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In Search For New p53 Regulated Genes

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IN SEARCH FOR NEW p53 REGULATED GENES

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER BY SUPERVISION BY Meldrick Mpagi ENTITLED In Search For New p53 Regulated Genes BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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The p53 tumor suppressor protein has the ability to transactivate its target genes whose gene products are involved in carrying out cell cycle arrest, apoptosis, DNA repair, and senescence. Here, I report that two genes may be p53 regulated. Utilizing a microarray method to search for novel p53 target genes, I was able to identify a possible transcriptional target of p53 being solute carrier family 1a1 (SLC1a1). Along with that finding I also identified an E2F-target gene, minichromosome maintenance 10 (MCM10), as being p53 regulated. Gene expression profiling of MCF7 breast cancer cells treated with RNAi targeting Hdm2 and HdmX in order to reactivate p53 led to increased SLC1a1 transcript levels. DNA damage experiments in several cell lines and along with a p53 overexpression experiment established that p53 activation unexpectedly lead to no transcriptional increase in SLC1a1 expression. The results suggest that SLC1a1 is not a transcriptional target of p53 and may have been a false positive result from the microarray experiment.

Gene expression profiling of MCF7 breast cancer cells treated with RNAi targeting Hdm2 and HdmX in order to reactivate p53 led to a transcriptional decrease in MCM10 expression. DNA damage experiments along with siRNA targeting Hdm2 and HdmX

established that p53 activation leads to a reduction in MCM10 transcript levels. I am able to show that the p53-mediated reduction of MCM10 mRNA levels is due to p53-mediated transactivation of p21, a well-known p53 target involved in cell cycle arrest. These results suggest that p53 activation leads to a reduction in gene expression of E2F target genes involved in cell cycle progression through transactivation of p21.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

Table Page

I. Introduction

p53 also known as the "guardian of the genome" is a well-studied tumor suppressor protein. Its primary role is in conserving genome stability through controlling cellular proliferation. This is the mechanism by which it prevents genome mutation (7). The importance of p53 in higher level organisms can be found by its ability to regulate the cell cycle or induction of apoptosis (20, 29). In nearly all cases of cancer the p53 protein mutated and or is inactive in its function as a tumor suppressor. Inactivation of p53 can be due to a number occurrences like genetic mutations, or can be attributed to abnormal overexpression of p53's two negative regulators, Hdm2 and HdmX. Nonetheless, inactivation of p53 by mutations or its two negative regulators leads to a p53 protein which is unable to transactivate its target genes whose gene products are involved in carrying out cell cycle arrest, apoptosis, DNA repair, and senescence (18, 26, 40).

The list of known p53 target genes is vast and well-studied but there exist many novel p53 regulated genes whose identity and function still remain unknown (36). The importance of discovering novel p53 transcriptional targets stems from trying to establish a better understanding of the p53 signaling pathway. Some of the methods used to uncover potential p53 regulated genes are computational analysis, Chip on Chip arrays, and microarray technology (25, 37, 43). In this particular study we utilized a microarray method to search for novel p53 target genes.

In trying to identify novel target genes our laboratory employed a siRNA approach to lower levels of p53's negative regulators, Hdm2 and HdmX. Hdm2 possesses the ability to inhibit p53 transcriptional activity and decrease p53 protein levels through ubiquitin mediated proteosomal degradation (18). HdmX shares the ability to inactivate p53 transcriptional activity but does not possess the ability to destabilize the p53 protein via proteosomal degradation (14, 34). MCF7 breast cancer cells were chosen for this study because both Hdm2 and HdmX are found overexpressed in this cell line as compared to other human cell lines possessing wild-type p53 (50). Employing Affymetrix microarray technology, we evaluated changes in gene expression following the non-genotoxic activation of p53 in MCF7 cells through lowering levels of either HdmX or Hdm2.

During the course of analyzing the results from the microarray experiment, Solute Carrier Family Member 1a1 (SLC1a1) was identified as a putative p53 transcriptional target. The Solute Carrier group of membrane transport proteins includes 43 families with 298 transporter genes (9). The SLC group members transport a vast array of solutes including charged and uncharged organic molecules as well as inorganic ions (9). The solute carrier family 1 (SLC1) includes five high-affinity glutamate transporters which have a crucial role in protecting neurons against glutamate toxicity in the central nervous system (15). SLC1a1 localizes on the neuronal cell membrane and uptakes glutamate from the extracellular space. This function is crucial for the termination of glutamate-mediated signaling in synapse and also for prevention of neuronal death by suppressing glutamate toxicity (17). It was discovered that SLC1a1 might possess additional mechanisms in terms of neuronal protection. One group revealed a unique "rescue" function of SLC1a1

independent of removal of extracellular glutamate where SLC1a1 possesses an anti-apoptotic function in PC12 cells which have been NGF deprived and motor neurons that have been injured (16). Interestingly, another lab was able to show that nerve growth factor withdrawal-mediated apoptosis is in some part regulated by p53 (41). All of this information taken together may implicate a novel p53-mediated pathway in neuronal cell death.

Another alternative function was found by Aoyama *et al* who reported that genetically null SLC1a1(-/-) mice have reduced levels of glutathione and with aging develop brain atrophy as well as behavioral changes (1). SLC1a1 has a distinguishing feature which allows it to bind and transport cysteine more effectively than astrocyte glutamate transporters (48). Cysteine is the rate-limiting substrate for the synthesis of glutathione which is important for the metabolism of hydrogen peroxide, nitric oxide and other reactive oxygen species (5). In summary this group suggested that SLC1a1 deficiency leads to impaired glutathione metabolism, oxidative stress and age-dependent neurodegeneration (1). Loss of glutathione has been used as a marker in neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (2). It was found that glutathione depletion in neurons and fibroblast cells leads to increased expression and activation of p53 ultimately leading to cell death (6). Elucidating p53's possible transcriptional regulation on a gene with such diverse functions as well as links to neurodegenerative diseases may be a huge step in uncovering p53's role in brain.

In most cells p53 plays vital role in the regulation of cell proliferation where in response to cellular stress p53 acts as a transcription factor to control a large group of genes of which gene products are involved in DNA repair, apoptosis, and cell cycle arrest (42). p53's capacity to regulate the cell cycle comes from its ability to control expression of p21(or cyclin-dependent kinase inhibitor 1A) which functions by inducing G1-S arrest (33). p53 regulation of p21 connects p53 with the RB tumor suppressor pathway leading to cell cycle arrest in response to cellular stress (45). Retinoblastoma gene (RB) has the primary role of controlling excessive cellular growth by inhibiting cell cycle progression (28). Rb is a member of the pocket protein family which possesses a functional binding site for other proteins (19). Rb's role to impact the cell cycle comes through its ability to bind the E2F transcription factor leading to a complex that inhibits E2F transactivation of its target genes involved in cell cycle progression (38, 39). Sequences homologous to the E2F DNA binding site have been found upstream of cellular genes that encode proteins involved in the G1-S transition and in DNA synthesis, including thymidine kinase, dihydrofolate reductase, DNA polymerase alpha, *cdc2*, c-*myc*, N-*myc*, c-*myb*, B-*myb*, and cyclin D1 (10, 21).

Analysis of the microarray study revealed a group of E2F regulated genes involved in G1-S cell cycle progression that were found to be down-regulated after p53 activation via RNAi targeting Hdm2 and HdmX. Mcm10, an E2F targeted gene (46), was chosen as a putative p53 repressed gene. Mcm10 was first identified in *Saccharomyces cerevisiae* as an essential DNA replication factor whom participates in an important role in the pre-RC assembly (23). Mcm10 proceeds the loading of the MCM 2-7 complex onto replication

origins (13) and interacts genetically with Cdc45 and DNA polymerases (12). Knockdown of Mcm10 using RNA interference lead to G2 arrest caused by slow and incomplete replication (32).

In this present study, I examined two genes detected as potential p53 regulated genes. First, SLC1a1 represented a novel p53 induced gene. Consequently, a number of DNA damaging experiments were performed to monitor SLC1a1 expression in response to cellular stress. Alternatively, RNAi targeting Hdm2 and HdmX along with p53 overexpression were carried out to monitor SLC1a1 transcript levels. I show that SLC1a1 expression is increased in response to activation of p53 by RNAi targeting its two negative regulators but its expression does not respond like a well-known p53 target in terms of cellular stress or p53 overexpression.

Secondly, I examined a group of E2F-1 regulated genes (MCM10, E2F-1, and cyclin A2) that appeared uniformly repressed by p53 activation. I was able to show that p53 regulates E2F-1 targets involved in G1-S cell cycle progression. I was also able to show that p53 activation leading to down-regulation of E2F-1 targets is through transactivation of p21. Through knocking-down expression of p21, the down-regulation of E2F-1 targets by p53 activation is completely abrogated.

II. Materials and Methods

Cell lines, siRNA, and chemotherapeutic reagents

The human tumor cell lines, U87 (glioblastoma), MCF7 (breast carcinoma), HCT116-p53+/+ and HCT116-p53-/- (colorectal carcinoma), MCF10A (mammalian mammary epithelial), H1299 (non-small cell lung carcinoma), HCN2 (primary cortical neurons) were grown in Dulbecco's modified Eagle medium(DMEM) supplemented with 10% bovine growth serum (BGS) and 10 μg/mL gentamicin unless otherwise indicated. siGENOME duplex RNA targeting mRNA from HdmX, Hdm2, p53, p21, and a non-targeting control siRNA were obtained from Dharmacon Research, Inc. and siRNA transfection was using Oligofectamine or Lipofectamine 2000 (Invitrogen) as described below. Doxorubicin hydrochloride, bleomycin, and AraC were all purchased from Sigma, Inc. UV-C irradiation was performed using UV-C Stratalinker 1800 (Stratagene).

Reverse transfection

MCF7 and MCF10A cells were reverse transfected at the time of seeding plus transfected again 24 hours later. Cells were seeded at 200,000 cells per well in 6-well plates, in antibiotic free DMEM containing 1% BGS in a small volume. Cells were

reverse transfected with 100 nM siRNA (Dharmacon Research, Inc) using Lipofectamine 2000 (Invitrogen).

	24-well plate	**6-well plate**	**6-cm dish**
Cell Number	100,000/well	200,000/well	700,000/dish
Volume of siRNA + SFM	2.5 µL siRNA + 182.5 µL SFM	5 µL siRNA + 180 µL SFM	7.5 µL siRNA + 177.5 µL SFM
Volume of Lipofectamine + SFM	1.5 µL lipid + 113.5 µL SFM	3 µL lipid + 112 µL SFM	4.5 µL lipid + 110.5 µL SFM
Total Volume	0.5 mL	1.0 mL	1.5 mL

Table 1. Volumes of siRNA, lipid, and serum free media (SFM) for siRNA transfections. The table lists the appropriate volumes of each component necessary to setup a 100 nM siRNA transfection in the various cell culture vessels.

Upon a five hour incubation, media was removed and cells were re-feed with DMEM containing 10% BGS. Twenty hours later, cells were transfected again with 100 nM siRNA in a small volume of serum free media using Oligofectamine (Invitrogen). After a four hour incubation, an equal volume of 20% serum DMEM is added to each well/dish without removing the transfection mixture. Total RNA was isolated 24 hrs post the last siRNA transfection unless indicated otherwise.

Plasmids and Transfections

For ectopic expression of p53: pcDNA3.1-p53, pcDNA3.1-mutant p53(R175H), pcDNA3.1-EGFP. Transient transfections of these plasmids were carried out using

lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, with regards to cell density, DNA amounts, and DNA:lipid ratios.

Quantitative RT-PCR

Cells were harvested directly in the culture dish and total RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer's protocol. The RNA was quantified by spectrophotometer reading at 260 nm. Next, 1 μg RNA was reverse transcribed with random hexamers to create cDNA using the TaqMan Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed in a 96-well plate on an ABI Prism 7900HT sequence detection system using 1 μl cDNA per well, TaqMan Universal PCR master mix and Assay-On-Demand Gene Expression products (Applied Biosystems) specific for genes of interest. Each cDNA sample was analyzed in triplicate and fold change relative to control was calculated based on a PCR efficiency of two and normalized to GAPDH (endogenous control) RNA levels using SDS 2.0 Software (ABI). Average fold change and standard deviation were obtained from 2-3 biological replicate samples per treatment.

III. Results: Part 1

List of 13 known p53 targets up-regulated upon activation of p53. Microarray results provided a list of known p53 target genes that were all seen to be up-regulated by activation of p53 via usage of siRNA targeting Hdm2 or HdmX (Figure 1). As expected these genes were increased upon siHdm2 or siHdmX but had comparable or lower transcript levels than siCon when treated with siHdmX+sip53 or sip53. Results for SLC1a1 followed a similar pattern as compared to these known p53 target genes (Figure 2). So, it seemed feasible to consider SLC1a1 as a possible candidate for a novel p53 transcriptional target.

