Molecular mechanisms of drug resistance in a triple negative breast cancer cell model

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Abstract

p53 is a transcription factor activated by genotoxic stress. Dependent on the level of DNA damage, p53 can either trigger cell cycle arrest and DNA repair or programmed cell death. DNA damaging agents that activate p53 are commonly used in the treatment of cancer. If p53 inducible proteins that promote arrest and repair and inhibit apoptosis are up-regulated, tumours can become resistant to many types of treatment (multidrug resistance, MDR). MDR leads to treatment failure, metastases, and death in breast cancer patients. It is especially important in patients who rely solely on chemotherapy because they do not express hormone receptors and thus, do not benefit from endocrine therapies (e.g. tamoxifen). We have generated a triple-negative doxorubicin resistant breast cancer cell line (CALDOX) and shown by RNA array and qPCR analysis that p53-inducible cell survival factor (*p53CSV*), a p53-inducible inhibitor of apoptosis, is up-regulated in CALDOX and inducible by doxorubicin. Transient knockdowns of *p53CSV* sensitised CALDOX cells to doxorubicin, but stable knockdowns failed to support this preliminary data. However, CALDOX cells maintained expression of arrest and repair proteins p21 and p53CSV at high levels of genotoxic stress by doxorubicin—while CAL51 cells induced apoptotic protein p53AIP1 and underwent apoptosis. Although it is unlikely that p53CSV acts as a sole factor in MDR in CALDOX cells, p53CSV and other p53 arrest and repair proteins are likely an important factor in MDR through the evasion of apoptosis and promotion of DNA repair in CALDOX cells. Interestingly, we find that p53CSV is widely expressed in normal healthy tissues, conserved across eukaryotes, and in CAL51 cells, only partially p53-dependent—indicating that p53CSV may have an important role separate of p53, perhaps in the control of proliferating cells during development.

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Statement of Originality

All of the following results are the product of my experimentation, with the exception of the retroviral transfections which were kindly done by Dr. Selina Raguz. In addition, the qPCR of the RNA panel of human tissues and the drug resistance in CALDOX assays were carried out with the technical aid of Dr. Sabeena Rasul and Dr. Selina Raguz. All preliminary work was completed by Dr. Ernesto Yagüe, Dr. Selina Raguz, Dr. Harriet Unsworth and Owen Bain. CALDOX cells were generated by Dr. Ernesto Yague and MLET5 cells were generated by Drs. Simak Ali and Laki Buluwela.

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Abbreviations

ABC: ATP-binding cassette

AF: auto-fluorescence

Apaf-1: Apoptotic protease activating factor 1

BAK: Bcl-2 homologous antagonist killer protein

BAX: Bcl-2 associated X protein

Bcl-2: B-cell lymphoma 2 protein

Bcl-xL: Bcl-extra large protein

CAS: cellular apoptosis susceptibility protein

CBP: CREB-binding protein

CDK: cyclin-dependent kinase

CGH: comparative genome hybridization

CKI: CDK inhibitor

CsA: cyclosporin A

CT: extreme C-terminus

ERα: estrogen receptor alpha

ErbB-2: human epidermal growth receptor (also known as HER2/neu)

FACS: fluorescence-activated cell sorting

G0: gap 0 phase

G1: gap 1 phase

G2: gap 2 phase

HER2: human epidermal growth receptor (also known as ErbB-2/neu)

Hsp70: heat shock protein 70

HZF: haematopoietic zinc-finger

M: mitosis

MDR: multidrug resistance

p53CSV: p53-inducible cell survival factor

Pgp: P-glycoprotein

PgR: progesterone receptor

PRD: proline rich domain

p53AIP1: p53-regulated apoptosis-inducing protein 1

Rb: retinoblastoma protein

RE: response element

RPLP0: 60S acidic ribosomal protein P0

RPS6: Ribosomal protein S6

RPS14: 40S ribosomal protein S14

S: synthesis phase (cell cycle)

TAD: transactivation domain

TET: tetramerization domain

TN: triple-negative

Wt: wild type

YB1: Y-box factor 1

TE: Tris-EDTA buffer

18S: 18S ribosomal RNA

1. Introduction

1.1 Breast cancer and multidrug resistance

Breast cancer was responsible for the deaths of 519,000 people worldwide in 2004, and there were an estimated 1.3 million new cases of breast cancer in 2007 (World Health Organization, 2006).[1-2] In women, it is the most common type of cancer and the second most common cause of cancer death. U.S. women have a 1 in 8 chance of developing invasive breast cancer during their lifetime, and a 1 in 35 chance of breast cancer causing their death. Nevertheless, 5-year survival rates remain at nearly 88% in the U.S., in part due to early detection and diagnosis as well as successful endocrine and chemo therapies.[2]

Seventy percent of breast cancer patients express and are therefore positive for estrogen receptor α.[3] ERα is a nuclear receptor that binds to estrogen and regulates gene transcription. However, in breast cancer, ERα accumulation causes numerous changes in genomic expression, leading to a net effect where mitotic check-points are overridden and consequently, uncontrolled cell proliferation occurs.[4] Endocrine therapies include anti-estrogens that specifically targets and inhibits ERα. They are the most popular and successful treatment plan for ERα+ tumours. Similarly, breast cancers that are human epidermal growth receptor (ErbB-2/HER2/neu) positive are treated with target specific hormone therapies.

The remaining 10% of breast cancers, which are ER, PgR (progesterone receptor), and HER2 negative, are classified as triple negative (TN)—and endocrine therapies are unsuccessful in patients with these cancers. TN breast cancer is highly aggressive, and despite adjuvant chemotherapy, has a distant metastatis-free survival rate of only 71% after 5 years.[5] TN patients are treated with a chemotherapeutic regimen consisting of several drugs, and although the specific regimen is country and physician dependent, typically at least one drug is an anthracycline such as doxorubicin (adriamycin) or epirubicin.[6]

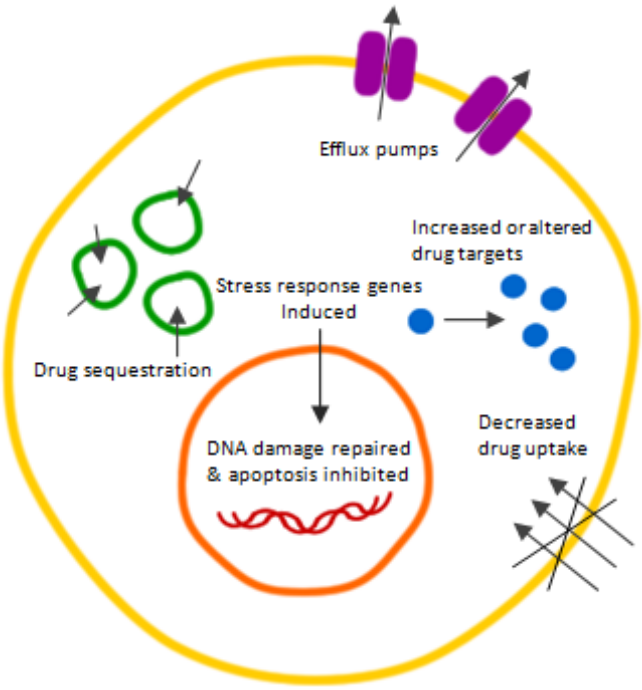


Figure 1.1 Schematic overview of multiple mechanisms of multidrug resistance
(modified from Gottesman et al. 2008)[7]

Multidrug resistance (relapse) arises when the cancer becomes resistant to a wide array of chemotherapeutic drugs—regardless of whether the patient has been previously exposed to the drug. Numerous mechanisms at the cellular level have been implicated, and resistance in numerous cancer cells lines has been linked to the overexpression of ATP-binding cassette (ABC) transporters, including P-glycoprotein (Pgp), MRP1, and BRCP1, because of their ability to pump drugs out of the cell. Other mechanisms include changes to DNA topoisomerase II (a target of anti-cancer anthracyclines), changes in intracellular and extracellular tumour pH, and an increase in oxidizing enzymes.[8] Apoptotic pathways have also been implicated in multi-drug resistance. Up-regulation of anti-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2) and Bcl-extra large (Bcl-xL), or the down-regulation of pro-apoptotic proteins such as Bcl-2 associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK) can mediate multi-drug resistance in vitro—and phase I/II clinical trials using inhibitors against these anti-apoptotic pathways are currently underway.[9] Anti-apoptotic proteins such as p21 that induce cell cycle arrest and DNA repair following

genotoxic stress are also potential therapeutic targets.[10] Due to the serious nature of TN breast cancer and the likelihood of relapse, it is crucial that we understand the mechanisms of chemotherapy resistance in TN multidrug resistant cells. Understanding the mechanism behind chemotherapy resistance will help to elucidate specific pathways that could be targeted *in vivo* to counter drug resistance in TN patients.

1.2 The cell cycle

The cell cycle is the orderly process by which cells replicate their genetic material and divide. There are two general stages: mitosis and interphase. Mitosis occurs when the nucleus divides in four subsequent steps: prophase, metaphase, anaphase and telophase. Interphase phase is essential for the preparation of cell division and growth, and consists of three phases: Gap 1 (G1), Synthesis (S), and Gap 2 (G2).[11] During G1, the cell resumes normal biological activities following mitosis, growing and preparing for the duplication of DNA during the S phase. DNA is replicated during the S phase, and the ploidy of the cell doubles, and other biological processes are slowed during this stage. At G2, the cell resumes important biological processes that will prepare it for mitosis. Furthermore, cells in G1 can undergo temporary arrest, where they do not replicate, entering a stage called G0. However, this decision to ~~r~~est" must occur before the restriction point, because after this checkpoint has passed, the cell is committed to division.[12]

There are multiple ~~c~~heckpoints" that a cell must proceed through at each stage, to ensure accurate replication of DNA and equal division of the cell. Cyclin dependent kinases (CDKs) act at these checkpoints, where they must be bound by cyclins in order to phosphorylate their downstream targets that trigger progression through the cell cycle.[13] CDKs transcription levels remain stable through the cycle, but transcription of the cyclins that bind them vary with the cell cycle and environmental conditions.[14-15] CDKs and CDK-

cyclin complexes can be inhibited by CDK inhibitors (CKIs) in the event of DNA damage, genotoxic stress, or oncogene activation.[16]

When a cell is signalled to divide, cyclin-dependent kinases 4 or 6 bind to cyclin D, which phosphorylates the tumour suppressor retinoblastoma (Rb).[17] Rb interrupts the inhibition of transcription factors E2F1 and Dp-1, allowing them to induce transcription of genes necessary for DNA replication in the S phase (e.g. cyclins A/E).[14, 18] Transcription of cyclin E allows it to bind to CDK2 and proceed through the G1-S phase transition. During and following the S phase, cyclin-CDK checkpoints are passed only when the DNA has been accurately replicated, and if necessary, repaired. Cyclin A/B and CDK1 complexes are involved in the late G2 and mitotic phases, where they phosphorylate and activate a multitude of proteins necessary for mitosis, and induce the assembly of the mitotic spindle and division of the nucleus.[14]

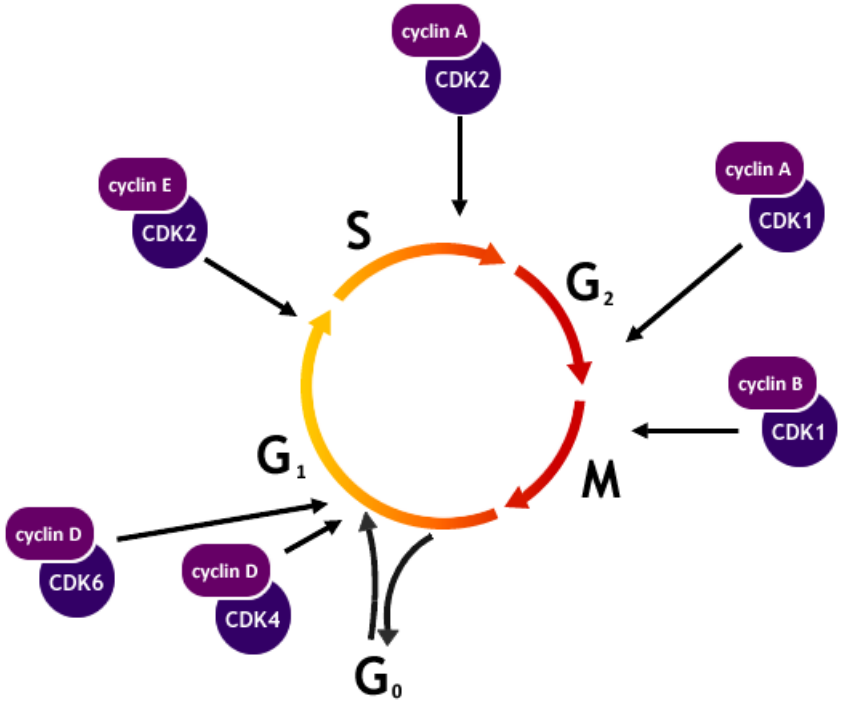


Figure 1.2 Overview of the cell cycle and its regulation by cyclin-CDK complexes
(modified from Vermeulan et al. 2003)[14]

1.3 p53: "guardian of the genome"

Transcription of genomic DNA to RNA occurs when RNA polymerase and its co-factors are recruited to the promoter site of a gene. Transcription factors are nuclear proteins that by binding to the promoter site of their target genes either inhibit or induce RNA polymerase recruitment and transcription of DNA. The induction of one transcription factor can have widespread effects throughout the cell, as most transcription factors bind to and regulate a multitude of genes. p53 is a transcription factor that at normal physiological levels plays a role in a number of key cellular processes (e.g. cell metabolism, mitochondrial respiration, cell adhesion, stem cell maintenance, and development).[10] However, p53 is most well known as a key regulator in the cellular response to cancer-associated stress signals—such as DNA damage and oncogene activation. First discovered in 1979, it has been nicknamed ~~"t~~he guardian of the genome" for its ability to differentiate between normal and neoplastic growth, and to trigger and choose between temporary arrest and DNA repair or programmed cell death (i.e. apoptosis) in response to cellular stress. [10, 19-21]

The p53 family of transcription factors, which includes p53, p63 and p73, stems from an ancient family of transcription factors.[22-23] p53-like proteins were, until recently, believed to be unique to the multicellular Animalia kingdom.[24] However, new evidence suggests the presence of p53-like proteins and sequences in multiple unicellular protists.[22-23] Although the original function of p53 is unclear, it is unlikely that p53 arose first as a tumour suppressor. Its presence in protists and other simple, short-lived organisms suggests that ancestral p53 predated the need to suppress uncontrolled cell proliferation. Similarly, early primordial organisms would not have had the longevity or size to accumulate genetic mutations necessary to induce neoplastic growth. More likely, p53 originally protected immortal germ lines against DNA damage and cellular stress in the Cambrian ocean, and it was later co-

opted for tumour suppression because of its ability to induce DNA repair and apoptosis. Interestingly, the ability of p53 to induce cellular arrest (through p21) is unique to vertebrates, and p53-independent induction of autophagy and senescence is limited to mammals alone.[10, 24]

However, this evolutionary heritage has led to two separate, and at times contradictory, responses to cellular stress, where a cell either survives and repairs damage, or undergoes programmed death. p53 is involved at every stage of cancer. It acts as a tumour suppressor in tumourigenesis: cell arrest and repair proteins fix DNA damage before mitosis occurs and apoptotic proteins induce cell death in irreparable cells. Both pathways eliminate the proliferation of oncogenic cells. Thus, it is unsurprising that tumourigenesis often relies on the down-regulation of the apoptotic pathway, and *p53* mutations occur in 50% of cancers.[25-26] However, most *p53* mutations are single point (missense) mutations in the DNA binding domain and thus, may not abrogate p53 activity completely.[27] During the treatment of cancer, p53 apoptotic pathways of rapidly dividing cells (e.g. tumour cells, but also cells in the gastrointestinal tract, hair follicles, and bone marrow) are targeted through chemo and radiation therapies. However, it is at this stage that the two separate pathways of the p53 response become disparate. p21 and cell repair pathways may help tumour cells recover and become resistant to treatments, such as UV radiation and doxorubicin; while pro-apoptotic pathways eradicate the tumourgenic cells. Understanding each pathway will be essential for restoring drug sensitivity in resistant tumours.

1.4 Structure of p53

Human p53 is composed of 393 amino acids, and it forms homotetramers in order to bind DNA. Each monomer has five separate domains, each playing an instrumental role in p53 function. The N-terminal region contains a trans-activation domain (TAD) that is divided into two subdomains (TAD1: residues 1-40 and TAD2: residues 40-61) and also a proline-

rich region. The TAD binds to proteins that modify the stability and activity of p53, including proteins that target p53 for ubiquination and degradation but also co-activators that induce its transcriptional function.[28] The proline-rich region is poorly understood, but it may act as a spacer between the TAD and DNA-binding domains, as its length is crucial for p53-dependent transcription.[29-30] The central DNA binding domain is responsible for the binding of p53 to DNA specific sequences of its target genes.[31] Near to the C-terminus, the tetramerization domain allows for a monomer to bind to another p53 abrogate to form a primary dimer, which binds to a second primary dimer—forming an active p53 tetramer that binds to and transcribes DNA.[32-33] The extreme C terminus regulates p53 activity, down-regulating or up-regulating its ability to either bind to DNA and its co-factors, through post-transcriptional modifications and protein-protein interactions.[28]

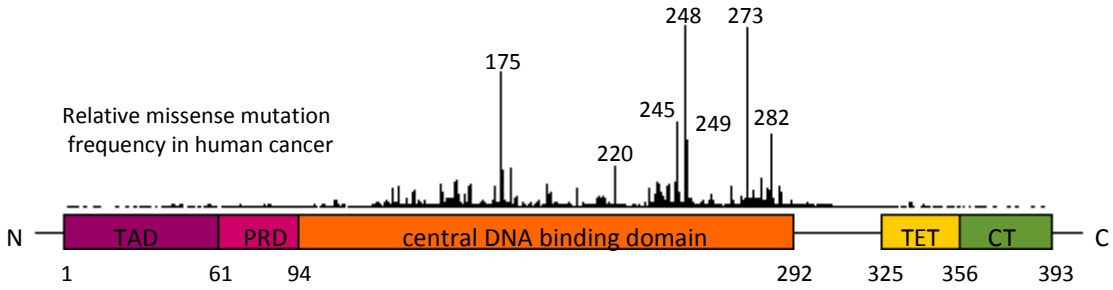


Figure 1.3 The functional domains of human p53. At the N-terminus, the transactivation domain (TAD) is necessary for p53 degradation or activation by protein co-factors. The proline-rich domain (PRD) sits between the TAD and the DNA binding domain, but its function is poorly understood. The central DNA binding domain is responsible for sequence-specific DNA binding of target genes. At the C-terminus post-translation modifications regulate p53 activity in the tetramerization domain (TET) and the extreme C-terminus (CT) (adapted from Joerger et al.)[28]

1.5 Choosing between cell cycle arrest and repair, or programmed cell death

The cell constitutively transcribes p53, but under normal conditions, p53 is down regulated at the protein level through ubiquitylation by MDM2—an E3 ubiquitin ligase that tags p53 for degradation by the 26S proteosome in the cytoplasm.[34-37] p53 is activated and up-regulated when DNA damage and other cellular stress signals

trigger post-translational modifications of p53 (e.g. phosphorylation, acetylation), inhibiting its interaction and degradation by MDM2 and enabling it to promote or inhibit the transcription of target genes.[38-39] p53 can either induce the transcription of genes known to play a role in cell cycle arrest and DNA repair (e.g. *p21*, *GADD45*, and *14-3-3-σ*) which lead to cell survival, or genes that lead to cell death (apoptosis) such as *APAF1*, *p53AIP1*, *NUMA*, *PUMA*, *PIG3*, and *BAX*.[40]

The choice made by p53 to induce arrest and repair or apoptosis is not only determined by its concentration levels, but also by changing its ability to bind apoptotic genes versus repair genes, through modifications such as phosphorylation and acetylation, in response to particular stress signal. p53 has at least 17 phosphorylation sites, primarily within its N-terminus.[39] The N-terminus of p53 is necessary for MDM2 binding, but also for binding with its transcriptional co-factors. Phosphorylation of p53 by its co-factors is correlated with its increased stability and activation, and in many cases, phosphorylation of one or multiple sites may be necessary for further post-translational modifications.[41] The numerous post-translational modifications as well as the many kinases that bind each site may represent redundant functions to ensure the response of p53, but also suggests the ability for a specific and fine-tuned p53 response to a multitude of separate stress signals.[39]

p53 is phosphorylated by the ATM kinase at serine 15, and the serine/threonine kinase CHK2 acts downstream to phosphorylate serine 20.[42-44] These two phosphorylations stabilize p53 and allow its binding to promoters of genes that induce G_1 arrest and DNA repair.[45] Other modifications of p53 enhance repair pathways too. At the carboxy-terminal lysine (Lys) 320 can be acetylated by the acetyltransferase PCAF and promotes p53-dependent growth arrest through the recruitment of co-activators to the promoter site, such as CREB-binding protein (CBP) and TRRAP.[38] Acetylated lysine 320 p53 binds more efficiently to *p21* than non-acetylated p53, and in mice, a single point mutation at 317 (the mouse

equivalent of lysine 320) induces p53-dependent transcription of pro-apoptotic target genes *NOXA* and *PUMA*.[46-47] Similarly, ubiquitination at Lys 320 by the ubiquitin ligase E4F1 induces the transcription of cell cycle arrest and DNA repair proteins such as p21, Gadd45, and cyclin G1, but not of apoptotic proteins. Similarly, on a mechanistic level, p53 ubiquitylated at serine 320 binds to the promoter of *p21*, but not to the apoptotic gene *NOXA*.[45, 48]

