studies have shown that SIRT2 downregulation in glioma cells leads to the induction of cell death via both necrosis and apoptosis (He et al., 2012; Yuan et al., 2013). Additionally, the inhibition of SIRT2 results in apoptosis of Hela cells by increasing the accumulation of p53 (Li et al., 2011). SIRT2 together with HDAC6 potentiates cancer cells migration and invasion by targeting contactin in the bladder (Zuo et al., 2012). Furthermore, SIRT2 is upregulated in neuroblastoma and pancreatic cancer. In these cancers, SIRT2 helps to stabilise the oncogenes, *C-Myc* and *N-Myc* by repressing the expression of ubiquitin-protein ligase NEDD4 (Liu et al., 2013).

The result of SIRT2 overexpression has been shown to increase drug resistance, especially in response to microtubule inhibitors including paclitaxel in cancer cells (Matsushita et al., 2005). To date no publication has reported that SIRT2 on its own is able to deacetylate p53. However, there has been a report of the functional redundancy between SIRT1 and SIRT2 by targeting p53 and its family members. In this study, the authors have identified inhibition of both SIRT1 and SIRT2 simultaneously is required in order to increase the acetylation status of p53 in breast cancer cells (Peck et al., 2010). Moreover, the downregulation of SIRT1 and SIRT2 can lead to a significant increase in the activation of p53 and its related factor p73 in response to ionising radiation, suggesting a role for SIRT1 and SIRT2 in the inhibition of p53 in DNA damage response (Bosch-Presegue and Vaquero, 2011). FOXO proteins have also been shown to be targeted by SIRT2. SIRT2 deacetylates

FOXO3a leading to an increase in the expression of the FOXO target gene *p27^{Kip1}*, *SOD2* (*MnSOD*), and *BIM* (Wang et al., 2007b). The induction of the mitochondrial *MnSOD* results in a reduced number of ROS induced by chemotherapeutic agents. This could explain the role of SIRT2 in conferring resistance in cancer cells. However, when the oxidative stress levels are highly elevated, SIRT2 could induce cell death by upregulating the FOXO1-induced proapoptotic gene, *BIM* (Olmos et al., 2011b). In line with this, the deacetylation of FOXO1 has also been shown to be mediated by SIRT2, and was shown to have a role in the adipocyte differentiation (Jing et al., 2007). In human cancer cells, cytoplasmic FOXO1 is primarily acetylated in response to oxidative stress. In turn, this acetylated FOXO1 specifically binds to Atg7, an E1-like protein, which leads to the induction of autophagy, cell death and the prevention of cancer initiation (Zhao et al., 2010). All of these events suggest that SIRT2 can act as a tumour suppressor and a modulator for cancer progression by the modulation of the function of FOXO1.

1.5.3 SIRT6 and cancer

Due to its ability to maintain genomic stability and DNA repair, SIRT6 have been proposed to act as a tumour suppressor in human cells. As expected, SIRT6 is found to be downregulated in many cancers, including pancreatic, colon and lung cancers (Cha and Kim, 2013; Sebastian et al., 2012). It has been reported that SIRT6 prevents liver cancer initiation by repressing the expression of survivin through the deacetylation of H3K9 and the inhibition of NF-κB (Min et al., 2012). In agreement with this finding, the lower expression of SIRT6 has been identified in cirrhotic livers and liver cancer compared to normal liver cells via microarray, confirming that the

downregulation of SIRT6 leads to the development of liver cancer (Marquardt et al., 2013). SIRT6 also targets HIF1α, leading to the modulation of its targets in the regulation of glycolysis. In this study SIRT6 was shown to act as a tumour suppressor by repressing the transcriptional activities of *Hif1α* and oncogene *C-Myc*. This suggests that SIRT6 is involved in tumourigenesis by regulating cancer cell metabolism (Cha and Kim, 2013). Moreover, it has been demonstrated that SIRT6 mediates the secretion of TNF-α by removing the fatty acyl group on Lys9 and Lys 20 of TNF-α, further supporting the tumour suppressive function of SIRT6 in cancer cells (Cha and Kim, 2013; Zhong et al., 2010).

Conversely, SIRT6 is also found to be involved in cancer progression. SIRT6 was shown to facilitate cytokine production and the migration in pancreatic cancer cells. SIRT6 increases the expression of pro-inflammatory cyto- and chemo-kines, such as IL8 and TNF-α, and promotes cell migration as well as angiogenesis by enhancing Ca^{2+} response (Bauer et al., 2012). This study suggests that the inhibition of SIRT6 can help to prevent cancer progression. Similarly, SIRT6 mRNA levels have been found to be upregulated in chronic lymphocytic leukaemia (CLL) patients compared to healthy ones, further supporting the involvement of SIRT6 in cancer initiation (Wang et al., 2011).

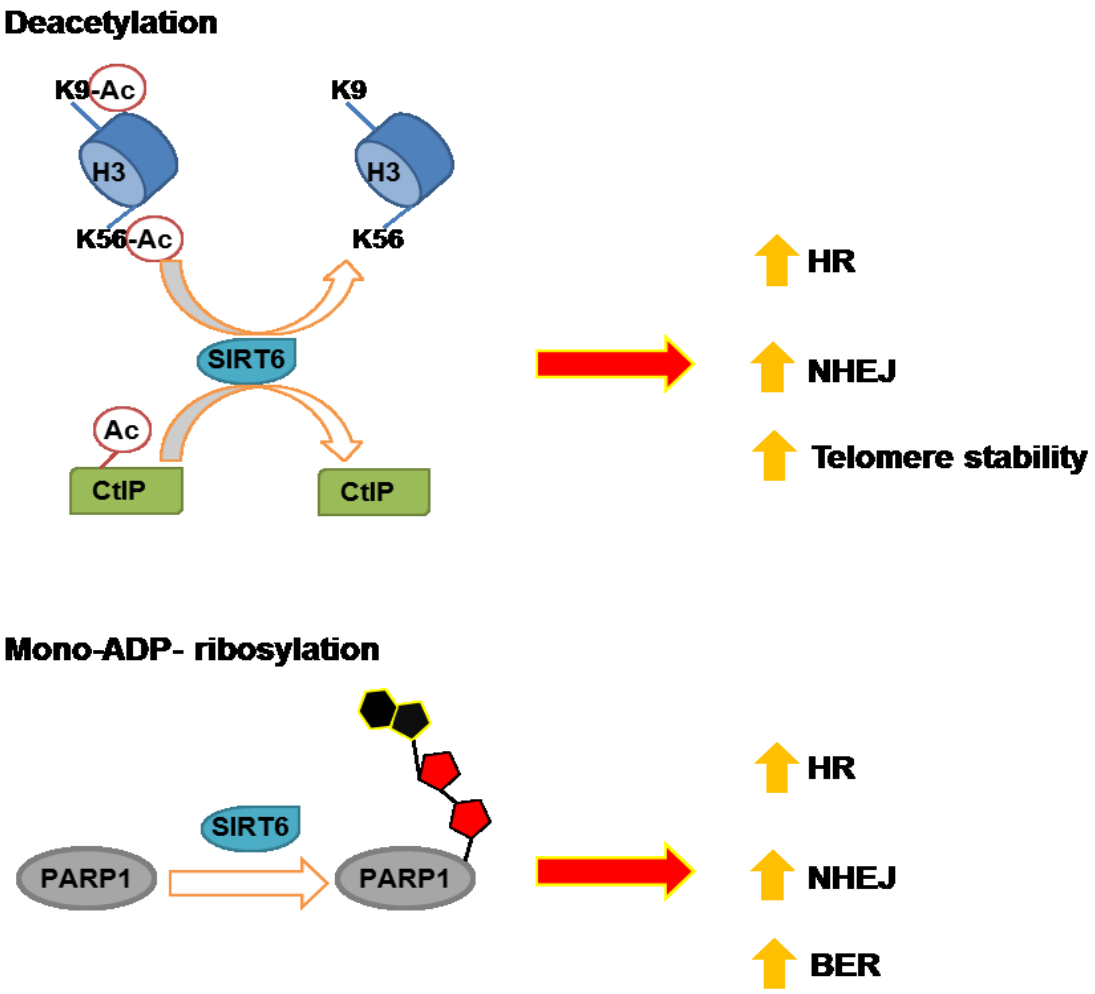


Figure 1.4 Schematic representation of the involvement of SIRT6 in DNA damage repair pathways (Adapted from (Van Meter et al., 2011). Ac: Acetyl group (-$CoCH_3$)

Moreover, SIRT6 has been proposed to be involved in the development of drug resistance in cancer cells due to its ability to control DNA damage repair and metabolism (Figure 1.4). *Sirt6*$^{-/-}$ MEFs exhibit greater sensitivity to DNA damaging agents when compared with WT MEFs (Mostoslavsky et al., 2006). Furthermore, SIRT6 regulates the deacetylation of H3K9 at *NF-kB* promoter to silence the activity of its target genes that induce cellular senescence and apoptosis in response to genotoxic agents (Cardus et al., 2013).

1.6 Tumour suppressors

Tumour suppressors are proteins that prevent normal healthy cells from transforming into cancer cells. Their activation leads to the activation of normal cellular processes, including cell proliferation arrest, DNA damage response, cellular senescence and apoptosis. Mutations or inactivation of these sets of genes often result in cancer initiation and progression.

1.6.1 p53

p53 is the best studied tumour suppressor gene, as its mutation has been found in more than half of all human cancers. In mammalian cells, p53 displays its primary functions in response to cellular stress by maintaining genomic stability through the activation of cell cycle arrest, DNA damage repair, cellular senescence and apoptosis (Gu and Roeder, 1997; Luo et al., 2001). In fact, both the loss of p53 function and its mutation have been reported to be involved in the development of many human tumours, including those of brain, breast, and adrenal gland origins (Lee et al., 2001).

Like other proteins, the function of p53 is modulated by its activation status. Thus, the dysregulation of p53 can lead to a decrease in its function, resulting in the initiation of cancer. The E3 ubiquitin ligase murine double minute2 (MDM2) has been demonstrated to negatively mediate p53 cellular expression, by blocking its *trans*activation domain, initiating its ubiquitination and subsequently its proteasomal degradation (Vassilev, 2007; Vousden, 2002). In response to DNA damage, p53 is dissociated from MDM2 binding, leading to an increase in its activation as well as its cellular expression. This results in the *trans*activation of its targeted genes involved

in DNA damage repair. Moreover, p53 also participates in the transcriptional regulation of MDM2, which is in turn negatively regulated by itself. This feedback regulation is in place to restrain the hyperactivation of p53 (Batchelor et al., 2009).

Since MDM2 functions as a negative regulator of p53, it has been suggested that this protein is a proto-oncogene by repressing the activity of p53. In agreement, MDM2 is found to be upregulated in 7% of human tumours (Momand et al., 1998).

The mutations of p53 are frequently identified in cancers, denoted as tumour protein (tp53). These mutations generally occur in two main regions, 95% on the DNA binding domain (DBD), and the other 5% corresponding to mutations at C- and N-termini (Vousden and Lu, 2002). The majority of DBD mutations are missense mutations, generally producing full-length p53 proteins in which their DNA binding, protein structure and *trans*activation domains are affected. This results in the loss of function by p53 as a genomic stability regulator and causes the accumulation of DNA damage, promoting cancer development and progression (Brown et al., 2009).

1.6.2 p53 regulation and cancer

The regulation of p53 expression is tightly controlled by different posttranslational modifications, such as phosphorylation, acetylation, and ubiquitination, which will ultimately modulate its transcriptional activity, stability and function. p53 is the first non-histone protein that was shown to be regulated by acetylation and deacetylation (Gu and Roeder, 1997; Luo et al., 2001). Wild-type p53 is activated by acetylation which is predominantly regulated by CBP/p300 and is generally triggered by different environmental stress stimuli, also including activation of oncogenes (Goodman and Smolik, 2000). The acetylation of p53 at eight different key lysine residues has been

shown not to affect its DNA-binding ability; however, this help to promote a dissociation of p53 and MDM2 binding, resulting in increased *trans*activation potential of p53. This indicates that acetylation enhances the function of p53 by stabilising its expression and increasing its *trans*activation activity (Carter and Vousden, 2009). Specifically, acetylation of p53 at Lys320 and Lys382 has been proved to increase its protein stability as well as its *trans*activation to induce genes involved in apoptosis (Carter and Vousden, 2009).

p53 can be also deacetylated causing the repression of its transcriptional activities by distinct HDACs, including SIRT1 and SIRT2. It has been shown that SIRT1 directly deacetylates p53 at Lys382 of its C-terminus (Luo et al., 2001; Vaziri et al., 2001). The interaction of SIRT1 and p53 generates a decrease in anti-proliferative function of p53, thus leading to tumorigenesis and development of drug resistance in cancer cells (Luo et al., 2001). Conversely, the induction of mutant SIRT1 expression in cancer cells can re-sensitise these cells to the cytotoxic effects of chemotherapeutic drugs (Luo et al., 2001; Vaziri et al., 2001). In agreement with this finding, it has been reported that hyperacetylation of p53 in *Sirt1*-deficient mice enhances the sensitivity of radiation-induced apoptosis (Cheng et al., 2003) suggesting a role for SIRT1 as an inhibitor of p53-mediated apoptosis.

Since p53 activation in response to genotoxic agent can be inhibited by SIRT1 and SIRT2, it has been suggested that a SIRT inhibitor could be a potential candidate for anticancer drug treatment (Olmos et al., 2011b). The use of tenovins, a specific inhibitor of SIRT1 and SIRT2, results in the induction of p53 transcriptional activity and subsequent reduction in the proliferation of cancer cells (Lain et al., 2008b). Additionally, sirtinol and salermide, the pan-inhibitors of SIRTs with higher specificity

for SIRT1/2, have been reported to increase the sensitivity of breast cancer cells to the cytotoxic effects of chemotherapeutic drugs by increasing the acetylation of p53 and causing cell cycle arrest and cell death (Peck et al., 2010).

1.7 Forkhead box protein family

Forkhead box (FOX) is a superfamily of proteins with a conserved transcriptional regulatory region defined by a common DBD known as the forkhead box or winged helix domain (Kaestner et al., 2000). To date, 19 subfamilies of this superfamily, ranging from FOXA-S have been identified. Despite the highly conserved forkhead box DBD, the functions and the activities of FOX proteins differ between the subfamilies due to differences in the other domains (Myatt and Lam, 2007). This family of proteins has a wide range of roles in biological processes, including development, metabolism, differentiation, apoptosis and longevity. Because of their functions, the deregulation of FOX proteins has been proposed to play a role in tumorigenesis and cancer progression (Lehmann et al., 2003). For example, FOXM1 proteins are crucial for G2/M cell cycle progression. The loss of *Foxm1* in mice is embryonic lethal. In the cancer biology context, ectopic overexpression of FOXM1 accelerates cell growth and tumour progression. Moreover, FOXM1 is involved in DNA damage repair by regulating the MRN complexes to activate DNA repair via HR (Khongkow et al., 2013b; Monteiro et al., 2013). Therefore, FOXM1 has been proposed to act as an oncogene and leads to the development of drug resistance in breast cancer. Conversely, the activation of FOXO3a leads to the regulation of cell cycle arrest and apoptosis in response to drug treatment. As a result, FOXO3a has been proposed to be a tumour suppressor.

1.7.1 FOXOs

Mammalian Forkhead box O (FOXO) is the orthologue of Daf-16, the only isoform that have been identified in *C. elegans*. FOXO belongs to of the subfamilies of the FOX superfamily, which is constituted by four members in mammals (FOXO-1, -3a, -4 and -6). These proteins share similarities in their DBD sequences and other functional motifs (van der Horst and Burgering, 2007). FOXO proteins are primarily described as potent transcriptional activators through their binding to the conserved motif, TTGTTAC (Calnan and Brunet, 2008; Furuyama et al., 2000). FOXO proteins have a variety of cellular functions, including cell proliferation, apoptosis, differentiation and resistance to oxidative stress and DNA damage (Arden, 0000; Burgering, 2008; Fu and Tindall, 2008; Ho et al., 2008; Myatt and Lam, 2007). FOXO-induced cell cycle arrest plays a crucial role in tumour suppression, whereas FOXO mediated stress resistance has an important function towards life span extension, especially in *C. elegans*. Moreover, FOXOs are also involved in the development as well as the regulation of cell differentiation in blood, muscle and adipose tissues (Calnan and Brunet, 2008). Finally, FOXO proteins also have a metabolic role through the regulation of gluconeogenesis and food intake (Salih and Brunet, 2008).

1.7.2 FOXO protein modifications and its function

FOXO proteins have a wide spectrum of cellular functions in specific tissues and in response to different stimuli. FOXO3a has been proposed to function as a tumour suppressor due to its ability to control cell cycle arrest and cell death. Particularly, the loss of FOXO3a may result in a decrease in the ability of cells to arrest at key cell cycle checkpoints, contributing to the development of tumours (Bouchard et al.,

2007). Therefore, the FOXO3a protein has been suggested to be a new therapeutic target for many cancers (Karen C, 2006). However, mutations to the *FOXO* gene have seldom been identified in human cancers (Bois and Grosveld, 2003; Karen C, 2006).

FOXO transcriptional factors are regulated by a wide range of external stimuli including insulin, insulin-like growth factor (IGF-1), nutrients, cytokines and oxidative stress. These stimuli cause changes in the expression of FOXO, its cellular localisation, DNA-binding and transcriptional activity. Additionally, the expression and activity of FOXO proteins is also tightly regulated by post-transcriptional modifications, including phosphorylation, acetylation/deacetylation and ubiquitination. Thus, loss of FOXO or disruption in these modifications could play a role in carcinogenesis (Weidinger et al., 2008). Some evidence suggests that FOXO proteins, especially the FOXO3a isoform, have an important role in anti-cancer drug sensitivity by mediating the cytostatic and cytotoxic effects of different chemotherapeutic agents, including taxanes, platinum compounds, anthracyclines and molecular targeted therapeutics (Fernández de Mattos et al., 2004; Olmos et al., 2011b). Conversely, it has also been proposed that FOXO may be involved in the development of acquired drug resistance. For example, the high levels of active nuclear FOXO3a found in multi-drug resistant leukaemic cells induced survival signals leading to the development of drug resistance (Hui et al., 2008). However, the mechanism underlying this resistant is still unclear. It has been suggested the distinct changes in its post-translational modifications seem to play a role in the development of drug resistance.

1.7.2.1 Phosphorylation/dephosphorylation of FOXOs

FOXOs are negatively regulated by PI3K-Akt signalling pathway. In the presence of Insulin, growth factors and cytokines, FOXOs are phosphorylated at three conserved sites by the Akt and SGK protein kinases. This phosphorylation leads to the cytoplasmic sequestration of FOXOs which disables the transactivation of their downstream target genes and accelerates the degradation of FOXO proteins. Phosphorylation of FOXOs also facilitates chaperone protein 14-3-3 binding, resulting in the prevention of FOXO nuclear reentry and the initiation of polyubiquitination with subsequent proteasome-dependent protein degradation. So these modifications lead to a completely deactivation of FOXO proteins. To activate FOXOs and recruit them into nucleus, dephosphorylation by protein phosphatases is required. The characteristics of phosphatases that dephosphorylate FOXOs at Akt/SGK sites have not been fully identified. However, it has been shown that Protein phosphatase 2A (PP2A) is one of the candidate phosphatases responsible for FOXO3a dephosphorylation (Calnan and Brunet, 2008; Singh et al., 2010).

In contrast to FOXOs phosphorylation by Akt/SGK, phosphorylation of FOXOs by Mammalian Ste20-like kinase (MST1) and c-Jun kinase (JNK1) under oxidative stress causes FOXO nuclear localisation even in the presence of growth factors/cytokines. This nuclear relocalisation is also independent of the dephosphorylation at Akt/SGK sites. For example, MST1 phosphorylates FOXO3a at Ser207 and leads to the disruption of 14-3-3 binding; as a result FOXO3a relocates from cytoplasm into the nucleus (Lehtinen et al., 2006). FOXO4 is also phosphorylated by JNK at Thr447 and Thr451, leading to its nuclear relocalisation under oxidative stress (Essers et al., 2004). Moreover, JNK can also phosphorylate

14-3-3 resulting in the dissociation of FOXO3a from the 14-3-3 anchors and its nuclear translocation (Sunayama et al., 2005).

1.7.2.2 Ubiquitination of FOXOs

FOXOs are also regulated by ubiquitination, which can be either reversible monoubiquitination or irreversible polyubiquitination. For instance, FOXO4 nuclear translocation is triggered by oxidative stress. The activation of FOXO4 transcription activity is initiated by monoubiquitination at Lys199 and Lys211 (van der Horst et al., 2006).

Polyubiquitination of FOXOs is an irreversible process since it leads to proteasome-dependent protein degradation (Vogt et al., 2005a). For instance, FOXO1 is phosphorylated by AKT, resulting in its nuclear exclusion and subsequently to an induction of the F-box protein Skp2 binding (E3 ubiquitin ligase), facilitating its polyubiquination and proteasome degradation.

FOXO3a is also regulated by proteosome degradation via the E3 ubiquitin ligase mdm2. The phosphorylation of FOXO3a by ERK at the Ser294, Ser388 and Ser425 leads to its cytoplasmic translocation and results in an increase of its interaction with MDM2 to initiate polyubiquitination. FOXO3a is then degraded by the proteasome pathway (Yang et al., 2008).

1.7.2.3 Acetylation/deacetylation of FOXOs

The acetylation status of FOXOs is regulated by the opposing actions of protein acetylases-CBP (CREB-binding protein), p300 and p300/CBP-associated factors (PCAF) and protein deacetylases, including Sir2/Sirtuins (Calnan and Brunet, 2008).

Acetylation of FOXO1 by p300 and CBP occurs at Lys242, Lys245 and Lys262 (Daitoku et al., 2011). In pancreatic B-cells, the acetylation of FOXO1 increases its interaction with promyelocytic leukaemia protein (PML) and also increases its nuclear accumulation in PML bodies (Calnan and Brunet, 2008).

FOXO proteins can be also deacetylated. For instance, SIRT1 can bind to FOXO1 and FOXO3a and remove the acetyl groups which in turn down-regulate these FOXO activities. As a result, SIRT1 is a pro-survival regulator of FOXO proteins that diverts its activity away from apoptotic-induced genes (*FASL* and *BIM*) towards genes involved in stress resistance (*MnSOD* and *GADD45*) and cell cycle arrest ($p27^{Kip1}$) (Vogt et al., 2005a).

The crosstalk between post-translation modifications (PTMs) of FOXOs results in the different range of functions of FOXOs in the regulation of its downstream targets.

1.7.3 FOXO protein regulation

FOXO protein degradation is initiated by Akt phosphorylation in response to insulin, growth factors and cytokines. In the cytoplasm, FOXOs are then polyuquitinated by the activity of the E3 ubiquitin ligase enzyme family; for example, the SCFskp2 (Skp1/Cu11/F-box) polyubiquitination complex targets FOXO1 (Huang et al., 2005) to undergo proteasome degradation. It has been proposed that permanent FOXO degradation is triggered by insulin stimulation and could be a potential cause of cell transformation and tumorigenesis (Hu et al., 2004b; Huang et al., 2005).

Moreover, FOXO degradation is also triggered by cytokine stimulation. FOXO3a is phosphorylated at Ser644 by I-kappa-B kinase (IKK) in response to TNF1-α culminating in its polyubiquination and protein degradation. Overexpression of

FOXO3a results in a reduction of cell transformation and cancer cell proliferation in the presence of IKK, further supporting the role of FOXO proteins as tumour suppressors (Hu et al., 2004b). Additionally, acetylation of FOXO1 induced by oxidative stress increases FOXO1 stability by preventing FOXO1 polyubiquitination (Kitamura et al., 2005). The mechanism of how FOXO1 acetylation leads to the enhancement of FOXO1 stability by preventing FOXO1 degradation is still unclear, as the lysine residues that are responsible for these two modifications are different.

With respects to the DNA binding activity, phosphorylation of FOXOs (such as the phosphorylation of Ser256 of FOXO1) by Akt leads to a decrease in the FOXO-DNA interaction (Zhang et al., 2002). This is because phosphorylation introduces a negative charge to FOXO leading to a neutralisation of positive charge in the DNA binding domain, thus resulting in the inhibition of FOXO-DNA binding. Acetylation of FOXOs also decreases their DNA binding capacity (Matsuzaki et al., 2005). Generally, acetylation of transcription factors leads to a change in their activity; however, this depends on the functional regions that are acetylated (Chen et al., 2001; Daitoku et al., 2011). Three acetylation sites of FOXO1 are located in wing 2 (W2) in C-terminus of the forkhead domain. This C-terminus is directly involved in DNA recognition and stabilisation of the FOXO-DNA complexes. Therefore, neutralising the positive charges at this domain by acetylation might lead to a reduction in the FOXO-DNA binding potential (Brent et al., 2008; Lai et al., 1993; Matsuzaki et al., 2005). Similarly, acetylation of FOXO3a at Lys242 and Lys245 reduces the affinity of FOXO3a-DNA binding. According to the 2.7A^{o} crystal structure of FOXO3a DNA binding domain, the phosphate group of DNA interacts directly with Lys245. In addition, the substitution of Lys 242,245 with Ala attenuates the DNA-

binding affinity of FOXO3a, further suggesting that acetylation inhibit FOXO-DNA interaction (Tsai et al., 2007).

Moreover, acetylation of FOXO1 leads to an increase in its phosphorylation by Akt at Ser253, which further reduces the FOXO1-DNA binding interaction (Matsuzaki et al., 2005). On the other hand, deacetylation of FOXO1 and FOXO3a proteins by Sir/Sirt deacetylase extend life span in a range of organism including *C. elegans* (Kasibhatla et al., 1999; Tissenbaum and Guarente, 2001). This suggests that the modification of the DNA binding domain of FOXO proteins regulates their function to activate a specific set of target genes.

Together, all these findings suggest that the function of FOXO proteins are tightly regulated by PTMs which control their interaction with specific protein partners and target genes via the regulation of its DNA binding domain (Calnan and Brunet, 2008), (Figure 1.5).

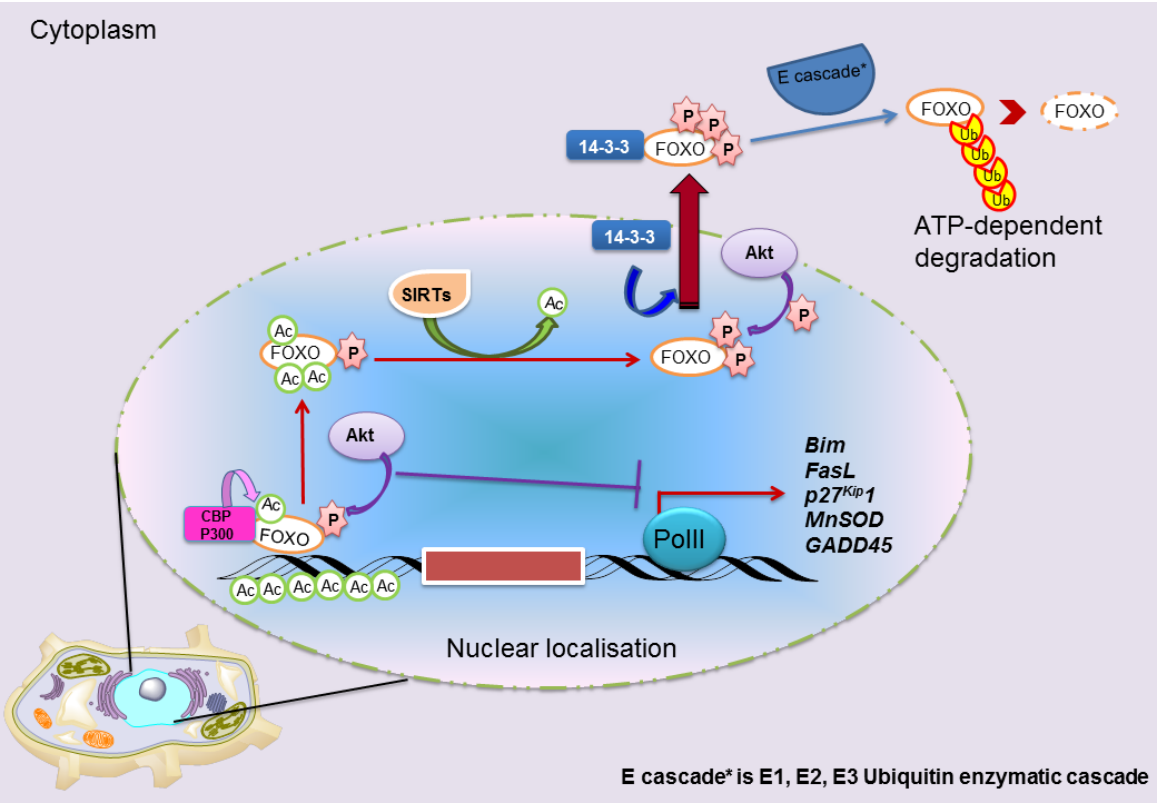


Figure 1.5 Schematic representation of the regulation of FOXOs via acetylation, phosphorylation and ubiquitination which leads to a change in FOXOs localisation and cellular functions. Acetylation activates FOXO *trans*activation on different target promoters, whereas phosphorylation inhibits the FOXO *trans*activation and promotes cytoplasmic translocation with subsequent inactivation of FOXOs via ubiquitination and proteasomal degradation.

1.7.4 FOXO functions and its targets

1.7.4.1 FOXOs in the Cell cycle

FOXOs are negative regulators of cell cycle progression. Cell cycle progression begins with G1/S transition. This transition is dependent on the activation of E2F transcription factors by phosphorylation of the retinoblastinoma proteins (pRB) and is believed to control by cyclin-D/E-CDK complexes. The complexes of CyclinD1/D2 and CDK4/6 are crucial for driving cells through the restriction point at G1 phase. Overexpression of FOXO1 and FOXO3a has been shown to repress CDK4 activity and subsequently induce G1 cell cycle arrest (Ho et al., 2008; Ramaswamy et al., 2002). Therefore, the mechanism by which FOXOs induce cell cycle inhibition at G1 phase is through the down-regulation of D-type cyclins activity (Ramaswamy et al., 2002). Additionally, FOXO proteins indirectly inhibit G1/S transition by upregulating the expression of the transcriptional repressor, Bcl-6, of the *cyclin D2* gene (Fernandez de Mattos et al., 2004).

Moreover, the regulation of G1/S transition by FOXOs is also strongly associated with the activity of Cyclin-dependent kinase inhibitors (CKIs). CKIs consists of 2 subclasses, namely Cip/Kip class ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) and INK4 class ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) (Ho et al., 2008). The Cip/Kip class of CKIs can form heterotrimeric complexes with CDK-cyclin A, E and D to inhibit G1/S cell cycle progression, whereas INK class only targets the CDK4/6 complexes. There is accumulating evidence showing that FOXOs regulate $p21^{Cip1}$ and $p27^{Kip1}$ at the transcriptional level (Gomis et al., 2006; Katayama et al., 2007; Medema et al., 2000). $p15^{INK4b}$ and $p19^{INK4d}$ have also been reported to be downstream targets of FOXOs and induce G1 cell cycle arrest (Katayama et al., 2007). In agreement with

this finding, the deactivation of Akt signalling by PI3K inhibitor, LY294002 could not induce G1 arrest in the absence of $p15^{INK4b}$ and $p19^{INK4d}$ in mice, confirming that FOXOs regulate G1/S transition by the upregulation of both classes of CKIs (Ho et al., 2008; Katayama et al., 2007). The inhibition of cyclin-CDK complex formation can lead to a decrease in phosphorylation of the retinoblastoma protein (pRB). This will result in the downregulation of E2F transcriptional activity and lead to the inhibition of cell cycle progression. In addition, FOXO3a and FOXO4 have been demonstrated to activate *p130* gene at transcriptional level leading to the induction of cell cycle arrest and cellular quiescence (Kops et al., 2002).

G2/M transition is a crucial phase of the cell cycle when DNA damage is repaired. The deregulation of G2/M arrest is often associated with genomic instability and tumorigenesis. The role of FOXOs in the regulation of G2/M is still unclear. Yet, a study showed that FOXO proteins regulate the expression of *cyclin B1* gene, which is important for G2/M checkpoint (Alvarez et al., 2001). Conversely, later microarray results revealed that the overexpression of FOXO1 leads to the downregulation of CDK2, *cyclin B1*, *cyclin B2* and CDC2 genes at the transcrpirtional level (Takano et al., 2007). FOXOs have been shown to regulate Cyclin G2, the protein that directly induce G2/M arrest by inhibiting the formation of cyclin B/CDC2(CDK1) complexes (Martinez-Gac et al., 2004). In response to stress, FOXOs induce G2/M arrest by the induction of growth-arrest and DNA-damage inducible protein α of 45 kDa (GADD45α) to allow DNA damage repair via BER (base excision repair) (Tran et al., 2002). All of these findings suggest that FOXO proteins regulate multiple changes in cell cycle progression in cooperation with many other transcriptional factors and/or co-factors. The deregulation of the transforming growth factor β (TGFβ) superfamily has been found to be involved in tumorigenesis. TGFβ functions as a tumour

suppressor by inhibiting cell proliferation of primary malignant epithelial cells through the induction of cell cycle arrest. This cell cycle arrest is initiated by the activation of the Smad transcription factor group. The formation of Smad-FOXO3a complexes has been shown to have an important role in tumour suppression by regulating TGFβ function. These Smad-FOXO3a complexes lead to the induction of cell cycle inhibitors, $p21^{Cip1}$ gene (Seoane et al., 2004) as well as the $p15^{INK4b}$ gene (Gomis et al., 2006).

The interaction of FOXO proteins with the other Forkhead box proteins has also been reported and shown to play a role in cell cycle regulation. For example, the study of Delpuech in 2007 revealed that FOXM1 is repressed by the FOXO3a target gene, *Mxil1* (Delpuech et al., 2007). FOXM1 is a crucial protein for G2/M checkpoint by mediating many cell cycle regulators, including *polo-like kinase* (*Plks*), *cyclin A*, *cyclin B*, *CDC2* and *CDC25B* (Ho et al., 2008; Wang et al., 2002a; Wang et al., 2002b; Yoshida et al., 2007). The overexpression of FOXM1 through Gli1 transcriptional activation leads to the initiation and development of many cancers including breast, lung (Kim et al., 2006) and prostate (Ho et al., 2008; Kalinichenko et al., 2004; Teh et al., 2002). The inhibition of FOXO3a by DYRK1 also leads to the activation of FOXM1 by enhancing the transcriptional activity of Gli1 on *FOXM1* promoter (Woods et al., 2001). Additionally, FOXO-dependent induction of CKIs may cause CDK-cyclin complex inhibition, which is required for FOXM1 activation and nuclear localisation (Luscher-Firzlaff et al., 2006; Wierstra and Alves, 2006). All of these examples suggest that FOXOs negatively regulate cell cycle progression through the inhibition of FOXM1.

1.7.4.2 FOXOs in Cellular senescence

Cellular senescence has been proposed to have a crucial role in tumour suppression. The function of FOXOs in the cell cycle regulation is negatively correlated with PI3K pathway activation. Inhibition of this pathway by chemical inhibitors, wortmannin or LY294002 results in the induction of cellular senescent phenotypes in primary mouse embryonic fibroblast cells through the FOXO3a-induced *p27^{Kip1}* activation. The ability of FOXO3a to induce G0/G1 cell cycle arrest also relies on the function of p130. It was shown that p130 can compensate for the absence of p27^{Kip1}. Therefore, p27^{Kip1} and p130 are two main players that trigger FOXO-induced cellular senescence in human cells (Collado et al., 2000). In agreement with this finding, *p27^{Kip1}* and *p130* double knocked out fibroblasts display a decrease in the number of cells that undergo cellular senescence, confirming that p27^{kip1} and p130 are important modulators of FOXO-induced G0/G1 cell cycle arrest and subsequent cellular senescence (Kops et al., 2002). Moreover, a different study revealed that the repression of PI3K pathway and the activation of FOXO have a crucial role in oncogene-induced cellular senescence (Courtois-Cox et al., 2006). The hyperactivation of oncogene, *Ras in vivo* can lead to a decrease in the number of senescent population in benign tumours, further supporting the role of cellular senescence in cancer prevention. They found that the expression of constitutively activated FOXO1, in which three phosphorylated residues are mutated to alanines to prevent Akt mediated phosphorylation and nuclear export, can result in a rapid increase in cellular senescent phenotypes by the activation of pRB, a modulator of G0/G1 cell cycle arrest (Courtois-Cox et al., 2006).

1.7.4.3 FOXOs in programmed cell death or apoptosis

Apoptosis or programmed cell death is a process that plays a crucial role in the embryonic development and health maintenance in human body. It is a well-programmed process that eliminates old cells without interfering with the surrounding healthy cells. There are two main apoptotic pathways that have been identified to date: the extrinsic and intrinsic pathways.

The extrinsic pathway is crucially dependent on the activation of death receptors. Death receptor protein families are defined by the highly conserved domain, consisting of an 80 amino acid sequence, called as “death domain”. This family of proteins include Death Receptor 4 (DR4) and Fas Receptor (FasR) (Elmore, 2007). The activation of these receptors is associated with the presence of their ligands. For example, the Tumour necrosis factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL) can initiate the activation of DR4, whereas Fas Ligand (FasL) is responsible for FasR activation. The activation of these receptors leads to the induction of caspase-8 and -3-dependent apoptosis.

The intrinsic pathway is non-receptor mediated intracellular signalling process to induce apoptosis by mitochondrial activity and is located around inner membrane of the mitochondria. The initiation of this pathway is triggered by induction of proteins involved in the integrity of mitochondrial membrane such as *Bcl-2* (B cell lymphoma gene 2). Bcl-2 family consists of two main sets of members –one group that favours pro-survival and another one family are pro-apoptotic. Pro-survival genes include *Bcl-XL* and *MCL-1* that share a conserved domain, called BH4, whereas the pro-apoptotic genes include *Bim*, *Bax*, *Bad* and *Puma* and contain only the BH3 domain (Gross et al., 1999).

As mentioned earlier, FOXOs (especially the FOXO3a isoform) have been proposed to act as a tumour suppressor due to its ability to induce cell cycle arrest and apoptosis. FOXOs induce programmed cell death in a tightly regulated manner in response to different stimuli. Fas ligand has been shown to be the first target of FOXO-induced apoptosis in Jurkat T cell, as three of the putative FOXO-binding sites have been identified at its promoter (Brunet et al., 1999; Burgering and Medema, 2003). However, the later study revealed that the full-length version of *Fas* ligand promoter cannot be activated by FOXO3a in BaF3 pre-B-cell. Instead, the FOXO3a induced cell death occurs by the activation of a proapoptotic Bcl-2 family member containing a single BH3 domain, Bim (Burgering and Medema, 2003; Dijkers et al., 2002). Moreover, FOXO3a and FOXO4 have been shown to bind to *Bcl-6* promoter and activate its transcription. *Bcl-6* in turn negatively regulates the expression of the anti-apoptotic gene *BCL-XL*. Therefore, FOXOs can mediate apoptosis through the repression of *BCL-XL* by Bcl-6 (Burgering and Medema, 2003; Schmidt et al., 2002a; Tang et al., 2002). TRAIL has been shown to be a target of both FOXO1 and FOXO3a in prostate cancer, as its gene promoter contains a FOXO-responsive region (Burgering and Medema, 2003; Rokudai et al., 2002). In this study it was also found that FOXO1 partially can induce cell death via the activation of Tumour necrosis factor receptor type 1-associated DEATH domain protein (TRADD) in HT1080 human fibroblasts. The activation of the other key pro-apoptotic genes by the intrinsic pathway - mediated by the mitochondria - such as *BAD*, *CASP9* (encoding caspase 9) have been also identified as cellular targets of FOXOs (Lam et al., 2013).

1.7.5 Targeting FOXOs in cancer

FOXO transcription factors have been proposed to be "*bona fide"* tumour suppressors. The involvement of FOXOs in human cancers was found by their involvement in chromosomal translocation. For example, *FOXO1*, *FOXO3a* and *FOXO4* genes have been found in some break points of chromosomes in leukaemia such as myeloid/lymphoid or mixed lineage leukaemia (MLL) and alveolar rhabdomyosarcomas. In rhabdomyosarcomas, *FOXO1* gene has been found to be targeted by the t(2,13) (q35;q14) and t(1,13)(p36;q14) chromosomal translocations. *FOXO3a* has been identified at t(6;11)(q21;q23) the chromosomal translocation in acute myeloid leukaemia, whereas *FOXO4* was identified at t(x;11)(q13;q23) in the patients. Additionally, further studies have revealed that the fusion of MLL-FOXO4 leads to the repression of wild-type FOXO function in the remaining intact allele. Together, all of these findings propose that the loss of FOXO function by gene chromosomal alterations leads to the development of cancer (Borkhardt et al., 1997; Davis et al., 1994; Zhang et al., 2011).

FOXO overexpression inhibits both cancer cell proliferation *in vitro* and tumour size *in vivo* in breast cancer. Moreover, nuclear FOXO expression is correlated with a better survival in breast cancer patients (Hu et al., 2004a; Yang et al., 2008; Zhang et al., 2011). Cell growth is inhibited by nuclear-FOXO induced cell cycle arrest and apoptosis, which further supports the role of FOXOs as tumour suppressors.

The crosstalk between FOXO and p53 is another other key mechanism for controlling cell cycle progression of cancer cells. FOXOs control the metabolic stability by the regulation of cell cycle arrest, cell death and cellular oxidative. Similarly, the tumour suppressor p53 is also involved in the same pathway to

regulate DNA repair and to insure genomic stability (Dansen and Burgering, 2008; Zhang et al., 2011). FOXOs and p53 regulate cell cycle arrest by the induction of their similar target genes, such as *p21*$^{WAF/Clip1}$ and *p27*^{Kip1} (Levine et al., 2004; Zhang et al., 2011). There are various FOXO target genes also regulated by p53, including *p16*^{INK4b}, *p21*^{Clip1}, *GADD45*, *WIP1* and *PA26*, suggesting that FOXOs and p53 function together for the tumour suppressive response (Fu and Tindall, 2008; Zhang et al., 2011). The repression of *Sirt1* gene is mediated by the binding of p53 to two sites at the promoter region of the *Sirt1* gene. In response to nutrient deprivation, this repression is attenuated by releasing of FOXO-p53 binding, independently of the presence of FOXO-binding site in the *Sirt1* promoter (Calnan and Brunet, 2008). Moreover, the interaction of FOXO and p53 has been found *in vitro* in response to stress stimuli. Collectively, these findings suggest that FOXO and p53 work together as tumour suppressors in similar pathways by regulating sets of overlapping target genes.

PTEN is the other tumour suppressor that has been found to be mutated in many cancers. The PI3K/Akt signalling pathway is negatively regulated by PTEN. Thus, the loss of PTEN results in the hyperactivation of PI3K/Akt pathway and subsequently in the development of cancer initiation and progression. Hyperactivation of this pathway causes the downregulation of FOXOs by increasing its phosphorylation levels. PTEN has been shown to be one of FOXO targets. Its mRNA level was upregulated after the inhibition of Akt survival pathway (Luo et al., 2013; Zhang et al., 2011). Thus, FOXOs can increase the transcription of PTEN to reinforce its tumour suppressive action, which in turn counteracts the hyperactivity of PI3K/Akt pathway (Zhang et al., 2011). This finding further suggests that targeting FOXOs could be one of potential strategy to overcome cancer.

FOXO proteins also function together with nuclear transcription factor Y (NFY) and the SP1 transcription factor to stimulate the expression of the tumour suppressor LKB1 or STK11 (Lam et al., 2013; Lutzner et al., 2012). FOXO3a also exerts its tumour suppressive function by targeting *MXI1* gene, which in turn inhibits the proto-oncogene *MYC* function (Delpuech et al., 2007).

The activation of cell cycle checkpoint is the most common response in the use of chemotherapeutic treatment. Cell cycle arrest can also be triggered by the genotoxic stress and result in apoptosis. Induction of DNA damage is one of the mechanisms of action of chemotherapeutic agents, which leads to the activation of cell cycle arrest or the inhibition of cell cycle progression. If the damaged DNA persists, cells either undergo senescence or apoptosis. The deregulation of cell cycle checkpoints is one of the main mechanisms by which cancer cells tolerate chemotherapeutic agents-induced oxidative stress. For instance, the deactivation of FOXO proteins by c-JNK phosphorylation could be one of possible mechanisms underlying this oxidative stress resistance induced by chemotherapeutic drugs (Essers et al., 2004; Huang et al., 2005; Vogt et al., 2005b).

Furthermore, the reintroduction of FOXO3a into cells can increase the number of cell death in response to chemotherapeutic treatment (Modur et al., 2002). FOXO3a has also been identified as a crucial target of gefitinib (Iressa), an inhibitor of EGFR, leading to a reduction in proliferation of cancer cell lines and tumour xenografts. Krol *et al.* found that reintroduction of FOXO3a in breast cancer resistant cells partially resensitises them in response to gefitinib treatment (Krol et al., 2007). FOXO3a is activated upon paclitaxel treatment in breast cancer carcinoma. This results in an arrest at the G2/M cell cycle phase and the subsequent apoptosis via the activation of *Bim*. In line with this, higher expression levels of FOXO3a have been found to be

correlated with a better survival in breast cancer patients following paclitaxel treatment (Sunters et al., 2003; Sunters et al., 2006).

Similarly, Imatinib or STI is a small molecule tyrosine kinase inhibitor (TKI) of BCR-ABL, which is used to treat patients with chronic myelogenos leukaemia (CML). This inhibitor acts by repressing the PI3K-AKT signalling pathway, resulting in FOXO3a-mediated cell cycle arrest by increasing the transcriptional levels of $p27^{Kip1}$ (Corrado et al., 2008).

Another chemotherapeutic drug that activates FOXO3a is cisplatin. Treatment with this drug has been shown to lead to an increase in the dephosphorylation levels of FOXO3a in sensitive colon cancer cells. This culminates in the nuclear translocation and transcriptional activation of FOXO3a, and induction of cell death by the upregulation of $p27^{Kip1}$ and *Bim* gene expression (Fernandez de Mattos et al., 2008). In accordance, the function of FOXO3a-induced cell death following cisplatin treatment is diminished in resistant colon cancer cells, suggesting that FOXO3a is a key regulator of the cytotoxic effects of cisplatin in colon cancer. Moreover, the ectopic expression of FOXO3a resensitises colon carcinoma cells to cisplatin-induced cell death (Fernandez de Mattos et al., 2008). Additionally, the acetylation of FOXO3a has also been shown to be involved in the cytotoxic effects of cisplatin. In colon cancer cells, cisplatin treatment results in a reduction of the levels of FOXO3a acetylation. FOXO3a acetylation status is lower in cisplatin resistant cells compared to the parental counterparts, suggesting that inducing the activation of FOXO3a by increasing its acetylation status could be of therapeutic use (Shiota et al., 2010).

Anthracyclines, including doxorubicin and epirubicin, are yet other examples of chemotherapeutic agents that function through FOXO3a to exert their cytotoxic

effects in cancer cells. In CML leukaemic cells, doxorubicin treatment leads to the induction of FOXO3a expression, its nuclear accumulation and FOXO3a-mediated cell death by activating *p27^{Kip1}* and *Bim* genes. However, sustained activation of FOXO3a in these cells also leads to the upregulation of MDR1 (Hui et al., 2008a; Hui et al., 2008b).

Finally, besides being a crucial modulator of the cytotoxic effects of many chemotherapeutic agents, FOXO proteins also play a role in the prevention of metastasis. FOXOs prevent cancer progression by controlling the expression of many key cell cycle and apoptotic genes. For instance, FOXO3a inhibits cancer progression, including invasiveness and angiogenesis by downregulating the expression of matrix metalloproteinase 9 (*MMP9*), *MMP13* and vascular endothelial growth factor (*VEGF*) (Delpuech et al., 2007; Karadedou et al., 2012; Lam et al., 2013).

1.7.6 Sirtuins as regulators of FOXO acetylation

The regulation of FOXO proteins by sirtuins became the topic of interest because of a study in *C.elegans* that demonstrated that *Sir2.1* (orthologue of human SIRT1 and 2) promotes longevity and survival by regulating DAF-16 (orthologue of human FOXOs) signalling pathway (Tissenbaum and Guarente, 2001). SIRT1 was found to directly interact with FOXO3a (Brunet et al., 2004). It counteracts the function of HAT p300 and mediates the acetylation status of FOXO3a and also FOXO4 (North et al., 2005). Motta *et al.* showed that the deacetylation of FOXO3a by SIRT1 reduces the binding to its downstream targets, *p27^{Kip1}* and *Bim*, thus inhibiting the FOXO-induced apoptosis (Motta et al., 2004). However, the later study by Brunet *et al.* demonstrated that SIRT1 deacetylates FOXO3a in response to oxidative stress,

leading to the activation of genes involved in the regulation of cell cycle arrest and DNA damage repair, like *p27^{Kip1}* and *GADD45* (Brunet et al., 2004). These studies report the different aspects of how FOXO3a regulates p27^{Kip1}, however both studies consolidate the notion that deacetylation of FOXO3a by SIRT1 leads to a prevention of FOXO3a to induce apoptosis.

Moreover, the deacetylation of FOXO1 by Sir2 in mouse has been found to increase its transcriptional activity with the enhancement of *p27^{kip1}* and *MnSOD* expression (Daitoku et al., 2004).

Subsequent studies revealed that SIRT2 and SIRT3 also directly interact with FOXO3a and mediate its acetylation levels and result in the accumulation of ROS. Interestingly, the overexpression of SIRT3 can rescue cardiac dysfunction in *Sirt3$^{-/-}$* deficient mice by increasing the activation of genes involved in the reduction of cellular oxidative stress (Sundaresan et al., 2009).

Together, all these studies suggest that deacetylation of FOXOs by sirtuins promotes FOXOs–mediated cell survival and oxidative stress and inhibit the function of FOXOs to induce apoptosis. This indicates that acetylation of FOXOs could enhance the apoptotic function (see also Figure 1.16 and Table 1.1).

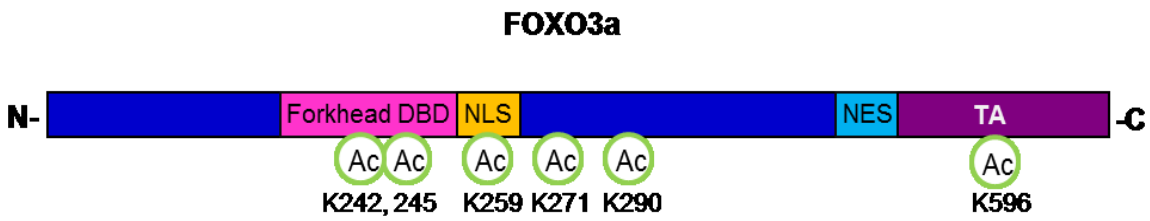


Figure 1.6: Schematic representation of acetylation sites of FOXO3a protein. The position of the forkhead DNA-binding domain (DBD -pink), nuclear localisation sequence (NLS - orange), nuclear export sequence (NES - blue) and transactivation domain (TA- purple) are represented diagrammatically. The reported acetylation sites (Ac) are shown in Green. Adapted from (van der Horst and Burgering, 2007).

Lysine residues	Regulating proteins (Acetylation, Deacetylation)	Effect of FOXO3a deacetylation at this site
Lys 242, 245, 259 and 271	CBP/p300, SIRT1	Inhibition of apoptosis induction by repression of p27 and Bim transcription (Motta et al., 2004).
		Increasing in DNA damage and oxidative stress response by upregulation of p27 and Gadd45 (Brunet et al., 2004).
	CBP/p300, SIRT2	Increasing in oxidative stress resistance by enhancing the expression of $p27^{Kip1}$ and MnSOD (Wang et al., 2007a).
	CBP/p300, SIRT3	Increasing in the activation of genes involved in cellular oxidative stress, MnSOD (Sundaresan et al., 2009).
Lys 242 and 245	CBP/p300, SIRT1	Reducing in DNA-binding affinity of FoxO3a (Tsai et al., 2007).
Lys 242, 259, 290 and 569	CBP/p300, SIRT1/SIRT2	Promoting FOXO3a poly - ubiquitination and degradation leading to a permanent inactivation of FOXO3a (Wang et al., 2012).

Table 1.1: The acetylation sites of FOXO3a, their regulators with their counter activities for acetylation or deacetylation at each site and the subsequent FOXO3a function

1.8 Thesis aim

The aim of this thesis was to investigate the role of sirtuins, especially SIRT6, in the development of drug resistance. The role of post-translation modifications, in particular acetylation, in the regulation of FOXO3a and in the development of cancer and drug resistance was also investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

2.1.1 MCF-7 (Michigan cancer foundation 7) cells

The human breast cancer cell line MCF-7 originated from the American Type Culture Collection and was acquired from the Cell Culture Service, Cancer Research UK, where it was tested and authenticated. MCF-7 epirubicin resistant cells (MCF-7-Epi^R) and MCF-7 paclitaxel cells (MCF-7-Tax^R) were generated in our lab by culturing the parental MCF-7 drug sensitive cells with small increases in the concentrations of both drugs until cell acquired resistance to 10 μM and 0.05 μM, respectively. For MCF-7-Epi^R and MCF-7-Tax^R cells, 10 μM epirubicin and 0.05 μM paclitaxel was added to maintain the resistant phenotypes of the cells.

2.1.2 Mouse embryonic fibroblasts (MEFs)

MEFs isolated from *wild-type* (WT) and *Sirt6*$^{-/-}$ mice were transformed by SV40 Large T antigen expression (Schwer et al., 2010). *p53*$^{-/-}$ MEFs have previously been described (Millour et al., 2011). *Foxo1/3/4*$^{-/-}$ MEFs were kind gifts from Professor Boudewijn Burgering, UMC, Utrecht, the Netherlands and have been described (van der Vos et al., 2012).

2.1.3 Hela cells

Hela cells harbouring DR-GFP reporter system (both DR-GFP and EJ-7-NHEJ) were stabilised as described (Bennardo et al., 2008) and were a gift from Dr. Maria Jasin (Memorial Sloan-Kettering Cancer Center, New York, USA) (Delacôte et al., 2002; Weinstock et al., 2006). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine, and 100

units/ml penicillin and 100 µg/ml streptomycin in a humidified incubator at 37ºC with 5% CO_2.

2.1.4 The maintenance of cell lines

Cells were cultured and split at approximately 80% confluency, twice a week. Media was aspirated and the adherent cell monolayer was washed once by warm PBS and then detached by adding 1×trypsin-EDTA (Sigma). After detached, cells were then resuspended in the completed media to stop trypsinisation and transferred into a new flask or into the appropriate cell culture dishes. Cells were cultured for approximately 35 passages before discarding.

2.1.5 Long term maintenance of cells

Healthy cells with a low passage number were detached from the monolayer by trypsinisation, as described above, and resuspended in media. Cells were then spun at 300×*g* for 3 min. The supernatant was discarded and the cell pellet was resuspended in 10% dimethyl sulphoxide (DMSO)/FCS. Cell suspension were then transferred to cryotubes and slowly frozen in a Mr. Frosty (VWR International) at -80°C overnight before being transferred for storage in liquid nitrogen.

For defrosting cells, cryotubes were rapidly defrosted in a water bath (~37°C) and the defrosted solution was added to complete media and spun at 300×*g* for 3 min. The DMSO containing supernatant was removed and cell pellets were resuspended in fresh supplemented medium and placed in a culture flask to grow.

2.2 Transfection

2.2.1 Plasmid DNA: Fugene 6 transfection

One day prior to the transfection, cells were seeded into 6-well plates or 100 mm dishes and left overnight to get at least 70-80% confluence. Plasmid DNA overexpressing gene of interest was transfected by using FuGENE 6 (Roche Diagnostics, West Sussex, UK) in 3:1 ratio (µl of FuGENE : µg of plasmid DNA). The ratio was recommended by the manufacture and was previously optimized by the people in our lab. Briefly, FuGENE 6 was diluted in DMEM serum free medium and incubated at room temperature for 5 min. The plasmid DNA was then gently added into the mixture and incubated up to 45 min. After incubation, the complexes were slowly dropped into the culture plates and left at least 24 h before proceeding further experiments.

2.2.2 Gene silencing: small interfering RNA (siRNA) transfection

Cells were transiently transfected with ON-TARGETplus SMARTpool siRNAs (Dharmacon) using Oligofectamine (Invitrogen) following the manufacturer's instructions. Briefly, cells were seeded into 6-well plates and left overnight to get 70-80% confluence. For two wells, 10 µl of Oligofectamine was added into 120 µl of OptiMEM (Life Technologies, UK), vortexed briefly and incubated at room temperature for 10 min.

2.2.3 Co-transfection

Co-transfection of plasmid DNA and siRNA was performed in Hela DR-GFP-reporter cells by using the Lipofectamine 2000 transfection reagent (Life Technologies, UK) according to manufacturer's protocol.

Briefly, Hela cells were seeded into 6-well plates and left overnight to get at least 80-90% confluences. For each well, 6 µl of Lipofectamine was added into 244 µl of OptiMEM (Life Technologies, UK) and incubated at room temperature for 5 min. One micro gram of plasmid DNA and 2-4 µg of siRNA pool was added into the mixture and toped up with OptiMEM to obtain final volume of 250 µl . The mixture was then incubated at room temperature for 20 min. The DNA-siRNA complex was then gently dropped into each well. Cells were then put back into 37^{o}C incubator. Medium was changed after 6 h of transfection.

2.3 RNA analysis

2.3.1 Total RNA extraction

RNA was extracted from cells by using the RNeasy Kit (Qaigen, Crawley, UK). Cell pellets were resuspended by adding 350 µl of RLT buffer followed by pipetting up and down. Three hundred and fifty µl of 70% ethanol were added. Seven hundred µl of total sample were then transferred into a RNeasy spin column placed in 2 mL collection tube and spun down in a desktop centrifuge for 15 s at 13,000 x*g*. The flow through was discarded and 700 µl of RW1 buffer were added into the spin column and spun down for 15 s at ≥8,000 x*g* and discarded. Then, 500 µl of RPE buffer were added into the spin column and spun down for 15 s at 13,000 x*g*, this step for 2 times and followed by 2 min centrifugation at 13,000 x*g*. The spin column was then transferred to a new collection tube. Thirty micro litre of TE buffer were added directly into the spin column and the column was microcentrifuged for 1 min at 13,000 x*g* to elute RNA. The concentration and purity of the RNA sample was then measured by the spectrophotometric absorption (NanoDrop ND-1000, Thermo Scientific, UK) at 260 nm and 280 nm.

2.3.2 First strand cDNA synthesis

The reverse transcription of 2 µg RNA to first stand cDNA was performed by using Superscript III first strand cDNA synthesis system (Invitrogen, Paisley, UK). The mixture of 1 µl of random primers and 1 µl of 10mM dNTPs mix (containing the four bases adenine, cytosine, guanine and thymine) were added into 2 µg of total RNA and filled with sterile RNase free water to obtain total volume of 12 µl . The sample was then heated at 65^{o}C for 5 min and immediately placed on ice for 1 min. The mixture of 4 µl of 5×first strand buffer, 1 µl of 0.1M DTT, 1 µl RNaseOUT and 1 µl of the reverse transcriptase Superscript III were then added into the RNA mixture to obtain a total volume of 20 µl . The mixture was spun down at 300 x*g* for 15 s and placed on a thermocycler (GeneAmp PCR system 9700, Applied Biosystems, UK). Each mixture was subjected to 5 min incubation at 25ºC, heating up to 50ºC for 50 min. To terminate the reaction, the mixture was heated to 70ºC for 15 min.

2.3.3 Real-time quantitative PCR (RT-qPCR)

cDNA was employed as a template and gene expression was determined by RT-qPCR using SYBR Green Mastermix (Applied Biosystems, UK) according the manufacture instructions and was performed using the 7900HT fast real-time PCR system (Applied Biosystems, UK). Gene specific primers were designed using the Primer Express Software (Applied Biosystems). This software provided an amplicon between 100-200 bp, based on the sequence of genes from PubMed (National Centre for Biotechnology Information: NCBI, USA).

Primers: The primers for qPCR designed by Primer Express 3.0 were listed in the Table 2.1 as below

Human	Forward	Reverse
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
SIRT1	TGCGGGAATCCAAAGGATAA	CAGGCAAGATGCTGTTGCA
SIRT2	CCTCGCCTGCTCATCAACA	CCCATAATCATCCCCAGGAA
SIRT3	CTCCCAGTTTCTTCTTTTCGAGTATT	TTGAAGCACCCACAGAAAAAGAT
SIRT4	TGGATGCTTTGCACACCAA	GAGTCTGTTCCCCACAATCCAA
SIRT5	TCCACACGAAACCAGATTTGC	CTTTCGAAAATCTGCCATACTTGA
SIRT6	AGGAGCTGGAGCGGAAGGT	TGGAACACCACACTGGAAGACT
SIRT7	CGTCCGGAACGCCAAATA	ACGCTGCCGTGCTGATTC

Table 2.1:List of Human primers

Mouse	Forward	Reverse
mnSOD2	GCCCCCTGAGTTGTTGAATA	AGACAGGCAAGGCTCTACCA
Gadd45	GGGCTCAGAGATGACTTTGC	TTTTTGTCCCTTTTGCCTTG
Bim	CACCTGCTGTGTGCTTCCTA	GCTGGCCTAAAGCAGTGAAC
$p21^{cip1}$	GCCTTAGCCCTCACTCTGTG	AGGGCCCTACCGTCCTACTA
$p27^{kip1}$	GGACCAAATGCCTGACTCGT	CGCTTCCTCATCCCTGGAC
sirt6	CCCAACAGCCCTATACTCCA	GCCTGGGCTATAGCAGTGAG
cyclinD1	CGAGGAGCTGCTGCAAATGG	GGTATCAAAATGCTCCGGAGAGG
cyclinD2	AGCAGCGGGAGAAGCTGTCTCTGA	ATGGACGCGTCTCTCTCTTTCGG

Table 2.2: List of mouse primers

Gene expression analysis was performed by using the relative standard curve method, in which 2 µg of cDNA from each sample was mixed and diluted into serial dilutions (1/4, 1/16, 1/64 and 1/256). L19 was used as the established housekeeping gene. Each gene expression was normalised by the L19 value. Each reaction mix consists of 2 µl of sample and 23 µl of the mixture of SYBR Green master mix, primers and water to obtain a final volume of 25 µl was used to measure gene expression was measured by using the TAQMAN 7700 real-time PCR machine. All measurements were performed in triplicates. Once, the preparation of samples was

done, plate was firmly sealed and spun down at 1,200 x*g* for 5 min at 4ºC. The real-time PCR machine was programmed for 10mins of 95ºC incubation for enzyme activation**,** followed by denaturing of samples at 95ºC for 10 min and primer annealing/extension at 60ºC for 30 s. These two steps were repeated for 40 cycles.