SLC1a1 transcript levels are increased upon activation of p53. Based on microarray results I hypothesized that SLC1a1 might be a possible transcriptional target of p53. To test this hypothesis, the first experiment performed was to validate the microarray results showing up-regulation of SLC1a1 following RNAi induced p53 activation. MCF7 cells were reverse transfected (See Materials and Methods) with various RNAi combinations and upon completion of the protocol cells were harvested for RNA extraction ultimately leading to RT-qPCR. qPCR results showed that SLC1a1 transcript levels were increased greater than two-fold upon simultaneously knocking down Hdm2 and HdmX (Figure 3). Intriguingly, knock-down of either Hdm2 or HdmX did not result in a statistical significant increase in SLC1a1 mRNA levels. This may be due to the effectiveness of

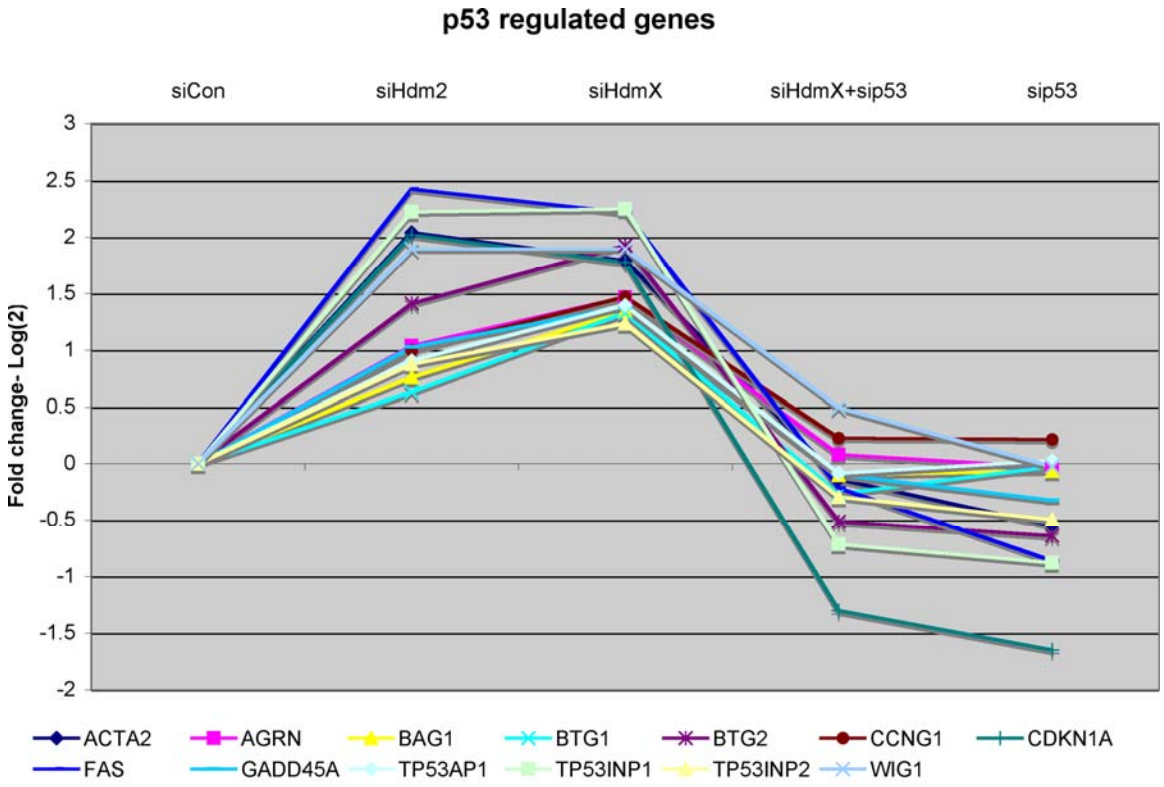


Figure 1: GeneChip expression of 13 known p53-regulated genes that were induced by knockdown of either siHdmX or siHdm2. Y-axis represents the average fold change (log2) for each of the genes in the indicated RNAi transfections relative to siCon (X-axis, labels at the top of the chart). Data analyzed by Dr. Michael Markey.

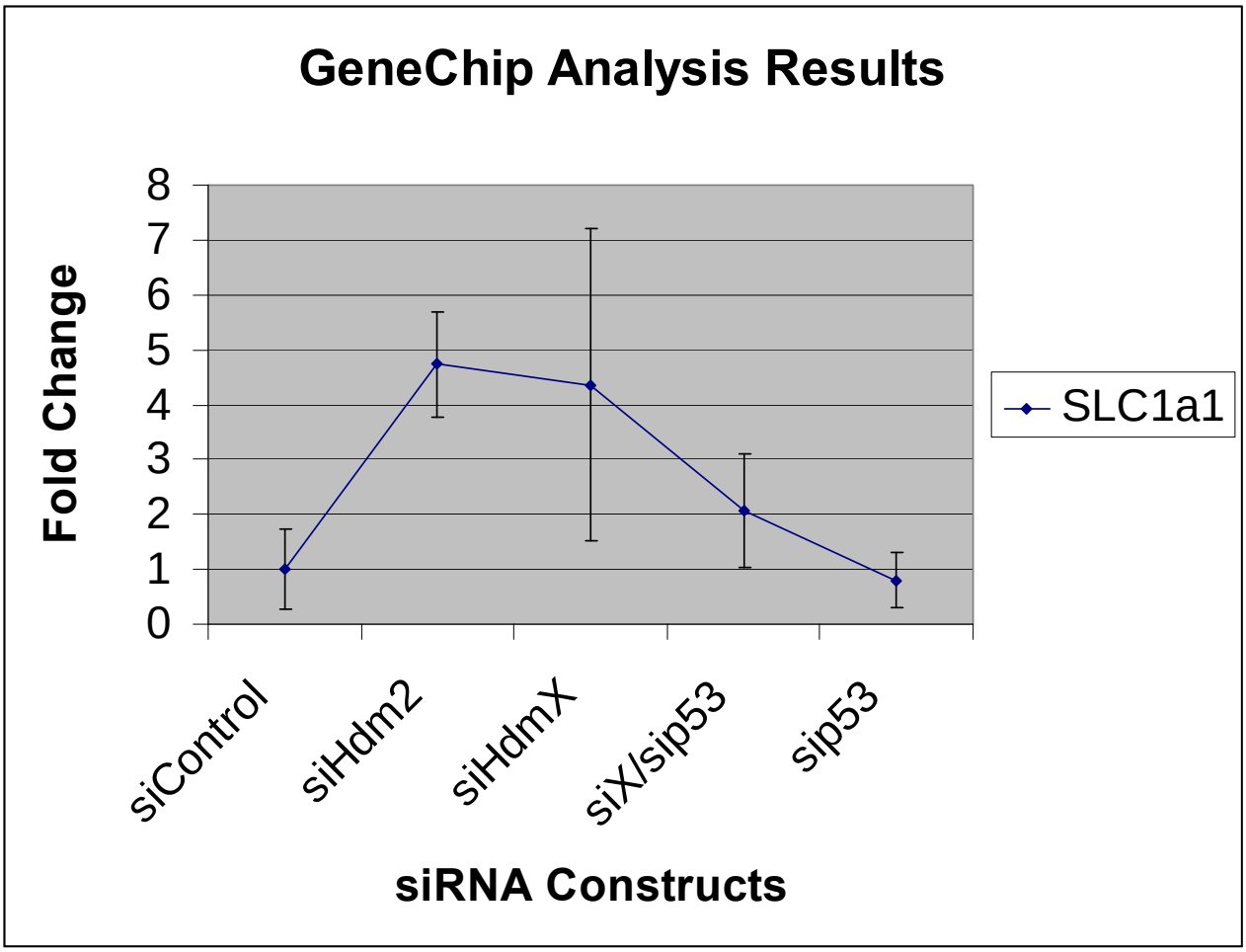


Figure 2: GeneChip relative expression of SLC1a1 in MCF7 cells treated with the indicated RNAi. Y-axis represents the average fold change for SLC1a1 in the indicated RNAi transfections relative to siCon.

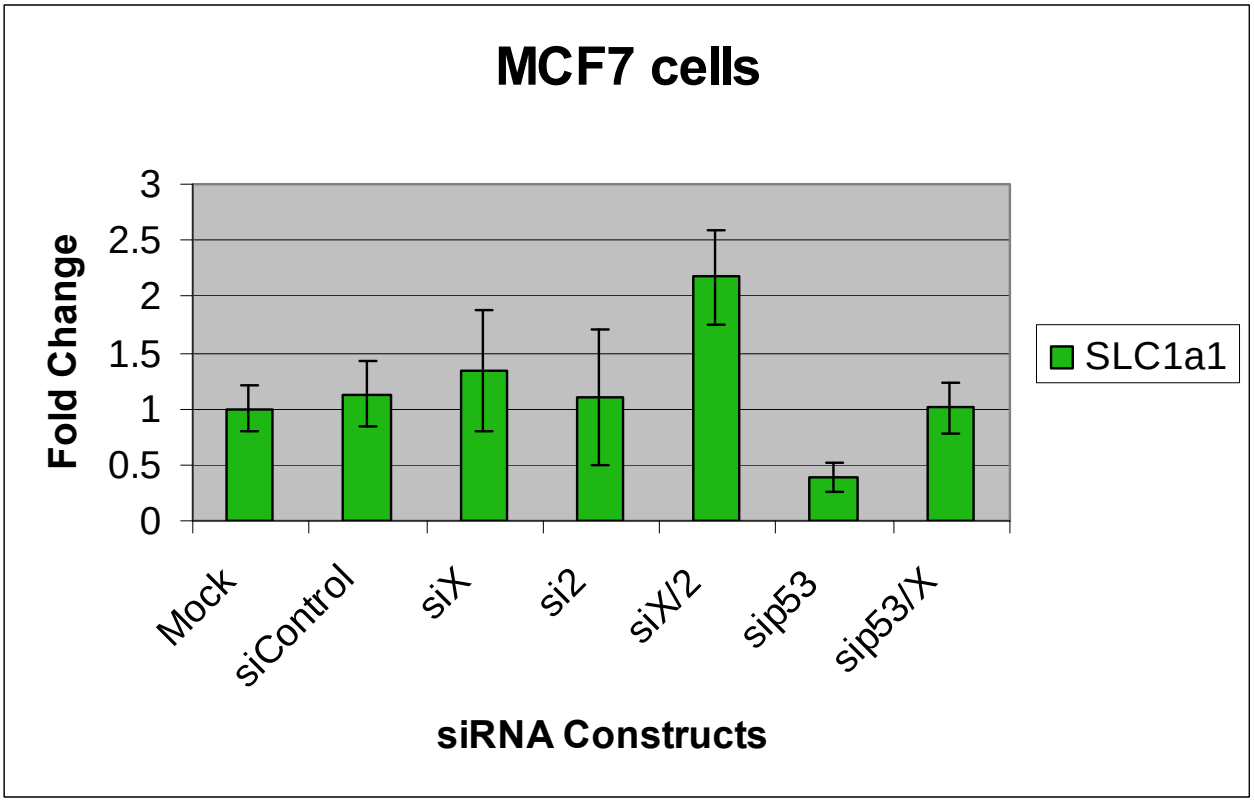


Figure 3: Induction of SLC1a1 by hdm2/hdmX knockdown using RNAi. MCF7 cells were reverse transfected (see Materials and Methods). Twenty-four hours after transfection of RNAi combinations, RNA was isolated and subjected to RT-qPCR to quantify expression of SLC1a1 using GAPDH expression for normalization. Expression levels (Y-axis) were relative to Mock and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

knocking-down expression of Hdm2 and HdmX. In terms of the microarray experiment, RNAi transfections resulted in a greater than 80% reduction of Hdm2 and HdmX expression (data not shown) where as I was only able to achieve a reduction of 64% for Hdm2 and 48% for HdmX (Figure 4). Also interestingly, SLC1a1 transcript levels were reduced by 60% upon knocking down expression of p53. Expression levels of p21, a well-known p53 target, were also examined. p21 transcript levels were increased (Figure 5) upon knocking down expression of Hdm2 (~4-fold) or HdmX (~4-fold) along with the combination of Hdm2 and HdmX (greater than 8-fold). p21 levels also decreased by 40% upon knocking-down p53. Similarly to p21, SLC1a1 transcript levels are increased upon activation of p53 by concurrently knocking down p53's negative regulators Hdm2 and HdmX.

SLC1a1 transcript levels are decreased upon activation of p53 via doxorubicin treatment in U87 cells. Doxorubicin is a DNA damaging agent that has the ability to intercalate into the DNA and cause double-strand DNA breaks eliciting a p53 response (22). Testing the effect of doxorubicin on SLC1a1 expression, U87 glioblastoma cells were treated with increasing amounts of doxorubicin over a twenty-four hour period. At the end of twenty-four hours cells are harvested and RNA was extracted in order to perform RT-qPCR. While, p21 transcript levels were increased (Figure 6) around 7-fold upon addition of 0.25 μg/mL doxorubicin, SLC1a1 transcript levels were decreased upwards of 50% after the same doxorubicin treatment (Figure 7). It was observed that administering more than 0.5 μg/mL doxorubicin was toxic to these U87 cells. The observation was represented by a drop off of p21 transcript levels along with visible cell

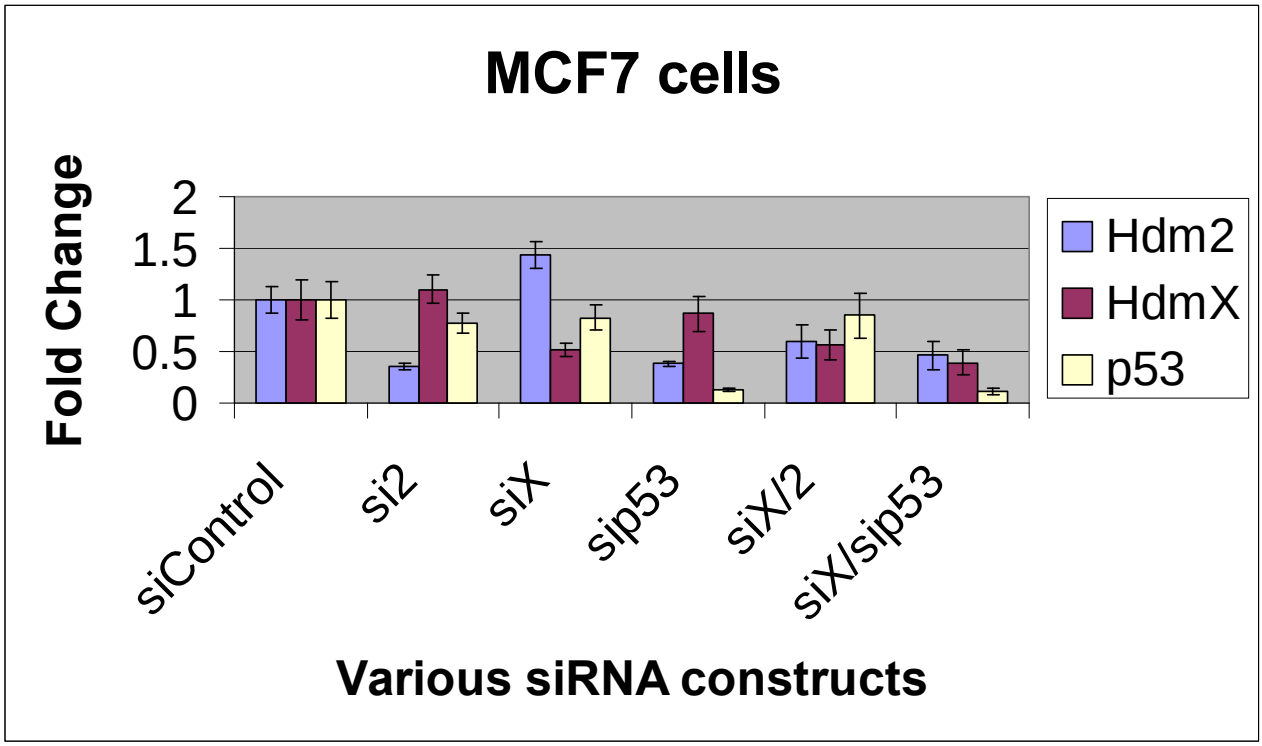


Figure 4: hdm2, p53, and hdmX knockdown using RNAi. MCF7 cells were reverse transfected (see Materials and Methods). Twenty-four hours after transfection RNA was isolated and subjected to RT-qPCR to quantify expression of hdm2, hdmX, and p53 after normalization to GAPDH. Expression levels (Y-axis) were relative to Mock and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