Numerous modifications of p53 have also been implicated in the apoptotic pathway. Phosphorylation of serine 46, for example, promotes the induction of the p53-regulated apoptosis-inducing protein 1 (p53AIP1) and downregulates the expression of p21—leading to p53 dependent programmed cell death.[49] Acetylation of Lys 373 by p300/CBP enhances phosphorylation at the N-terminus, which helps to stabilize p53 and allows it to bind to promoters of pro-apoptotic genes including *PIG3*, *BAX*, and *p53AIP1*.[45-46] Acetylation at lysine 120, in the DNA binding region of p53, also enhances p53's ability to bind to and transcribe apoptotic genes (e.g. *PUMA* and *BAX*)—and Lys 120 mutants have trouble inducing apoptosis, but their ability to induce cell arrest remains intact.[50]

Human p53 binds to target genes at a p53 sequence specific site called a p53 response element (RE). The location of the RE to the transcription start site is likely significant in p53-dependent transcription, as 50% of established REs are located in the promoter region of a gene, and approximately 25% are located in the first intron.[51] However, there is evidence that p53 binding sites also exist large distances from the start site. Many p53 post-translational modifications exert their effect by changing the way p53 binds to its target genes at their RE. RE sequences vary between genes, and determine a target gene's affinity for p53 binding. The accepted consensus sequence contains two 10-base decamers and a spacer: RRRCWWGYYY…n(0-2)…RRRCWWGYYY (R is a purine, Y is a pyrimidine, W is an A or T).[52] Each p53 unit of the p53 tetramer binds to three of nucleotides within the RRRCW or

WGYYY pentamers, causing conformational changes to the DNA and p53 monomers. However, some studies suggest that p53 can bind to REs with longer spacers (as many as 13), or with half or three-quarter sites.[53] Genes associated with the arrest and repair pathway typically have high affinity REs that respond to lower levels of p53. Pro-apoptotic genes generally contain low affinity REs that are activated following high levels of genotoxic stress and increased concentrations and modifications to p53.[52]

In addition to post-translational modifications and varying affinity to target gene REs, the choice between arrest and repair or apoptosis can be made by which of many co-factors are present to bind to p53. Co-factors influence promoter-selective p53 transcription and post-translational modifications can alter which co-factors can bind to p53. For example, p53 can recruit a number of co-factors that induce the transcription of pro-apoptotic genes and repression of cell cycle arrest, including prolyl isomerase PIN1, cellular apoptosis susceptibility protein (*CAS*), and transcription factors such as NF-κB, p63, and p73.[54-56] These factors can act at multiple levels, and can be involved in recruitment of p53 to promoters, in post-translational modification of p53, and in the inhibition or stabilization and activation of p53. PIN1 for example, is recruited to chromatin by p53 and allows p300 to acetylate p53 and induce the transcription of pro-apoptotic genes. Furthermore, PIN1 helps p53 dissociate from the apoptosis inhibitor (iASPP) after the phosphorylation of serine 46.[54] In contrast, at low levels of stress, p53 can interact with co-factors that promote transcription of cell cycle arrest and DNA damage repair, such as Y-box factor 1 (YB1), hematopoietic zinc-finger (HZF), or co-factors that inhibit apoptosis (e.g. iASPP).[52]

1.6 p53CSV: a novel gene with a role in the p53 pathway

In 2005, Park and Nakamura reported a new gene, p53-inducible cell survival factor (*p53CSV*), which they identified in cells subjected to UV radiation or doxorubicin treatment.[57] p53CSV is a small protein (8786 daltons) that consists of 76 amino acids. In *p53*$^{-}$

$^{/-}$ cell lines, Park and Nakamura claimed that p53CSV was not induced following genotoxic stress, and although this was largely the case, their data suggested the possibility of some p53-independent induction of p53CSV. However, subsequent studies have suggested, but failed to test, alternative RE sites nearer to the transcription start site; and the transcriptional regulation of *p53CSV* by p53 remains poorly understood.[58] Nevertheless, p53CSV was much more strongly induced in p53 wild type (wt) cell lines. Furthermore, Park and Nakamura identified a putative p53 binding site in exon 2 and confirmed binding *in vitro* with chip analysis and a luciferase reporter assay. The down-regulation of *p53CSV* in p53wt cell lines increased the percentage of apoptotic cells following treatment with either UV radiation or doxorubicin, while stable overexpression of *p53CSV* in $p53^{-/-}$ cells decreased the percent of apoptotic cells following treatment with UV radiation or doxorubicin.[57]

Because p53CSV prevented apoptosis but was induced by p53, they postulated that it played a role in the cellular arrest and repair pathway. In support of this hypothesis, they found that when the arrest and repair pathway was activated, determined by the expression of p21 and phosphorylation of p53 serines 15 and 20, p53CSV was highly expressed. Moreover, when apoptosis was induced, confirmed by the expression of apoptotic protein p53AIP1, phosphorylation of p53 serine 46, and down-regulation of p21, p53CSV was not expressed. Co-immunopreciation experiments with a p53CSV antibody determined that p53CSV bound to apoptotic protease activating factor 1 (Apaf-1) and heat shock protein 70 (Hsp70). Apaf-1 is a protein that cleaves pro-caspase 9 to become caspase-9, which triggers downstream caspases (e.g caspase-3) and apoptosis. Hsp70 is a protein that helps to mediate cellular stress, and in the case of Apaf-1, it inhibits its function and prevents apoptosis. The authors found that in $p53^{-/-}$ cells expressing p53CSV, cleavage of pro-caspase-9 decreased following genotoxic stress compared to those cells that did not express p53CSV—confirming a role for

p53CSV in the arrest and repair pathway by inhibiting apoptosis in order to give the cell time to repair DNA damage.[57]

p53CSV remains largely unstudied. A recent in vitro study found that *p53CSV* is up-regulated following camptothecin treatment in two separate glioblastoma cell lines; however, both cell lines ultimately progressed to senescence or apoptosis, and the screenings failed to discern whether *p53CSV* was induced as part of the early or late stress response.[59]

p53CSV has also been found to be up-regulated in several genomic screenings of human cancers. In a cohort of 50 patients with multiple melanoma, *p53CSV* was upregulated in 50%.[60] In patients with follicular lymphoma, *p53CSV* and other p53-inducible genes were up-regulated following irradiation treatment.[61] However, as both anti- and pro-apoptotic genes were induced, we can only infer that p53CSV plays a role in the p53 response, but not as what stage, following irradiation in vivo. In patients with AML, *p53CSV* was up-regulated shortly after the administration of chemotherapy, but again, both anti- and pro-apoptotic p53-dependent genes were up-regulated in this screening, and a role for p53CSV in the arrest and repair or the apoptotic pathway could not be distinguished *in vivo*.[62]

Another screening found that p53CSV was part of a "Poised Gene Cassette" of 48 genes that experience tighter regulation and less variance in six different tumour types compared to non-malignant controls.[63] Although this suggests that p53CSV plays an important role in tumours, these authors found that silencing p53CSV increased invasion in HCT116 cells—likely increasing their metastatic potential. However, such tight regulation in cancer suggests that the protein is carefully balancing two disparate pathways, and it is possible that p53CSV plays a negative role in invasion, but a pro-survival pathway in response to therapy.[63] Recently, *p53CSV* was found to be expressed in all breast cancer cell lines.[64]

Interestingly, p53CSV is highly conserved in vertebrates (Table 1.1, Appendix A for full sequences) and has orthologs in *X. laevis, C. elegans, S. cerevisieae* and *A. thaliana* (Appendix A). MDM35, the p53CSV ortholog in budding yeast, was shown to mediate doxorubicin resistance.[65] Its high level of conservation suggests that p53CSV has an essential role in eukaryotic cells, perhaps as part of the cell's response to genotoxic stress.

Table 1.1 Conservation of p53CSV in vertebrates

Organism	Amino Acid Sequence (51-76)
Human	AIKEKEIPIEGLEFMGHGKEKPENSS
Chimpanzee	AIKEKEIPIEGLEFMGHGKEKPENSS
Dog	AIKEKEIPIEGLEFMGHGKEKPESSS
Cow	AIKEKEIPIEGLEFMGHGKEKPESSS
Mouse	AIKEKEIPIEGLEFMGHGKEKPENSS
Rat	AIKEKEIPIEGLEFMGHGKEKPENSS
Chicken	AIKEKDIPIEGLEFMGPSKGKAENSS
Zebra Fish	AIKEKDIPIEGVEFMGPNSEKADS - -

In summary, p53CSV is likely to play an important role in the p53 arrest and repair pathway. Previous data suggests that it assists cells in surviving low doses of irradiation or chemotherapy treatment. Thus, it may play a role in the development of MDR in vivo, and could be a potential target for restoration of drug sensitivity. However, its role in the p53 response pathway remains poorly understood, and further studies are necessary.

2. Preliminary Work

CAL51 is a triple-negative breast cancer cell line originally derived from a malignant pleural effusion of a 45-year old patient with invasive adenocarcinoma with extensive intraductal involvement. It is epithelial, clonogenic in soft agar, and tumourigenic in nude mice. It has a normal diploid karyotype and it is phenotypically negative for ER, PgR, and ERBB2—making it a good cell model to study TN breast cancer chemotherapy resistance.[66-67]

Dr. E Yagüe and his laboratory, in collaboration with Dr. S Raguz, generated a doxorubicin resistant CAL51 derivative (CALDOX) by selection of CAL51 cells in the presence of 0.4 μM doxorubicin. Although microscopically indistinguishable from CAL51, CALDOX cells have a slower proliferation rate than their parental progenitor. Yagüe and colleagues determined 400 genes which were differentially expressed in CALDOX cells by Affymetrix hybridization. They compared these to the changes in genomic DNA between CALDOX and CAL51 cell lines, as determined by Comparative Genomic Hybridization (CGH), performed by Dr. Nigel Carter's group at Cambridge University. Of these 400 genes, ten that had been previously associated with chemotherapy resistance in other cell lines were selected for further investigation. Transient down-regulation by siRNA or up-regulation by cDNA expression was performed in CALDOX cells (Table 2.1). Controls were obtained by transfection of either empty cDNA expression vectors, or *EGFP* siRNA. The drug response of the transfected cells was examined by sulforhodamine B staining (SRB).[68] Although genes such as catalase, metallothionein, and glutathione peroxidise, all of which were overexpressed in CALDOX cells and have been previously associated with doxorubicin resistance, their experimental down-regulation with siRNA did not change CALDOX cells' sensitivity to doxorubicin.[69-71] In contrast, down-regulation of p53CSV, also overexpressed in

CALDOX, increased CALDOX cells sensitivity to doxorubicin as an increase in cell death upon exposure to doxorubicin was observed (Figure 2.1).

Table 2.1 Changes in drug sensitivity following experimental up or down regulation of candidate genes obtained from the Affymetrix array analysis

Gene	Product	Expression in CALDOX	Strategy	Change in Drug Sensitivity
BCAT1	Amino acid transporter	Up	RNAi	No
MT1M	Metallothionein	Up	RNAi	No
p53CSV	Anti-apoptotic	Up	RNAi	Yes
CAT	Catalase	Up	RNAi	No
GPX1	Glutathione peroxidase	Up	RNAi	No
EMP1	Epithelial membrane protein 1	Down	Overexpression	No
SLC2A3	Glucose transporter	Down	Overexpression	No

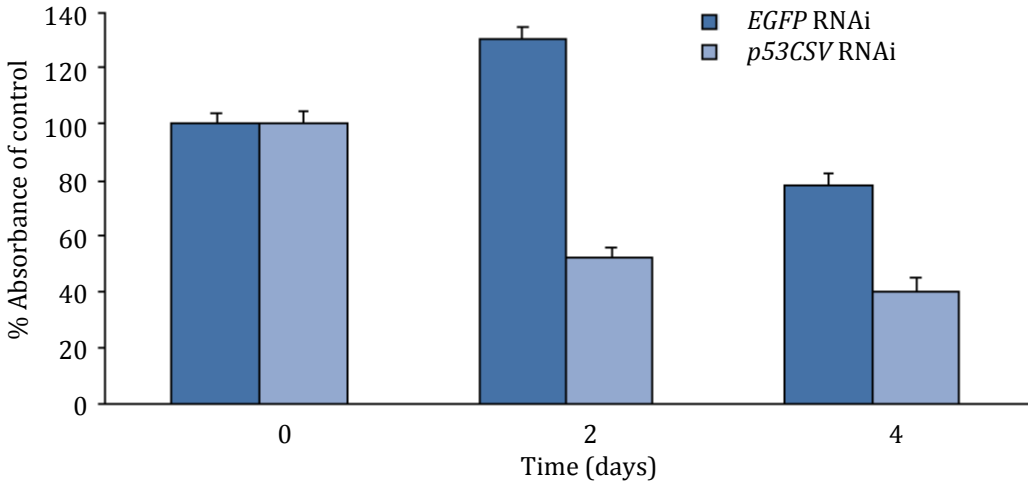


Figure 2.1 Transient knockdown of *p53CSV* sensitises CALDOX cells to doxorubicin. CALDOX cells were transiently transfected with a siRNA targeting p53CSV mRNA and then left to grow for 4 days in the presence of 0.4 µM doxorubicin. Change in cell numbers were determined by SRB growth assays. A siRNA targeting *EGFP* mRNA, a protein absent in human cells, was used as a negative control. CALDOX cells transfected with transient siRNA against *p53CSV* were more sensitive to and grew more slowly in 0.4 µM doxorubicin than cells transfected with control siRNA against EGFP (at 2 and 4 days). Data represents a single experiment with 3 internal replicates ± SD. Values are expressed relative to the absorbance obtained from cells growing in the absence of doxorubicin.

As previous studies implicated p53CSV in the p53 arrest and repair pathway, it was important to better understand the role of p53 in CALDOX and CAL51 cells. Western blot data indicated that the apoptotic pathway, measured by the presence of acetylated p53, was

induced at lower doses (0.05 μM+) of doxorubicin in CAL51 cells than in CALDOX cells—where acetylated p53 was only detected at high (10 μM) concentrations (Figure 2.2). Furthermore, p21, indicative of the arrest and repair pathway, appeared to be constitutively activated in CALDOX cells, as it was present with or without the addition doxorubicin. In contrast, although p21 was induced at low levels of damage in CAL51 cells, by 10 μM it had been completely down-regulated as CAL51 cells underwent apoptosis. This was not the case in CALDOX cells, where p21 also remained active at high doses of genotoxic stress.

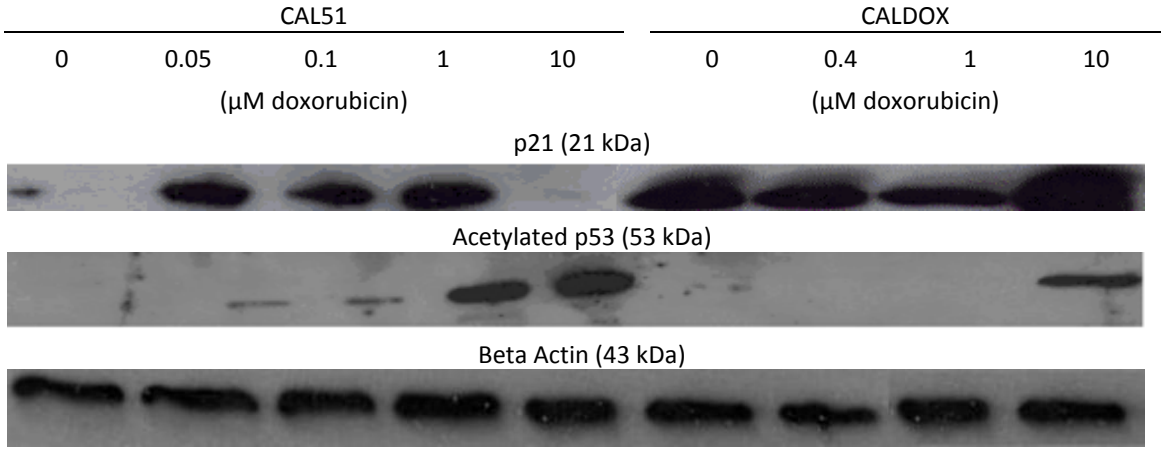


Figure 2.2 The expression of p53 and p21 differs between CALDOX and CAL51 cells. Protein levels of p21 and acetylated p53 in CALDOX and CAL51 cells at increasing concentrations of doxorubicin (0 – 10 μM) were determined by western blot analysis. p21 (indicative of the arrest and repair pathway) was expressed at all concentrations in CALDOX cells, but in CAL51 cells it was only induced at low concentrations of doxorubicin (0 – 1μM). Acetylated p53 (indicative of the apoptotic pathway) was expressed in CAL51 cells at all doses (0.05 – 10 μM) but was only induced at 10 μM in CALDOX cells. β-actin is used as a loading control.

In addition, caspase activity assays showed that caspase 9 and downstream caspases 3 and 7 were induced at low levels of stress in CAL51 cells, and that activity plateaued between 1 and 10 μM of doxorubicin (Figure 2.3). Caspases are involved in the early stages of apoptosis, and their high activity at low doses of genotoxic stress supports our hypothesis that CAL51 undergoes apoptosis earlier than CALDOX. CALDOX cells, despite an unexplained peak in activity at 0 μM, had low levels of caspase activity at low doses of doxorubicin, and activity increased but remained low at 10 μM doxorubicin.

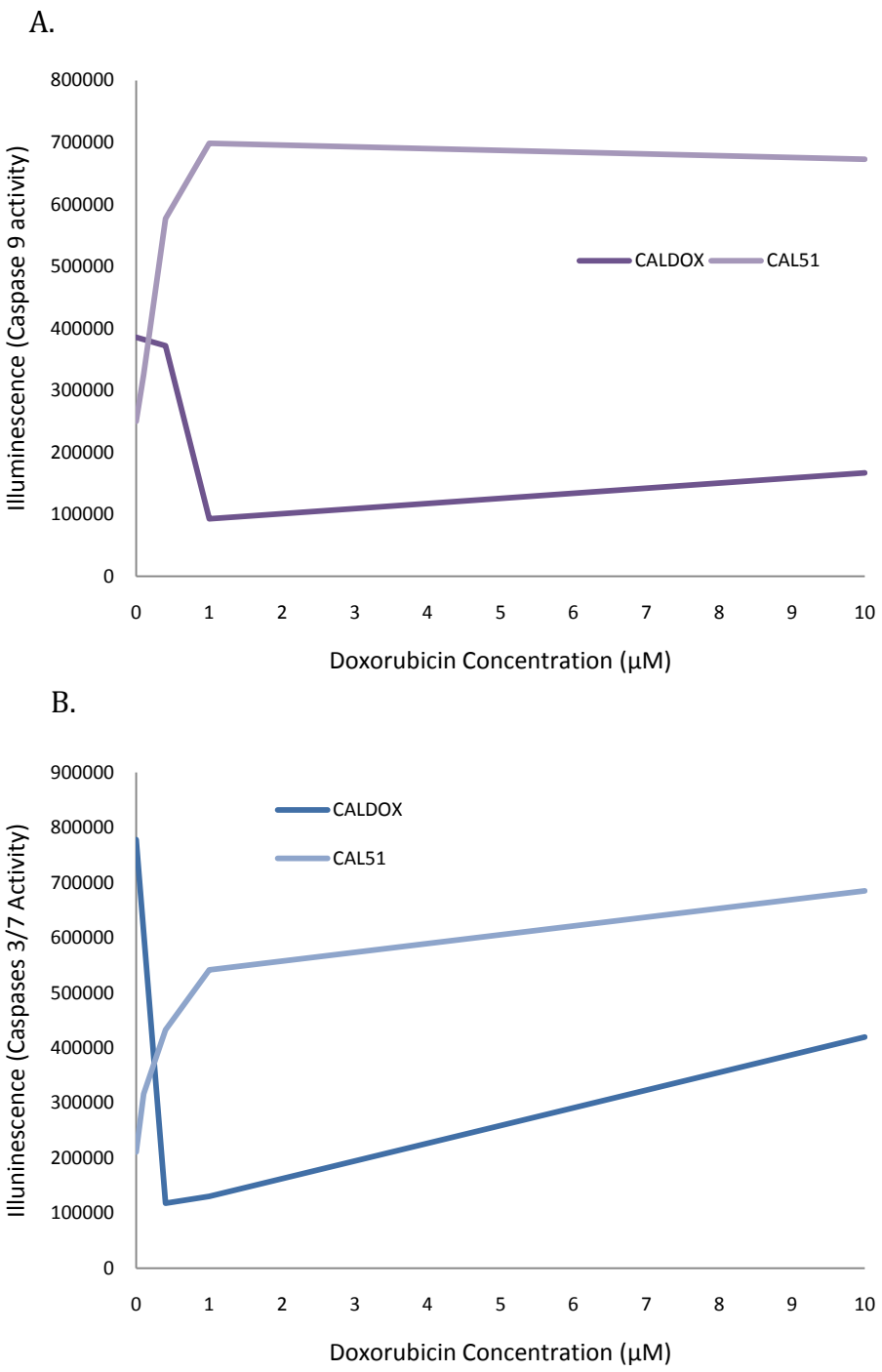


Figure 2.3 Activation of caspases in CAL51 and CALDOX cells following doxorubicin treatment is different. Apoptotic activity in CAL51 and CALDOX cells was measured using Caspase-glo activity assays following genotoxic stress by doxorubicin (0 – 10 µM). Higher levels of caspase 9 (a) and caspases 3/7 (b) activity were observed in CAL51 cells than in CALDOX cells following treatment with doxorubicin.