2.4 Protein analysis

2.4.1 Cell lysate preparation

Cells were washed with warm PBS to remove the excess medium. Trypsin was then added to detach adherent cells. Cells were then re-suspended with completed medium and spun down at 300 x*g* for 4 min. Cells were re-suspends with PSB and transferred to an eppendoff tube. Total cell lysates were extracted by lysis buffer (1 M HEPES, 5 M NaCl, 0.5 M ethylenediaminetetracetic acid (EDTA), 1% (v/v) Nonidet P-40, 1mM sodium fluoride (NaF), 2mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate (Na_3VO^4) and protease inhibitors ("Complete" protease inhibitor mixture, as instructed by the manufacturer, Roche Applied Science) on ice for 10 min. Insoluble debris was removed by centrifugation (13000 x*g*, 4ºC, 10 min). The supernatant was then transferred to a clean eppendorf tube and protein concentration was determined by Bio-Rad Dc protein assay.

2.4.2 Determination of protein concentration

Protein concentration was determined using the Bio-Rad D_c protein assay (Bio-Rad Laboratories), as instructed by the manufacturer. Reagent S and reagent A were mixed with 1:50 ratio (20µl of reagent S was added into 1000 µl of reagent A). Twenty-five µl of this mixture were added to 2 µl of protein solution. After 2 min, 200 µl of reagent B was added to the mixture. Samples were shaken gently and then

covered with foil to protect them from direct sunlight and incubated for a further 15 min at room temperature. After incubation, absorbance of samples was read at 700nm. Protein concentrations were determined by the equation absorbance x 25 = µg/µl .

2.4.3 Western blotting or Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein expressions were investigated by using gel electrophoresis. Gel was made of the acrylmide/bis stock solution with 25% of ammonium persulphate (APS) and Tetramethylethylenediamine (TEMED) as polymerisation catalysts. Each SDS Gel contained two parts, which are resolving gel (bottom) and stacking gel (top). The percentage of the resolving gel used depended on the size of the protein of interest. Higher percentage of gels is suitable for analysing proteins with lower molecular weight (mW), whereas small protein will be suitable for bigger proteins. Table 2.3 showed the components of each gel percentage.

Reagents (mL)	Resolving gel				Stacking gel
	7%	10%	12%	14%	5%
dH_20-ml	5.02	4.02	3.35	2.68	3.67
1.5M Tris pH 8.8	2.5	2.5	2.5	2.5	-
1.5M Tris pH 6.8	-	-	-	-	0.42
30%Acrylamide/ 0.8% Bis mix (37.5:1)	2.33	3.33	4.00	4.67	0.83
10% SDS (µl)	100	100	100	100	50
25% APS (µl)	40	40	40	40	20
TEMED (µl)	10	10	10	10	10
Total	10	10	10	10	5

Table 2.3: SDS-PAGE percentages and their components

For protein expression analysis, 20-25 µg of protein lysate were diluted in PSB to obtained final volume 20 µl. Then, 10 µl of 2xSDS loading buffer (4% (w/v) SDS, 62.5mM Tris-HCl (pH 6.8), % (v/v) glycerol, 0.01% (w/v) bromophenol blue) was added into each sample to obtain 1xconcentration and boiled for 5 min at 100ºC. Samples were then briefly spun down and 20 µl of each sample was then loaded into the sacking wells. Protein separation was obtained by running SDS-PAGE gels in running buffer (25mM Tris-Base, 250mM glycine, 0.1% (w/v) SDS) at 70V through the stacking gel and then 120V through the resolving gel.

Once the protein separation had finished, proteins were then electro-transferred onto Protran nitrocellulose membranes (Perkin Elmer) using a wet tank blotting system (Bio-Rad Laboratories Trans-Blot Cell) in transfer buffer (25mM Tris, 190 mM glycine and 20% (v/v) ethanol) for 90 min at 90V. Ice block was put into the wet tank in order

to make sure that the transferring process does not overheat. Membranes were then blocked in 2.5% of Bovine Serum Albumin (BSA) in TBS (Tris-buffered saline) with 0.5% Tween (TBST) solution for 1 h at room temperature and then incubated with a specific primary antibody (Table 3) at 4ºC in cold room overnight (Table 2). Membranes were then washed 3 times with TBST at room temperature for 10 min each. Membranes were then incubated with their respective horseradish peroxide conjugated secondary antibody (anti-rabbit or anti-mouse (1:2000) - Table 3) for 45 min at room temperature. The membranes were then washed 3 times with TBST for 10 min each. The membranes were then developed using the enhanced chemiluminescence (ECL) detection system (PerkinElmer SheerGreen, UK and the SRX-101A X-ray developer (Konica Minolta, Tokyo, Japan).

Antibody	Product number	Species	Company
FOXO1 (C29H4)	2880	rabbit	Cell signalling technology
FOXO3a	H-144	rabbit	Santa Cruz
	07-702	rabbit	Millipore
FOXO4	834	rabbit	Gift - Prof. BM Burgering
Acetyl-FOXO3a (K242/5)	Homemade	rabbit	Prof.Eric Lam
Total-Acetyl-lysine			
	9441L	rabbit	Cell signalling technology
FOXM1 (C-20)	(sc502)	rabbit	Santa Cruz
p53	Sc-98	mouse	Santa Cruz
p21	Sc-397	mouse	Santa Cruz
MDR1 (C219)	517310	mouse	Calbiochem
p27 (C-19)	sc-528	rabbit	Santa Cruz
Histone 3 (H3)	Ab61251	rabbit	Abcam
Histone 3 acetyl K9	Ab61231	rabbit	Abcam
Histone 3 acetyl K56	2134-1	rabbit	Epitomics
SIRT1	Ab32441	rabbit	Abcam
SIRT2	sc-20966	rabbit	Santa Cruz
SIRT3	2627	rabbit	Cell signaling
SIRT4	s0948	rabbit	Sigma Aldrich
SIRT5	SA464	rabbit	Biomol
SIRT6	2950	rabbit	Cell signaling
SIRT7	Ab62748	rabbit	Abcam
FLAG	F1804	mouse	Sigma Aldrich
BRCA1	Ab-1/MS110	mouse	Calbiochem
Lamin B	F1/119D5	mouse	Abcam
Beta-tubulin	Sc-9104	rabbit	Santa Cruz
Rabbit HRP	P0448	goat	Dako
Mouse HRP	P0447	goat	Dako

Table 2.4: Antibodies used for western blotting

2.5 Sulforhodamine B (SRB) assay

Three thousand cells were seeded in each well of a 96-well plate and cultured overnight. Cells were treated with different final concentrations of chemotherapeutic agents. At a specific time point, cells were fixed with 100 µl of 40% trichloroacetic acid and incubated for 1 h at 4ºC. Plates were then rinsed 5 times with slow running tap water and 100 µl of SRB solution (0.4% SRB in 0.1% acetic acid) was added and incubated at room temperature for 1 h. Plates were washed three times with 1% acetic acid and left for air-dried overnight. A hundred microlitre of 10mM Tris base solution was then added to each well to dissolve the protein bound dye, and optical density was measured at 492 nm with a microplate reader (Sunrise, Tecan).

2.6 Clonogenic assay

Cells were seeded into 6-well plates and cultured overnight. Cells were then treated with either epirubicin or paclitaxel and cultured up to 15 days until colonies were formed. Cells were washed 3 times with PSB and fixed with 4% PFA for 20 min at room temperature. Visible colonies consisting of at least 50 cells were stained with 0.5% crystal violet (Sigma) for 30 min and submersed in water to remove the excess of crystal violet. Plates were then left air dried at room temperature for a few days. Acetic acid 33% (v/v) was added to solubilise the bound crystal violet and the optical density (OD) was then measured at 592 nm using a microplate reader (Sunrise, Tecan).

2.7 SA-β-gal staining

Cells were seeded in 6-well plates at a density of approximately 20,000 cells/well before treatment with epirubicin for 72 h. Medium was then changed and left them grow further for 5 days. Cells were fixed and stained using a Senescence β-Galactosidase Staining Kit #9860 purchased from Cell Signalling Technology (Beverley, MA, USA). Briefly, cells were fixed with fixative solution for 15 min and then were rinsed with PSB for 2 times. Cells were then stained with staining solution, containing 2% of β-galactosidase solution (w/v). Plates were incubated overnight at 37ºC in a dry incubator (no CO_2). Cells were then detected for blue staining under a bright-field microscope. The percentage of SA-β-gal-positive cells was calculated by counting the cells in five different fields.

2.8 Subcellular fractionation

The subcellular fractionation assay was performed using NE-PER Nuclear and cytoplasmic Extraction Reagents (Perbio, Belgium). Cell pellets were re-suspended in 200 µl of CERI with EDTA-free protease inhibitors, vortexed vigorously with the highest setting for 15 s, and incubated for 10 min on ice. Then, 11 µl of CERII solution were added into each tube. These suspensions were then vortexed for 5 s and incubated on ice for 1 min. Samples were then vortexed with the highest setting speed for the other 5 s and spun down at 13,000 x*g* of a desktop centrifuge for 5 min at 4ºC. The supernatant was collected in pre-chilled tubes as the cytoplasmic fraction. The insoluble fraction was then re-suspended in 100 µl ice-cold NER solution with EDTA-free protease inhibitor, vortexed for 15 s with the highest setting and incubated on ice. The vortexing and incubating on ice was repeated in every 10 min for 40 min in total. The suspensions were then spun down with the maximum

speed for 10 min at 4ºC. The supernatants were collected as a nuclear fraction in a pre-chilled tube.

2.9 Immunofluorescent staining

Cells were placed onto 4-chamber slides and cultured overnight. Cells were treated and harvested at specific time points. Cells were then fixed with 4% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X-100 in PBS for 10 min. The samples were then blocked with 5% goat serum for 30 min and then incubated overnight with specific antibodies. Samples were then washed 3 times with PBS and incubated with secondary antibodies IgG-FITC (1:250; Invitrogen) for an hour in the dark. The cells were washed again 3 times with PSB and counterstained with DAPI (Sigma) before mounting. The images were captured from 5 different fields by using the Zeiss Axiovert 100 confocal laser scanning microscope and software Zeiss LSM 500 or ImageXpress (Molecular Devices) using the Nikon, Eclipse E400 fluorescent microscope (UK Labs-Direct, Ltd, Kilnhurst, UK).

Antibody	Product numbers	Species	Company	dilution
FOXO3a	H144	rabbit	Santa Cruz	1:200
γH2AX	20 E3/9718	rabbit	Cell signalling	1:250
Alexa Fluor 488 anti-rabbit	A-21070	goat	Life technology	1:500
Alexa Fluor 488 anti-mouse	A-10680	goat	Life technology	1:500
Alexa Fluor 555 anti-rabbit	A-21428	goat	Life technology	1:500
Alexa Fluor 555 anti-mouse	A-11078	goat	Life technology	1:500

Table 2.5: List of antibodies for immunofluorescent staining

Foci counter software was used to quantify the level of H2AX foci formation. The all parameters, including crown radius, rim radius and brightness difference was optimised by my colleague, Miss Pasarat Khongkow, shown in Supplemental data 1.

2.10 Comet assay

The alkaline single-cell gel electrophoresis assay was a modified method published in Singh, N. *et al* (Singh et al., 1988). Briefly, cells were harvested and suspended in PSB. Cells were then re-suspended in 0.7% low melting point agarose (LMA) (Promega) and were spread over a double frosted microscope slide (Thermo Scientific), which was pre-coated with 1% normal melting point agarose (NMA) (Promega). After adding a third layer of 0.7% LMA, the slides were immersed in freshly prepared ice-cold lysis buffer (2.5M NaCl, 100mM Na_2EDTA, 250mM NaOH, 10mM Tris, 2.5% N-lauroylsarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4ºC. The slides were then placed in an electrophoresis tank to unwind DNA for 20 min in an alkaline solution (250 mM NaOH and 1.5 mM Na_2EDTA, pH 13). Electrophoresis was then performed at 25 V and 300 mA for 15 min. The slides were neutralized by washing gently with a neutralization buffer (0.4M Tris, pH 7.5) three times for 5 min and then stained and mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Pictures of approximately 100 cells per sample were captured from 5 different fields using the fluorescent microscope Nikon Eclipse E600 (Nikon, Japan), with an excitation filter of 340-380nm, connected to a digital CCD camera C8484 (Hamamatsu, Japan). DNA damage was quantified by measurement of the percentage of DNA in tail, tail moment and olive moment with the software Comet Assay IV (Sumer Perceptive Instruments Ltd. Suffolk, UK).

Pseudocolour of isolated nuclei was attributed by the program, reflecting the condensation state of the chromatin.

2.11 Immunoprecipitation

Total cell lysates were extracted by lysis buffer (1 M HEPES, 5 M NaCl, 0.5 M EDTA, 1% Nonidet P-40). 10-20 µg of crude lysates was collected as a positive control. All lysates were then pre-cleaned by incubating with protein A/G magnetic beads (invitrogen) for 2 h at 4ºC. After incubation, supernatants were collected and divided into 2 different tubes to subject to either a specific antibody or IgG as a negative control (invitrogen) overnight at 4ºC. Twenty micro litre of protein A/G magnetic beads were added and incubated for 2 h at 4ºC. Magnetic beads with immunoprecipitates (IPs) were then washed, eluted in 1×SDS loading buffer, and processed for Western blotting, performed with standard protocols. Co-immunprecipitation was investigated by immunoblotting with specific antibodies incubation.

2.12 Cell cycle analysis

The cell cycle was analysed using flow cytometry. The cultured media was removed and kept in 15 mL falcon. Cells were then detached by adding trypsin as earlier described. After trypsinisation, completed media was added to stop the reaction. Cell suspension was then mixed with the kept media and spun down again at 300 x*g* for 2 min. Supernatant was then discarded. Cell pellets were resuspended and washed in 500 µl PBS and spun at 300 x*g* (4ºC) for 2 min and then resuspended and fixed with 5 ml of 90% ethanol at 4ºC for overnight. Cells were then spun at 300 x*g* (4ºC) for 5 min. The supernatant was discarded and the cells washed twice in 2 ml PBS

and then spun at 300 x*g* (4ºC) for 5 min. The supernatant was discarded and then the cells were stained using 600 µl propidium iodide (50 µmol/l) for 1 h in the dark. Cell cycle analysis was then analysed using a FACS canto and FACSDiva software (BD Biosciences).

2.13 Repair assay

Hela cells harbouring DR-GFP-HR or GFP-NHEJ reporter systems were transfected with plasmid DNA or siRNA alone or in combination (See section 2.2 for transfection protocol). Cells were left in incubator for 24 h and then transfected again with pCMV-I-SceI plasmids after 24 h or 48 h of transfection, cells were then harvested by trypsinisation. Cells were then spun down at 300 x*g* for 2 min after stopping reaction with completed medium. Cells were then resuspended in 1 mL cold PSB. Then, 700 µl of cell suspension were transferred to FACS tubes and analysed for GFP-positive cells, and the remaining of 300µl suspension was collected and analysed for the transfection efficiency by Western blotting. The number of GFP-positive cells was investigated by the BD FACSCanto II flow cytometer system (BD Biosciences, Oxford, UK) with the FACSDiva acquisition software (BD Biosciences, UK). The results were examined by either the plotting of green fluorescence (FL1-H or A) on y-axis and auto orange fluorescence (FL2-H or A) on the x-axis or the plotting of cells count on the y-axis and green fluorescence on the x-axis. The repair efficiency of HR and NHEJ of each condition was calculated relative to control (empty vector or siControl).

2.14 Clinical data analysis

2.14.1 Tissue Microarray

The same panel of patients sample described in Chen et al, 2010 was used. Briefly, one hundred and thirty-three cases of breast cancer diagnosed between the years 1992 to 2001 together with clinical follow up data were retrieved from the records of the Department of Pathology, Queen Mary Hospital of Hong Kong. This is with approval of the Institutional Review Board of The University of Hong Kong. The summary of patients 'profile is presented in Figure 3.20 (page 134). Histological features of all cases were reviewed by the pathologist, the representative paraffin tumour blocks were chosen as donor block for each case and the selected areas were marked for construction of tissue microarray (TMA) blocks. A total of 118 was assessed and scored for SIRT6 expression. The expression pattern and subcellular localization were analysed and correlated with histological type, histological grade, clinical stage, estrogen and progestrogen receptor status, lymph node metastasis and survival time.

2.14.2 Immnohistochemistry

The deparaffinization and rehydration of TMA sections was achieved by incubating with xylene and decreasing concentrations of ethanol. Citrate buffer (0.01M, pH 6.0) was used for antigen retrieval. To quench endogenous peroxidase, the slides were then immersed into 3% H_2O_2/methanol for 10 min at room temperature. After rinsing in 0.05% Tween in PBS (PBST) twice, 200µl of 1:50 primary mouse monoclonal SIRT6 specific antibody dilution (Abcam, Cambridge, UK) was added to each section

and incubated at 4°C overnight. The slides were then washed in PBST and 4-5 drops of DAKO Polymer were added onto each sample and incubated at room temperature for 30 min. After washing, Chromogen DAB/substrate reagent was applied on the slides and further incubated for 3 min. Finally, the dehydration of slides was performed using increasing concentrations of ethanol solution, followed by clearing in xylene and slides were then mounted.

Aperio ScanScope ® system (Aperio technology, USA) was employed to visualize and assess for SIRT6 expression. The SIRT6 stained TMA slides were scanned by ScanScope scanners. The high resolution tissue section images captured by ScanScope were automatically recored in the Spectrum Database. Individual stained TMA spots were assessed and scored by two independent individuals and the average scores were used for calculations.

2.14.3 Staining scoring and Statistical Analysis

Tissue sections were scored according to their intensity and percentage of stained tumour cells in the whole core. As SIRT6 was detectable in both the cytoplasm and the nucleus, separate evaluation on cytoplasm and nucleus was done. The intensity of staining was scored as follows: 1 = weak, 2 = moderate, 3 = strong. The percentage of cells positively stained was scored as follows: 1 ≤ 25%, 2 ≤ 50%, 3 ≤ 75%, 4 > 75%. For each case, a final score was obtained by multiplying the score of intensity with the score of percentage.

To minimize interference of cytoplasmic staining with the scoring of nuclear staining, and vice versa, nuclear and cytoplasmic localization scores were derived by dividing the nuclear or the cytoplasmic score by the total score (i.e. nuclear localization score = nuclear score/nuclear + cytoplasmic score; cytoplasmic localization score =

cytoplasmic score/nuclear + cytoplasmic score.) These localization score values are a more accurate measure nuclear or cytoplasmic expression and were used for statistical analysis.

Statistical analysis was performed using the SPSS programme version 17. The correlation between SIRT6 expression and clinical-pathological features data were obtained by bi-variate Pearson Correlation analysis. Survival analysis was analysed by Kaplan Meier analysis with log-rank test. Correlation of SIRT6 expression and clinical-pathological parameters and patient survival were also examined by Cox-regression. P values of less than or equal to 0.05 were considered statistically significant.

CHAPTER 3

THE ROLE OF SIRT6 IN THE DEVELOPMENT OF DRUG RESISTANCE IN BREAST CANCER

3.1 Introduction

Anthracyclines, including doxorubicin and epirubicin, and taxanes, including paclitaxel and docetaxel, are the most common chemotherapeutic agents for treating hormone receptor-negative breast cancer patients (Piccart, 2003; Wu et al., 2011). These agents are also important for treating advance and metastatic breast cancer, typically resulting in 30-70% of response rate. Most of the treatments eventually fail due to the development of resistance to one or both drugs (Cortes and Baselga, 2007; Moreno-Aspitia and Perez, 2009). Therefore, an understanding of the mechanism underlying the development of either intrinsic or acquired resistance is essential for improving the efficacy of these treatments for many cancers, including breast cancer.

Sirtuins (SIRTs) are histone deacetylase enzyme class III that used NAD+ as a cofactor. They have been proposed to have a crucial role in cancer progression and drug resistance (Horio et al., 2011). SIRT1 is a nuclear protein and has been reported to overexpress in many cancers, including non-melanoma skin cancer (Teh et al., 2002), lung (Yeung et al., 2004), prostate (Kuzmichev et al., 2005) and pancreatic cancer (Zhao et al., 2011). SIRT1 depletion leads to the downregulation of multidrug resistant molecule P-glycoprotein (Pg-P/MDR1) (Chu et al., 2005). Thus, SIRT1 could be a potential target in order to overcome drug resistance in some cancers.

SIRT6 is the other nuclear sirtuin that has been described to have a crucial role in the maintenance of genomic stability (Liszt et al., 2005; Michishita et al., 2008). SIRT6 depletion leads to premature ageing in mice (Mostoslavsky et al., 2006), and as a consequence, SIRT6 is proposed to have a role in pathologies associated with

ageing. Interestingly, there is accumulating evidence reporting a role of SIRT6 in DNA damage repair through promoting base excision repair (BER) (Mostoslavsky et al., 2006) and DNA double-strand breaks (DSBs) repair, including both non-homologous end joining (NHEJ) and homologous recombination (HR) (Kaidi et al., 2010; Mao et al., 2011; McCord et al., 2009). SIRT6 is recruited to DSB sites where it interacts and stabilises the DSB repair factor DNA-PK to promote DNA repair (McCord et al., 2009). It also promotes DNA end resection, a crucial step in DSB repair by homologous recombination, through CtIP deacetylation (Kaidi et al., 2010). In addition, mono-ADP-ribosylation of PARP1 by SIRT6 leads to the enhancement of DNA DSB repair under oxidative stress (Mao et al., 2011). Moreover, SIRT6 has also been shown to be the main modulator for DNA damage repair via chromatin rearrangement. It binds to the damage site and recruits chromatin remodeller (SNF2H) to initiate the repair processes (Toiber et al., 2013).

3.2 Hypothesis

As SIRT6 has a role in DNA damage repair, I hypothesise that SIRT6 is involved in the development of drug resistance upon chemotherapeutic treatment in cancer.

3.3 Aim

The objective is to investigate the role of sirtuins, especially SIRT6, in the development of drug resistance in breast cancer.

3.4 Results

3.4.1 The overexpression of SIRT6 is observed in both epirubicin and paclitaxel resistant MCF-7 cells.

Sirtuins (SIRTs) have been suggested to play a crucial role in the development of chemotherapeutic drug resistance (Olmos et al., 2011b). To test this hypothesis, the expression levels of all SIRT proteins were compared between the sensitive MCF-7 cells with either the MCF-7-epirubicin resistant cells (MCF-7-Epi^R) or the MCF-7-paclitaxel resistant cells (MCF-7-Tax^R). To optimize the drug concentrations to be used, cell proliferation was investigated by SRB assay. As shown in Figure 3.1A, the optimum concentrations for epirubicin and paclitaxel were 1 μM and 10 nM, respectively. Cells were treated with either epirubicin or paclitaxel and harvested at 0, 6, 24 and 48 h timepoints. Whole cell lysates were prepared and the protein expression levels of human SIRTs were investigated by Western blotting. The upregulation of SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 expression levels were observed in MCF-7-Epi^R compared to MCF-7 cells, whereas SIRT2 was down-regulated in MCF-7-Epi^R cells (Figure 3.1B). There were no differences in the expression of SIRT1 observed between these two cell lines. For paclitaxel treatment, there were no significant differences in SIRT1, SIRT4 and SIRT7 expression levels in MCF-7 compared to MCF-7-Tax^R cells (Figure 3.1C). Similar to MCF-7-Epi^R, the expression levels of SIRT2 in MCF-7-Tax^R cells were lower compared to those in MCF-7 cells, whereas SIRT3, SIRT5 and SIRT6 protein levels were upregulated in MCF-7-Tax^R compared to the MCF-7 sensitive cells. Moreover, the expression levels of all SIRTs remained constant after epirubicin and paclitaxel treatment. Notably, the overexpression of SIRT6 in both MCF-7-Epi^R and MCF-7-

Tax^R is consistently maintained at high levels, supporting my hypothesis that SIRT6 has a role in the development of drug resistance in breast cancer.

3.4.2 The overexpression of SIRT6 is due to post-translational modification.

Next, I then investigated whether this overexpression of SIRT6 is regulated at transcriptional level or post-translational level. The *RT*-qPCR was performed to assess and compare the mRNA levels of SIRT6 (Figure 3.2A and B). The *SIRT6* mRNA levels were significantly lower in both resistant cells compared to MCF-7 sensitive cells in the absence of treatment. Notably, both epirubicin and paclitaxel treatments caused a noticeable increase in the mRNA levels of SIRT6 in MCF-7 cells. Similar result was obtained in paclitaxel treated MCF-7-TaxR cells; however there was any change in SIRT6 mRNA level in MCF-7-EpiR cells after epirubicin treatment.

These results reflect that the upregulation of SIRT6 protein level in both types of resistant cells is possibly due to post-transcriptional as well as post-translational modification of this protein. Therefore, it would be interesting to investigate the post-translation modification of SIRT6 in response to epirubicin and paclitaxel treatment to understand if SIRT6 post-translational would be involved in resistant phenotypes of these two cell lines. For instance, intramolecular mono-ADP-ribosylation of SIRT6 have been suggested to contribute to self-regulating SIRT6 function (Beauharnois et al., 2013) and it could be therefore investigated after epirubicin and paclitaxel treatment.

However, this thesis was mainly focus on how SIRT6 overexpression mediates drug resistance in breast cancer. Therefore, this research was established to investigate the role of SIRT6 in the development of chemoresistance in breast cancer.

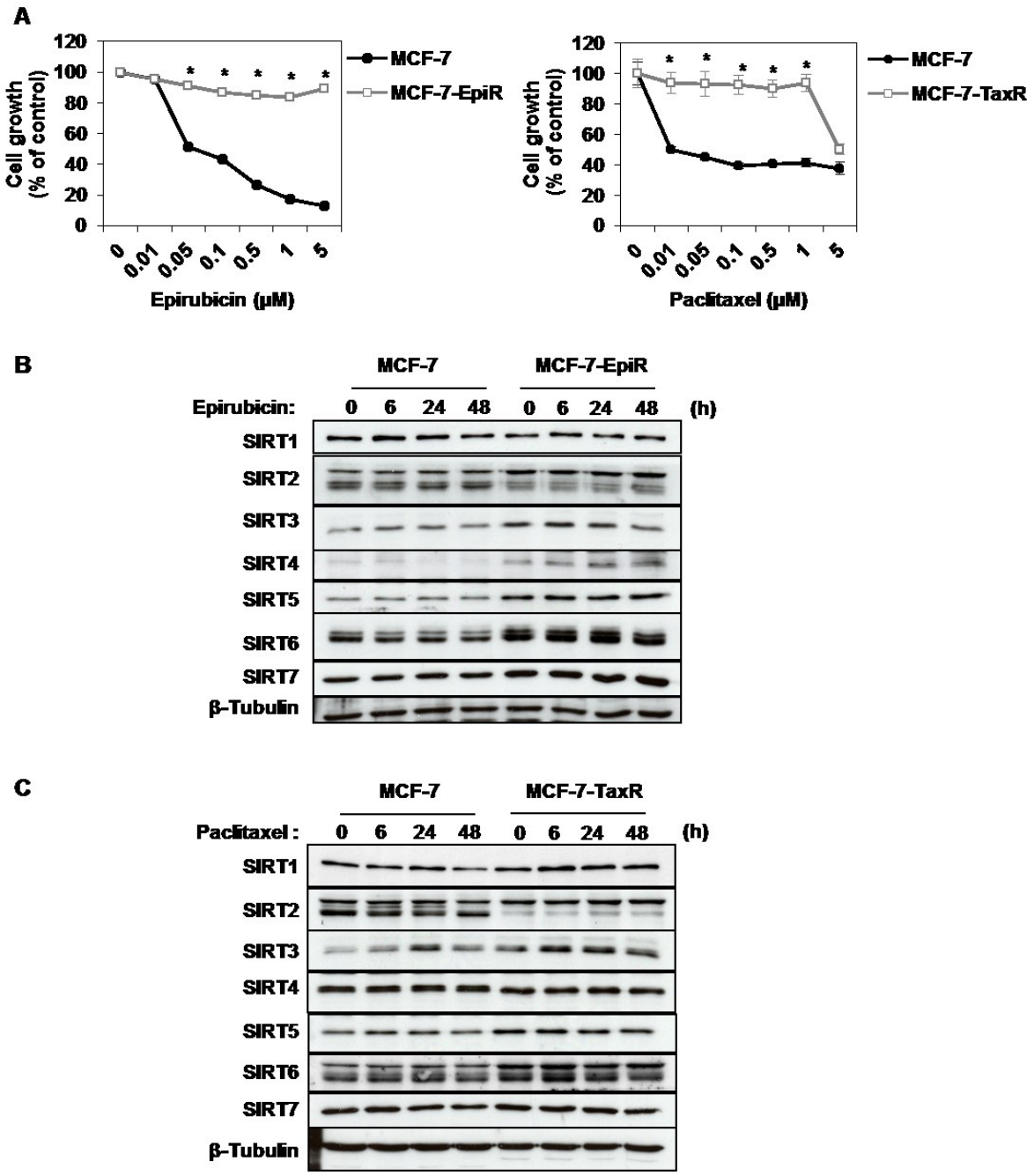


Figure 3.1 SIRT6 overexpression is observed in both epirubicin and paclitaxel resistant cells. A. SRB assay showed the cell proliferation of MCF-7 and MCF-7-Epi^R cells (**left panel**) or MCF-7 and MCF-7-Tax^R cells (**right panel**) after 72 h treatment with increasing concentrations of paclitaxel or epirubicin. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant). **B.** Western blot analysis of MCF-7 and MCF-7-Epi^R cells were treated with 1 μM epirubicin for 0, 6, 24 and 48 h. **C.** MCF-7 and MCF-7-Tax^R cells were treated with 10 nM paclitaxel for 0, 6, 24 and 48 h in order to compare the levels of SIRT1-7 protein expressions.

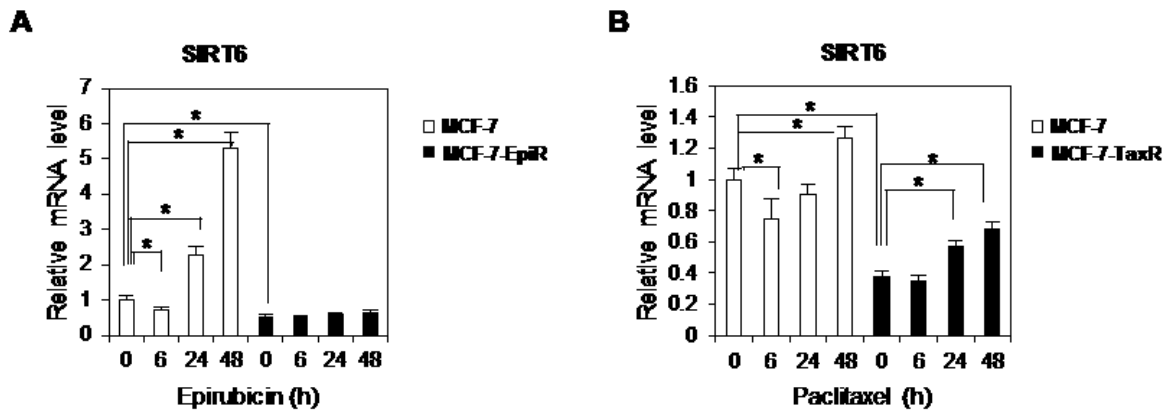


Figure 3.2 SIRT6 upregulation is not primarily due to transcription regulation. **A.** MCF-7 and MCF-7-Epi^{R} cells were treated with 1 μM epirubicin for 0, 6, 24 and 48 h. **B.** MCF-7 and MCF-7-Tax^{R} cells were treated with 10 nM paclitaxel for 0, 6, 24 and 48 h. At the indicated times, Cells were harvested and analysed by *RT*-qPCR to determine *SIRT6* mRNA expression levels. Representative data from 3 independent experiments are shown. Bars represent average ± s.d. Statistical significance was determined by Student's t test (* significant, $p \leq 0.05$; n.s. not significant).

3.4.3 SIRT6 mediates epirubicin and paclitaxel resistance in breast cancer

As my hypothesis is that SIRT6 has a role in the development of epirubicin and paclitaxel resistance in breast cancer, so I investigated whether knocking down SIRT6 expression in MCF-7-Epi^R and MCF-7-Tax^R can re-sensitise these cells in response to epirubicin and paclitaxel treatment. MCF-7-Epi^R and MCF-7-Tax^R cells were transfected with siSIRT6 or siControl (non-silencing siRNA). Cell proliferation was examined by SRB assay after treating with 8 different drug concentrations. As shown in Figure 3.3A, the proliferation rates of SIRT6 depleted cells were significantly lower compared to the controls for both epirubicin (left panel) and paclitaxel treatment (right panel). The SIRT6 expression efficiency was investigated by Western blotting, as depicted in Figure 3.3B. These results revealed that the absence of SIRT6 expression in MCF-7-Epi^R and MCF-7-Tax^R can re-sensitise these resistant cells in response to the drug treatments, suggesting a role of SIRT6 in conferring epirubicin and paclitaxel resistance in MCF-7 cells.

To further investigate this hypothesis, the MCF-7 cells were transiently transfected with ectopic pSIRT6 or pControl overexpressing vectors (Figure 3.3D). The proliferation rate of these cells were then analysed by SRB. The proliferation rate of SIRT6 overexpressing cells was significantly higher compared with the control cells after both treatment (Figure 3.3C). This result further supports the notion that SIRT6 confers epirubicin and paclitaxel resistance in breast cancer.

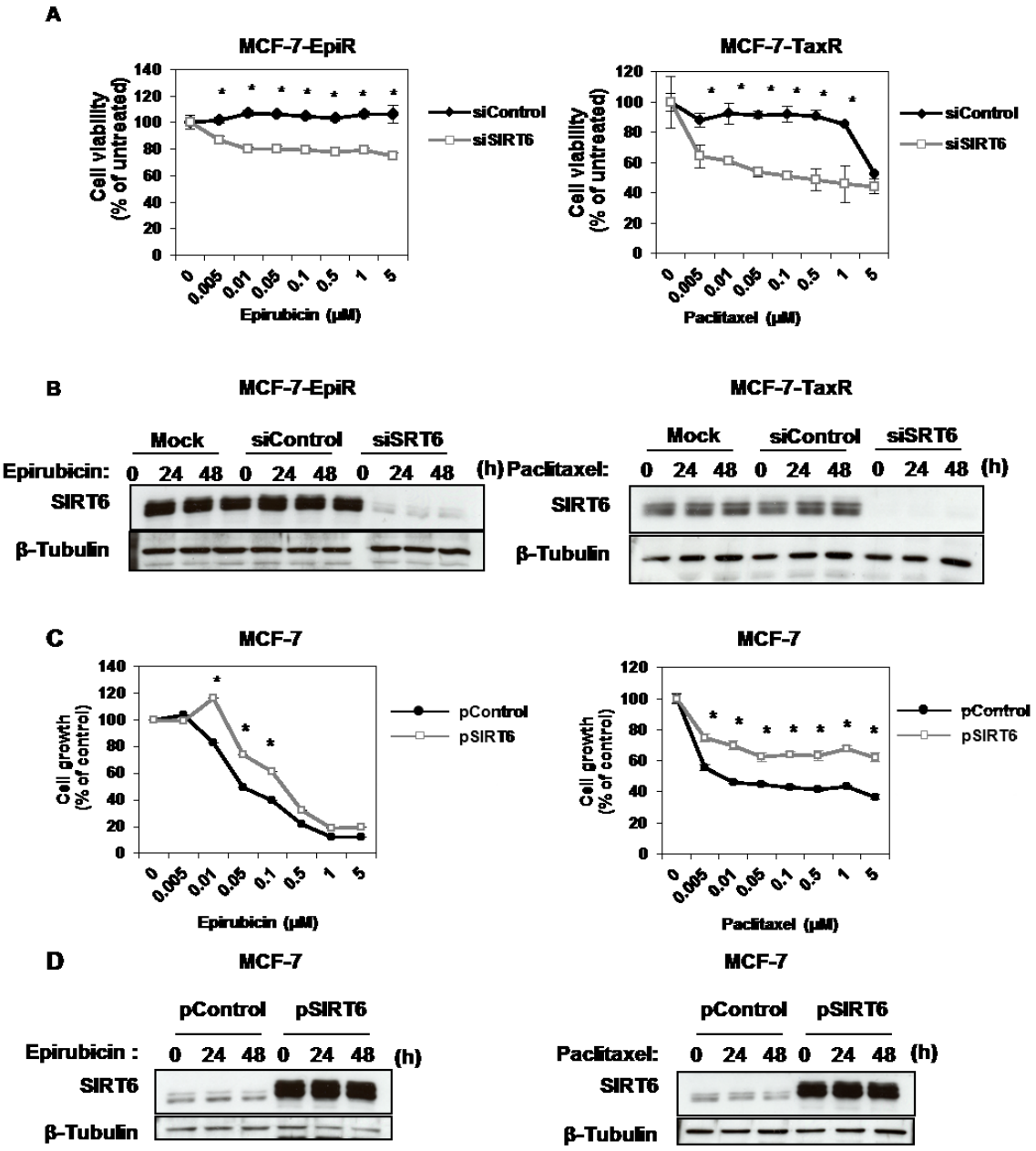


Figure 3.3 SIRT6 partly mediates epirubicin and paclitaxel resistance in breast cancer. A. MCF-7-Epi^R and MCF-7-Tax^R cells were transfected with siControl or siSIRT6 and treated with 8 different concentrations of epirubicin or paclitaxel. SRB assays showed the cell proliferation of these cells at 72 h treatment (**left:** MCF-7-Epi^R and **right:** MCF-7-Tax^R). **B**. Western blot analysis of MCF7-Epi^R (**left panel**) and MCF-7-Tax^R cells (**right panel**) transfected with siSIRT6 or siControl and treated with 1 μM epirubicin or 10 nM paclitaxel. **C**. MCF-7 cells were transfected with pSIRT6 or pControl (empty vector) overexpressing vectors. SRB assays show the cell proliferation of these cells at 72 h treatment (**left:** epirubicin, **right**: paclitaxel). **D**. Western blot analysis was performed to assess the transfection efficiency of MCF-7 cells transfected with pSIRT6 or pControl (empty vector) overexpressing vectors. Cells were treated with 1 μM epirubicin (**left panel**) or 10 nM paclitaxel (**right panel**). Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

3.4.4 SIRT6 mediates epirubicin and paclitaxel resistance in MEFs

After defining that SIRT6 is involved in the development of drug resistance in breast cancer cells, SV40 large T antigen-transferred WT and *Sirt6*$^{-/-}$ MEFs were next used in order to understand and confirm the function of SIRT6 in response to these treatments. Again, cell proliferation of WT and *Sirt6*$^{-/-}$ MEFs was investigated by using SRB assay after treating with 8 different drug concentrations. As shown in Figure 3.4, the proliferation rate of *Sirt6*$^{-/-}$ MEFs was slightly lower compared to WT MEFs, inferring that *Sirt6*$^{-/-}$ MEFs were more sensitive to the treatments of epirubicin (Figure 3.4, left panel) and paclitaxel (Figure 3.4, right panel) than WT MEFs. These results can support the hypothesis that SIRT6 partially have a role in conferring drug resistance. An increase in expression levels of known targets of SIRT6, H3K9Ac and H3K56Ac (Michishita et al., 2008; Yang et al., 2009a), was also observed and found to be increased after treatment, as shown in Figure 3.4B.

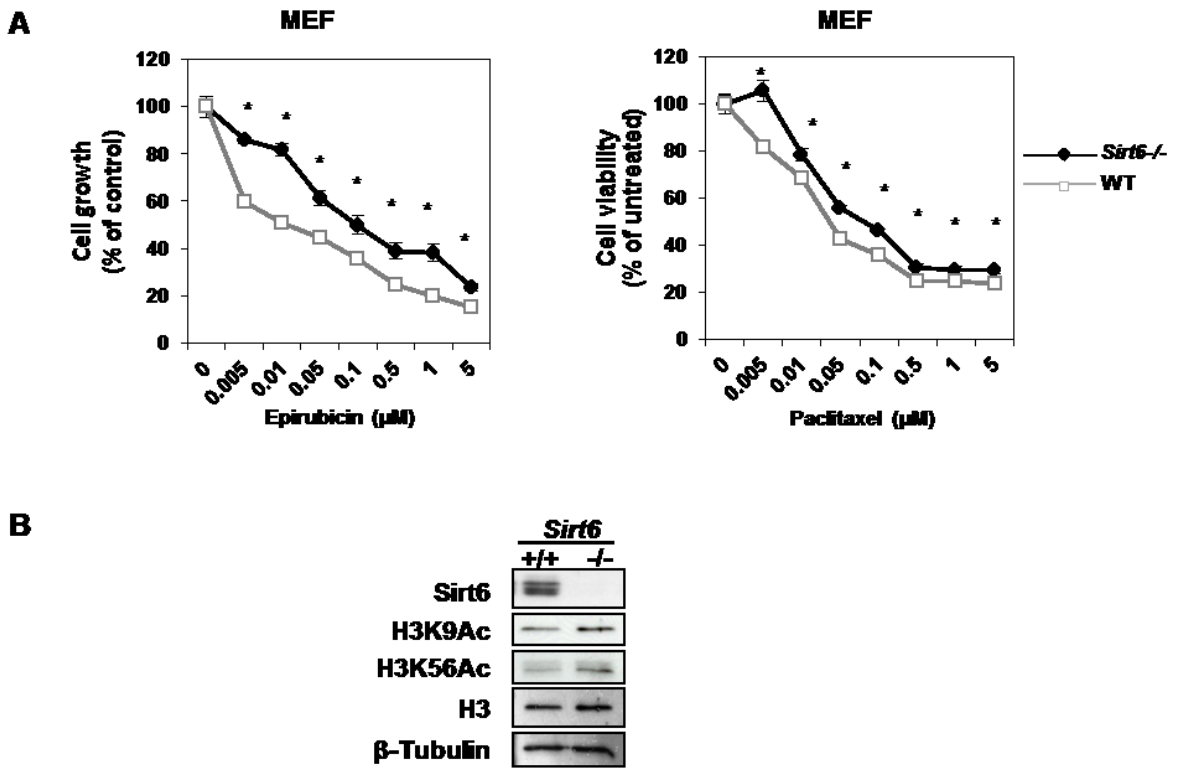


Figure 3.4 *Sirt6*$^{-/-}$ MEFs are more sensitive to epirubicin and paclitaxel treatment. A. WT and *Sirt6*$^{-/-}$ MEFs were treated with increasing concentrations of paclitaxel or epirubicin for 72 h and proliferation rates were measured by SRB assay. **B**. Western blot analysis was performed to assess the levels of SIRT6, H3K9Ac, H3H56Ac, total H3 and β-tubulin protein in SV40 large T antigen-transformed WT and *Sirt6*$^{-/-}$ MEFs. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

3.4.5 *Sirt6*$^{-/-}$ MEFs exhibit higher levels of DNA damage in response to epirubicin.

Epirubicin is an anthracycline, which is known to cause DNA damage through different mechanisms, which include its intercalation into topoisomerase II that leads to DNA double strand breaks and the induction of oxidative stress (Gewirtz, 1999; Hurley, 2002; Jung and Reszka, 2001). SIRT6 has been shown to be a main player in the initiation of DNA damage response (Kaidi et al., 2010). Therefore, I hypothesised that SIRT6 confers epirubicin resistance by enhancing DNA damage repair. To test this hypothesis, the levels of the nuclear foci formation of phosphorylated histone H2AX (γH2AX), a marker for DSBs to recruit repair factors to the break sites (Zhou et al., 2006), were investigated by immunofluorescent staining. WT and *Sirt6*$^{-/-}$ MEFs were treated with 0, 0.05, 0.1, 0.5, 1 and 5 μM of epirubicin for 2 h. Cells were then harvested, fixed, stained for γH2AX foci formation (Figure 3.5A). According to the quantification of γH2AX staining in Figure 3.5B, the levels of epirubicin-induced foci formation were similar in WT and *Sirt6*$^{-/-}$ MEFs for different epirubicin concentrations, reflecting that epirubicin can equally induce DNA DSBs in WT and *Sirt6*$^{-/-}$ MEFs. I then treated WT and *Sirt6*$^{-/-}$ MEFs with 100 nM epirubicin for 0, 2, and 24 h (Figure 3.6A). Similar to the previous results, there were no significant differences in the formation of γH2AX foci at the earlier time point of 2 h (Figure 3.6B). However, *Sirt6*$^{-/-}$ MEFs displayed significantly higher numbers of γH2AX formation compared to WT MEFs at the later time point of 24 h. This result suggests that the absence of SIRT6 results in the impairment of DNA damage repair response after epirubicin treatment.

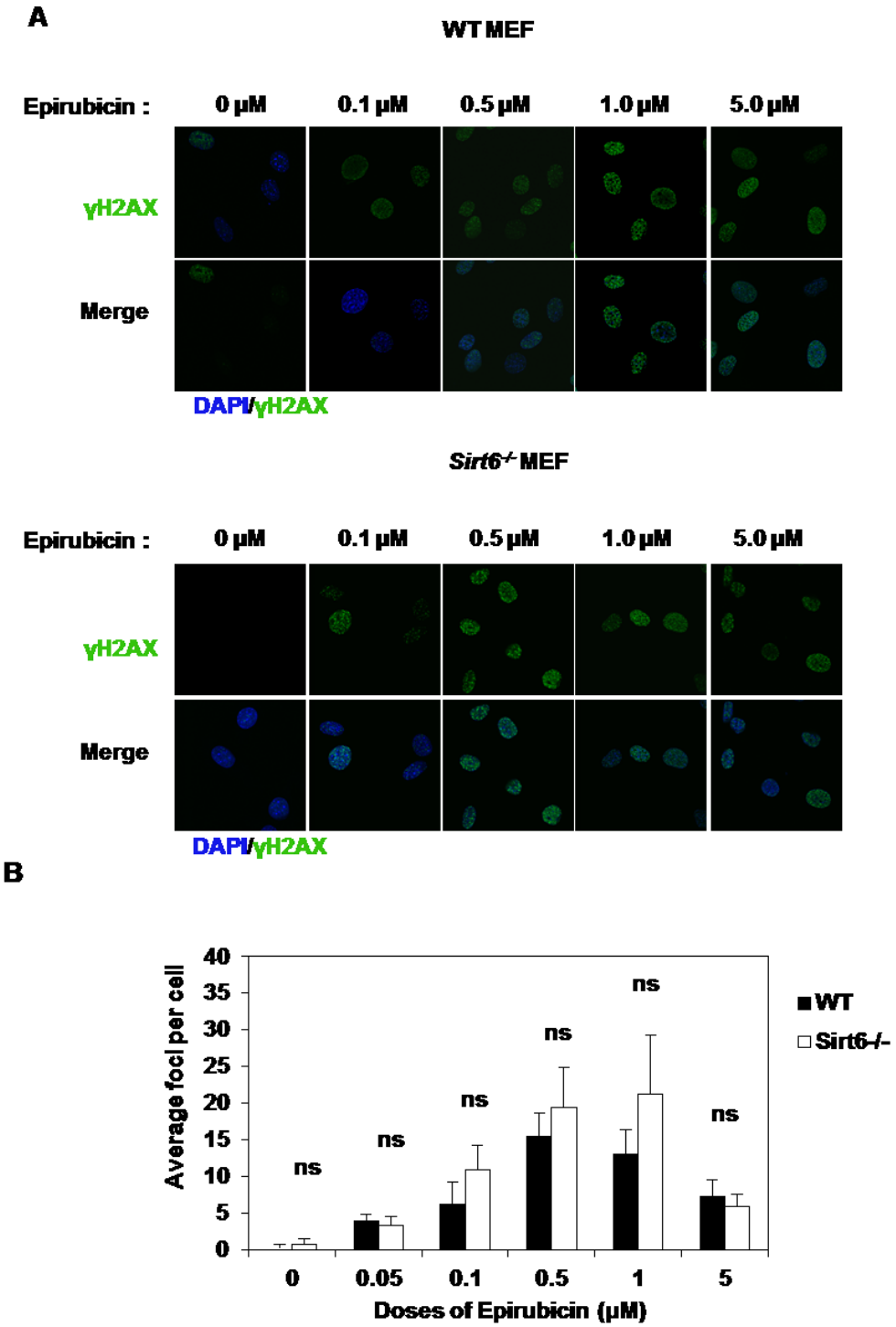


Figure 3.5 Epirubicin induces the formation of γH2AX foci equally in WT and *Sirt6*$^{-/-}$ MEFs at 2 h. **A.** WT and *Sirt6*$^{-/-}$ MEFs were treated with different concentrations of epirubicin for 2 h. Representative images showing γH2AX foci formation induced by 2 h treatment with different concentrations of epirubicin as shown. **B.** Quantification of the average number of γH2AX foci per cell induced at 2 h treatment with different concentrations of epirubicin. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

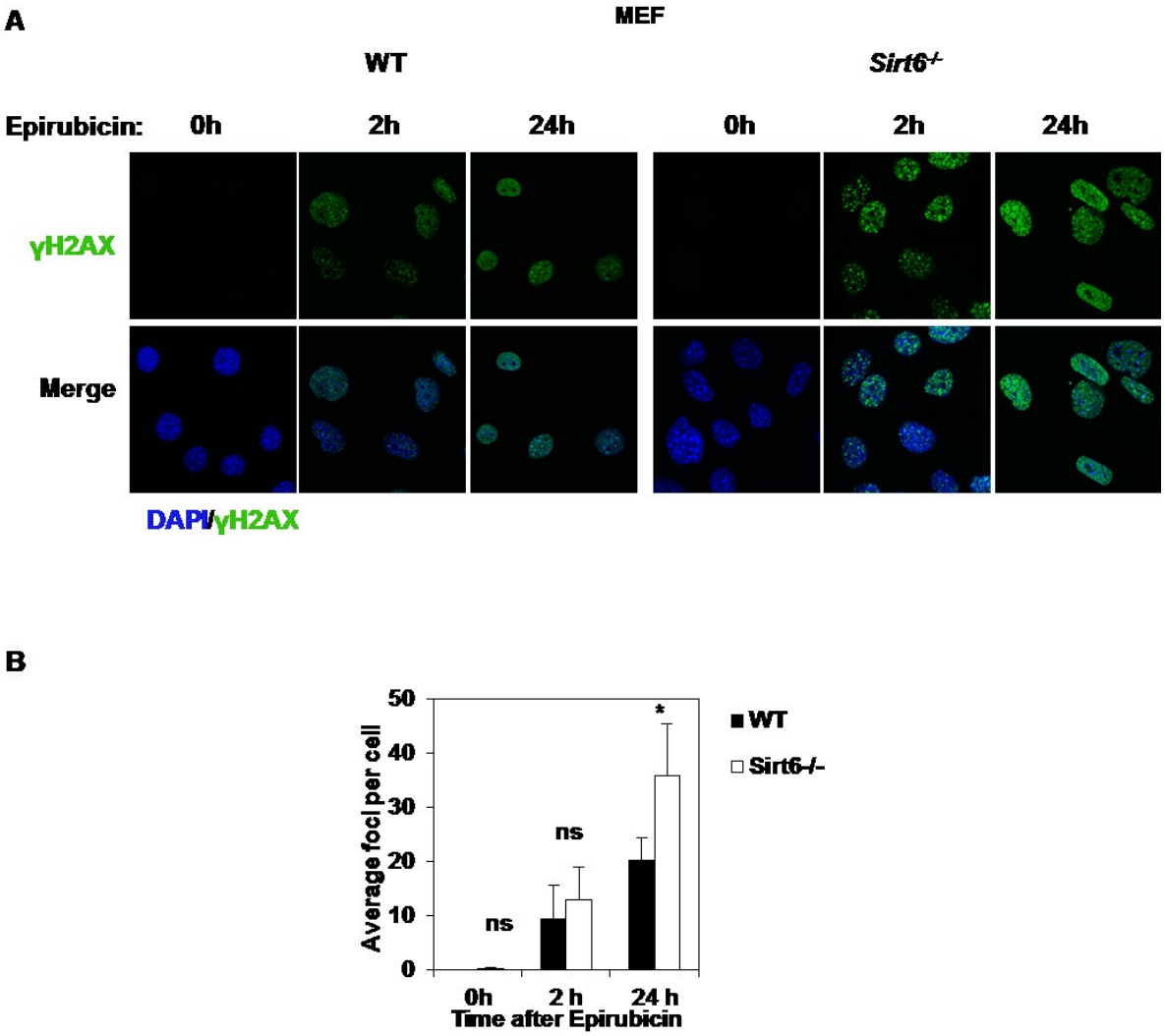


Figure 3.6 *Sirt6*$^{-/-}$ MEFs exhibit a higher degree of DNA damage compared with WT. WT and *Sirt6*$^{-/-}$ MEFs were treated with 1 μM epirubicin for 0, 2 and 24 h. **A.** Representative images showing γH2AX foci formation induced with 1 μM epirubicin at 0, 2 and 24 h. **B.** Quantification of the average number of γH2AX foci per cell induced by 1 μM epirubicin treatment with different time points. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

To further analyse the role of SIRT6 in DNA damage response after epirubicin treatment, alkaline comet assay was performed to validate the degree of DNA damage in a single cell. WT and *Sirt6*$^{-/-}$ MEFs were treated with 0.5 or 1 μM epirubicin for 0, 6 and 24 h. Cells were then harvested and performed comet assays. The degree of DNA damage, including tail moment and olive moment, was measured and analysed by Comet Assay IV image analysis software (Figure 3.7A). The numbers of visible comet tails in WT and *Sirt6*$^{-/-}$ MEFs were increased after epirubicin treatment, showing the induction of DNA damage in both cells. The percentage of DNA in tail, tail moment and olive moment was depicted in Figure 3.7B. The quantification showed that *Sirt6*$^{-/-}$MEFs displayed a higher degree of DNA damage in response to epirubicin treatment compared to the control WT MEFs. This result suggests that the depletion of SIRT6 leads to the diminishing of DNA damage repair, resulting in the accumulation of broken DNA in response to epirubicin treatment.

To further confirm whether SIRT6 has a key role in epirubicin resistance of breast cancer, MCF-7-EpiR cells were transfected with siControl or siSIRT6, treated with 1, 5 and 20 μM epirubicin for 0, 6 and 24h and performed COMET assay. Unfortunately, there was no COMET tail observed in this experiment (data not shown). This is possibly because MCF-7-Epi^{R} cells display higher degree of resistance to epirubicin and this set of epirubicin concentration could not induce DNA damage in this resistant cell. Therefore, WT and *Sirt6*$^{-/-}$ MEFs were used as a template to study the function of SIRT6 in my experiment.

Together, all of these results suggest that SIRT6 confers the epirubicin-induced DNA damage resistance by improving DNA damage repair response.

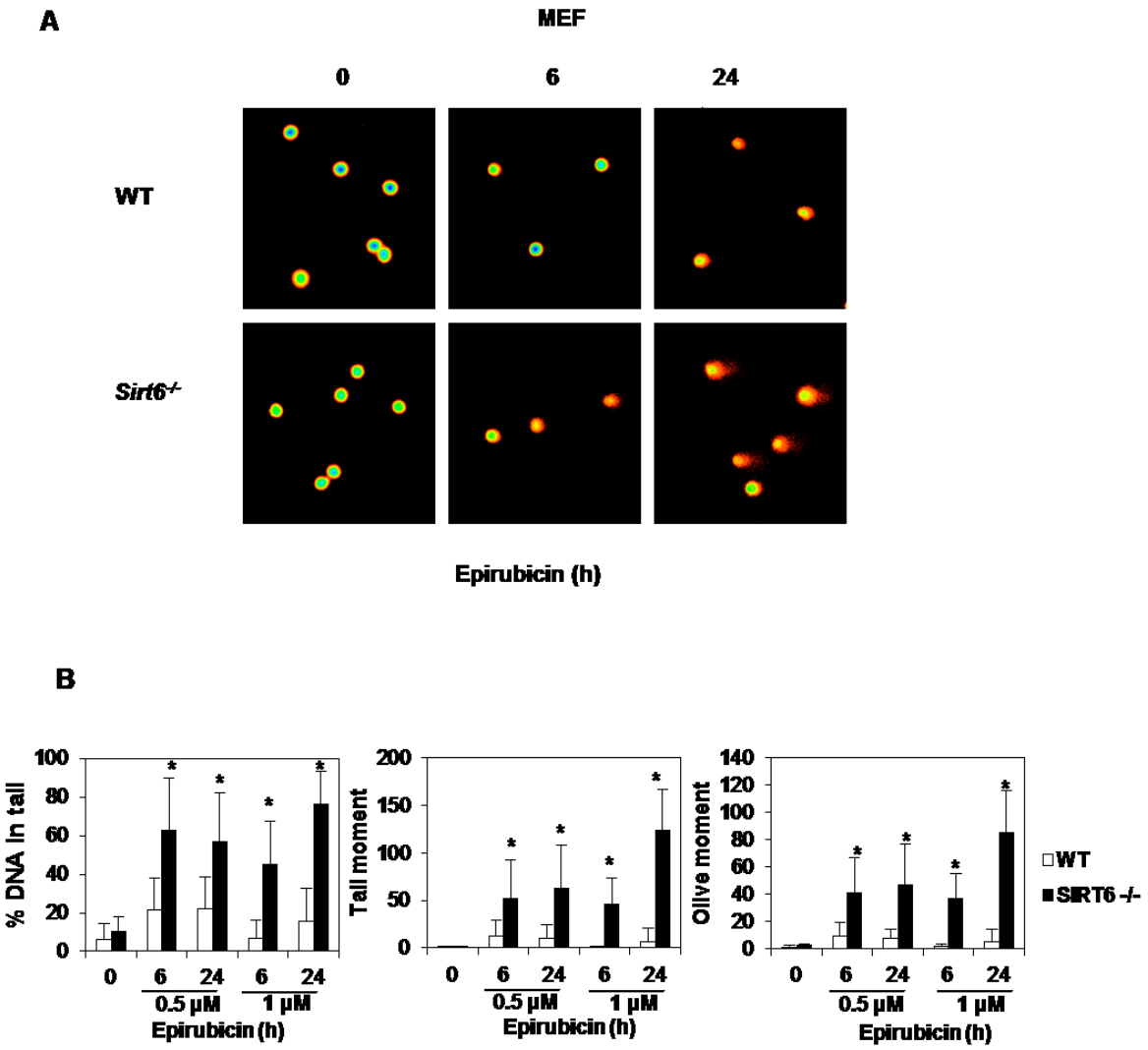


Figure 3.7 *Sirt6*$^{-/-}$ MEFs exhibit the impairment of DNA damage repair. WT and *Sirt6*$^{-/-}$ MEFs were treated with 0.5 or 1 µM epirubicin for 0, 6 and 24 h. **A**. Representative images of comet assay showing DNA migration pattern of *Sirt6*$^{-/-}$ or WT MEFs following with 1 µM epirubicin treatment for 0, 6 and 24 h. **B.** DNA damage represented by various comet assay parameters: average % of DNA in tail (**left panel**), average tail moment (**middle panel**) and average olive tail moment (**right panel**) after treatment with 0.5 or 1 µM epirubicin for 6 and 24 h. These parameters were measured with Comet Assay IV image analysis software for at least 100 cells per dose. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

3.4.6 SIRT6 prevents G2/M cell cycle arrested inducing by paclitaxel.

Paclitaxel is the other common chemotherapeutic agent used for treating hormone-negative breast cancer patients. The mechanism of action of these agents is through binding to and preventing the depolymerisation of microtubules, leading to a transient block at G2/M phase, and subsequently cell death (Manfredi and Horwitz, 1984). It has been reported that MCF-7 treated with paclitaxel causes G2/M cell cycle arrest and apoptosis (Choi and Yoo, 2012). SIRT6 is described as a main factor in DSB repair. Surprisingly, SIRT6 was found to have a role in paclitaxel resistance in breast cancer in this study. To further investigate the role of SIRT6 as a mediator of paclitaxel resistance, cell cycle analysis was performed by Propidium iodide (PI) staining. The effects of silencing or overexpressing of SIRT6 in MCF-7 cells on cell cycle distribution were investigated. MCF-7 cells were transfected with siSIRT6 or siControl, and treated with 1 μM epirubicin for 0, 24, 48 and 72 h. Figure 3.8A demonstrated that silencing of SIRT6 expression in MCF-7 cells did not show a dramatic change in cell cycle distribution. It might be because the expression of SIRT6 is relatively low in MCF-7 cells. Notably, SIRT6 knocked down cells displayed marginally higher number of cells in sub-G1 phase compared to the control cells (Figure 3.8B).

Conversely, the overexpression of SIRT6 in MCF-7 cells resulted in a reduction of cells accumulated in sub-G1 and G2/M phase after paclitaxel treatment (Figure 3.8C). These results suggest that SIRT6 prevents the paclitaxel induced apoptosis by deregulating the G2/M checkpoint, leading to the development of paclitaxel resistance.