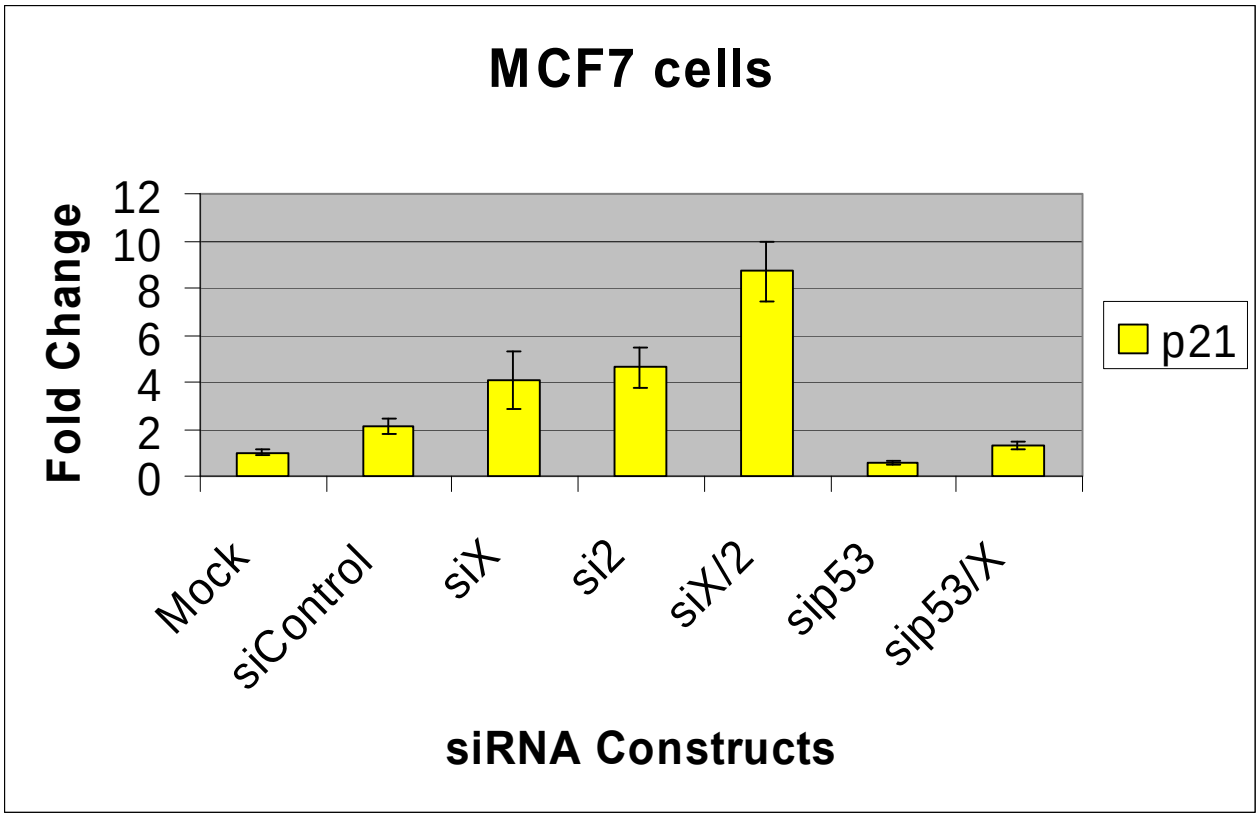


Figure 5: Induction of p21 by hdm2 or hdmX knockdown using RNAi. MCF7 cells were reverse transfected (see Materials and Methods). Twenty-four hours after transfection RNA was isolated and subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to Mock and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

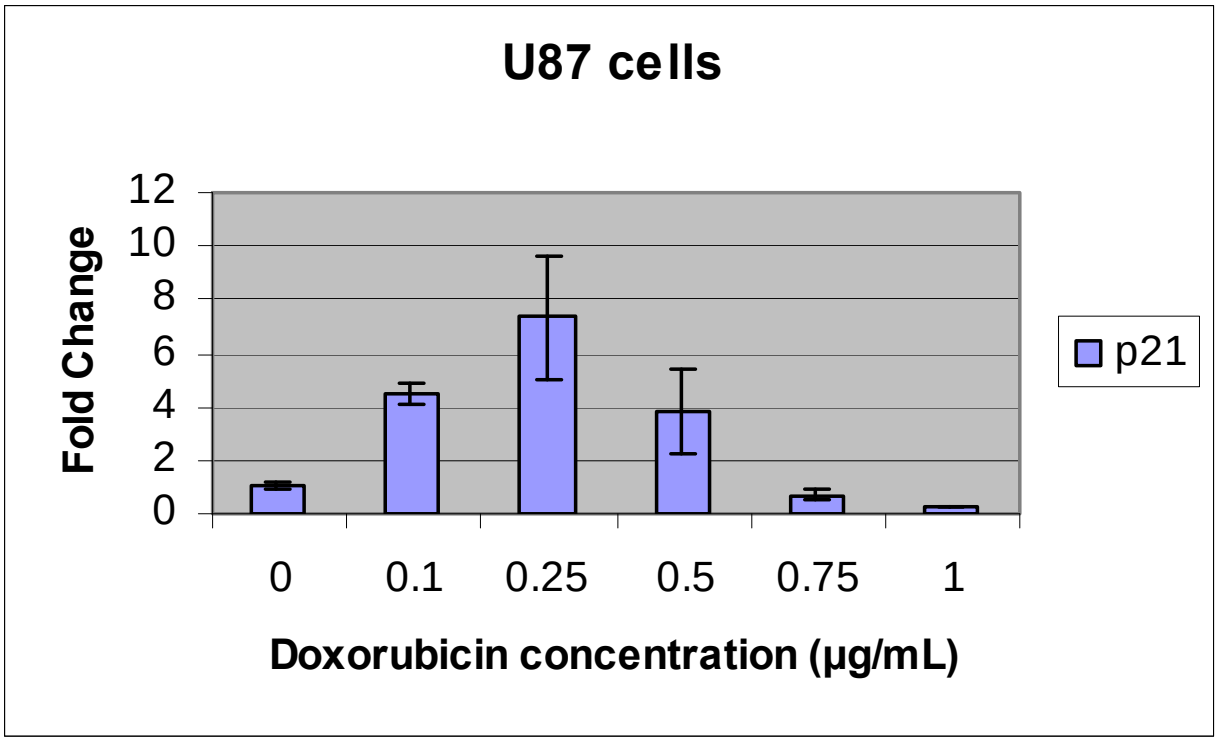


Figure 6: p21 transcript levels are increased upon doxorubicin treatment. U87 cells were treated with increasing amounts of doxorubicin. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

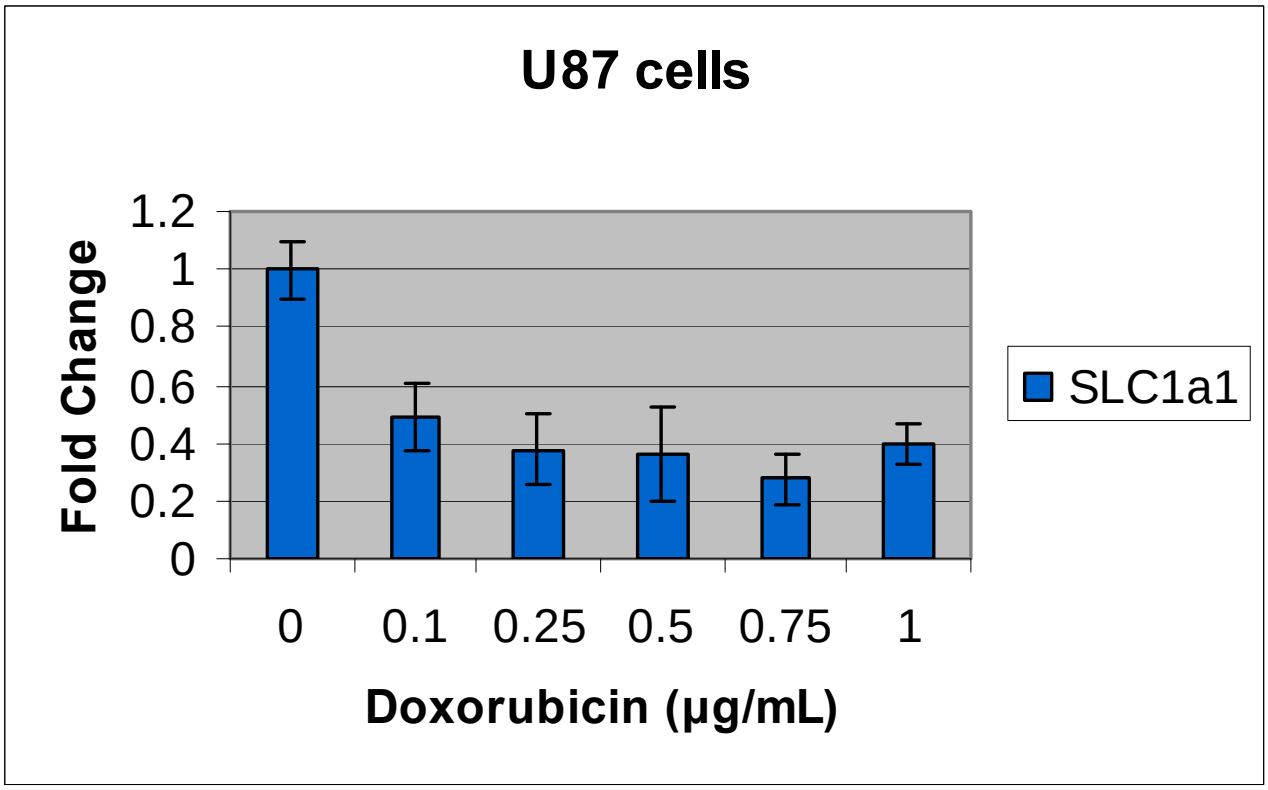


Figure 7: SLC1a1 transcript levels are decreased upon doxorubicin treatment. U87 cells were treated with increasing amounts of doxorubicin. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of SLC1a1 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

death. As expected, p21 transcript levels were increased upon activation of p53 via DNA damage. Conversely, doxorubicin treatment in U87 cells did not lead to an induction of SLC1a1 as proposed, in fact SLC1a1 transcript levels were decreased upon doxorubicin treatment in U87 cells.

SLC1a1 transcript levels are not altered after addition of doxorubicin in MCF7 cells. Using the same damaging agent but different cell line, I sought out to determine whether cell line specificity had an effect on SLC1a1 gene expression. MCF7 breast cancer cells were treated with increasing doses of doxorubicin over a twenty-four period. After drug treatment, cells were harvested for RNA in order to perform qPCR to monitor the transcript levels of SLC1a1. Here, I used a lower range of doxorubicin in an attempt to avoid cellular toxicity. Unlike my previous result showing a decrease in SLC1a1 transcript levels upon doxorubicin treatment in U87 cells, SLC1a1 transcript levels in MCF7 cells treated with doxorubicin showed no change at the two lowest doses of damage where at 0.3 μg/mL SLC1a1 mRNA levels did not increase 2-fold or more (Figure 8). p21 transcript levels were increased 4-fold after addition of 0.3 μg/mL doxorubicin (Figure 8). Taken together, MCF7 cells treated with increasing doses of doxorubicin lead to a p53 response that unaltered increased SLC1a1 gene expression.

SLC1a1 is non-responsive to DNA damage in mammary epithelial cells. Using a mammalian mammary epithelial cell line wild-type for p53 that is immortialized but not transformed, I sought out to test whether DNA damage in a non-cancerous cell line would lead to any modulation of SLC1a1 gene expression. MCF10A cells were treated

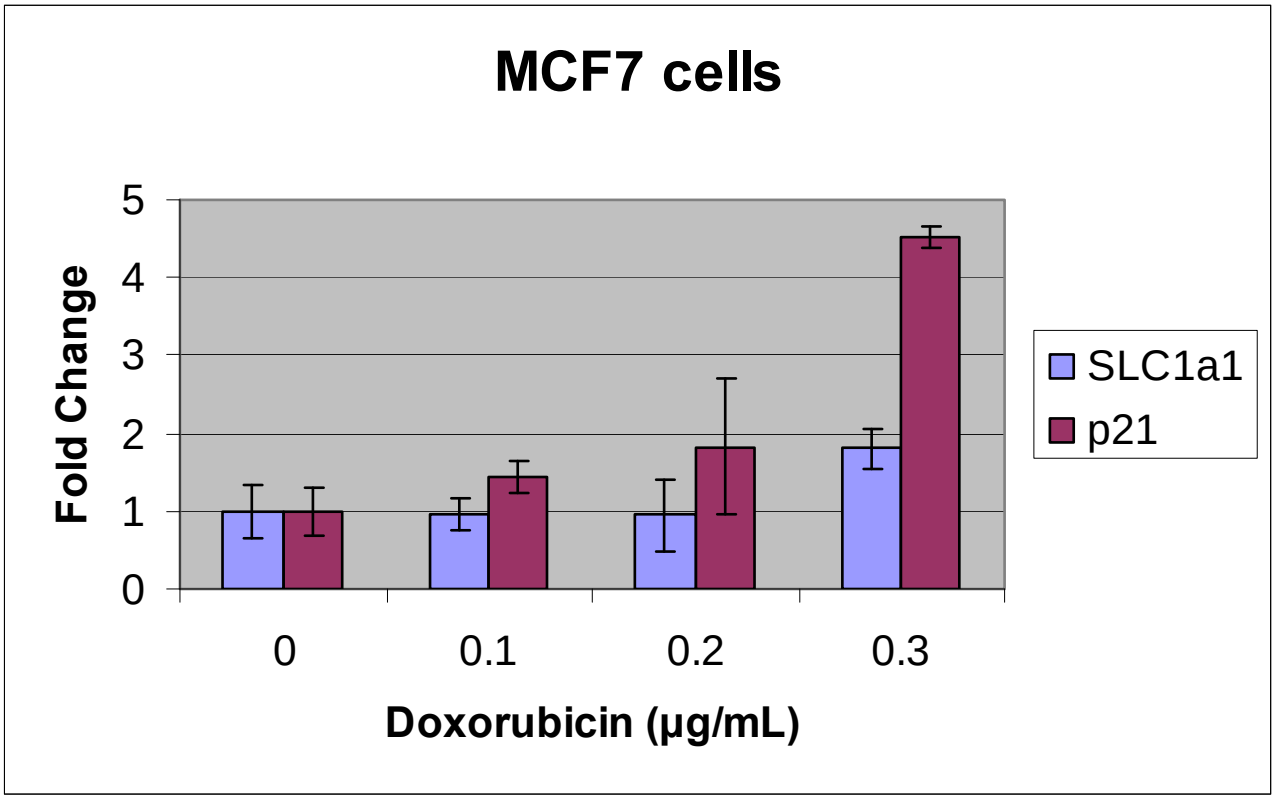


Figure 8: SLC1a1 transcript levels are unaffected upon doxorubicin treatment.