Cell cycle analysis with fluorescence-activated cell sorting (FACS) showed a higher percentage of cells at G_1 in CALDOX cells than in CAL51 cells (Figure 2.4). When 0.4 µM doxorubicin was added for either 24 or 48 hours, the number of cells at the G_1 checkpoint did

not significantly change in CALDOX cells, while in CAL51 cells many cells underwent apoptosis (evidenced by the increasing sub-G_1 population in P4) or entered G_2 arrest.

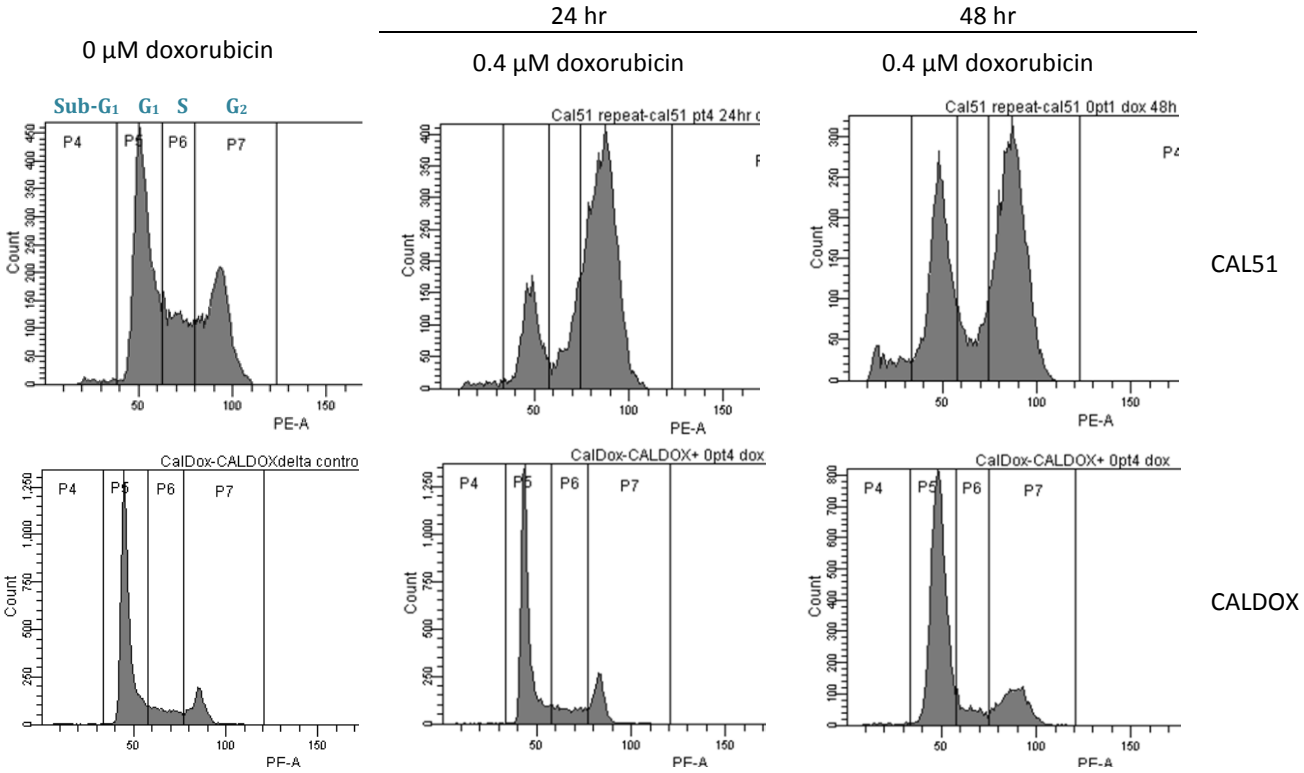


Figure 2.4 CALDOX cells remain in G_1 while CAL51 undergo G_2 arrest and apoptosis following treatment with doxorubicin. Cell cycle analysis by flow cytometry was performed in CALDOX and CAL51 cells treated with 0 µM or 0.4 µM doxorubicin for 24 and 48 hours. P4: cells in sub-G_1; P5: cells in G_1; P6: cells in S; P7: cells in G_2. A larger percent of CALDOX cells were observed at the G_1 checkpoint than in CAL51. In response to doxorubicin at 24 and 48 hours, a large percent CAL51 cells underwent G_2 arrest or apoptosis (sub-G_1); meanwhile, CALDOX cells did not alter their cell cycle profile.

Lastly, because P-glycoprotein and other ABC transporters are well known for their role in multi-drug resistance, we ruled out the possibility of efflux pumps in mediating MDR in CALDOX cells. Flow cytometry experiments showed that fluorescent antibodies against Pgp did not bind to CALDOX or CAL51 cells (Figure 2.5a). Additionally, Calcein AM-Efflux, an assay where Calcein AM, a non-fluorescent transporter substrate, that fluoresces when cleaved by intracellular esterases, also indicated the absence of a cyclosporin-inhibitable pump in CALDOX cells (Figure 2.5b). Lastly, doxorubicin, which is naturally fluorescent, accumulated to the same extent in both CAL51 and CALDOX cells after 1 hr—

but did not accumulate in NCI cells (positive control cells expressing Pgp, Figure 2.5c). Thus, we confirmed that drug resistance in CALDOX cells is not mediated by a known transporter.

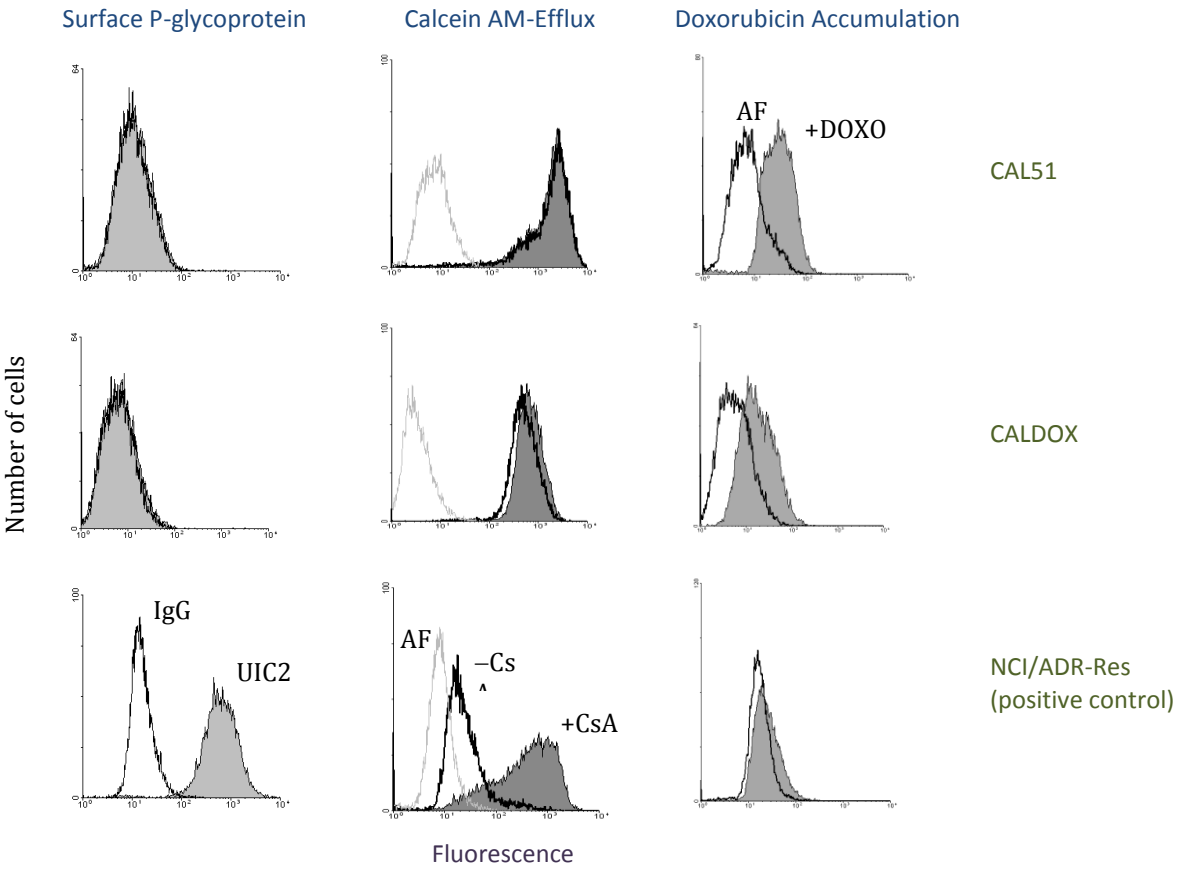


Figure 2.5 Absence of efflux pumps in CALDOX cells. A. Flow cytometry analysis of Pgp expression using the UIC2-phycoerythrin-conjugated antibody (filled peaks) and the corresponding IgG isotype control (clear peak). In CAL51 and CALDOX cells, these peaks overlap, indicating that Pgp is not present in the cellular membrane. Pgp positive NCI-ADR-Res cells were used as a positive control. B. Flow cytometry analysis of calcein-AM efflux. Cells that were incubated in the presence of a pump inhibitor (cyclosporin A, filled peaks) produced a fluorescent peak as they could no longer efflux the fluorescent substrate. AF, autofluoresecnce. In CAL51 and CALDOX cells, the presence of CsA did not affect the intracellular concentrations of calcein, indicating the absence of membrane pumps. NCI/ADR-Res cells were used as a positive control. C. Flow cytometry analysis of intracellular accumulation of doxorubicin. Cells were incubated in 0.4 μM doxorubicin for 15 minutes. In CAL51 and CALDOX cells, doxorubicin accumulates intracellularly and the cells fluoresce (filled peaks). NCI/ADR-Res positive controls did not fluoresce as they pumped out the fluorescent drug.

In summary, our previous data suggested that doxorubicin resistance in CALDOX cells was due to their ability to inhibit and evade apoptosis and induce the p53-dependent cellular repair pathway. Transient knock-down of p53CSV indicated that this protein was important for resistance in CALDOX, and we postulate that it plays a role in inhibiting apoptosis, allowing for DNA repair by p21, in response to genotoxic stress.

3. Methods and Materials

3.1 Cell line maintenance

Breast cancer cell lines CAL51, MCF7, and CALDOX were grown in low glucose GIBCO® Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™-1 (Invitrogen) containing 10% fetal calf serum, and 1% Antibiotic Antimycotic Solution (Invitrogen). In addition, CALDOX was maintained in media containing 0.4 μM doxorubicin. The estrogen-independent MCF7 derivative cell line, MLET5, was grown in phenol red free, low glucose GIBCO® DMEM (Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine and 1% Antibiotic Antimycotic Solution.

3.2 RNA extraction

Cells were trypsinized, washed with PBS (phosphate buffer saline), centrifuged, and stored at −80°C until further use. Frozen cell pellets were thawed by the addition of 450 μL of RNAzol **(**Biogenesys, Poole, United Kingdom**)** and 50 μL of chloroform. Pellets were resuspended by gentle mixing with a Pipetman followed with vigorous mixing by a vortex for 15 seconds. Samples were stored on ice for 15 minutes and then spun at 17,000 g for 15 minutes at 4°C. The aqueous upper phase containing RNA was carefully collected and transferred to a fresh tube where an equal volume of isopropanol was added. The homogenate was inverted several times and left on ice for 15 minutes. Samples were then spun for 15 minutes at 17,000 g at 4°C; and the supernatant was removed and the RNA pellet washed with 70% ethanol. Samples were left to dry on ice before being resuspended in 30 μL of Analar autoclaved water. The RNA pellet was rehydrated for 1 hr on ice. The sample was mixed by pipetting and the RNA concentration was measured using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific). RNA was stored for short periods at −20°C, but for long-term storage it was kept at −80°C.

3.3 Reverse transcription: preparation of cDNA

Reverse transcription reactions were performed with sterile filter tips (StarLabs) and a First Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche Applied Sciences). To each 10 µL reaction, 0.4 µL (≥10 units) AMV reverse transcriptase, 1 µL of 1.6 µg/µL random hexamers, 1µL of 10 mM dNTPs, 2 µL of 25 mM $MgCl_2$, 1 µL of 10x reaction buffer, 1 µg of sample RNA and Analar autoclaved water (as necessary, to make 10 µL) were added. Samples were incubated at 42°C for 1 h and the reverse transcriptase was then denatured by incubation 95°C for 5 min. Once cool, cDNA was diluted in 90 µL of Analar autoclaved water to a final concentration of approximately 10 ng/µL.

3.4 Quantitative PCR

Quantitative PCR (qPCR) reactions were prepared with a SensiMix™ SYBR® Kit (Bioline) and sterilized filter tips (Starlabs) on MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) in a PCR laminar air flow cabinet. For each gene, a master mix containing 50 µL SensiMix, 2 µL SYBR® Green, and 0.5 µL of 100 µM of each primer (i.e. forward and reverse) was prepared. To each well 5 µL of cDNA and 5 µL of master mix were added. For standard curves, 0%, 1%, 10%, and 100% neat cDNA of either untreated CAL51 or CALDOX cDNA was prepared and aliquoted into appropriate wells. Standards were prepared by serial dilution using sterile Analar water. All samples and standards were performed in triplicate. For each experiment, a minimum of two normalisers were included in order to account for the varying amounts of cDNA between samples.

Table 3.1 Primer Sequences for qPCR

Gene Amplified	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
p53AIP1	CACAGATGTGCAGGAGGAGA	TCACCGAGAGGTTCTGGTCT
p21	CCTGTCACTGCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT
p53CSV	AGGATTTCGCAAGTCCAGAA	GCTGATTCCACCCAAGTAT
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
RPS6	AGGGTTATGTGGTCCGAATCA	TGCCCCTTACTCAGTAGCAGG
RPS14	TCACCGCCCTACACATCAAACT	CTGCGAGTGCTGTCAGAGG
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

Reactions were performed on a 7900 Fast Real-Time PCR System (Applied Biosystems). Primers were selected by their ability to amplify under the pre-determined reaction conditions (95°C 10 m hot-start, followed by 40 cycles: 95°C 30s denaturation step, 60°C 30s annealing step, 72°C 30s elongation step), and primer sequences are listed in Table 3.1. Dissociation curves were run for each primer set (Table 3.1) to make certain that primer dimers did not form and prevent the accurate quantification of cDNA.

3.5 Quantitative PCR (qPCR) analysis

Quantitative PCR analysis was done with SDS 2.3 Taqman software. The cycle threshold (Ct) values were manually set to the steepest part of the amplification slope and outlying wells were omitted from data analysis. Standard curves, mean DNA quantities, and standard deviations were calculated by the Taqman software. In order to account for varying amounts of cDNA between RNA samples (e.g. CALDOX and CAL51), the gene of interest in each sample was divided by a house-keeping gene (i.e. "normaliser") from that sample (Equation 3.1). Standard deviations of these ratios were calculated using Excel (Equation 3.2). Ratios were used to compare the expression of the gene of interest across multiple test conditions (e.g. between cell lines, or at different drug concentrations).

Equation 3.1

$$Relative\ quantity\ of\ gene\ x\ in\ Condition\ A = \frac{mean\ quantity\ of\ gene\ x\ in\ Condition\ A}{mean\ quantity\ of\ normalizer\ y\ in\ Condition\ A}$$

Equation 3.2

$$STD\ of\ gene\ x = \sqrt{\left(\frac{STD\ of\ gene\ x}{mean\ quantity\ of\ gene\ x}\right)^2 + \left(\frac{STD\ of\ normalizer\ y}{mean\ quantity\ of\ normalizer\ y}\right)^2}$$

3.6 Drug assays

Cells (2000-4000) were seeded in 200 μL of medium per well in flat-bottomed 96-well plates. In stable cell lines, the antibiotic used for selection (i.e. G418 or puromycin) was added, but for CALDOX cells, the medium was doxorubicin free. The cells were permitted to adhere to the plate for 24 hours. The following day, the medium was removed and 200 μL of medium containing the experimental conditions (e.g. drug(s) at various concentrations) was added. In stable cell lines, cells continued to be grown in the presence of their selection factor throughout the experiment. Each condition (i.e. drug concentration) was performed with six replicates.

Cells were left to grow in the presence of the drug(s) for approximately three doubling times before a sulforhodamine B (SRB) colourimetric assay was performed.[68] In some experiments, time points were collected by seeding the plates in triplicate, and performing an assay at days 0, 2 and 4. Cells were first fixed by adding 100 μL of ice-cold 40% (w/v) trichloroacetic acid (TCA) to each well. After 1 hr, the TCA was removed by gentle washing under running tap water. Once the plate had been washed five times, 100 μL of SRB was added to each well, in order to bind with intracellular proteins, for 30 minutes. Excess SRB dye was removed by carefully washing the plates five times with 1% (v/v) acetic acid. Plates were left to air-dry for 2-3 days. SRB was solubilized by adding 100 μL of 10 mM Tris-base and leaving the plate on an orbital shaker for 30 minutes. Blank samples were prepared by pipeting 100 μL of 10 mM Tris-base into several empty wells on each plate. Absorbance was measured at 492 nm by a Tecan Sunrise microplate reader (Tecan Group Ltd.).

3.7 RNA interference: transient transfections

CALDOX ($1x10^5$) cells were seeded in flat bottomed 6-well plates and left to adhere overnight. Cells were grown in medium under normal conditions. The next day, transient knock-downs were prepared by sequentially adding neat media (without FCS or antibiotic),

HiPerFect transfection reagent (Qiagen), and siRNA (EGFP from Ambion, p53CSV from Dharmacon) to an eppendorf tube and leaving the reaction to incubate for 10 minutes. Concentrations of siRNA and HiPerFect are listed in Table 3.2. p53CSV siRNA is a mixture of 4 siRNAs targeting different regions of the *p53CSV* mRNA. Media was removed from the 6 well plates and cells were resuspended in 2.3 mL neat media. The 100 µL siRNA reactions were added drop-wise to each well. One control well received HiPerfect without siRNA and another control received neither HiPerFect nor siRNA.

The cells were left to incubate with siRNA for 24 hours. Cells were washed with PBS, trypsinised, and counted using an haemocytometer. Treated cells were seeded (6000 per well) with six replicates in 3 flat-bottomed 96 well plates. On one plate, cells were resuspended in normal medium (containing FCS and antibiotic), but without drug. This plate was named day 0, and an SRB assay was performed after 24 hours, once the cells had adhered to the plate. On the two remaining plates, treated CALDOX cells (control, control with HiPerfect, *EGFP* siRNA cells, *p53CSV* siRNA cells) were resuspended in 0.4 µM doxorubicin in normal medium. These plates were left to incubate for 2 and 4 days before an SRB assay was performed (see drug assays).

Table 3.2 SiRNA Reagents and concentrations

Reagent	**50 µM *EGFP* siRNA (Ambion)**	***p53CSV* siRNA smartpool (Dharmacon)**
Neat media (µL)	86.1	83.2
HiPerFect (µL)	12.0	12.0
siRNA (µL)	1.92	4.80

3.8 RNA interference: stable transfections

Small hairpins targeting *p53CSV* mRNA and a scrambled mRNA that acted as a negative control were cloned into pGFP-V-RS from OriGene (catalogue #TG318755, Figure 3.1). DNA preparations from the above plasmids were performed using a Maxiprep kit (Qiagen). Retroviral transfections were performed by Dr. Selina Raguz (MRC) following standard protocols. In brief, retroviral supernatants of infected HEK293T cells (transfected

with retroviral plasmid and helper virus using polyethylenimine) were added to CALDOX cells and stably transfected cells selected by puromycin (0.5 μg/mL) resistance. Stable CALDOX lines expressing small hairpins to p53 and p21 were generated in the same way by Dr. Selina Raguz.

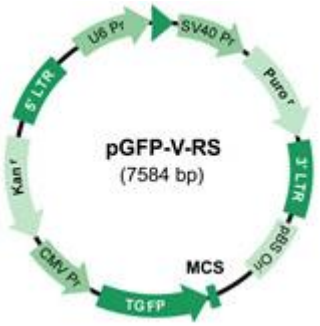


Figure 3.1 Vectors used in stable *p53CSV* knockdown in CALDOX cells. Small hairpin RNAs targeting *p53CSV* or a scrambled negative control were cloned into PGFP-V-RS (Origene, catalogue number #TG318755).