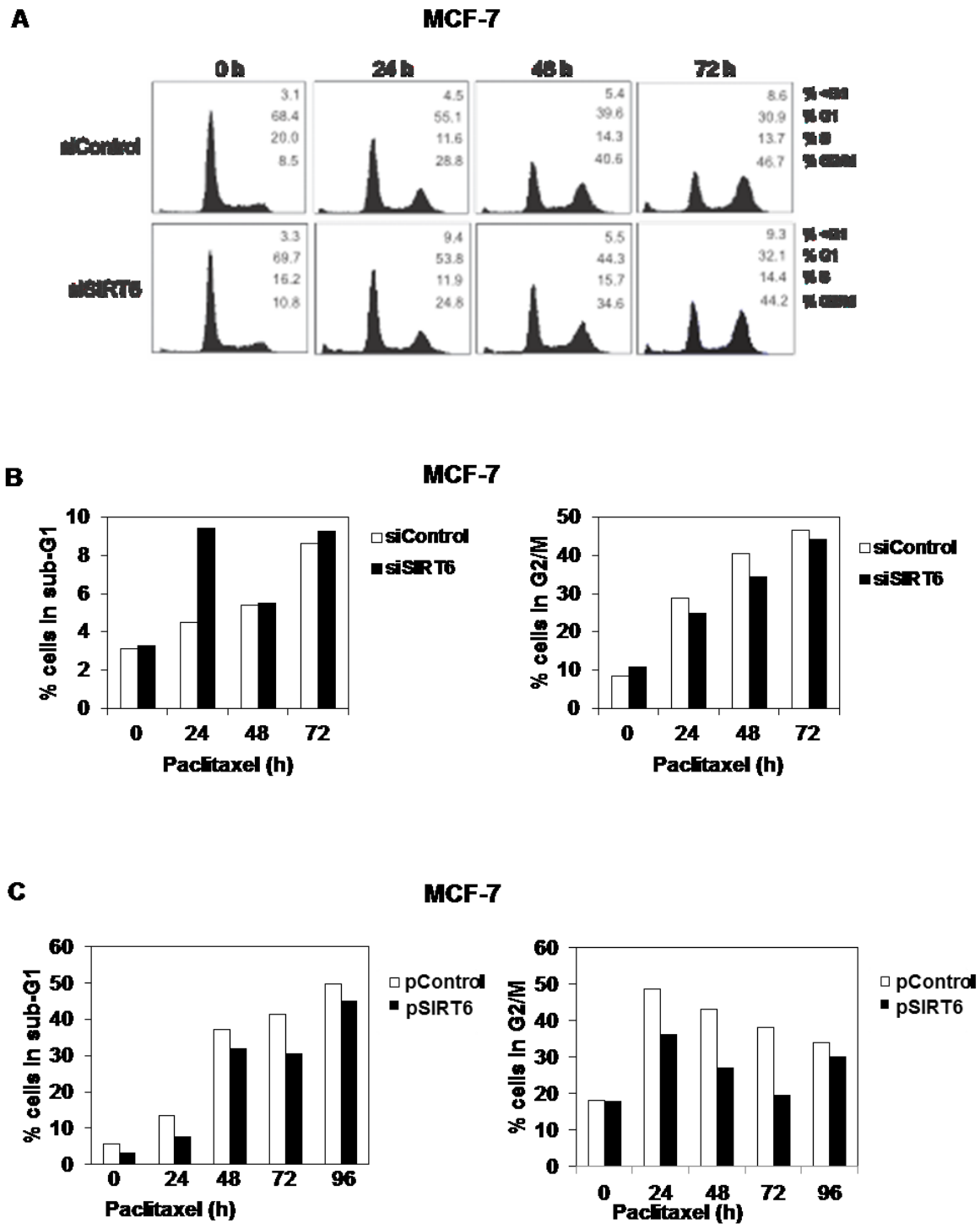


Figure 3.8 SIRT6 prevents G2/M cell cycle arrested induced by paclitaxel A. MCF-7 cells were transfected with siSIRT6 or siControl and treated with 10 nM paclitaxel for the times indicated. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S and G2/M) is indicated. **B.** Percentage of cells in the sub-G1 (**left panel**) or the G2/M (**right panel)** phase of the cell cycle is shown. **C.** MCF-7 cells were transfected with pSIRT6 or pControl overexpressing vectors and treated with 10 nM paclitaxel for the indicated times. Percentage of cells in the sub-G1 (**left panel**) or the G2/M (**right panel**) phase of the cell cycle is shown.

To further validate the role of SIRT6 in paclitaxel resistance, MCF-7-Tax^R cells were transfected with siSIRT6 or siControl, and treated with 10 nM paclitaxel for 0, 24, 48, 72 and 96 h. Cell cycle profiles were again monitored by FACs analysis with PI staining. As shown in Figure 3.9A, paclitaxel treatment did not induce G2/M arrest in MCF-7-Tax^R cells. This might be because the deregulation of G2/M checkpoint is one of the resistant phenotypes of MCF-7-Tax^R. However, the absence of SIRT6 in MCF-7-Tax^R cells led to an increase in the number of dying cells accumulated in sub-G1 compared to the controls (Figure 3.9B). This result suggests that SIRT6 depletion improves the efficiency of paclitaxel-induced cell death in MCF-7-Tax^R cells, supporting the role of SIRT6 as one of mediators of paclitaxel resistance in breast cancer.

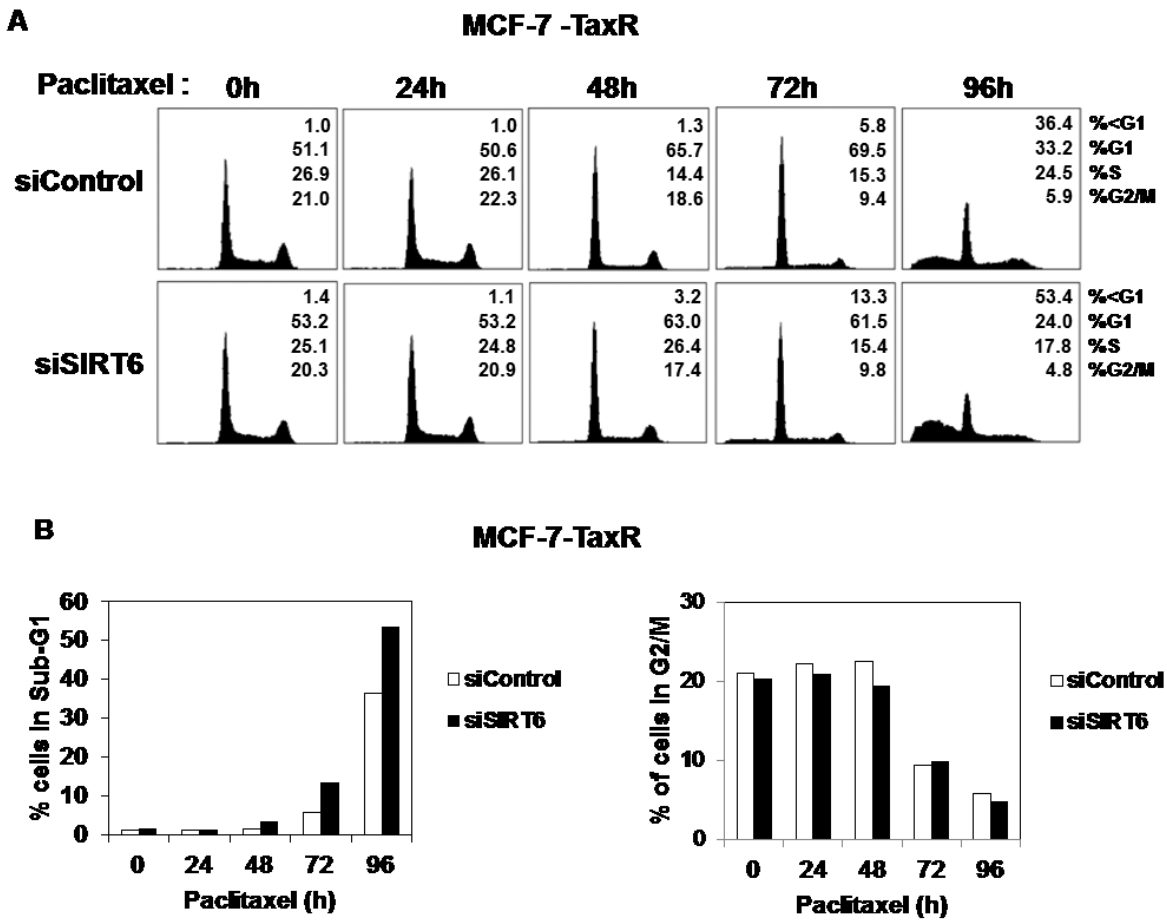


Figure 3.9 SIRT6 depletion in MCF-7-Tax^R cells increases the sensitivity of these cells to paclitaxel treatment. A. MCF-7-Tax^R cells were transfected with siSIRT6 or siControl. Cells were treated with 10 nM paclitaxel for the indicated times and FACS analysis was performed after propidium iodide staining. Percentage of cells in sub-G1, G1, S and G2/M phases is indicated**. B.** Percentage of cells in the sub-G1 (**left panel)** or the G2/M (**right panel**) phase of the cell cycle is shown.

3.4.7 *Sirt6*$^{-/-}$ MEFs are more sensitive to γ–irradiation and display higher degrees of DNA damage compared with WT MEFs.

I next assessed whether the multidrug-resistant protein (Pg-P/MDR1) has a role in the resistant phenotypes of MCF-7-Epi^{R} and MCF-7-Tax^{R} cells. As depicted in Figure 3.10, the overexpression of Pg-P/MDR1 was observed in both MCF-7-Epi^{R} and MCF-7-Tax^{R} cells compared to the sensitive MCF-7 cells, suggesting that this multidrug-resistant protein partly regulates the resistant phenotypes of these cell lines.

To confirm the role of SIRT6 in mediating genotoxic drug resistance and DNA damage repair, and also to eliminate the effect of the multidrug-resistant protein, WT and *Sirt6*$^{-/-}$ MEFs were used. These cells were subjected to γ–irradiation to directly induce DSBs. The proliferation of WT and *Sirt6*$^{-/-}$ MEFs was again investigated by SRB assays after exposing to 5 different doses of γ–irradiation. Figure 3.11A demonstrated that *Sirt6*$^{-/-}$ MEFs were more sensitive to γ–irradiation than WT MEFs. The levels of DNA damage response upon γ–irradiation were also investigated by γH2AX staining. As illustrated in Figure 3.11B, *Sirt6*$^{-/-}$ and WT MEFs displayed similar levels of γH2AX foci formation at the earlier time point, 4h. However, there were significant higher numbers of γH2AX foci in *Sirt6*$^{-/-}$ MEFs compared to WT MEFs at the later time point of 48 h. These data confirm that the depletion of SIRT6 leads to the impairment of DNA DSB repair.

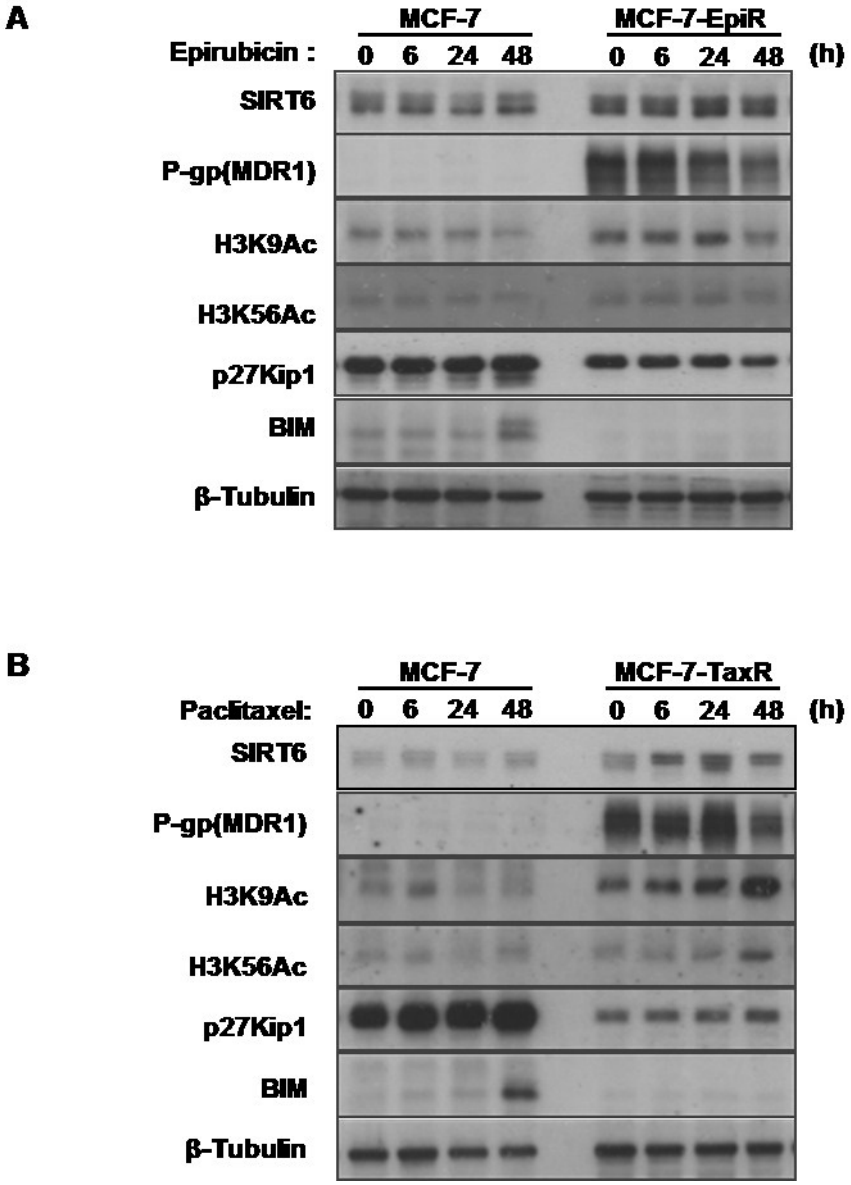


Figure 3.10. Overexpression of multidrug resistant marker Pg-P/MDR1 is involved in the resistant phenotypes of both epirubicin and paclitaxel resistant cell lines. A. MCF-7 and MCF-7-Epi^{R} cells were treated with 1 μM epirubicin for 0, 6, 24 and 48 h. **B.** MCF-7 and MCF-7-Tax^{R} cells were treated with 10 nM paclitaxel for 0, 6, 24 and 48 h. At the indicated times, cells were harvested and analysed by Western blot analysis to determine expression levels of SIRT6, FOXO3a, p27^{Kip1}, Ac-p53, p53, H3K9Ac, H3H56Ac, Bim, Ac-α-Tubulin and β-Tubulin

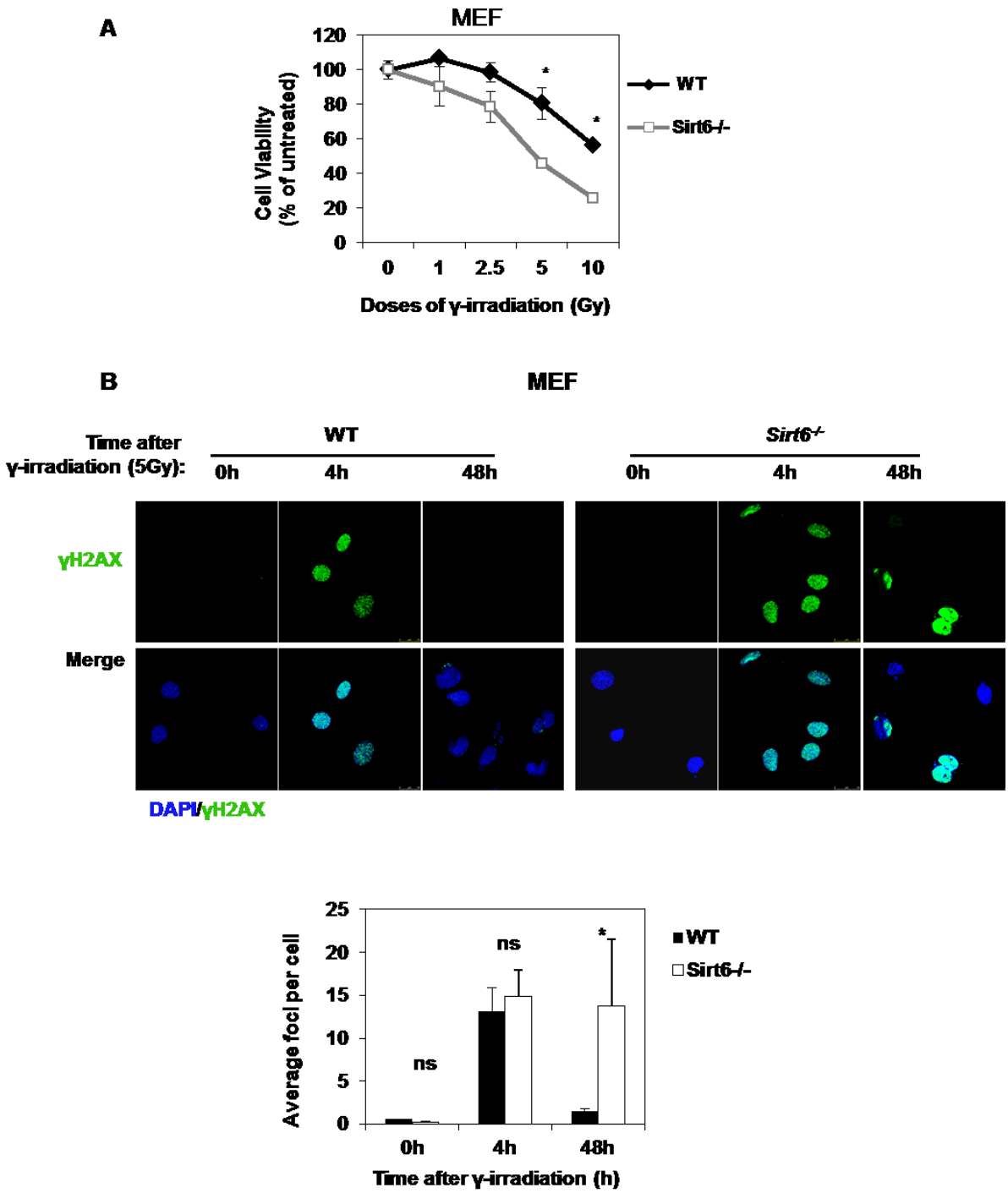


Figure 3.11 *Sirt6*$^{-/-}$ MEFs display a higher degree of sensitivity to γ-irradiation compared with WT MEFs. A. WT and *Sirt6*$^{-/-}$ MEFs were subjected to 5 different doses of γ-irradiation and cultured for 72 h. Cell proliferation was investigated by SRB. **B.** DNA damage analysis in WT and *Sirt6*$^{-/-}$ MEFs. **Top:** Representative images showing γH2AX foci formation induced with 5Gy γirradiation at 0, 4 and 48 h. **Bottom:** Quantification of the average number of γH2AX foci per cell induced by 5Gy γ-irradiation with different time points. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

The depletion of SIRT6 decreases long-term cell survival.

I then studied the role of SIRT6 in long-term survival by using clonogenic assay. Ten thousand cells of WT and *Sirt6*$^{-/-}$ MEFs were seeded into 6-well plates, and treated with 0, 50 and 75 nM of epirubicin and cultured for 2 weeks. Cells were then harvested, fixed and stained with 5% crystal violet solution. Consistent with the SRB results, *Sirt6*$^{-/-}$ MEFs displayed lower ability to survive and form colonies compared with WT cells at 50 and 75 nM epirubicin, inferring that SIRT6 also has a role in the long-term survival of these cells (Figure 3.12). This result further supports a role of SIRT6 in the development of epirubicin resistance.

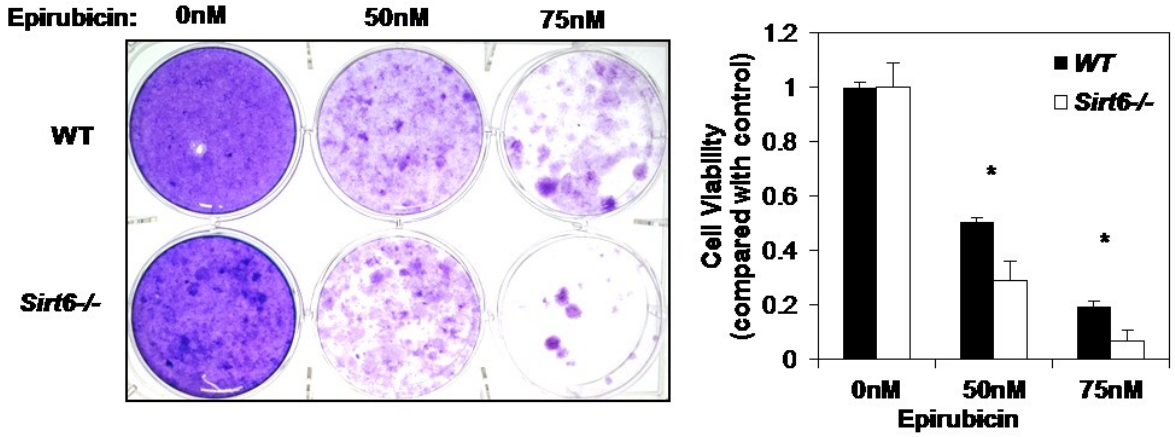


Figure 3.12 Absence of SIRT6 decreases long-term cell survival after epirubicin treatment. WT and *Sirt6-/-* MEFs were treated with 50 and 75nM epirubicin and cultured for 2 weeks. Long-term survival relative to control was investigated by clonogenic assay. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (***, *p≤0.005* **, *p≤0.01 versus* control).

3.4.8 SIRT6 regulates the expression of p53 FOXO3a, and other cell cycle regulators.

Because of the homology and function redundancy in this protein family, I next investigated whether some targets of SIRT1 and SIRT2 such as FOXO3a and p53 might be regulated by SIRT6. To validate if FOXO3a is a possible target of SIRT6, the expression levels of FOXO3a and its downstream target, $p27^{Kip1}$ were investigated and compared in WT and *Sirt6*$^{-/-}$ MEFs in response to epirubicin treatment. As shown in Figure 3.13, the expression levels of FOXO3a and $p27^{Kip1}$ in the absence of epirubicin were slightly higher in *Sirt6*$^{-/-}$ MEFs compared to WT MEFs. Additionally, a strong upregulation of H3K9Ac and H3K56Ac (known targets of SIRT6) was also observed in *Sirt6*$^{-/-}$ MEFs at 6h of epirubicin treatment.

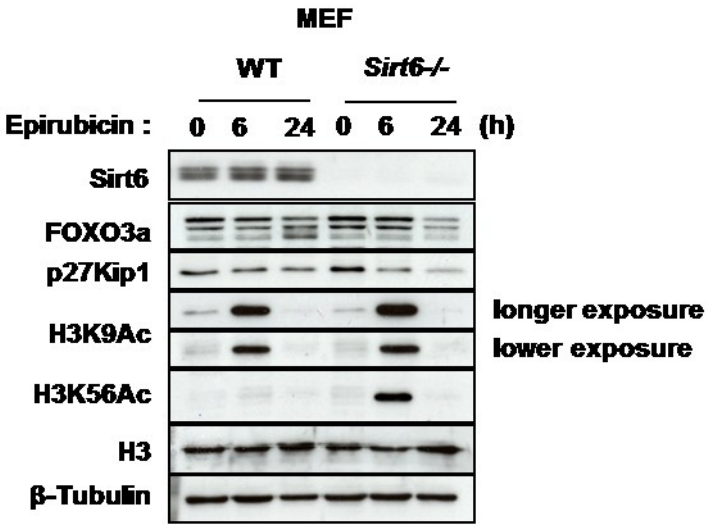


Figure 3.13 Absence of SIRT6 results in the upregulation of FOXO3a and its downstream target, $p27^{Kip1}$. WT and *Sirt6*$^{-/-}$ MEFs were treated with 1 μM epirubicin for 0, 6 and 24 h. The expression of FOXO3a, $p27^{Kip1}$, H3K9Ac and H3K56Ac was investigated by Western blotting.

I next performed the overexpression of SIRT6 in MCF-7 cells and the expression levels of FOXO3a and $p27^{Kip1}$ were investigated. In contrast to the obtained results in MEFs, FOXO3a expression was down-regulated after SIRT6 overexpression in MCF-7 cells, which led to a downregulation of $p27^{Kip1}$expression, in a comparison to the control (Figure 3.14A). Conversely, SIRT6 depletion in MCF-7-Epi^{R} and MCF-7-Tax^{R} cells resulted in the induction of FOXO3a expression, and the subsequent activation of $p27^{Kip1}$ (Figure 3.14B). Together, these results suggest that FOXO3a could be partly regulated by SIRT6 in breast cancer cells.

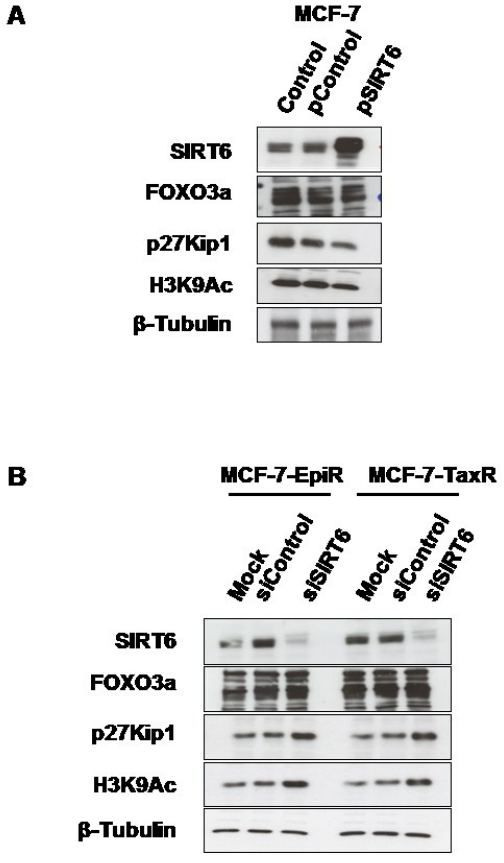


Figure 3.14 Absence of SIRT6 results in the upregulation of FOXO3a and its downstream target, $p27^{Kip1}$. A. MCF-7 cells were transfected with pSIRT6 or pControl overexpressing vectors. **B.** MCF-7-Epi^{R} and MCF-7-Tax^{R} cells were transfected with siSIRT6 or siControl. The expression of FOXO3a, $p27^{Kip1}$, H3K9Ac and H3K56Ac was investigated by Western blotting.

3.4.9 SIRT6 directly interacts with FOXO3a and p53 in cancer cells.

As I shown previously that FOXO3a could be partly regulated by SIRT6, immunoprecipitation was performed in order to investigate whether SIRT6 directly bind to FOXO3a and also p53. MCF-7-Epi^{R} cells were treated with 1 μM epirubicin and harvested at 0, 6 and 24 h post-treatment. Whole cell lysates were extracted and subjected to immunoprecipiation with anti-SIRT6 antibody and IgG as a negative control. As demonstrated in Figure 3.15, p53 and FOXO3a were specifically immunoprecipitated with the anti-SIRT6 antibody, which was not observed in the IgG negative control. This result suggests that SIRT6 might directly interact with p53 and FOXO3a in MCF-7 cells.

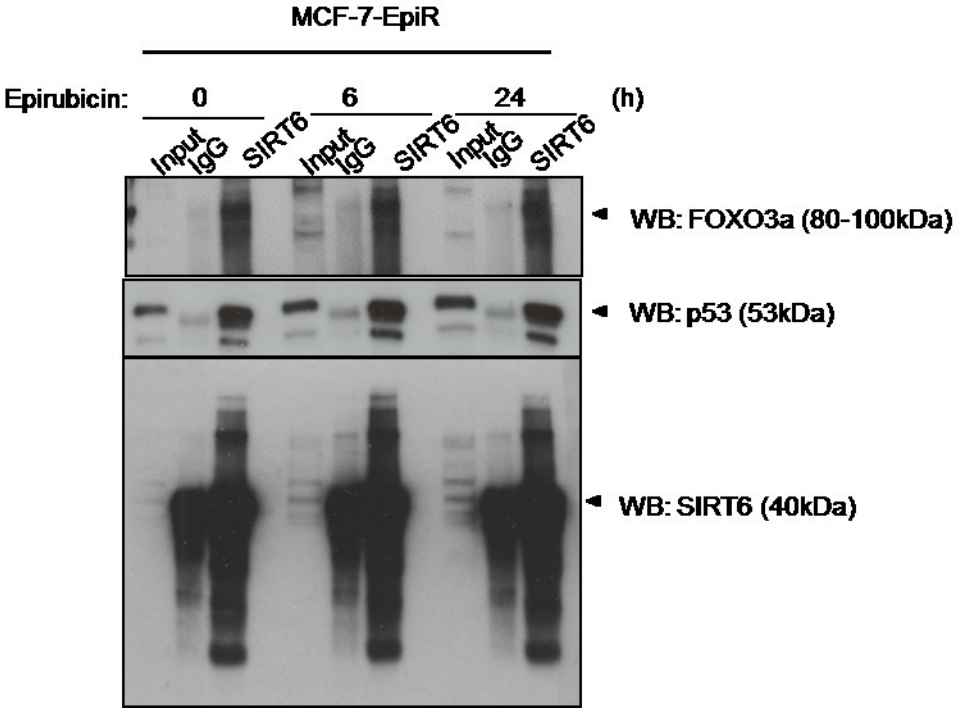


Figure 3.15 SIRT6 binds to p53 and FOXO3a in cells. MCF-7-Epi^{R} cells were treated with 1 μM epirubicin for 0, 6 and 24 h. Cells were then harvested and performed immunoprecipitation with anti SIRT6 antibody. Western blot analysis of eluted protein was performed to investigate co-immunoprecipitation with anti-FOXO3a and p53 antibodies.

3.4.10 SIRT6 is a modulator of FOXO3a and p53 acetylation in cells.

To understand the mechanism of action of SIRT6 in the regulation of FOXO3a and p53, immunoprecipitation was performed. As SIRT6 is described to have both deacetylase and mono-ribosylase activities, I next examined whether the acetylation status of FOXO3a and p53 is regulated by SIRT6 in cells. Immunoprecipitation was firstly performed in WT and *Sirt6*$^{-/-}$ MEFs using anti-total acetyl lysine antibody. As shown in Figure 3.16A (left panel), the immunoprecipitation-western blot analysis showed that the acetylation levels of FOXO3a and p53 were markedly higher in *Sirt6*$^{-/-}$ MEFs compared to WT MEFs. These patterns were similar to the expression levels of known target of SIRT6, H3K9Ac. Similar results were observed for SIRT6 depletion in MCF-7 cells, Figure 3.16A (right panel). The levels of the acetylation and expression of FOXO3a were predominantly higher in SIRT6 knocked down cells compared to control cells.

To further confirm these results, the reverse immunoprecipitation using anti-FOXO3a antibody was then performed. Figure 3.16B (left panel) demonstrated that *Sirt6*$^{-/-}$ MEFs exhibited higher levels of FOXO3a acetylation compared to WT MEFs. Again, similar results were obtained after SIRT6 depletion in MCF-7 cells. As illustrated in Figure 3.16B (right panel), the acetylation levels of FOXO3a were noticeably higher in SIRT6 depleted cells compared to the levels in control cells. All of these results confirm that SIRT6 regulates acetylation status of FOXO3a and p53 in cells.

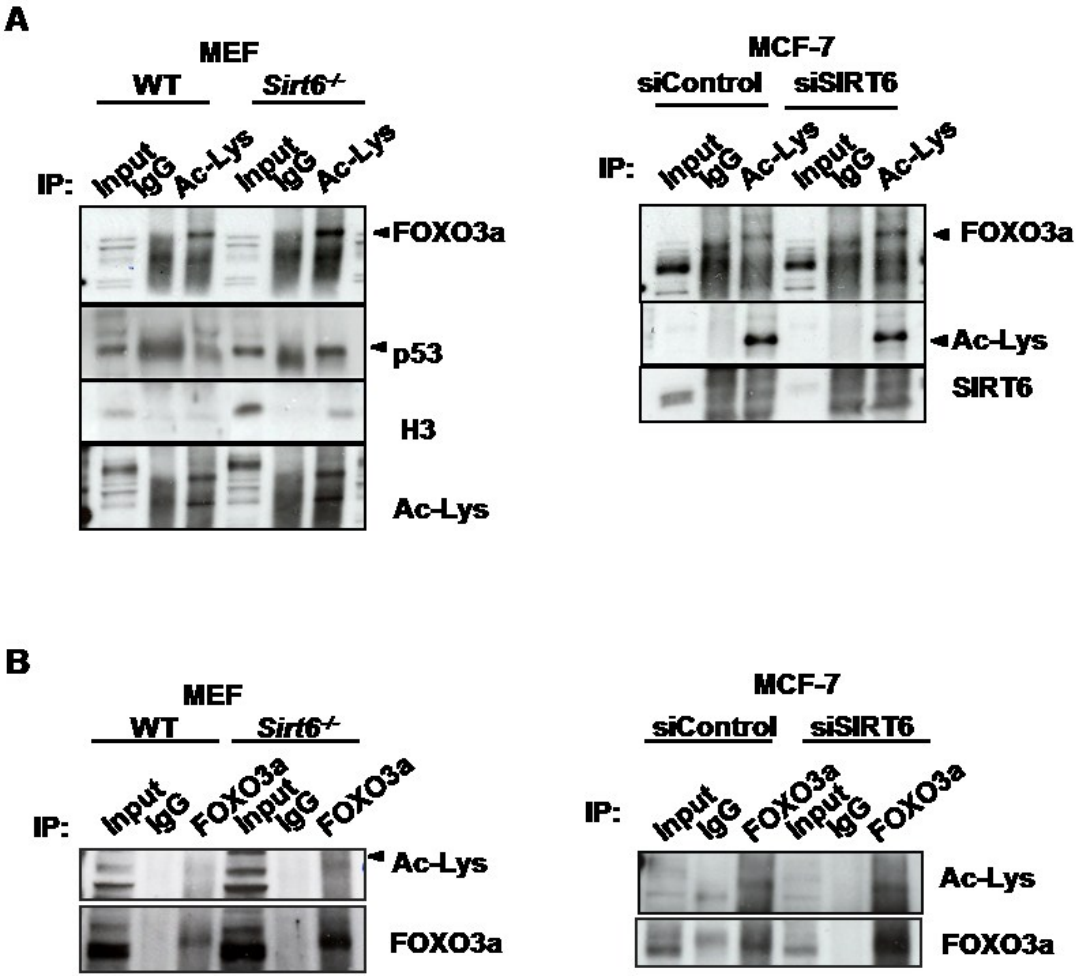


Figure 3.16 SIRT6 regulates the acetylation levels of FOXO3a and p53 in cells. A. WT and *Sirt6*$^{-/-}$ MEFs **(left panel)**, MCF-7 cells were transfected with siSIRT6 or siControl **(right panel**) and were extracted for whole cell lysates and subjected to immunoprecipitation using total-acetylated lysine antibody. The acetylation levels of FOXO3a were investigated by incubating with anti-FOXO3a antibody. **B.** WT and *Sirt6*$^{-/-}$ MEFs (**left panel**), MCF-7 cells were transfected with siSIRT6 or siControl (**right panel**) and were extracted for whole cell lysates and subjected to immunoprecipitation using anti-FOXO3a antibody. The acetylation levels of FOXO3a were investigated by incubating with anti- total acetyl lysine antibody.

3.4.11 Role of SIRT6 in the regulation of acetylation of its downstream targets

To further analyse the role of SIRT6 in the regulation of acetylation and the expression of some targets, SIRT6 was the ectopically overexpressed in MCF-7 cells and the expression levels of potential downstream targets, including p53 and FOXO3a, were investigated. SIRT6 overexpression in MCF-7 cells led to a decrease in the expression levels of FOXO3a after both epirubicin (Figure 3.17: top panel) and paclitaxel treatment (Figure 3.17: bottom panel). Similar to FOXO3a, acetylated p53 and p53 expression were significantly higher in control cells compared to SIRT6 overexpressing MCF-7 cells. In agreement with FOXO3a and p53, the expression of H3K9Ac and H3K56Ac was also lower in SIRT6 overexpressing cells compared to the controls. In contrast, the depletion of SIRT6 in MCF-7-Tax^{R} cells resulted in higher levels of Ac-p53, p53 and FOXO3a expressions compared to control cells for paclitaxel treatments (Figure 3.18 right panel). An increase in FOXO3a expression was also observed in SIRT6-depleted MCF-7-Epi^{R} cells compared to control cells (Figure 3.18 left panel). However, the expression of Ac-p53 and p53 could not be detected in MCF-7-Epi^{R} cells because p53 is absent in this resistant cell line. Additionally, the expression levels of H3K9Ac and H3K56Ac were also slightly higher in SIRT6 silenced MCF7-Epi^{R} and MCF-7-Tax^{R} cells compared to the control cells. Further experiment was done in WT and *Sirt6*$^{-/-}$ MEFs. Consistently, the expression levels of FOXO3a, Ac-p53 and p53 were noticeably higher in *Sirt6*$^{-/-}$ MEFs in comparison with the levels in WT MEFs. Notably, there was an induction of p27^{Kip1} and BIM expressions, FOXO3a downstream antiproliferative effectors, in *Sirt6*$^{-/-}$ MEFs after epirubicn and paclitaxel treatment (Figure 3.18B).

Together, all of these results suggest that SIRT6 partly mediates epirubicin and paclitaxel resistance in breast cancer by targeting and repressing the activities of FOXO3a and p53 via its deacetylation.

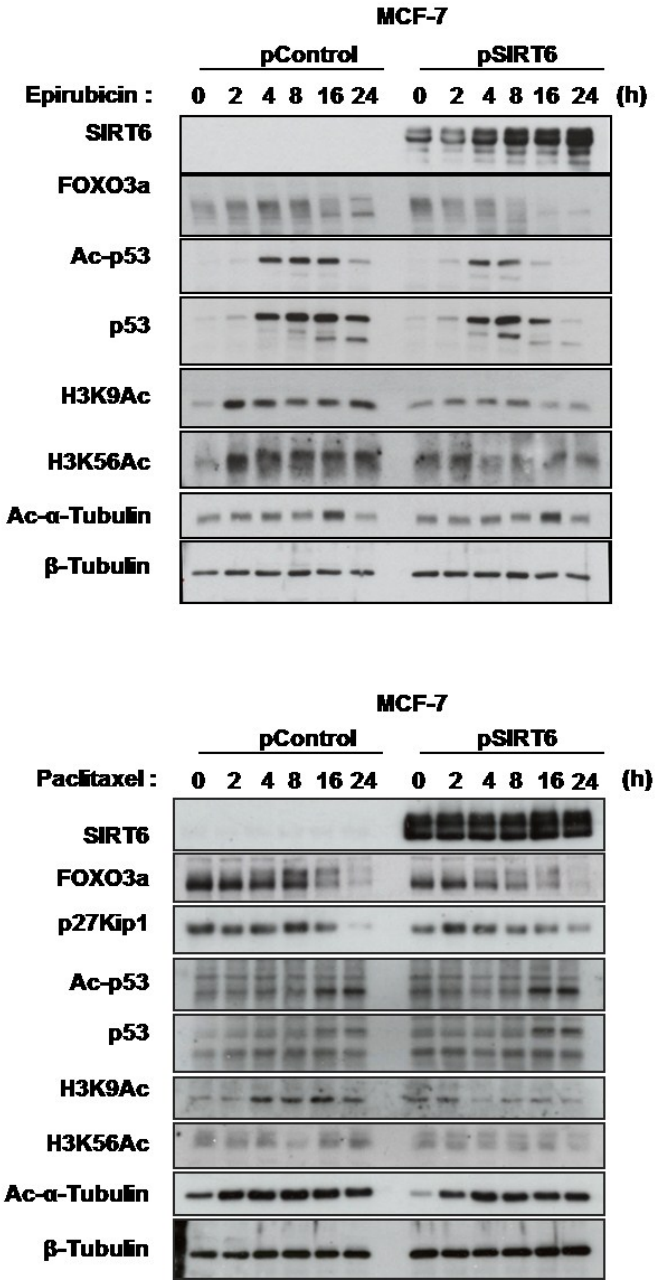


Figure 3.17 SIRT6 overexpression results in a decrease in FOXO3a, Ac-p53 and p53 expression. MCF-7 cells were transfected with pSIRT6 or pControl overexpressing vectors. Cells were treated with 1 μM epirubicin (**top panel**) or 10 nM paclitaxel (**bottom panel**) for the indicated times and harvested for Western Blot analysis of SIRT6, FOXO3a, $p27^{Kip1}$, Ac-p53, p53, H3K9Ac, H3H56Ac, Ac-α-Tubulin and β-Tubulin.

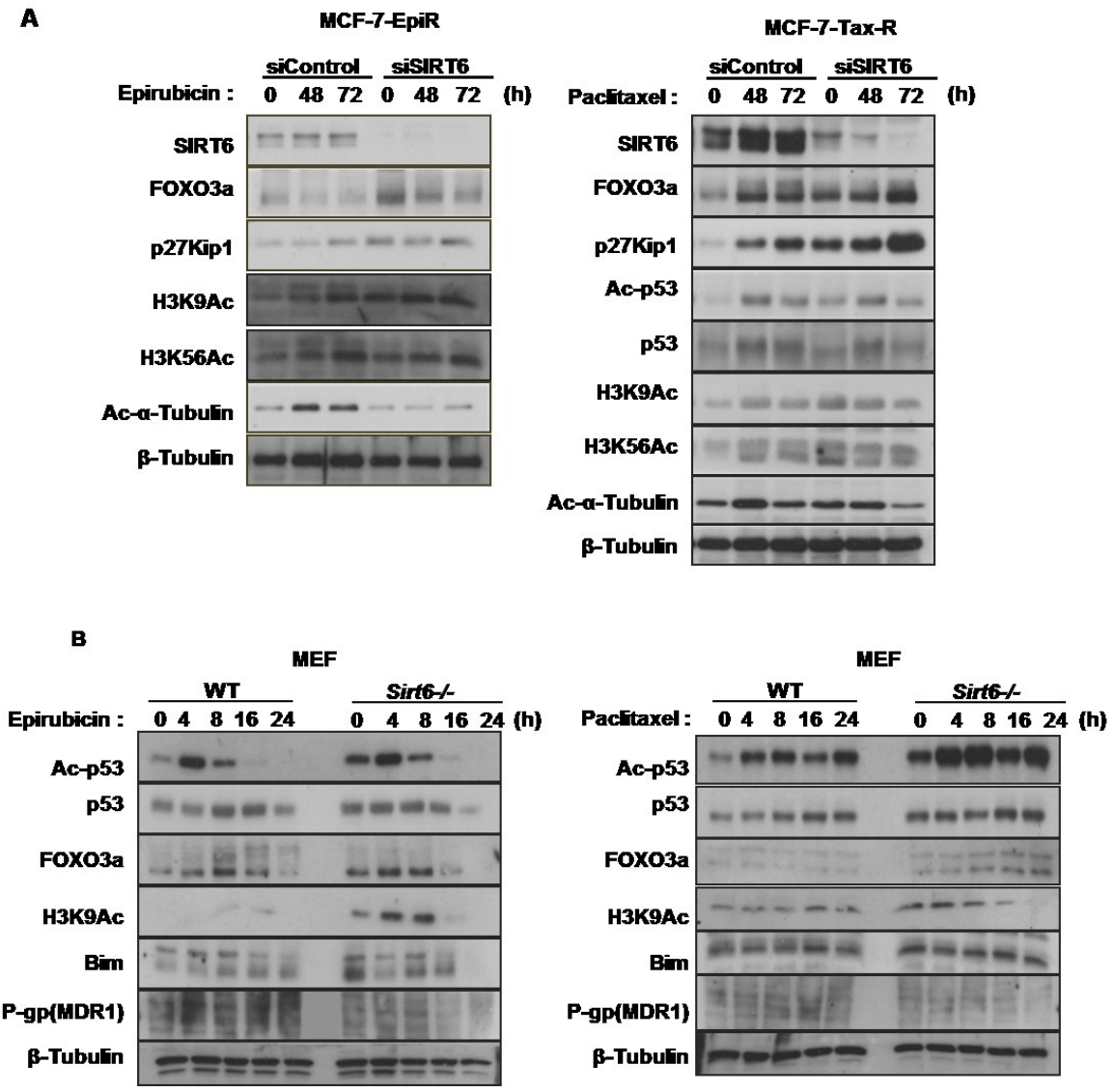


Figure 3.18 SIRT6 depletion results in the upregulation of FOXO3a, Ac-p53 and p53 expressions. A. MCF-7-Epi^{R} cells and MCF-7-Tax^{R} cells were transfected with siSIRT6 or siControl. Cells were treated with 1 μM epirubicin (**left panel**) and 10 nM paclitaxel (**right panel**) for 0, 4, 8, 16, and 24 h and harvested for Western Blot analysis. **B.** WT and *Sirt6*$^{-/-}$ MEFs were treated with 0.1 μM epirubicin (**left panel**) and 10 nM paclitaxel (**right panel**) for the times indicated, and harvested for Western Blot analysis. * It is notable that some of the proteins in the *Sirt6*$^{-/-}$ MEFs appeared at lower levels. It is likely to be due to the high levels of apoptosis.

3.4.12 Absence of Foxo1/3/4 and p53 expression in MEFs results in the higher degrees of drug resistance.

To further analyse the role of SIRT6 in the regulation of FOXO3a and p53 and to validate the functions of FOXOs and p53 proteins in drug resistance, WT, *p53*$^{-/-}$ and *Foxo1/3/4* knocked out MEFs were used as a template. The sensitivity of WT, *p53*$^{-/-}$ and *Foxo1/3/4* –deficient MEFs to epirubicin and paclitaxel was then investigated using SRB. As shown in Figure 3.19A, *Foxo1/3/4* null MEFs displayed significant higher levels of drug resistance compared to WT MEFs from range of 0.05-5 μM of epirubicin and paclitaxel concentrations. This result reveals that Foxo1/3/4 expression is essential for the sensitivity of these cells in response to epirubicin and paclitaxel treatments. In contrast, similar sensitivity of WT and *p53*$^{-/-}$ MEFs was observed in response to both epirubicin and paclitaxel treatments. However, there was a marginally higher rates of cell proliferation in *p53*$^{-/-}$ MEFs compared to WT MEFs especially at the lower drug concentrations (*e.g.* 0.005 and 0.01 μM) suggesting that the absence of p53 expression in MEFs leads to epirubicin and paclitaxel resistance.

All of these results could further support the role for SIRT6 in the downregulation of FOXO3a and p53 expressions, leading to the development of drug resistance.

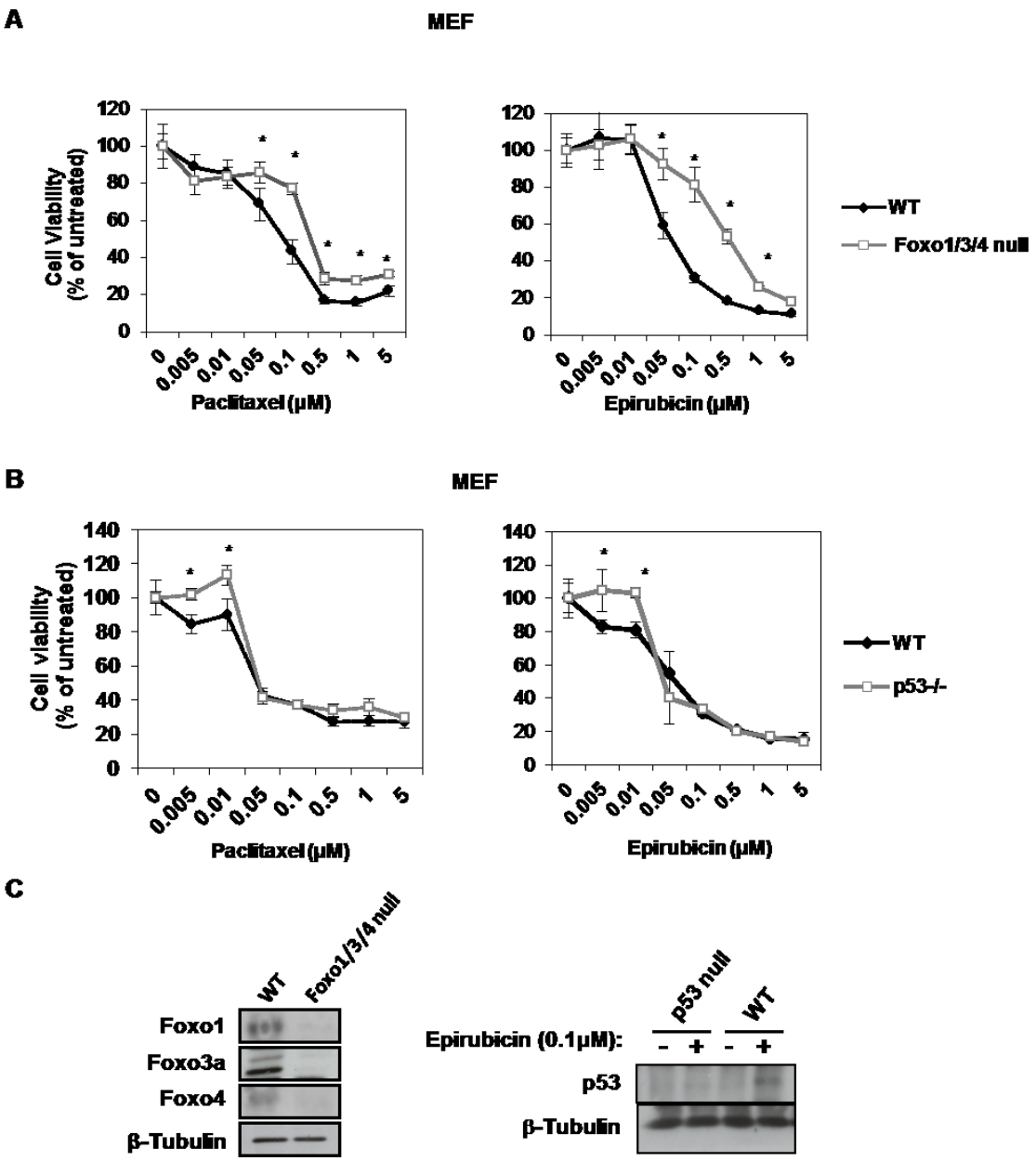


Figure 3.19 Absence of Foxo and p53 in MEFs results in a higher degree of drug resistance. WT, *Foxo1/3/4* null, and *p53*$^{-/-}$ MEFs were treated with 8 different concentrations of epirubicin (**right panel**) or paclitaxel (**left panel**) for 72 h. **A.** Cell viability of WT and *Foxo1/3/4* null MEFs, **B.** Cell viability of WT and p53$^{-/-}$ MEFs was investigated by SRB. **C.** Western blot analysis was performed to assess the expression levels of Foxo1, Foxo3, Foxo4 and β-tubulin protein in WT and *Foxo1/3/4* null MEFs (**left panel**). The levels of p53 and β-tubulin in WT and *p53*$^{-/-}$ MEFs were depicted on the right panel. Bars represent average ± s.d. Statistical significance was determined by Student's t test from 3 independent experiments (* significant, $p \leq 0.05$; n.s. not significant).

3.4.13 Nuclear SIRT6 expression in breast cancer is significantly associated with poorer prognosis.

After identifying the role for SIRT6 in development of drug resistance in breast cancer cells, I next validated the role of SIRT6 in patient samples. The expression pattern of SIRT6 was recorded and studied by Ms Chun Gong at the department of Pathology, Queen Mary Hospital of Hong Kong. SIRT6 expression was investigated by using immunohistochemical staining. A panel of breast cancer samples (118 cases) with a high proportion of invasive ductal carcinoma (93 cases) as previously described in Chen et al. (Chen et al., 2010b) was accessed and scored for SIRT6 expression. The ages of patients ranged from 30 to 90 years old, with a mean of 53 years. The expression pattern and subcellular localisation were analysed and correlated with histological type, histological grade, clinical stage, estrogen and progestrogen receptor status, lymph node metastasis, survival time and chemotherapeutic treatment (Figure 3.20A). Approximately 80% of the patients were in tumour stage I and II and about half of the patients were with Grade 3 tumours. Moreover, half of them had lymph node metastasis. Within the cohort, 67 patients have received chemotherapy, including 34 cases for either epirubicin or paclitaxel. The remaining 33 patients were treated with CMF (Figure 3.20A). For these patients, chemoresistance was significantly associated with poor survival (overall survival; p = 0.003, disease specific survival; p= 0.025 in Figure 3.20B). As the majority of the histological types of the tumour samples were invasive ductal carcinoma, for consistency, only cases of this type were used for statistical analysis in this study. SIRT6 staining was observed in both nuclear and cytoplasmic localisation with representative expression patterns as depicted in Figure 3.21A. Figure 3.21B demonstrated that SIRT6 nuclear staining was significantly associated with poorer

survival (p=0.018 for overall survival, log-rank test, Kaplan-Meier estimate analysis). After correcting for tumour stage and lymph-node involvement, SIRT6 nuclear staining was still associated with death on multivariate analysis (RR = 2.477, p=0.033) (Figure 3.21C). Cytoplasmic staining, on the other hand, was significantly related to better survival (p=0.014 for overall survival, Kaplan Meier estimate analysis, Figure 3.22). Even though, it was not statistically significant, a strong correlation of SIRT6 nuclear staining associated with disease specific survival was observed in both Kaplan-Meier estimate analysis ($p=0.057$) (Figure 3.23A) and Cox univariate regression analysis (RR = 2.143, $p=0.063$) (Figure 3.23B). However, Cox multivariate regression analysis was shown no significant correlation between SIRT6 and disease specific survival after correcting for tumour stage and lymph-node involvement (Figure 3.23B). We therefore conclude that higher SIRT6 nuclear expression is associated with poorer survival in breast cancer patients. This result further suggests that SIRT6 expression is associated with cytotoxic chemotherapeutic drug resistance, as at least half of these patients received treatments and monitored after diagnosis.

A

	Number of cases (%)
Patients	118
Median age of first diagnosis	52 (30-90)
Mean of survival months	76.66 (0-182) months
Tumor stage	
I	30 (25)
II	64 (54)
III	16 (14)
IV	3 (3)
Missing	5 (4)
Tumor grade	
1	11 (9)
2	30 (25)
3	55 (47)
Missing	22 (19)
Lymph node metastasis	
Positive	62 (53)
Negative	43 (36)
Missing	13 (11)
Estrogen receptor status	
Positive	66 (56)
Negative	35 (30)
Missing	17 (14)
Progesterone receptor status	
Positive	52 (44)
Negative	50 (42)
Missing	16 (14)
Chemotherapy	
Yes	67 (57)
TAX	7 (10)
EPI	27 (40)
CMF	33 (49)
No	27 (23)
Missing	24 (20)

Figure 3.20A The summary of described patient sample features in Chen et al, 2010 was accessed and scored for SIRT6 expression. One hundred and eighteen cases of breast cancer diagnosed between the years 1992 to 2001 together with clinical follow up data were retrieved from the records of the Department of Pathology, Queen Mary Hospital of Hong Kong. The ages of patients ranged from 30 to 90 years old, with a mean of 53 years. The expression pattern and subcellular localisation were analysed and correlated with histological type, histological grade, clinical stage, estrogen and progestrogen receptor status, lymph node metastasis and survival time.

B

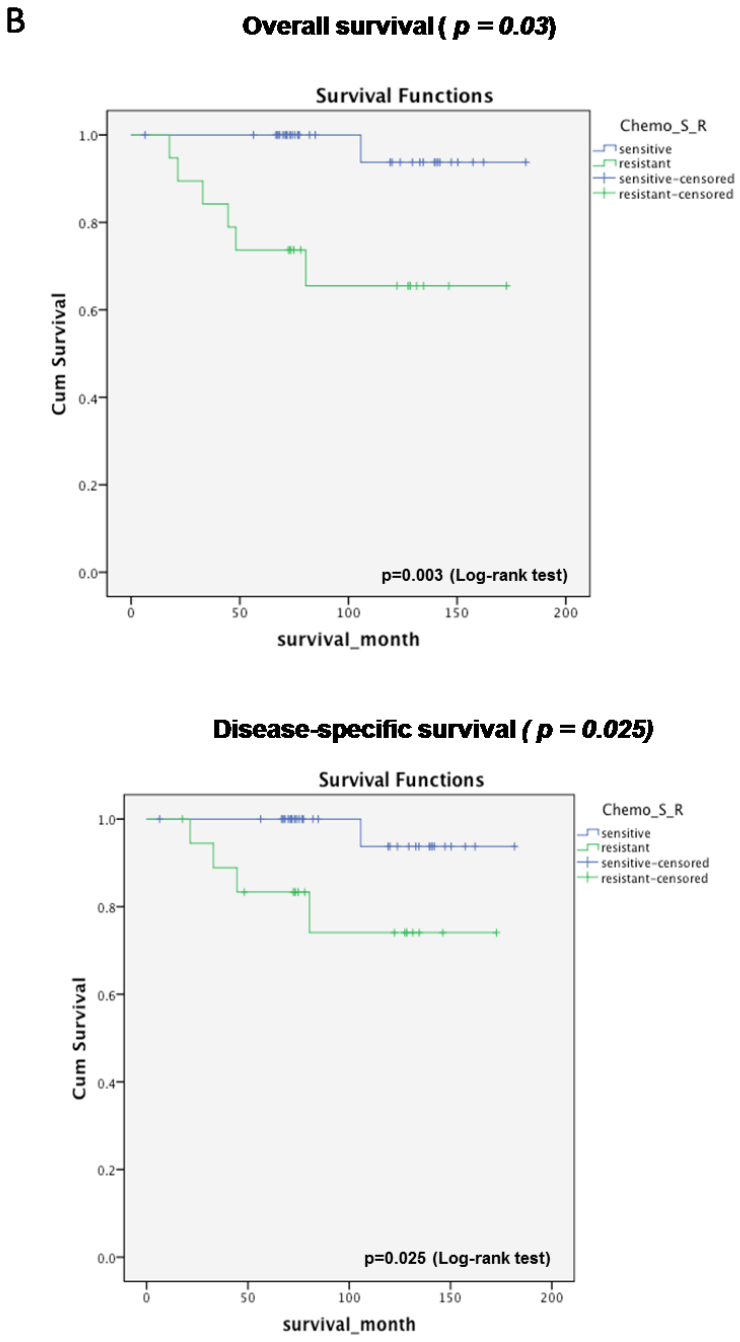


Figure 3.20B Kaplan-Meir analysis comparing patients with chemosensitive with chemoresistant tumors for overall survival and disease free survival
Analysis of survival data of the patients in TMA samples with paclitaxel and epirubicin resistance did indeed show statistical significant correlation. There were a total of 67 patients who had been recorded to have received either neoadjuvant or adjuvant chemotherapy. Chemoresistance was defined as those who received chemotherapy, and subsequently developed local relapse or distant metastases. Chemosensitivity was defined as those who remained disease free on follow-up having received chemotherapy. Kaplan-Meir analysis comparing patients with chemosensitive with chemoresistant tumors for overall survival and disease free survival showed there was significant association between chemoresistance with overall survival (*p=0.003*) and with poor disease-free survival (*p=0.025*)

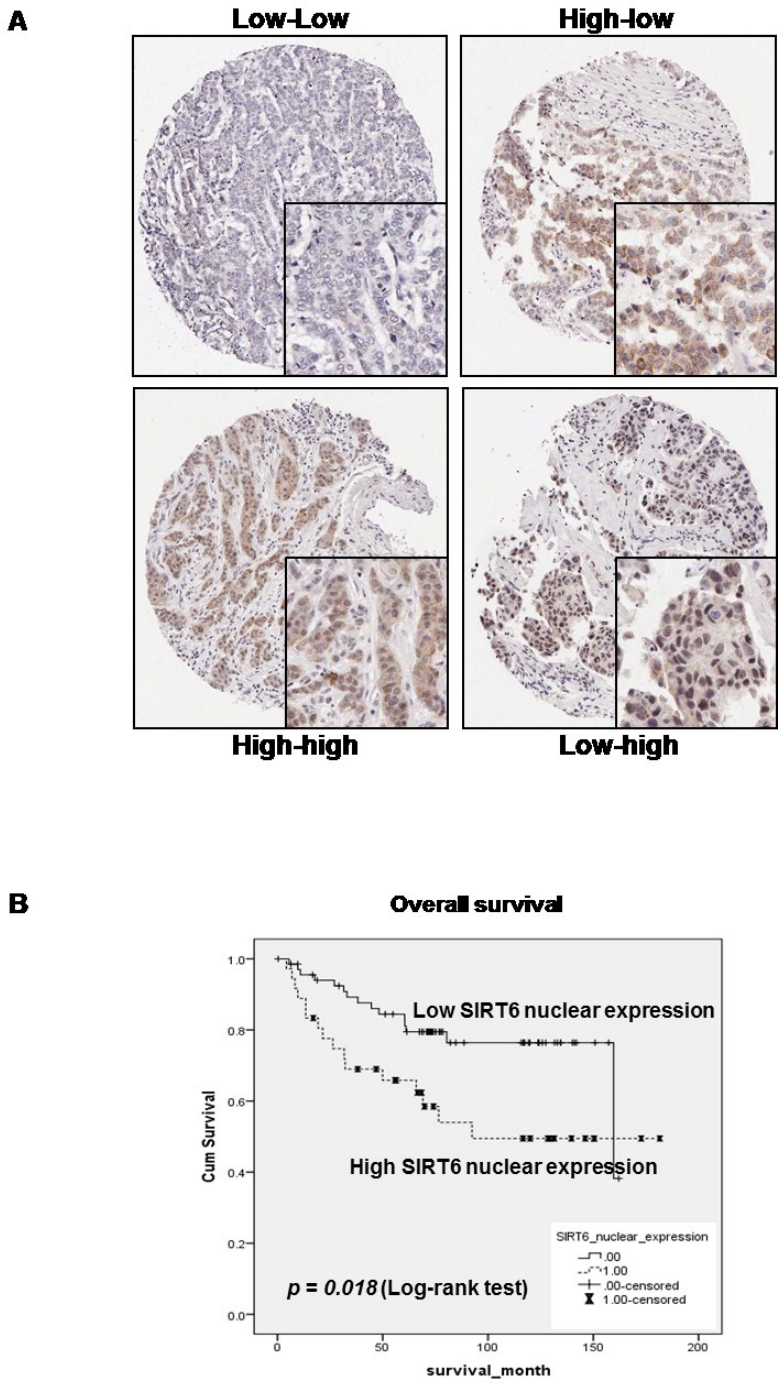


Figure 3.21 Higher nuclear SIRT6 expression is associated with poorer clinical outcome in breast cancer patients. Tissue samples were fixed with formalin, embedded with paraffin and immunohistochemically stained with anti-SIRT6 antibody. The clinical and pathological features of patients used in the study: high-high, high-low, low-high and low-low corresponding cytoplasmic-nuclear staining pattern. **A** Four representative tumour cases showing high-high, high-low, low-high and low-low corresponding SIRT6 cytoplasmic-nuclear staining patterns (magnification: x 100; insets x400). **B.** Kaplan–Meier analysis of overall survival for nuclear SIRT6 staining with log-rank test P-values indicated. High expression of nuclear SIRT6 significantly inversely correlated with poor overall and disease-specific survival in breast cancer patients.