MCF7 cells were treated with increasing amounts of doxorubicin. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of SLC1a1 and p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

with increasing amounts of cytosine arabinoside also known as AraC. AraC is a DNA damaging agent that inhibits DNA and RNA polymerases. Twenty-fours after AraC treatment, cells were harvested for RNA. qPCR results illustrates that increasing amounts of AraC led to the expected dose-dependent increase of p21 transcripts (Figure 9). Where as, SLC1a1 transcripts were unaffected by the increasing amounts of AraC (Figure 10). In a similar experiment MCF10A cells were treated with increasing amounts of bleomycin, which is a DNA damaging agent that acts as a radiomimetic to cause DNA strand breaks. Transcript levels were monitored by qPCR and illustrate a similar trend as before, where p21 transcripts are increased in a dose-dependent manner (Figure 11) and SLC1a1 transcripts are unaltered (Figure 12). These results along with previous results clearly suggest that SLC1a1 gene expression is unaffected by DNA damage in this mammary epithelial cell line.

SLC1a1 transcripts are reduced upon doxorubicin treatment in human cortical neurons. Though SLC1a1 is expressed in multiple tissues throughout the body (kidney, liver, etc.) its relation to disease is found predominantly in the brain, neurons in particular. When compared to the previously described cell line (MCF7, MCF10A, U87) HCN2 cells which are wild-type for p53 express over 100-fold higher level of SLC1a1 mRNA (data not shown). Doxorubicin administration to HCN2 cells was used to assess the effect of DNA damage on SLC1a1 gene expression in this particular cell line. qPCR results showed damage response that lead to a 4-fold induction of p21 transcripts at 0.25 μg/mL (Figure 13). Results also indicate a greater than 60% loss of SLC1a1 transcripts at

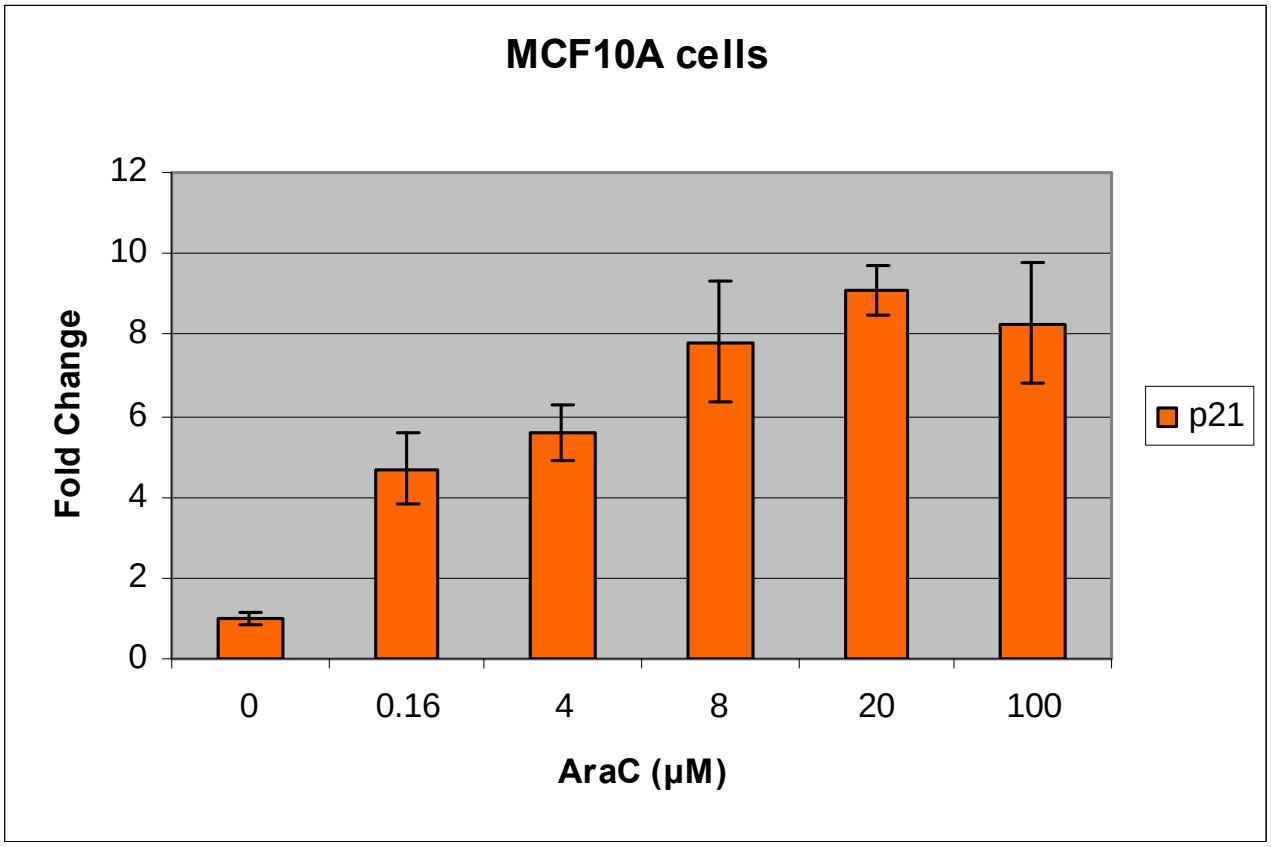


Figure 9: p21 transcript levels are increased upon AraC treatment. MCF10A cells were treated with increasing amounts of AraC. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

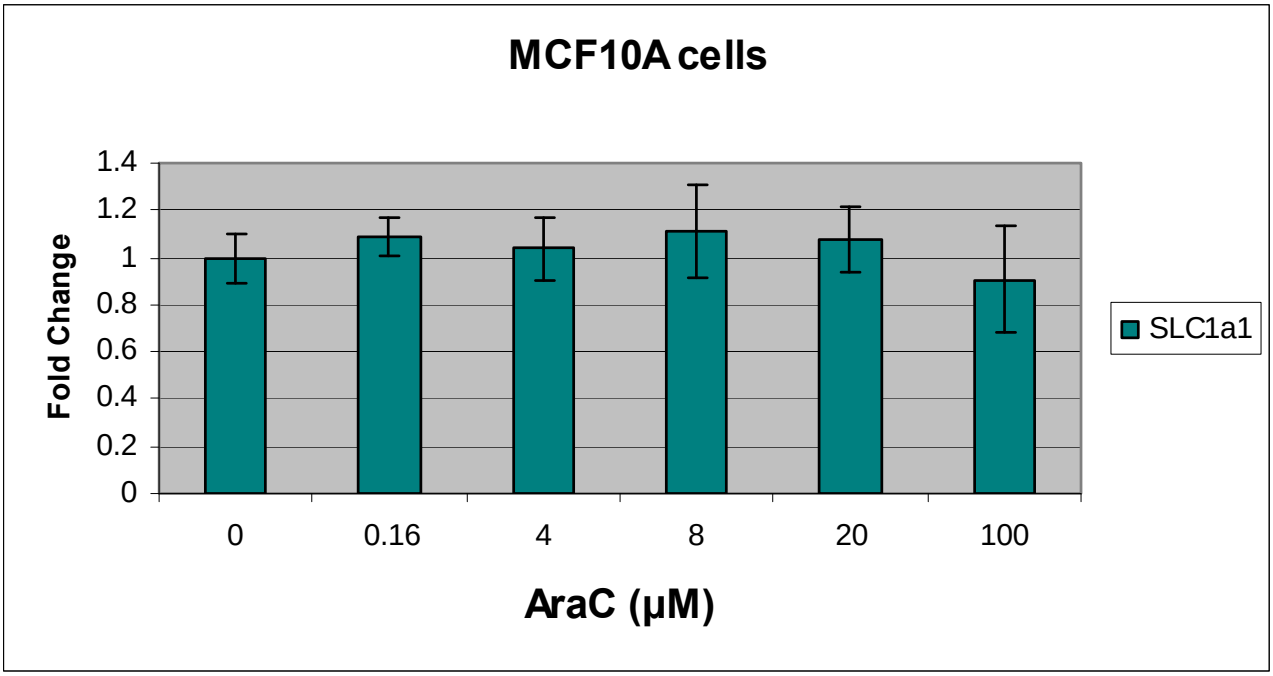


Figure 10: SLC1a1 transcript levels are unaffected upon AraC treatment. MCF10A cells were treated with increasing amounts of AraC. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of SLC1a1 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

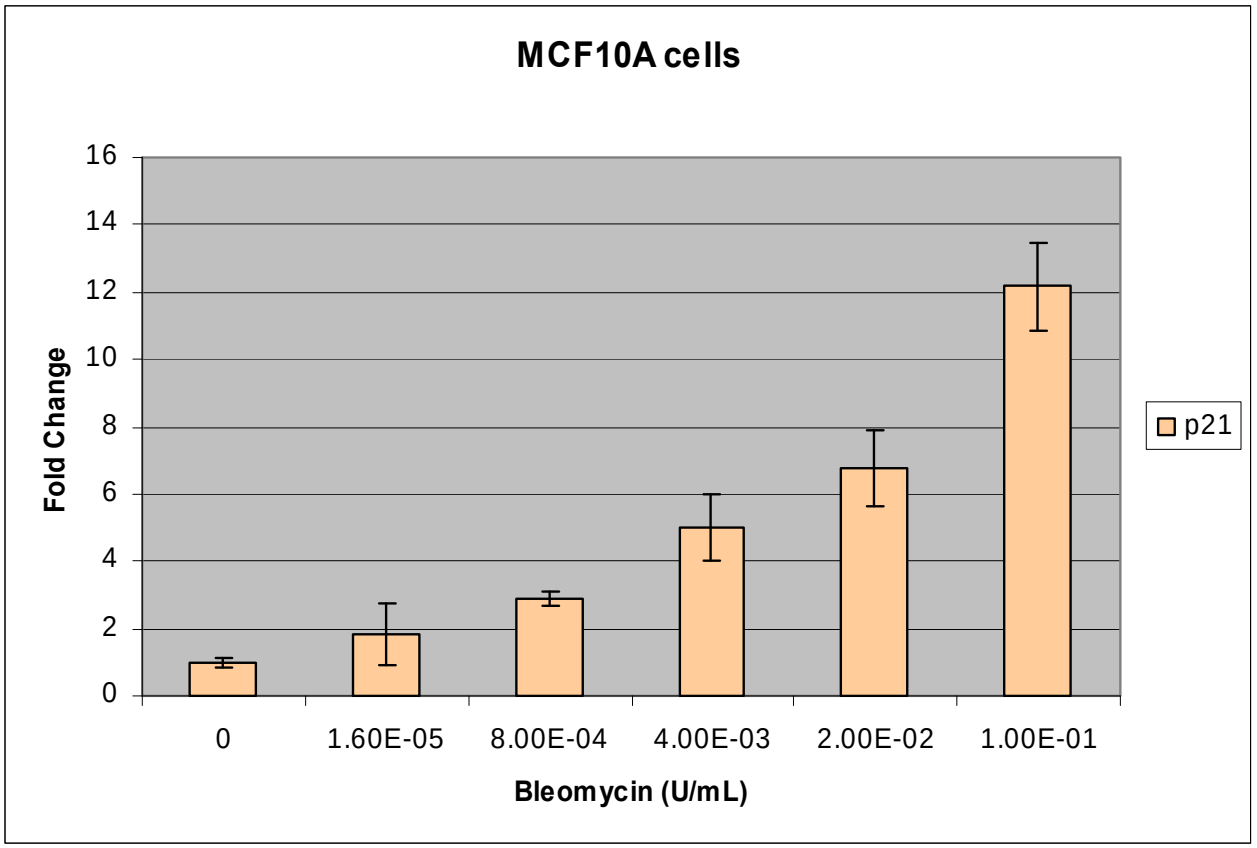


Figure 11: p21 transcript levels are increased upon AraC treatment. MCF10A cells were treated with increasing amounts of AraC. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

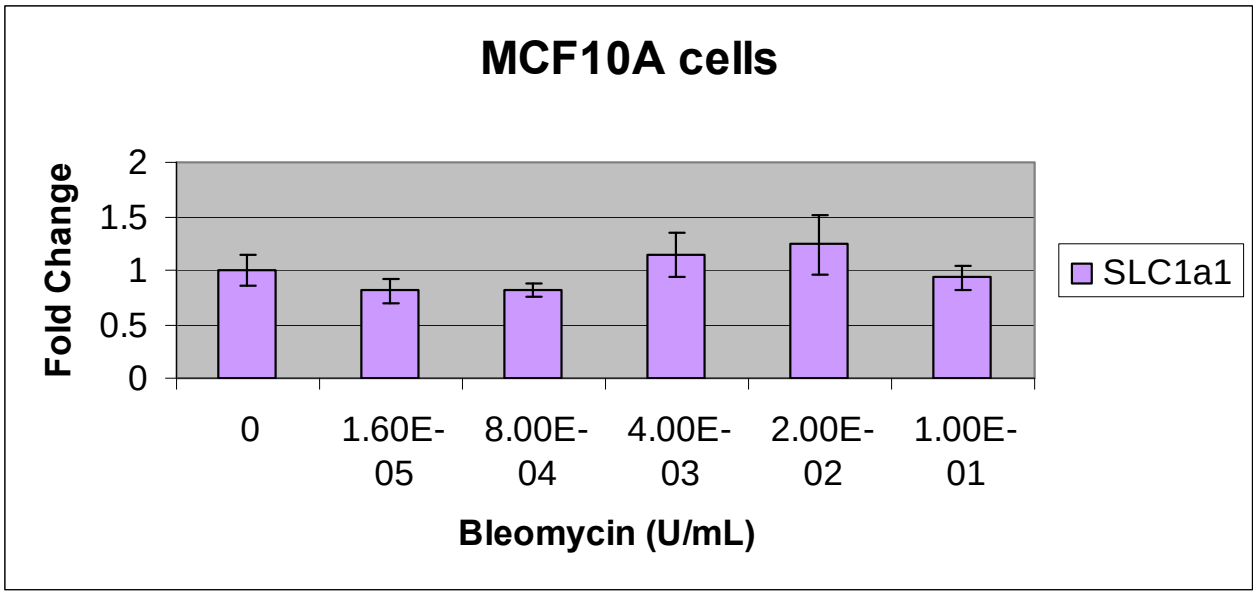


Figure 12: SLC1a1 transcript levels are unaffected upon bleomycin treatment. MCF10A cells were treated with increasing amounts of bleomycin. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of SLC1a1 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