3.9 Overexpression of p53CSV in CAL51 cells

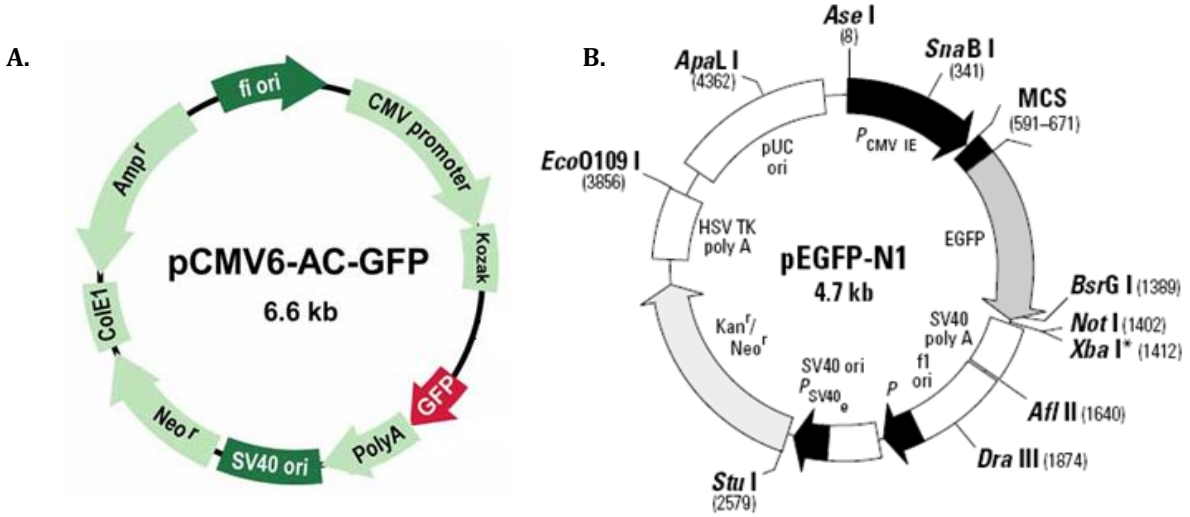


Figure 3.2 Vectors used in *p53CSV* overexpression in CAL51 cells. A. *p53CSV* cDNA was cloned into vector pCMV6-AC-GFP by Origene. B. Vector pEGFP-N1 expresses *EGFP* cDNA and was obtained as a negative control.

p53CSV (513, pCMV6-AC-GFP, Origene, Figure 3.2a) and *EGFP* plasmids (D161, pEGFP-N1, Clonetech Laboratories, Figure 3.2b) were linearised with ScaI and ApaLI, respectively, and confirmed by gel electrophoresis. Linear DNA was purified by phenol-

chloroform extraction. First, the linearised DNA was made up to 400 µL with Tris EDTA (TE) buffer and 400 µL of phenol-chloroform was added in order to denature the restriction enzymes from the previous digestion step. The samples were vortexed vigorously for 15 seconds, incubated on ice for 5 minutes and then centrifuged at 17,000 g for 15 minutes at 4°C. The homogenate had separated into two separate phases, and the top, aqueous layer containing DNA was transferred to a new Eppendorf tube—great care was taken not to disturb the interphase which contained denatured protein. To the aqueous phase, 1/10 volume of 3 M NaCl and 2.5 volumes 100% ethanol were added. After gently mixing with a Pipetman, the samples were left at –20°C for 30 minutes. The DNA was centrifuged at 17,000 g for 15 minutes at 4°C. A DNA pellet was observed and washed with 500 µL of 70% ethanol. The ethanol was removed and the pellet was left to dry in a sterile tissue culture hood. When all ethanol droplets had evaporated, the pellet was resuspended in 60 µL of sterile Analar water. The DNA concentration was measured using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific). DNA was stored at –20°C.

CAL51 cells were trypsinized, counted, and seeded (800,000 cells) in 4 x 25 cm^2 flasks, in 10% FCS GIBCO® DMEM with GlutaMax™ (Invitrogen) without antibiotic. Cells were permitted to adhere to the flasks for 24 hours. Next, in one Eppendorf tube, 24 µg of purified linear EGFP or p53CSV cDNA was incubated in 1.8 mL of neat DMEM for 5 minutes. In a second Eppendorf tube, 72 µL of Lipofectamine™ transfection reagent (Invitrogen) was simultaneously incubated in 1.8 mL of neat DMEM. The two were gently mixed in a 15 mL Falcon tube and incubated at room temperature for another 20 minutes. Cells were then resuspended in this new mixture, with two replicates for each gene (i.e. EGFP and p53CSV). Cells were left in this suspension overnight, and an additional 3.6 mL of antibiotic-free media, but containing 20% FCS, was added to each flask. Three days after the addition of Lipofectamine and cDNA, each flask was passaged and cells were resuspended in

medium containing 10% FCS and antibiotic. In the next passage, G418 (700 µg/mL, Sigma) was added and the formation of numerous colonies was observed after approximately 2-3 weeks. Colonies were mixed in the next passage and stably transfected pools of cells were maintained in 350 µg/mL G418.

4. Results

4.1 Expression of p53CSV in CALDOX and CAL51 cells

Previous RNA array data indicated that *p53CSV* was overexpressed in CALDOX cells compared to CAL51 (1.48 fold, 2.33 fold in doxorubicin). We sought to confirm this data using qPCR. CALDOX (grown in 0.4 μM doxorubicin) and CAL51 cells were harvested and RNA extractions, reverse transcription reactions, qPCR with two normalisers, 18S ribosomal RNA (*18S*) and Ribosomal protein S6 (*RPS6*), were performed (Figure 4.1). CALDOX cells were found to overexpress *p53CSV* by 1.76-2.73 fold compared to CAL51 ($p<0.001$).

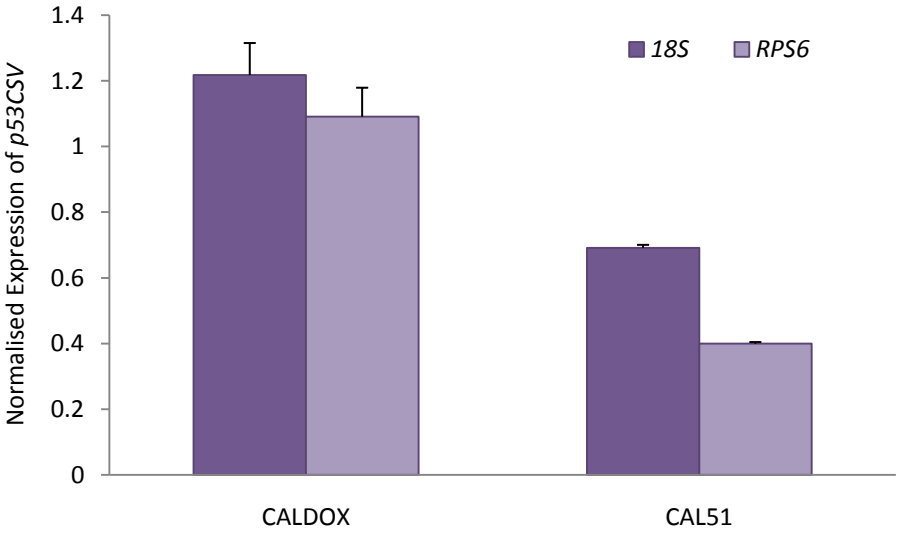


Figure 4.1 *p53CSV* is up-regulated in CALDOX cells. RNA was extracted from CALDOX cells grown in 0.4 μM doxorubicin and from untreated CAL51 cells. qPCR measured *p53CSV* RNA levels that were normalised with expression levels of *18S* and *RPS6*. Data represents a single experiment with 4 internal replicates (± SD). Experiment was twice repeated with similar results (data not shown).

4.2 Transient knock-down of p53CSV in CALDOX cells

In a previous experiment that transiently knocked-down *p53CSV* expression in CALDOX cells, an increase in sensitivity to doxorubicin after 2 and 4 days was observed. We repeated this experiment, using the same siRNAs (a 4 siRNA cocktail, as described in methods and materials), to knock-down p53CSV in CALDOX (Figure 4.2). Following 2 and

4 days in 0.4 µM doxorubicin, CALDOX cells transfected with *p53CSV* siRNA grew significantly less (64% and 66%, respectively) than those transfected with control *EGFP* siRNA (both $p<0.0001$). We repeated the experiment with similar results (data not shown). From this data, we confirmed that transiently knocking down *p53CSV* partially re-sensitised cells to doxorubicin.

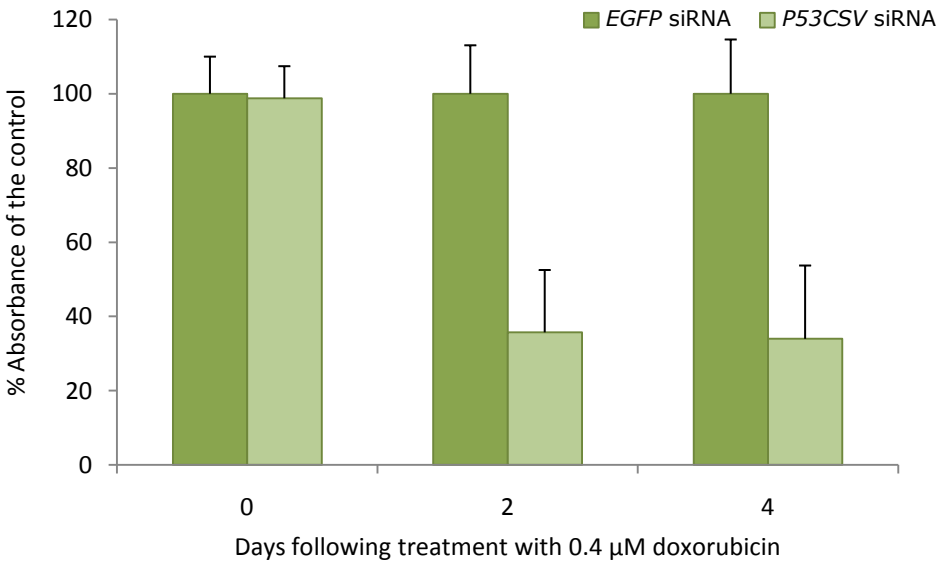


Figure 4.2 Transient knockdown of *p53CSV* sensitises CALDOX cells to doxorubicin. CALDOX cells were transiently transfected with a siRNA targeting *p53CSV* mRNA and then left to grow in the presence of 0.4 µM doxorubicin. Changes in cell numbers were determined by SRB growth assays. siRNA targeting *EGFP* mRNA, which is absent in human cells, was used as a negative control. CALDOX cells transfected with transient siRNA against *p53CSV* were more sensitive to and grew more slowly in 0.4 µM doxorubicin than cells transfected with control siRNA against *EGFP* (at 2 and 4 days). Data represents a single experiment with 6 internal replicates ± SD. Values are expressed relative to the absorbance obtained in cells transfected with *EGFP* siRNA.

4.3 p53CSV is up-regulated in CALDOX cells in the absence of doxorubicin

Our RNA array and qPCR data indicated that *p53CSV* is up-regulated in CALDOX cells. As cells were grown in the absence of doxorubicin for five days prior to the array, it is likely that *p53CSV* plays a role in resistance separately of the immediate response to the drug. We grew CALDOX cells in the absence of doxorubicin for 5, 23, and 38 passages (approximately 15, 69, and 114 days). RNA was extracted from these cells in addition to

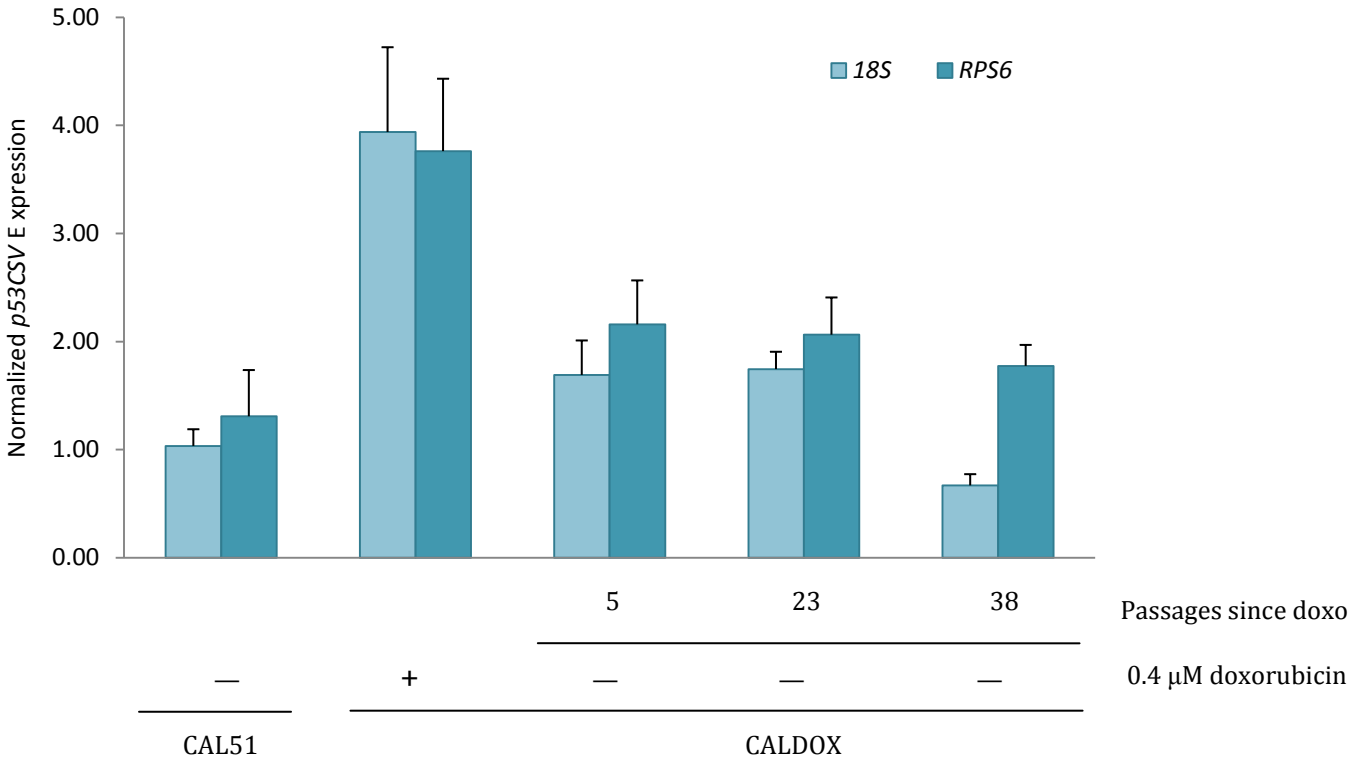


Figure 4.3 *p53CSV* is up-regulated in CALDOX cells in the absence of doxorubicin. RNA was extracted from CALDOX cells grown in 0.4 μM doxorubicin or in the absence of doxorubicin for 5, 23, and 38 passages and from untreated CAL51 cells. qPCR measured *p53CSV* RNA levels that were normalised against expression levels of *18S* and *RPS6*. Data represents a single experiment with 4 internal replicates (± SD).

CALDOX cells grown in doxorubicin and CAL51 cells. cDNA was obtained through reverse transcription reactions. Quantitative PCR with SYBR Green was performed, and the expression of *p53CSV* was measured and normalised with two house-keeping genes, *RPS6* and *18S*. CALDOX cells grown in doxorubicin had the highest levels of *p53CSV* RNA (*RPS6*: 2.88 fold; *18S*: 3.81 fold), likely as *p53CSV* is also part of immediate response to genotoxic stress. However, *p53CSV* remained up-regulated in the absence of doxorubicin, compared to CAL51 cells, for up to 23 passages ($p<0.05$). At 38 passages, the data was inconclusive between two normalisers, but suggest that *p53CSV* levels are similar to those in CAL51 cells at this point. Collectively, the data suggests that *p53CSV* is part of both the p53 response to doxorubicin and the long-term mechanism that CALDOX has developed against doxorubicin.

4.3 Expression of p53CSV in CALDOX and CAL51 in response to doxorubicin

Previous work in our laboratory suggested that in CALDOX cells, the p53 cell repair and arrest pathways remained activated at high doses of doxorubicin (5-10 μM) while CAL51 cells responded to the same doses with apoptosis. We aimed to confirm these results by ascertaining RNA levels of *p53CSV*, *p21* and apoptotic gene *p53AIP1* at different concentrations of doxorubicin using qPCR (Figure 4.4).

CAL51 and CALDOX cells were grown in increasing concentrations of doxorubicin for 24 or 48 hours. RNA was extracted and cDNA was obtained through reverse transcription reactions. Quantitative PCR with SYBR® Green was performed, and the expression of *p53CSV*, *p21*, and apoptotic *p53AIP1* was measured and normalised with two "house-keeping" genes, *RPS6* and 60S acidic ribosomal protein P0 (*RPLP0*). In one case, two normalisers did not agree and a third normaliser was used as indicated (40S ribosomal protein S14 or *RPS14*). The expression of a gene at each concentration of doxorubicin was made relative to its expression in untreated (0 μM) cells (e.g. the expression of each gene in CAL51 and CALDOX cells at 0 μM is set to 1). CAL51 gene expression was the combination of two separate experiments, where cells were grown in low doses (0, 0.1, 0.2, 0.3 μM) or high doses (0, 0.4, 1, 2, 5 μM) of doxorubicin but have been combined to observe trends.

As p53AIP1 is part of the p53-dependent apoptotic pathway, it was not expressed in untreated CAL51 cells, or at low doses of doxorubicin (up to 0.2 μM). *p53AIP1* expression experienced a 5000 fold increase at 0.3 μM doxorubucin. However, *p53AIP1* was a difficult gene to measure, as in our cells, it was either highly upregulated, or not expressed at all. Thus, although we attempted to measure *p53AIP1* with several different standards (CAL51 untreated or CAL51 + 1 μM doxorubicin), we could not produce a standard curve that could accurately measure both high and low expression levels of *p53AIP1*. If untreated CAL51 cells were used as a standard, cells expressing high levels of *p53AIP1* fell outside the linear

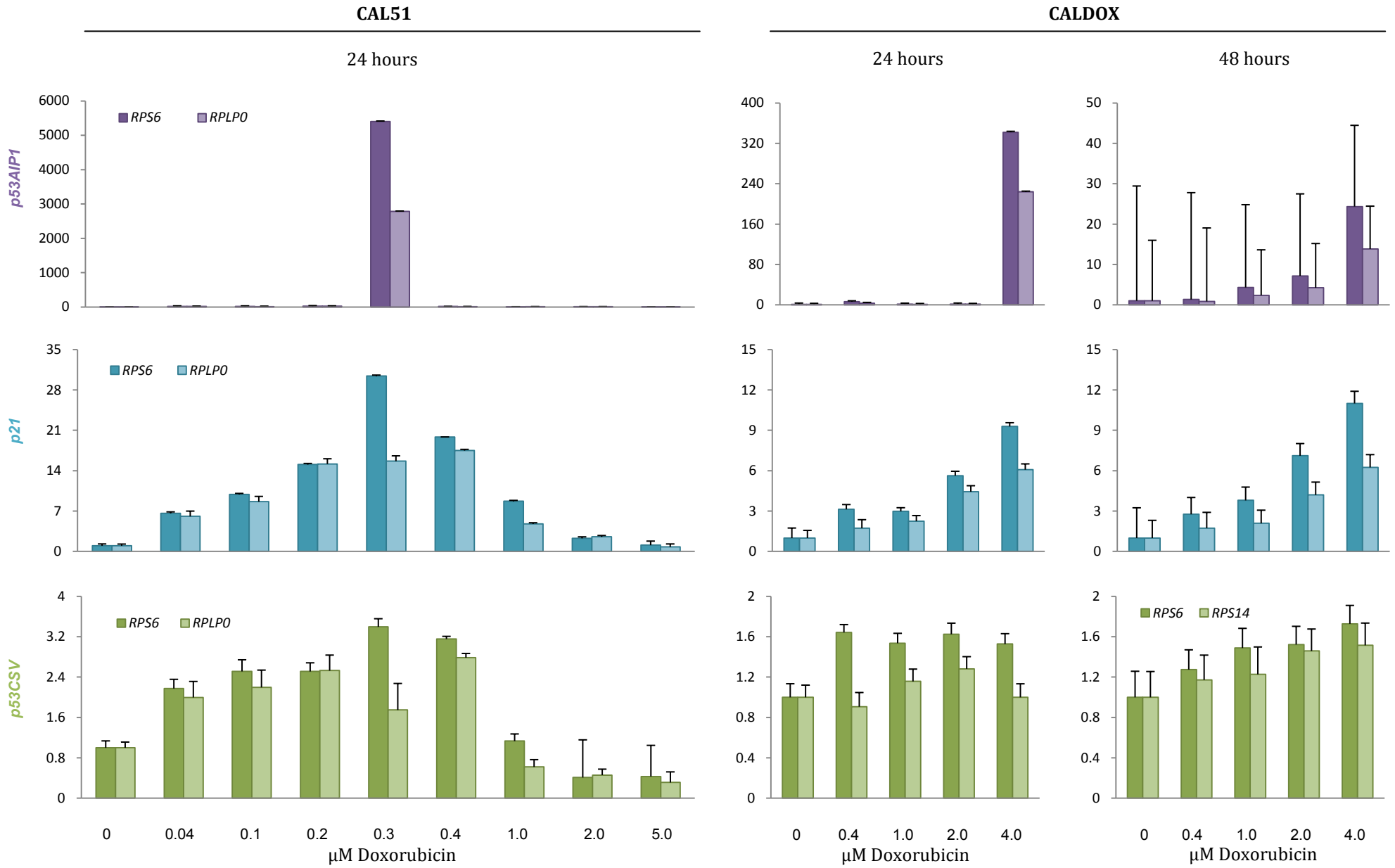

CAL51
CALDOX
24 hours
24 hours
48 hours
p53AIP1
p21
p53CSV
RPS6
RPLP0
RPS14
0 0.04 0.1 0.2 0.3 0.4 1.0 2.0 5.0
0 0.4 1.0 2.0 4.0
µM Doxorubicin

Figure 4.4 *p53CSV* and *p21* are expressed in CALDOX cells at low and high concentrations of doxorubicin. CALDOX cells were grown in 0, 0.4, 1, 2. and 4 μM doxorubicin for 24 and 48 hours. CAL51 cells were grown for 24 hours in 0, 0.04, 0.1, 0.2, and 0.3 μM doxorubicin and in separate experiment, 0, 0.4, 1, and 5 μM doxorubicin. RNA was extracted and expression levels of *p21*, *p53AIP1*, and *p53CSV* mRNA were quantified by qPCR and normalised with *RPS6* and *RPLP0*. A third normaliser *RPS14* was used in one case. Data represent single experiments with 3 internal replicates ± SD. Values are expressed relative to the mRNA levels obtained in cells growing in the absence of doxorubicin.

range of the standard curve. Similarly, if treated CAL51 cells were used, samples with low levels of *p53AIP1* fell outside the linear range. Thus, *p53AIP1* levels should be looked at to observe a trend, rather than as an accurate measure of fold increase. The two CAL51 experiments have been separated in Figure 4.5, to better observe the expression of *p53AIP1* at both low and high doses: it was most up-regulated at 0.3-0.4 μM and its expression decreased in cells treated with higher cytotoxic concentrations (1-5 μM).