C

Cox regression analyses of Overall Survivial on all cases

Clinical-pathological parameters	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
Age (n=118)	1.387 (0.714, 2.695)	0.334		
T-stage (n=118)	2.960 (1.362, 6.431)	***0.006***	4.040 (1.655, 9.861)	***0.002***
Lymph-node involvement (n=118)	2.882 (1.166, 7.122)	***0.022***	3.429 (1.248, 9.424)	***0.017***
Tumor-Grade (n=118)	1.061 (0.496, 2.268)	0.879		
Estrogen receptor status (n=118)	1.152 (0.527, 2.517)	0.722		
SIRT6 nuclear epxression (n=118)	2.297 (1.133, 4.659)	***0.021***	2.477 (1.077, 5.697)	***0.033***
SIRT6 cytoplasmic expression (n=118)	0.402 (0.189, 0.855)	***0.018***		

Figure 3.21 (Continued.) Higher nuclear SIRT6 expression is associated with poorer outcome in breast cancer patients. C. Cox univariate and multivariate regression analysis of SIRT6 staining and clinical parameters.

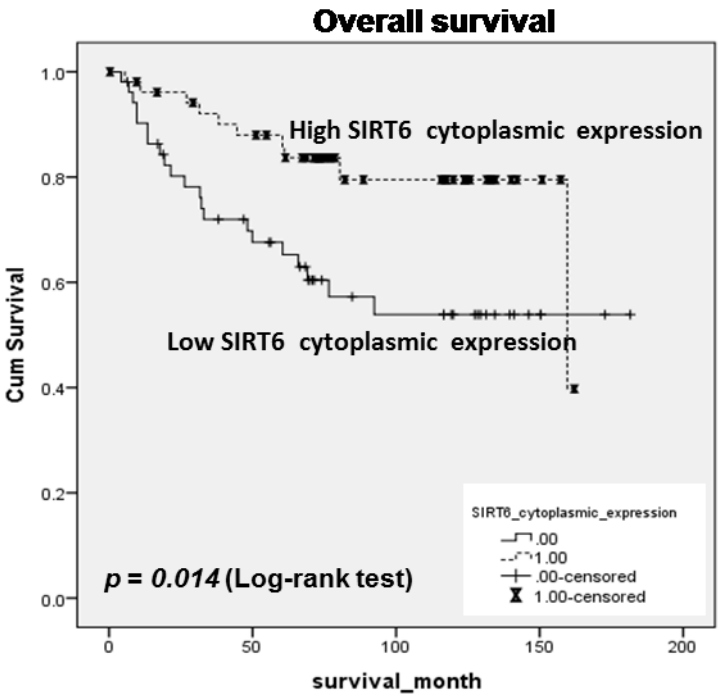
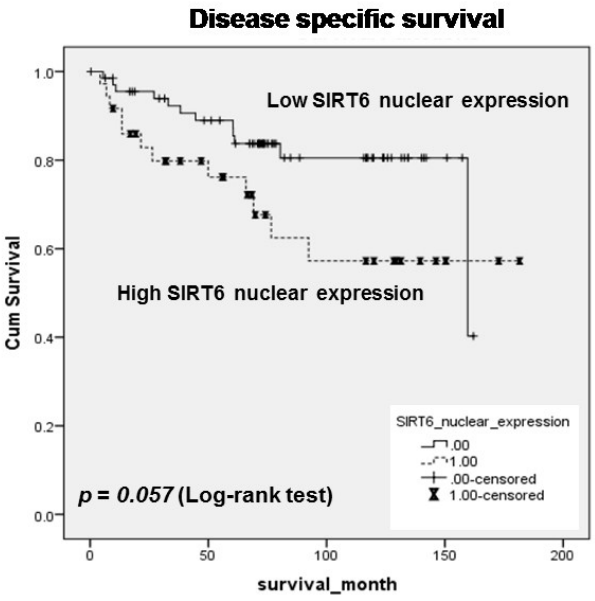


Figure 3.22 Examination of cytoplasmic SIRT6 staining in breast cancer patient samples by Kaplan–Meier analysis. Kaplan–Meier analysis of overall survival for cytoplasmic SIRT6 staining with log-rank test P-values indicated. High expression of cytoplasmic SIRT6 significantly correlated with poor overall survival in breast cancer patients.

A



B Cox regression analysis of disease specific survival

Clinical-pathological parameters	Univariate analysis		Multivariate analysis	
	RR (95% CI)	p	RR (95% CI)	p
Age (n=118)	0.858 (0.401, 1.834)	0.692		
T-stage (n=118)	4.034 (1.787, 9.103)	0.001	5.346 (2.084, 13.713)	<0.001
Lymph-node involvement (n=118)	3.931 (1.341, 11.525)	0.013	4.056 (1.315, 12.509)	0.015
Tumor-Grade (n=118)	1.487 (0.623, 3.549)	0.371		
Estrogen receptor status (n=118)	0.995 (0.426, 2.327)	0.991		
PR status (n=118)	1.028 (0.461, 2.296)	0.945		
Nuclear localization Score of SirT6 (n=118)	2.143 (0.959, 4.788)	0.063	2.232 (0.914, 5.453)	0.078
Cytoplasmic localization Score of SirT6 (n=118)	0.507 (0.221, 1.159)	0.107		

Figure 3.23 Examination of nuclear SIRT6 staining in breast cancer patient samples by Kaplan–Meier and Cox regression analysis. A. Kaplan–Meier analysis of disease specific survival for nuclear SIRT6 staining with log-rank test P-values indicated. High expression of nuclear SIRT6 significantly inversely correlated with poor disease-specific survival in breast cancer patients. **B.** Cox univariate and multivariate regression analysis of SIRT6 staining and clinical parameters.

3.5 Discussion

The overexpression of sirtuins, the family of deacetylase enzymes, has been proposed to be involved in the development of drug resistance and cancer progression in many cancers including lung, colon, and breast cancer (Olmos et al., 2011b). SIRT1 and SIRT2 have been intensively studied and has been shown to repress the expression and function of p53 (Peck et al., 2010) and FOXO3a (Wang et al., 2012), which are known tumour suppressors, via their deacetylation activities. In this research, I studied the role of sirtuins in the development of drug resistance in breast cancer and focused mainly on SIRT6. The regulation of FOXO3a and p53 by acetylation was also investigated.

To this end, all of human sirtuin levels in MCF-7 sensitive cells were investigated and compared to the levels in MCF-7-Epi^R and MCF-7-Tax^R cells. Interestingly, the expression of SIRT6 is upregulated in both MCF-7-Epi^R and MCF-7-Tax^R cells compared with drug sensitive cells. This upregulation of SIRT6 seems to be due to post-transcription modification as *RT*q-PCR shows SIRT6 *mRNA* levels in both resistant cells are in fact lower than the level in MCF-7 sensitive cells. However, the other sirtuins, SIRT3 and SIRT5, are also upregulated, whereas SIRT2 is down-regulated. These results suggest that these sirtuins could contribute to the resistant phenotypes of MCF-7-Epi^R and MCF-7-Tax^R cells and the development of drug resistance in breast cancer. Surprisingly, there is no significant difference in SIRT1 expression, which has been previously shown to be overexpressed and involved in drug resistance in many cell lines (Chu et al., 2005; Kojima et al., 2008). It has been shown to induce doxorubicin resistance by modulating multidrug-resistance molecule P-glycoprotein expression in osteosarcoma SaOS2 cells (Chu et al., 2005). This

result proposes the role for SIRT1 in drug resistance may vary in different cell lines, because each cell line exhibits different genetic background and different mechanisms of response to different chemotherapeutic drugs used. SIRT6 is a nuclear protein that has a role in the regulation of chromatin structure, gene expression and genomic stability. SIRT6 is initially described as a mono-ADP ribosylase and has been shown to regulate histone H3 lysine 9 and 56 by its NAD^+-dependent deacetylase activity (Michishita et al., 2008; Yang et al., 2009a). This modification has been shown to be involved in the regulation of telomeric chromatin, gene expression and the chromatin dynamics associated with DNA damage repair factors (Michishita et al., 2008; Yang et al., 2009a). Because of its functions and the consistent upregulation of SIRT6 expression in MCF-7-Epi^R and MCF-7-Tax^R cells, this led me to investigate the role of SIRT6 in the chemotherapeutic resistance in breast cancer.

SIRT6 depletion in MCF-7-Epi^R and MCF-7-Tax^R cells cannot completely reverse drug resistance of these cells. However, it leads to a noticeable increase in the sensitivity of these resistant cells in response to epirubicin and paclitaxel treatment, thus suggesting that SIRT6 partly involves in drug resistance in MCF-7 cells. In agreement to the knockdown experiment, *Sirt6*$^{-/-}$ MEFs also displays a higher degree of sensitivity to epirubicin and paclitaxel in comparison with WT MEFs. Additionally, the ectopic overexpression of SIRT6 is sufficient to confer epirubicin and paclitaxel resistance in MCF-7 sensitive cells. All of these results could support the notion that SIRT6 mediates epirubicin and paclitaxel resistance in breast cancer.

The mechanism of action of epirubicin is to intercalate into DNA topoisomerase II and induce DSBs. SIRT6 is reported to have a crucial role to maintain genomic

stability and to enable DSB repair (Kaidi et al., 2010; Mao et al., 2011; McCord et al., 2009; Mostoslavsky et al., 2006) I hypothesised that SIRT6 mediates epirubicin resistance by enhancing in DNA DSBs repair to counteract with epirubicin-induced DNA damage in breast cancer cells. Phosphorylated H2AX (γH2AX) is a marker that has been reported to be induced and accumulated at the DNA damaged sites after exposing to DNA damaging agents. The accumulation of γH2AX foci is shown to enable the recruitment of the other repair factors and checkpoint proteins to the break sites and initiate the repair processes (Zhou et al., 2006). These foci formation have been suggested as an indicator of DNA repair centre (Paull et al., 2000). Immunostaining results show that *Sirt6*$^{-/-}$ MEFs exhibits significant higher numbers of epirubicin-induced γH2AX foci formation compared to WT MEFs ,suggesting that SIRT6 deletion leads to the impairment of DNA damage repair in response to epirubicin treatment. Alkaline comet assay reveals higher levels of broken DNA in *Sirt6*$^{-/-}$ MEFs compared to WT MEFs, further supporting the mechanism by which SIRT6 mediates epirubicin resistance by improving the DNA damage repair response. The upregulation of multidrug resistance P-glycoprotein (P-Pg/MDR1) has been reported to be one of the characteristic of resistant phenotypes of cancer cells (Baguley, 2010). The overexpression of this protein leads to the development of resistance to anti-cancer drug-induced apoptosis. I found that MCF-7-Epi^{R} and MCF-7-Tax^{R} displays higher levels of P-Pg/MDR1 compared to MCF-7 sensitive cells. This result suggests that the overexpression of P-Pg/MDR1 protein could account for drug resistant phenotypes of these two cell lines. To eliminate the effect of this multidrug resistant marker and to confirm the role of SIRT6 in drug resistance, WT and *Sirt6*$^{-/-}$MEFs were used and subjected to γ-irradiation. I found that *Sirt6*$^{-/-}$ MEFs are more sensitive to γ-irradiation compared to WT MEFs, confirming that SIRT6 at

least has a role in the modulation of drug resistance in cells by enhancing DNA damage repair.

The role of SIRT6 in paclitaxel resistance was also investigated. Paclitaxel is an antibiotic agent that binds to and prevents the depolymerisation of microtubules. Paclitaxel-induced cytotoxic is believed to trigger mitotic arrest and subsequently cell death (Wang et al., 2000). Surprisingly, SIRT6 is found to be involved in conferring paclitaxel resistance. SIRT6 depletion in MCF-7-Tax^{R} cells increased apoptotic cells accumulated in sub-G1 phase after paclitaxel treatment, whereas the ectopic overexpression of SIRT6 in MCF-7 cells led to a decrease in cells accumulated in both sub-G1 and G2/M phase. These results suggest that SIRT6 overexpression may deregulate G2/M checkpoint and prevent cells to undergo apoptosis induced by paclitaxel treatment. However, the mechanism underlying this deregulation remains unclear and still needs further investigation. The expression of some proteins involved in the regulation of G2/M cell cycle transition, such as FOXOM1, should be examined.

The acetylation levels of two important cell cycle regulators, FOXO3a and p53, are found to be regulated by SIRT6. We have previously shown that FOXO3a and p53 are the crucial modulators for epirubicin and paclitaxel sensitivity (Millour et al., 2011; Sunters et al., 2003; Sunters et al., 2006). Thus, SIRT6 could confer epirubicin and paclitaxel resistance by repressing the activities of FOXO3a and p53 proteins through its deacetylase. However, SIRT6 is not only protein that involve in conferring epirubicin and paclitaxel resistance in breast cancer. The other SIRTs could also partly involve, especially SIRT1 and SIRT2 to set the optimal threshold for lysine acetylate activity to counteract the activity of acetyl transferase activity induced by drug treatment. Therefore, SIRT6 overexpression can confer significant levels of

drug resistance to these chemotherapeutic agents. However, SIRT6 could also be a useful marker to screen cancer patients that are likely to develop drug resistance during the treatment. In agreement, SIRT6 expression in a panel of breast cancer patient samples was analysed and I found that the high levels of nuclear SIRT6 expression are associated with poorer prognosis. This investigation, therefore, supports the role of SIRT6 in the development of poor clinical response to cytotoxic chemotherapy and that SIRT6 is a nuclear protein that functions within the nucleus (Michishita et al., 2008; Mostoslavsky et al., 2006).

In conclusion, I identify the novel role of SIRT6 in the mediation of epirubicin and paclitaxel resistance in breast cancer. SIRT6 can prevent epirubicin-induced DNA damage and deregulate G2/M arrest-induced by paclitaxel. SIRT6 also represses the function of p53 and FOXO3a via its deacetylase activity and it could be targeted in order to improve the efficacy of epirubicin and paclitaxel treatment and to prevent the acquired resistance of these agents in breast cancer.

CHAPTER 4

THE ROLE OF SIRT6 IN CELLULAR SENESCENCE AND IN THE REGULATION OF FOXO3A

4.1 Introduction

Cellular senescence, including replicative and oncogene-induced senescence, is the process by which cells are irreversibly arrested. Even in the presence of growth factors, these cells remain arrested and metabolically active. The display of senescence-associated β-galactosidase (SA-β-Gal) is one of most common characteristics of senescent cells. Other senescence characteristics include the upregulation of cell cycle inhibitors ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$), expression of secretomes (IL6, IL8, and CXCR2), distinctive change in cell morphology, increase in the number of lysosomes, and chromatin reorganisation called senescence-associated heterochromatin foci (SAHF) (Acosta and Gil, 2012; Cairney et al., 2012; Coppé et al., 2010; Kuilman and Peeper, 2009). Replicative senescence is triggered by two main factors, DNA damage response and the inhibition of INK/ARF locus, resulting in the activation of tumour suppressor pathways such as the p53 and $p16^{INK4b}$ pathways. Senescence can be also initiated by the activation of oncogenes, including Ras^{G12V}, BRAF and NRAS, and it is termed oncogene-induced senescence (OIS). OIS is believed to cause cancer progression and it could be exploited for the therapeutic prevention of cancer progression (Acosta and Gil, 2012). Additionally, stress induced senescence or premature senescence can also be induced by DNA-damaging insults, oxidative stress as well as chemotherapeutic agents. All of these senescence types result in a change in cellular organisation and gene expression. It has been documented that senescence plays an important role in limiting cancer progression and has been employed as a template for conventional anticancer therapeutic processes (Acosta and Gil, 2012). Reactivation of p53 pathway, inactivation of C-MYC or cyclin-dependent kinase (CDKs) have been developed and proven to be successful tools for cancer treatment by inducing senescence. Imatinib,

HER2 and BRAF inhibitors are successful examples that introduce therapy-induced senescence (TIS) as a mechanism of action by inducing the activation of the $p16^{INK4a}$ and p53 pathways (Acosta and Gil, 2012). Moreover, senescent phenotypes have been found in cancer cells subjected to some conventional chemotherapeutic agents and irradiation (Acosta and Gil, 2012; Chang et al., 1999). Therefore, the induction of cellular senescence in cancer could be a potential therapeutic strategy for improving cancer treatment efficiency and for overcoming drug resistance.

4.2 Aims

In the previous chapter I have shown that SIRT6 is involved in the development of epirubicin and paclitaxel resistance, the objective of this chapter is to investigate the role of SIRT6 in cellular senescence.

4.3 Results

4.3.1 SIRT6 depletion decreases long-term survival in MCF-7 cells after epirubicin and paclitaxel treatments.

I previously found that SIRT6 has a role in the development of epirubicin and paclitaxel resistance in breast cancer. SIRT6 depletion in MCF-7 cells led to a decrease in short-term cell viability after epirubicin and paclitaxel treatments. I also showed that SIRT6 regulates the acetylation status of FOXO3a and p53, two main cell cycle regulators. In this chapter, the role of SIRT6 in long-term survival and cellular senescence was further investigated. To this end, transiently siRNA mediated knockdown of SIRT6 expression in MCF-7 was performed followed by treatment with either 0-50 nM of epirubicin or 0-5 nM of paclitaxel. Long-term survival of these cells was then examined by clonogenic assay. As shown in Figure 4.1A and 4.2A and C, SIRT6 depleted cells exhibited significantly lower ability to form colonies compared to control cells (at 20, 30 and 40nM of epirubicin and at 2, 2.5 and 3nM of paclitaxel).

To further validate these results, the ectopic overexpression of SIRT6 in MCF-7 was then analysed. MCF-7 cells were transfected with an empty (pControl) or a SIRT6-overexpressing vector (pSIRT6) and then treated with either epirubicin or paclitaxel. The overexpression of SIRT6 in MCF-7 significantly increased long-term cell survival compared to control cells at 30 and 40 nM of epirubicin (Figure 4.1B) and 3 and 4 nM of paclitaxel (Figure 4.2B). The range of concentrations used in MCF-7 regarding SIRT6 overexpression were slightly higher than the concentrations used in the siRNA mediated knockdown experiment. This result further confirms the role of

SIRT6 in conferring drug resistance in breast cancer established in the previous chapter.

Notably, the epirubicin and paclitaxel concentrations used in the clonogenic assay were relatively lower than the one previously used in SRB assay and might not be able to cause considerable cell death. In order to address this, cell cycle analysis was performed by FACs using PI staining. MCF-7 cells were transiently transfected with siRNA against SIRT6 (siSIRT6) or non-silencing control (siControl) and followed by treatment with either epirubicin or paclitaxel. As depicted in Figure 4.3, SIRT6 depletion in MCF-7 cells resulted in higher numbers of cells accumulated in sub-G1 phase, in the absence of treatment. Epirubicin and paclitaxel treatment led to a negligible increase in cell death, as the percentage of sub-G1 cells at 48 h was marginally increased by approximately 6% and 10% for epirubicin and paclitaxel, respectively. This result suggests that the mechanism of action of epirubicin and paclitaxel at these concentrations does not encompass the induction of cell death but acts by inducing cellular senescence. Therefore, I hypothesised that the function of SIRT6 in regulating and controlling long-term cell survival could be due to the prevention of cellular senescence mediated by epirubicin and paclitaxel treatments.

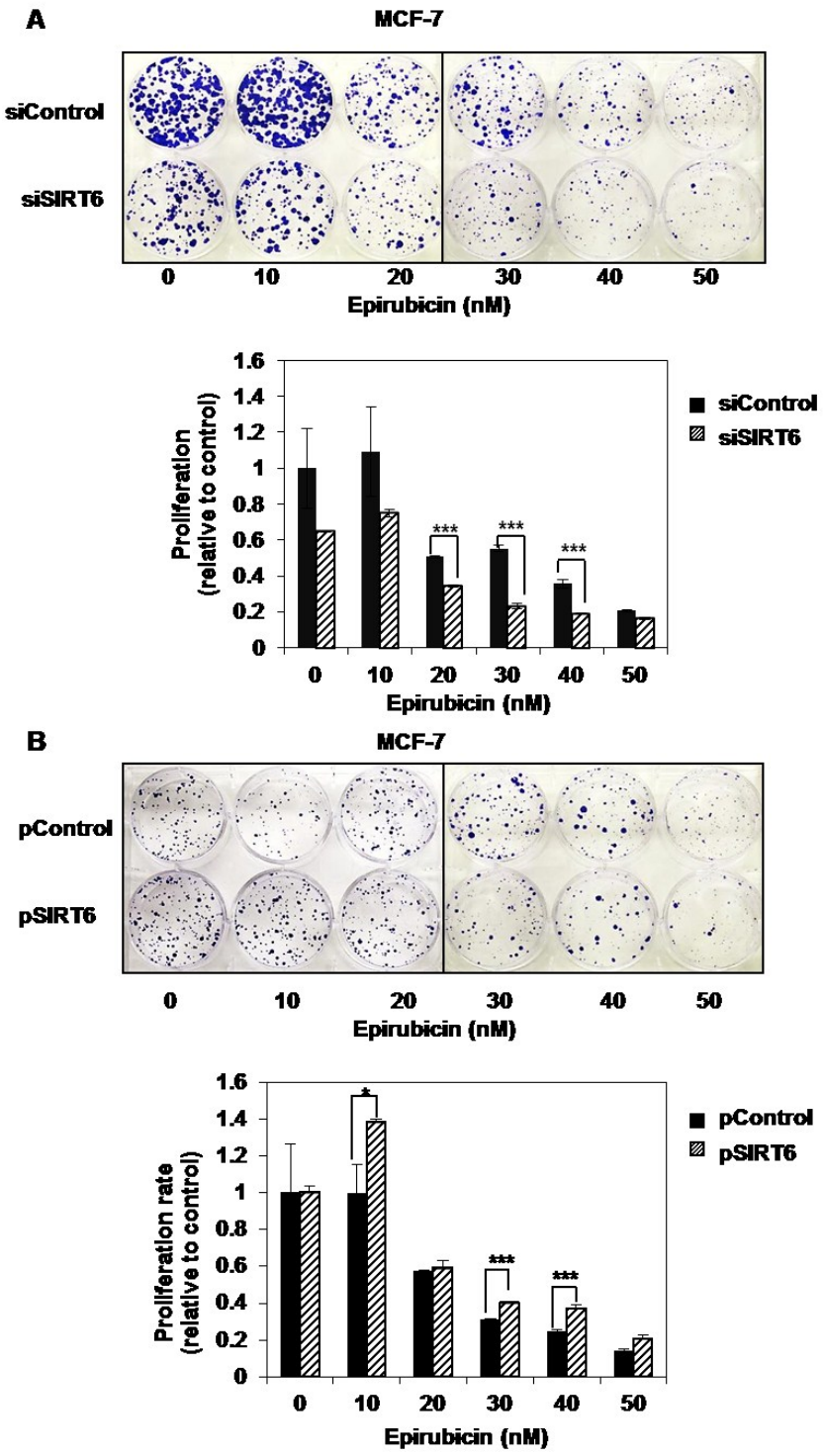


Figure 4.1 SIRT6 depletion in MCF-7 cells decreases long-term survival after epirubicin treatment. A. MCF-7 cells were transfected with siRNA against SIRT6 (siSIRT6) or non-silencing control (siControl). **B.** MCF-7 cells were transfected with ectopic SIRT6 overexpressing vector (pSIRT6) or empty vector (pControl) and followed by 0-50nM epirubicin treatment. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

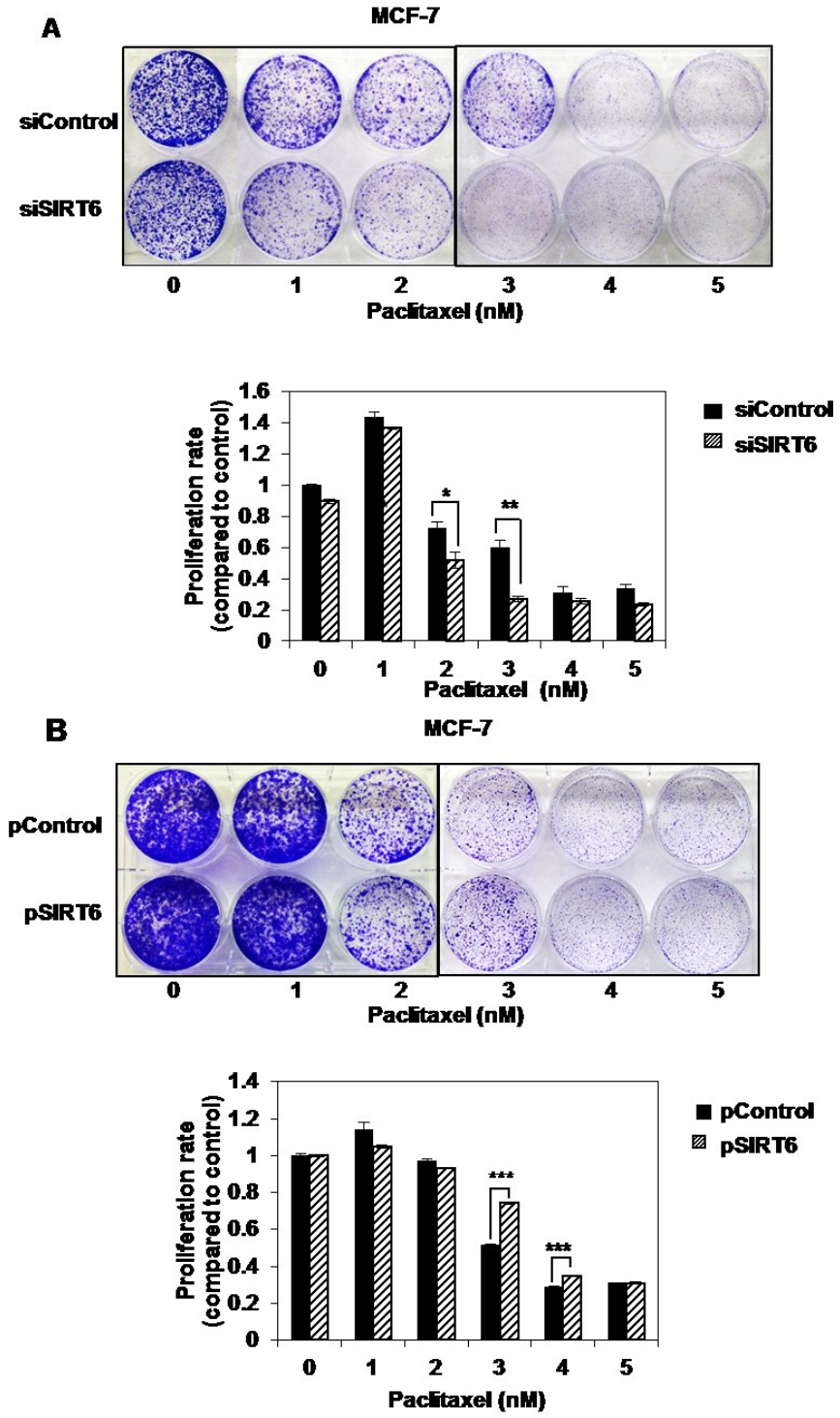


Figure 4.2 SIRT6 depletion in MCF-7 cells decreases long-term survival after paclitaxel treatment. A. MCF-7 cells were transfected with siSIRT6 or siControl. **B.** MCF-7 cells were transfected with pSIRT6 or pControl and followed by 0-5nM paclitaxel treatment. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

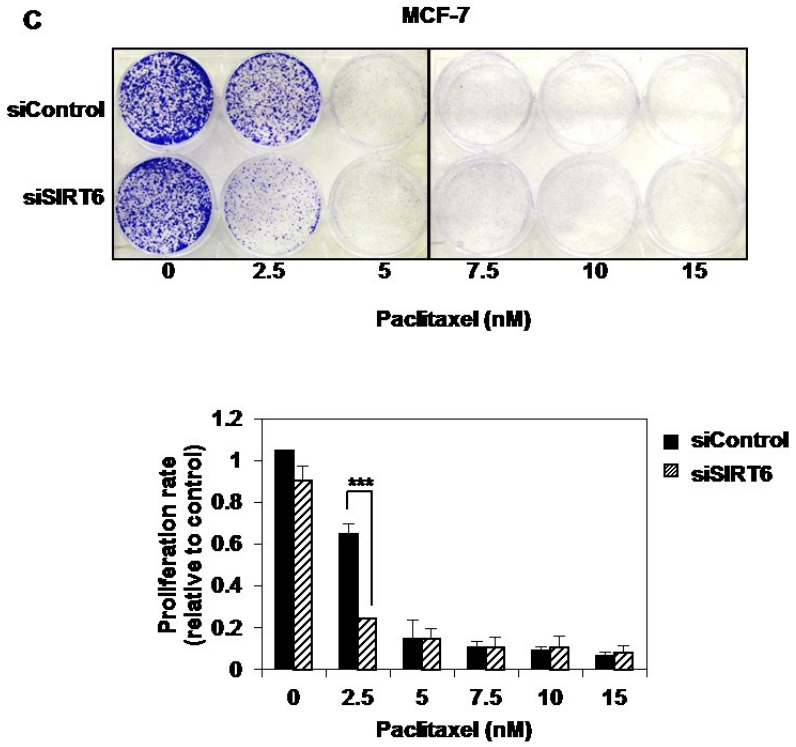


Figure 4.2 (Continued) SIRT6 depletion in MCF-7 cells decreases long-term survival after epirubicin treatment. C. MCF-7 cells were transfected with siSIRT6 or siControl and followed by higher paclitaxel concentrations (0-15 nM) treatment. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

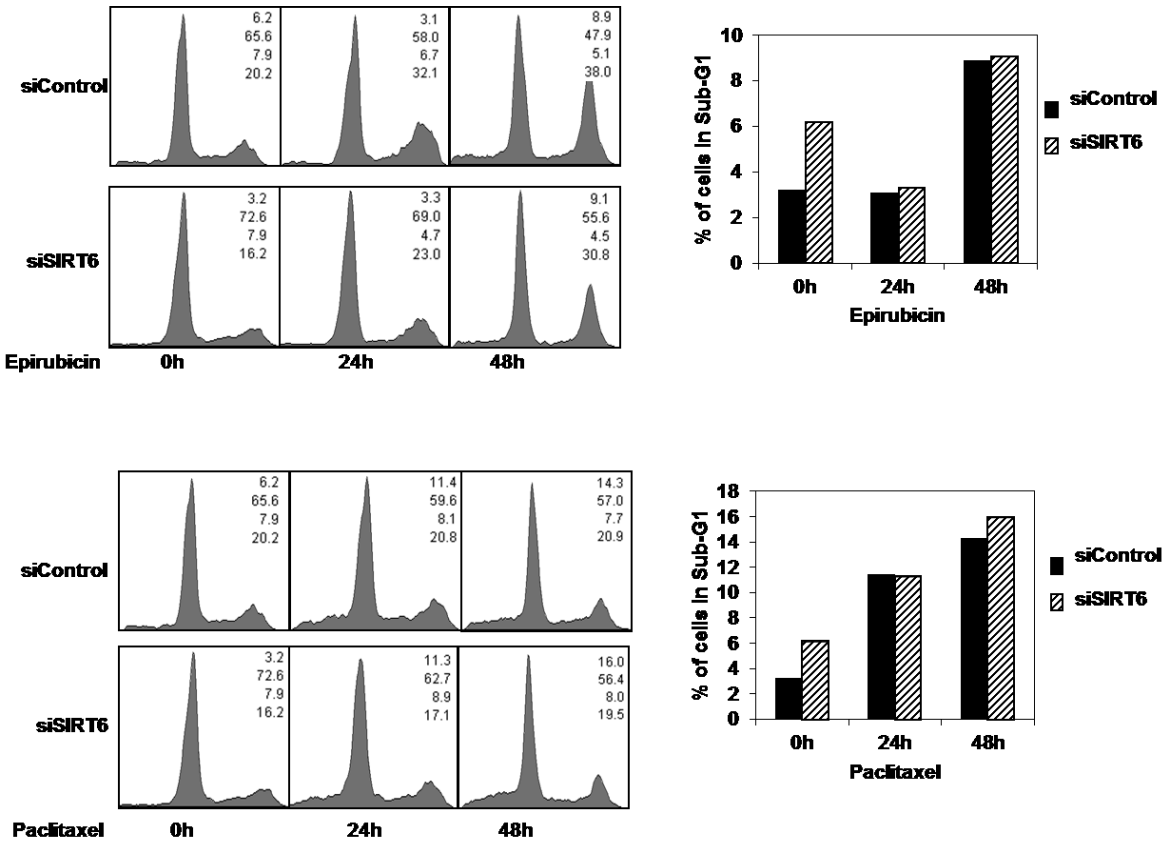


Figure 4.3 Treatment with 50 nM epirubicin and 5 nM paclitaxel does not cause a considerable cell death in SIRT6 depleted MCF-7 cells. MCF-7 cells were transfected with siSIRT6 or siControl and **Top:** followed by 50 nM epirubicin for the times indicated. **Bottom:** followed by 2.5 nM paclitaxel treatment. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle is indicated. The bar chart represents the percentage of cells in sub-G1.

4.3.2 Absence of SIRT6 in MCF-7 cells enhances cellular senescence

To test this hypothesis, senescent associated phenotypes in these cells were then elucidated. First, the status of cellular senescence was examined for β-galactosidase expression and morphology changes. As shown in Figure 4.4A and 4.5A, the level of senescence-associated β-galactosidase (SA-β-gal) in the absence of treatment was markedly higher in SIRT6 knocked down MCF-7 compared to control cells. In addition, higher numbers of cells with large flattened-morphology were also observed in SIRT6 depleted group in comparison to control. The lack of SIRT6 in MCF-7 cells displayed a significant higher level of SA-β-Gal expression compared to control cells after epirubicin treatment for all the tested concentrations (0-40 nM), whereas similar results were obtained with paclitaxel at concentrations of 2.5 and 5 nM.

To investigate this further, SIRT6 was ectopically overexpressed in MCF-7 cells and followed by epirubicin or paclitaxel treatment. A notable decrease in the activity of SA-β-Gal was detected in SIRT6 overexpressing cells compared with the controls. The significant differences were observed at 10-30 nM of epirubicin and 2.5 nm of paclitaxel as shown in Figure 4.4B and 4.5B, respectively. All of these results propose a role for SIRT6 in the inhibition of cellular senescence mediated by epirubicin and paclitaxel in breast cancer cells.

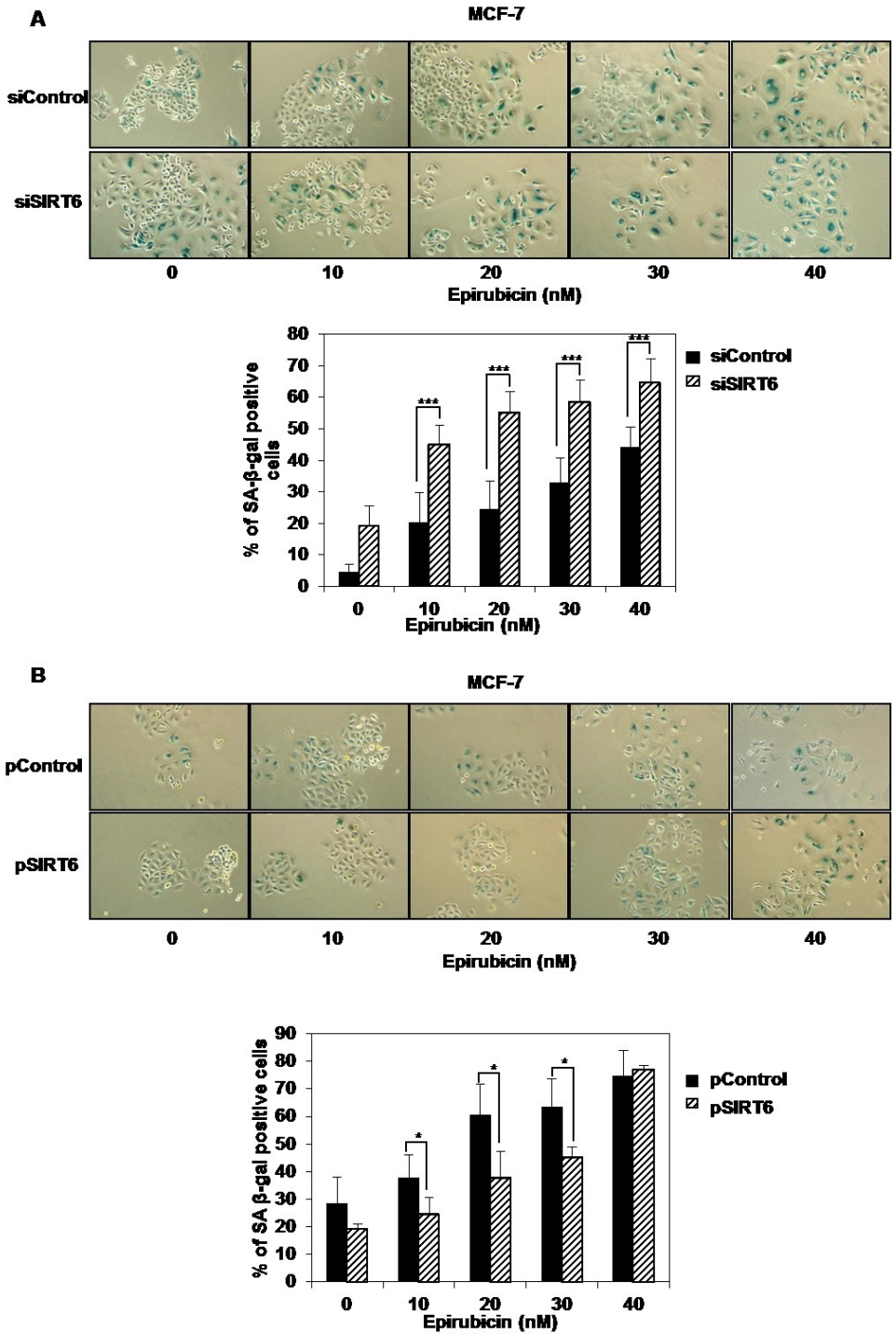


Figure 4.4 Depletion of SIRT6 in MCF-7 cells reduces SA-β-Gal activity after epirubicin treatment. A. MCF-7 cells were transfected with siSIRT6 or siControl. **B.** MCF-7 cells were transfected with pSIRT6 or pControl and followed by epirubicin treatment (0-40 nM). The graph (lower panel) represents the percentage of SA-β-gal positive cells compared with controls from 5 different fields from 3 independent experiments. Bars represent average ± s.d. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

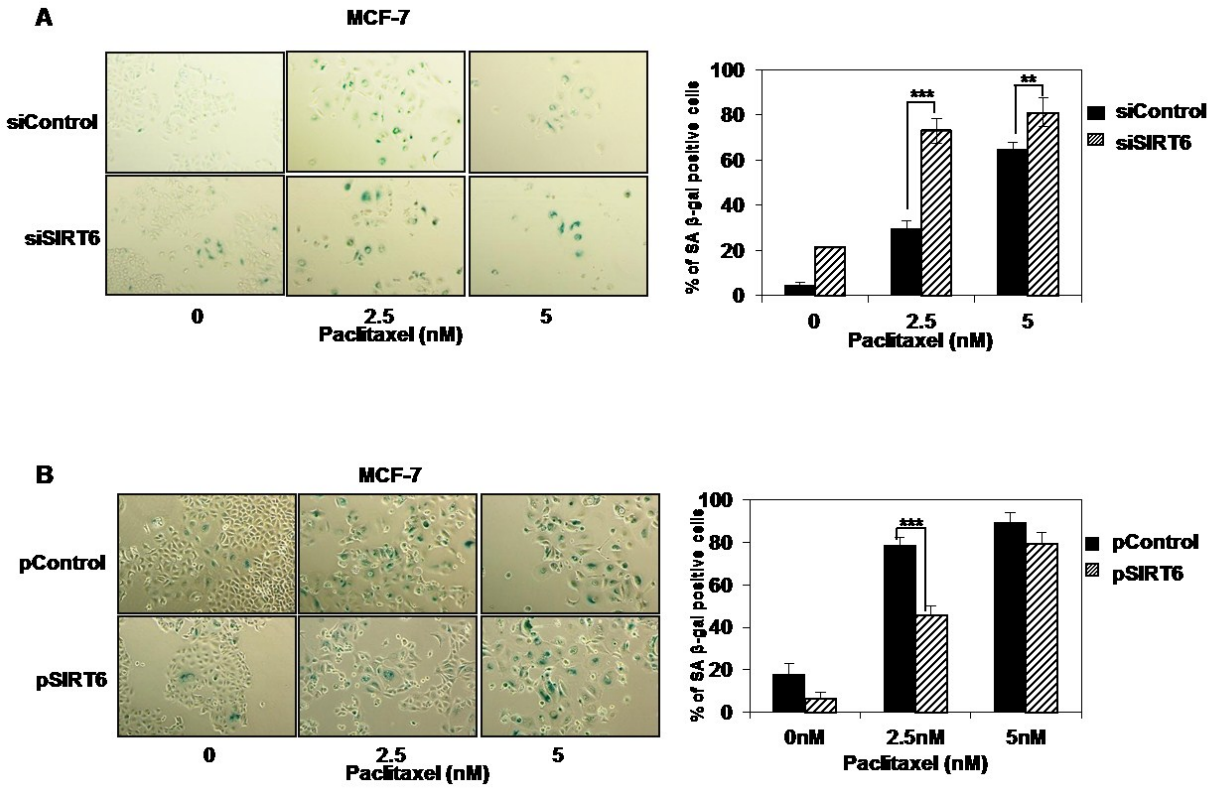


Figure 4.5 Absence of SIRT6 in MCF-7 cells reduces SA-β-Gal activity in MCF-7 cells after paclitaxel treatment. **A**. MCF-7 cells were transfected with siSIRT6 or siControl. **B.** MCF-7 cells were transfected with pSIRT6 or pControl and followed by paclitaxel treatment (0, 2.5 and 5 nM). The graph (lower panel) represents the percentage of SA-β-gal positive cells compared with controls from 5 different fields from 3 independent experiments. Bars represent average ± s.d. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

4.3.3 The senescent phenotypes induced by epirubicin and paclitaxel are not attributed to a marked change in cell cycle distribution.

After establishing that SIRT6 inhibits epirubicin and paclitaxel induced cellular senescence, I next investigated if the senescent phenotypes observed previously affect cell cycle distribution. To that end, cell cycle analysis was performed by flow cytometry analysis after PI staining. As presented in Figure 4.6A, in the SIRT6 siRNA- transfected cells, there were a considerably higher number of cells in the G1 phase when compared to the control cells. However, there were no noticeable changes in the number of cells accumulated in G1 after epirubicin treatment. This result reflected that knocking down of SIRT6 expression is sufficient to induce G1 cell cycle arrested in MCF-7 cells, suggesting that SIRT6 plays a role in the regulation of G1 phase. Additionally, SIRT6-depleted cells exhibited a lower proportion of cells in G2/M phase in comparison with the controls in the absence of the treatment. Interestingly, there was a considerable increase in the number of cells arrested at G2/M after epirubicin treatment in the SIRT6-depleted MCF-7 cells, whereas the number of G2/M cells remained consistent for the control group. This result implicates SIRT6 in the regulation of G2/M checkpoint and confirms the results obtained in the previous chapter, showing that SIRT6 mediates paclitaxel resistance in MCF-7 cells. Noteworthy, the proportion of cells in S phase was relatively lower in the SIRT6 depleted cells when compared to controls, indicating that SIRT6 helps to overcome epirubicin-induced cell cycle arrest.

On the other hand, the ectopic overexpression of SIRT6 did not cause a noticeable change in the number of cells accumulated in G1 and G2/M in the absence of epirubicin treatment, as depicted in Figure 4.6B. SIRT6 overexpression resulted in remarkably lower number of cells accumulated in G2/M phase compared to control

cells at 24 h of epirubicin treatment. Interestingly, the induction of G2/M arrest induced by epirubicin was observed at 24 h for control cells, whereas SIRT6-overexpressing cells accumulated later at 72 h after epirubicin treatment. In addition, the overexpression of SIRT6 resulted in higher numbers of cells in S phase in comparison to control cells after epirubicin treatment. These results suggest that SIRT6 can promote cell cycle progression and prevent MCF-7 cells from undergoing epirubicin-mediated cellular senescence. Together, all of these results indicate that overexpression of SIRT6 may inhibit the induction of epirubicin-mediated cellular senescence by deregulation of both G1 and G2/M check-points of the cell cycle.

In agreement with the results obtained with epirubicin treatment, SIRT6 depletion in MCF-7 followed by 2.5 nM paclitaxel treatment also caused a marginal increase in the number of cells accumulated in G2/M, whereas there were no appreciable changes in the control cells (Figure 4.6C). This result further confirms the role of SIRT6 in G2/M checkpoint regulation and in the prevention of paclitaxel–mediated cellular senescence in MCF-7 cells.

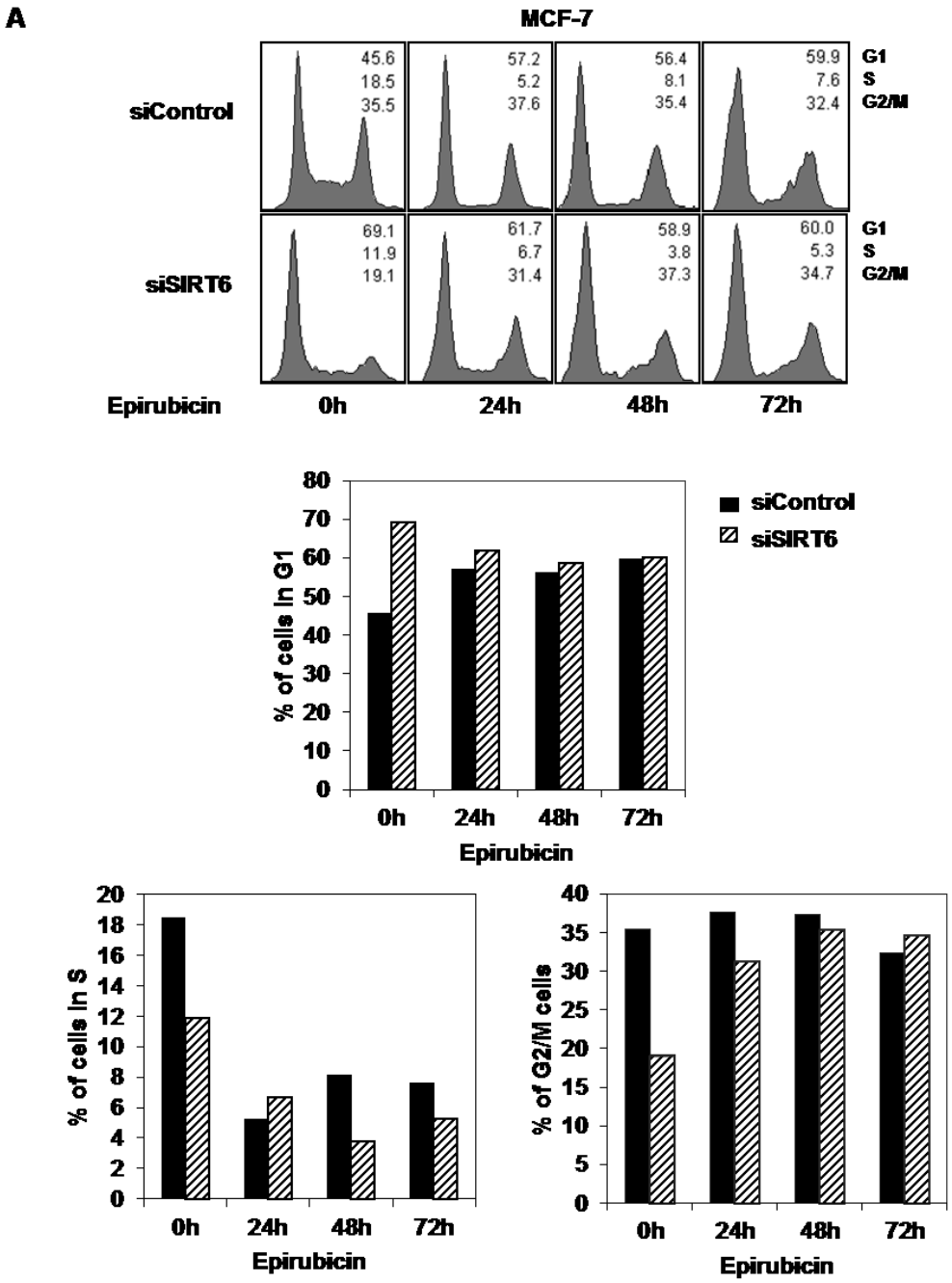


Figure 4.6 The induction of senescent phenotypes upon epirubicin treatment is not associated with a marked change in cell cycle distribution profile. SIRT6 overexpression results in the deregulation of G1 and G2/M phases. **A.** MCF-7 cells were transfected with siSIRT6 or siControl and treated with 50 nM epirubicin for the times indicated. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G1, S, and G2/M) is indicated.

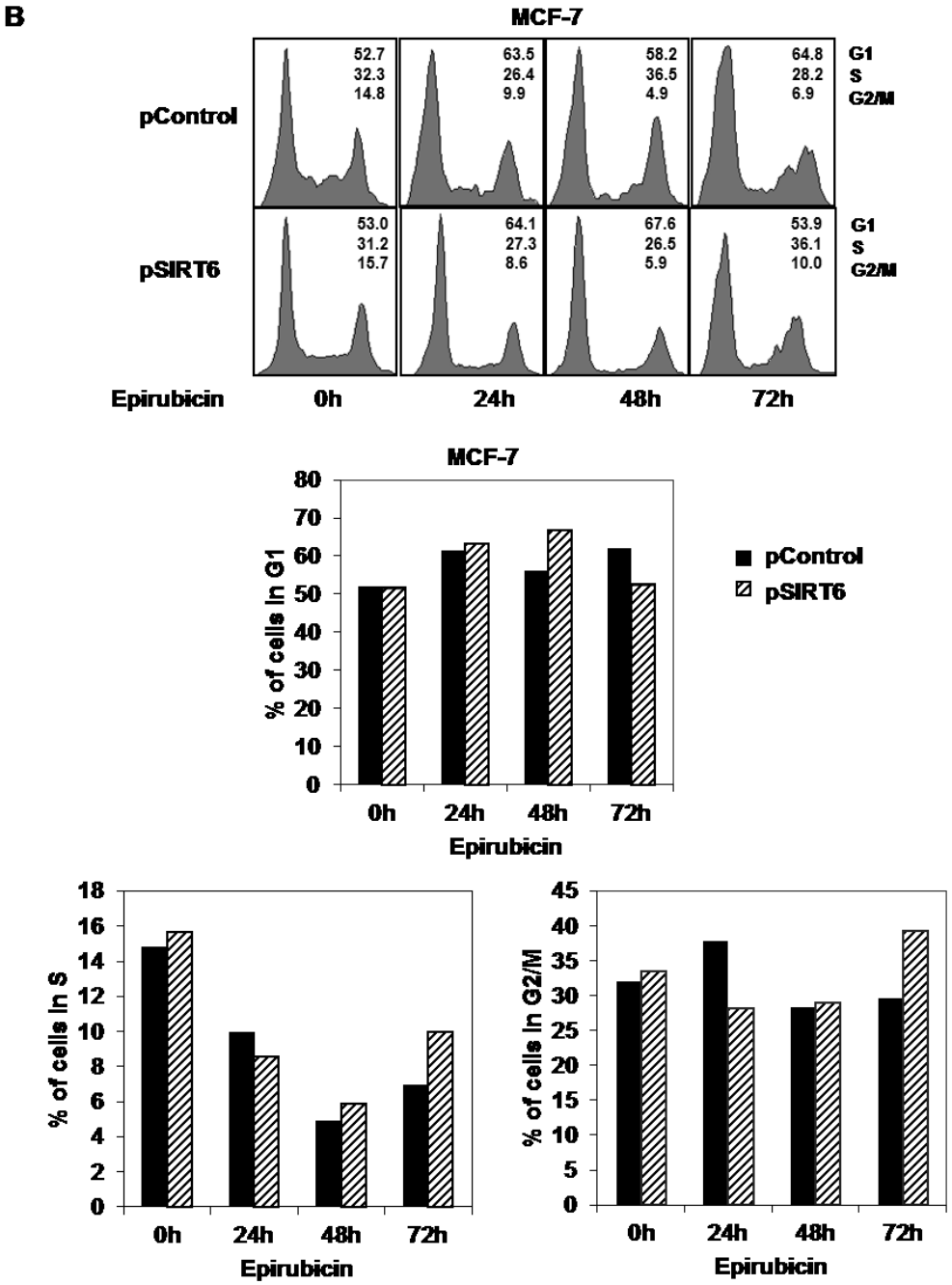


Figure 4.6 (Continued) Induction of senescent phenotypes upon epirubicin treatment is not associated with a marked change in cell cycle distribution profile. SIRT6 overexpression results in the deregulation of G1 and G2/M phases. **B**. MCF-7 cells were transfected with pSIRT6 or pControl and treated with 50 nM epirubicin for the times indicated. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G1, S, and G2/M) is indicated.

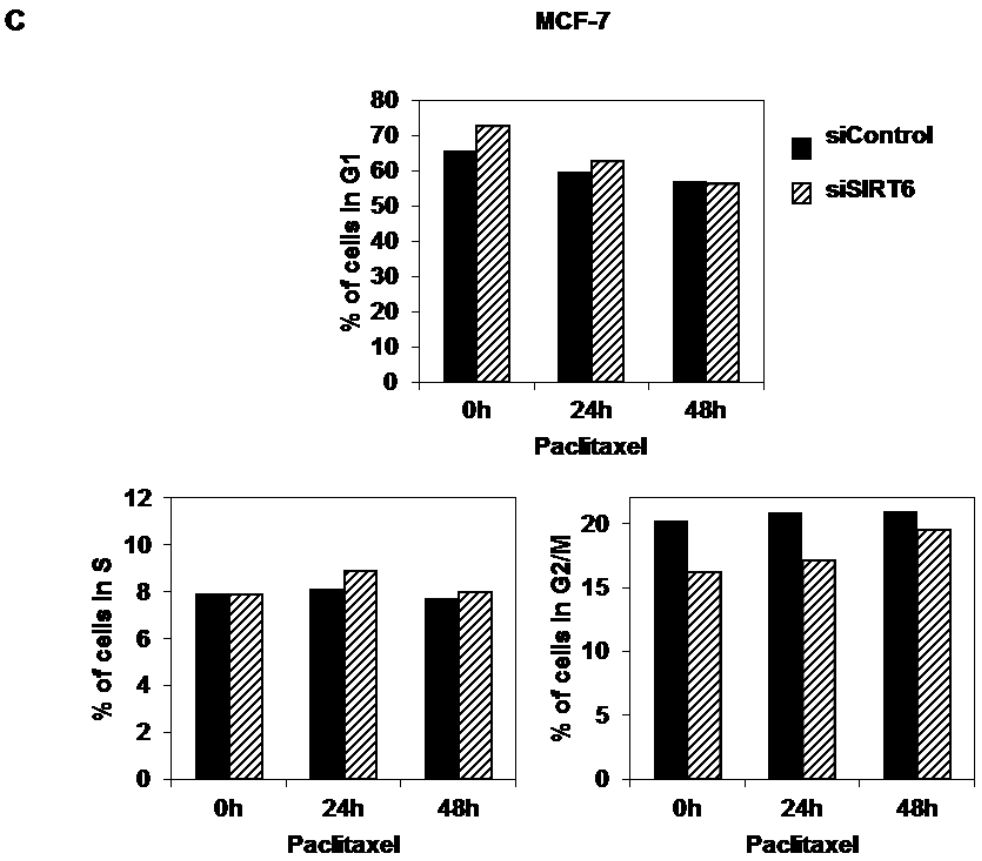


Figure 4.6 (Continued) Induction of senescent phenotypes upon paclitaxel treatment is not due to a marked change in cell cycle distribution profile. SIRT6 overexpression results in the deregulation of G1 and G2/M phases. **C**. MCF-7 cells were transfected with siSIRT6 or siControl and treated with 2.5 nM paclitaxel for the times indicated. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G1, S, and G2/M) is indicated.

4.3.4 SIRT6 overexpression in MCF-7 cells down-regulates some cellular senescence mediators.

Given the previously proposed role of SIRT6 in the prevention of cellular senescence following epirubicin and paclitaxel treatment, the expression levels of some cellular senescence markers were investigated by Western blotting. MCF-7 cells were transfected with siSIRT6/siControl or pSIRT6/pControl, followed by treatment with either 50 nM epirubicin or 2.5 nM paclitaxel treatment. Cells were then harvested at specific time points, 0, 24, 48 and 72 h, and extracted for total cell lysates. The expression levels of p53, FOXO3a and their downstream cell cycle regulatory effectors, including $p21^{Cip1}$ and FOXM1, were investigated by Western blotting. As depicted in Figure 4.7 (upper panel A and B), the absence of SIRT6 in MCF-7 cells resulted in a noticeable induction in p53 after both epirubicin and paclitaxel treatment over the time course. Similarly, the expression of FOXO3a was also induced after both treatments and was markedly higher in SIRT6 depleted cells compared to control cells. As a consequence, the expression of $p21^{Cip1}$, p53-and FOXO3a-dependent cell cycle-dependent kinase inhibitor (CKI) was upregulated, providing the explanation for the accumulation of SIRT6-depleted cells in G1 phase. Notably, p53 and FOXO3a are stress-dependent response proteins. The activation of p53 and FOXO3a leads to an induction of their downstream effectors to mediate cell cycle arrest and apoptosis, such as $p21^{Cip1}$. Therefore, the induction of $p21^{Cip1}$ after epirubicin and paclitaxel treatment indicates that oxidative stress could be one of the mechanisms triggered by these two chemotherapeutic agents. Interestingly, the expression of p53 and FOXO3a was predominantly higher in SIRT6-depleted cells compared to control ones, further confirming that p53 and FOXO3a are regulated by SIRT6.

On the other hand, the ectopic overexpression of SIRT6 resulted in a decrease in the expression and activity of p53 and FOXO3a, as it resulted in the downregulation of $p21^{Cip1}$ after the treatment (Figure 4.7C and D). The expression of p53 and FOXO3a was markedly lower in SIRT6 overexpressing cells compared to the control ones in the absence of treatment. Interestingly, SIRT6 overexpression led to a complete abolishment of $p21^{Cip1}$ induction after paclitaxel treatment even in the presence of p53. This result implicates that the activation of $p21^{Cip1}$ after epirubicin and paclitaxel treatment potentially depends on the function and the activity of FOXO3a, as its expression was also markedly repressed by SIRT6 overexpression in MCF-7 cells.

Moreover, FOXM1 expression was also investigated. FOXM1 is a transcription factor that plays an important role in mitosis by mediating G2/M transition. Its overexpression has been demonstrated to be involved in the development of epirubicin resistance by enhancing DNA damage repair and preventing epirubicin-mediated cellular senescence in MCF-7 cells (Khongkow et al., 2013b; Monteiro et al., 2013). Moreover, the depletion of FOXM1 in cancer cells is reported to increase the sensitivity of these cells in response to cytotoxic agents by increasing the G2/M cell cycle arrest (Barsotti and Prives, 2009). As shown in Figure 4.7C and D, FOXM1 expression was noticeably upregulated in SIRT6 overexpressing cells compared to controls at 24 h of both epirubicin and paclitaxel treatment. This upregulation of FOXM1 could prevent epirubicin and paclitaxel permanent induction of G2/M cell cycle arrest and subsequently cellular senescence. All of these results further contribute to the notion that SIRT6 has a role in the repression of senescent phenotypes of cancer cells in response to epirubicin and paclitaxel treatments.

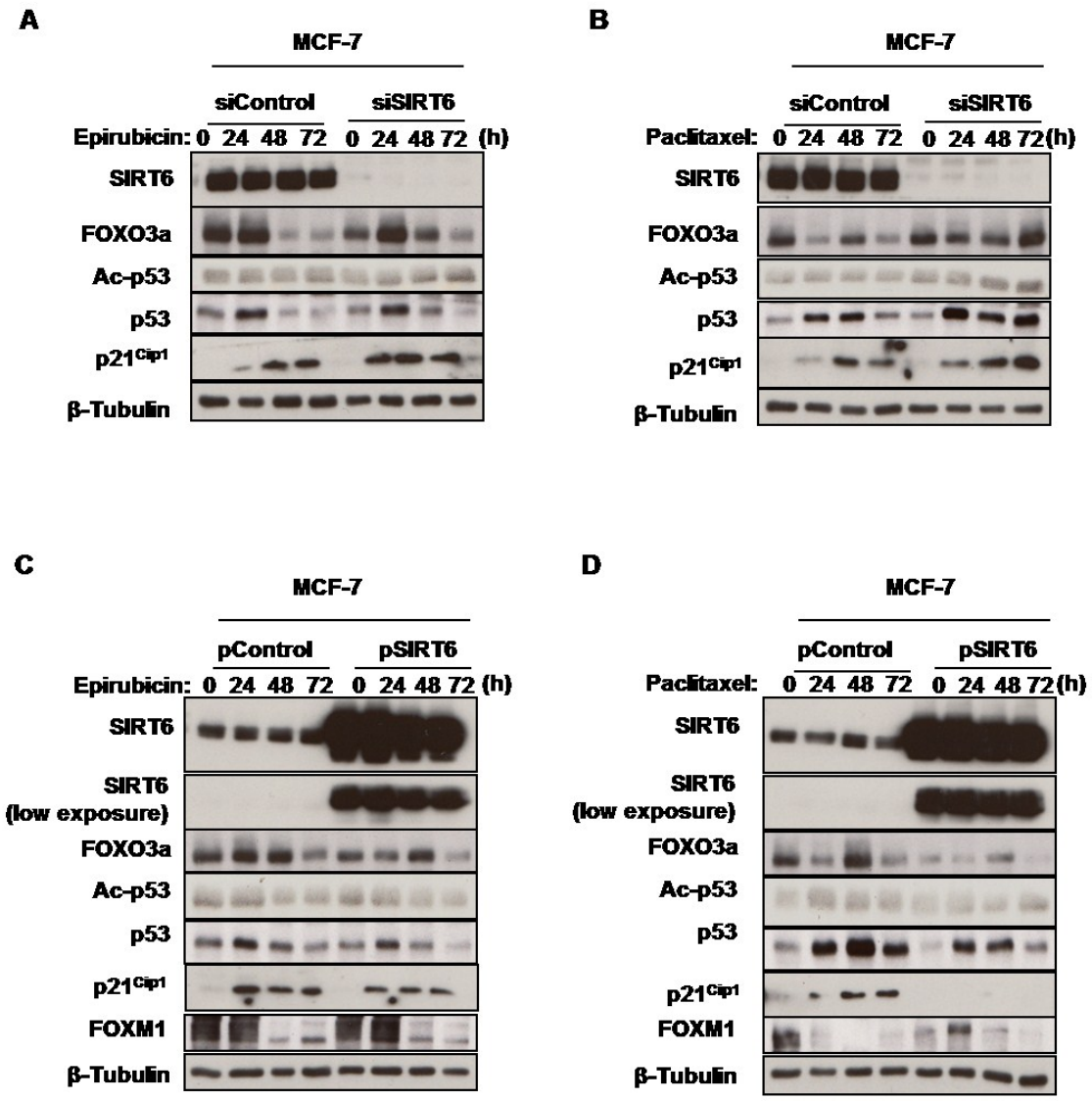


Figure 4.7 SIRT6 depletion leads to the induction of some cellular senescent markers such as p53, FOXO3a and their downstream targets. The levels of these cell cycle regulators were investigated by Western blotting. **A** and **C** MCF-7 cells were transfected with siSIRT6 or siControl and followed by epirubicin or paclitaxel treatment for 24, 48 and 72 h, respectively. **B** and **D** Cells were transfected with pSIRT6 or pControl and followed by epirubicin or paclitaxel treatment for 24, 48 and 72 h. β-Tubulin was used as a loading control.