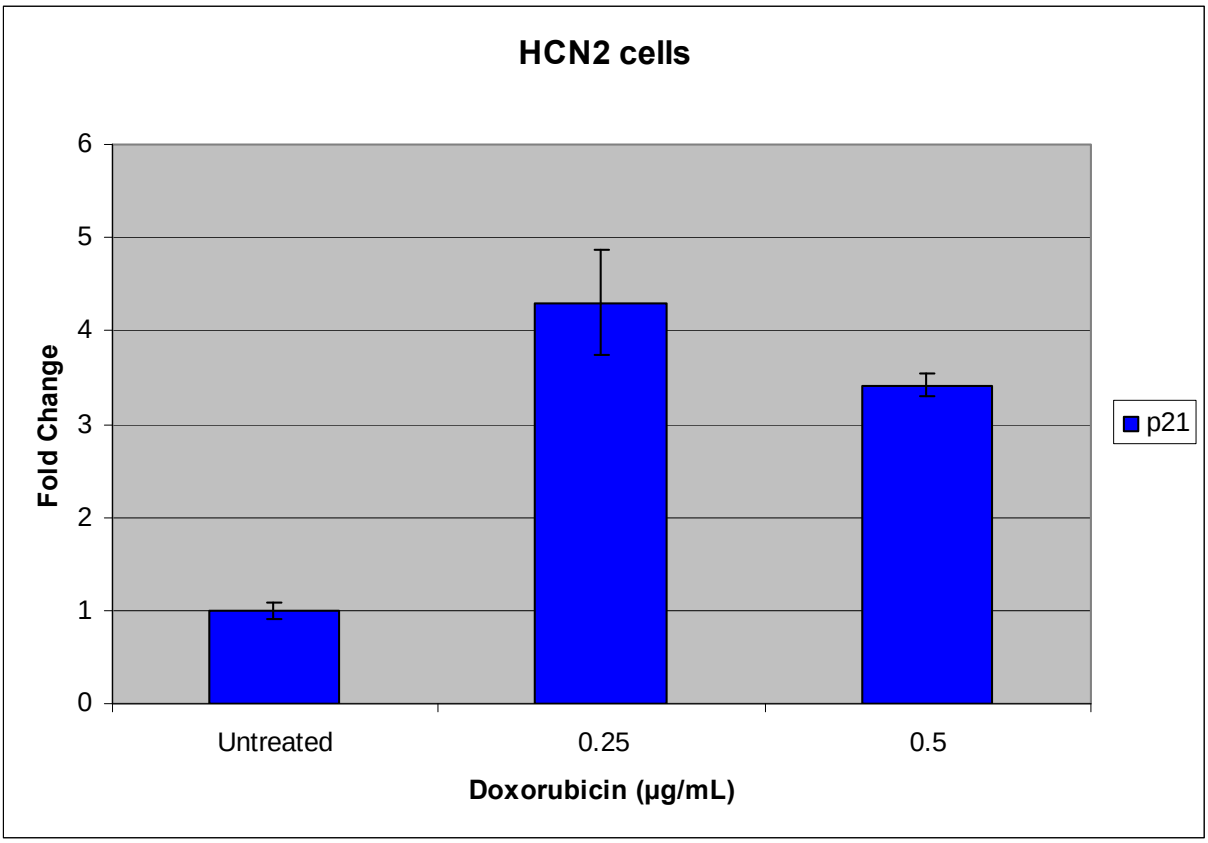


Figure 13: p21 transcript levels are increased upon doxorubicin treatment. HCN2 cells were treated with increasing amounts of doxorubicin. Twenty-four hours after treatment of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

0.25 μg/mL (Figure 14). As in previous experiments, DNA damage of HCN2 cells led to an expected p21 induction but unexpectedly led to a reduction in SLC1a1 transcripts.

Genotoxic stress is known to initiate a p53 response which leads to transactivation of its target genes (30). My results from these DNA damaging experiments suggest that if indeed SLC1a1 is a direct transcriptional target of p53, then genotoxic stress is not the mechanism for its transactivation.

Overexpression of p53 does not lead to an increase in SLC1a1 transcript levels. After testing a number of DNA damaging agents in several cell lines, I next wanted to assess the effect of overexpression of p53 on SLC1a1 gene expression. Using H1299 cells (non-small cell lung carcinoma which are null for p53), expression plasmids containing either a empty vector, wild-type p53, mutant p53, and the combination of both were transfected (See Materials and Methods) into these cells. Upon conclusion of the protocol, RNA is isolated and subjected to RT-qPCR. As shown, p21 transcripts are increased 25-fold (Figure 15) upon overexpression of p53. Mutant p53 results proved to be inconclusive due to high levels of variability (Figure 15) while the combination of both wild-type and mutant p53 lead to a decrease in p21 transcript levels when compared to H1299 cells overexpressing wild-type p53 (Figure 15). SLC1a1 transcript levels were shown be unaffected by overexpression of either wild-type or mutant p53 (Figure 16). In summary, overexpression of p53 led to a robust induction of its known target p21 while overexpression of p53 had little to no influence on SLC1a1 gene expression.

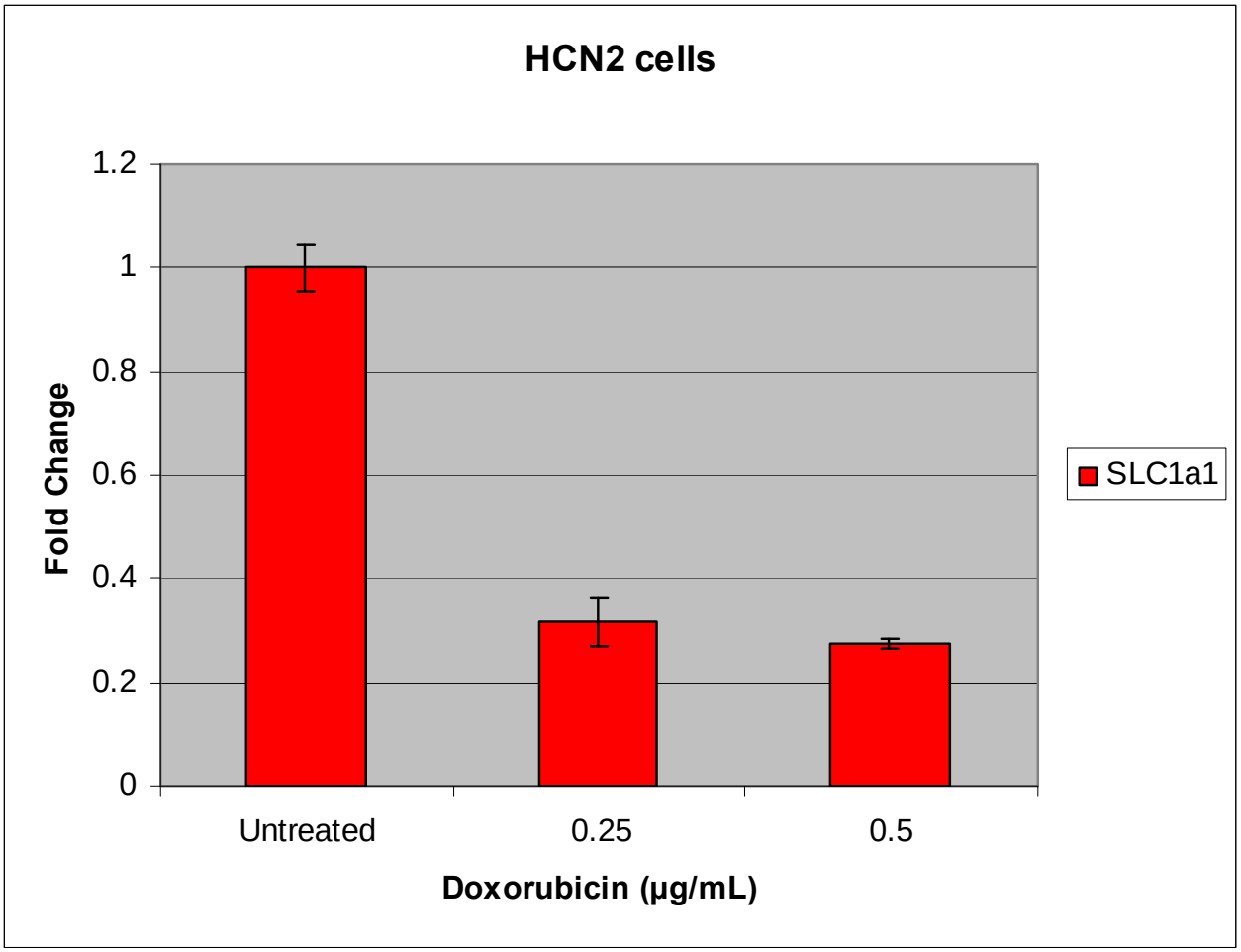


Figure 14: SLC1a1 transcript levels are decreased upon doxorubicin treatment in HCN2 cells. HCN2 cells were treated with increasing amounts of doxorubicin. Twenty-four hours after treatment with the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of SLC1a1 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

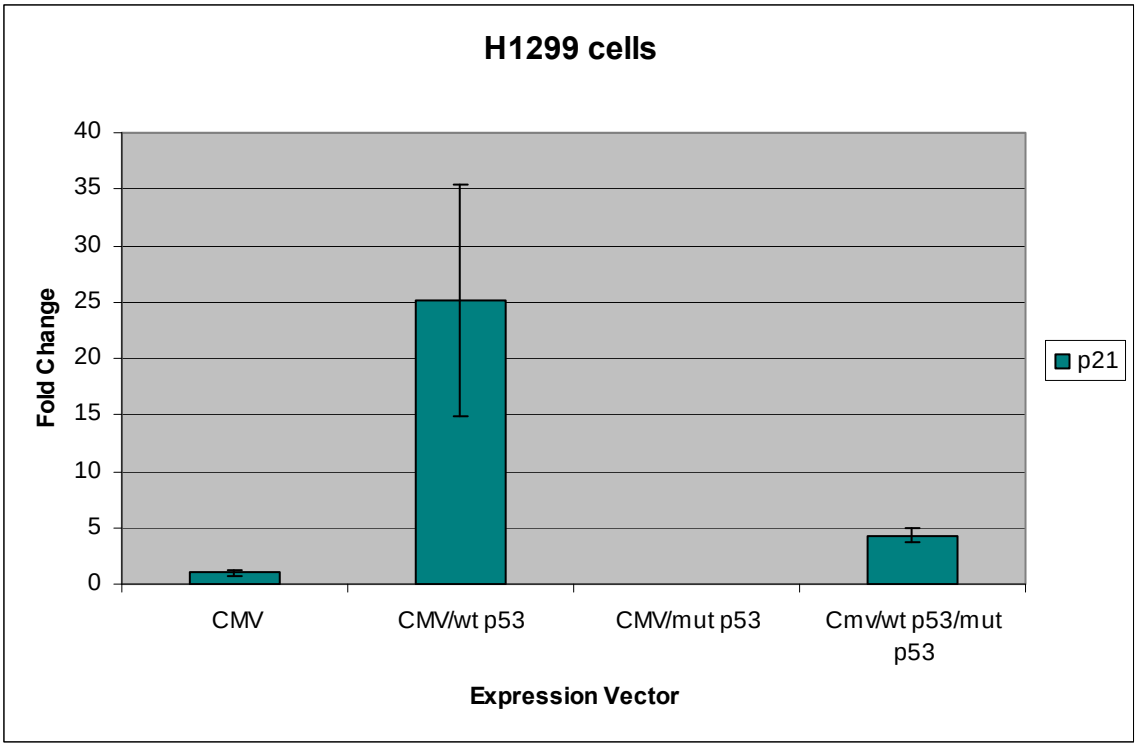


Figure 15: Overexpression of p53 leads to an increase in p21 transcript levels. H1299 were transfected (See Materials and Methods) with the indicated expression plasmids. Upon completion of transfection cells were harvested for RNA which was subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to CMV and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