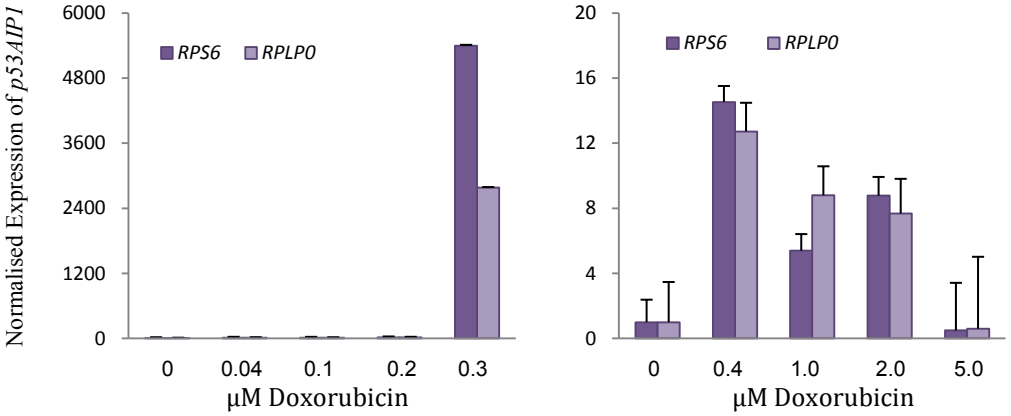


Figure 4.5 Pro-apoptotic *p53AIP1* is expressed at moderate doses of doxorubicin in CAL51 cells. Experiment as described in Figure 4.4: *p53AIP1* mRNA levels were measured by qPCR in CAL51 cells were grown for 24 hours in 0, 0.04, 0.1, 0.2, and 0.3 μM doxorubicin (A), and in a second experiment 0, 0.4, 1.0, 2.0, and 5.0 μM doxorubicin (B). *p53AIP1* mRNA was up-regulated at 0.3 μM and slowly decreased as cells underwent apoptosis following 1-5 μM doxorubicin treatment. Data represent two single experiments with 3 internal replicates (± SD). Values are expressed relative to the mRNA levels of two normalisers: RPS6 and RPLP0.

As expected, *p21* was not expressed in untreated CAL51 cells. Starting at low concentrations of doxorubicin, *p21* was up-regulated and its expression peaked at 0.3 μM before it was down-regulated at high concentrations of doxorubicin (0.4-5 μM), when cells,

through visual observation, had reached an apoptotic state. A similar pattern was observed for the expression of *p53CSV* was measured. *p53CSV* was up-regulated at 0.04 μM (2.0-2.2 fold) and its expression continued to increase up to 0.4 μM (2.8-3.2 fold) before decreasing as cells entered apoptosis (1-5 μM).

However, in CALDOX cells, *p53AIP1* was not highly expressed in cells treated with 0 - 2 μM doxorubicin. It was only after 24 hours with 4 μM doxorubicin that CALDOX cells up-regulated *p53AIP1*. Like the 24 hour time point, *p53AIP1* was not highly expressed between 0 - 1 μM doxorubicin at the 48 hour time point. However, it was up-regulated at slightly lower concentrations of doxorubicin than the 24 hour time point (7.7-8.8 fold at 2 μM) likely due to the fact that cells had spent an additional 24 hours under stress in doxorubicin.

p21 was up-regulated in CALDOX cells as doxorubicin concentrations increased at both 24 and 48 hour time points. *p53CSV* appeared to be induced by doxorubicin at 24 hours, although the data was less clear in this case. With normalization by *RPS6*, it experienced a 1.6 fold increase at 0.4 μM, but it was not further induced at increasing concentrations of doxorubicin. In contrast, *RPLP0* suggests that doxorubicin induced the expression of *p53CSV* from 1-2 μM, but not at concentrations between 0.4 and 4 μM. Previous experiments have shown that *p53CSV* is induced at 0.4 μM and it appears that *RPS6* is the more reliable normaliser because it agrees with previous observations. Furthermore, at 48 hours, three normalisers were used, with *RPLP0* being in disagreement with *RPS6* and *RPS14* (data not shown). Our protocol calls for the use of a third normaliser to decide between disputing data, and thus, we were able to conclude, using normalisers *RPS6* and *RPS14*, that *p53CSV* was induced with increasing doses of doxorubicin. Unlike in CAL51 cells, *p53CSV* and *p21* expression levels did not decrease as doxorubicin doses increased, and the majority of CALDOX cells did not appear to be undergoing apoptosis.

In summary, the data suggests that the arrest and repair pathway (p53CSV and p21) remains activated at higher concentrations in CALDOX than in CAL51 cells. In the parental cells, *p53CSV* and *p21* are up-regulated at low doses, but are not expressed at higher concentrations of doxorubicin. Furthermore, in CAL51 cells, apoptotic *p53AIP1* is up-regulated at lower doses of doxorubicin (0.3 μM) than in CALDOX (2-4 μM)—indicative of an earlier activation of cell death in the more sensitive parental cells.

4.4 Stable overexpression of p53CSV in CAL51

Transient knock-downs of *p53CSV* resensitised CALDOX cells to doxorubicin. Therefore, we postulated that stably over-expressing p53CSV in CAL51 would render cells more resistant to doxorubicin. CAL51 cells were transfected with linear vectors containing either *p53CSV* (513) or *EGFP* cDNA (D161) and a marker for selection with G418. Over-expression of *p53CSV* was checked using qPCR using two normalisers: *RPS6* and *RPS14*. We expected to see significant overexpression of *p53CSV* in transfected cells. However, CAL51 513a did not significantly up-regulate *p53CSV* compared to either control transfection (Figure 4.6; D161a and D161b; $p>0.05$). CAL51 513b showed slight, but significant up-regulation compared to both controls (1.49 fold, $p<0.05$), but an insignificant change compared to naive CAL51 cells.

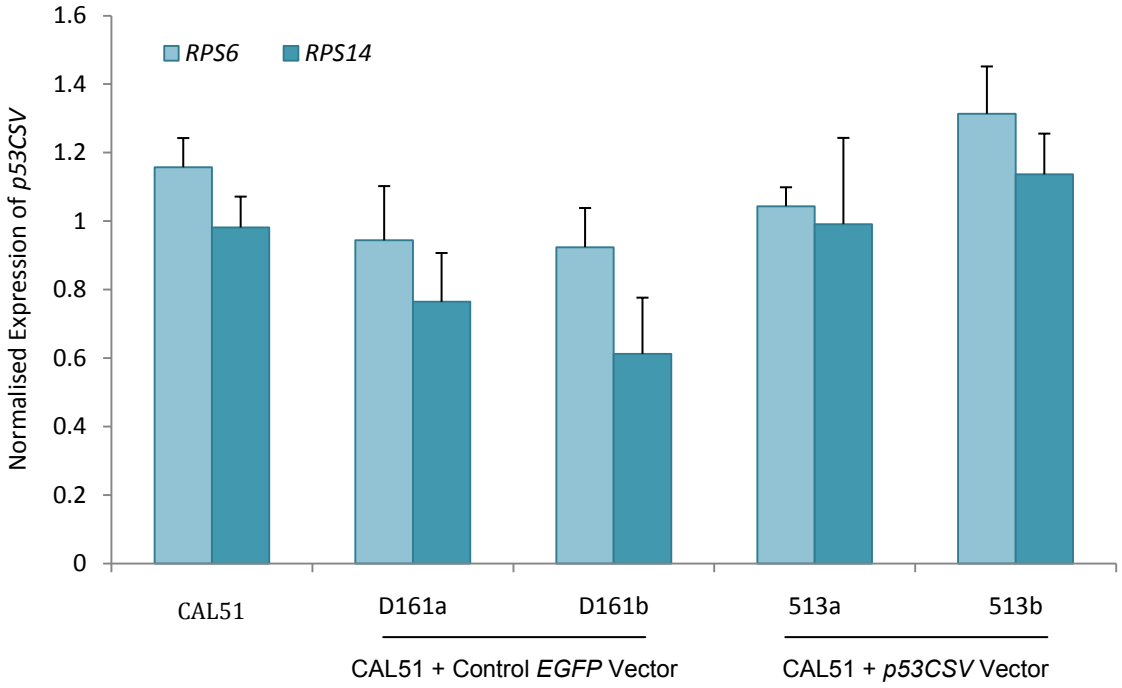


Figure 4.6 *p53CSV* is not overexpressed in CAL51 cells transfected with *p53CSV* cDNA. CAL51 cells were stably transfected with linear vectors containing a selection marker and either *p53CSV* or control (*EGFP*) cDNA. *p53CSV* mRNA levels were measured by qPCR. *p53CSV* values are expressed relative to the mRNA levels of normalisers *RPS6* and *RPS14*. Data represents a single experiment with 4 internal replicates ± SD.

As 513b was thought to overexpress *p53CSV* at levels similar to CALDOX cells, we proceeded with the drug sensitivity assay. We plated cells containing *p53CSV* (513a-b), their controls (D161a-b), and non-transfected CAL51 in 96 well plates. After 24 hours, cells were resuspended in medium containing doxorubicin at ten different concentrations (serial dilutions from 0 - 2.5 μM). Cells were left to grow in the presence of doxorubicin for four days, before an SRB assay was performed to measure cell growth (Figure 4.7). For each cell line, the concentration of doxorubicin that inhibited growth by 50% compared to untreated cells was calculated (Table 4.1). We did not see a significant difference between CAL51 cells transfected with *p53CSV* and those transfected with *EGFP*.

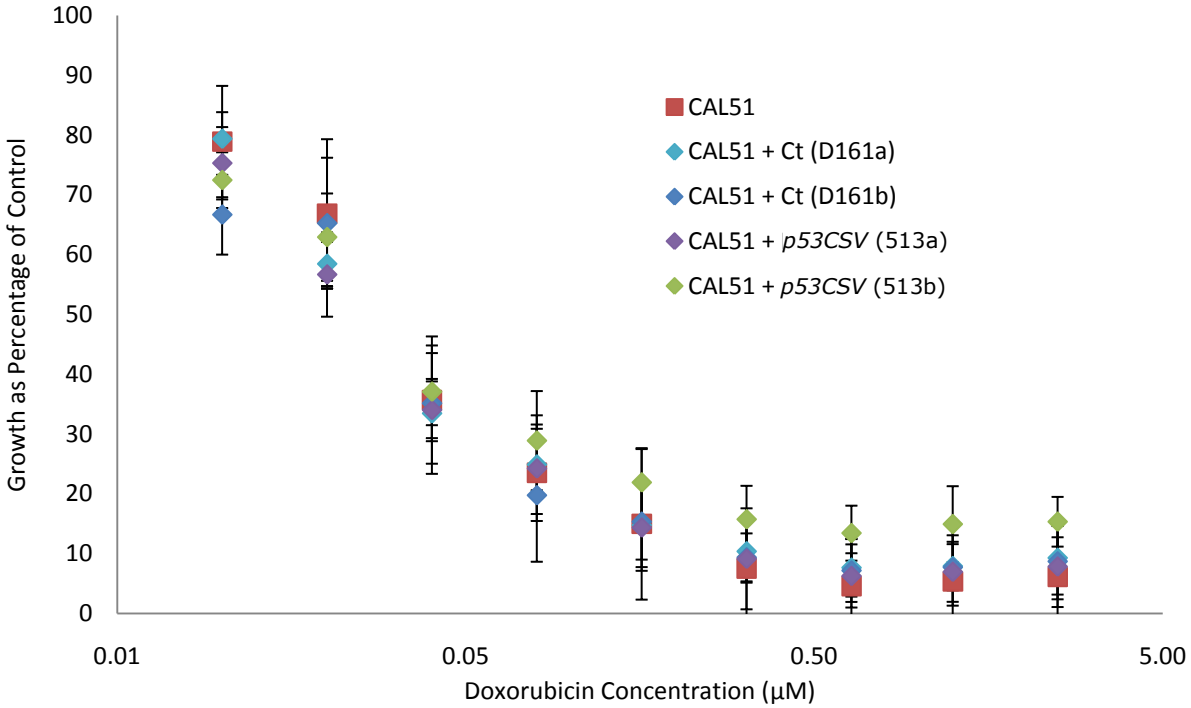


Figure 4.7 Doxorubicin sensitivity is unchanged in CAL51 cells transfected with *p53CSV* cDNA. CAL51 cells were stably transfected with linear vectors containing a selection marker and either *p53CSV* or control (*EGFP*) cDNA. Cells were grown in the presence 0 – 2.5 µM doxorubicin for 4 days. Changes in cell numbers were determined by SRB growth assays. Data represents a single experiment with 6 internal replicates ± SD. Values are expressed relative to the absorbance obtained in cells growing in the absence of doxorubicin.

Table 4.1 Half maximal inhibitory concentration (IC_{50}) of doxorubicin in CAL51 cells transfected with p53CSV cDNA

Cell Line	IC_{50} (µM)
CAL51	0.030
CAL51 + control vector (D161a)	0.027
CAL51 + control vector (D161b)	0.025
CAL51 + *p53CSV* (513a)	0.026
CAL51 + *p53CSV* (513b)	0.029

We sought to confirm poor *p53CSV* up-regulation through FACS analysis (Figure 4.8). As cells transfected with *p53CSV* were expected to express *p53CSV-EGFP* fusion proteins, we expected to observe fluorescence these cells, as well as in the *EGFP* controls. Fluorescence was observed in a sub-population of each of the *EGFP* control cell lines D161a (23.5%, Figure 4.8d) and D161b (20.7%, Figure 4.8e). In *p53CSV* transfected cell lines, no

fluorescent shift or sub-population was observed (Figure 4.8b-c), and we concluded that *p53CSV* had been unsuccessfully transfected in CAL51. Thus, we concluded that the lack of change in drug sensitivity was the result of unsuccessful transfections, rather than the inability of p53CSV to mediate drug resistance.

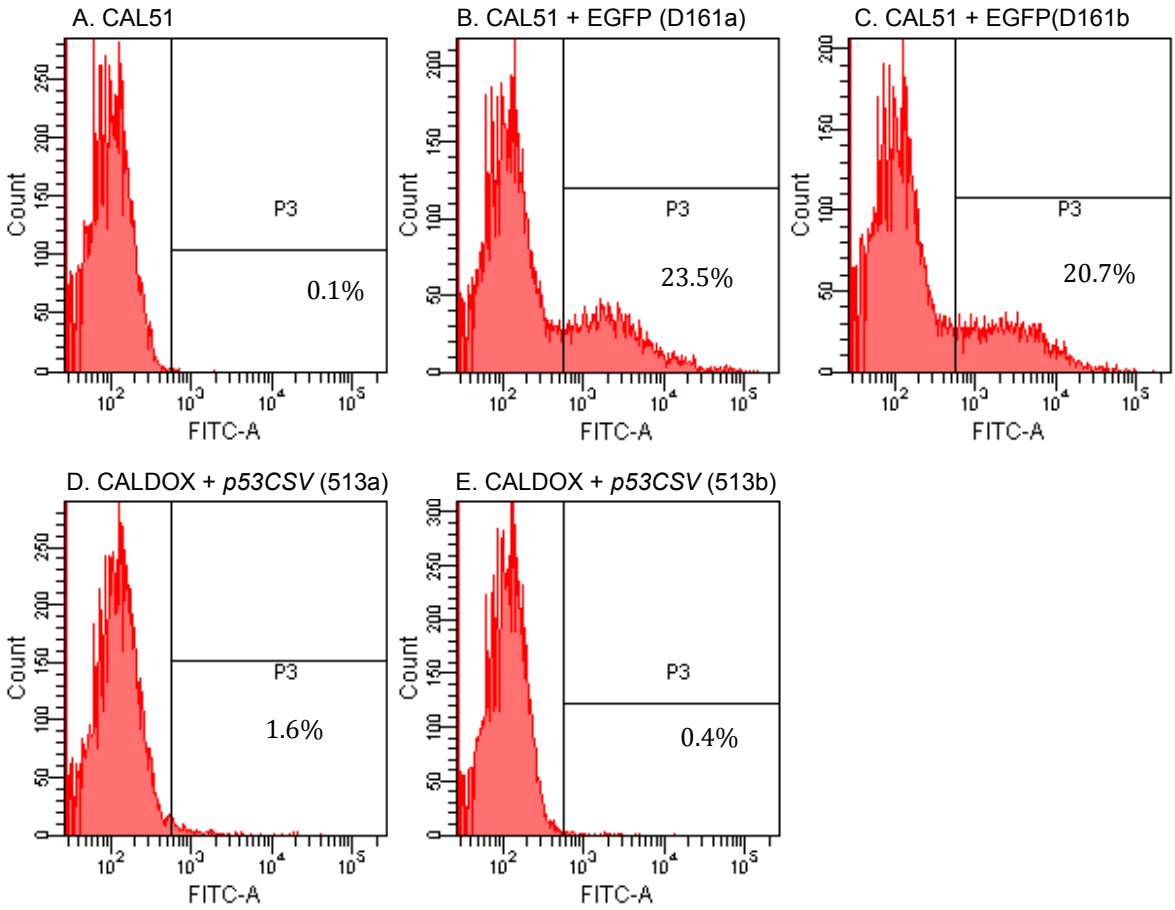


Figure 4.8 CAL51 cells transfected *p53CSV-GFP* cDNA do not fluoresce. Flow cytometry was utilized to observe fluorescence in CAL51 cells transfected with linear vectors that expressed either *p53CSV-GFP* or *EGFP* cDNA alone. Fluorescent cells (% in P3) were observed in the control vectors (B-C), but not in the *p53CSV* expressing cells (D-E), indicating that transfection had failed.

4.5 Stable knock-down of p53CSV, p21 and p53 in CALDOX cells: first attempt

To confirm that p53CSV mediated drug resistance in CALDOX, we stably transfected CALDOX cells with four different short hairpin RNAs (516-9) against *p53CSV* using a retrovirus. Down-regulation of *p53CSV* was confirmed with qPCR using two normalisers

(*RPS6* and *RPLP0*; Figure 4.9). A decrease in *p53CSV* RNA levels was observed in all four cell lines (516-519) compared to a cell line transfected with a scrambled short hair pin control (515). CALDOX cell lines 516 (*RPS6* 61.5% p=0.0015; *RPS14* 76.4% p=0.0036) and 517 (*RPS6* 73.0% p<0.0001; *RPS14* 78.5% p=0.0017) were selected for drug assays, as they experienced the greatest down-regulation of p53CSV.

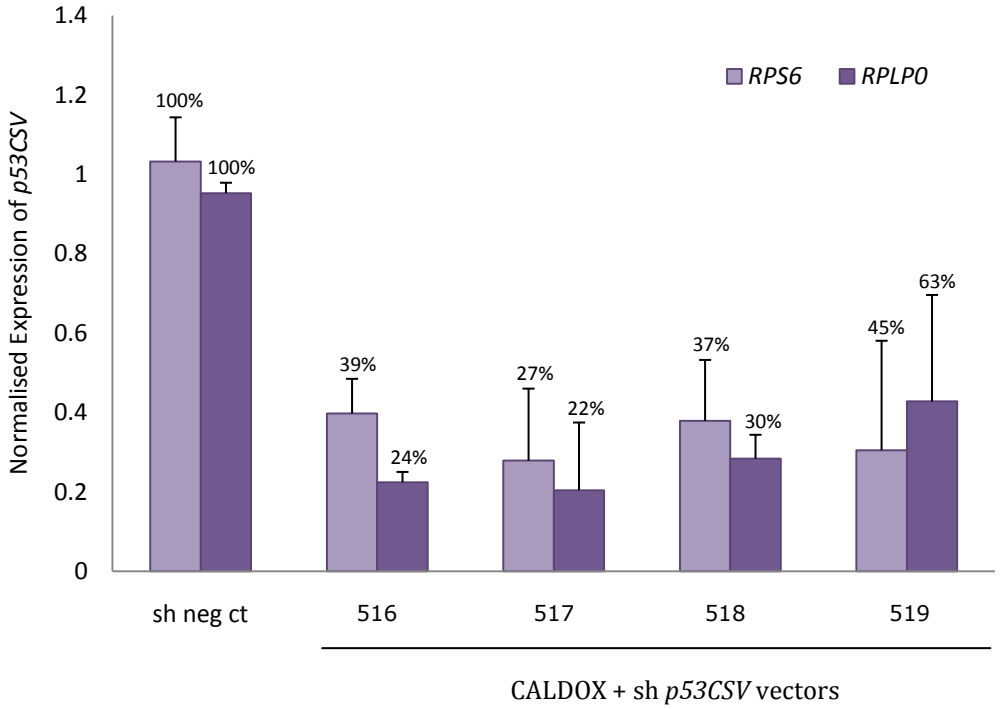


Figure 4.9 *p53CSV* is successfully down-regulated in CALDOX cells transfected with shRNA that targets *p53CSV*. CALDOX cells were stably transfected with short hairpin RNAs that targeted *p53CSV* (516-519) or scrambled control RNA. *p53CSV* mRNA levels were measured by qPCR. *p53CSV* values are expressed relative to the mRNA levels of normalisers *RPS6* and *RPLP0*. Data represents a single experiment with 3 internal replicates ± SD. This experiment was repeated at a later passage with the same result (data not shown).