4.3.5 SIRT6 overexpression prevents cellular senescence by enhancing of DNA damage repair via both HR and NHEJ.

Since I previously showed that SIRT6 inhibits epirubicin and paclitaxel-mediates cellular senescence, I hypothesised that this could be attributed to the role of SIRT6 in DSB repair response. SIRT6 has been shown to have a crucial role in DSB repair and I have previously shown that SIRT6 mediates epirubicin and paclitaxel resistance in breast cancer by enhancing DNA damage response in Chapter 3. To further validate whether SIRT6 is in fact crucial for DNA damage repair response, DNA damage repair assays were performed. Hela cells harbouring an integrated direct repeat green fluorescent protein (DR-GFP) reporter for both homologous recombinant (HR) and non-homologous end joining (NHEJ) were employed to assess DNA damage repair response (Bennardo et al., 2008; Delacôte et al., 2002; Monteiro et al., 2013). The endonuclease I-SCEI enzyme was co-transfected into DR-GFP Hela cells with siControl, siSIRT6, siBRCA1 or siNBS1. Cells were then harvested and analysed for GFP-positive cells by flow cytometry shown in Figure 4.8A. The quantification of relative DNA repair efficiency is depicted in Figure 4.8B. The result showed that silencing of SIRT6 expression significantly decreased the number of GFP-positive cells compared with non-silencing controls (siControl). The HR repair efficiency of SIRT6 depleted cells was approximately 3 fold lower than in control. siBRCA1 and siNBS1 were used as positive controls, as they are two crucial DNA damage repair factors (Khongkow et al., 2013b; Monteiro et al., 2013). The depletion of BRCA1 and NBS1 also resulted in a significant reduction of HR repair compared to the siControl group.

Conversely, overexpression of SIRT6 in these Hela cells improved HR repair efficiency by approximately 2.5 fold in comparison to the non-silencing controls. In

agreement with this result, BRCA1 and NBS1 overexpression enhanced HR repair efficiency by 2 and 1.75 fold, respectively. Moreover, Figure 4.9 shows that the overexpression of NBS1 cannot rescue HR repair efficiency in the absence of SIRT6. All of these results confirm the crucial role of SIRT6 in DNA damage repair, specifically in HR mediated DNA DSB repair.

In agreement with HR, SIRT6 depletion reduced the efficiency of NHEJ repair by approximately 75%. This was similar to the effect of BRCA1 and NBS1 silencing as shown in Figure 4.10A. Conversely, NHEJ repair efficiency was prominently enhanced by approximately 1.5 fold after SIRT6 overexpression. Similar results were obtained with the overexpression of BRCA1 and NBS1, as Figure 4.10B illustrates. NHEJ repair efficiency was improved by BRCA1 and NBS1 by 2- and 1.75-fold, respectively. Collectively, these results confirm that SIRT6 mediates DSB repair mainly through NHEJ pathway. Therefore, the overexpression of SIRT6 led to the improvement of DNA damage repair, suggesting the contribution of SIRT6 in the resistant phenotypes of cancer cells. As a consequence, it results in the prevention of cellular senescence induced by epirubicin and paclitaxel.

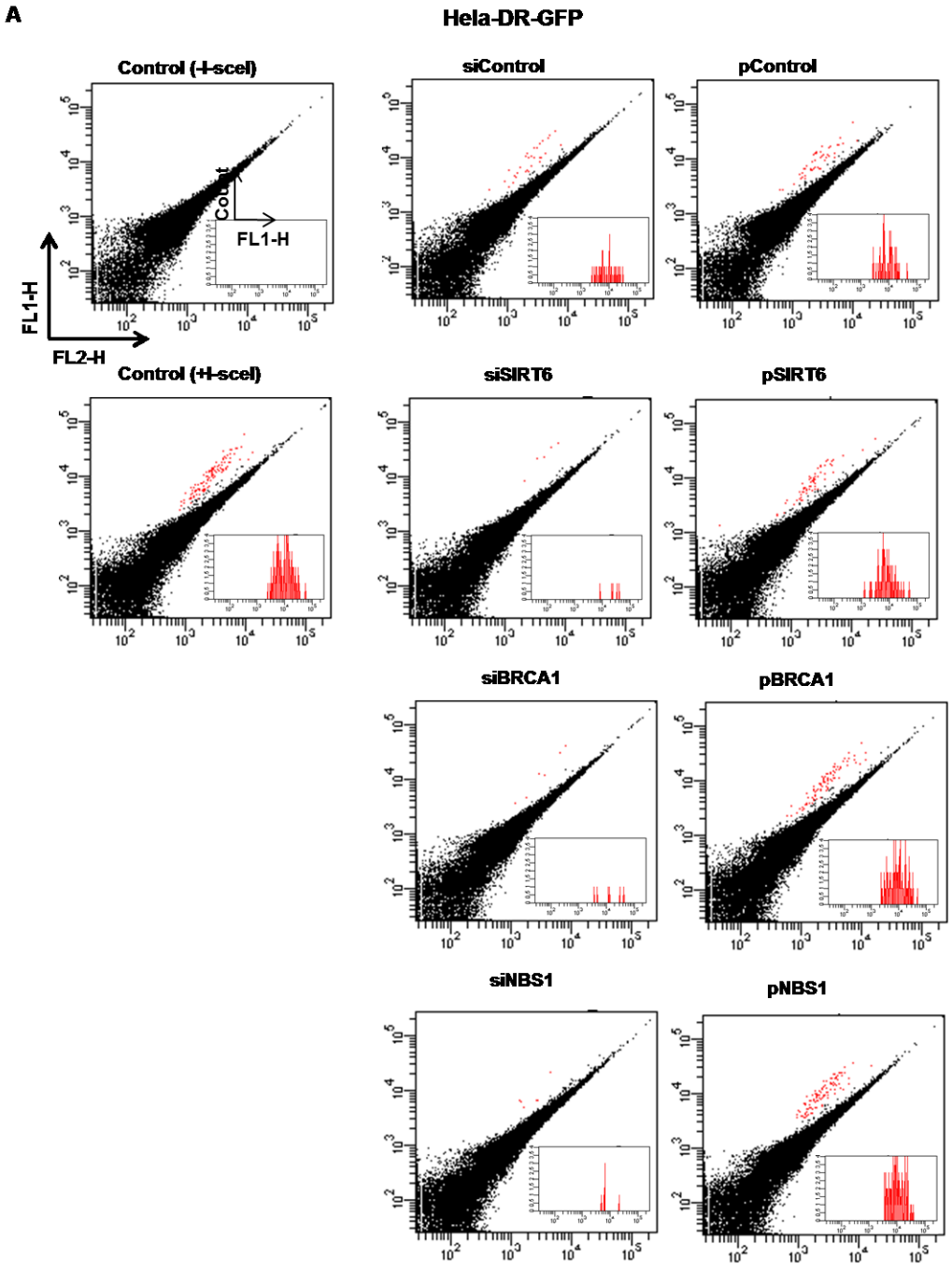


Figure 4.8 SIRT6 overexpression prevents cellular senescence by enhancing of DNA damage repair through HR. HR repair assay was performed by using Hela cells harbouring an integrated DR-GFP reporter for HR repair (Hela-DR-GFP). Cells were transfected with siSIRT6 or siControl, siBRCA1 and siNBS1 as positive controls or pSIRT6, pBRCA1, pNBS1 and pControl. After 24 h, cells were co-transfected with 1 µg of I-SCEI and left for 48h before analysis.

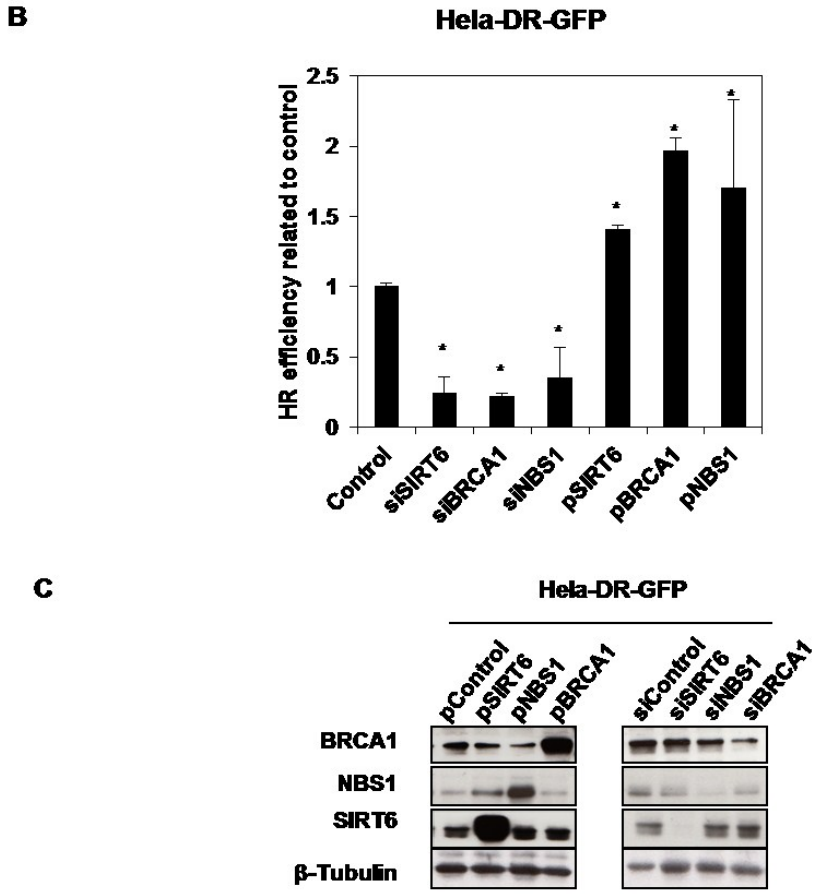


Figure 4.8 (Continued) SIRT6 overexpression prevents cellular senescence by enhancing of DNA damage repair through HR. B. The repair efficiency was determined relative to control (50,000 events were analysed from each experiment). Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control). **C.** The transfection efficiency was investigated by Western blotting.

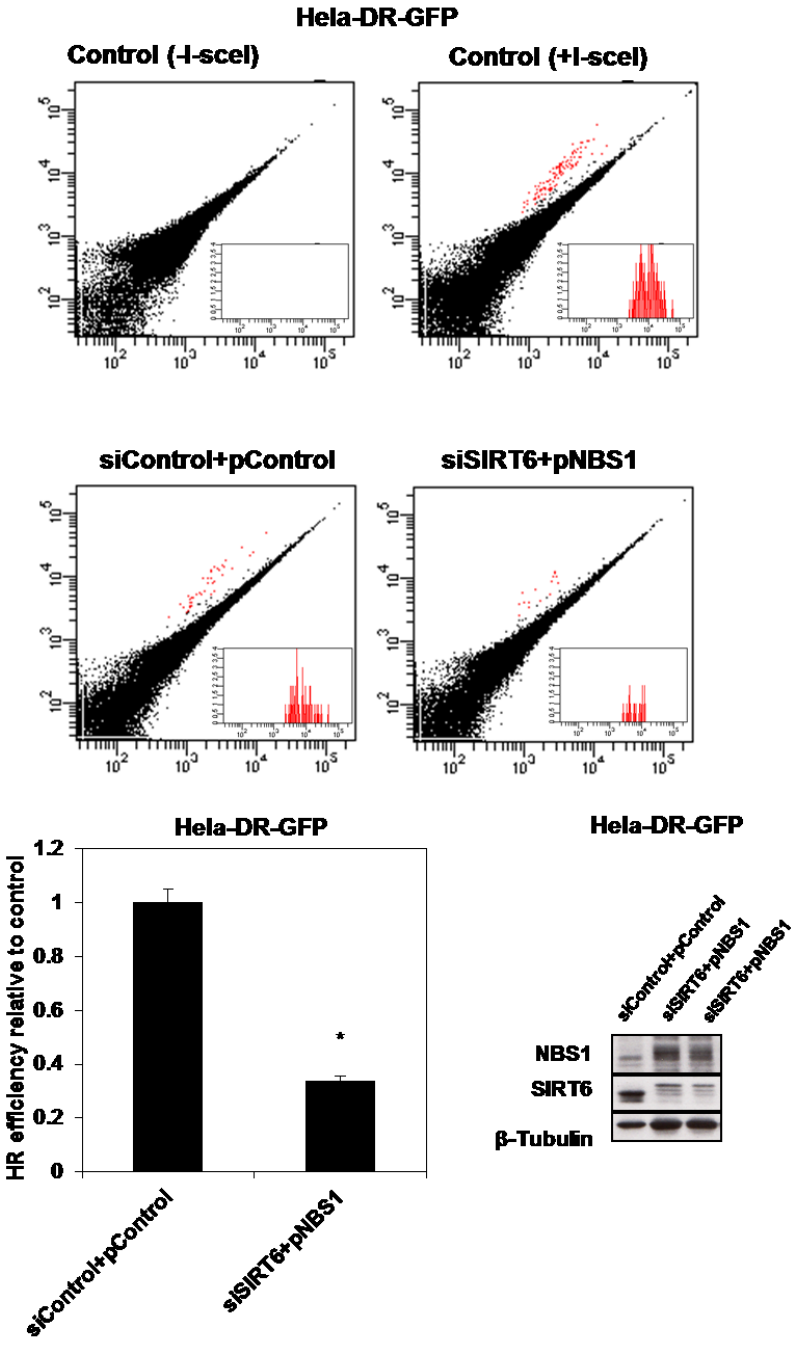


Figure 4.9 Overexpression of NBS1 in the absence of SIRT6 cannot rescue HR repair in Hela cells. HR repair assay was performed by using Hela cells harbouring an integrated DR-GFP reporter for HR repair (Hela-DR-GFP). Cells were co-transfected with siSIRT6 and pNBS1. siControl and pControl were used as controls. The repair efficiency was determined relative to control (50,000 events were analysed from each experiment). Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, and ***, *p≤0.005 versus* control).

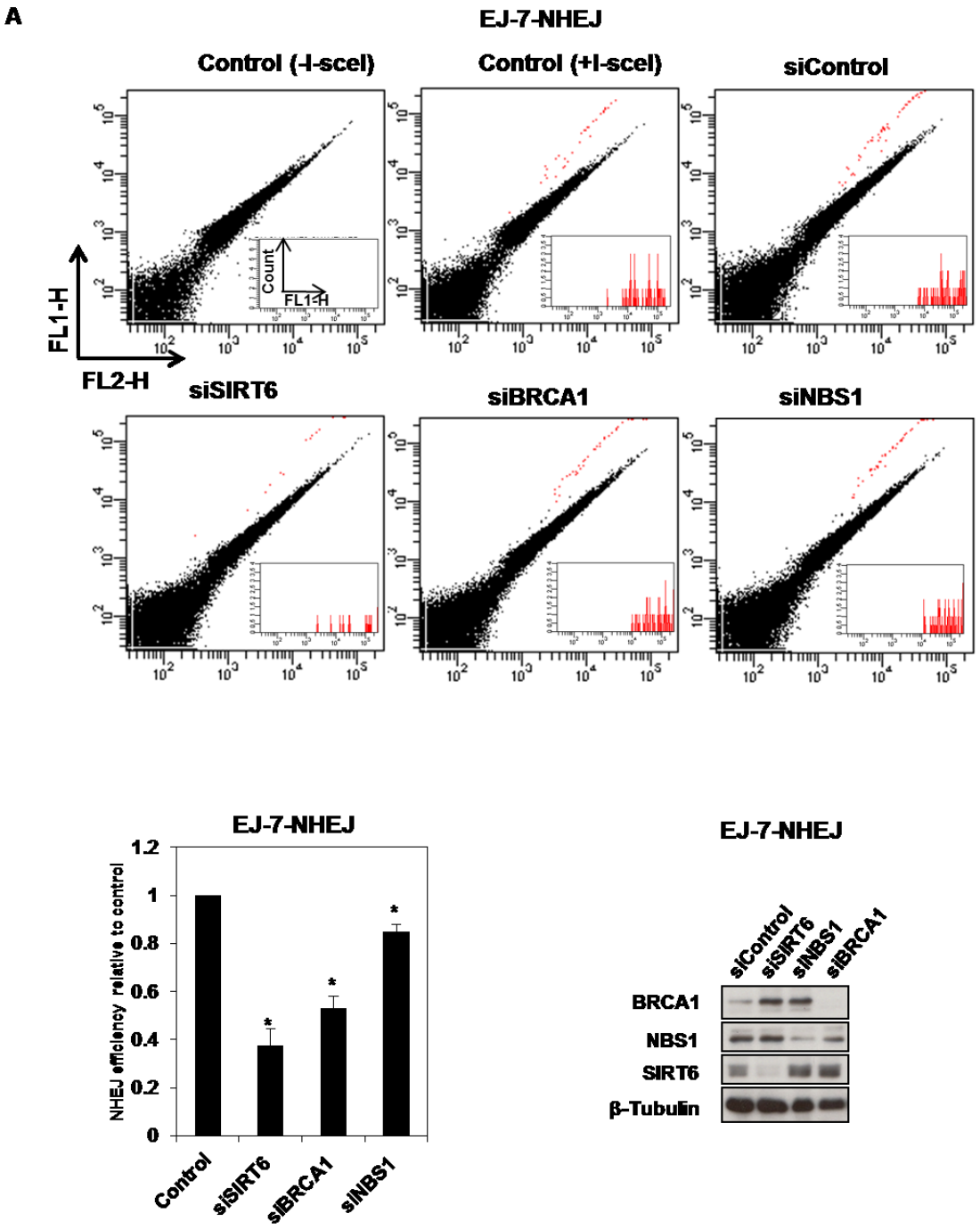


Figure 4.10 Overexpression of SIRT6 improves NHEJ repair and prevents cancer cells from undergoing senescence. NHEJ repair assay was performed by Hela cells harbouring an integrated GFP reporter for NHEJ repairs (EJ-7-NHEJ). **A.** Cells were transfected with siSIRT6, siBRCA1, siNBS1 or siControl. After 24 h, cells were co-transfected with 1 µg of I-SCEI and left for 48 h before analysis. The repair efficiency was determined relative to control (50,000 events were analysed from each experiment). Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05* **, *p≤0.01* ***, *p≤0.005* *versus* control). The transfection efficiency was investigated by Western blotting.

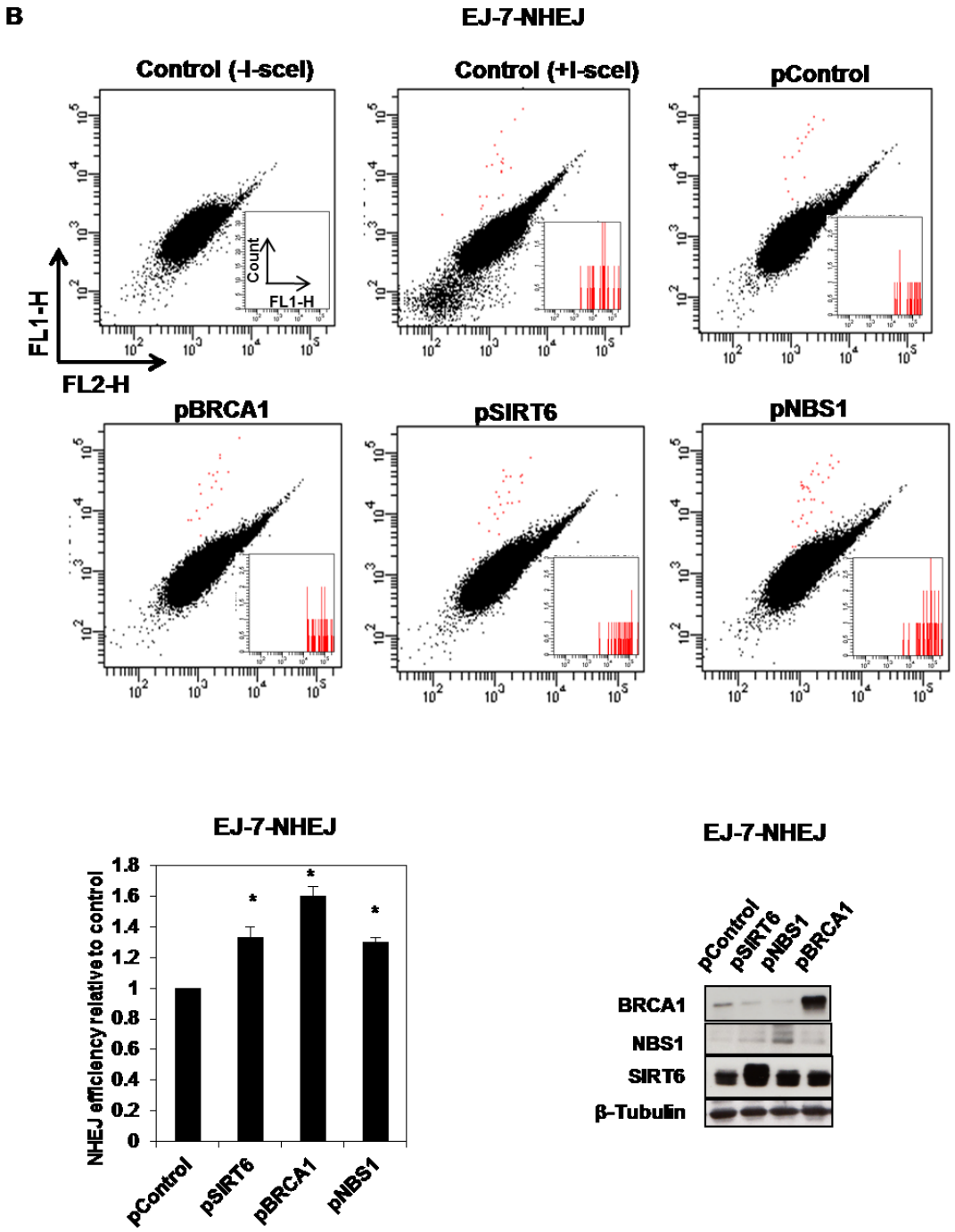


Figure 4.10 (Continued) Overexpression of SIRT6 improves NHEJ repair and prevents cancer cells from undergoing senescence. NHEJ repair assay was performed by using Hela cells harbouring an integrated DR-GFP reporter for NHEJ repair (EJ-7-NHEJ). **B.** Cells were transfected with pSIRT6, pBRCA1, pNBS1 or pControl. After 48 h, cells were co-transfected with 1 µg of I-SCEI and left for 48 h before analysis. The repair efficiency was determined relative to control (50,000 events were analysed from each experiment). Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05* **, *p≤0.01* and ***, *p≤0.005 versus* control). The transfection efficiency was investigated by Western blotting.

4.3.6 SIRT6 binds to and regulates the acetylation status of NBS1 in response to epirubicin treatment.

NBS1 is a part of the MRN complex, which has a crucial role in DNA damage repair. It has been reported that NBS1 is an acetylated protein and its deacetylation is regulated by SIRT1. Hyperacetylation of NBS1 renders cells more sensitive to γ–irradiation (Yuan and Seto, 2007). Moreover, *Sirt1*$^{-/-}$ MEFs exhibit a higher degree of γ–irradiation sensitivity compared to WT MEFs, indicating that the deacetylation of NBS1 by SIRT1 causes the induction of resistance to γ–irradiation. To investigate whether SIRT6 interacts with NBS1 in the same context of SIRT1, I performed co-immunoprecipitation assays. Whole cell lysates extracted from MCF-7 were immunoprecipitated using antibodies against SIRT6, SIRT1 antibodies (positive control), or IgG as a negative control. Eluted proteins were then processed, and analysed by using Western blotting. As depicted in Figure 4.11A, endogenous NBS1 was specifically immunoprecipitated with the anti-SIRT6 and anti-SIRT1 antibodies, and this was not observed in the IgG negative control fraction. This result suggests that NBS1 directly interacts with SIRT1 and SIRT6 in MCF-7 cells. Notably, endogenous SIRT1 and SIRT6 bind to NBS1 in the absence of epirubicin. This SIRT6-NBS1 interaction disappeared after 24 h epirubicin treatment in MCF-7, whereas the SIRT1-NBS1 interaction was still present after the treatment.

I next questioned whether SIRT6 deacetylates NBS1 in a similar way as SIRT1. Immunoprecipitation was performed by using anti-total-acetyl lysine antibody. WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM of epirubicin and the acetylation status of NBS1 investigated by incubation with anti-NBS1 antibody. As shown in Figure 4.11B, I found that the acetylation status of NBS1 was increased in response to epirubicin treatment, whereas total NBS1 expression was decreased at 6 h of epirubicin

treatment in both WT and *Sirt6*$^{-/-}$ MEFs. These results indicate that SIRT6 deacetylates NBS1 in these cells. Importantly, the basal acetylation status of NBS1 in *Sirt6*$^{-/-}$ MEFs was noticeably higher than the ones in WT MEFs. This indicates that SIRT6 might negatively regulate the acetylation status of NBS1 resulting in an improvement of repair of DSBs mediated by epirubicin, and subsequently in the halt of cellular senescence in these cells.

Consistently with the immunoprecipitation results, the expression levels of NBS1 were gradually decreased in WT MEFs, whereas a dramatic reduction of NBS1 was observed in *Sirt6*$^{-/-}$ MEFs. This suggests that SIRT6 expression is required in order to maintain the DNA damage response via the regulation of NBS1.

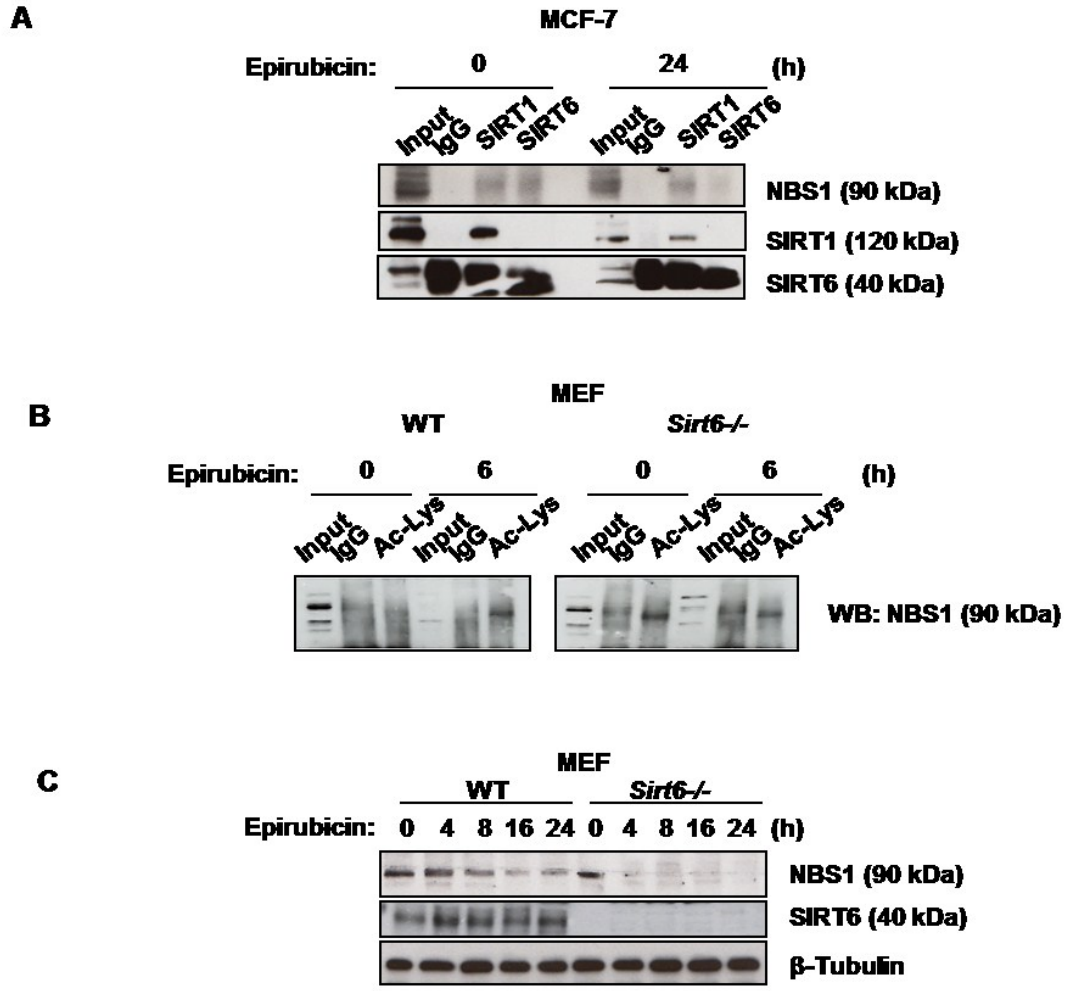


Figure 4.11 SIRT6 directly binds to and deacetylates NBS1 in MCF-7 cells after epirubicin treatment. A. MCF-7 cells were treated with epirubicin for 0, 6 and 24 h. Whole cell lysates were subjected to immunoprecipitation with specific antibodies against SIRT1, SIRT6 or rabbit IgG as a negative control. Co-immunoprecipitation was investigated by Western blotting using anti-NBS1 antibodies. **B.** WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM epirubicin, harvested and extracted for total cell lysates. These lysates are subjected to immunoprecipitation with specific antibody against total-acetyl-lysine. Acetylation status of NBS1 was investigated by Western blotting by using antibodies against NBS1. **C.** WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM epirubicin, harvested at indicated time points. The expression of NBS1 was investigated by Western blotting.

4.3.7 SIRT6 binds to and deacetylates FOXO3a in response to epirubicin.

I previously showed that SIRT6 regulates FOXO3a acetylation in chapter 3. I found that acetylated FOXO3a was higher in *Sirt6*$^{-/-}$ MEFs in comparison with WT MEFs. To further investigate the role of SIRT6 in the regulation of FOXO3a acetylation, co-immunoprecipitation experiments were performed in both MCF-7 and MCF-7-Epi^{R} cells. Similar to the results obtained in Figure 3.15 in Chapter 3, I found that SIRT6 directly binds to FOXO3a in MCF-7 cells prior to treatment, while there is a weak interaction between SIRT6 and FOXO3a in MCF-7-Epi^{R} (Figure 4.12A). Interestingly, the binding of SIRT6 and FOXO3a in MCF-7 cells totally disappeared after 24 h of epirubicin treatment, whereas there was a significant increase in the binding of SIRT6 and FOXO3a response to epirubicin in MCF-7-Epi^{R}.

Next, the acetylation levels of FOXO3a were also investigated. WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM epirubicin, harvested at 0, 6 and 24 h. Immunoprecipitation was performed using anti-FOXO3a antibodies. Figure 4.12B showed that both the levels of FOXO3a acetylation and its total expression levels in *Sirt6*$^{-/-}$ MEFs was elevated compared to WT MEFs at 0 h and 6 h after epirubicin treatment. These results definitely show that SIRT6 directly binds to and deacetylates FOXO3a in cells.

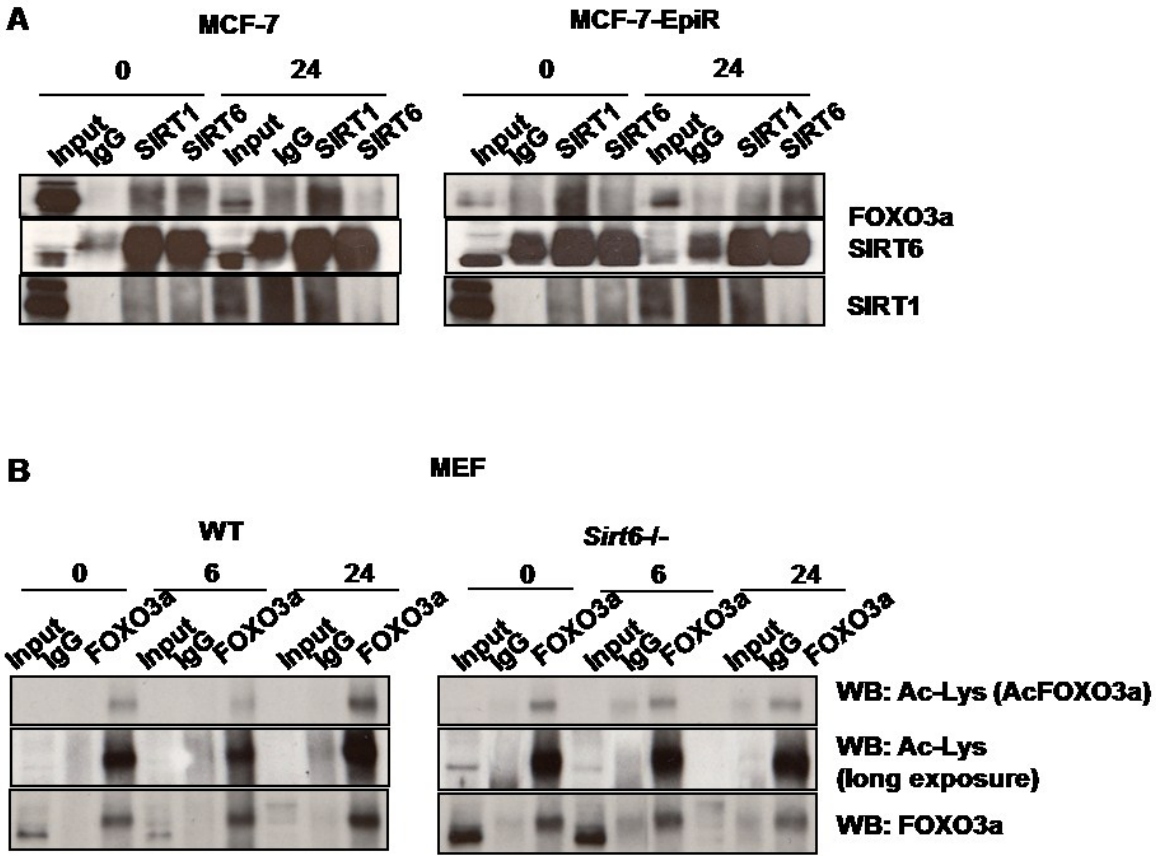


Figure 4.12 SIRT6 directly binds to and deacetylates FOXO3a in MCF-7 cells after epirubicin treatment. A. MCF-7 and MCF-7-Epi^{R} cells were treated with 50 nM epirubicin for 0, 6 and 24 h. Whole cell lysates were subjected to immunoprecipitation with specific antibodies against SIRT1, SIRT6 or rabbit IgG as a negative control. Co-immunoprecipitation was investigated by Western blotting using anti-FOXO3a antibodies. **B.** WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM epirubicin, harvested and extracted for total cell lysates. These lysates are subjected to immunoprecipitation with a specific antibody against FOXO3a. Acetylation status of FOXO3a was investigated by Western blotting by using an anti-total acetyl lysine antibody.

4.3.8 Depletion of SIRT6 leads to the FOXO3a nuclear localisation upon epirubicin treatment.

To further investigate the role of SIRT6 in the modulation of FOXO3a acetylation, WT and *Sirt6*$^{-/-}$ MEFs were used as a model. To check the expression levels of FOXO3a and its localisation, immunofluorescent staining was performed. WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM epirubicin for 0, 6, and 24 h. Cells were then harvested, fixed, permeabilised with 2% triton-X, stained with anti-FOXO3a antibody and then counter-stained with DAPI. The images of stained cells were acquired by confocal microscopy. Figure 4.13A clearly demonstrated that in the absence treatment, *Sirt6*$^{-/-}$ MEFs exhibited a higher level of FOXO3a expression in comparison with WT MEFs. There was an increase in FOXO3a expression levels in both *Sirt6*$^{-/-}$ and WT MEFs after epirubicin treatment. Yet, nuclear FOXO3a was markedly higher in *Sirt6*$^{-/-}$ MEF in comparison with WT, whereas the expression of cytoplasmic FOXO3a was noticeably higher in WT compared to *Sirt6*$^{-/-}$ MEFs. These results suggest that SIRT6 deacetylates FOXO3a and lead to a reduction of its nuclear localisation after epirubicin treatment. To further investigate this result, the subcellular distribution of FOXO3a in *Sirt6*$^{-/-}$ MEFs compared with WT MEFs was examined after 6 and 24 h of epirubicin treatment. β-Tubulin and Lamin B were used as controls for cytoplasmic and nuclear fractions, respectively. Similar to the immunofluorescent staining result, nuclear FOXO3a expression was elevated in *Sirt6*$^{-/-}$ compared with WT MEFs following epirubicin treatment. These results indicate that the acetylation of FOXO3a results in an increase in FOXO3a nuclear relocation.

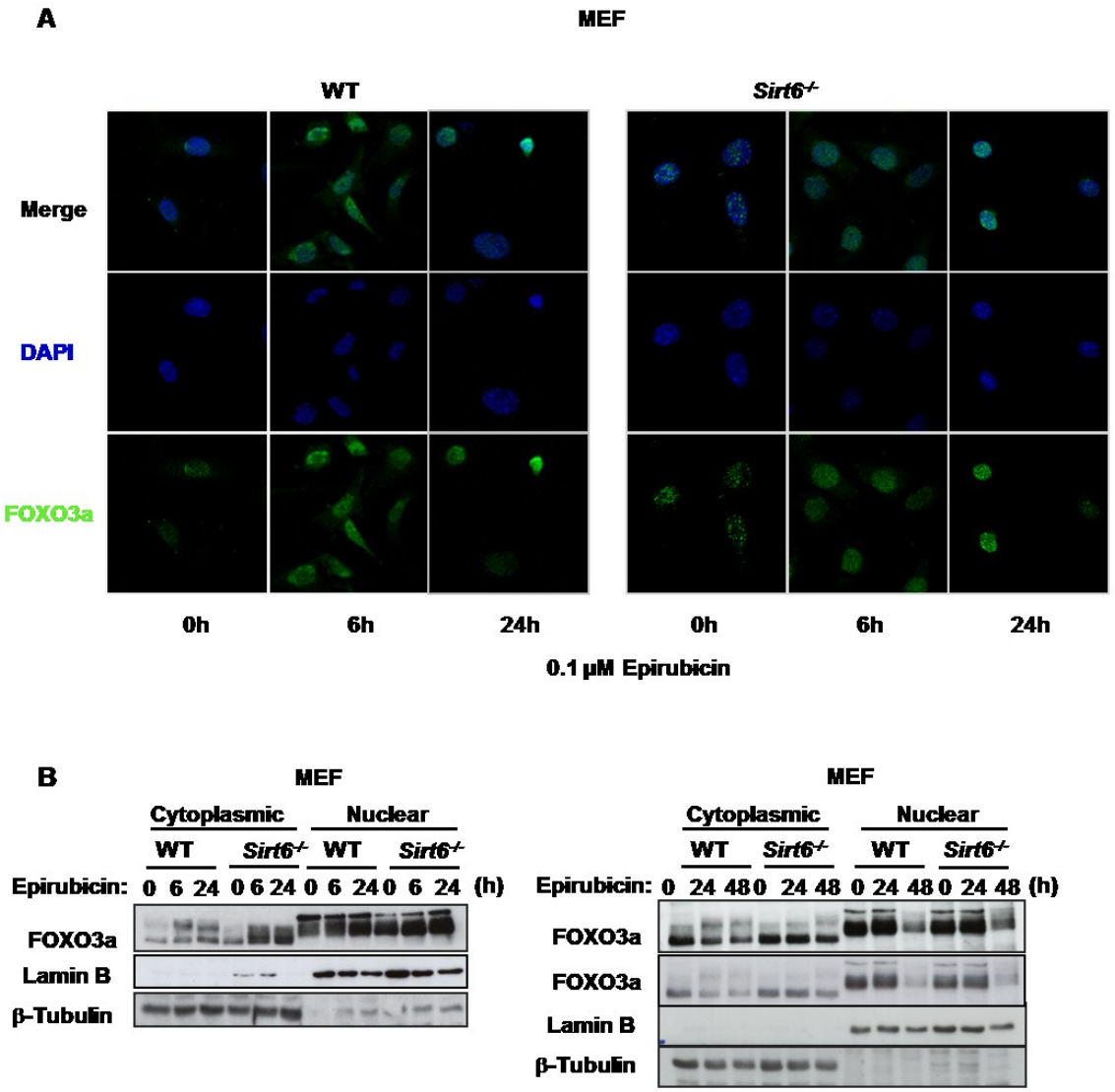


Figure 4.13 Absence of SIRT6 induces FOXO3a nuclear relocation. WT and *Sirt6*$^{-/-}$ MEFs were treated with 0.1 μM epirubicin for 0, 6 and 24 h. **A.** Cells were fixed, stained with anti-FOXO3a antibody and counter-stained with DAPI. All of images shown were acquired by confocal microscopy and obtained from at least 5 different fields. **B.** Cells were harvested for preparation of subcellular fractionation. 30 μg of nuclear and cytoplasmic lysates were analysed by Western blotting. FOXO3a expression was investigated; Lamin B and β-Tubulin were used as controls for nuclear and cytoplasmic fractions, respectively.

4.3.9 Inhibition of sirtuins increases the acetylation status of FOXO3a and its nuclear localisation.

The preliminary data from Prof. Eric Lam group found that the use of sirtuin inhibitors, sirtinol and salermide in MCF-7, MCF-7-EpiR and MCF-7-TaxR cells resulted in a repression of cell growth in response to epirubicin and paclitaxel treatment (Di Fruscia et al., 2012). Also the previous results from my colleague, Dr. Berry Peck, showed that sirtinol and salermide treatments in MCF-7 cells increased FOXO3a stability and expression in response to Laptinib treatment. These experiments possibly suggest that an increase in FOXO3a acetylation after sirtinol and salermide treatments could contribute to treatment response in MCF-7 cells.

To further investigate this hypothesis and also confirm the effect of FOXO3a acetylation on its cellular localisation, sirtuin inhibitors, sirtinol and salermide, were therefore used to inhibit all sirtuins activities. MCF-7 cells were treated with 20 μM of either sirtinol or salermide for 24 and 48 h. Cells were then harvested and analysed by immunoprecipitation. As shown in Figure 4.14A, FOXO3a acetylation was significantly increased after sirtinol treatment compared to untreated cells, whereas a marked induction of FOXO3a acetylation was observed for the treatment of salermide. Then, FOXO3a localisation after sirtinol and salermide treatment was investigated by subcellular fractionation followed by Western blotting. As depicted in Figure 4.14B, a noticeable increase in nuclear FOXO3a expression levels was observed after sirtinol and salermide treatment. These subcellular localisation studies suggest that an increase in the acetylation status of FOXO3a leads to an induction of its nuclear localisation.

Together, all of these results suggest that SIRT6 regulates the activity of FOXO3a by inhibiting its acetylation. This decreased acetylation of FOXO3a leads to the

reduction of cellular senescence and apoptosis. The role and regulation of FOXO3a by acetylation in the context of drug resistance and cell survival will be further discussed in the next chapter.

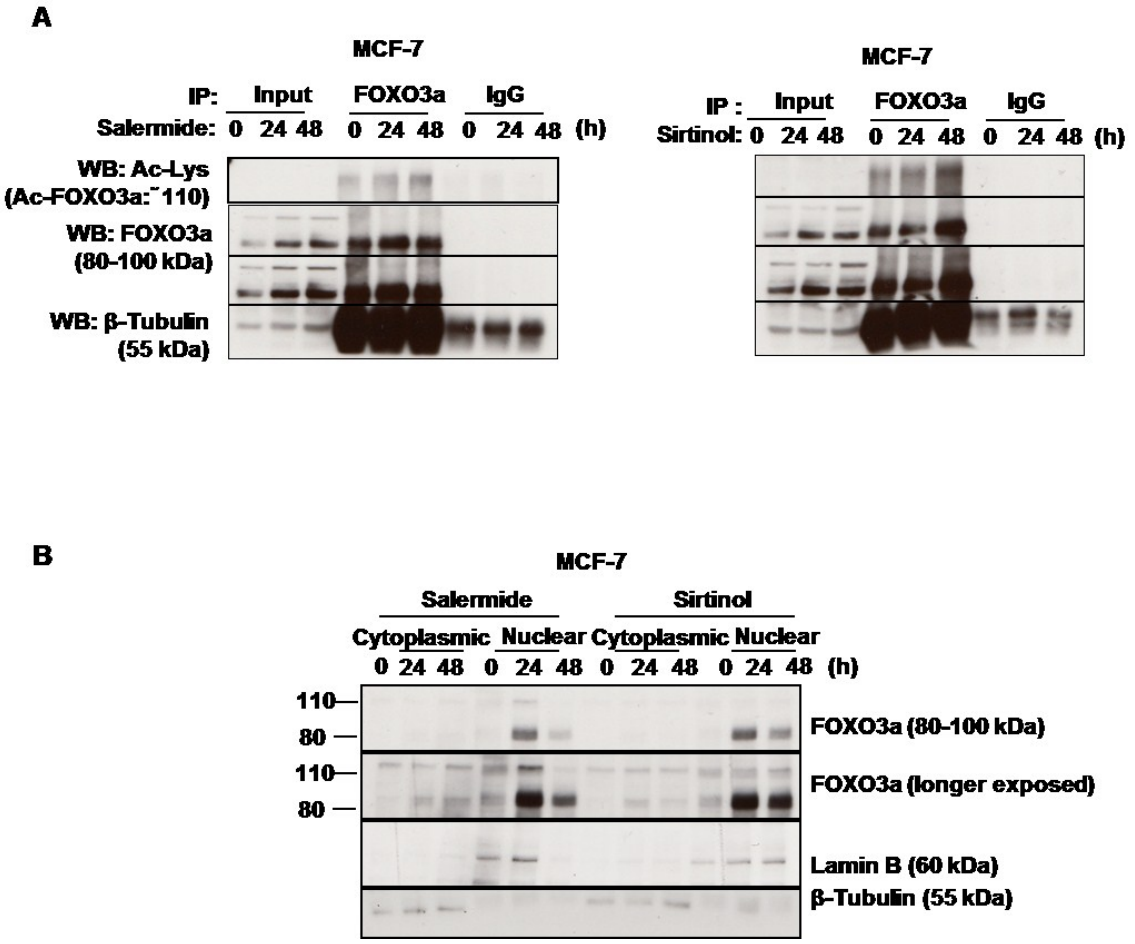


Figure 4.14 Sirtuin inhibitors, sirtinol and salermide, promote FOXO3a nuclear localisation. MCF-7 cells were treated with either 20 μM of sirtinol or salermide for 0, 24, and 48 h. Cells were then harvested and extracted for total cell lysates. **A**.These lysates are subjected to immunoprecipitation with specific antibody against FOXO3a. Acetylation status of FOXO3a was investigated by Western blotting using anti-total acetyl FOXO3a antibody. **B**. Cells were then harvested for the preparation of subcellular fractionation. The expression of FOXO3a was investigated by Western blotting. 30 μg of nuclear and cytoplasmic lysates were analysed by Western blotting. FOXO3a expression was investigated; Lamin B and β-Tubulin were used as controls for nuclear and cytoplasmic fractions, respectively.

4.3.10 Increased levels of nuclear FOXO3a and its acetylation in *Sirt6*$^{-/-}$ MEFs results in the upregulation of specific downstream targets of FOXO3a.

After identifying that SIRT6 regulates FOXO3a acetylation and results in a decrease in its nuclear accumulation, I next examined the expression levels of some downstream targets of FOXO3a that control cell cycle arrest, oxidative stress and apoptosis. WT and *Sirt6*$^{-/-}$ MEFs were treated with epirubicin, harvested and analysed for mRNA transcript levels of some FOXO3a targeted genes.

Cyclin D, p21^{Cip1} and p27^{Kip1} have been reported to be downstream targets of FOXO3a that regulate G1 progression. *p21*^{Cip1} and *p27*^{Kip1} transcription is positively regulated by FOXO3a, whereas FOXO3a represses the transcription of *Cyclin D1/D2*. The downregulation of cyclin D1/D2 contributes to the anti-proliferative function of FOXO3a and is reported to be independent of p27^{Kip1} (Schmidt et al., 2002a). As depicted in Figure 4.15, I found that there was a downregulation of *Cyclin D1* mRNA levels but not *Cyclin D2* in *Sirt6*$^{-/-}$ MEFs compared to WT after epirubicin treatment. Similar to the result obtained by Western blotting in Figure 4.6, the transcriptional levels of *p21*^{Cip1} were significantly higher in *Sirt6*$^{-/-}$ MEFs compared to control cells, whereas no difference in mRNA levels of *p27*^{Kip1} was observed.

Additionally, the expression levels of FOXO3a target genes in response to oxidative stress and DNA damage repair, such as *MnSOD2* and *Gadd45* were also assessed. No differences were found in the transcriptional levels of *MnSOD2*, whereas *Gadd45* mRNA levels were significantly higher in WT MEFs in comparison to *Sirt6*$^{-/-}$ MEFs. This indicates that the lack of SIRT6 in these cells leads to an impairment of DNA damage repair. Moreover, the transcriptional level of the proapototic gene, *Bim*, was significantly elevated in *Sirt6*$^{-/-}$ MEFs in comparison to WT cells. These results indicate that the absence of SIRT6 in cells results in higher acetylation levels of

nuclear FOXO3a and can cause a shift of FOXO3a function from the regulation of oxidative stress and DNA damage response towards the regulation of genes involved in cell cycle arrest, cellular senescence and apoptosis.

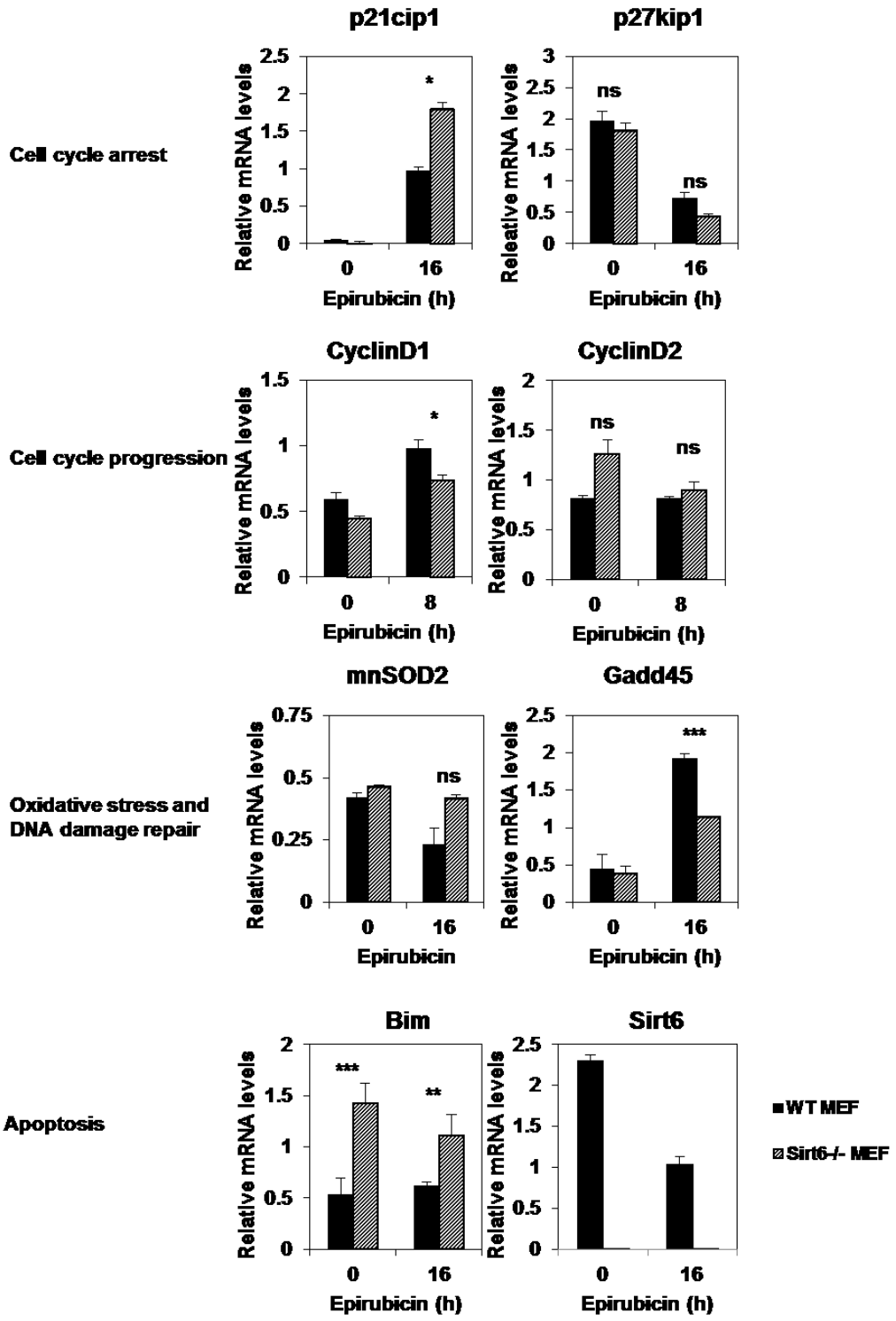


Figure 4.15 Absence of SIRT6 leads to an upregulation of FOXO3a targeted genes involving in cell cycle arrest and apoptosis. WT and *Sirt6*$^{-/-}$ MEFs were treated with 100 nM epirubicin, harvested and examined for mRNA levels of some FOXO3a targeted genes. Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05* **, *p≤0.01*, ***, *p≤0.005 versus* control)

4.4 Discussion

Cellular senescence is the mechanism that induces permanent cell cycle arrest. It can be induced by many mechanisms including DNA damage response, downregulation of cell cycle inhibitors INK/AFR, as well as oncogene activation. When DNA damage occurs, cells can arrest in order to activate DDR and repair the broken DNA to preserve genomic stability. If the damaged DNA still persists, these cells then undergo senescence or apoptosis, depending on the level and nature of the damage. Senescent cells never go back to cell cycle progression even if they are stimulated by growth factors. Due to these characteristics, senescent phenotypes have been used as markers for following the action of conventional anti-cancer drugs (Acosta and Gil, 2012). SIRT6 has been shown to have a crucial role in DNA damage repair, genomic maintenance and has been suggested to be a tumour suppressor gene (Sebastian et al., 2012). However, there is also evidence showing that SIRT6 is one of the main players to confer drug resistance in cancer (Khongkow et al., 2013a; Olmos et al., 2011b). I previously demonstrated that SIRT6 has a role in epirubicin and paclitaxel resistance in breast cancer in Chapter 3. In this study, I showed that the substantially lower doses of epirubicin and paclitaxel can induce cellular senescence in MCF-7 cells. These concentrations of epirubicin and paclitaxel are significant lower than the concentrations required to cause short-term cell cycle arrest and apoptosis (Khongkow et al., 2013b; Monteiro et al., 2013). The optimum concentrations of epirubicin that trigger senescence phenotypes in MCF-7 cells were determined to be between 10-40 nM, whereas 2-5 nM were the optimal concentrations for paclitaxel-induced senescence. This demonstrates that lower doses of these chemotherapeutic agents are sufficient for treating cancer cells

without inducing cell death. This might help to improve the efficiency of cancer treatment by reducing the toxicity and side effects of chemotherapy.

SIRT6 is chromatin-associated nuclear protein that exhibits both deacetylase and mono-ribosylase activities. I previously showed that SIRT6 confers epirubicin resistance in breast cancer by enhancing DNA damage repair. SIRT6 also has a role in paclitaxel resistance by deregulating the G2/M checkpoint. Here I found that SIRT6 depletion in MCF-7 cells resulted in a decrease in the long-term cell proliferation and enhanced not only higher SA-β-Gal expression but also and higher number of large-flattened cells – traits that characterised cellular senescence. Conversely, the overexpression of SIRT6 in MCF-7 led to an increase in long-term cell survival and a reduction in the senescent phenotypes. These results indicate that SIRT6 can promote cell cycle progression and prevent epirubicin and paclitaxel–mediated cellular senescence in MCF-7 cells. This is achieved by deacetylating and inhibiting p53 and FOXO3a, two major cell cycle regulator proteins (Millour et al., 2011; Sunters et al., 2003; Sunters et al., 2006).

SIRT6 is described as a genomic stability modulator. It has a role in single strand break by deacetylasing CtIP to regulate DNA-binding protein, replication protein A (RPA) and initiate ATR pathway (Kaidi et al., 2010). SIRT6 is the one of the first group of proteins that is recruited to the break sites and initiates DSB repair via both NHEJ and HR. SIRT6 recruits SNF2H -a chromatin remodeler - to the damaged site and deacetylatess H3K56. This leads to the recruitment of the other repair factors that will auxiliate in the repair processes (Toiber et al., 2013). SIRT6 has also been reported to activate PARP-1 by mono-ADP ribosylation with a subsequent activation of NHEJ pathway under oxidative stress conditions (Shrivastav et al., 2008). The absence of SIRT6 impaired the HR repair efficiency by 3 folds, to a similar extent of

BRCA1 and NBS1 depletion. BRCA1 and NBS1 were used as controls since they are the most important proteins that regulate DNA damage repair. BRCA1 is a protein that is involved in both HR and NHEJ pathways. It regulates MRN complexes and recruits other repair factors that initiate either NHEJ or HR pathways. In HR, it has been reported that BRCA1 interacts with BRCA2 to facilitate the recruitment of other repair factors to the damaged sites. NBS1 is a protein that binds to MRE11 and Rad50 to form MRN complexes and works as a DNA damage sensor (d'Adda di Fagagna, 2008; D'Amours and Jackson, 2002; Mao et al., 2011; Mao et al., 2012). These complexes are responsible for the mediation of DNA end resection, a crucial step of DNA damage repair, to recruit the other factors and initiate DNA damage response via both HR and NHEJ.

My results also showed that SIRT6 overexpression significantly increased HR repair efficiency by approximately 2.5 folds. This observation is in accordance to the study performed by Mao *et al*, 2011 reporting that SIRT6 overexpression enhances HR repair efficiency by 3 times and 7 times compared to control, in the presence and absence of oxidative stress, respectively (Mao et al., 2011). The overexpression of BRCA1 and NBS1 improved the HR repair efficiency by 2 folds and 1.75 folds, respectively. Interestingly, in the absence of SIRT6, the overexpression of NBS1 failed to rescue HR repair, confirming that SIRT6 is a crucial protein to process DNA damage repair through HR. Similarly to HR, NHEJ repair efficiency was reduced by approximately 75% following SIRT6 depletion. In contrast, overexpression of SIRT6 enhanced NHEJ repair by 1.5 folds. Collectively, these results demonstrated that overexpression of SIRT6 results in the enhancement of DSB repair and subsequently lead to the inhibition of epirubicin and paclitaxel-induced cellular senescence in breast cancer cells.

In order to understand the mechanism by which SIRT6 impairs the induction of senescent phenotypes in MCF-7 cells, the downstream targets of SIRT6 were also investigated. It has been shown that SIRT1 is a NBS1 regulator. SIRT1 directly binds to and deacetylates NBS1. Acetylation of NBS1 leads to the inhibition of its phosphorylation, which results in a decrease in cell survival after γ-irradiation. Additionally, *Sirt1*$^{-/-}$ MEFs are more sensitive to γ-irradiation than WT MEFs (Yuan and Seto, 2007). Similarly to SIRT1, endogenous SIRT6 was able to bind and regulate the acetylation status of NBS1 without epirubicin treatment. This result denotes that overexpression of SIRT6 might decrease the acetylation status of NBS1 leading to the enhancement of DSB repair induced by epirubicin. These results further support the role of SIRT6 in the prevention of epirubicin induced cellular senescence in MCF-7 cells.

Earlier, I reported that the acetylation of FOXO3a is also partly regulated by SIRT6. In the current chapter, I found that SIRT6 directly binds to and deacetylates FOXO3a in MCF-7 cells. The acetylation status of FOXO3a results in its nuclear relocalisation. This acetylation of FOXO3a stabilises its protein levels and prevents ubiquitination (Wang et al., 2012) leading to a nuclear retention of FOXO3a. Nuclear FOXO3a can positively activate its downstream target effectors crucial for cell cycle arrest such as *p21*^{Cip1} and also repress the transcription of *Cyclin D1.* These two genes display contrary roles in the regulation of G1 phase progression. p21^{Cip1} inhibits G1 cell cycle progression, whereas Cyclin D1 works together with CDK4 to promote cell cycle progression from G1 to S phases (Schmidt et al., 2002a). In addition, SIRT6 overexpression leads to the activation of FOXM1 after epirubicin and paclitaxel treatment. The upregulation of FOXM1 is possibly due to the repression of p53 and FOXO3a function by the deacetylase activity of SIRT6, as FOXM1 has been

reported to be negatively regulated by p53 and FOXO3a in response to DNA damage agents (Barsotti and Prives, 2009).