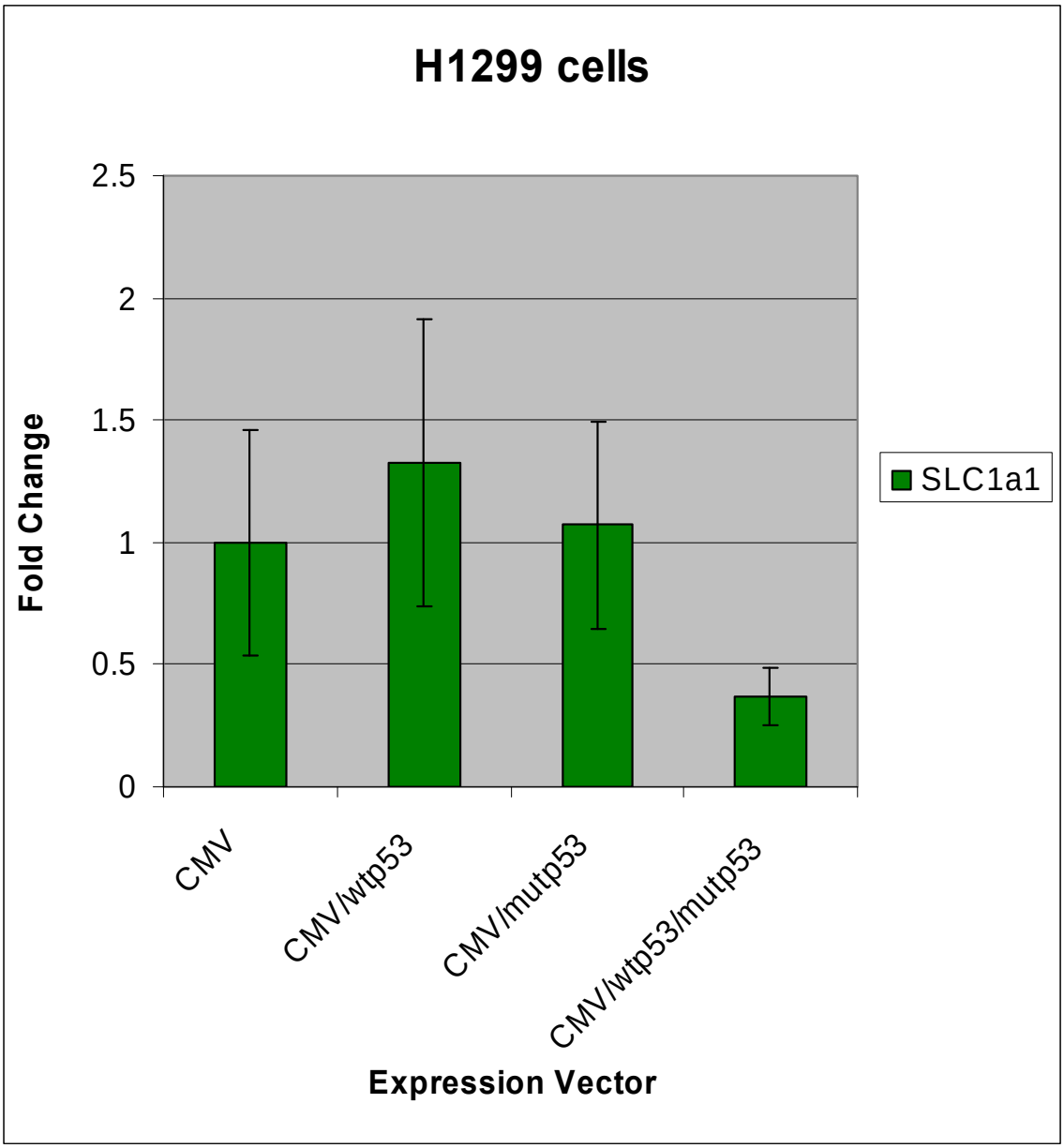


Figure 16: Overexpression of p53 has little to no effect on SLC1a1 transcript levels. H1299 were transfected (See Materials and Methods) with the indicated expression plasmids. Upon completion of transfection cells were harvested for RNA which was subjected to RT-qPCR to quantify expression of SLC1a1 after normalization to GAPDH. Expression levels (Y-axis) were relative to CMV and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

IV. Results: Part 2

List of genes that are down-regulated upon activation of p53 by RNAi targeting HdmX and Hdm2. While p53 activation through Hdm2 and HdmX knockdown was expected to result transcriptional up-regulation of target genes, we also uncovered a group of genes demonstrating what appeared to be a p53-dependent downregulation (Figure 17). Comprised within this list of genes was a subset of genes with gene products that are involved in the G1-S transition of the cell cycle. Most interestingly was that, the all of these genes, downregulated following HdmX and Hdm2 knockdown had the commonality of being E2F1 target genes.

MCM10 transcript levels are decreased upon activation of p53 by RNAi targeting Hdm2 and HdmX. To further examine this subset list of genes that were down-regulated after p53 was activated, an E2F1 target gene-MCM10 (46) was used to assess p53's ability to repress this group of E2F1 targets. The MCM family consist of a number of genes (2-7 including 10) all in which are involved in DNA replication, specifically, the MCM2-7 complex encode proteins that are seen to act as the DNA helicase in initiation of DNA replication (3**)**. MCM10 has been found to be an essential component of the replication fork along with being required to maintain DNA polymerase alpha on the chromatin (35). The MCM genes encode proteins that are involved in G1-S phase transition (44) and are known E2F target genes (24, 31, 46).

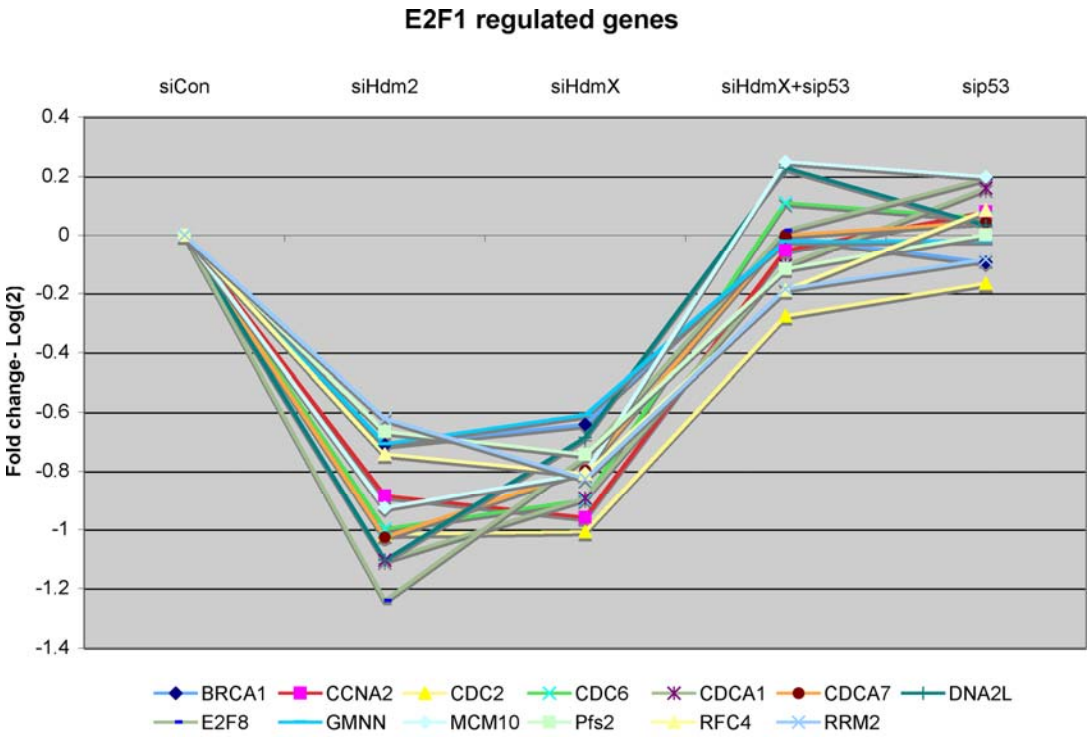


Figure 17. GeneChip expression of 13 reported E2F1-regulated genes that were repressed by knockdown of either siHdmX or siHdm2. Y-axis represents the average fold change (log2) for each of the genes in the indicated RNAi transfections relative to siCon (X-axis, labels at the top of the chart). Data analyzed by Dr. Michael Markey

To test if loss of Hdm2 or HdmX results in repression of E2F-1 regulated genes involved in cell cycle progression, I monitored MCM10 and how its transcript levels are modulated in relation to p53 activation. Having identified MCM10 as potentially repressed by activation of p53, I next set out to validate this microarray finding (Table 2) by repeating the siRNA transfections in MCF7 breast cancer cells and employing RT-qPCR to examine the expression of MCM10 relative to GAPDH. siRNA targeting the indicated gene was reverse transfected into MCF7 cells as described in the Materials and Methods section. Subsequent RT-qPCR from RNAi transfected MCF7 cells suggests that indeed MCM10 transcipt levels decrease upon activation of p53: 25% reduction by siHdm2, 60% reduction by siHdmX, 66% reduction by siHdm2/HdmX (Figure 18). Also, MCM10 transcript levels were increased upon sip53 and had levels comparable to siCon in terms of siHdmX/sip53. I propose that the repression of MCM10 may be p53-dependent shown by the fact that simultaneous removal of p53 and HdmX blocks the decrease one achieves by removing HdmX alone. Activation of p53 by knocking-down expression of its two negative regulators leads to a decrease in MCM10 transcript levels.

MCM10 transcript levels are reduced following of DNA damage in MCF7 breast cancer cells. Cellular stress or DNA damage has been shown to elicit a p53 response in cells harboring a functional p53. In order to test if MCM10 gene expression was inhibited following DNA damage, I exposed MCF7 cells to increasing amounts of doxorubicin. qPCR results illustrate that increasing amount of doxorubicin leads to a reduction in MCM10 mRNA levels (Figure 19). In another experiment, I treated MCF7 cells with UV-C irradiation (generates pyrimidine dimmers) and monitored MCM10 transcript

Affymetrix GeneChip Results for MCM10

	Relative Expression	Relative Expression Range
Treatment type Mock	0.958	(.808 to 1.095)
Treatment type siCon	1.004	(.873 to 1.094)
Treatment type siHdm2(A)	0.431	(0.411 to 0.451)
Treatment type siHdm2(B)	1.063	(1.007 to 1.094)
Treatment type siHdmX(A)	0.546	(0.469 to 0.614)
Treatment type siHdmX(B)	0.61	(0.599 to 0.616)
Treatment type siX(A)/sip53	1.349	(1.306 to 1.406)
Treatment type Treatment type sip53	1.159	(1.041 to 1.25)

Table 2: GeneChip relative expression of MCM10 under indicated RNAi combinations. Note, A and B denote different siRNA constructs targeting the same gene.

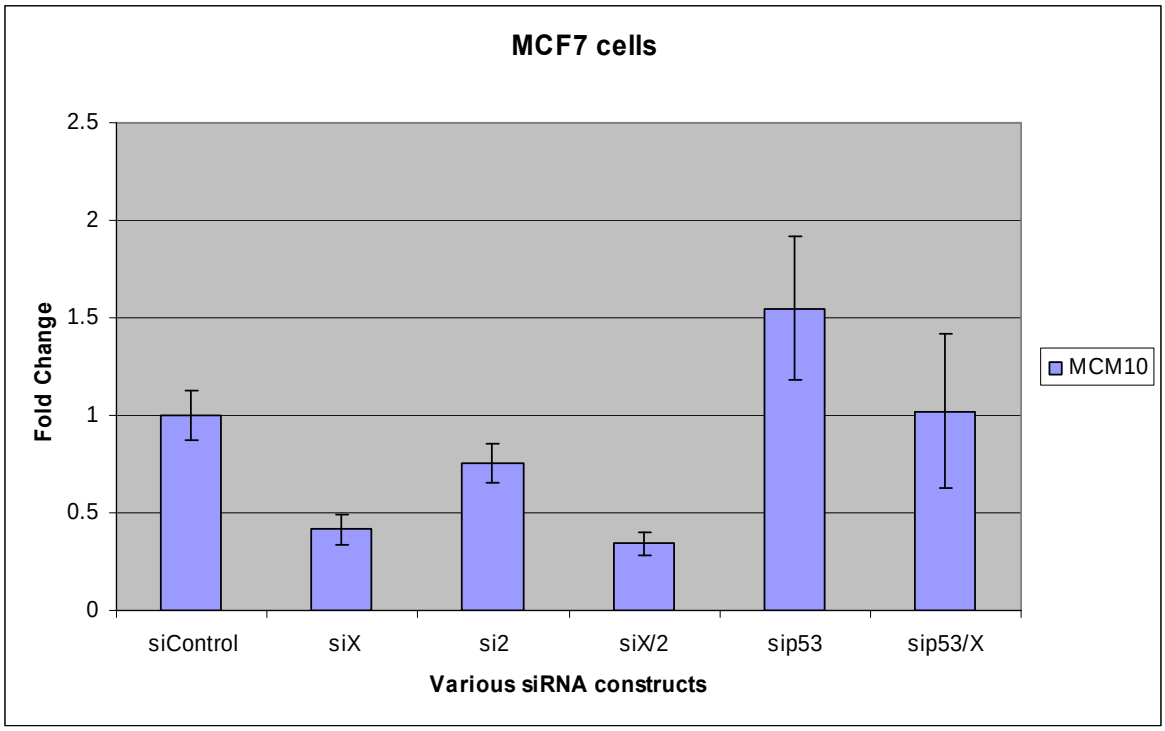


Figure 18: Repression of MCM10 by hdm2 or hdmX knockdown using RNAi. MCF7 cells were reverse transfected (see Materials and Methods). Twenty-four hours after transfection RNA was isolated and subjected to RT-qPCR to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to siControl and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

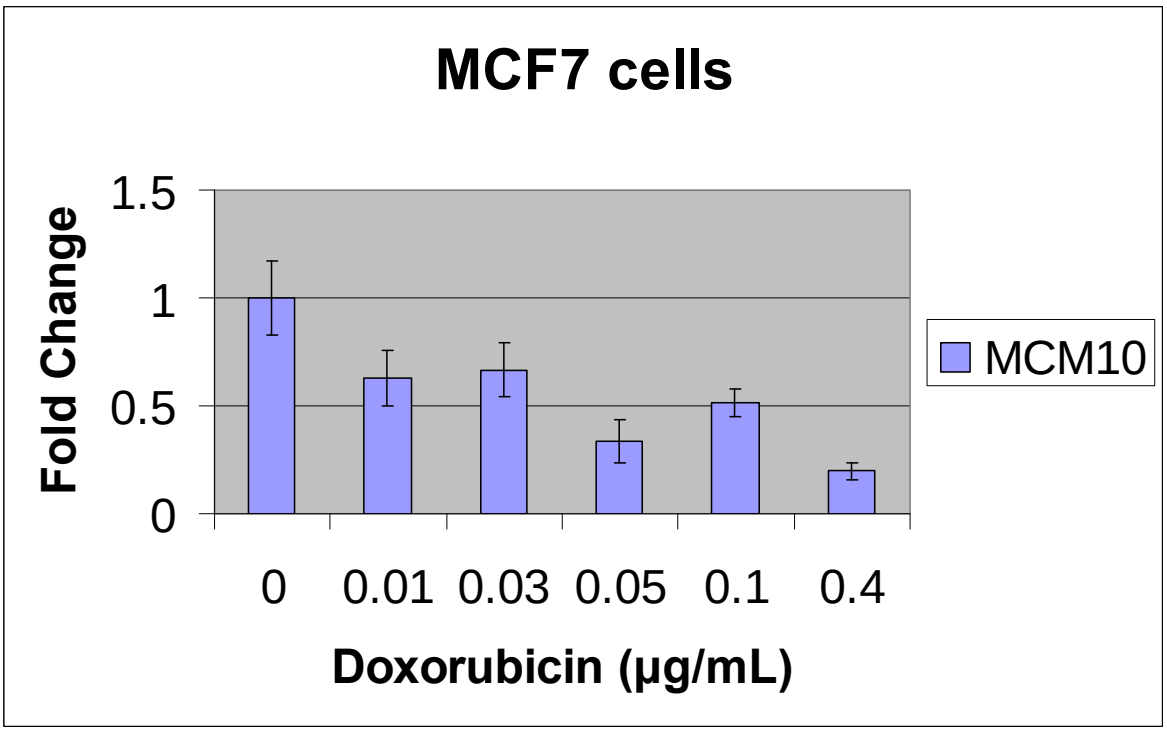


Figure 19: MCM10 transcript levels are reduced following doxorubicin treatment. MCF7 cells were treated with increasing amounts of doxorubicin. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

levels. Here, qPCR results again showed that increasing amounts of UV-C irradiation lead to decrease in MCM10 transcript levels (Figure 20). As seen in both cases, increasing amounts of either doxorubicin or UV-C irradiation caused a reduction in MCM10 transcript levels. Both cases clearly indicate that DNA damage in MCF7 cells leads to a decrease in MCM10 mRNA levels.