Changes in sensitivity to doxorubicin following *p53CSV* knock-down were measured by SRB growth assays. Cells were plated overnight before resuspension in doxorubicin (0-2.5 μM) for four days. The absorbance at each concentration, representative of cell number and growth, was plotted as a percentage of the untreated control (Figure 4.10). The half-maximal inhibitory concentration (IC_{50}) were calculated (Table 4.2) for CALDOX (no transfection), CALDOX 515 (sh *EGFP*), and the two CALDOX *p53CSV* knock-downs (516-7). CALDOX

cells transfected with sh *p53CSV* were slightly more sensitive to doxorubicin than the negative control, but were similar in sensitivity to non-transfected CALDOX. Thus, these results are unlikely to be representative of a change in drug sensitivity.

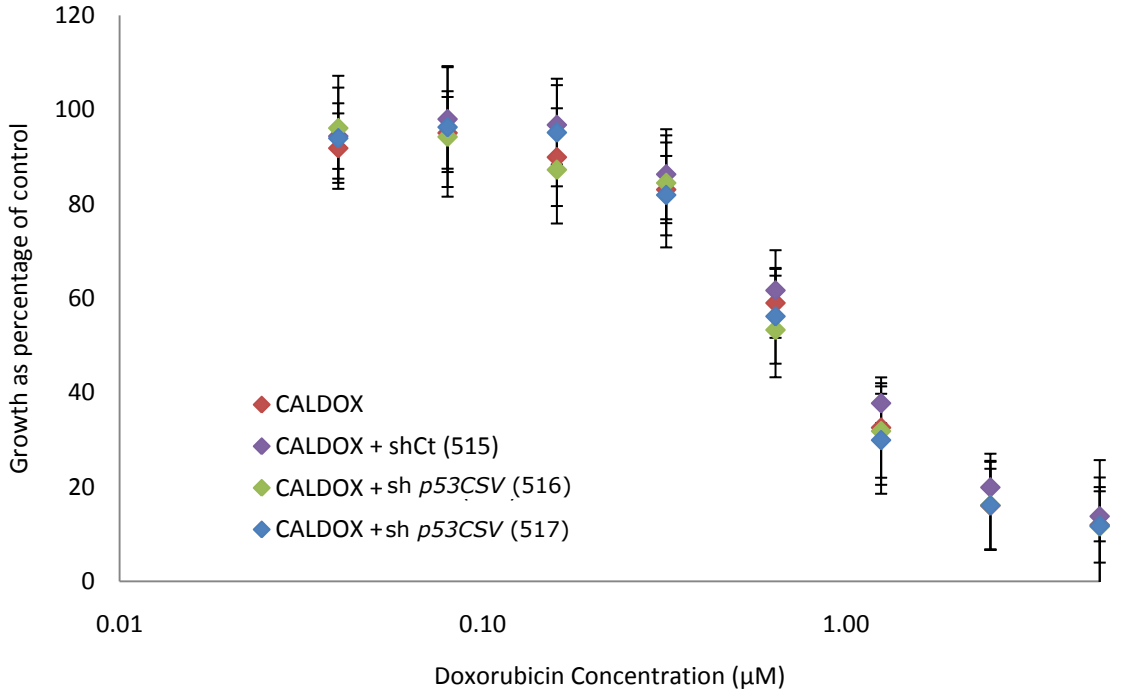


Figure 4.10 Doxorubicin sensitivity is unchanged in CALDOX cells that stably down-regulate *p53CSV*. CALDOX cells were stably transfected with short hairpin RNA that targeted either *p53CSV* (516-7) or scrambled control RNA. Cells were grown in the presence 0 – 5 µM doxorubicin for 4 days. Changes in cell numbers were determined by SRB growth assays. Data represents a single experiment with 6 internal replicates ± SD. Values are expressed relative to the absorbance obtained in cells growing in the absence of doxorubicin.

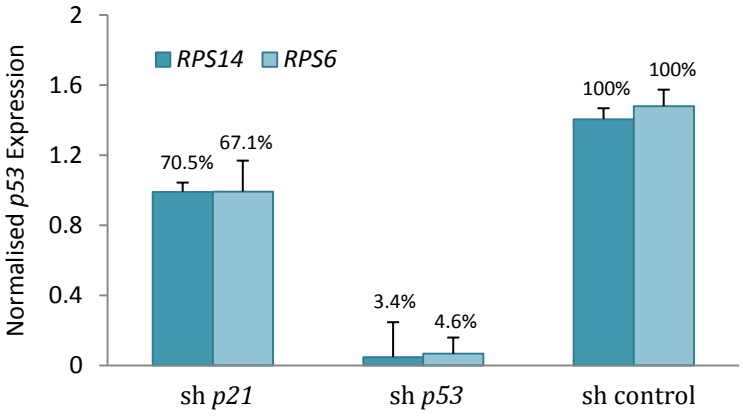
Table 4.2 Half maximal inhibitory concentration (IC_{50}) of doxorubicin in CALDOX cells transfected with short hairpin mRNAs that down-regulate *p53CSV*

Cell Line	IC_{50} (µM)
CALDOX	0.83
CALDOX + control vector (515)	0.93
CALDOX + sh *p53CSV* (516)	0.80
CALDOX + sh *p53CSV* (517)	0.79

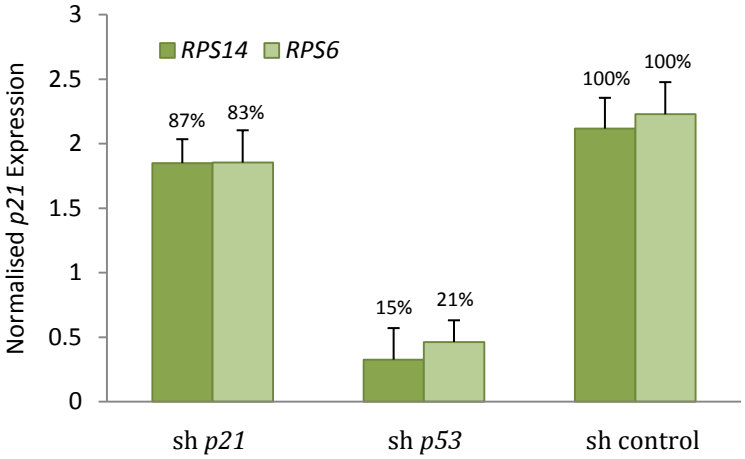
Array data indicated that *p21* was up-regulated in CALDOX cells, as were other p53-inducible genes. We postulated that the p53 pathway plays a role in drug resistance in

CALDOX and that knocking-down the expression of p53 and its targets could mediate drug resistance in CALDOX cells. CALDOX cells were stably transfected with short hairpin RNAs against *p53* or p21 using retroviral transfection. Quantitative PCR (using *RPS6* and *RPS14* as normalisers) confirmed that *p53* was knocked-down in CALDOX cells (Figure 4.11a). CALDOX cells transfected with shRNA against *p21* did not show decreased expression of *p21* (*RPS14* 12.6% p=0.1992; *RPS6* 16.8% p=.1397; Figure 4.11b), and the transfection was assumed to be a failure. Interestingly, the p53 stable knock-down showed some, but incomplete, down-regulation of *p53CSV* (*RPS14* 50.3% p=0.0095; *RPS6* 33% p=0.0109; Figure 4.11c). As expected, *p21* expression was significantly decreased in the *p53* stable knock-down (*RPS14* 84.6% p=0.0008; *RPS6* 79.2 p=0.0005; Figure 4.11b).

A.



B.



C.

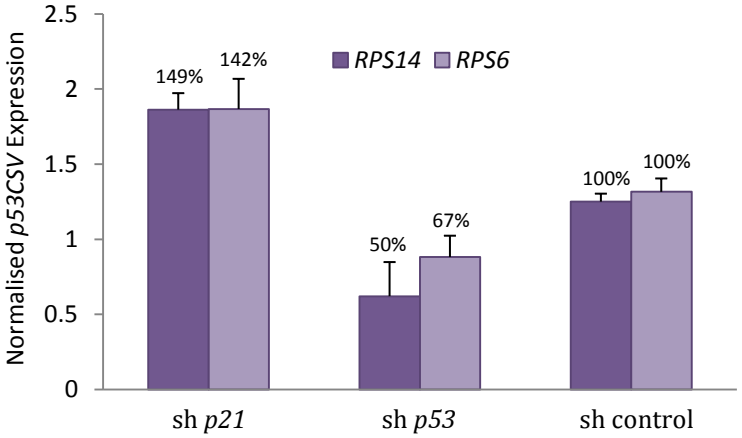


Figure 4.11 *p53* but not *p21* is successfully down-regulated in CALDOX cells transfected with shRNA that targets *p53* or *p21*. CALDOX cells were stably transfected with short hairpin RNAs that targeted *p53*, *p21*, or scrambled control RNA. *p53* (A), *p21*(B), *p53CSV* (C) mRNA levels were measured by qPCR. A. *p53* was successfully down-regulated in CALDOX transfected with sh *p53*. B. *p21* was down-regulated in CALDOX cells transfected with sh *p53* but not in cells transfected with sh *p21*. C. *p53CSV* was also down-regulated in cells transfected with sh *p53*.Values are expressed relative to the mRNA levels of normalisers R*P*S6 and *RPS14*. Data represents a single experiment with 3 internal replicates ± SD.

Changes in doxorubicin sensitivity in CALDOX cells transfected with sh *p53* were measured by an SRB assay as previously described (Figure 4.12). Growth was inhibited by 50% at 6.5 µM doxorubicin in CALDOX sh *p53* and 5.6 µM (Table 4.3) in the negative scrambled control—suggesting that CALDOX sh *p53* cells were 1.16 fold more resistant to doxorubicin. However, there was significant overlap in error, as observed in Figure 4.12, and we cannot conclude a significant change in drug sensitivity in CALDOX as a result of *p53* knock-down.

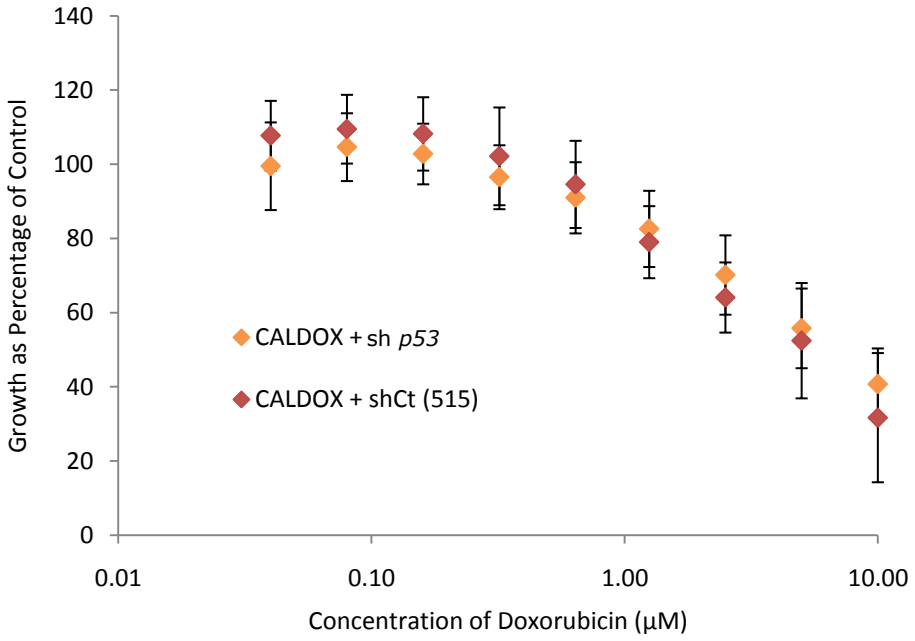


Figure 4.12 Doxorubicin sensitivity is unchanged in CALDOX cells that stably knock-down *p53*. CALDOX cells were stably transfected with short hairpin RNA that targeted either *p53* or control scrambled RNA. Cells were grown in the presence 0 – 5 µM doxorubicin for 4 days. Changes in cell numbers were determined by SRB growth assays. Data represents a single experiment with 6 internal replicates ± SD. Percentages are relative to the absorbance obtained in cells growing in the absence of doxorubicin.

Table 4.3 Half maximal inhibitory concentration (IC_{50}) of doxorubicin in CALDOX cells transfected with short hairpin mRNAs that down-regulate *p53*

Cell Line	IC_{50} (µM)
CALDOX + control vector (515)	5.64
CALDOX + sh *p53*	6.51

4.6 Stable knock-down of p53CSV, p21 and p53 in CALDOX: second attempt

As previous *p53CSV* and *p53* stable knock-downs in CALDOX cells did change not drug sensitivity and because the *p21* knock-down did not depress *p21* levels, we chose to repeat the short hair pin transfections for *p21*, *p53*, and *p53CSV*. Retroviral transfections were performed as described above by Dr. S. Raguz. Quantitative PCR was used to confirm *p21*, *p53CSV*, and *p53* expression with two house-keeping genes (*RPS6*, *RPS14*).

In CALDOX cells transfected with sh p53, p53 was successfully down-regulated (*RPS6* 92.4%, $p<0.001$; RPS14 93.0%, $p<0.001$; Figure 4.13a), as were *p21* (*RPS6* 82.2% $p=0.0001$; *RPS14* 83.5% $p=0.0001$; Figure 4.13b), and *p53CSV* (*RPS6* 15.4%, $p=0.051$; *RPS14* 21.6%, $p=0.027$; Figure 4.13c). In CALDOX cells transfected with shRNA against *p21*, *p21* was successfully down-regulated (*RPS6* 51.7% $p=0.0001$; *RPS14* 59.6% $p=0.0001$; Figure 4.13b). In CALDOX cells transfected with sh *p53CSV*, *p53CSV* was knocked down (Figure 4.13c) in both 516 (*RPS6* 81.1% $p<0.0001$; *RPS14* 76.9% $p<0.001$) and 517 (*RPS6* 81.1% $p<0.0001$; *RPS14* 84.1% $p<0.001$).

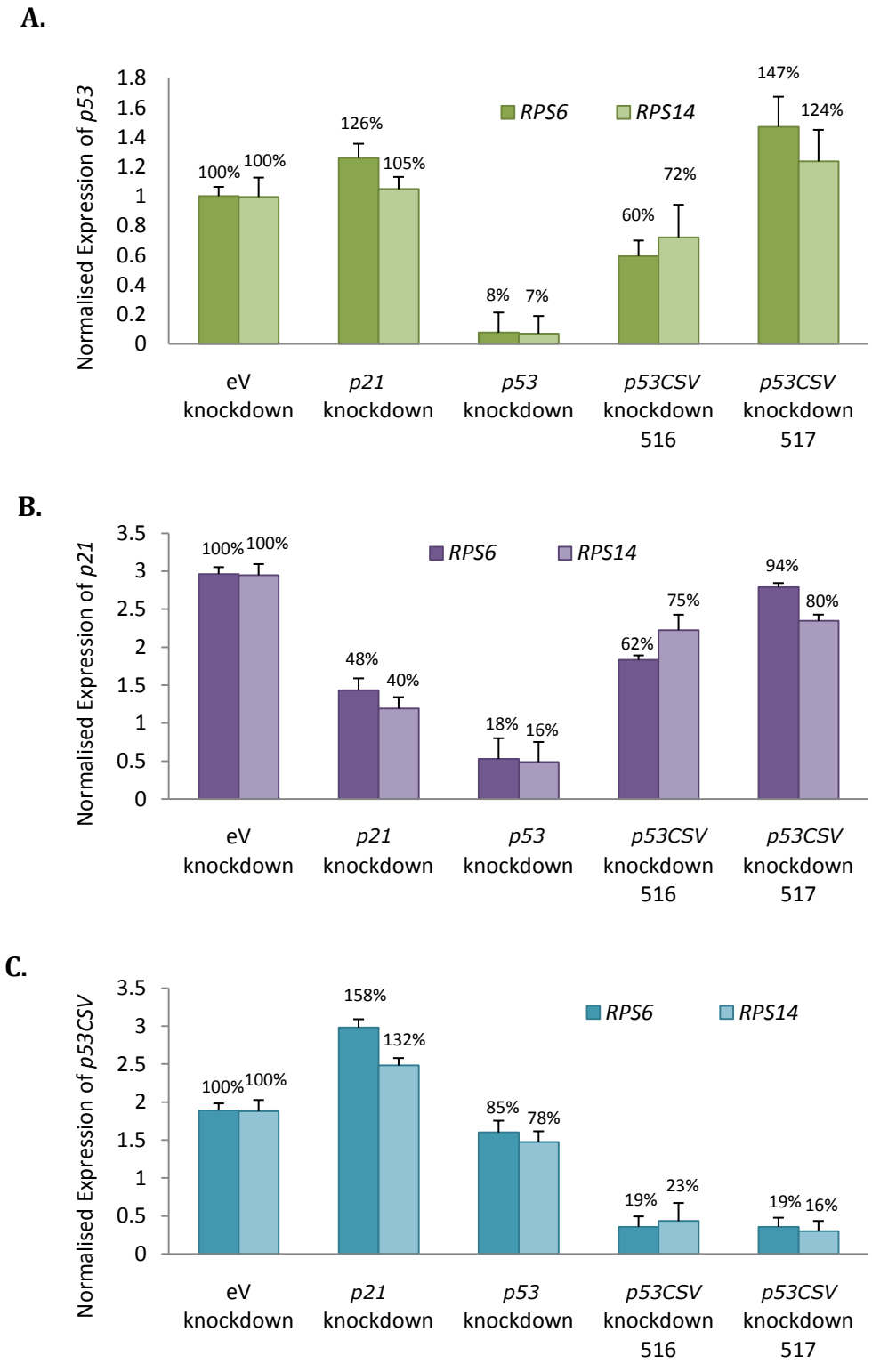


Figure 4.13 *p53*, *p21*, and *p53CSV* (516-7) are successfully down-regulated in CALDOX cells transfected with shRNA against *p53*, *p21*, or *p53CSV* (second transfection attempt). CALDOX cells were stably transfected with short hairpin RNAs that targeted *p53*, *p21*, or scrambled control RNA. *p53* (A), *p21*(B), *p53CSV* (C) mRNA levels were measured by qPCR. A. *p53* was successfully down-regulated in CALDOX transfected with sh *p53*. B. *p21* was down-regulated in CALDOX cells transfected with sh *p53* and sh *p21*. C. *p53CSV* was down-regulated in cells transfected with sh *p53* or sh *p53CSV*.Values are calculated against the mRNA levels of normalisers *RPS6* and *RPS14*. Data represents a single experiment with 3 internal replicates ± SD.

SRB drug assays were used to determine differences in doxorubicin sensitivity between stable cell lines. Two separate drug assays were performed to keep the experiment manageable. In the first, CALDOX shRNA against *p21*, *p53CSV* (517), and *p53* were compared to CALDOX cells transfected with negative controls (empty vector 514 and scrambled sh 515). The percent growths compared to untreated controls were plotted for each cell line, and significant overlap can be observed (Figure 4.14). Furthermore, calculated IC_{50}s did not significantly differ between cell lines (Table 4.4), suggesting that stably knocking down *p21*, *p53*, or *p53CSV* alone does not mediate drug resistance in CALDOX cells.

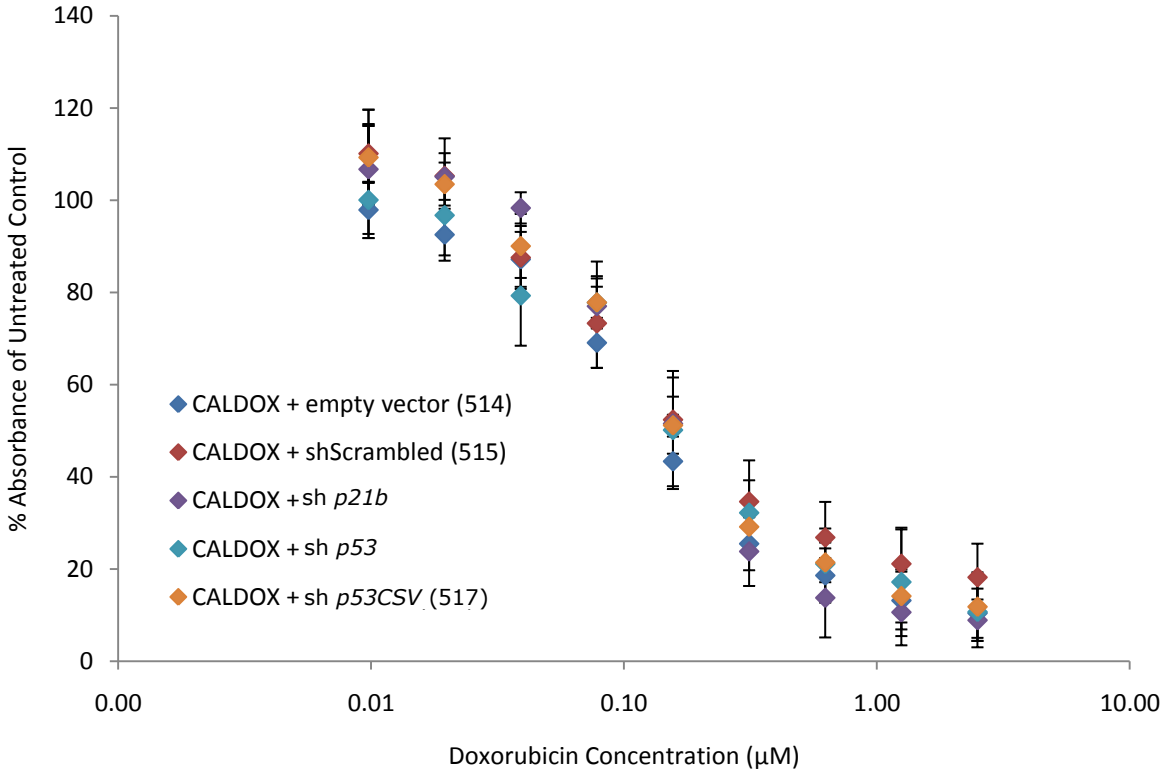


Figure 4.14 Doxorubicin sensitivity is unchanged in CALDOX cells that stably knock-down *p53CSV* (517), *p53*, or *p21* (second transfection attempt). CALDOX cells were stably transfected with short hairpin RNA that targeted *p53CSV*, *p53*, *p21*, or control (scrambled or *EGFP*) RNA. Cells were grown in the presence 0 – 5 µM doxorubicin for 4 days. Changes in cell numbers were determined by SRB growth assays. Data represents a single experiment with 6 internal replicates ± SD. Values are expressed relative to the absorbance obtained in cells growing in the absence of doxorubicin.