Collectively, in this study, I identified a novel role for SIRT6 in the inhibition of epirubicin and paclitaxel induced cellular senescence in breast cancer. SIRT6 represses FOXO3a activity by deacetylating it and leading to its cytoplasmic relocalisation with a reduction in the transcriptional functions of FOXO3a in the activation of downstream targets. Acetylated nuclear FOXO3a preferentially activates the transcription of target genes that are involved in the regulation of cell cycle arrest and cellular senescence, such as $p21^{Cip1}$ and apoptosis, such as Bim, rather than genes involved in oxidative stress and DNA damage response, such as MnSOD2 GAAD45, respectively. Additionally, overexpression of SIRT6 also improves the function of NBS1 in DSB repair induced by epirubicin, which can inhibit epirubicin induced cellular senescence in breast cancer.

CHAPTER 5

THE ROLE OF FOXO3A ACETYLATION IN DRUG RESISTANCE AND LONG-TERM CELL SURVIVAL IN BREAST CANCER CELLS

5.1 Introduction

FOXO proteins have a crucial role in longevity by regulating targets genes involved in stress resistance, DNA damage, cell cycle arrest, metabolism and apoptosis. They have been proposed to be tumour suppressors due to their ability to control cell survival and cell death. Particularly, the loss of FOXO3a leads to the deregulation of cell cycle progression, which results in tumour progression (Bouchard et al., 2007). Therefore, FOXO proteins could represent new targets in the treatment of many cancers (Karen C, 2006). The expression and cellular functions of FOXOs are tightly regulated by post-translational modifications, including phosphorylation, acetylation, deacetylation and ubiquitination. Consequently, the disruption of FOXOs by these modifications could have a potential role in carcinogenesis (Weidinger et al., 2008). There is mounting evidence suggesting that FOXO proteins, especially the FOXO3a isoform, have a central role in anti-cancer drug sensitivity. FOXO3a mediates the cytostatic and cytotoxic effects of different chemotherapeutic agents, such as paclitaxel (Sunters et al., 2003; Sunters et al., 2006), cisplatin (Fernandez de Mattos et al., 2008), doxorubicin and epirubicin (Hui et al., 2008) as well as molecular targeted therapeutics including Lapatinib (Krol et al., 2007). However, FOXO3a has also been shown to be involved in the development of acquired drug resistance. High levels of active nuclear FOXO3a were found in multi-drug resistant leukaemic cancer cells and shown to induce cell survival signalling, leading to the development of drug resistance (Hui et al., 2008). However, the mechanism by which FOXO3a induces drug resistance in cancer cells is still unclear. The distinct changes in the post-translational modifications could play a role in the development of drug resistance. FOXOs are acetylated by p300 and CBP, lead to an increase in their stability and enhancing their genotoxic stress response (Calnan and Brunet, 2008;

Matsuzaki et al., 2005). On the other hand, the deacetylation of FOXOs is controlled by HDACs including HDAC class III, Sirtuins. For instance, SIRT1 can bind to FOXO3a and remove its acetyl groups which in turn impair the FOXO-mediated cell death. Therefore, SIRT1 is a pro-survival regulator of FOXO that shifts its functions away from the regulation of pro-apoptotic genes towards the regulation of genes involved in stress resistance such as *GADD45* and *MnSOD* (Vogt et al., 2005a).

5.2 Aims

As FOXO3a has been shown to be regulated and repressed by SIRT6 deacetylase activity, the aim of this chapter is to study the impact of acetylation of FOXO3a in the development of drug resistance and cell survival.

5.3 Hypothesis

We previously reported that higher levels of nuclear FOXO3a are associated with poorer prognosis in the clinical outcome in breast cancer patients (Chen et al., 2010a). In the same set of patient samples, we also observed that higher levels of nuclear SIRT6 confer poorer survival for breast cancer patients (Khongkow et al., 2013a). As FOXO3a has been shown to regulate both epirubicin and paclitaxel response and acts as a tumour suppressor, it seems contradictory that higher levels of nuclear FOXO3a result in poorer survival in breast cancer patients. These apparently paradoxical results lead us to hypothesise that nuclear SIRT6 might deacetylase nuclear FOXO3a resulting in the downregulation of FOXO3a activity. This would explain why FOXO3a-mediated response to epirubicin and paclitaxel treatment in breast cancer patients is impaired.

5.4 Results

5.4.1 FOXO3a depletion increases the long-term survival in MCF-7 cells.

The role of FOXO3a in the long-term cell survival was firstly investigated. Clonogenic assays were performed using 5% crystal violet staining. MCF-7 cells were transiently transfected with siRNA against FOXO3a (siFOXO3a) and non-silencing (siControl) as a control, followed by treating with 0-50 nM epirubicin or 0-5 nM paclitaxel and cultured for 2 weeks. As shown in Figure 5.1A, FOXO3a depletion in MCF-7 cells led to a noticeably increase in cell proliferation compared to control in the absence of treatment. FOXO3a knocked down cells displayed higher levels of long-term survival compared to control after the treatment with epirubicin at 20-50 nM and paclitaxel at 1 nM. Conversely, ectopic overexpression of FOXO3a in MCF-7 cells did not cause a noticeable change in long-term cell survival (Figure 5.2). One possible explanation for this observation is the fact that MCF-7 cells already express FOXO3a at high levels. This result confirms that FOXO3a has an important role in epirubicin and paclitaxel responses in breast cancer cells.

To further investigate the role of the FOXO isoforms in long-term cell survival, clonogenic assays were performed again using the triple knock-out MEFs for *Foxo1*, *Foxo3a*, and *Foxo4* (*Foxo* triple MEFs). As demonstrated in Figure 5.3, *Foxo* triple MEFs exhibited a greater ability to form colonies compared to WT MEFs following treatment with epirubicin (50 nM) and paclitaxel (40-100 nM). This result indicates that the absence of the three FOXO isoforms in cells enhances long-term cell survival in response to epirubicin and paclitaxel treatments.

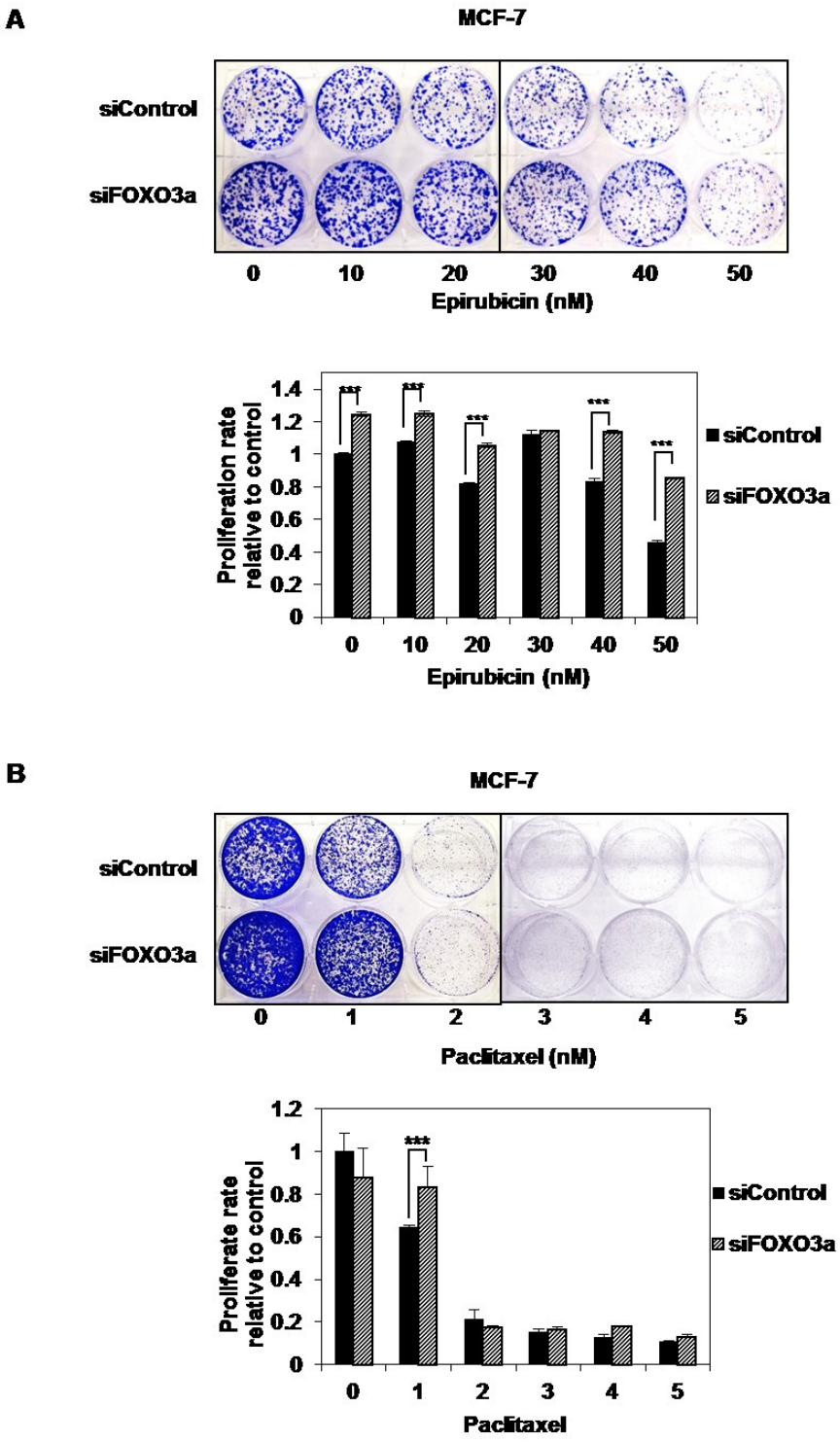


Figure 5.1 Absence of FOXO3a in MCF-7 cells increases long-term survival after epirubicin and paclitaxel treatments. MCF-7 cells were transfected with siRNA against FOXO3a (siFOXO3a) or non-silencing control (siControl). **A.** Cells were then treated with 0-50 nM epirubicin. **B.** Cells were treated with 0-5 nM paclitaxel. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

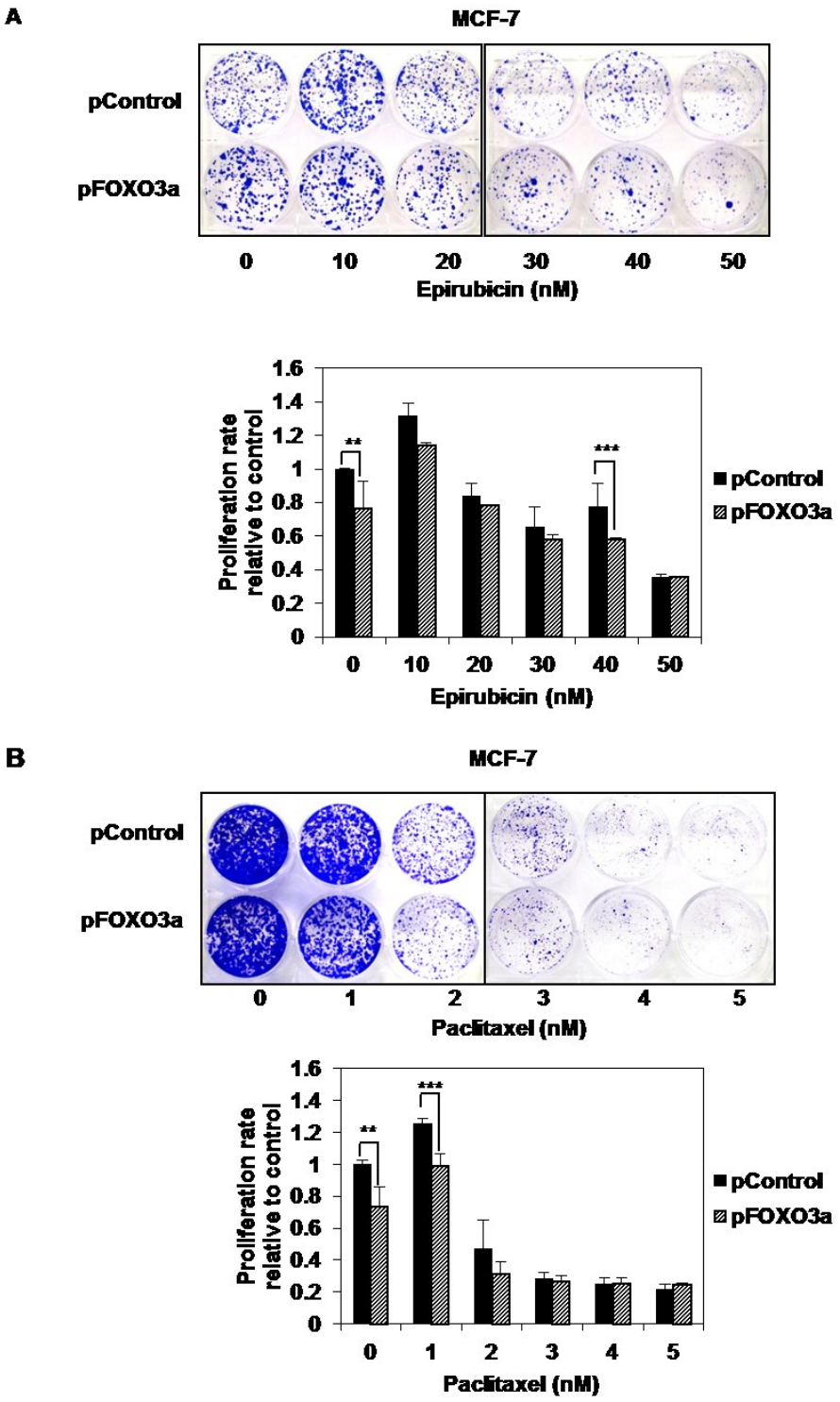


Figure 5.2 Overexpression of FOXO3a in MCF-7 cells did not change long-term cell survival after epirubicin and paclitaxel treatments. MCF-7 cells were transfected with pFOXO3a or pControl overexpressing vectors. **A.** Cells were treated with 0-50 nM epirubicin. **B.** Cells were treated with 0-5 nM paclitaxel. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

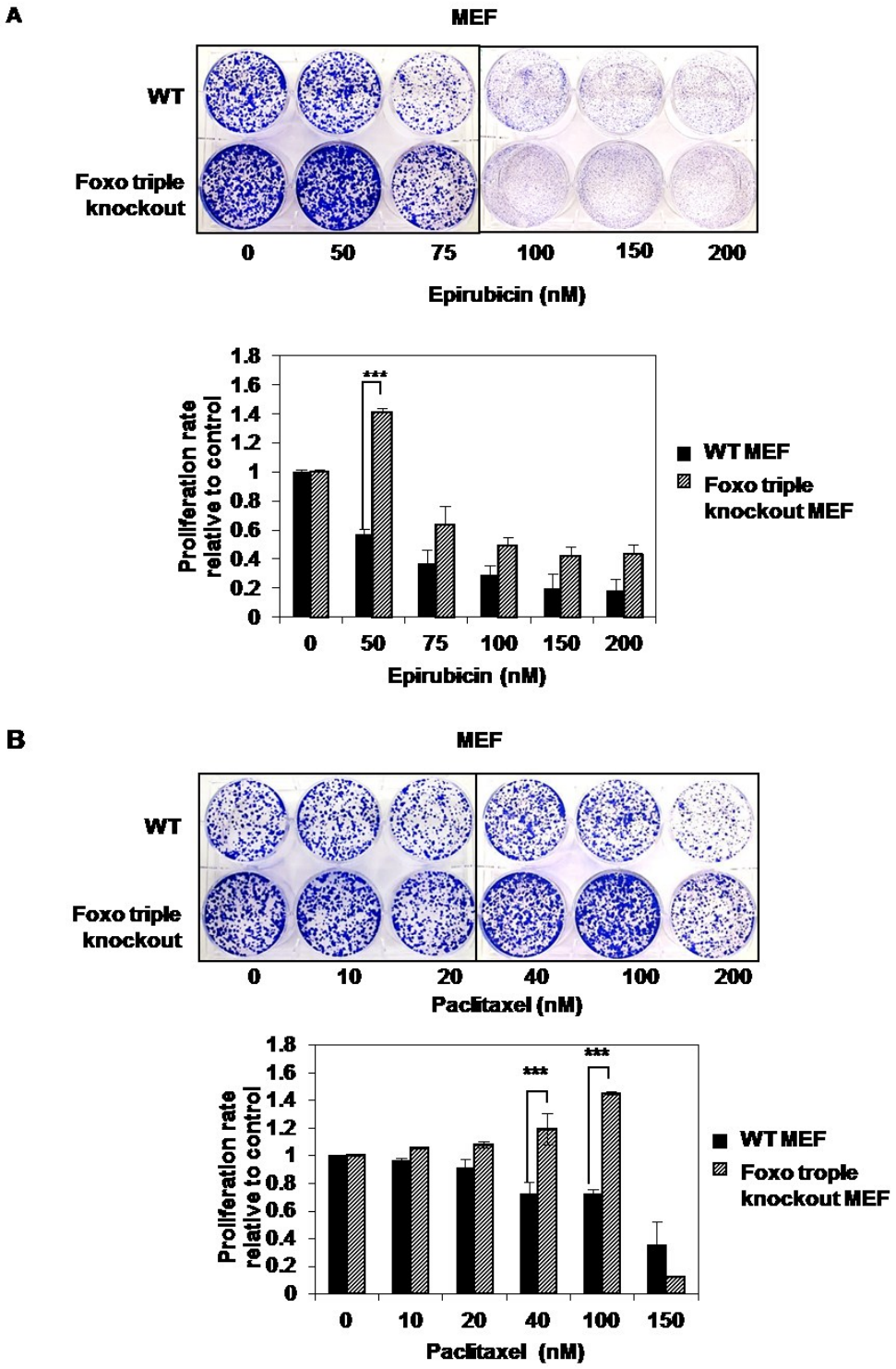


Figure 5.3 Absence of Foxo proteins in MEFs increases long-term cell survival after both epirubicin and paclitaxel treatments. A. *Foxo* triple knocked out and WT MEFs were treated with 0-200 nM epirubicin. **B.** *Foxo* triple knocked out and WT MEFs were treated with 0-150 nM paclitaxel. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

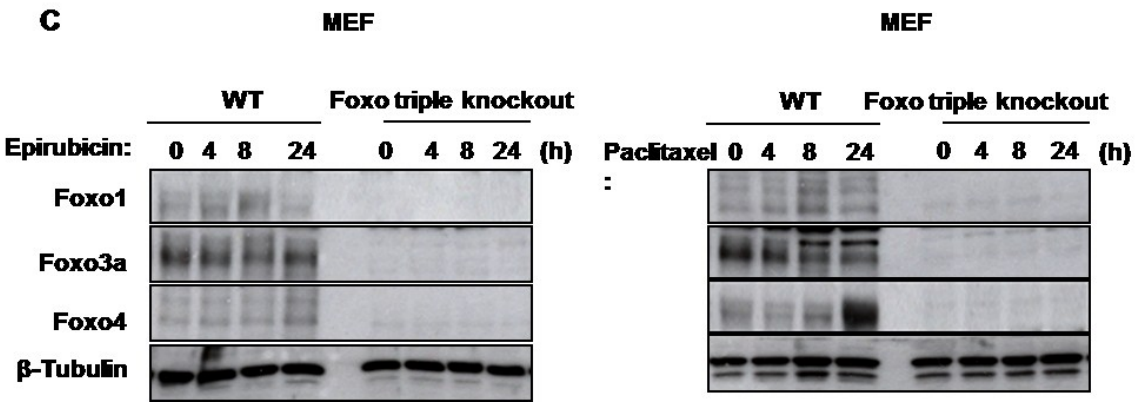


Figure 5.3 (**Continued**) **Absence of Foxo proteins in MEFs increases long-term cell survival after both epirubicin and paclitaxel treatment. C**. Western blot analysis was performed to check the expression levels of Foxo1, Foxo3 and Foxo4.

5.4.2 FOXO3a depletion impairs G2/M arrest induced by epirubicin and paclitaxel treatments.

In Chapter 4, I have shown that not only SIRT6 overexpression contributes to a downregulation of G2/M arrest induced by epirubicin and paclitaxel but also that FOXO3a is a downstream target of SIRT6. Based on this, I then decided to investigate whether the absence of FOXO3a in MCF-7 cells leads to the downregulation of G2/M checkpoint induced by epirubicin and paclitaxel. MCF-7 cells were transfected with siFOXO3a or siControl. Cells were then treated with 50 nM epirubicin or 5 nM paclitaxel and cell cycle profiling was obtained by FACS. Notably, this epirubicin concentration did not cause considerable cell death, and there was a mild increase in the number of cells accumulated in sub-G1 (Figure 5.4). Therefore, I reasoned if cellular senescence could be the mechanism responsible for the action of these drugs as these specific drug concentrations will be discussed in the next topic.

As depicted in Figure 5.5A, there were no significant changes in the cell cycle distribution after epirubicin treatment. However, FOXO3a depletion resulted in lower number of cells accumulated in G2/M phase compared to control after epirubicin treatment. Moreover, the proportion of cells in S phase was marginally higher in FOXO3a depleted cells compared to control cells. This result suggests that knocking down FOXO3a expression may promote cell cycle progression and lead to the deregulation of G2/M cell cycle arrest induced by epirubicin. In agreement with epirubicin treatment, depletion of FOXO3a in MCF-7 cells considerably reduced paclitaxel induced G2/M cell cycle arrest at 72 h (Figure 5.5B). Additionally, the number of cells in S phase was also markedly higher in FOXO3a depleted cells in comparison with control after paclitaxel, suggesting that FOXO3a is crucial to

mediate cell cycle arrest in response to cytotoxic treatment. Specifically, FOXO3a is the main regulator of G2/M cell cycle arrest induced by epirubicin and paclitaxel in MCF-7 cells.

In contrast, FOXO3a overexpression in MCF-7 cells led to a considerable decrease in the number of cells accumulated in S phase in the absence of treatment (Figure 5.6A). These results together with the data from the proliferation assay (Figure 5.2) indicate that the overexpression of FOXO3a in MCF-7 inhibits the proliferation of these cells in the absence of drug treatment, supporting the role of FOXO3a as a tumour suppressor. Moreover, a noticeable increase in the number of cells accumulated in G2/M after epirubicin and paclitaxel treatment was also observed, as depicted in Figure 5.6A and 5.6B. Together, these results indicate an important role of FOXO3a in mediating the cytotoxic responses of epirubicin and paclitaxel in MCF-7 cells.

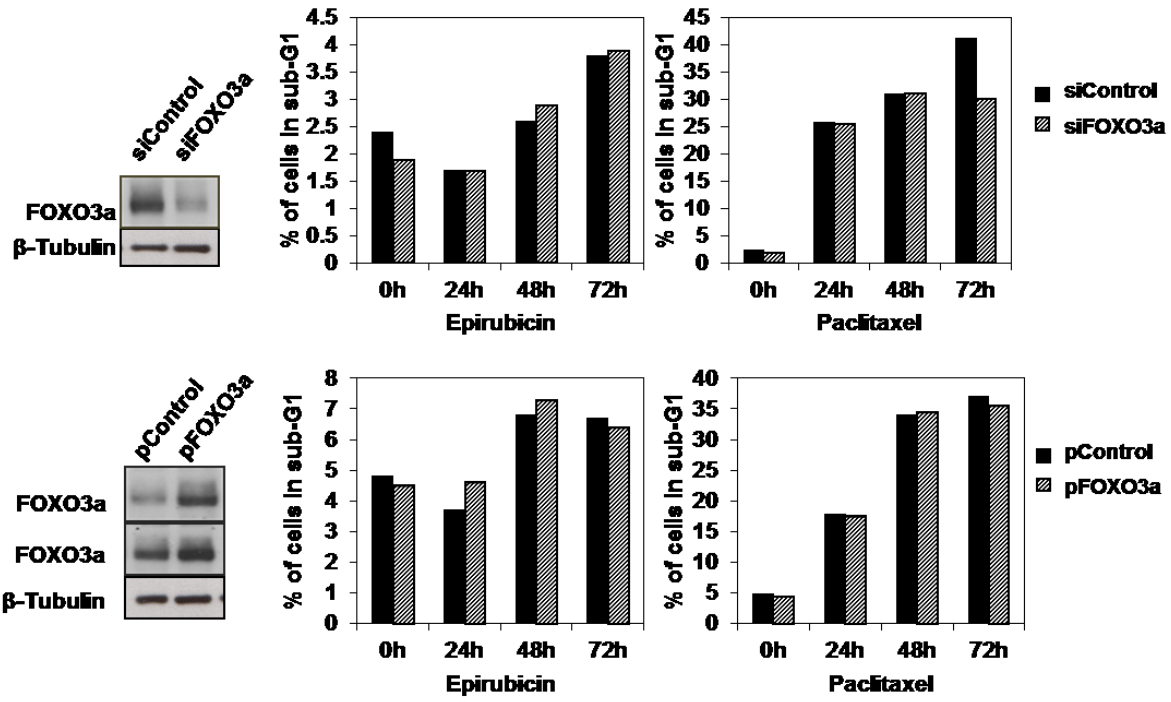


Figure 5.4 Treatment of 50 nM epirubicin and 5 nM paclitaxel treatment does not cause considerable cell death in FOXO3a-depleted MCF-7 cells. Top: MCF-7 cells were transfected with siFOXO3a or siControl. **Bottom**: MCF-7 cells were transfected with pFOXO3a or pControl overexpressing vectors and followed by 50nM epirubicin (**left**) or followed by 5 nM paclitaxel treatment (**right**). FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle is indicated. The bar chart represents the percentage of cells in sub-G1.

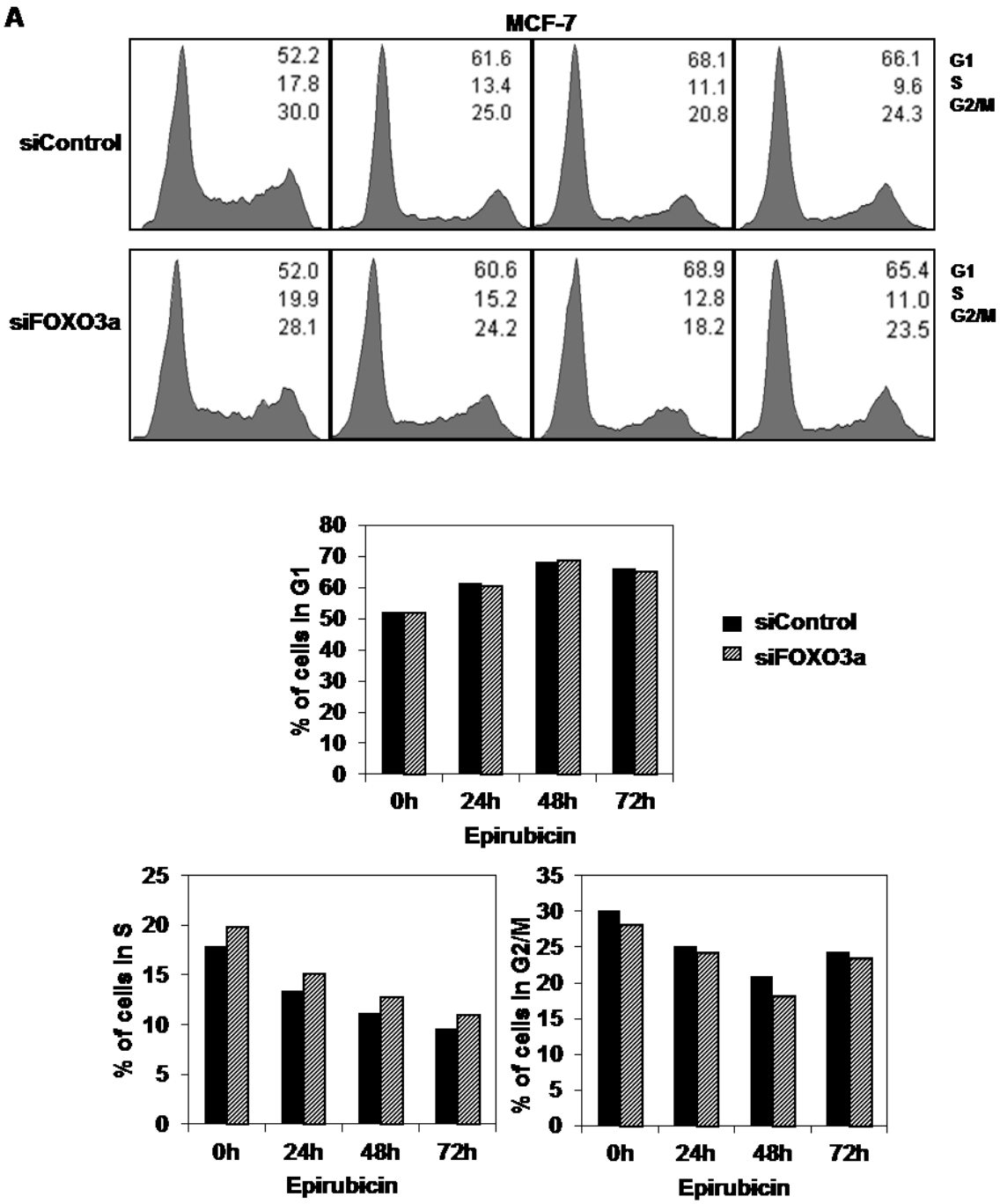


Figure 5.5 Depletion of FOXO3a results in a mild change in the regulation of cell cycle distribution after epirubicin treatment. MCF-7 cells were transfected with siFOXO3a or siControl. **A**. Cells were treated with 50 nM epirubicin for the indicated times. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G1, S and G2/M) is indicated.

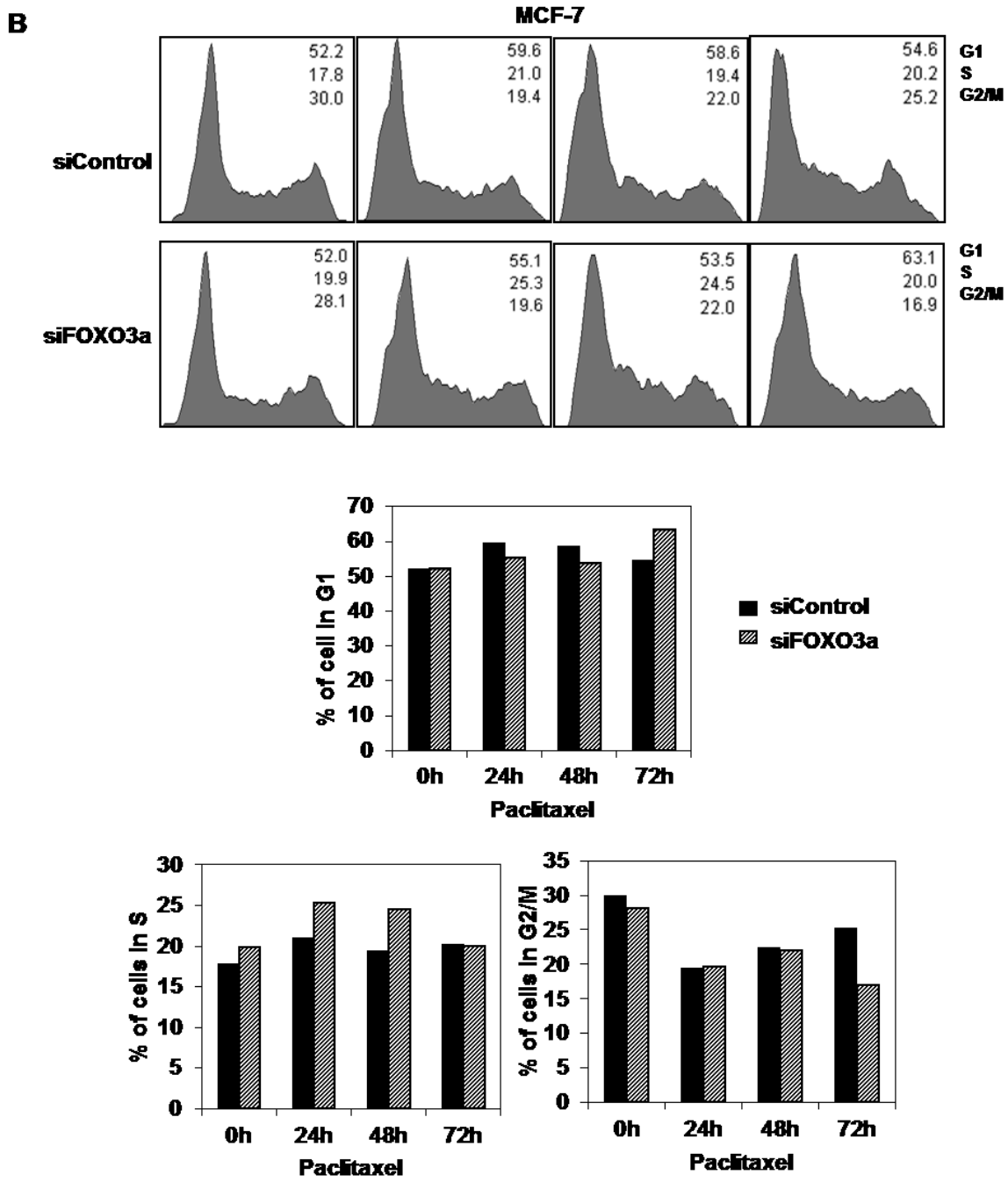


Figure 5.5 (Continued) Depletion of FOXO3a results in a mild-change in the regulation of cell cycle distribution after paclitaxel treatment. MCF-7 cells were transfected with siFOXO3a or siControl. **B**. Cells were treated with 5 nM paclitaxel for the indicated times. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G1, S and G2/M) is indicated.

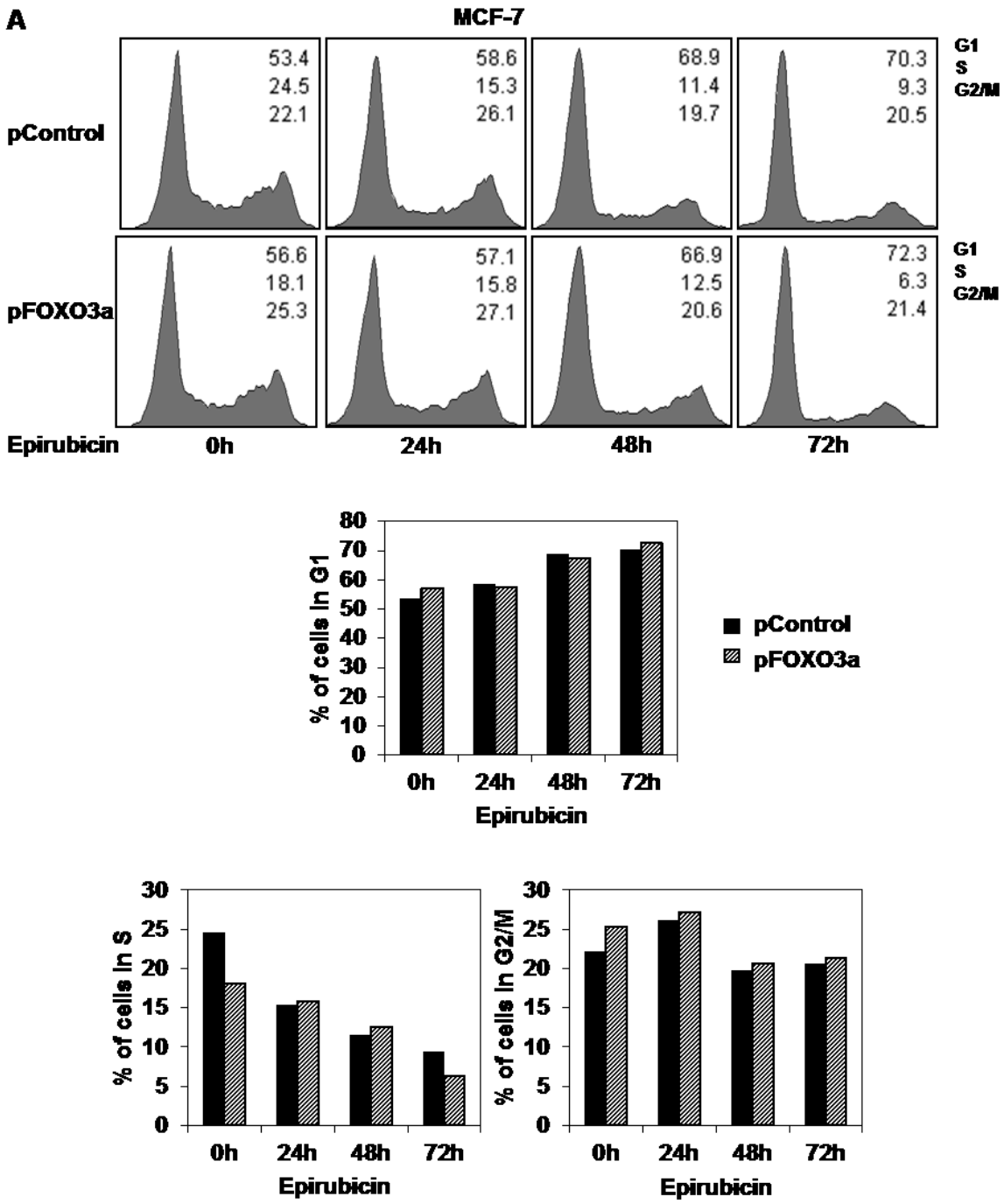


Figure 5.6 Overexpression of FOXO3a does not alter the cell cycle distribution. MCF-7 cells were transfected with pFOXO3a or pControl overexpressing vectors and **A.** Cells were treated with 50 nM epirubicin for the indicated times. **B.** Cells were treated 5 nM paclitaxel for the indicated times. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, and G2/M) is indicated.

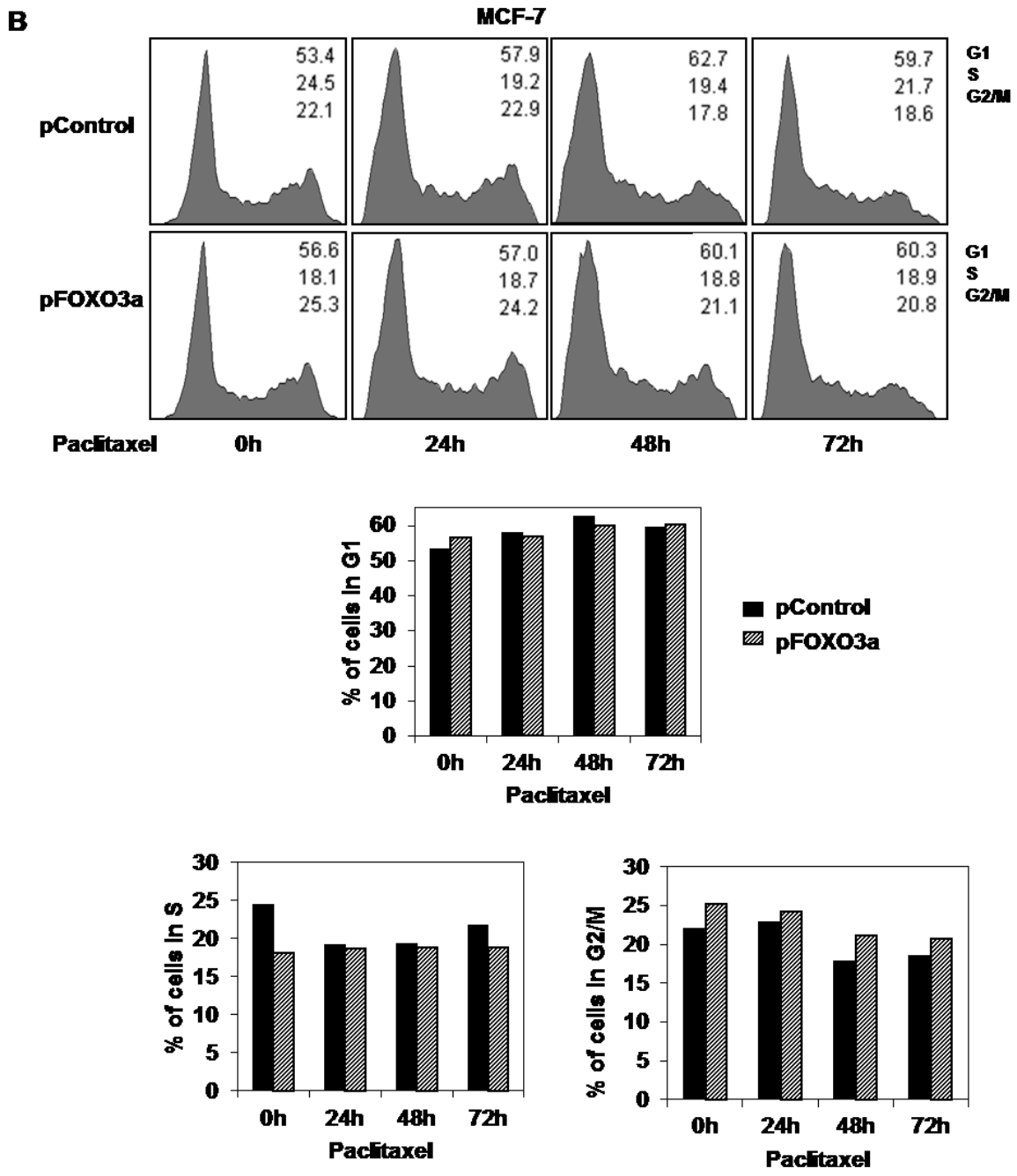


Figure 5.6 (Continued) Overexpression of FOXO3a does not alter cell cycle distribution. MCF-7 cells were transfected with pFOXO3a or pControl overexpressing vectors **B.** Cells were treated with 5 nM paclitaxel for the indicated times. FACS analysis was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G1, S, and G2/M) is indicated.

5.4.3 Silencing of FOXO3a inhibits cellular senescence in MCF-7 cells.

As previously shown in chapter 4, overexpression of SIRT6 decreases the senescent phenotypes of MCF-7 cells in response to epirubicin and paclitaxel treatments. This might be because SIRT6 diverts its functions of FOXO3a away from cellular senescence, apoptosis and cell death. Therefore, senescent associated phenotypes were examined after FOXO3a depletion in MCF-7 cells. Firstly, the status of cellular senescence was elucidated by the measurement of β-galactosidase expression and the changes in cell morphology. As show in Figure 5.7, there were no differences in the expression of SA-β-gal in FOXO3a knocked down cells compared to control cells in the absence of the treatment. However, the number of senescent cells were significantly increased after both epirubicin and paclitaxel treatment. The depletion of FOXO3a led to a significant reduction in the expression of SA-β-gal in cells treated with epirubicin from 10-40 nM (Figure 5.7A) and 1-2 nM of paclitaxel (Figure 5.7B), in comparison with the control cells. Notably, at 3 nM of paclitaxel treatment, both FOXO3a depleted and control cells displayed similar levels of senescent phenotypes, suggesting that 3 nM of paclitaxel is the maximum concentration to induce cellular senescence in MCF-7 cells. Similarly, a reduction in the number of 'flat' cells was also observed in the absence of FOXO3a compared to the control conditions in both Figures 5.7A and 5.7B. In summary, in FOXO3a-silenced conditions, there are more proliferating cells in comparison with controls following treatment with either epirubicin or paclitaxel. This indicates that FOXO3a plays a role in the cellular senescence induced by epirubicin and paclitaxel treatments.

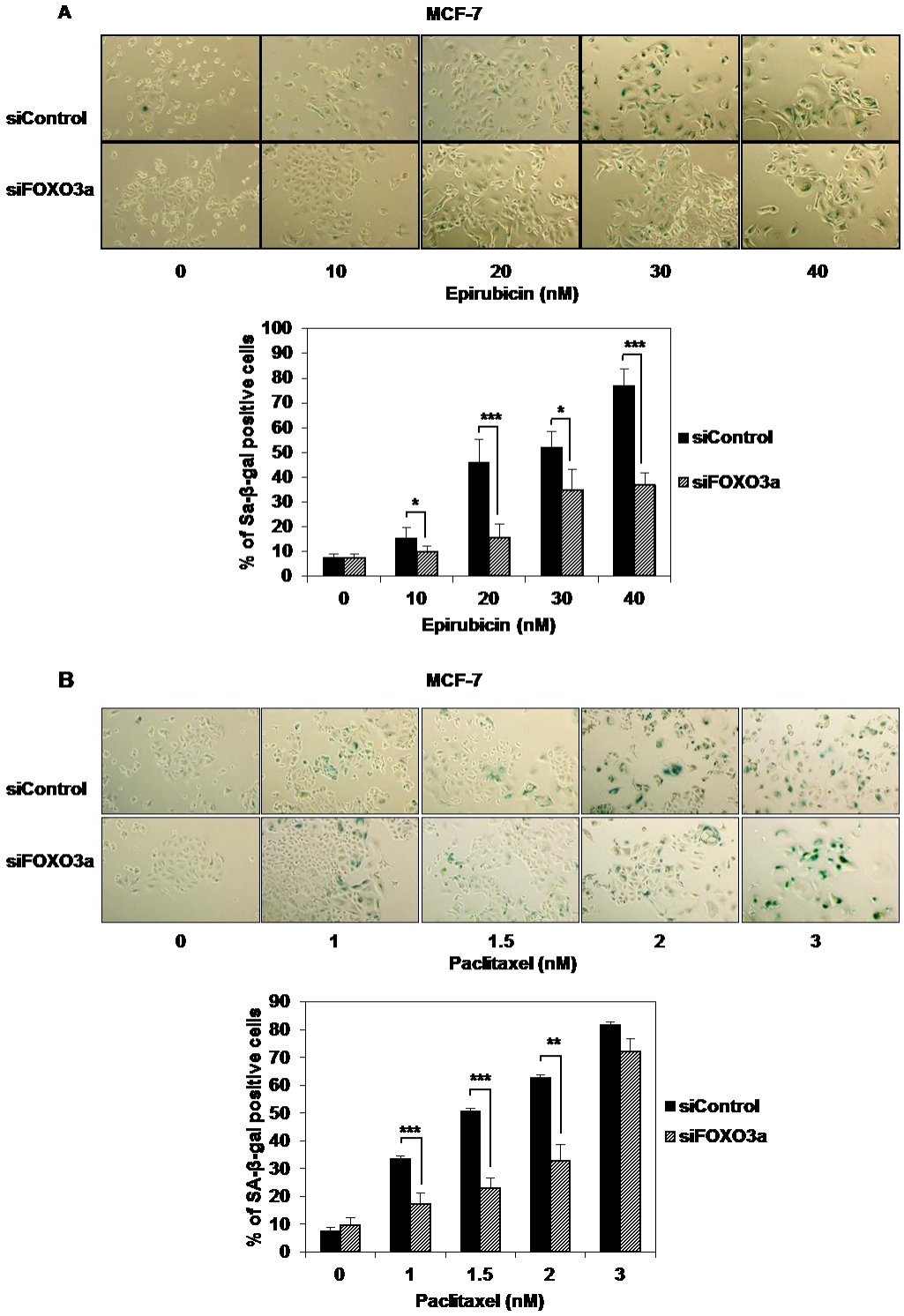


Figure 5.7 Depletion of FOXO3a reduces of SA-β-Gal expression in MCF-7 cells after treatments. MCF-7 cells were transfected with siFOXO3a or siControl. **A**. Cells were treated with epirubicin (0-50 nM). **B.** Cells were treated with paclitaxel (0-3 nM). The graph (lower panel) represents the percentage of β-gal positive cells compared with control from 5 different fields from 3 independent experiments. Bars represent average ± s.d. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

5.4.4 FOXO3a expression is down-regulated in MCF-7-Epi^R and MCF-7-Tax^R cells.

To validate the role of FOXO3a in drug resistance, I first assessed the expression levels of FOXO3a in MCF-7-Epi^R and MCF-7-Tax^R cells compared with the sensitive MCF-7 cells. Figure 5.8A demonstrated that FOXO3a expression was down-regulated in both resistant cells compared to sensitive MCF-7 cells. This suggests that FOXO3a could play a role as an important mediator for the cytotoxic effects of these two chemotherapeutic drugs in cancer cells (Sunters et al., 2003; Sunters et al., 2006). Together, these denote that FOXO3a expression is partly involved in the drug sensitivity of cancer cells in response to the treatment. However, we previously reported in Chen *et al* 2010 that nuclear FOXO3a corresponds to a poorer prognosis in terms of the clinical outcome of breast cancer patients (Chen et al., 2010b). In the same set of clinical data, we also found that higher nuclear SIRT6 expression results in poorer survival. Therefore, I hypothesised that nuclear FOXO3a might be hypoacetylated and this would impact on the function of FOXO3a in drug sensitivity after epirubicin and paclitaxel treatments.

To address this question, the levels of FOXO3a acetylation was determined and compared between MCF-7 and MCF-7-Epi^R as well as MCF-7-Tax^R cells. Whole cell lysates were extracted and immunoprecipitation performed using anti-FOXO3a antibody. As depicted in Figure 5.8B, the acetylation levels of FOXO3a in MCF-7 cells were higher than in MCF-7-Epi^R and MCF-7-Tax^R cells. The total levels of FOXO3a also exhibited the same pattern as its acetylation status.

To analyse this result further, the reverse immunoprecipitation was performed using anti-total acetyl lysine antibodies. In agreement with the previous result, acetylated FOXO3a as well as total FOXO3a expression was noticeably higher in MCF-7

compared to MCF-7-Epi^R and MCF-7-Tax^R cells (Figure 5.8C). All of these results confirm that FOXO3a expression is deregulated in both MCF-7-Epi^R and MCF-7-Tax^R cells and that acetylation of FOXO3a plays a role in these drug response mechanisms.

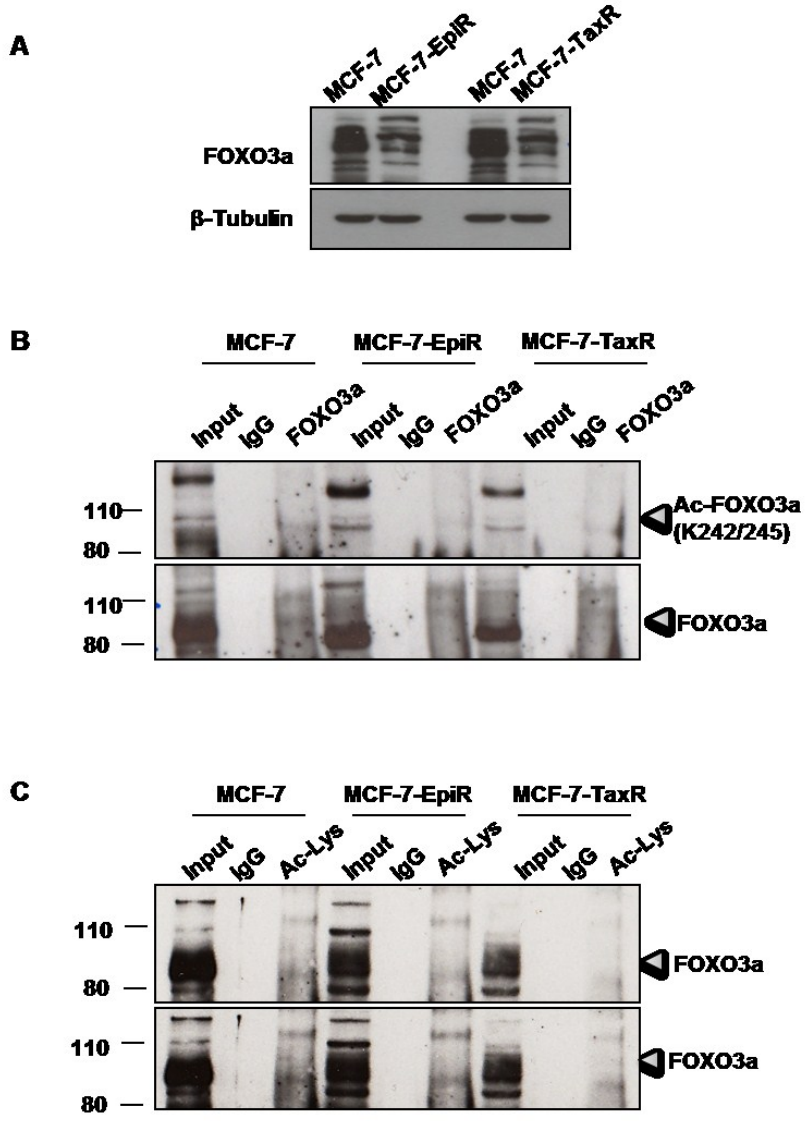


Figure 5.8 MCF-7 cells exhibit a higher level of FOXO3a acetylation compared to MCF-7-Epi^R and MCF-7-Tax^R cells. MCF-7, MCF-7-Epi^R and MCF-7-Tax^R cells were lysed for total cell lysates. **A.** The expression levels of FOXO3a were examined and compared between MCF-7, MCF-7-Epi^R, and MCF-7-Tax^R cells by Western blotting. **B.** Cell lysates were subjected to immunoprecipitation using anti-FOXO3a antibody and **C.** Cell lysates were subjected to immunoprecipitation using anti-total Ac-Lysine antibodies. The expression levels of acetylated FOXO3a were investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-lysine and anti-total FOXO3a antibodies.

5.4.5 MCF-7 cells express higher levels of acetylated nuclear FOXO3a compared to MCF-7-Epi^R and MCF-7-Tax^R cells.

In order to trigger a response to oxidative and cytotoxic stress, FOXO3a needs to be located in the nucleus to activate its downstream targets. To investigate whether the acetylation of FOXO3a has a role in drug response, subcellular fractionation and immunoprecipitation was performed using anti-FOXO3a antibodies to compare the levels of acetylated FOXO3a in the nucleus and in the cytoplasmic fractions. MCF-7 and MCF-7-Epi^R were treated with 1 μM epirubicin for 0, 6 and 24 h. Cells were then subjected to subcellular fractionation. Ten μg of nuclear and cytoplasmic fractions were collected for each sample as inputs. Cell lysates were then immunoprecipitated using anti-FOXO3a antibodies, and IgG was used as a negative control.

Figure 5.9A showed that MCF-7 cells (top) displayed higher acetylated nuclear FOXO3a expression when compared to MCF-7-Epi^R cells (bottom). However, the acetylation levels of cytoplasmic FOXO3a in MCF-7-Epi^R were noticeably higher than the levels in MCF-7 cells. There was an increase in the acetylation status of nuclear FOXO3a after epirubicin treatment in MCF-7 cells, whereas the level in MCF-7-Epi^R remained constant. Acetylated cytoplasmic FOXO3a levels were decreased in MCF-7 cells, while an increase in the acetylation of FOXO3a was observed in MCF-7-Epi^R cells. These results suggest that acetylation could be partly involved in mediating drug sensitivity response induced by epirubicin in MCF-7 cells.

To further confirm this result, reverse immunoprecipitation was performed using anti-total acetyl lysine antibody. In agreement with the previous results, (Figure 5.9B) MCF-7 cells (top) exhibited higher levels of FOXO3a acetylation compared to MCF-7-Epi^R cells (bottom). Additionally, acetylated FOXO3a was predominantly located in the nucleus in MCF-7 cells, whereas similar levels of acetylated FOXO3a were

observed in the cytoplasmic and nuclear fractions of MCF-7-Epi^R cells. These results further suggest that acetylation of nuclear FOXO3a could have a crucial role in the drug sensitivity of epirubicin treatment.

Next, to extend these findings to MCF-7-Tax^R, a similar experiment was performed comparing MCF-7 and MCF-7-Tax^R in response to paclitaxel treatment. This set of experiments was conducted with the kind help of my colleague, Miss Sasiwan Laohasinnarong. Cells were treated with 10 nM paclitaxel and harvested at 0, 6 and 24 h. Cells were then subjected to subcellular fractionation and immunoprecipitation using anti-FOXO3a antibody. The acetylation levels of nuclear FOXO3a were predominantly higher in MCF-7 cells compared to MCF-7-Tax^R cells (Figure 5.10A). There was a marked increase in the nuclear FOXO3a acetylation levels in MCF-7 cells following paclitaxel treatment, whereas a marginal increase was observed for the acetylation levels of nuclear FOXO3a in MCF-7-Tax^R cells. Notably, FOXO3a acetylation in MCF-7 cells was predominantly higher in the nuclear compared to the cytoplasmic fractions. In contrast, similar levels of acetylated FOXO3a in both nuclear and cytoplasmic compartments were observed in MCF-7-Tax^R cells.

Figure 5.10B illustrated the results obtained from the reverse immunoprecipitation using anti-total acetyl lysine antibody. In this figure, I found that the acetylated levels of nuclear FOXO3a were noticeably higher in the nuclear compared to the cytoplasmic levels in MCF-7 cells. Nuclear FOXO3a acetylation levels in MCF-7 were considerably higher when compared to MCF-7-Tax^R cells. Similar to the results obtained in Figure 5.9, these results further support that the acetylation of nuclear FOXO3a could also play a role in the sensitivity of MCF-7 cells in response to paclitaxel treatment.

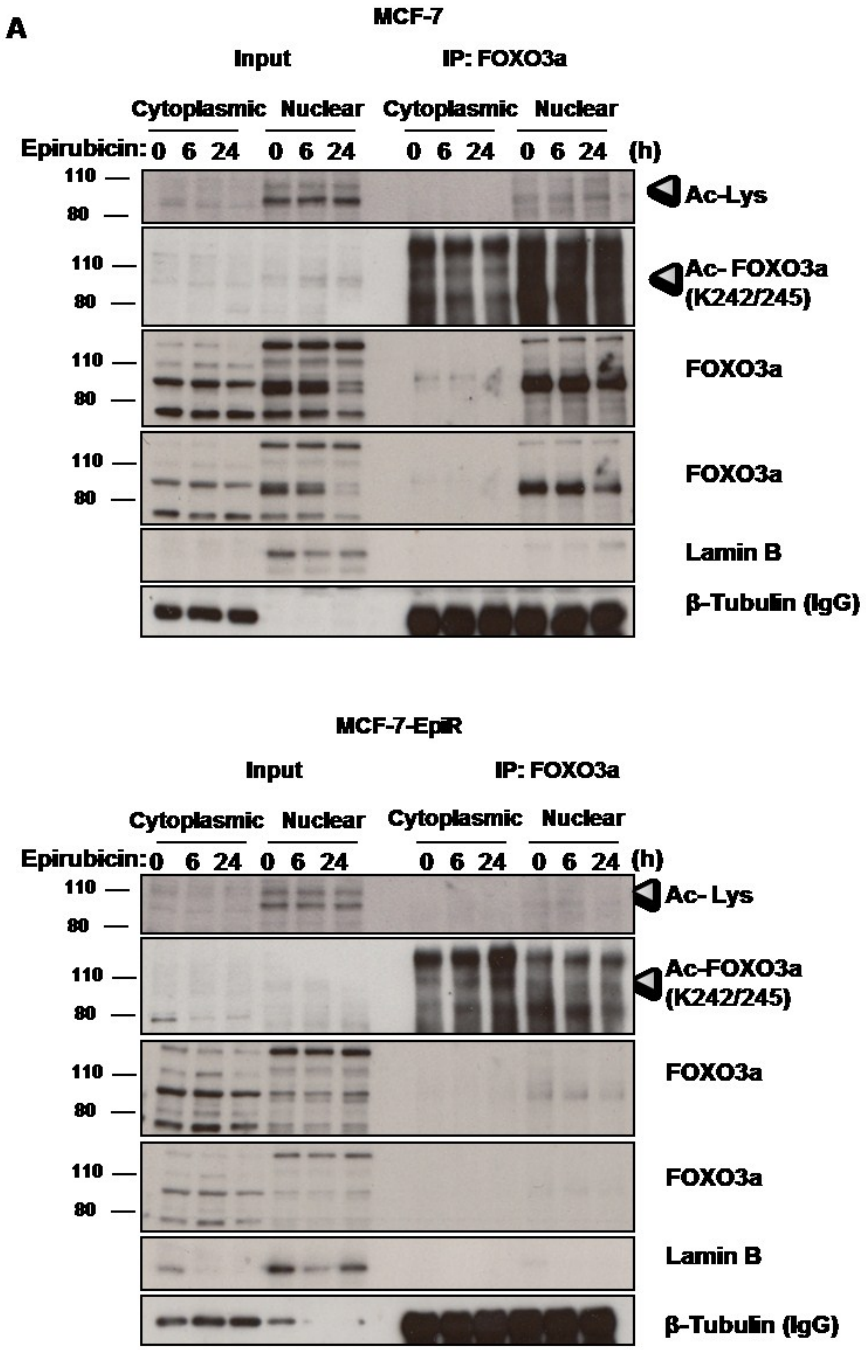


Figure 5.9 Acetylated FOXO3a in MCF-7 cells predominantly localises in the nucleus, whereas it is mostly located in the cytoplasm in MCF-7-Epi^R cells after epirubicin treatment (see also Supplemental Figure 7.1). MCF-7 and MCF-7-Epi^R cells were treated with 1 μM and cells were then harvested at 0, 6 and 24 h for preparation of subcellular fractions. **A.** Each fraction was then immunoprecipitated using anti-FOXO3a antibody. The expression of acetylated FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-lysine and anti-total FOXO3a antibodies. Lamin B and β-Tubulin were used as a control for nuclear and cytoplasmic fractions, respectively.