MCM10 transcript levels are reduced upon UV-C irradiation in MCF10A mammalian mammary epithelial cells. The next experiment performed was one intended to answer the question of whether DNA damage induced repression of MCM10 is limited to MCF7 breast cancer cells and to test if the repression can be observed in a non-transformed epithelial cell line. To address these questions MCF10A cells (which are immortalized, non-transformed mammalian mammary epithelial cells) were treated with increasing amounts of UV-C irradiation. From the RT-qPCR results it was concluded that indeed MCM10 transcript levels are reduced at high levels of UV-C (47% reduction at 250 J/m^2 and 80 % reduction at 500 J/m^2 UV-C irradiation) in MCF10A breast epithelial cells (Figure 21). In two cell lines derived from the breast tissue, one being cancerous (MCF7) and other being mammary epithelial (MCF10A) MCM10 transcripts levels are decreased upon increasing amounts of UV-C irradiation.

MCM10 transcript levels are not reduced in HCT116 -/-p53 upon UV-C irradiation. Activation of p53 by either DNA damage or by RNAi targeting its two negative regulators Hdm2 or HdmX leads to a reduction in the transcript levels of MCM10. The next step was to use a cell line which did not possess wild-type p53 and perform a DNA

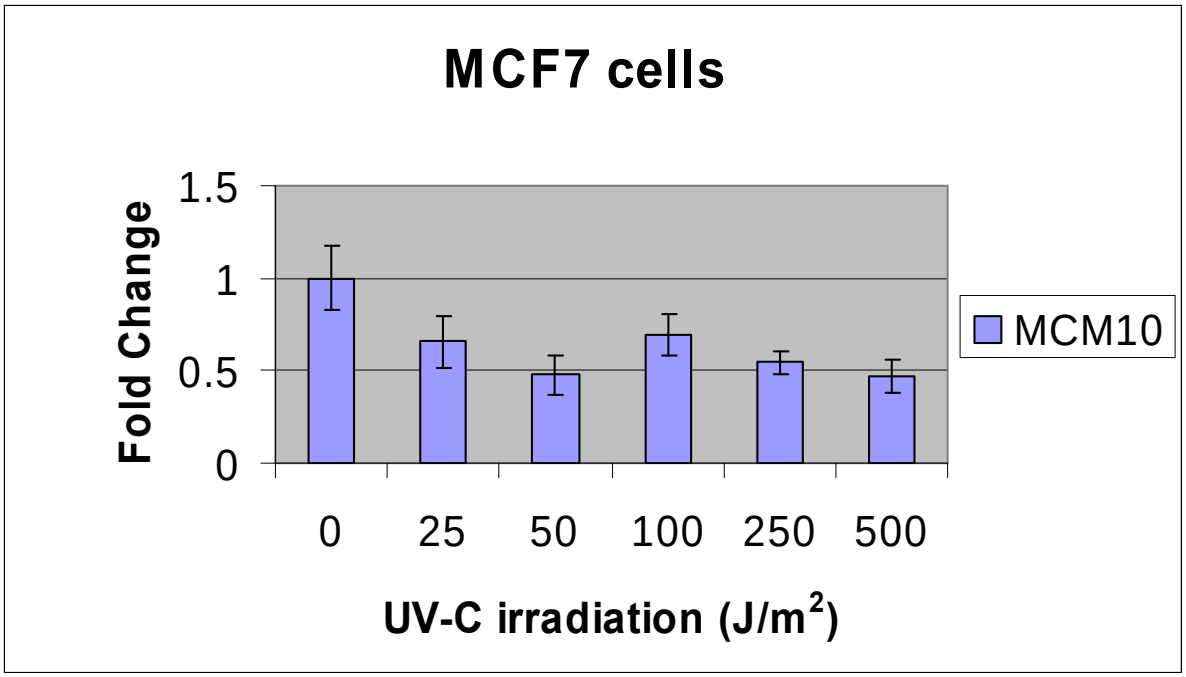


Figure 20: MCM10 transcript levels are reduced upon UV-C irradiation. MCF7 cells were treated with increasing amounts of UV-C irradiation. Twenty-four hours after application of the damaging agent, RNA was isolated and subjected to RT-qPCR to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

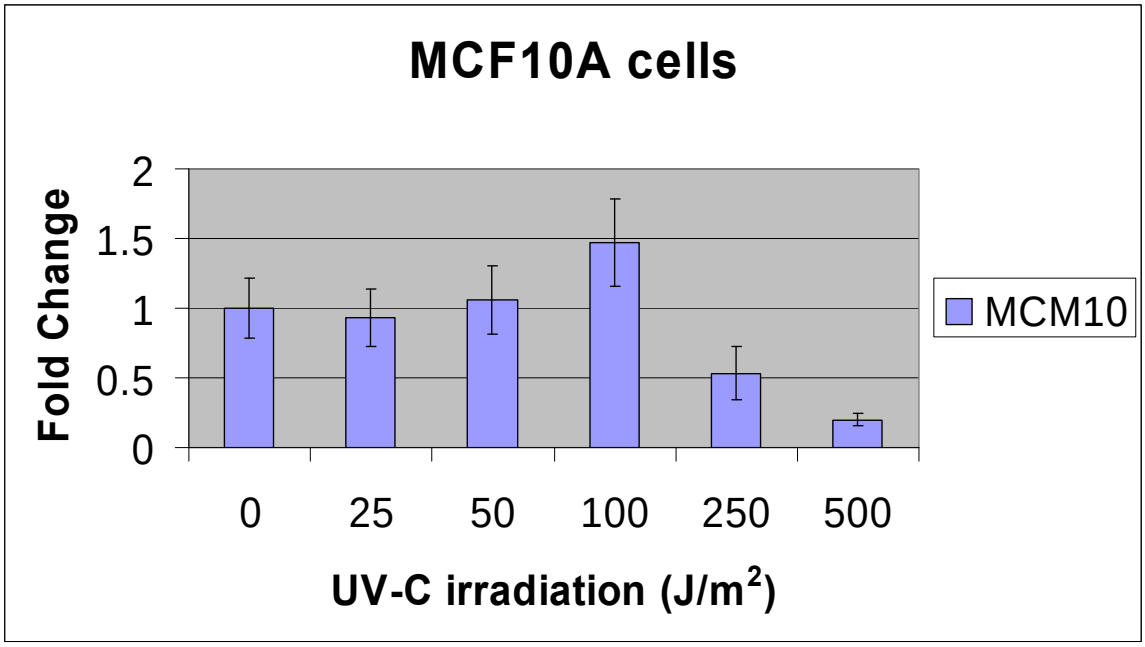


Figure 21: MCM10 transcript levels are decreased upon UV-C irradiation in mammary epithelial cells. MCF10A cells were subjected to increasing amounts of UV-C irradiation. Twenty-four hours after irradiation, cells were harvested and RNA was isolated. RT-qPCR was then performed to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

damaging experiment assessing MCM10 transcript levels. The cell line chosen was HCT116 -/-p53 human colorectal cancer cells. These cells were treated with increasing amounts of UV-C irradiation. Results exemplify that MCM10 transcript levels are not reduced in HCT116 -/-p53 cells treated with increasing amounts of UV-C irradiation (Figure 22). In a separate experiment, HCT116 cells that possess wild-type p53 were treated with increasing amounts of UV-C irradiation. In contrast with the previous result, MCM10 transcript levels were reduced 60% at the lowest dose of irradiation (Figure 23). Thus, the reduction of MCM10 transcript levels by DNA damage may be p53-dependent.

p53 activation by siRNA targeting Hdm2/HdmX or by DNA damage leads to an induction of its well known target gene p21. p21, also known as cyclin-dependent kinase inhibitor 1A, can inhibit CDK/cyclins involved in the phosphorylation of Rb which relieves E2F-1 from the Rb-E2F1 complex allowing E2F-1 transactivation of its target genes (4). Thus, p53 activation through DNA damage or RNAi targeting HdmX or Hdm2 would lead to increased p21 levels which in turn may lead to repression of E2F-1 target genes through inhibiting Rb phosphorylation.

siRNA targeting p21 has little to no effect on MCM10 transcript levels. Employing multiple siRNA constructs targeting p21, I set out first to test whether knocking-down endogenous expression of p21 has any effect on steady-state MCM10 transcript levels. sip21 constructs 1 and 3 led to over 95% reduction of p21 transcript levels (Figure 24). Interestingly, these siRNA constructs did not lead to a pronounce change in MCM10 transcript levels (Figure 25). A possible explanation is that endogenous p21 does not regulate the basal levels of MCM10. Thus, activation of p53 (whether that be through

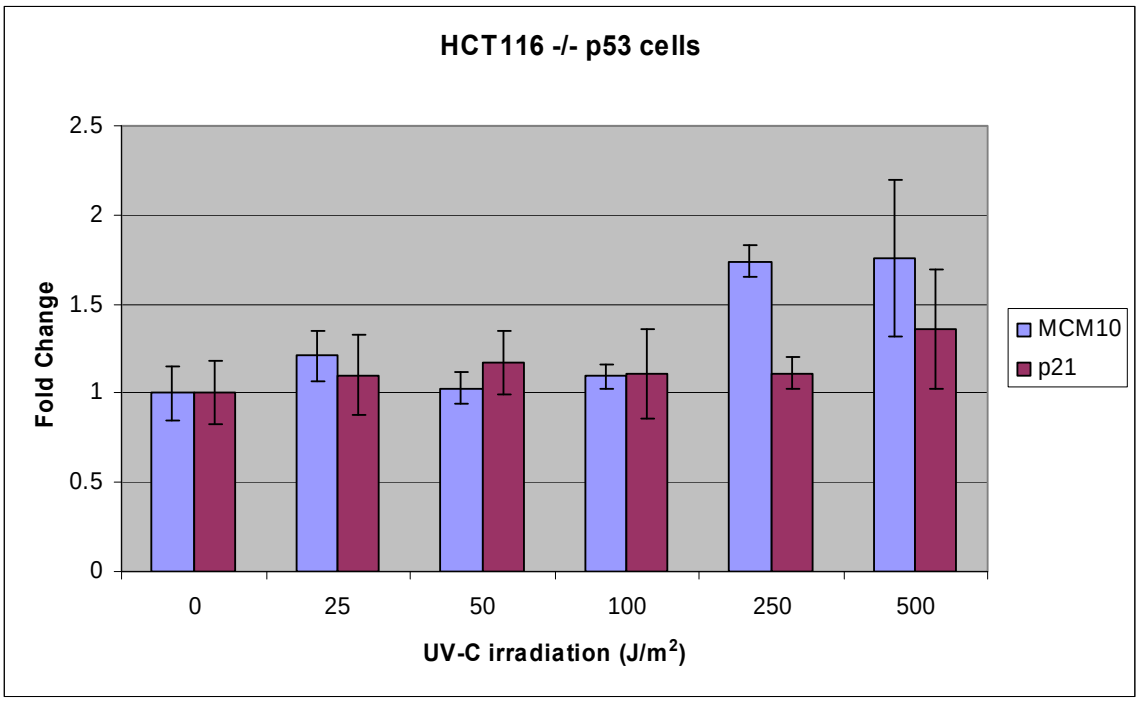


Figure 22: MCM10 transcript levels are not decreased upon UV-C irradiation in HCT116 -/-p53 colorectal cancer cells. HCT116 -/- cells were subjected to increasing amounts of UV-C irradiation. Twenty-four hours after irradiation, cells were harvested and RNA was isolated. RT-qPCR was then performed to quantify expression of MCM10 and p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

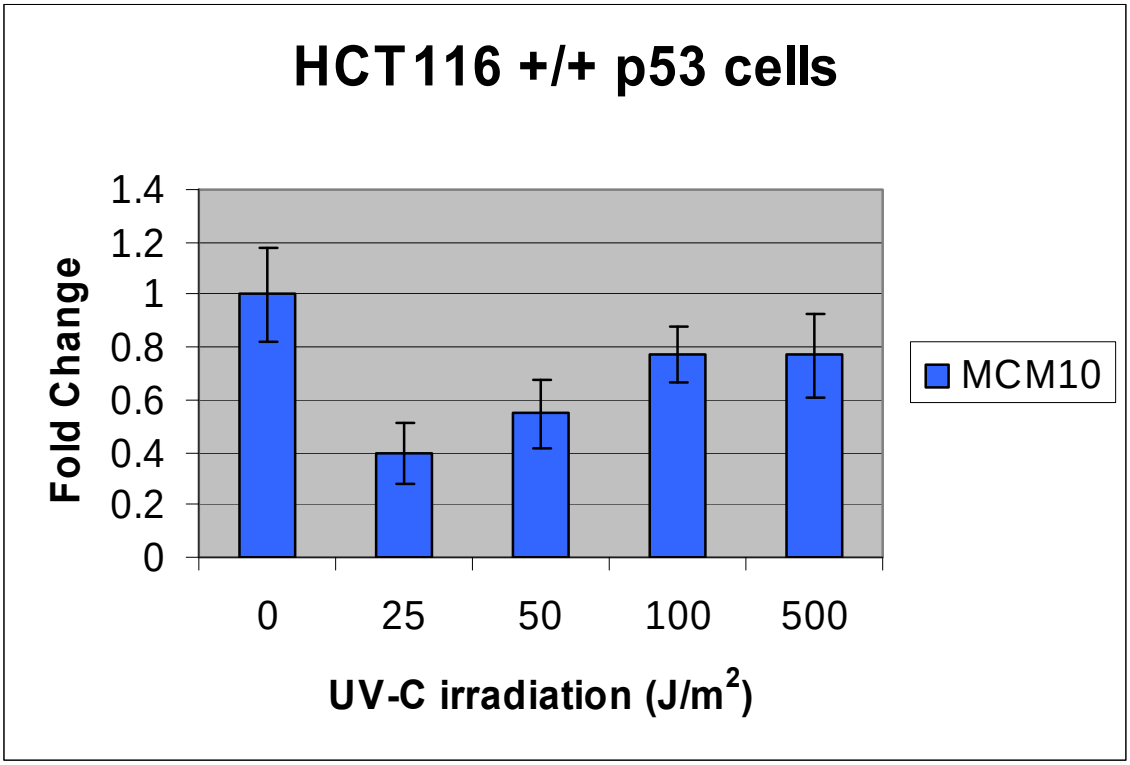


Figure 23: MCM10 transcript levels are decreased upon UV-C irradiation in HCT116 +/+ p53 colorectal cancer cells. HCT116 +/+ cells were subjected to increasing amounts of UV-C irradiation. Twenty-four hours after irradiation, cells were harvested and RNA was isolated. RT-qPCR was then performed to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to untreated and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