Table 4.4 Half maximal inhibitory concentration (IC_{50}) of doxorubicin in CALDOX cells transfected with short hairpin mRNAs that down-regulate *p53CSV* (517), *p21*, or *p53* (second attempt)

Cell Line	IC_{50} (µM)
CALDOX + control vector (514)	0.14
CALDOX + control vector (515)	0.18
CALDOX + sh *p21*	0.16
CALDOX + sh p53	0.19
CALDOX + sh *p53CSV* (517)	0.18

A second SRB assay was performed, to measure the differences in doxorubicin sensitivity between CALDOX transfected with sh *p53CSV* (516) or sh *p53* and negative sh controls. As before, the empty vector control appeared to be more sensitive than the other scrambled control and the experimental knock-downs. However, the scrambled control vector is more appropriate to use as a negative control, as it contains scrambled shRNA that can account for cellular changes due to sh transfections. Again, we did not observe significant changes between CALDOX sh *p53* or sh *p53CSV* (516) and the negative scrambled control (Figure 4.15 and Table 4.5).

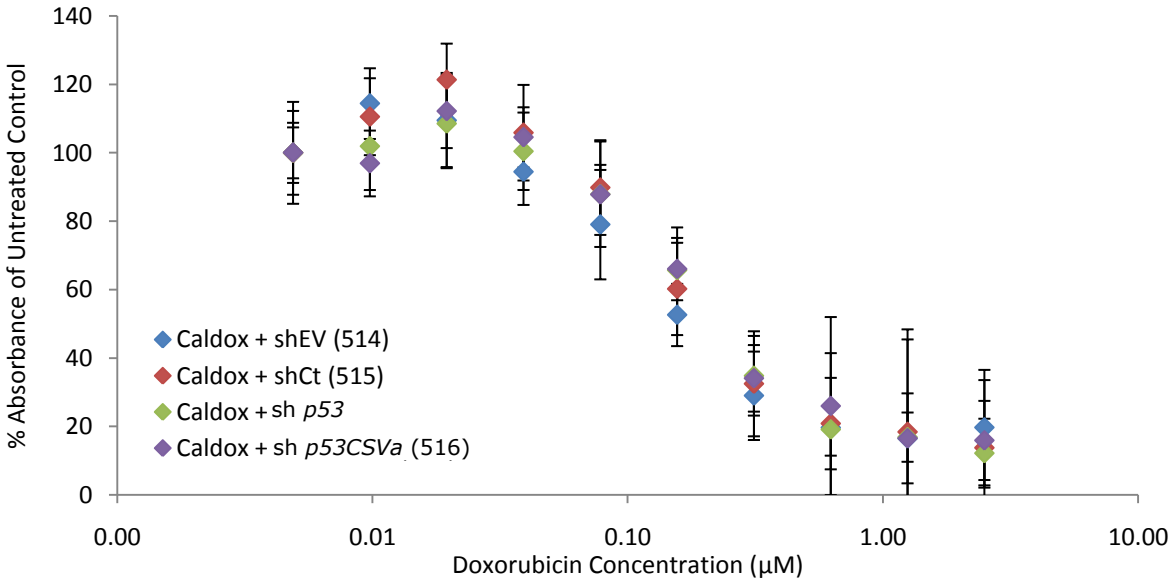


Figure 4.15 Doxorubicin sensitivity is unchanged in CALDOX cells that stably knock-down *p53CSV* (516) or *p53* (second transfection attempt). CALDOX cells were stably transfected with short hairpin RNA that targeted either *p53CSV* or control (*EGFP*) RNA. Cells were grown in the presence 0 – 5 µM doxorubicin for 4 days. Changes in cell numbers were determined by SRB growth assays. Data represents a single experiment with 6 internal replicates ± SD. Values are expressed relative to the absorbance obtained in cells growing in the absence of doxorubicin.

Table 4.5 Half maximal inhibitory concentration (IC_{50}) of doxorubicin in CALDOX cells transfected with short hairpin mRNAs that down-regulate *p53CSV* (516) or *p53* (second attempt)

Cell Line	IC_{50} (µM)
Caldox + empty vector (514)	0.19
Caldox + control vector (515)	0.23
Caldox + sh *p53*	0.23
Caldox + sh *p53CSVa* (516)	0.25

A final SRB assay was performed to confirm the role of p53 in drug sensitivity, as previous experiments yielded disparate results (Figure 4.16). Interestingly, CALDOX cells in which *p53* was down regulated were observed to be more sensitive to doxorubicin compared to the scrambled control in this case (0.63 fold resistant; Table 4.6). However, the observed error makes this result non-significant. In total, the role for p53 in CALDOX was inconclusive, as in three different experiments, shRNA against *p53* mRNA rendered cells more sensitive, indifferent, or more resistant to doxorubicin.

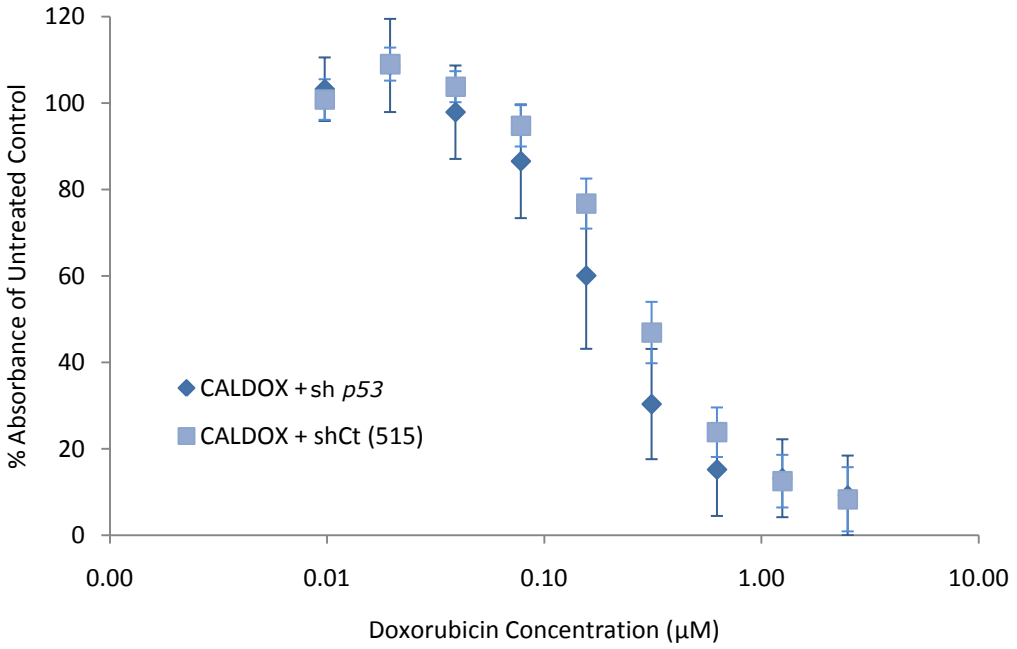


Figure 4.16 Doxorubicin sensitivity is not significantly different in CALDOX cells that stably knock-down *p53* (second transfection attempt). CALDOX cells were stably transfected with short hairpin RNA that targeted either *p53* or scrambled control RNA. Cells were grown in the presence 0 – 5 µM doxorubicin for 4 days. Changes in cell numbers were determined by SRB growth assays. Data represents a single experiment with 6 internal replicates ± SD. Values are expressed relative to the absorbance obtained in cells growing in the absence of doxorubicin.

Table 4.6 Half maximal inhibitory concentration (IC_{50}) of doxorubicin in CALDOX cells transfected with short hairpin mRNAs that down-regulate *p53* (second attempt)

Cell Line	IC_{50} (µM)
CALDOX + control vector (515)	0.61
CALDOX + sh *p53*	0.39

4.7 p53CSV expression in normal tissues

Our data indicated that *p53CSV* is not completely down-regulated in the absence of p53. Thus, it is possible that it has a role outside of the p53 response to cellular stress, and that it could be expressed in normal, non-tumourigenic tissues. We measured the gene expression of *p53CSV* in healthy human tissues using an RNA panel (Invitrogen). Following reverse transcription, qPCR was performed with four normaliser genes: *18S*, *RPLP0*, *RPS6* and *RPS14*. Normalisers *RPS6* and *RPS14* showed the most consistent pattern of expression and were considered reproducible.

p53CSV was widely expressed in normal human tissues (Figure 4.17). However, most tissues did not express *p53CSV* to the levels that it was expressed CAL51 and CALDOX cells. *p53CSV* was expressed at levels similar to CAL51 in the brain (fetal, whole, and cerebellum), placenta, skeletal muscle, and testis; however, *p53CSV* was most highly expressed in CALDOX cells by at least 1.5 times more (mean: 4.3 fold, *RPS6*) than normal tissues. *p53CSV* was least expressed in the heart, kidneys, and bone marrow.

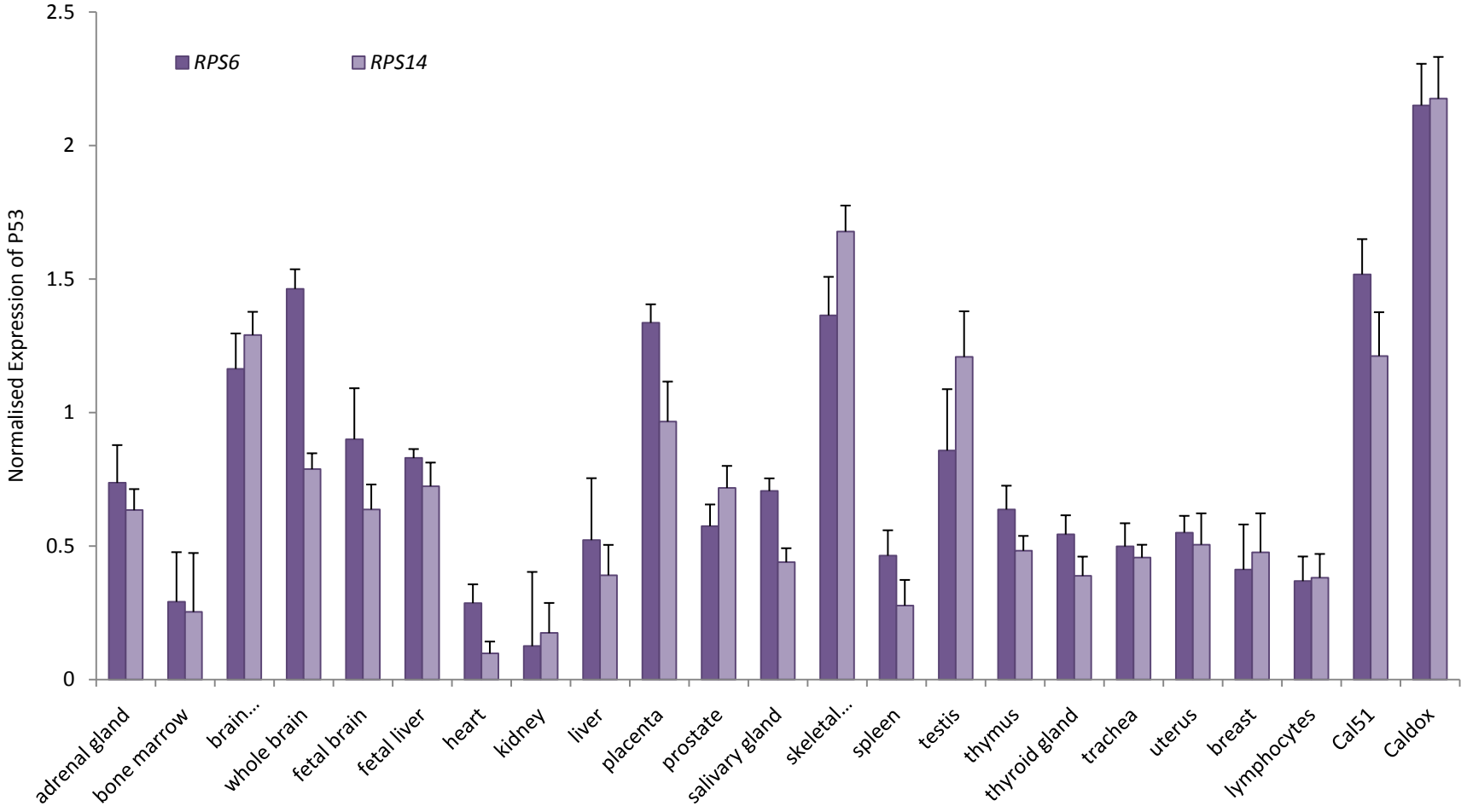


Figure 4.17 *p53CSV* is widely expressed in normal tissue. cDNA was obtained by reverse transcription from an RNA panel of healthy human tissues (Invitrogen). CAL51 and CALDOX RNA were included for comparison. Quantitative PCR was performed with four normalisers (*18S*, *RPLP0*, *RPS6*, *RPS14*) and the two that agreed most were considered reproducible. Data represents a single experiment with 3 internal replicates ± SD. Values are expressed relative to the mRNA levels of normalisers *RPS6* and *RPS14*.

4.8 p53CSV expression in estrogen-independent ER+ MLET5 and parental MCF7 cells

We next investigated the role of p53CSV in survival following DNA cellular stress in a second breast cancer cell line provided by Drs Ali Simak and Laki Bulawela. Their array data suggested that in their estrogen independent cells, ER+ MLET5, *p53CSV* was approximately 8 times up-regulated compared to the parental estrogen dependent MCF7 cell line. In both MCF7 and their MLET5 cells, the presence of estrogen or its absence did not change the expression of p53CSV. We aimed to confirm their data with qPCR. MLET5 and MCF7 cells were grown in medium with or without estrogen for 3 days. cDNA was obtained following RNA extractions and reverse transcription reactions. Quantitative PCR was performed with two normaliser genes (*RPS6* and *RPS14*), and p53CSV and p21 expression levels were measured.

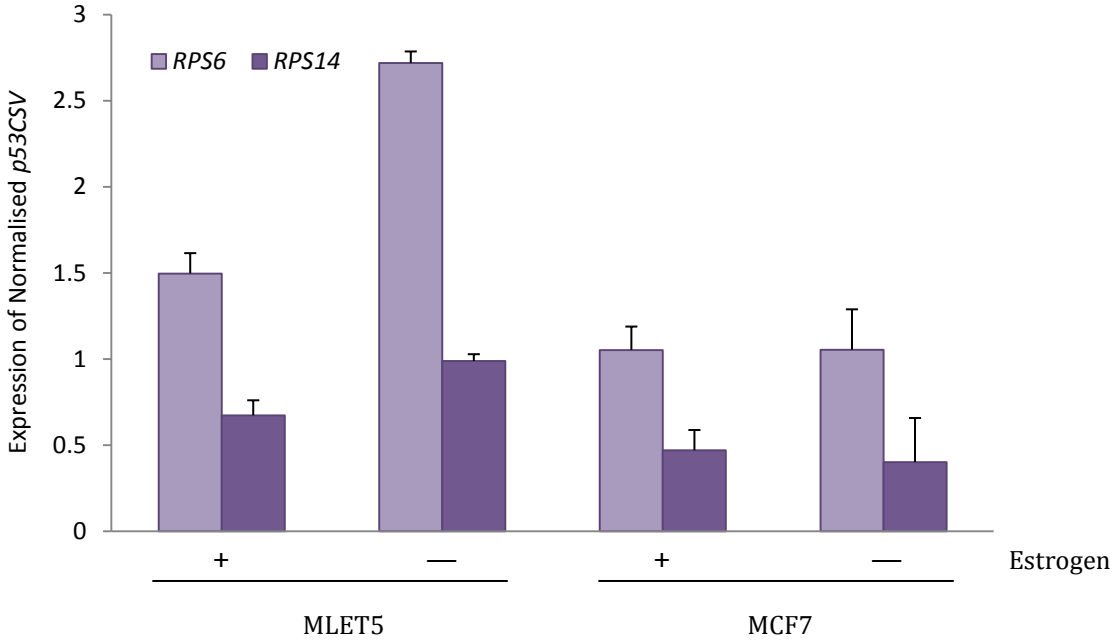


Figure 4.18 *p53CSV* is up-regulated and can be further induced by estrogen withdrawal in MLET5 cells. In MCF7 cells, *p53CSV* expression levels were unaffected by estrogen withdrawal. MLET5 and MCF7 cells were grown in the presence or absence of estrogen for 3 days. *p53CSV* mRNA levels were measured by qPCR. *p53CSV* values are expressed relative to the mRNA levels of normalisers *RPS6* and *RPLP0*. Data represents a single experiment with 3 internal replicates ± SD. MLET5 and parental MCF7 cells were kindly provided by Drs Laki Buluwela and Simak Ali.

In concordance with array data, *p53CSV* (Figure 4.18) was up-regulated in MLET5 cells (*RPS6* 1.41 fold, p=0.015; *RPS14* 1.43 fold, p=0.075). Yet, the absence of estrogen strongly induced p53CSV expression in MLET5 (*RPS6* 1.82 fold, p=0.0001; *RPS14* 1.47 fold, p=0.005), but not in MCF7 (*RPS6* 1.00 fold, p=0.99; *RPS14* 0.87 fold, p=0.7). We expected that *p53CSV* levels would parallel *p21* expression, as both are part of the p53-dependent arrest and repair pathway. However, although *p21* (Figure 4.19) was up-regulated in MLET5 cells (RPS6 1.94 fold, p=0.003; RPS14 1.96 fold, p=0.04), it was not strongly inducible in the absence of estrogen (i.e. cellular stress; *RPS6* 1.10 old, p=0.15; *RPS14* 0.89 fold, p=0.4). In MCF7 cells, *p21* was expressed at low levels in the presence of estrogen, but when cells were starved from estrogen, *p21* was induced and up-regulated (*RPS6* 2.51 fold, p=0.001; *RPS14* 2.15 fold, p=0.05).

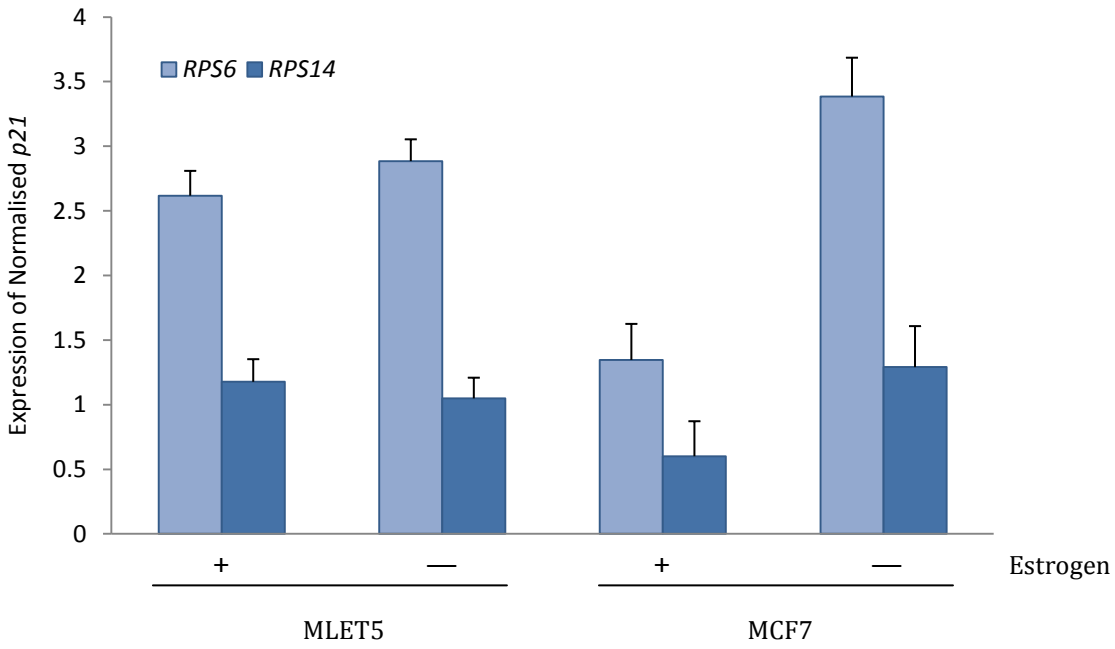


Figure 4.19 *p21* expression is up-regulated but unaffected by estrogen withdrawal in MLET5 cells. In MCF7 cells, *p53CSV* was significantly induced by estrogen withdrawal. MLET5 and MCF7 cells were grown in the presence or absence of estrogen for 3 days. *p21* mRNA levels were measured by qPCR. *p21* values are expressed relative to the mRNA levels of normalisers *RPS6* and *RPLP0*. Data represents a single experiment with 3 internal replicates ± SD. MLET5 and parental MCF7 cells were kindly provided by Drs Laki Buluwela and Simak Ali.