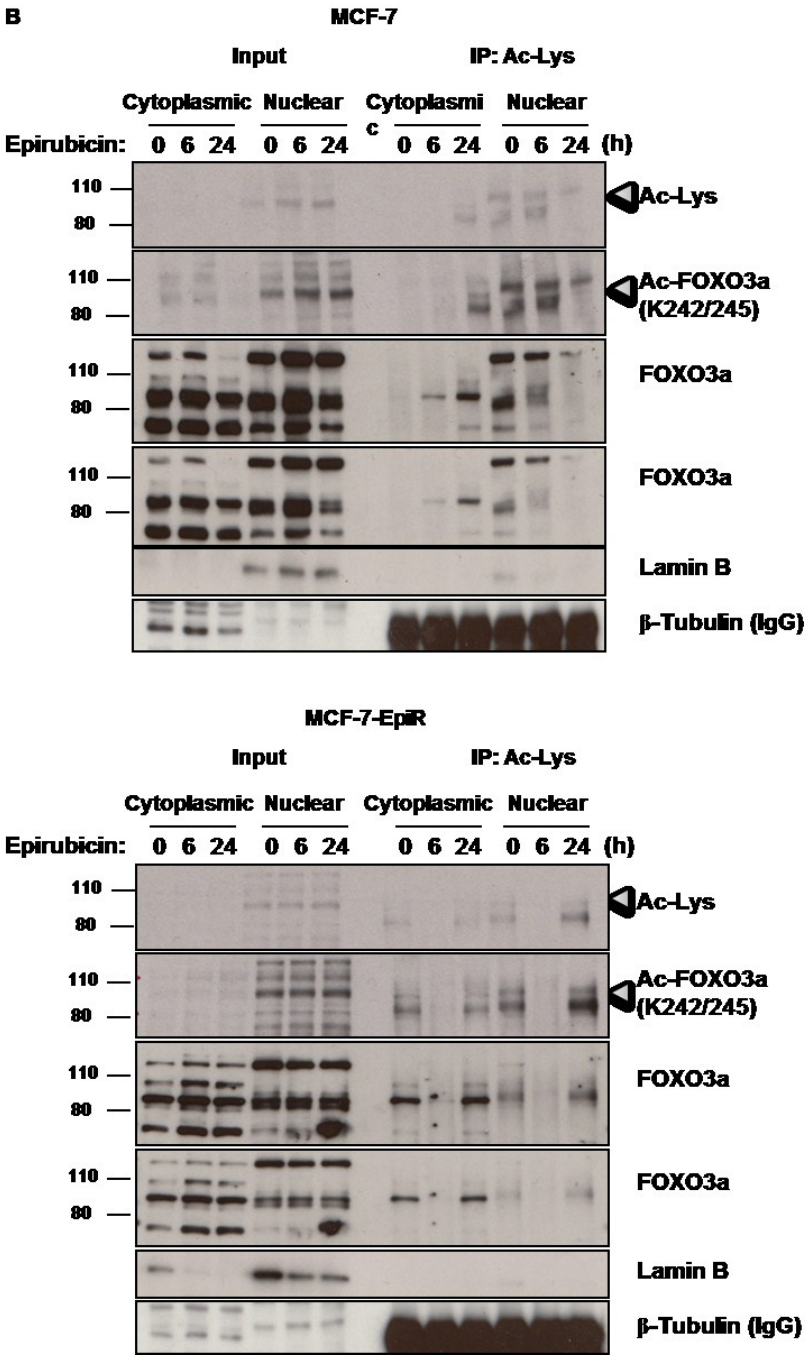


Figure 5.9 (Continued) Acetylated FOXO3a in MCF-7 cells is predominantly localised in the nucleus, whereas it is mostly located in the cytoplasm in MCF-7-Epi^R cells after epirubicin treatment. MCF-7 and MCF-7-Epi^R cells were treated with 1 μM and cells were then harvested at 0, 6 and 24 h for preparation of subcellular fractions. **B.** Reverse immunoprecipitation was performed using anti-total Ac-Lys antibody. The expression of FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-lysine and anti-total FOXO3a antibodies Lamin B and β-Tubulin were used as a control for nuclear and cytoplasmic fractions, respectively.

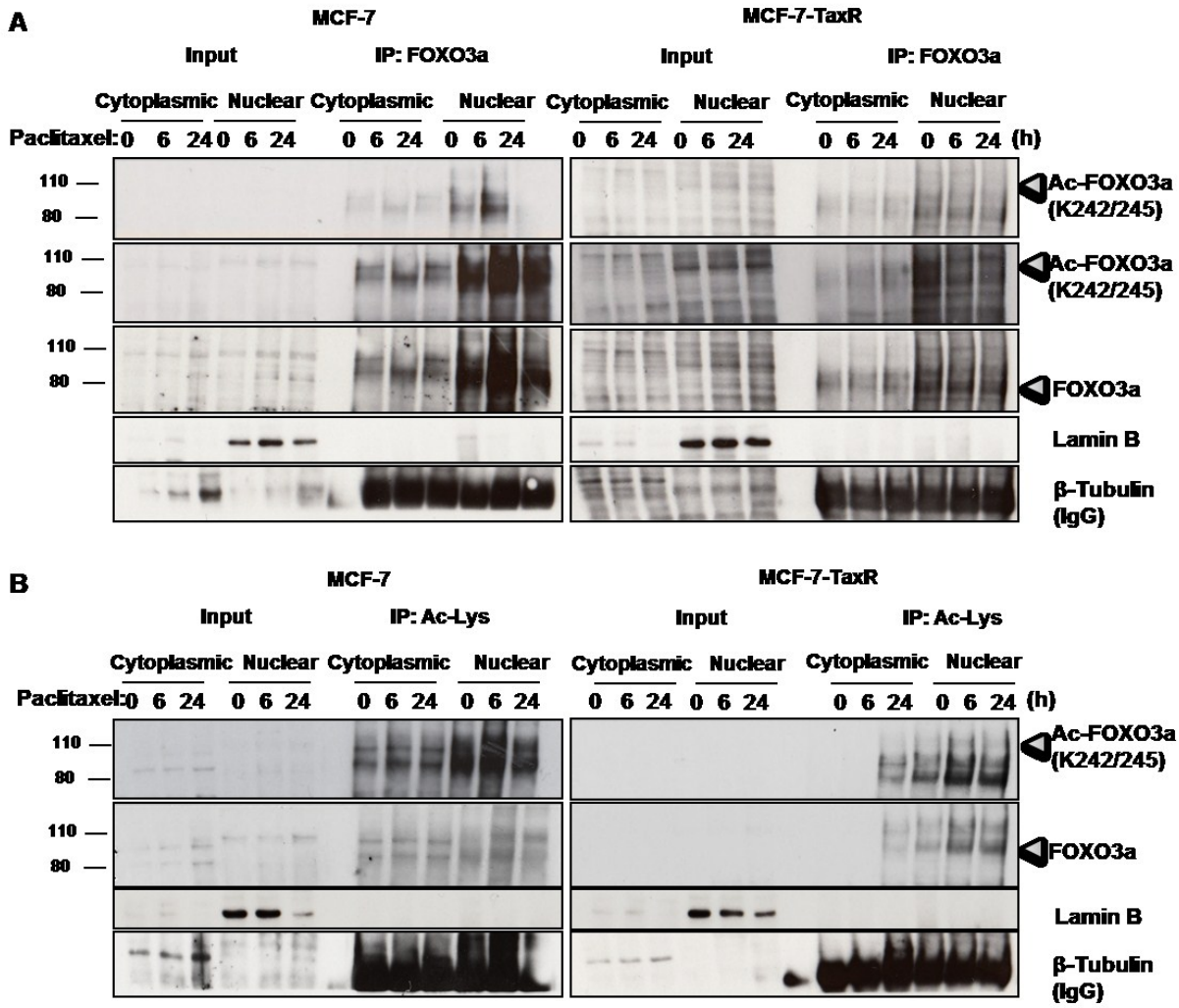


Figure 5.10 Acetylated FOXO3a in MCF-7 cells was predominantly localised in the nucleus, whereas it is mostly located in the cytoplasm of MCF-7-Tax^R cells after paclitaxel treatment. MCF-7 and MCF-7-Tax^R cells were treated with 10 nM and cells were then harvested at 0, 6 and 24 h for preparation of subcellular fractions. **A.** Each fraction was then immunoprecipitated using anti-FOXO3a antibodies. **B.** Reverse immunoprecipitation was performed using anti-total Ac-Lys antibodies. The expression of acetylated FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-Lysine and anti-total FOXO3a antibodies. Lamin B and β-Tubulin were used as controls for nuclear and cytoplasmic fractions, respectively.

5.4.6 FOXO3a is a target of sirtuins and its deacetylation is associated with the development of drug resistance in cancer cells.

Functional redundancy amongst sirtuin members has been elucidated by the identification of their overlapping downstream targets, including p53 and FOXO3a. Many studies have showed that FOXO3a is a cellular target of SIRT1 (Brunet et al., 2004; Motta et al., 2004) and SIRT2 (Wang et al., 2012; Wang et al., 2007a). These sirtuins regulate the activity of FOXO3a through deacetylation. Sirtuins have been proposed to play a role in drug resistance and cancer progression through the downregulation of p53, E2F1 and FOXO3a activity (Olmos et al., 2011b). SIRT1 overexpression has been shown to be involved in drug resistance in many cancers, including prostate, and colon cancers as well as leukaemia (Kojima et al., 2008).

In order to investigate the role of SIRT1 and SIRT2 in breast cancer drug resistance, MCF-7 cells were transfected with pSIRT1 or pSIRT2 overexpressing vector or the empty vector control (pControl). The proliferation rate of these cells was assessed by SRB assay after treatment with eight different concentrations of epirubicin. As expected, the overexpression of SIRT1 and SIRT2 in MCF-7 cells resulted in a significant increase in cell proliferation compared to control cells, in the presence of epirubicin ranging from 0 to 0.1 μM (Figure 5.11A). These results indicate that the overexpression of SIRT1 and SIRT2 in MCF-7 cells can lead to the development of epirubicin resistance. As FOXO3a has been reported to be regulated by SIRT1 and SIRT2, the expression levels of FOXO3a were then investigated by Western blotting. In agreement with previous reports (Brunet et al., 2004; Motta et al., 2004; Wang et al., 2007a), the expression levels of FOXO3a were down-regulated in SIRT1- and SIRT2 overexpressing cells after 24 h of epirubicin treatment (Figure 5.11B and C). Together, these results confirm that FOXO3a is a target of SIRT1 and SIRT2 and

that SIRT1 and SIRT2 overexpression contributes to the development of epirubicin drug resistance in MCF-7 cells.

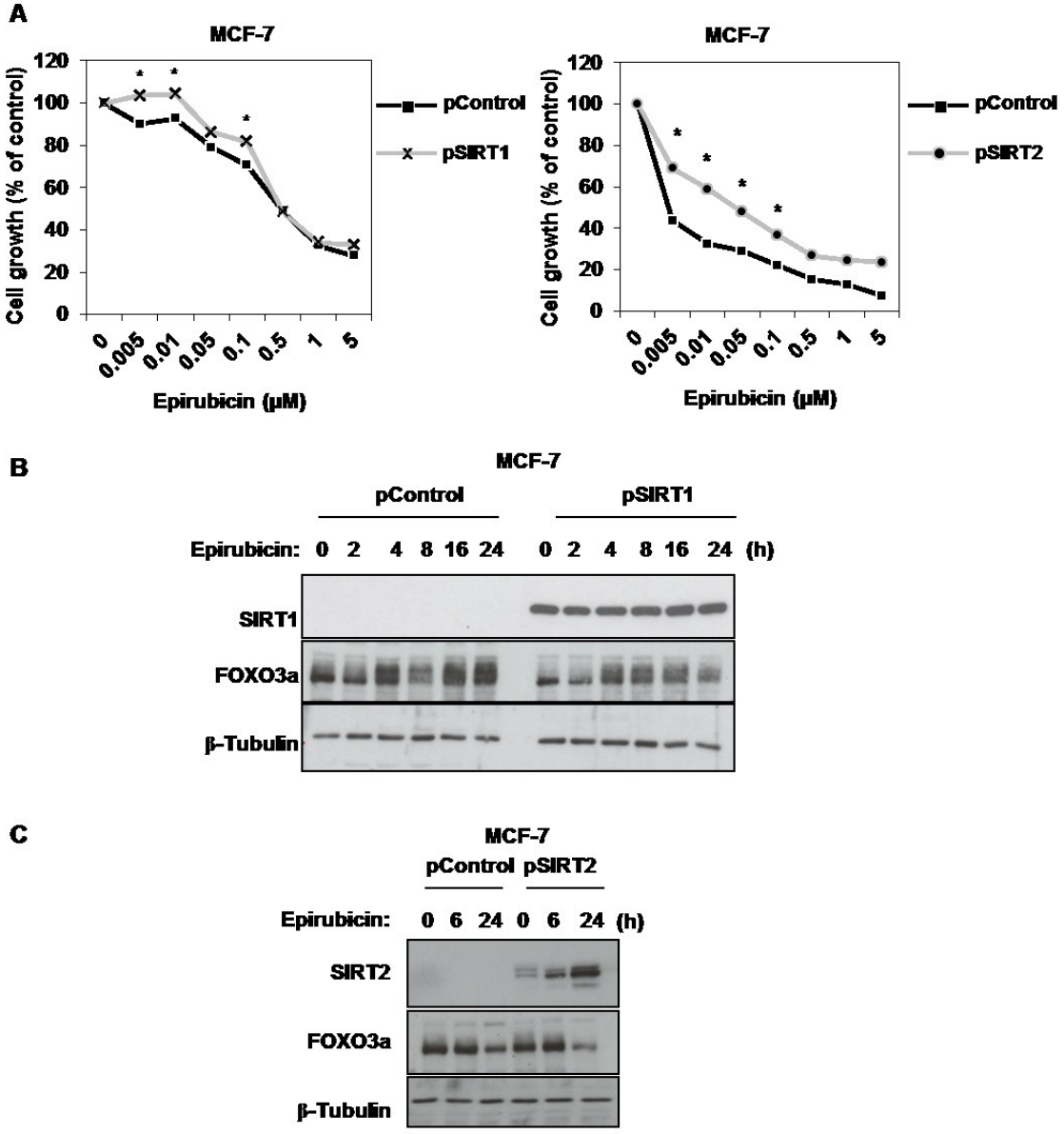


Figure 5.11 Overexpression of SIRT1 and SIRT2 leads to the development of epirubicin resistance via the downregulation of FOXO3a. A. MCF-7 cells were transfected with pSIRT1- (**left)**, pSIRT2- overexpressing vectors (**right**), together with pControl. Cells were then treated with 8 different concentrations of epirubicin for 72 h. Cell proliferation was measured by SRB assay. Bars represent average ± s.d. Statistical significance was determined by Student's t test (* significant, $p \leq 0.05$; n.s. not significant). **B.** Western blot analysis of SIRT1 overexpressing or control overexpressing MCF-7 cells treated with 1 μM epirubicin for 0, 2, 4, 8, 16 and 24 h to assess transfection efficiency and the expression of FOXO3a. **C.** Western blot analysis of SIRT2 overexpressing or control MCF-7 cells treated with 1 μM epirubicin for 0, 6 and 24 h to assess the transfection efficiency and the expression of FOXO3a.

5.4.7 SIRT1, SIRT2 and SIRT6 depletion increases the acetylation levels of FOXO3a in MCF-7 cells.

To investigate the role of sirtuins in the downregulation of FOXO3a through their deacetylase activity, the acetylation levels of FOXO3a in MCF-7 cells was assessed following the silencing of SIRT1, SIRT2 and SIRT6. The transfection efficiency was investigated by Western blotting. Cells were then subjected to immunoprecipitation using anti-total acetyl lysine and anti-FOXO3a antibodies. As shown in Figures 5.12A and 5.12B, the depletion of SIRT1, SIRT2 and SIRT6 increased the acetylation levels of FOXO3a in MCF-7 cells. This result confirms that sirtuins regulate the deacetylation of FOXO3a. Notably, the acetylation levels of FOXO3a were dramatically increased in SIRT6, SIRT1 and SIRT2-depleted cells when compared with control cells. Of note, the acetylation levels of FOXO3a were considerably higher in SIRT6 depleted cells compared to SIRT1- and SIRT2 depleted cells. Therefore, I reasoned that SIRT6 could work as the main regulator of the FOXO3a acetylation status in MCF-7 cells.

Interestingly, SIRT1 depletion resulted in the upregulation of SIRT6, whereas a downregulation of SIRT6 was observed after the silencing of SIRT2 (Figure 5.12C). This set of results suggests that there is a compensatory mechanism between sirtuin proteins. Similar results were observed in Figure 5.12D for SIRT6 deletion in MCF-7-Epi^{R} cells. In these cells, there was an increase in the basal levels of SIRT1 expression and a decrease in the expression levels of SIRT2. However, there were no changes to the expression levels of both proteins after epirubicin treatment. This result could further confirm that sirtuins compensate for the lack of the other family members in the regulation of their downstream targets.

The acetylation levels of FOXO3a were also investigated in MCF-7-Epi^R (Figure 5.13A) and MCF-7-Tax^R cells (Figure 5.13B) after SIRT1, SIRT2, and SIRT6 depletion. The absence of SIRT1, SIRT2, and SIRT6 expression in both types of resistant cells led to an increase in the FOXO3a acetylation status compared to control cells. Notably, this increase in the acetylation of FOXO3a was higher in the SIRT6-depleted MCF-7-Epi^R cells and MCF-7-Tax^R cells when compared to the SIRT1 and SIRT2 knocked down cells. Accordingly, the total FOXO3a expression levels were also increased considerably in SIRT6 depleted cells compared to the SIRT1 and SIRT2 knocked down cells. These results further indicate that the absence of sirtuins in MCF-7, MCF-7-Epi^R and MCF-7-Tax^R cells leads to an increase in the acetylation and expression levels of FOXO3a. Since all of the results obtained so far seem to favour a crucial role for SIRT6 in deacetylating FOXO3a, my study will specifically focus on the role of SIRT6 in the regulation of the FOXO3a acetylation in drug resistance.

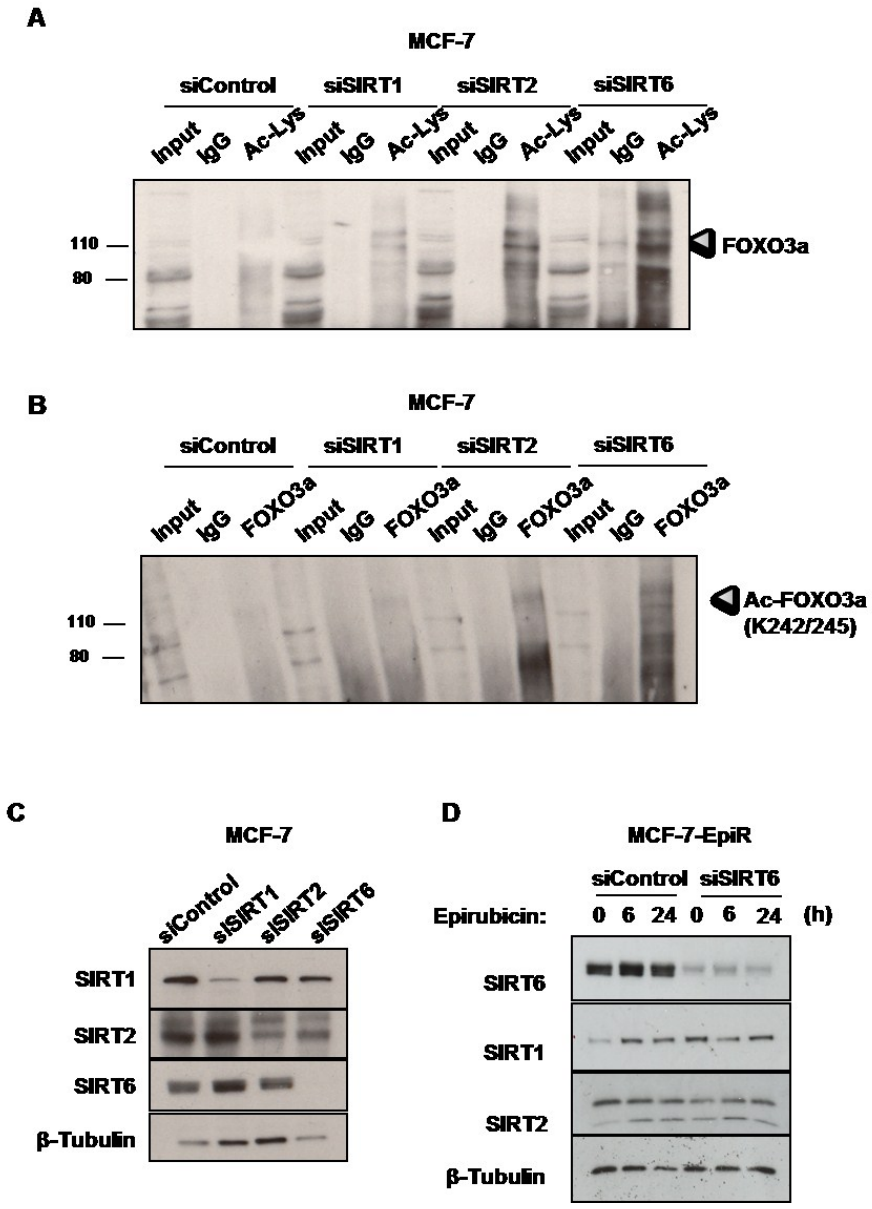


Figure 5.12 Absence of SIRT1, SIRT2 and SIRT6 expression in MCF-7 cells leads to an increase in the acetylation levels of FOXO3a. MCF-7 cells were transfected with siSIRT1, -2, -6 and siControl. **A**. Whole cell lysates were extracted and subjected to immunoprecipitation using anti-total Ac-Lysine antibody. **B.** Whole cell lysates were extracted and subjected to immunoprecipitation using anti-FOXO3a antibody. The acetylation levels of FOXO3a were investigated by incubating with anti-total Ac-Lysine and anti-total FOXO3a antibodies. **C**. Western blot analysis of transfected MCF-7 cells was performed to assess transfection efficiency. **D**. MCF-7-Epi^{R} cells were transfected with siSIRT6 or siControl, treated with 1 μM epirubicin and harvested at 0, 6 and 24h. The compensatory mechanism of SIRT1,-2, and -6 was investigated by Western blotting

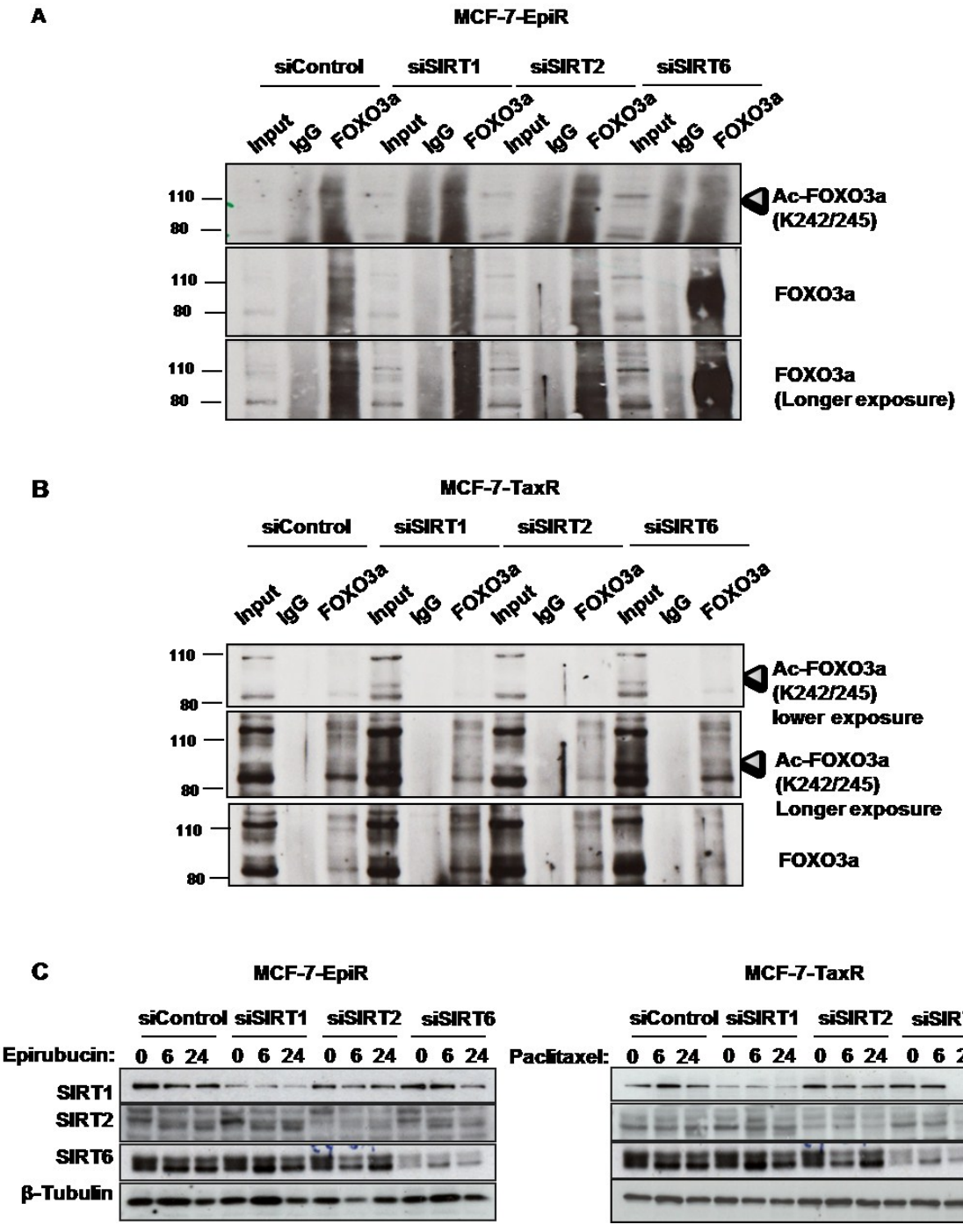


Figure 5.13 Depletion of SIRT1, SIRT2 and SIRT6 in MCF-7-Epi^R and MCF-7-Tax^R cells increases FOXO3a acetylation A. MCF-7-Epi^R and **B**. MCF-7-Tax^R cells were transfected with siSIRT1, -2, -6 and siControl. Whole cell lysates were extracted and subjected to immunoprecipitation by anti-FOXO3a antibodies. The acetylation levels of FOXO3a were investigated by incubating with anti-Ac-FOXO3a (K242/245) and anti-FOXO3a antibodies. **C.** Western blot analysis of transfected MCF-7-Epi^R (**left**) and MCF-7-Tax^R (**right**) cells was performed to assess transfection efficiency.

5.4.8 SIRT6 deacetylates FOXO3a and increases its cytoplasmic localisation leading to the suppression of its activity in drug sensitivity.

As previously shown, FOXO3a is one of the downstream targets of SIRT6. To validate the role of SIRT6 in the regulation of FOXO3a acetylation, I decided to silence SIRT6 expression in both epirubicin-treated MCF-7 and MCF-7-Epi^{R} cells. MCF-7 cells were transfected with siSIRT6 or siControl. Cells were then treated with 1 μM of epirubicin and harvested at 0, 6 and 24 h. Subcellular fractionation was performed and the protein expression analysed by Western blotting. The acetylation status of FOXO3a in nuclear and cytoplasmic fractions was assessed and compared between control and SIRT6 depleted cells. As depicted in Figure 5.14, SIRT6 depletion in MCF-7 cells increased not only the expression levels of nuclear acetylated FOXO3a but also the total FOXO3a levels in comparison to control cells. This result shows that the acetylated FOXO3a is predominantly localised in the nucleus.

To further validate these results, cell lysates were immunoprecipitated with anti-FOXO3a antibodies and IgG was used as a negative control. As presented in Figure 5.15 (top), SIRT6 depleted cells displayed higher levels of acetylated nuclear FOXO3a compared to control cells. Notably, there was a slight increase in the acetylation levels of nuclear FOXO3a in SIRT6 knocked down cells after epirubicin treatment, whereas a decrease in nuclear acetylated FOXO3a was observed in control cells. Additionally, the total FOXO3a levels were noticeably higher in SIRT6 depleted cells in comparison with control cells.

I obtained similar results in MCF-7-Epi^{R} cells. Unfortunately, acetylated FOXO3a was hardly detected in both control and SIRT6-depleted MCF-7-Epi^{R} cells. This might be

due to the lower level of FOXO3a acetylation. However, total FOXO3a expression levels were markedly elevated in SIRT6 knocked down cells compared to control MCF-7-Epi^{R} cells at 24h of epirubicin treatment.

In order to confirm these results further, reverse immunoprecipitation was performed once more using anti-total acetyl lysine antibody. SIRT6 depletion in MCF-7 cells resulted in the accumulation of acetylated FOXO3a in the nuclear fraction, whereas there was a moderate increase in the acetylation levels of nuclear FOXO3a compared to the cytoplasmic fraction in control cells (Figure 5.16 top). In agreement with the results for MCF-7 cells, Figure 5.16 (bottom) showed that knocking down SIRT6 in MCF-7-Epi^{R} increased the acetylation levels of FOXO3a accumulated in the nucleus compared to control cells. Additionally, SIRT6 depleted cells exhibited a significantly higher nuclear FOXO3a expression compared to the cytoplasmic compartment, while the expression of FOXO3a remained consistent in both compartments in the control cells. These results confirm that SIRT6 deacetylates FOXO3a leading to a decrease in its nuclear localisation and could deregulate its function in drug sensitivity after epirubicin treatment.

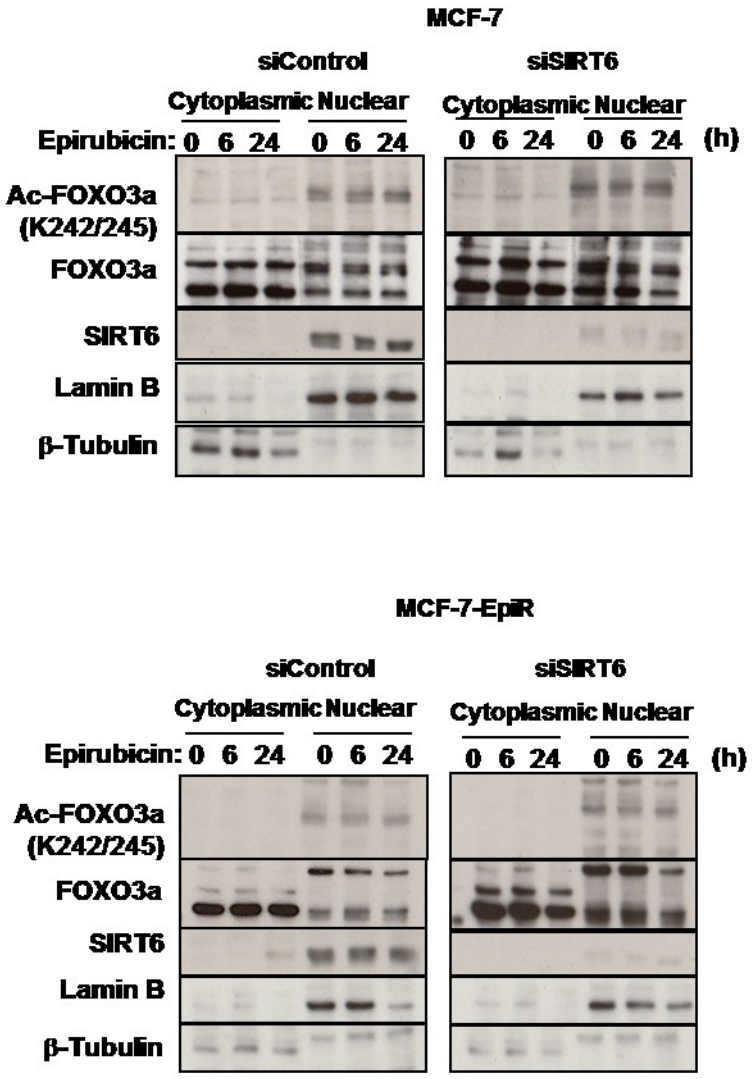


Figure 5.14 SIRT6 depletion in MCF-7 cells (top) and MCF-7-Epi^R cells (bottom) increases both the expression and the acetylation levels of FOXO3a in the nucleus after epirubicin treatment. MCF-7 cells were transfected with siSIRT6 or siControl. Cells were then treated with 1 μM epirubicin for 0, 6 and 24 h and harvested for subcellular fractionation. The acetylation of FOXO3a was investigated by Western blotting with anti-Ac-FOXO3a (K242/245), FOXO3a antibodies and Lamin B and β-Tubulin were used as controls for nuclear and cytoplasmic fractions, respectively.

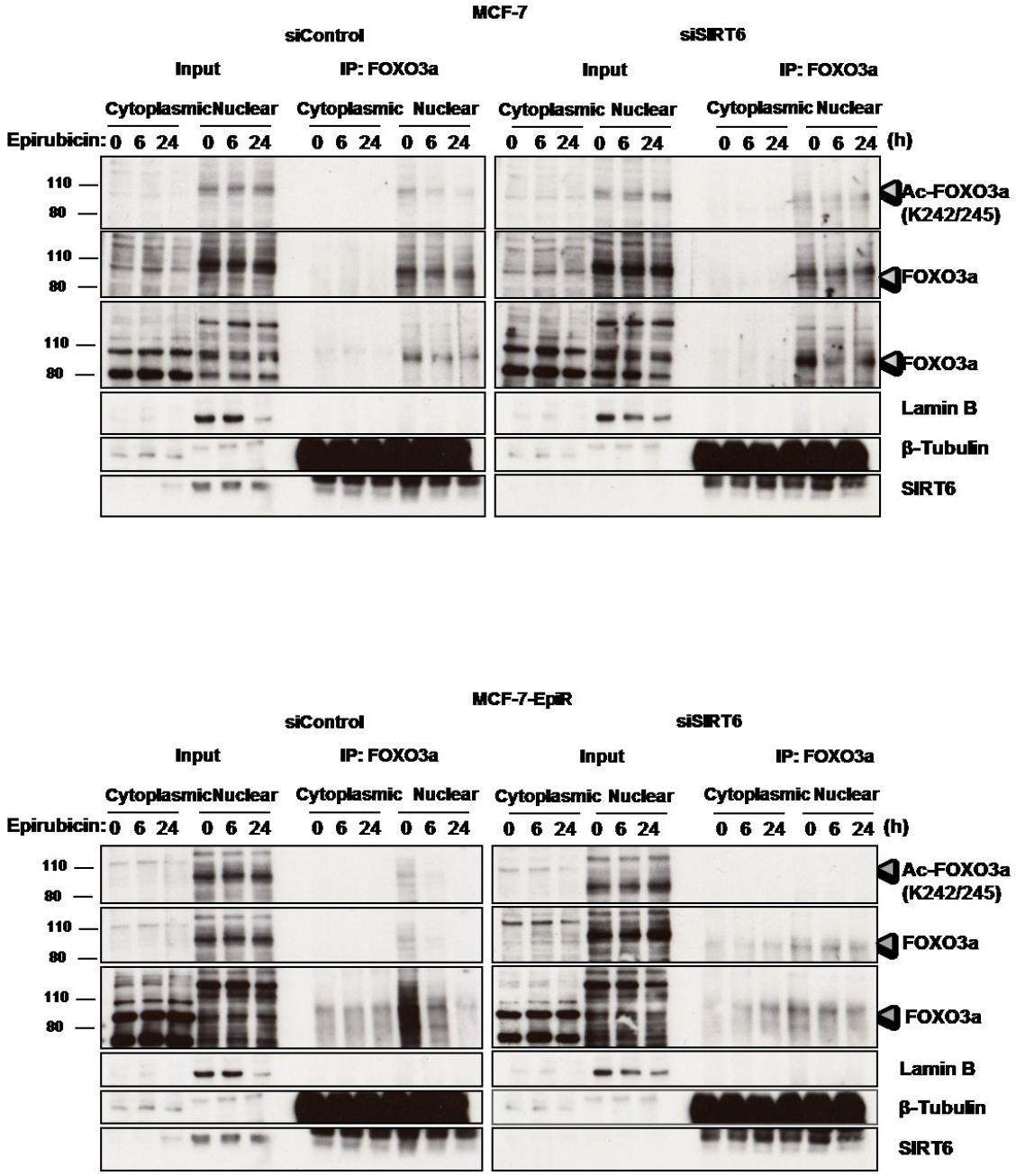


Figure 5.15 SIRT6 depletion in MCF-7 cells (top) and MCF-7-Epi^R cells (bottom) increases both the expression and the acetylation levels of FOXO3a in the nucleus after epirubicin treatment. MCF-7 cells were transfected with siSIRT6 or siControl. Cells were then treated with 1 μM epirubicin for 0, 6 and 24 h and harvested for subcellular fractionation. Each fraction was then immunoprecipitated using anti-FOXO3a antibodies. The acetylation levels of FOXO3a were investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-Lysine and anti-total FOXO3a antibodies. Lamin B and β-Tubulin were used as a control for nuclear and cytoplasmic fraction, respectively. SIRT6 expression was also investigated to assess the transfection efficiency.

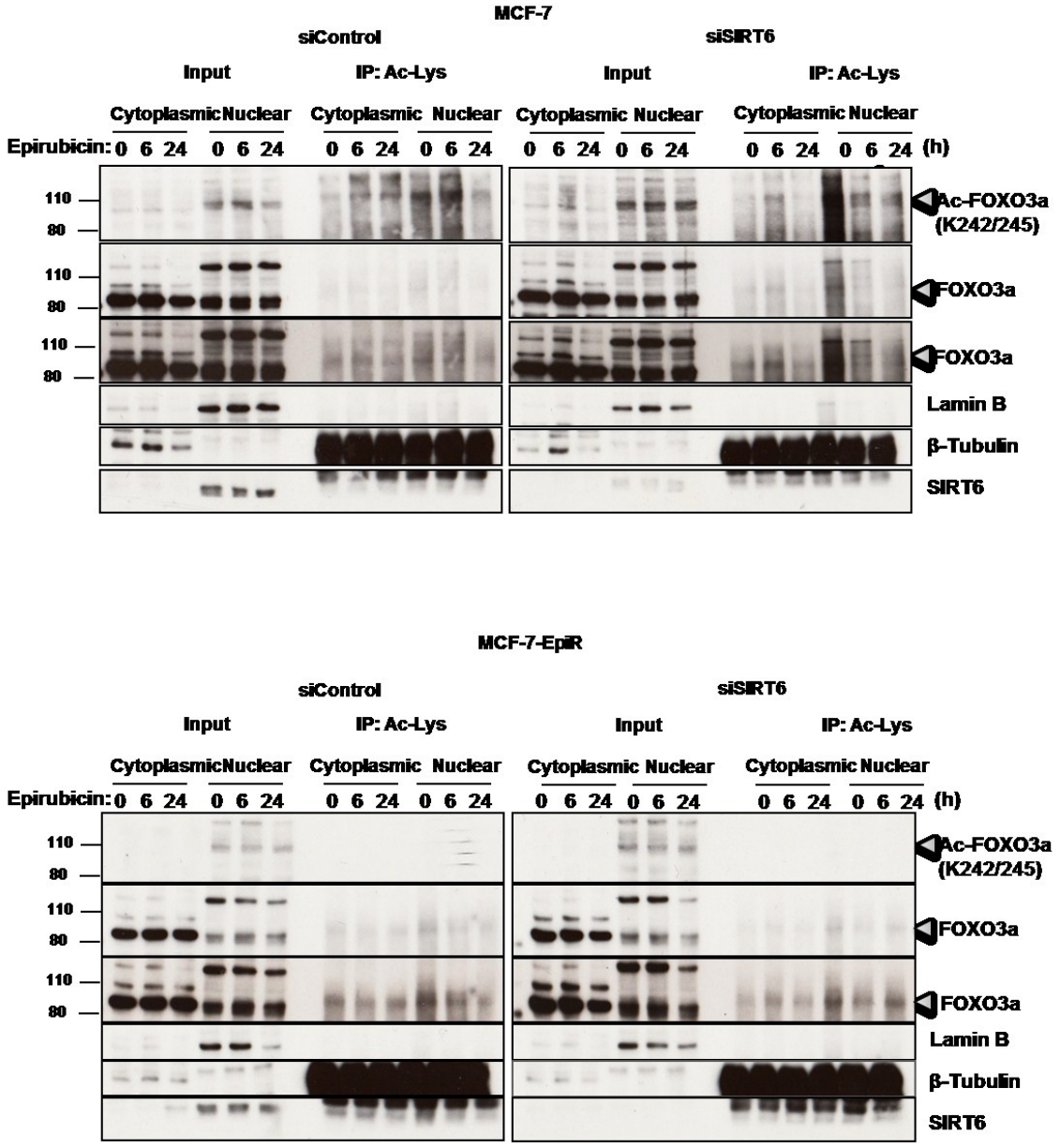


Figure 5.16 SIRT6 depletion in MCF-7 cells (top) and MCF-7-Epi^R cells (bottom) increases both the expression and the acetylation levels of FOXO3a in the nucleus after epirubicin treatment. MCF-7 cells were transfected with siSIRT6 or siControl. Cells were then treated with 1 μM epirubicin for 0, 6 and 24 h and harvested for preparation of subcellular fractionation. Each fraction was then immunoprecipitated using anti-total Ac-Lysine antibody. The acetylation level of FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-Lysine and anti-total FOXO3a. Lamin B and β-Tubulin were used as controls for nuclear and cytoplasmic fractions, respectively. SIRT6 expression was also investigated to assess the transfection efficiency.

In order to also assess the role of SIRT6 in the regulation of FOXO3a acetylation in MCF-7 and MCF-7-Tax^R cells, similar experiments to above were performed. To this end, MCF-7 and MCF-7-Tax^R cells were transfected with siSIRT6 or siControl. Cells were then treated with 10 nM of paclitaxel and harvested at 0, 6 and 24 h. Cells were then subjected to subcellular fractionation and immunoprecipitation using anti-FOXO3a antibodies. As shown in Figure 5.17 (top), the absence of SIRT6 in MCF-7 cells increased the acetylation levels of nuclear FOXO3a and also its expression levels compared to control cells. Notably, the acetylated FOXO3a was predominantly found in the nucleus of MCF-7 cells. Similar results were observed in Figure 5.18 (top), for the reverse immunoprecipitation with anti-total acetyl lysine antibodies. In here, SIRT6 depletion caused an increase in the nuclear FOXO3a acetylation levels as well as in FOXO3a expression in comparison with controls. Paclitaxel treatment did not cause any change in the acetylation and expression levels of FOXO3a in both SIRT6 depleted and control cells.

In the resistant MCF-7-Tax^R cells, there was a significant increase in both the acetylation of nuclear and total FOXO3a after SIRT6 depletion compared to control cells (Figure 5.17, bottom). Notably, acetylated FOXO3a was higher in the nuclear compared to the cytoplasmic fraction in SIRT6 depleted cells, whereas similar levels of acetylated FOXO3a were observed in both compartments for control cells. In agreement, reverse immunoprecipitation results in Figure 5.18 (bottom) showed that SIRT6 deletion in MCF-7-Tax^R cells exhibited higher levels of nuclear acetylated FOXO3a compared to control cells. Interestingly, nuclear FOXO3a acetylation was decreased in SIRT6 depleted cells, whereas the levels were generally unchanged in control cells. FOXO3a expression was detected mainly in the nuclear compartment in the absence of SIRT6 and was found to be induced after paclitaxel treatment. On

the other hand, FOXO3a expression levels in the control cells remained unchanged in both the nuclear and cytoplasmic compartments after paclitaxel treatment. Together these results indicate that SIRT6 affects the acetylation levels of FOXO3a in sensitive as well as resistant MCF-7 cells.

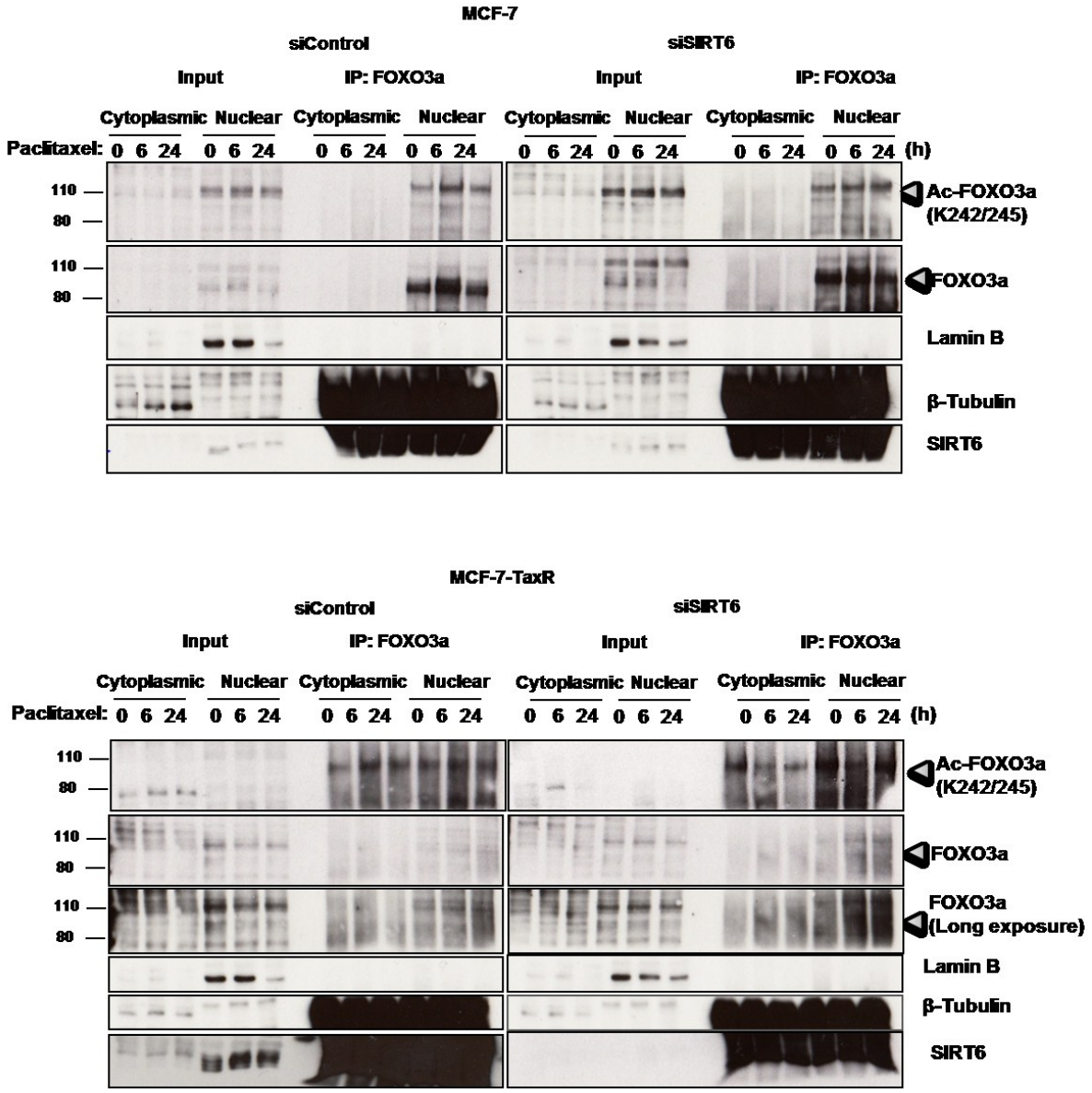


Figure 5.17 SIRT6 depletion in MCF-7 cells (top) and MCF-7-Tax^R cells (bottom) increases both the expression and the acetylation levels of FOXO3a in the nucleus after paclitaxel treatment. MCF-7 cells were transfected with siSIRT6 or siControl. Cells were then treated with 10 nM paclitaxel for 0, 6 and 24 h and harvested for subcellular fractionation. Each fraction was then immunoprecipitated using anti-FOXO3a antibodies. The acetylation levels of FOXO3a were investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-Lysine and anti-total FOXO3a antibodies. Lamin B and β-Tubulin were used as controls for nuclear and cytoplasmic fractions, respectively. SIRT6 expression was also investigated to assess the transfection efficiency.

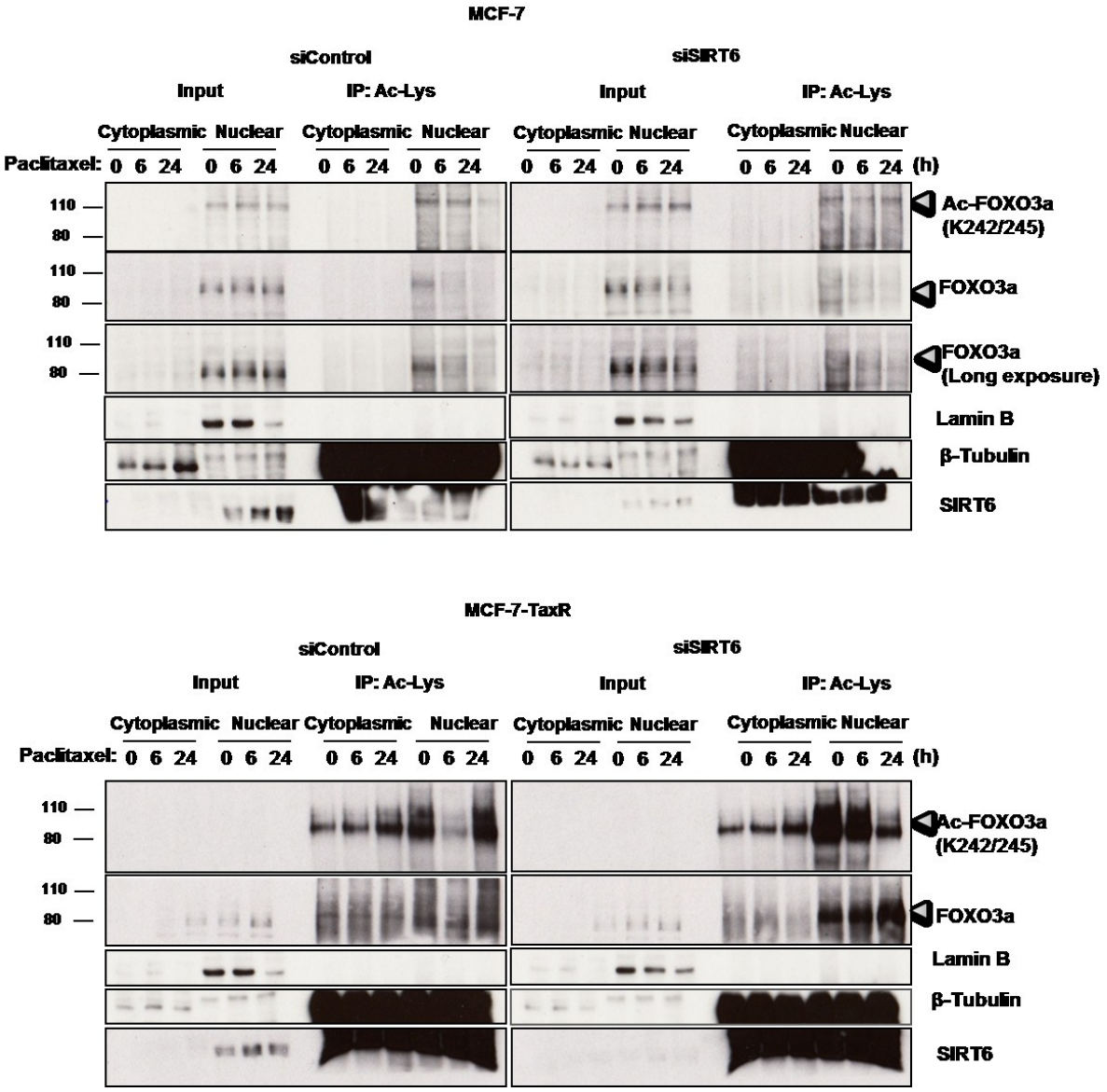


Figure 5.18 SIRT6 depletion in MCF-7 cells (top) and MCF-7-Tax^R cells (bottom) increases both the expression and the acetylation levels of FOXO3a in the nucleus after paclitaxel treatment. MCF-7 cells were transfected with siSIRT6 or siControl. Cells were then treated with 10 nM paclitaxel for 0, 6 and 24 h and harvested for subcellular fractionation. Each fraction was then immunoprecipitated using anti-total Ac-Lysine antibody. The acetylation level of FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-Lysine and anti-total FOXO3a. Lamin B and β-Tubulin were used as a control for nuclear and cytoplasmic fraction, respectively. SIRT6 expression was also investigated to assess the transfection efficiency.

5.4.9 Induction of FOXO3a acetylation in MCF-7 cells enhances epirubicin and paclitaxel sensitivity.

Since my previous results showed that FOXO3a acetylation levels impact on its subcellular localisation, I next questioned whether this nuclear acetylated form of FOXO3a could play a role in epirubicin and paclitaxel sensitivity in MCF-7 cells. To that end, a plasmid that mimics constitutively acetylation of FOXO3a (pFOXO3aAc) was used. In this acetylated mutant plasmid of FOXO3a, Lysine residues 242 (K242) and 245 (K245) were substituted by glutamine (Matsuzaki et al., 2005). This plasmid was previously generated by Prof. Eric Lam. To further investigate the role of FOXO3a acetylation in drug resistance, MCF-7 cells were transfected with pFOXO3aWT, pFOXO3aAc or pControl and clonogenic assays were performed. Cells were treated with either 0-50 nM of epirubicin or 0-5 nM of paclitaxel and left in culture for 2 weeks. In agreement with previous results in Figure 5.2, the overexpression of pFOXO3aWT in MCF-7 cells caused a marginal decrease in cell proliferation compared to controls after epirubicin treatment (depicted in Figure 5.19A). However, there was a significant reduction in cell proliferation in the pFOXO3aAc overexpressing cells compared to control at 20 and 30 nM of epirubicin. These results suggest that FOXO3a acetylation increases the sensitivity of MCF-7 cells in response to epirubicin treatment. The localisation of acetylated FOXO3a after epirubicin treatment was also investigated (Supplemental Figure 7.2).

Similarly, pFOXO3a and pFOXO3aAc overexpression significantly suppressed the proliferation of MCF-7 cells after paclitaxel treatment, in cells treated with 1-2 nM of paclitaxel (Figure 5.19B). There was a marginal difference in the proliferative rate of cells overexpressing pFOXO3aWT compared to those overexpressing pFOXO3aAc in response to treatment with 1 nM of paclitaxel, suggesting that the increased

FOXO3a acetylation inhibits the growth of MCF-7 cells. Notably, both the overexpression of pFOXO3aWT and pFOXO3aAc in MCF-7 cells led to a suppression of cell growth, as there was a mild drop in proliferation in the absence of treatment. Together, these results indicate that the acetylation of FOXO3a improves both epirubicin and paclitaxel responses in MCF-7 cells.

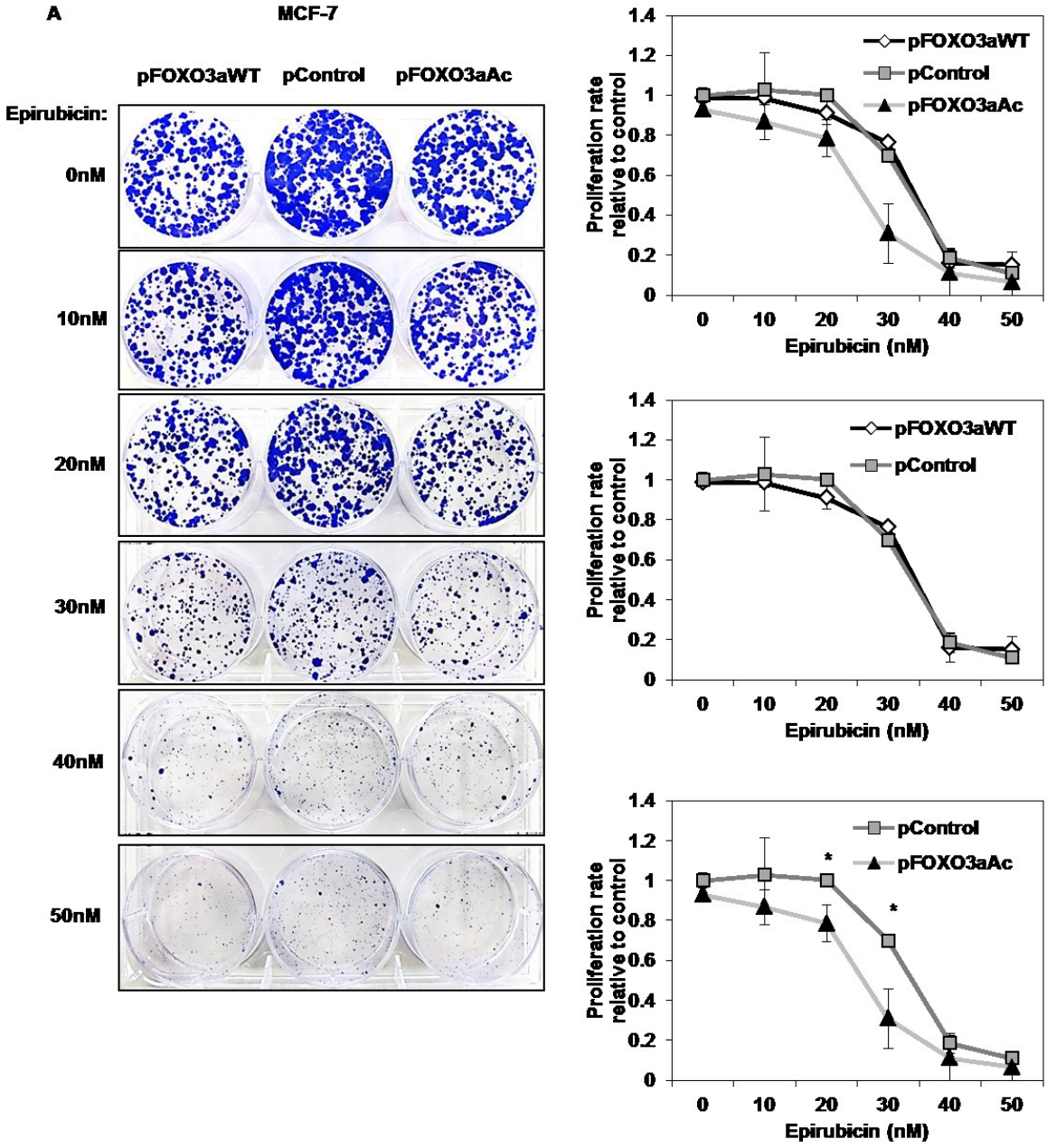


Figure 5.19 An induction in the acetylation of FOXO3a in MCF-7 cells decreases long-term survival after epirubicin treatment. MCF-7 cells were transfected with ectopic pFOXO3aWT, pFOXO3aAc and pControl overexpressing vectors. **A.** Cells were then treated with 0-50 nM epirubicin. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

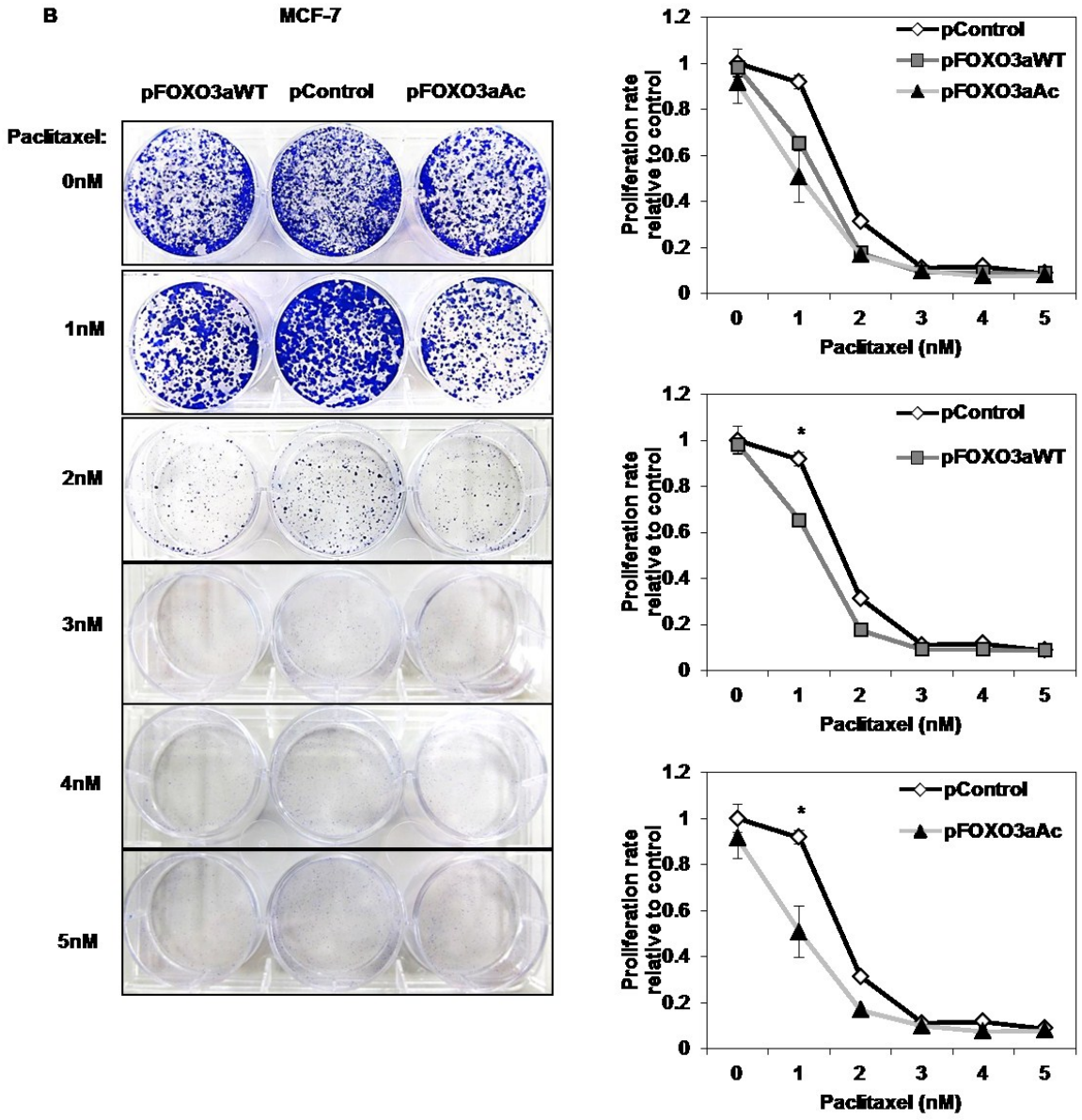


Figure 5.19 (Continued) An induction in the acetylation of FOXO3a in MCF-7 cells increases long-term survival after paclitaxel treatment. MCF-7 cells were transfected with ectopic pFOXO3aWT, pFOXO3aAc and pControl overexpressing vectors. **B.** Cells were then treated with 0-5 nM paclitaxel. Representative data from 3 independent experiments are shown. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01*, ***, *p≤0.005 versus* control).