4.9 Drug resistance in CALDOX cells

Cells that become resistant to one type of chemotherapy often become resistant to other chemotherapeutic regimens as well (i.e. multi-drug resistant). We compared the sensitivity of our CALDOX cell line to parental CAL51 to multiple chemotherapy drugs. CAL51 and CALDOX cells were plated in 96-well plates at two separate concentrations and were left in drug-free medium for 24 hours by Dr. Raguz. Doxorubicin, Etoposide, Cisplatin, Taxol, Hydrogen Peroxide, Camptothecin, or 5-Fluorouracil was added the next day by serial dilution at concentrations as established by previous literature (Table 4.7). After 3 doubling times, an SRB assay was performed to measure cell growth. The percent growth compared to the untreated control (100%) at each concentration was determined and plotted with the help of Dr. Yagüe (data not shown). The IC_{50} was determined by linear regression from the steepest part of the plotted slope. Fold regression for each drug was calculated by dividing the CALDOX IC_{50} by the CAL51 IC_{50}. Interestingly, CALDOX cells were most resistant to Etoposide (150 fold) compared to CAL51. CALDOX cells were, unsurprisingly, more resistant to doxorubicin than CAL51 (45 fold). CALDOX cells were weakly resistant to Hydrogen peroxide (1.41 fold), Cisplatin (1.47 fold) and Taxol (2.62 fold) compared to CAL51. CALDOX cells were neither resistant nor more sensitive to Camptothecin (1.25 fold). However, these cells were more sensitive to 5-fluorouracil than parental CAL51 (0.47 fold less resistant).

Table 4.7 Comparing drug sensitivity of CALDOX and CAL51*

Drug (cell line)	Drug Concentration Range (µM)	LD_{50} (µM)	Fold Resistance
Etoposide			150
CALDOX	0 – 100	>100	
CAL51	0 – 10	0.65	
Doxorubicin			45.0
CALDOX	0 – 20	0.675	
CAL51	0 – 5	0.015	
Cisplatin			1.47
CALDOX	0 – 100	30.50	
CAL51	0 – 100	20.75	
Taxol			2.62
CALDOX	0 – 100	9.25	
CAL51	0 – 100	3.53	
5-Fluorouracil			0.47
CALDOX	0 – 1150	12.0	
CAL51	0 – 1150	25.5	
Camptothecin			1.25
CALDOX	0 – 1	0.088	
CAL51	0 – 1	0.070	
Hydrogen Peroxide			1.41
CALDOX	0 – 8.8	0.56	
CAL51	0 – 8.8	0.40	

*Drug assays and data analyses were performed with help from Dr. Sabeena Rashied, Dr. Selina Raguz, and Dr. Ernesto Yagüe.

5. Discussion

5. 1 The role of p53CSV and the p53 pathway in CALDOX cells

p53 is a transcription factor that is stabilized and activated in cells experiencing genotoxic stress. At reparable levels of DNA damage, p53 activates genes that induce cell arrest and DNA repair. At high and irreversible levels of genotoxic stress, p53 instead promotes the transcription of genes which lead to programmed cell death (apoptosis).[40] In tumourigenesis, p53 has been co-opted as a tumour suppressor, as both its p21 repair and apoptotic pathways prevent the proliferation of uncontrolled, oncogenic cells.[10] However, p53 may have a role in chemotherapy resistance. Inhibition of its apoptotic pathway and up-regulation of its arrest and repair pathway have been implicated in multidrug resistance.[72] This is particularly important in triple-negative breast cancer patients who do not have alternative treatment options outside of chemotherapy. p53 is commonly mutated in cancers.[25-26] New therapies that reactivate p53 are in development, and thus it is especially crucial we understand the implications that p53 may have in chemotherapy response.[73]

Our laboratory has generated a triple-negative doxorubicin resistant breast cancer cell line, CALDOX, to better understand the biological mechanisms of drug resistance in breast cancer. An RNA array revealed an up-regulation of *p21*, and also a novel gene to mammalian drug resistance, *p53CSV*, in CALDOX cells (compared to parental CAL51 cells). As CAL51 cells were not treated with doxorubicin, CALDOX cells were also grown in the absence of doxorubicin prior to the array, in order to separate genes that were differentially expressed as part of the drug response from those that mediated drug resistance. *p53CSV* was first described in 2005 as a p53-inducible gene and it was suggested that it plays a role in the arrest and repair pathway by inhibiting the cleavage of pro-caspase-9 and the activation of apoptosis.[57]

Quantitative PCR confirmed the array results. *p53CSV* was overexpressed in CALDOX cells treated with doxorubicin (compared to untreated CAL51 cells). A second experiment was performed to separate whether *p53CSV* was up-regulated as part of the molecular changes that accompanied MDR or as part of the cellular reponse to genotoxic stress, and showed that *p53CSV* was up-regulated in CALDOX cells that were grown for up to twenty three passages in the absence of doxorubicin. In addition, *p53CSV* was further induced in CALDOX cells that were grown in 0.4 μM doxorubicin.

Analysis of the activation of the p53 pathway in CAL51 and CALDOX cells at different concentrations of doxorubicin (representative of differing levels of genotoxic stress) was conducted. *p21* and *p53CSV* were activated at low levels of DNA damage in CAL51 cells (0.01-0.4 μM doxorubicin). At moderate to high levels of DNA damage, *p21* and *p53CSV* expression levels decreased as cells became increasingly sick and apoptotic. As *p21* and *p53CSV* expression levels decreased, apoptotic *p53AIP1* was induced at 0.3 μM of doxorubicin. At the highest levels of DNA damage, RNA expression decreased for all measurable genes—which was to be expected, as cells undergoing apoptotsis experience programmed break-down of RNA. This pattern of arrest and repair, as evidenced by *p21* and anti-apoptotic *p53CSV* at low doses of doxorubicin, but apoptosis at high doses was strikingly different in CALDOX cells. CALDOX cells did not undergo apoptotsis, even at high levels of genotoxic stress. Instead, *p53CSV* and *p21* were induced at low doses of doxorubicin and remained up-regulated in concentrations as high as 10 μM. *p53AIP1* was only induced at the highest concentration (10 μM). This shift in p53 response appeared to protect CALDOX cells from doxorubicin induced death. *p53CSV* correlated well with the p21 response, providing further support for its role in p53-induced cell survival.

Thus, we can infer that p53CSV is part of the immediate response to cellular stress in CALDOX cells, that it is up-regulated in CALDOX cells as part of the p53 pathway and that

its continued activity at high doses of doxorubicin, play a role in resistance. However, as we did not measure *p21* and *p53AIP1* levels in CALDOX cells deprived of doxorubicin for multiple passages, we cannot firmly conclude that the long-term up-regulation of p53CSV in CALDOX is representative of a similar widespread change in the p53 pathway. However, array data suggests that *p21* is also up-regulated in CALDOX, independently of doxorubicin (data not shown); and thus p53CSV is likely part of a widespread p53 dependent mechanism that promotes MDR in CALDOX cells.

Stable knock-downs of *p21*, *p53*, and *p53CSV* were generated in order to directly assess the role of the p53 pathway in drug-resistant CALDOX cells. Our transient knock-down of *p53CSV* rendered CALDOX cells more sensitive to doxorubicin, and we expected our *p53CSV* stable knock-downs in CALDOX cells would experience a similar effect. However, repeated p53CSV knock-downs, confirmed by qPCR, did not show a change in drug sensitivity. Transient knock-downs were not confirmed with qPCR, and it is possible that the change in drug sensitivity was due to a separate effect; however, RNAi is normally highly successful in completely knocking-down the gene. If we make this assumption, it is possible that complete knock-down is required to observe an effect, as stable knock-downs typically (and in our case) only down-regulate gene expression by 60-70%. As multi-drug resistance is normally the result of a multitude of genetic, transcriptional, and translational changes, partial down-regulation of *p53CSV* was not enough to resensitise cells to genotoxic stress. Until we repeat transient experiments and verify *p53CSV* knock-down, we were unable to determine whether p53CSV alone can mediate drug resistance in CALDOX cells. However, stable knock-downs suggest that p53CSV is part of a larger picture in mediating MDR in CALDOX cells. We postulated that this picture included other p53-inducible genes, specifically the up-regulation of antiapoptotic and DNA repair genes, and the down-regulation of pro-apoptotic genes, such as *p53AIP1*. To this effect, we attempted to stably

knock-down *p21* in CALDOX. However, our transfections were largely unsuccessful, with complete failure in one case and only 50% reduction in another. In the latter case, we did not see an effect on drug sensitivity. Like with *p53CSV*, this could be because it does not have a significant effect until it is completely knocked down. Even more likely, multiple genes would need to be successfully knocked down to observe an effect on drug resistance (such as *p21* and *p53CSV* together). Unfortunately, over-expression of *p53CSV* in CAL51 cells failed, and could not be used to draw further conclusions about the role of p53CSV in doxorubicin resistance.

Finally, we were able to successfully down-regulate *p53* in CALDOX cells. However, the resulting change in drug sensitivity was inconsistent, and in most cases, non-significant. This was not surprising, as p53 is associated with increases in drug sensitivity in some cases, but also in decreases in other studies.[74-75] Although the expression of wildtype p53 is popularly associated with chemosensitvity, a single point mutation on wildtype p53 rendered cells more sensitive to docetaxel because the mutant p53 could more efficiently induce apoptotic genes.[72] These varied responses are likely due to the disparate effects that the two p53 response pathways have in chemotherapy.[10] Ultimately, the choice to undergo arrest and repair or programmed death will depend upon the balance of pro-apoptotic versus anti-apoptotic proteins in the cell.[76] In CALDOX cells, we can infer that the inability to trigger p53-induced repair (thus sensitising cells to doxorubicin) would be negated by the equal inability to trigger p53-dependent apoptosis. This especially highlights the importance of targeting or expressing particular pathways in p53, rather than the transcription factor as a whole.

In short, our data supports previous studies that implicate p53CSV in early response to manageable levels of genotoxic stress as part of the p53-dependent pathway. In CALDOX cells, p53CSV likely plays a role in drug-resistance and drug-response as part of the p53 cell

repair and arrest pathway. However, p53CSV alone is not enough to mediate drug resistance in our triple-negative breast cancer cell line.

5.2 Role of p53CSV in MLET5 cells

The deletion of p53CSV ortholog MDM35 in yeast increased sensitivity to doxorubicin and was part of a larger survey for genes that mediated drug resistance by improving G_1 and early S phase repair and checkpoint mechanisms.[65] Similarly, in our CALDOX cells, p53CSV has a potential role in the p53-dependent cell arrest and repair pathway by inhibiting apoptosis. As this pathway is not drug specific, as long as the chemo or hormone regimen induces p53 response pathways, we obtained array data for *p53CSV* in MLET5 cells. MLET5 cells are an ER+ MCF7 breast cancer cell line that can grow independently of estrogen, and are less sensitive to tamoxifen and etoposide.

Generated by Dr. Simak Ali and Dr. Laki Buluwela, MLET5 cells survive low estrogen conditions through up-regulation of anti-apoptotic Bcl-2 family members and down-regulation of pro-apoptotic Bcl-2 family members.[77] The Bcl-2 family plays a role in the stabilization of the mitochondrial membrane, and the balance of pro-apoptotic to anti-apoptotic members determines cell fate. Furthermore, p53 plays a role in Bcl-2 (the anti-apoptotic founder of this family) inactivation both by direct binding and transcription of pro-apoptotic Bcl-2 family members.[78] Thus, the p53 pathway could play a direct role in the survival of MLET5 in low estrogen conditions. Their array data indicated that *p53CSV* was up-regulated by a factor of eight in MLET5 cells. The presence of estrogen did not affect the expression of *p53CSV* in either MCF7 or MLET5. We postulated that p53CSV played a role in preventing apoptosis in MLET5 cells, perhaps independently of p53—as stress did not induce the expression of *p53CSV* in either cell line.

Quantitative PCR was used to confirm the array data. In accord with the array, we found that *p53CSV* was up-regulated in MLET5 cells compared to MCF7 cells. Furthermore,

the presence or absence of estrogen did not affect the expression of *p53CSV* in MCF7 cells. However, our data disagreed with the array as we observed a significant induction of *p53CSV* during estrogen withdrawal in MLET5 cells. This was different from the expression of *p21* in MLET5 and MCF7 cell lines. Although *p21* was also significantly up-regulated in MLET5 cells, it was not further induced during estrogen withdrawal. Meanwhile, MCF7 cells that were grown in the absence of estrogen significantly induced the expression of *p21*. From this data, we can infer that the p53 anti-apoptotic pathway is up-regulated in MLET5 cells. However, *p53CSV* could be induced separately from the p53 arrest and repair pathway in low estrogen conditions in MLET5 cells. Knock-down experiments of *p53* and *p53CSV* in MLET5 cells would help to elucidate the importance of p53 and p53CSV in MLET5 cells under low and normal estrogen conditions. Nevertheless, it is clear that p53CSV is a potential therapeutic target for increasing sensitivity to a number of different therapies in varying types of breast cancer.

5.3 p53-independent role of p53CSV

In their original paper, Park and Nakamura claimed that *p53CSV* was completely dependent upon activation by p53.[57] When in fact, their data suggest that *p53CSV* is expressed at low levels independently of p53 activation and genotoxic stress; and that p53 knock-downs do not completely eradicate the expression of *p53CSV*. *p53CSV* would not be the only p53-induced gene to have a function separate from the p53 pathway. For example, *p21* is known to be transcribed and function separately from p53 and our p53 knock-downs did not completely abrogate *p21* expression.[79] Similarly, we found that *p53* stable knock-downs (93%), confirmed by qPCR, did not substantially down-regulate the expression of *p53CSV* (20%). However, in CALDOX cells that have been selected by their ability to escape apoptosis, *p53CSV* could be partially up-regulated independently of p53.

We found that, *p53CSV* is expressed throughout normal healthy tissues, albeit at lower levels than in CALDOX cells. This suggests that its p53-independent transcription is not limited to CALDOX. A previous study has implicated the necessity of p53CSV in the development of the larval kidney in *Xenopus* (African frog).[80] Its high degree of conservation, not only in mammals but also in lower organisms such as yeast, could indicate that its ability to inhibit apoptosis and allow G_1 arrest and DNA repair may also be important for the regulation of proliferating cells during development. Our lab is currently cloning the promoter region of *p53CSV* with the aim to elucidate the transcriptional regulation of *p53CSV*.

5.4 Multidrug resistance in CALDOX cells

Multidrug resistance is defined by the ability of cells to become less responsive to more than one type of therapy, including ones that they may not have been exposed to previously. SRB assays were performed to measure the resistance of CALDOX cells to a wide array of chemotherapeutic drugs. Interestingly, CALDOX cells were most resistant to etoposide (150 fold) and doxorubicin (45 fold) compared to CAL51 cells. Importantly, these two drugs assert their genotoxic effect through inhibition of topoisomerase II, which is important for unwinding DNA during replication in the S phase.[81] The other drugs do not induce apoptosis and cellular stress in this way, and camptothecin inhibits topoisomerase I. Previous research has indicated that topoisomerase II alterations or changes in its expression play a role in MDR.[82] However, topoisomerase II was not differentially expressed in CALDOX cells, but it is possible that single point mutations (undetectable in our comparative genomic hybridazation assays) may alter the ability of these drugs to interact and inhibit topoisomerase II. Current work in our lab is underway to sequence topoisomerase II for mutations in CALDOX cells.

Finally, CALDOX cells were not, on the whole, more resistant to Pgp substrates (doxorubicin, taxol, etoposide, camptothecin) than drugs that are not effluxed by ABC transporters (hydrogen peroxide, cisplatin, 5-fluoruracil)—supporting our preliminary data that ruled out drug efflux as a significant mediator of MDR in CALDOX cells.

5.5 Conclusions

The up-regulation of the p53 arrest and repair pathway is likely one player in the multifactorial drug-resistance of CALDOX cells. p53CSV likely plays a p53-dependent role in CALDOX cells as an inhibitor of apoptosis. However, some of its effects may be independent of p53. Nevertheless, p53CSV as well as p21 and other DNA repair proteins are an important reminder of the possible pro-tumourigenic and pro-resistant role of p53 in cancer. It will be important in future therapies when considering p53 or its two disparate downstream pathways as potential therapeutic targets. Furthermore, p53CSV is well conserved in mammals, and could play a role in development independently of p53.

6. Appendix

Table 6.1 Full length amino acid sequences of P53CSV in select vertebrates*

Protein Acc.	Gene	Organism	Full Amino Acid Sequence
NP_057483.1	TRIAP1	*Homo sapiens* (Human)	MNSVGEACTDMKREYDQCFNRWFAEKFLKGDSSGDPCTDLF KRYQQCVQKAIKEKEIPIEGLEFMGHGKEKPENSS
XP_001160868.1	TRIAP1	*Pan troglodytes* (Chimpanzee)	MNSVGEACTDMKREYDQCFNRWFAEKFLKGDSSGDPCTDLF KRYQQCVQKAIKEKEIPIEGLEFMGHGKEKPENSS
XP_546335.2	TRIAP1	*Canis lupus* familiaris (Dog)	MNSVGEACTDMKREYDQCFNRWFAEKFLKGDGSGDPCTDLF KRYQQCVQKAIKEKEIPIEGLEFMGHGKEKPESSS
NP_001073244.1	TRIAP1	*Bos taurus* (Cow)	MNSVGEACTDMKREYDQCFNRWFAEKFLKGDGSGDPCTDLF KRYQQCVQKAIKEKEIPIEGLEFMGHGKEKPESSS
NP_081209.1	TRIAP1	*Mus musculus* (Mouse)	MNSVGEACTDMKREYDQCFNRWFAEKFLKGDGSGDPCTDLF KRYQQCVQKAIKEKEIPIEGLEFMGHGKEKPENSS
XP_001077518.1	TRIAP1	*Rattus norvegicus* (Rat)	MNSVGEACTDMKREYDQCFNRWFAEKFLKGDGSGDPCTDLF KRYQQCVQKAIKEKEIPIEGLEFMGHGKEKPENSS
XP_429464.2	TRIAP1	*Gallus gallus* (Chicken)	MNSVGEACTELKREYDQCFNRWFAEKFLKGENDGDPCGQLF KRYQLCVQKAIKEKDIPIEGLEFMGPSKGKAENSS
XP_699741.1	zgc:112025	*Danio rerio* (Zebra fish)	MNSVGEGCTELKREYDQCFNRWFAEKFLKGDRSADPCSELFN KYHTCVQKAIKEKDIPIEGVEFMGPNSEKADS--

Table 6.2 Full length amino acid sequences of P53CSV and its ortologs in eukaryotes*

ID	Gene	Organism	Sequence	#AA
O43715	TRIA1	*Homo sapiens* (human)	----------MN-SVGEACTDMKREYDQCFNRWFAEKFLKGDSSG---- DPCTDLFKRYQQCVQKAIKEK---EIPIEGLE--FMG--------HGKEKPEN--SS----	76
Q9D8Z2	TRIA1	*Mus musculus* (mouse)	----------MN-SVGEACTDMKREYDQCFNRWFAEKFLKGDGSG---- DPCTDLFKRYQQCVQKAIKEK---EIPIEGLE--FMG--------HGKEKPEN--SS----	76
Q6INR6	TRIAAA	*Xenopus laevis* (African frog)	----------MN-SVGEECTDMKRDYDQCFNRWFAEKFLKGAGSG---- DPCTELFRRYRECVQKAIKDK---DIPVDGVD--FMG-------- PSKSKTESDGSS----	78
Q96VG1	YBAI	*S. pombe* (fission yeast)	----------MSSSVSEECTPAKKKYDACFNDWYANKFLKGDLHN---- RDCDELFAEYKSCLLKALKTK---KIDP-LLE----------AARKED--------	69
O60200	MDM35	*S. cerevisiae* (budding yeast)	------ MGNIMSASFAPECTDLKTKYDSCFNEWYSEKFLKGKSVE---- NECSKQWYAYTTCVNAALVKQ---GIKPALDEAREEAP------ FENGGKLKEVDK-----	86
Q9SMZ9	Y4331	*A. thalina* (thale cress)	MGLLKKKDSTSARSSTSPCADLRNAYHNCFNKWYSEKFVKGQWDK ----EECVAEWKKYRDCLSENLDGK---LLTRILEVDGELN-------- PTKQATDSKESSS---	92
P45967	YNZ	*C. elegans*	-----MSDRHMS-SIFPECDHLKQIYDKCFTEFF- QKFITPNYRHQYAVNPCERLHDVYKRCVEERLATQRPFEIDLDEIRK EYLNTDDDKLKDRQNNQKTNSENKCSSS	103

**All amino acid sequences were obtained by E. Yague from Genbank*

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