5.4.10 Foxo1/3/4 null MEFs display higher levels of DNA damage response.

The main mechanism of action of epirubicin is to intercalate into DNA strands and induce DNA DSBs. FOXO3a has been reported to be involved in the regulation of DNA damage response (Tran et al., 2002). As a result, the role of FOXO proteins in DNA damage repair was then studied. Immunofluorescent staining was performed using WT and *Foxo1/3/4* null MEFs. *Foxo* null and WT MEFs were treated with 100 nM epirubicin for 0, 4 and 24 h. Cells were then harvested, fixed and stained with anti-γH2AX antibody to investigate the levels of DNA damage response. According to the foci formation quantification in Figure 5.20, *Foxo1/3/4* null MEFs exhibited a lower amount of foci formation compared to control cells as observed by the staining with γH2AX. This result suggests that the absence of Foxo proteins leads to the improvement of DNA damage repair response.

To further investigate the role of FOXO proteins, especially FOXO3a in DNA damage response, DNA damage repair assay was performed using Hela cells harbouring a (DR-GFP) reporter for HR repair (Hela-DR-GFP). These Hela cells were transfected with 1 μg of pFOXO3aWT or pFOXO3aAc overexpressing vectors or pControl. 24 h after transfection, cells were co-transfected with 1 μg of I-SCEI endonuclease. Cells were then harvested and analysed for GFP-positive cells by FACS. As depicted in Figure 5.21, both pFOXO3aWT and pFOXO3aAc overexpression reduced HR repair efficiency by approximately 50% compared to control cells. Specifically, pFOXO3aAc caused a significant reduction in the HR repair efficiency, whereas a marked decrease was also observed for pFOXO3aWT. This result suggests that acetylation of FOXO3a inhibits the function of FOXO3a in the regulation of DNA damage repair response.

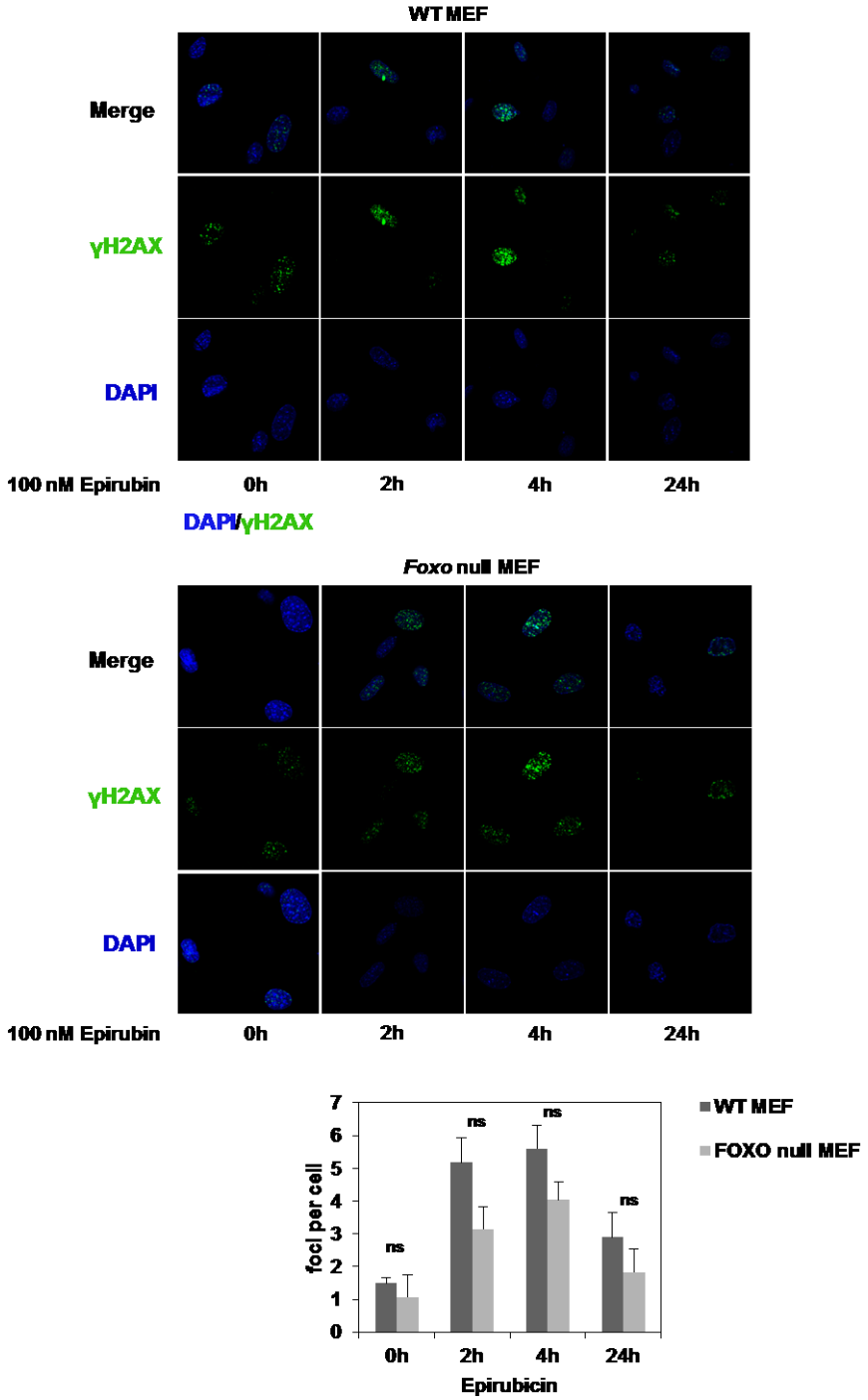


Figure 5.20 *Foxo1/3/4 null* MEFs exhibit higher levels of DNA damage compared with WT MEFs. *Foxo* triple knockout and WT MEFs were treated with 100 nM epirubicin, harvested at 0, 2, 4 and 24 h, fixed, stained with anti-γ -H2AX antibody and counter-stained with DAPI. All of images shown were acquired by confocal microscopy and obtained from at least 5 different fields. **A.** Representative images showing γ-H2AX foci formation induced with 100 nM epirubicin at 0, 2, 4 and 24h. **B.** Quantification of the average number of γ-H2AX foci per cell induced by 100 nM epirubicin treatment with different time points. Bars represent average ± s.d. Statistical significance was determined by Student's t test (* significant, $p \leq 0.05$; n.s. not significant).

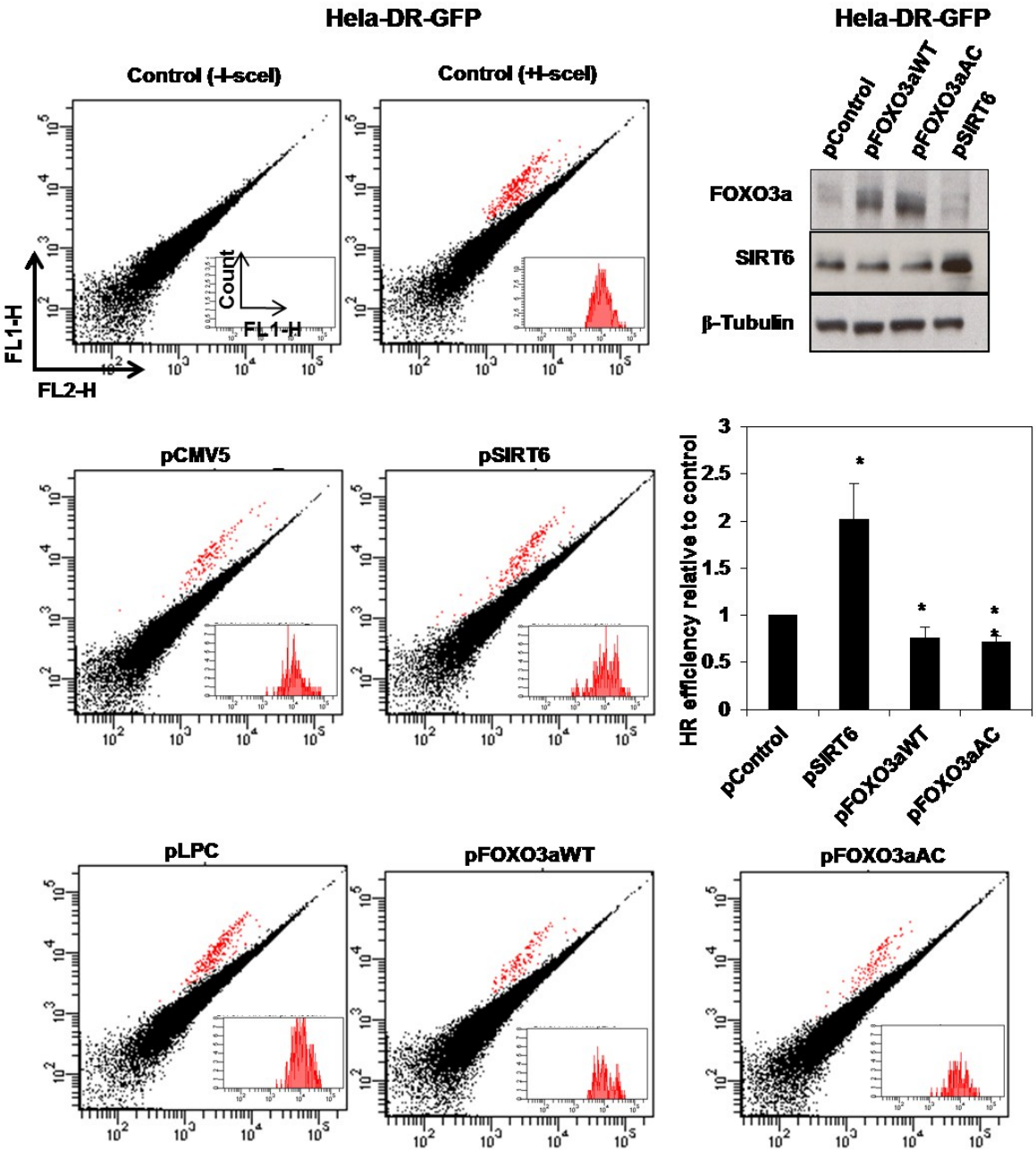


Figure 5.21 pFOXO3aWT and pFOXO3aAc overexpression reduces DNA damage repair via HR. HR repair assay was performed using Hela cells harbouring an integrated with DR-GFP reporters for HR repairs (Hela-DR-GFP). Cells were transfected with pSIRT6, pFOXO3aWT, pFOXO3aAc or pControl overexpressing vectors. After 24 h, cells were co-transfected with 1 µg of I-SCEI and left for 48 h before analysis. The repair efficiency was determined relative to the control (50,000 events were analysed from each experiment). Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.01 versus* control).

As SIRT6 has a role in DNA damage repair and FOXO3a is one of its downstream targets, I next checked whether the overexpression of SIRT6 can improve HR repair efficiency of cells transfected with pFOXO3aWT and pFOXO3aAc. Hela cells harbouring a DR-GFP reporter for HR repair were transfected with 0.5 μg of pFOXO3aWT or pFOXO3aAc together with 0.5 μg of pSIRT6. pCMV5 and pLPC empty vectors were also co-transfected and used as controls. As shown in Figure 5.22, co-transfection of pSIRT6 together with pFOXO3aWT and pSIRT6 with pFOXO3aAc increased the HR repair to levels similar to those of control cells. Specifically, the overexpression of pFOXO3aAc together with pSIRT6 displayed similar levels of HR repair efficiency to control. This efficiency was increased by approximately 15% compared to the overexpression of pFOXO3aAc alone. This result consolidates the notion that the acetylation of FOXO3a leads to the impairment of its function in DNA damage repair response and that this deacetylation is mediated by SIRT6.

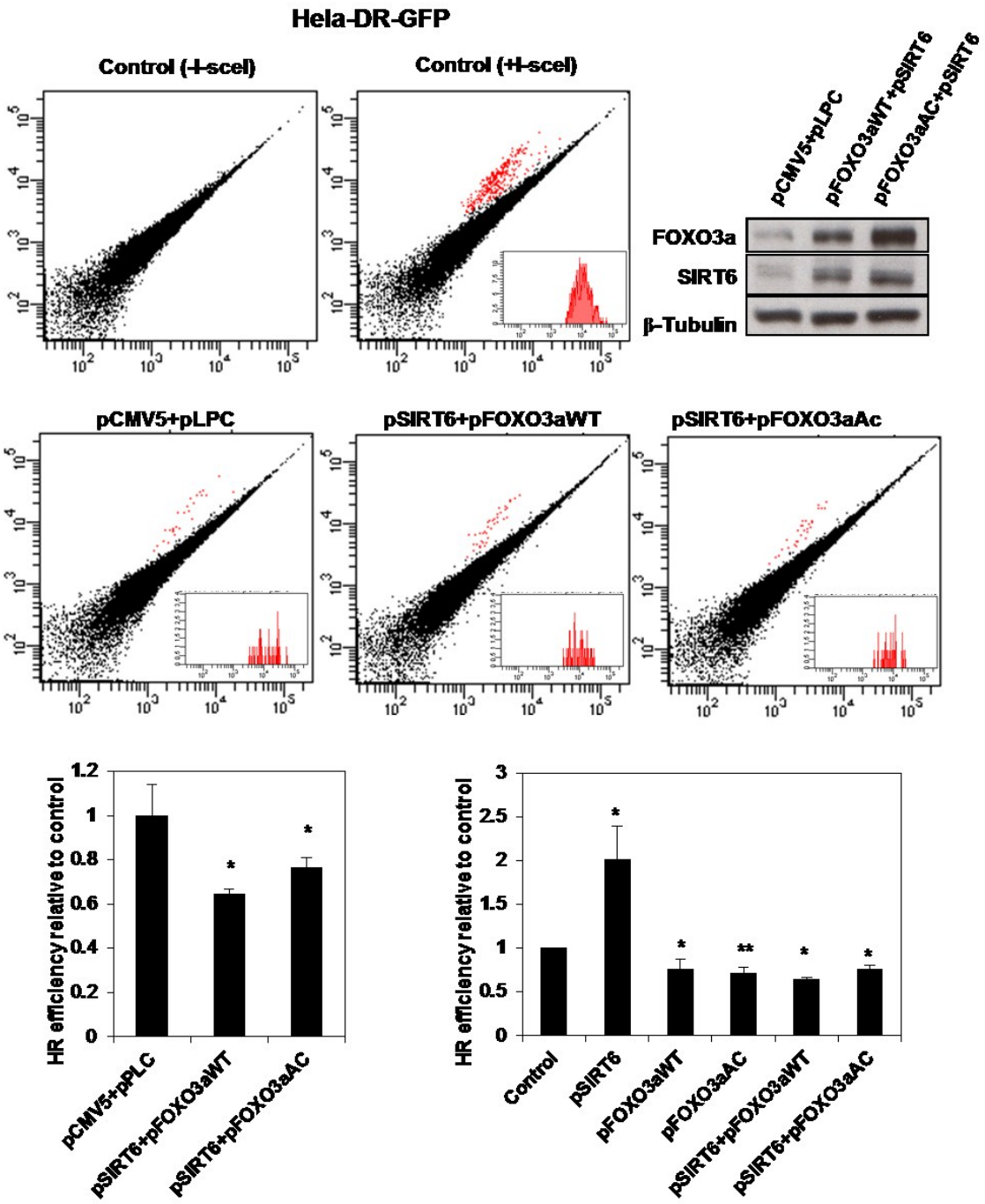


Figure 5.22 SIRT6 overexpression can rescue HR efficiency in acetylated FOXO3a and FOXO3a overexpressing cells. Hela cells harbouring an integrated DR-GFP reporter for HR repair were co-transfected with pSIRT6 together with pFOXO3aWT or pFOXO3aAc overexpressing vector. pCMV5 and pLPC empty vectors were co-transfected and used as controls. The repair efficiency was determined relative to the control (50,000 events were analysed from each experiment). Bars represent s.d. from 3 independent experiments. Statistical significance was determined by Student's t-test (*, *p≤0.05*, **, *p≤0.05*, ***, *p≤0.005 versus* control).

5.5 Discussion

I have previously shown that SIRT6 deacetylates FOXO3a, leading to the suppression of FOXO3a-mediated cell cycle arrest and apoptosis. In this Chapter, I studied the role of FOXO3a acetylation in drug sensitivity and resistance. To confirm that FOXO3a has a potential role in epirubicin and paclitaxel resistance, the effects of FOXO3a in cell survival was investigated. I found that FOXO3a deletion leads to an increase in long-term cell survival in the presence of epirubicin or paclitaxel treatment. The absence of FOXO3a in MCF-7 cells decreased the accumulation of cells in G2/M phase in response to both epirubicin and paclitaxel and promoted cell cycle progression by increasing the number of cells that progress from G1 to S phase. Similar to the results obtained for the cell cycle distribution analysis, SA-β-gal staining showed that FOXO3a depleted cells expressed lower levels of cellular senescence in response to both epirubicin and paclitaxel. This result confirmed an important role for FOXO3a as a mediator of the cytotoxic response to these chemotherapeutic agents (Wilson et al., 2011). Additionally, the expression levels of FOXO3a in MCF-7-Epi^{R} and MCF-7-Tax^{R} cells were considerably lower than the levels in MCF-7 cells, further suggesting that the presence of FOXO3a is crucial for mediating the chemotherapeutic response in MCF-7 cells. In this way, the absence or downregulation of FOXO3a in MCF-7 cells can lead to the development of drug resistance and promote cancer progression. However, Prof Iam's group have reported that both nuclear FOXO3a and nuclear SIRT6 correlate with poorer survival in breast cancer patients (Chen et al., 2010a). Therefore, I hypothesised that this nuclear FOXO3a might be deacetylated in the nucleus and hence unable to have a role in modulating epirubicin and paclitaxel drug sensitivity. This could be a mechanism by which cancer cells develop resistance to chemotherapeutic drugs in

the presence of nuclear FOXO3a. Indeed, the acetylation levels of FOXO3a were observed to be reduced in MCF-7-Tax^{R} cells in comparison with MCF-7 sensitive cells. Yet, the acetylation levels of FOXO3a were noticeably higher in MCF-7 sensitive cells compared to MCF-7-Epi^{R} cells. Acetylation of FOXO3a is regulated by the opposing activity of acetyl-transferases, such as p300/CBP and histone deacetylase (including class III, sirtuins) (Daitoku et al., 2011). The overexpression of SIRT1 and SIRT2 has been demonstrated to be involved in the development of drug resistance and cancer progression (Chen et al., 2013; Xie et al., 2011). It has also been reported that SIRT1 overexpression leads to an increase in tumour cell growth and cell survival in response to stress and drug treatments (Chu et al., 2005; Wang and Chen, 2013). Additionally, the upregulation of SIRT1 has been shown to be involved in the initiation of many cancers, including lymphomas, leukaemia and soft-tissue sarcomas, prostate cancer, lung and colon carcinomas (Fraga et al., 2007a; Fraga et al., 2007b; Lim, 2007). Similarly to SIRT1, SIRT2 overexpression also results in the induction of drug resistance, especially to agents that target microtubule, such as noconazole (Inoue et al., 2014; Matsushita et al., 2005). FOXO3a has been reported to be a substrate of both SIRT1 and SIRT2 by their deacetylase activity. The effects of FOXO3a deacetylation by SIRT1 and SIRT2 have been intensively studied. Most of the studies show that SIRT1 deacetylates FOXO3a resulting in a shift on the function of FOXO3a from the regulation of cell death towards the regulation of cell cycle arrest and oxidative stress (Bosch-Presegue and Vaquero, 2011). In agreement with this previous report, I found that the overexpression of SIRT1 and SIRT2 in MCF-7 led to the development of epirubicin resistance and a downregulation of FOXO3a expression after the treatment. These results suggest that deacetylation of FOXO3a by sirtuins could be

a potential cause of drug resistance in breast cancers. Interestingly, amongst the sirtuins family members that regulate nuclear FOXO3a, SIRT6 has been shown to be the most important regulator of the acetylation of FOXO3a, as the depletion of SIRT6 in MCF-7 resulted in a noticeable induction of the acetylation levels of FOXO3a in the absence of treatment. As a result, I investigated the role of SIRT6 in the regulation of FOXO3a acetylation and in the development of epirubicin and paclitaxel treatment. Previous results from our lab showed that the use of the inhibitor of sirtuins, sirtinol, in p53 deficient breast cancer cell lines results in the activation and stabilisation of FOXO3a (Peck et al., 2010). This in turn, re-sensitised the Her2-overexpressing cells to lapatinib-mediated cell death. This activation and stabilisation was independent of Akt-phosphorylation. Furthermore, a recent report from Wang F. et. al, 2012 (Wang et al., 2012) shows that FOXO3a deacetylation by SIRT1 or SIRT2 promotes Skp2-mediated FOXO3a polyubiquitination and subsequently proteasomal degradation. These results demonstrate that an induction of FOXO3a acetylation by the use of inhibitors of sirtuins could represent a potential approach to overcome drug resistance in breast cancer cells. Specifically, FOXO3a acetylation leads to an increase in the stability and activity of FOXO3a in response to the cytotoxic effects of chemotherapeutic agents. In this Chapter, I found that acetylated FOXO3a is predominantly localised in the nuclear compartment in MCF-7 cells, whereas acetylated FOXO3a is localised in both nuclear and cytoplasmic compartments in MCF-7-Epi^{R} and MCF-7-Tax^{R} cells.

SIRT6 depletion in MCF-7 cells increased the acetylation and expression of nuclear FOXO3a in the absence of treatment. Epirubicin and paclitaxel treatments caused a mild increase in the acetylation levels of FOXO3a in SIRT6 depleted cells, but not in control cells. Similarly, SIRT6-silenced MCF-7-Epi^{R} and MCF-7-Tax^{R} cells resulted in

an increase in the accumulation of FOXO3a expression and acetylation levels in the nucleus compared to control cells in the absence of treatment. Moreover, in the absence of SIRT6, epirubicin treatment led to a marginal induction of FOXO3a expression in MCF-7-Epi^{R} cells, whereas a significant increase in FOXO3a expression was observed in MCF-7-Tax^{R} cells after paclitaxel treatment. Additionally, the induction of FOXO3a acetylation led to an improvement of epirubicin and paclitaxel sensitivity in MCF-7 cells. I also found that the overexpression of the constitutively acetylated FOXO3a mutant (pFOXO3aAc) resulted in a significant decrease of cell growth in MCF-7 cells after both epirubicin and paclitaxel treatments. These results further confirm the involvement of FOXO3a acetylation in drug sensitivity.

Since the induction of DNA damage is the common mechanism of action of chemotherapeutic agents, including epirubicin, FOXO3a could improve the efficiency of these therapeutic drugs by preventing DNA damage repair via HR. In this study, I found that the overexpression of pFOXO3aWT and pFOXO3aAc significant reduces HR repair efficiency. Notably, pFOXO3aAc expression is more efficient to reduce HR repair compared to pFOXO3aWT. However, the repression of DSB repair by FOXO3a expression can be rescued by the overexpression of SIRT6, as the HR efficiency was improved after co-expressing pSIRT6 with either pFOXO3aWT or pFOXO3aAc. This set of results further confirms the importance of FOXO3a acetylation in mediating drug sensitivity by preventing DNA damage repair in response to epirubicin and paclitaxel.

Together, all these results indicate that the inhibition of sirtuins, especially SIRT6 in MCF-7 cells could represent an improvement to the chemotherapeutic treatment efficacy by activating the acetylation of FOXO3a.

CHAPTER 6

FINAL DISCUSSION

Discussion

Drug resistance is one of the most challenging problems that need to be addressed to improve treatment efficiency and to overcome cancer. For breast cancer, more than half of the patients are classified as hormone-positive (ERα+). For this type of cancer, endocrine treatment is the primary option used. However, patients who receive endocrine treatment eventually relapse due to the development of either intrinsic or acquired drug resistance. When patients become resistant to hormone therapy, cytotoxic agents, such as epirubicin and paclitaxel, are employed (Piccart, 2003; Wu et al., 2011). Moreover, cytotoxic treatment is sometimes the only option for treating patients with hormone negative breast cancer as well as metastatic or advanced cancer. It has been reported that the treatment with anthracyclins (*eg*. epirubicin) and taxanes (*eg*. Paclitaxel) for the metastatic or advanced breast cancer generally provides 30-70% of response rate (Cortes and Baselga, 2007; Moreno-Aspitia and Perez, 2009). This response, however, is not often durable due to the development of drug resistance to one or more agents. Therefore, the study of the mechanisms underlying the development of epirubicin and paclitaxel resistance is crucial for the improvement of novel chemotherapeutic treatment of breast cancer as well as other cancers.

The deregulation of transcription factors activity involved in the cytotoxic and cytostatic response has been proposed to be one of the crucial factors in the acquired resistance to chemotherapeutic drugs (Olmos et al., 2011a). Additionally, epigenetic and genetic changes have been reported to be involved in cancer initiation, progression, and subsequently in the development of drug resistance by altering global gene expression. The hypermethylation of gene promoters and also

histone deacetylation have been revealed to be a crucial event during tumourigenesis and cancer progression. For instance, the silencing of tumour suppressor genes, such as *p16*^{INK4A} and *p15*^{INK4B} is mediated by histone deacetylation and/or DNA methylation (Krug et al., 2002; Olmos et al., 2011a). Apart from epigenetic changes, the regulation of transcription factors, such as p53, E2F1 and p73 via deacetylation, independent of histone modifications, has been also documented to be involved in cancer progression and drug resistance (Brooks and Gu, 2003; Brunet et al., 2004; Glozak et al., 2005; Martinez-Balbas et al., 2000). Acetylation of proteins is regulated by counter-activities of acetyl-transferase and histone deacetylase enzymes. The imbalance of the activities of these enzymes in the regulation of transcription factors, like tumour suppressors, could be a possible mechanism behind cancer progression and drug resistance. Therefore, the aim of this thesis was to investigate the role of histone deacetylase, especially class III, (sirtuins) in the development of drug resistance in breast cancer.

In chapter 3, I identified a novel role for SIRT6 in epirubicin and paclitaxel resistance. I found that amongst the seven members of the sirtuin protein family, the overexpression of SIRT6 was consistently observed in both epirubicin and paclitaxel resistant MCF-7 cells. In resistant MCF-7 cells, SIRT6 depletion leads to a higher response to epirubicin and paclitaxel, whereas the ectopic overexpression of SIRT6 in sensitive cells results in a lower response to the both treatments. Similar to MCF-7 cells, mouse embryonic fibroblast lacking Sirt6 expression (*Sirt6*$^{-/-}$ MEF) displays a higher degree of sensitivity to epirubicin and paclitaxel, which could further confirm the involvement of SIRT6 in drug resistance. I also found that the depletion of SIRT6 in MCF-7 cells is associated with an increase in the senescent phenotypes in

response to lower doses of epirubicin and paclitaxel treatment in the long-term survival assays shown in chapter 4.

As the induction of DNA damage is the main mechanism of action of epirubicin, the role of SIRT6 in DNA damage repair response was investigated. In chapter 3, I found that the absence of *Sirt6* in MEFs results in a reduction of DNA damage repair response after epirubicin treatment. Consistently, the analysis of DNA damage in a single cell by the alkaline comet assay also revealed that *Sirt6*$^{-/-}$MEFs displayed a higher degree of DNA damage compared to WT MEFs after epirubicin treatment. Together with the use of Hela cells, harbouring DR-GFP reporters for both HR and NHEJ, I also demonstrated that SIRT6 depletion suppressed DSB repair efficiency via both HR and NHEJ approximately 2 times less than control. All these findings were in agreement with previous reports from Mao *et al*, 2011 showing that SIRT6 overexpression improved HR and NHEJ under oxidative stress via the mono-ADP-ribosylation of PRAP1 (Mao et al., 2011) and also from Kaidi et al, 2010, demonstrating that SIRT6 promote DNA end resection, a crucial step of DSB repair by HR, via CtIP deacetylation (Kaidi et al., 2010). Thus, the enhancement of DNA damage repair by SIRT6 overexpression could be one of the possible mechanisms of epirubicin resistance in breast cancer cells.

Disruption of mitosis through interfering with microtubule depolymerisation is one of the main mechanisms of action of paclitaxel-induced cytotoxicity in cancer cells. It has been reported that the cytotoxic effects of paclitaxel is to induce a transient block at G2/M and subsequently an induction of cell death (Wang et al., 2000). Therefore, the regulation of the G2/M cell cycle check point was investigated in order to study paclitaxel resistance. In chapter 3, I found that SIRT6 overexpression in MCF-7 cells leads to a decrease in cells accumulated in G2/M phase after paclitaxel treatment.

Additionally, SIRT6 depletion in MCF-7-Tax^{R} cells results in an increase in cell death (sub-G1 cells) after paclitaxel treatment. Thus, SIRT6 plays a role in the development of paclitaxel resistance by deregulating of G2/M arrest induced by paclitaxel.

To understand the role of SIRT6 in epirubicin and paclitaxel resistance, SIRT6 downstream targets were identified. FOXO3a and p53 have been reported to be mediators for the cytotoxic response of cancer cells to chemotherapeutic agents. Additionally, the deacetylation of FOXO3a and p53 has been shown to be regulated by sirtuins, including SIRT1, 2, 3 and 7 (Olmos et al., 2011a). These studies suggest the functional redundancy of sirtuins. Therefore, the acetylation status of p53 and FOXO3a was examined to establish whether they are SIRT6 dependent. As expected, I found that SIRT6 depletion enhances the acetylation levels of p53 and FOXO3a in MCF-7 cells as well as in MEFs in response to both epirubicin and paclitaxel, suggesting that p53 and FOXO3a are potentially regulated by SIRT6.

The function of p53 and FOXO3a are tightly controlled by their post-translational modification status. It is well established that p53 acetylation represents an activated form of p53 in response to environmental stress, oncogenic activation as well as geno- and cytotoxic response to chemotherapeutic drugs (Olmos et al., 2011a; Vousden and Lane, 2007). This activation leads to an enhancement of p53 binding to its downstream target promoters and leads to the induction of p53 transactivation potential in the regulation of cell cycle arrest and apoptosis. Additionally, p53 acetylation is believed to enhance p53 stability by preventing ubiquitination and MDM2 mediated proteasome degradation (Li et al., 2002). Thus, the induction of p53 acetylation has been proposed as a new approach for targeted cancer therapy by using small inhibitors. A previous report from our group suggested that the use of

sirtuin inhibitors, sirtinol and salermide, can result in an increase in p53 acetylation, leading to a repression of breast cancer cell growth (Peck et al., 2010). We found that an induction of p53 acetylation is achieved from the inhibition of SIRT1 and SIRT2. In agreement, the report of Lain *et al*, 2008, demonstrated that the use of tenovins, a SIRT1 and SIRT2 inhibitor, can lead to an enhancement of the transcriptional activity of p53 and subsequently results in the repression of cell proliferation of different tumour cell lines (Lain et al., 2008a). Similarly, I found that SIRT6 overexpression can result in a downregulation of p53 and its acetylation in response to epirubicin and paclitaxel treatments. This suggests that SIRT6 contributes to drug resistance in breast cancer by partly inhibiting the acetylation of p53 and its expression induced by chemotherapeutic agents.

FOXO3a has also been reported to be a modulator for paclitaxel and epirubicin response in cancer cells. In Chapter 3, I found that SIRT6 overexpression leads to a downregulation of FOXO3a in response to both epirubicin and paclitaxel treatments. Furthermore, *Sirt6*$^{-/-}$ MEFs displays a higher degree of FOXO3a acetylation and its nuclear expression in response to epirubicin in Chapter 4. Similar to p53 acetylation, the treatment of MCF-7 cells with Sirtinol and Salermide, increases the acetylation levels of FOXO3a, resulting in an induction of FOXO3a nuclear localisation. In agreement with this, I also found that epirubicin treatment of *Sirt6*$^{-/-}$ MEFs leads to an increase in the acetylation status of FOXO3a and its nuclear expression. As a consequence, there was an induction of some of the downstream targets of FOXO3a, such as *p21*^{Cip1} and *Bim*, whereas there is no the induction of *p27*^{Kip1}, *GADD45* and *MnSOD2* mRNA levels. A previous study by our group showed that there is a FOXO3a-independent activation of $p27^{Kip1}$ to control G1 arrest (Schmidt et al., 2002b). Our group also found that FOXO3a inhibits cell cycle progression at

G1/S transition by the downregulation of Cyclin D1 and D2. In agreement with this report, the downregulation of *Cyclin D1* mRNA levels was also observed in *Sirt6*$^{-/-}$ MEFs. This result suggests that SIRT6 represses the function of FOXO3a to inhibit of G1/S cell cycle progression at least partially by downregulating Cyclin D1expression.

The role of FOXO3a in the induction of G2/M arrest still needs further investigation. However, the expression of FOXM1, a direct transcriptional target of FOXO3a (Francis et al., 2009; Gomes et al., 2013), was upregulated after SIRT6 overexpression in MCF-7 cells. FOXM1 is a transcription factor thought to be crucial for G2/M transition by regulating the transcription of genes that stimulate G2/M progression as well as chromosome stability and segregation during mitosis. It is also involved in the regulation of genes involved in the mitotic spindle assembly checkpoint and cytokinesis (Laoukili et al., 2005). FOXM1 is also reported to be an antagonist of FOXO3a (Gomes et al., 2013) and has a crucial role in drug resistance by enhancing of DNA damage repair response induced by chemotherapeutic agents (Khongkow et al., 2013b; Monteiro et al., 2013). The inhibition of FOXM1 in breast cancer cells leads to an increase in G2/M cell cycle arrest and represses cancer cell proliferation (Kwok et al., 2010; McGovern et al., 2009). Thus, the upregulation of FOXM1 after SIRT6 overexpression in MCF-7 cells could be a consequence of the downregulation of FOXO3a activity via deacetylation, suggesting a crucial role of FOXO3a in G2/M cell cycle arrest. Moreover, this could explain the results obtained in Chapter 3 showing that the overexpression of SIRT6 in MCF-7 cells leads to a decrease in paclitaxel induced G2/M cycle arrest and cell death. In summary, I have demonstrated the involvement of SIRT6 in paclitaxel resistance via the activation of FOXM1, as a consequence of FOXO3a downregulation. However, the role of

FOXM1 in the regulation of paclitaxel resistance still needs further investigation. A study from Carr *et al*, 2010 reported that the overexpression of FOXM1 confers paclitaxel and Herceptin resistance in breast cancer (Carr et al., 2010). However, it would be interesting to identify some of the downstream targets of FOXM1 in the regulation of proteins involved in mitosis, such as members of the kinesin protein family. Moreover, the FOXO-FOXM1 axis has a crucial role in drug sensitivity and therefore, could be a potential candidate signalling axis to be targeted in order to improve cancer treatment efficiency (Wilson et al., 2011).

Together, these results further support that acetylated FOXO3a preferentially activates proapoptotic genes over the genes involved in cell cycle arrest, oxidative stress response and DNA damage repair. Particularly, the deacetylation of FOXO3a by sirtuins causes a shift of FOXO3a functions from the activation of apoptosis pathway towards the regulation of oxidative stress and DNA damage response (Brunet et al., 2004).

The regulation of FOXO3a by acetylation and its role drug sensitivity was therefore further investigated in Chapter 5. Our previous report from a panel of advanced breast cancer patients (118 cases) revealed that higher nuclear FOXO3a expression corresponds to poorer prognosis (Chen et al., 2010b). In this report we showed that the expression of FOXO3a upregulates the activity of Akt and PI3K pathway through the induction of *PIK3CA* (Hui et al., 2008). Moreover, we observed that there is nuclear accumulation of FOXO3a in sensitive and resistant MCF-7 cells. Therefore, the normal tumour suppressive functions of FOXO3a must be deregulated in resistant cells. One possible mechanism that might help to explain this is via the post-translational modifications of FOXO3a in these cells. It has been reported that FOXO3a phosphorylation via JNK pathway enhances FOXO3a nuclear localisation

(Sunters et al., 2006). This FOXO3a accumulation can in turn stimulate SIRT1 expression in a feedback mechanism (Brunet et al., 2004). As mentioned earlier, they found that SIRT1 is a FOXO3a modulator through its deacetylase activity, which can shift the function of FOXO3a from the induction of apoptosis towards the promotion of cell survival.

Additionally in the same set of samples reported in Chen *et al*, 2010, I found that higher nuclear SIRT6 expression is significantly correlated to poorer survival in Chapter 3. As a consequence, deacetylation of FOXO3a by sirtuins has been proposed to be another mechanism that accounts for resistant phenotypes in this set of breast cancer patient samples. Interestingly, I found that the acetylation levels of nuclear FOXO3a were significantly higher in MCF-7 cells compared to both the drug resistant MCF-7-Epi^{R} and MCF-7-Tax^{R} cells in Chapter 5. An induction of nuclear FOXO3a acetylation was increased in sensitivity cells after epirubicin and paclitaxel, whereas there were no such changes in acetylated nuclear FOXO3a levels in resistant cells. I also found that SIRT6 is the main modulator of FOXO3a acetylation compared to SIRT1 and SIRT2, as SIRT6 depletion in MCF-7 sensitive and MCF-7 resistant cells leads to a noticeable induction of nuclear FOXO3a acetylation with and without epirubicin or paclitaxel treatment. Together with the results obtained in Chapter 3, I have showed that SIRT6 depletion in MCF-7-Epi^{R} and MCF-7-Tax^{R} cells leads to a decrease in cell proliferation in response to epirubicin and paclitaxel treatments. These results led me to hypothesise that an induction of nuclear FOXO3a acetylation might increase the sensitivity of both MCF-7-Epi^{R} and MCF-7-Tax^{R} cells in response to epirubicin and paclitaxel treatments. As expected, I found that an increase in FOXO3a acetylation in MCF-7 cells by the overexpression of a constitutively active acetylated FOXO3a mutant leads to a decrease in long-term cell

survival in response to epirubicin and paclitaxel treatment in Chapter 5. This further supports my hypothesis that FOXO3a acetylation plays a key part in drug sensitivity of cancer cells. In agreement with my study, the role of FOXO3a acetylation was previously investigated in cisplatin resistance (Shiota et al., 2010). Similarly to my findings, this study showed that FOXO3a depletion leads to an increase in cell proliferation and cisplatin resistance. The acetylation status of FOXO3a was decreased in response to cisplatin treatment and was lower in cisplatin-resistant cells. This study further supports an essential role of FOXO3a acetylation in chemotherapeutic sensitivity.

Unlike FOXO3a, FOXO1 acetylation has been shown in many studies to be important in cancer treatment. Yang, Zhao et al demonstrated that the use of capsaicin can lead to an increase in FOXO1 acetylation and this subsequently leads to FOXO1-mediated induction of Bim in human lung cancer (Yang et al., 2009b). Recently, it has been also reported that targeting SIRT1 in pancreatic tumour cells can also result in an induction of FOXO1 acetylation, thus promoting cancer cell death via the activation of Bim (Pramanik et al., 2014). Nevertheless, the acetylation of FOXO1 has been reported to enhance the phosphorylation of FOXO1 by Akt resulting in its cytoplasmic sequestration. On the other hand, acetylation enhances FOXO stability, as it has been demonstrated that SIRT1 and SIRT2 inhibition prevents FOXO3a ubiquitination, and Skp2-mediated proteasome degradation (Wang et al., 2012). Together, this study reinforces the possible mechanism by which higher nuclear levels of FOXO3a correlate with low response of breast cancer patients to epirubicin and paclitaxel. FOXO3a acetylation might be required for triggering Akt-phosphorylation and subsequently cytoplasmic sequestration. Therefore, nuclear SIRT6 expression leads to a decrease in FOXO3a acetylation,

resulting in the retention of FOXO3a in the nuclear compartment. However, the functions of this nuclear FOXO3a are impaired, as I found that acetylation is crucial for FOXO3a activation to induce cell cycle arrest and apoptosis in response to epirubicin and paclitaxel treatments. Acetylation also enhances FOXO3a stability by preventing proteasome degradation. Therefore, this study demonstrated an important role for the post-translational modifications of FOXO3a, which will fine tune its functions in response to different stimuli to either act as a tumour suppressor or as a regulator of oxidative resistance and survival.

Acetylation levels of nuclear FOXO3a should be such that the classical FOXO3a tumour suppressive functions are activated but should not exceed such levels that will contribute to its phosphorylation by Akt in cancer cells in response to chemotherapy.

In conclusion, my study is the first report to identify FOXO3a as a downstream target of SIRT6 via its deacetylase activity. This deacetylation leads to a downregulation of the anti-proliferative and apoptotic FOXO3a-mediated responses (Figure 6.1). My study could open up the application of FOXO3a acetylation in the improvement of chemotherapeutic response and in the prevention of drug resistance in breast cancer. Additionally, this study also provides evidence supporting that SIRT6 could be used as a predictive biomarker in order to screen for the patients that are likely to become resistant to epirubicin and paclitaxel treatments

HDAC inhibitors, targeting class I, II and IV HDACs, have been reported to be in phase I and II clinical trials, such as Romidepsin, MGCD0130, and Panobiostat for the treatment of cancer (Kim and Bae, 2011). Vorinostat, called as SAHA, has already been approved by FDA for the treatment of elapsed and refractory

cutaneous T-cell lymphoma (Lemoine and Younes, 2010). Small molecules have been also developed to target Sirtuins, and they include EX527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide)(Kabra et al., 2009; Napper et al., 2005), sirtinol (2-[(2-hydroxynaphthalen-1-ylmethylene)amino]-N-(1-phenethyl)benzamide) (Grozinger et al., 2001; Ota et al., 2006), and salermide (Lara et al., 2008; Olmos et al., 2011b) For example, EX527 is a SIRT1 specific inhibitor, while salermide has specificity for SIRT1 and -2 (Peck et al., 2010). More specific molecules have also been designed to inhibit SIRT2 (Di Fruscia et al., 2014). Sirtinol is a pan-SIRT inhibitor, but no specific SIRT6 inhibitor has yet been identified. However, to date, there are no specific SIRT-inhibitors in clinical trials for treating cancer.

My study supports that a small molecule inhibitor could be generated in order to target specifically SIRT6 in breast cancer. It could be used as a single agent or in a combination with epirubicin and paclitaxel for improving the cancer treatment efficacy.

Furthermore, a proteomic screening should be performed in order to identify downstream targets of SIRT6. By studying these putative targets of SIRT6, we could have a better understanding of how deacetylation of key proteins drives cancer initiation, progression and even drug resistance. This would prove useful for unravelling the SIRT6-mediated acetylation signature in cancer.

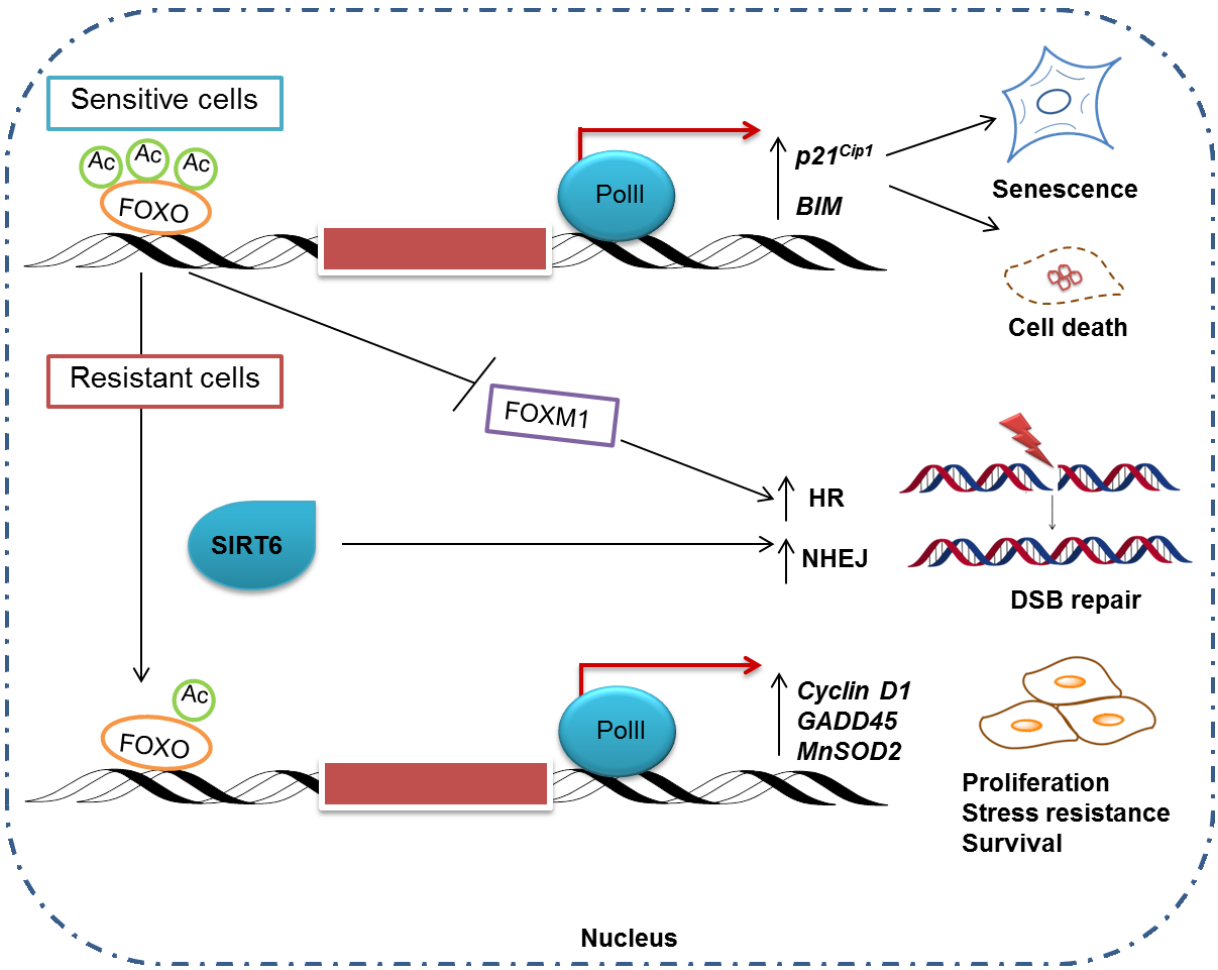
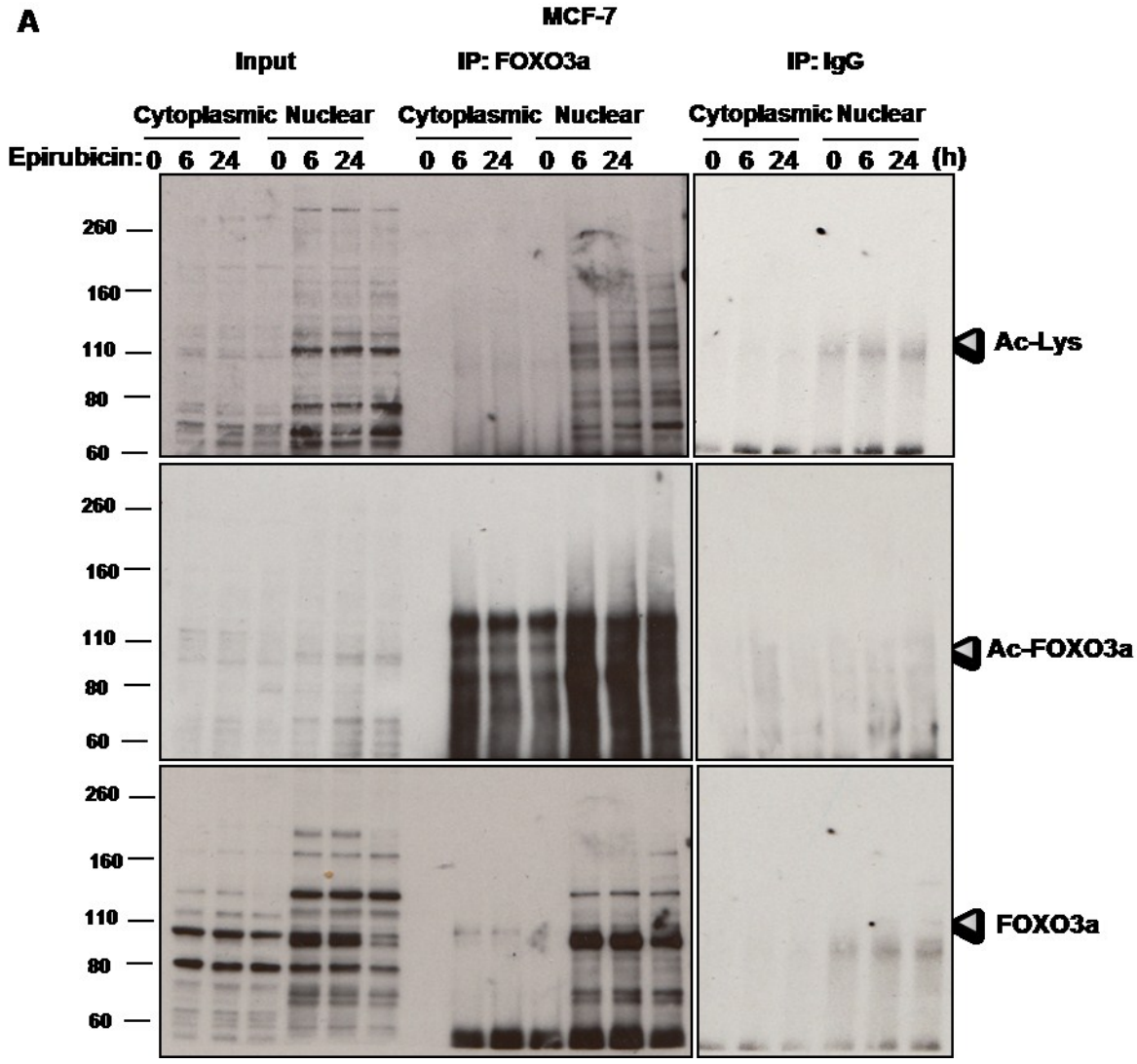


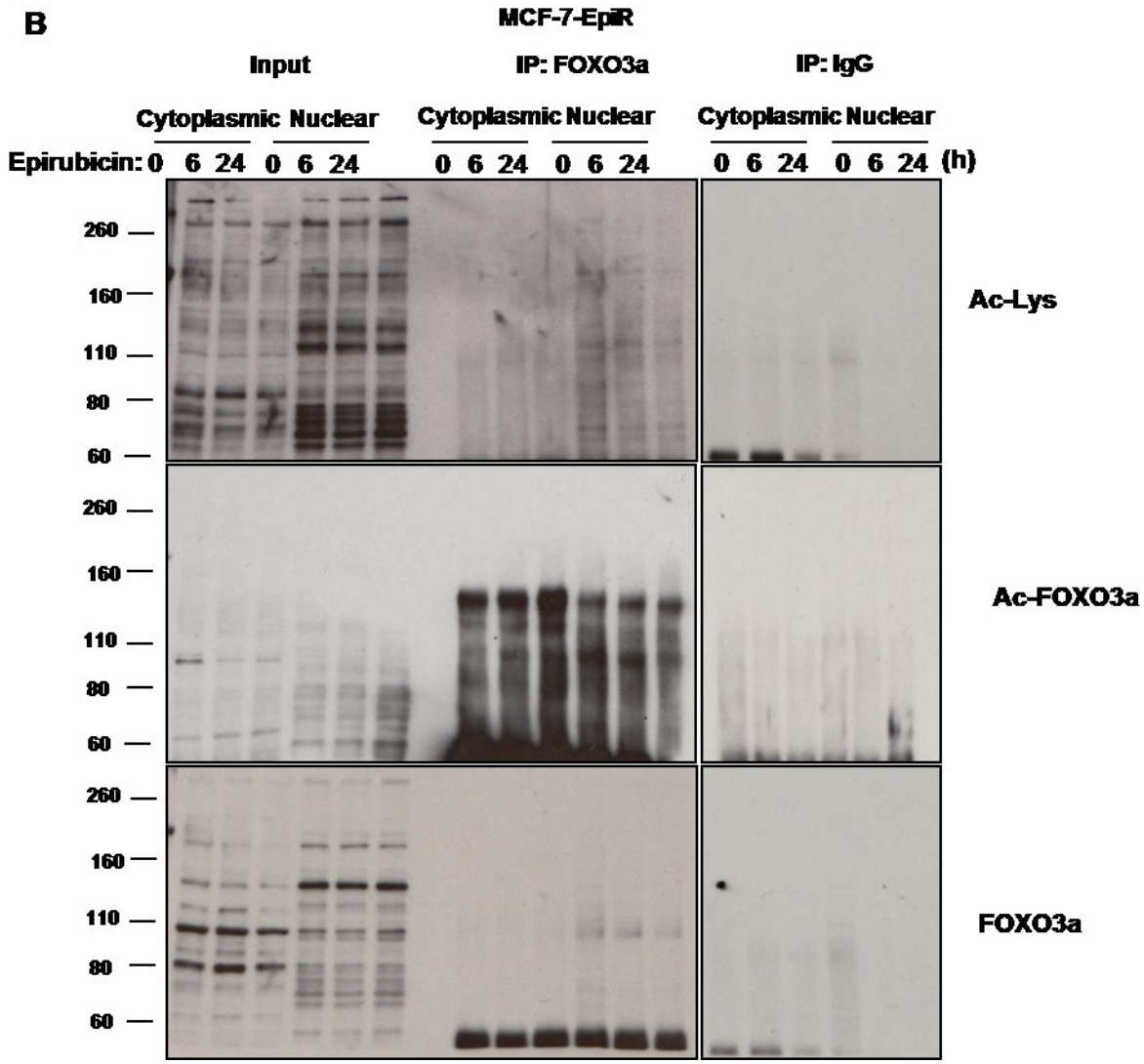
Figure 6.1 Schematic representation of the role for SIRT6 in the development of drug resistance. In sensitive cells, the expression of SIRT6 is decreased, leading to higher levels of FOXO3a acetylation and its mediated induction of cell cycle arrest and cell death. Conversely, SIRT6 is overexpressed in resistant cells leading to an enhancement in DNA damage repair and in the induction of proliferative and stress resistant genes.

CHAPTER 7

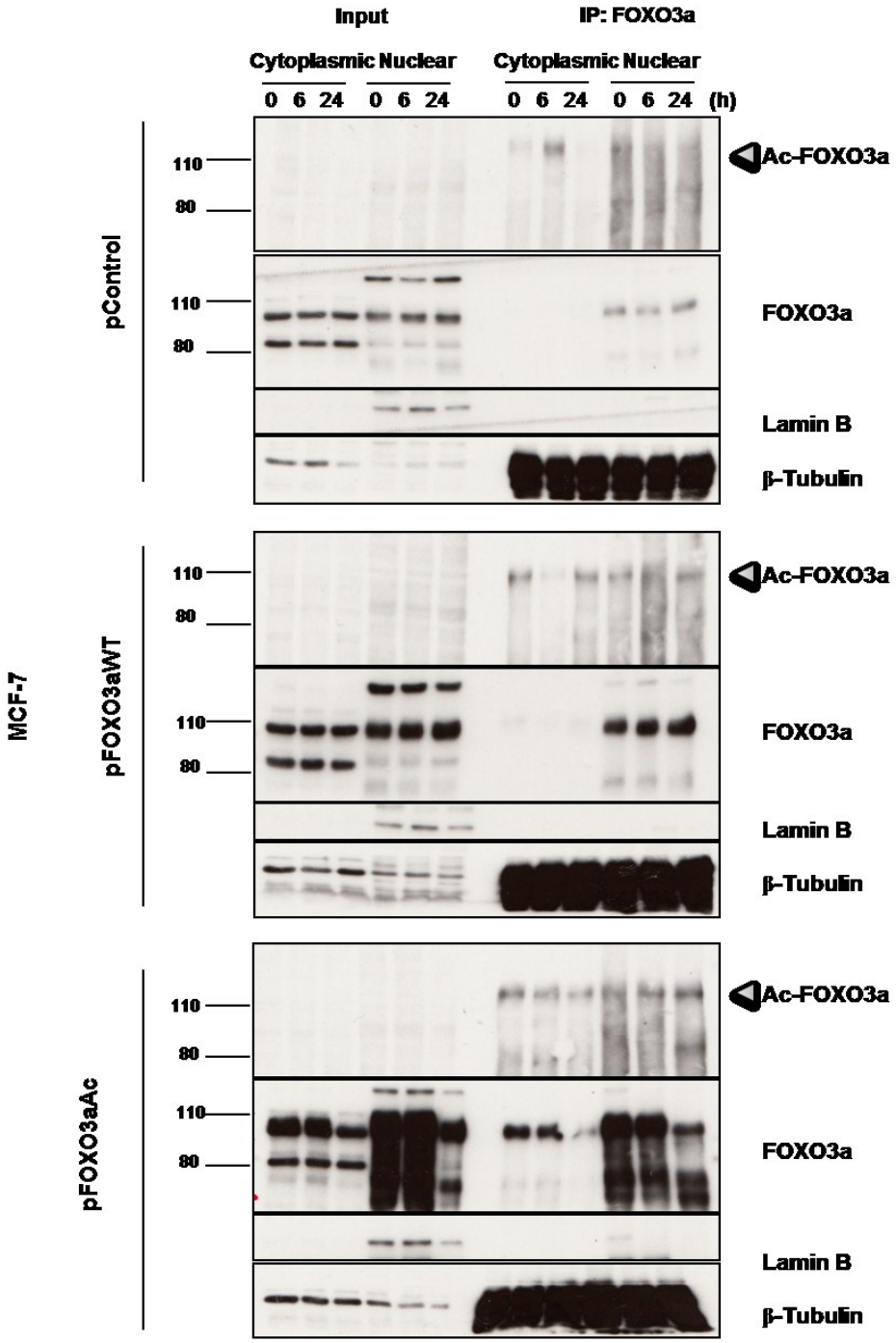
SUPPLEMENTAL DATA



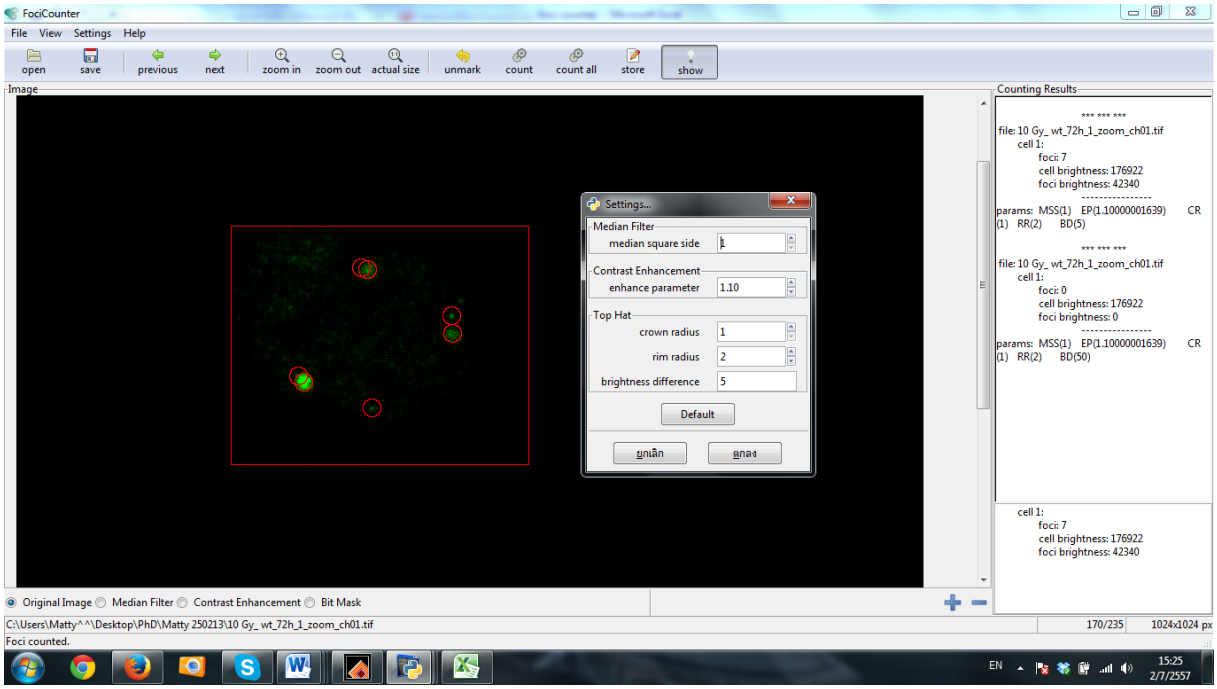
Supplemental Figure 7.1 Acetylated FOXO3a in MCF-7 cells predominantly localises in the nucleus, whereas it is mostly located in the cytoplasm in MCF-7-Epi^{R} cells after epirubicin treatment. A. MCF-7 cells were treated with 1 μM and cells were then harvested at 0, 6 and 24 h for preparation of subcellular fractions. Each fraction was then immunoprecipitated using anti-FOXO3a antibody. The expression of acetylated FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-lysine and anti-total FOXO3a antibodies.



Supplemental Figure 7.1 (Continued) Acetylated FOXO3a in MCF-7 cells predominantly localises in the nucleus, whereas it is mostly located in the cytoplasm in MCF-7-Epi^{R} cells after epirubicin treatment. B. MCF-7-Epi^{R} cells were treated with 1 μM and cells were then harvested at 0, 6 and 24 h for preparation of subcellular fractions. Each fraction was then immunoprecipitated using anti-FOXO3a antibody. The expression of acetylated FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), anti-total Ac-lysine and anti-total FOXO3a antibodies.



Supplemental Figure 7.2 Acetylated FOXO3a in MCF-7 cells is predominantly localised in the nucleus after epirubicin treatment. MCF-7 cells were transfected with ectopic pFOXO3aWT, pFOXO3aAc and pControl overexpressing vectors. Cells were treated with 1 μM and then harvested at 0, 6 and 24 h for preparation of subcellular fractions. Each fraction was then immunoprecipitated using anti-FOXO3a antibody. The expression of acetylated FOXO3a was investigated by incubating with anti-Ac-FOXO3a (K242/245), and anti-total FOXO3a antibodies.



Supplemental data 7.3 Foci counter software is a programme using to quantify the level of H2AX foci formation. It is the software used to quantify the level of Foci formation. This program was developed by Anna Jucha in the collaboration with Anna Lankoff.

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