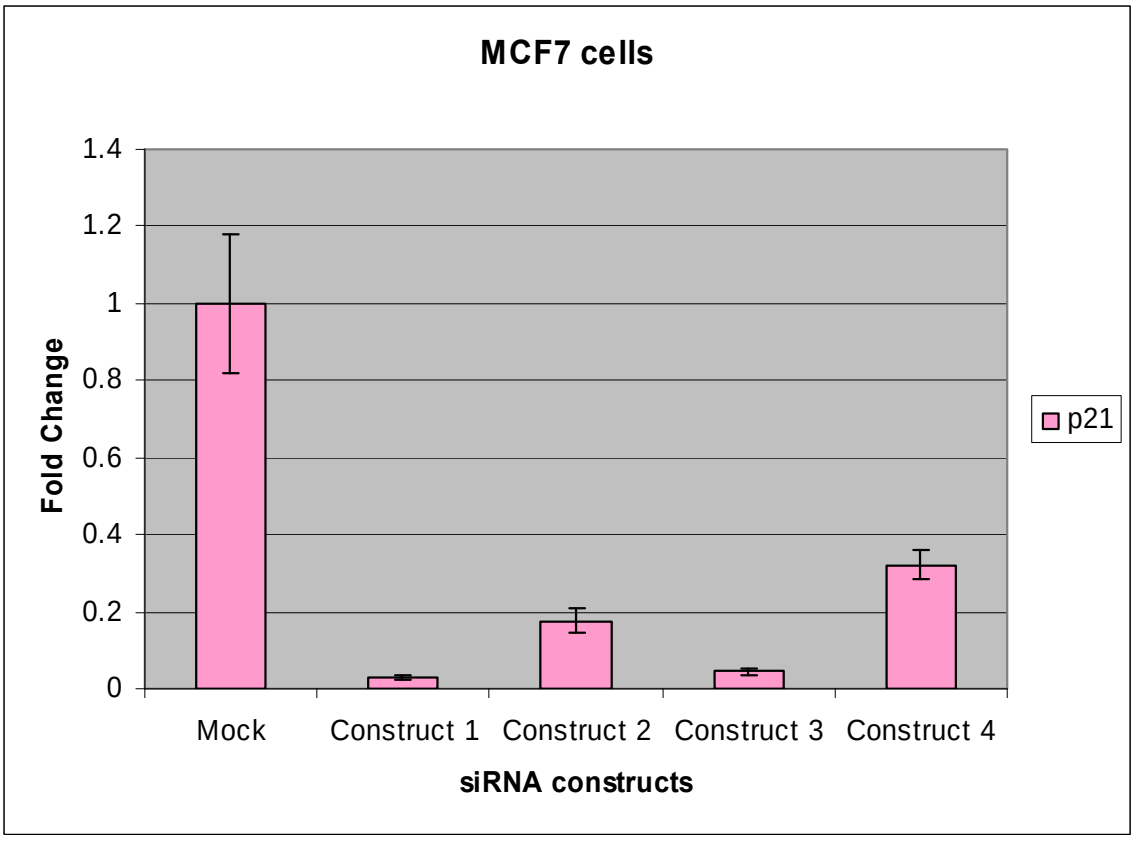


Figure 24: sip21 decreases p21 transcript levels. MCF7 breast cancer cells were reverse transfected(See Materials and Methods) with the indicated RNAi targeting p21. Twenty-four hours later, RNA was isolated and subjected to RT-qPCR to quantify expression of p21 after normalization to GAPDH. Expression levels (Y-axis) were relative to Mock transfection(MCF7 cells) and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

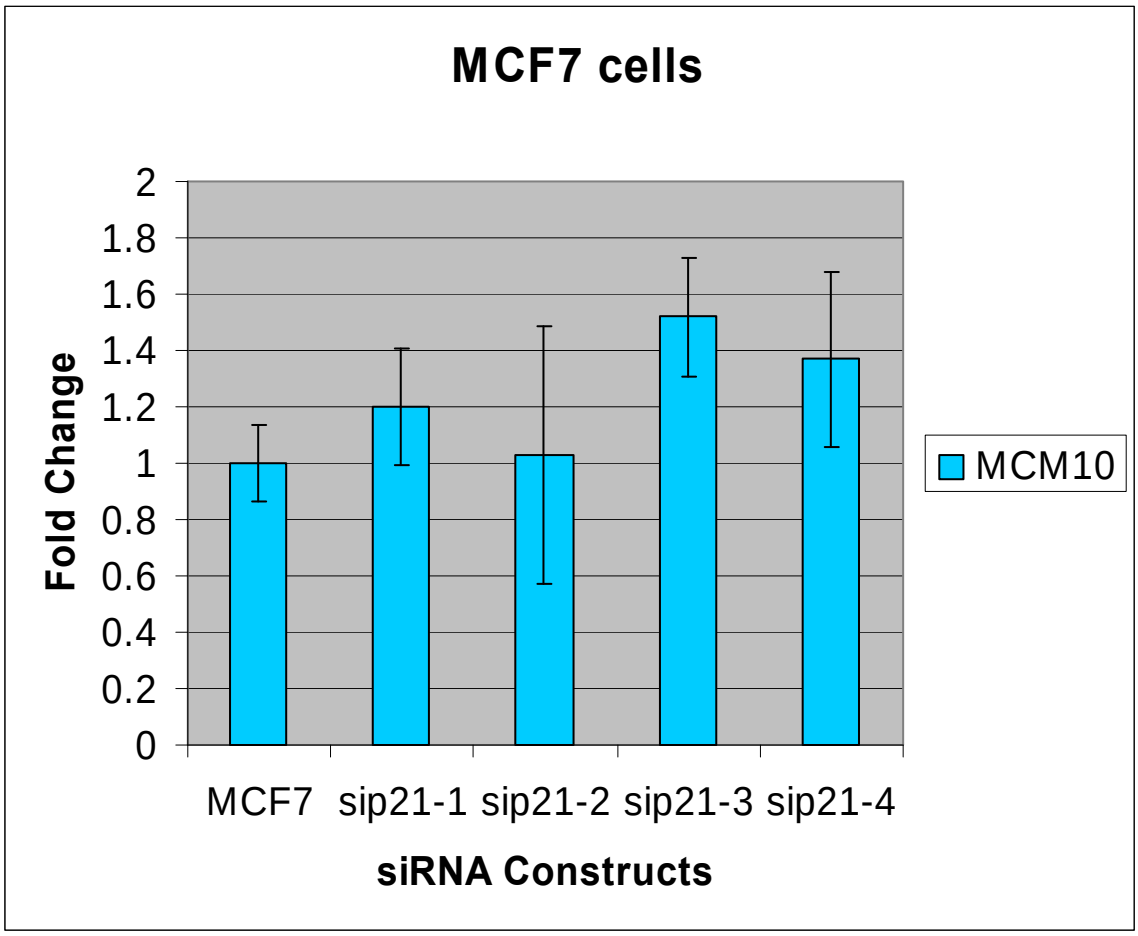
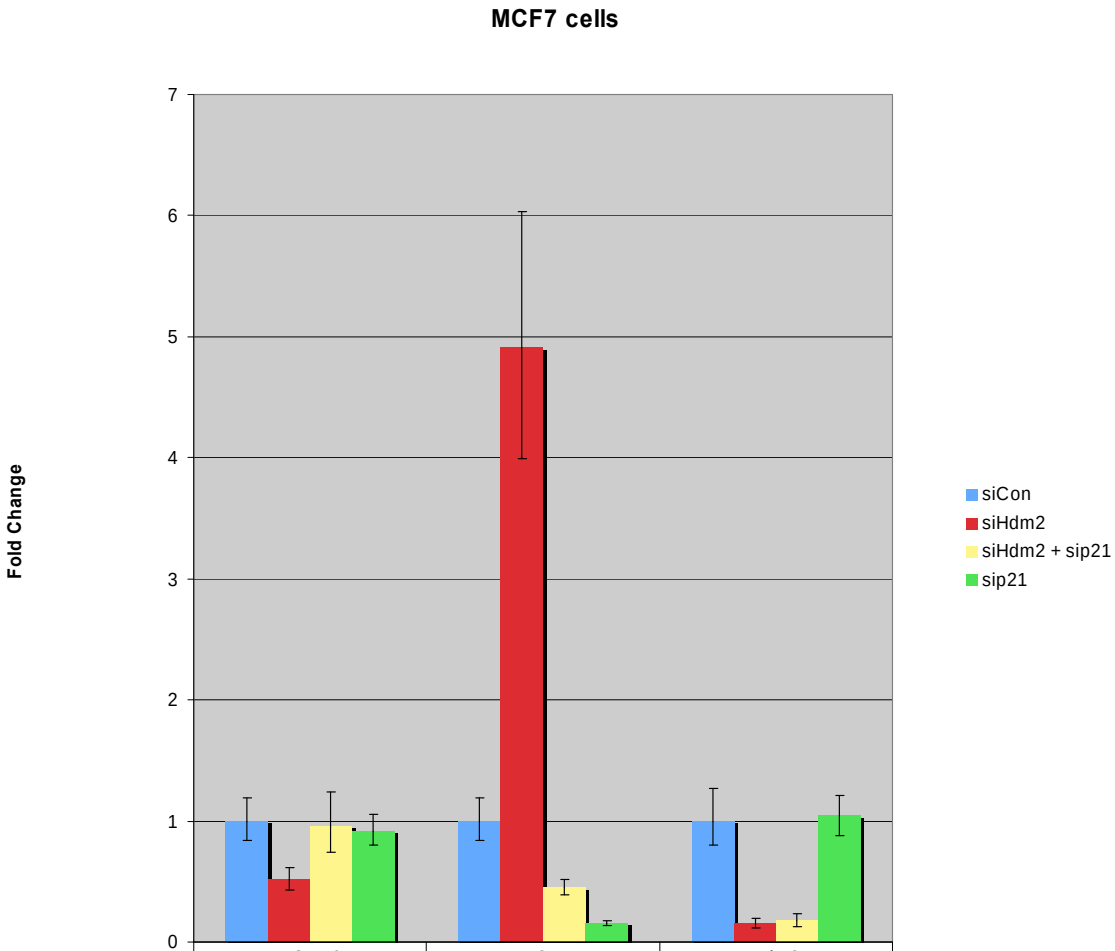


Figure 25: siRNA targeting p21 has little to no effect on MCM10 transcript levels. MCF7 breast cancer cells were reverse transfected(See Materials and Methods) with the indicated RNAi targeting p21. Twenty-four hours later, RNA was isolated and subjected to RT-qPCR to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to Mock transfection(MCF7 cells) and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

DNA damage or RNAi targeting HdmX/Hdm2) is essential in the down-regulation of MCM10 transcript levels stemming from increased levels of p21.

Repression of MCM10 by RNAi targeting Hdm2 is blocked by concurrent p21 knockdown. If in fact the increased levels of p21 (by p53 activation) is the critical effector in the down-regulation of E2F-1 targets, activation of p53 along with simultaneously knocking-down p21 expression should abrogate the repression seen by solely knocking down Hdm2 or HdmX. Therefore, MCF7 cells were reverse transfected as described in the Materials and Methods section with siHdm2 alone or in combination with sip21. As expected, MCM10 levels are reduced 50 % by knocking down Hdm2 with a concomitant increase in p21 levels (Figure 26). Conversely, the simultaneous knock-down of p21 and Hdm2 completely abrogated the repression of MCM10 gene expression. Similar results were seen when using siHdmX alone or in combination with sip21 (data not shown). These results suggest that p53 activation inactivates E2F-1 transactivation via p21 induction.

Repression of E2F-1 targets by RNAi targeting Hdm2 or HdmX is blocked by concurrent p21 knockdown. MCM10 was a member of a large list of E2F-1 targets that were repressed upon activation of p53. Next, I wanted to choose other known E2F-1 targets to determine if they were also possibly regulated by p53 through p21 induction. In MCF7 breast cancer cells, using E2F-1 (which targets itself), along with cyclin A2, and MCM4 (a family member of MCM10) I monitored their transcript levels in response to p53 activation. Upon application of RNAi targeting Hdm2, cyclin A2 transcript levels



	MCM10	p21	Hdm2
siCon	1	1	1
siHdm2	0.51571	4.908507	0.155
siHdm2 + sip21	0.95777166	0.45220748	0.173
sip21	0.9164914	0.1528942	1.04

Figure 26: Repression of MCM10 by hdm2 knockdown is blocked by concurrent knockdown of p21. MCF7 breast cancer cells were reverse transfected (See Materials and Methods) with the indicated RNAi combinations. Twenty-four hours later, RNA was isolated and subjected to RT-qPCR to quantify expression of MCM10 after normalization to GAPDH. Expression levels (Y-axis) were relative to siCon and reported as fold change. Error bars represent the 95% confidence interval of the relative expression.

were reduced by 47% while E2F-1 levels dropped off by 57% (Figure 27) MCM4 transcripts were reduced by 25% (Figure 28). RNAi targeting HdmX led to a reduction of cyclin A2 levels by 89% while decreasing E2F-1 levels by 58% (Figure 27), where MCM4 levels were also reduced by 45% (Figure 28). Just as before, concurrent knock-down of the Hdms and p21 completely abrogated MCM4, E2F-1, and cyclin A2 repression.

Repression of E2F-1 targets by siHdm2 is blocked by concurrent p21 knockdown in mammalian mammary epithelial cells. In the previous experiments I was able to illustrate that three E2F-1 target genes (MCM10, cyclin A2, E2F-1) are all repressed by p53 activation through p21 induction. These experiments were done in MCF7 breast cancer cells where both Hdm2 and HdmX are overexpressed. To test if the repression can be observed in a non-transformed epithelial cell line, I choose MCF10A cells to assess if activating p53 by targeting its negative regulator could lead to a repression of the same E2F-1 targets. Using RNAi targeting Hdm2 I was able to show repression of all three genes: MCM10 37%, E2F-1 46%, Cyclin A2 42% (Figure 29). The same as in MCF7 cells, concurrent knock-down of Hdm2 with p21 abrogated E2F-1 target gene repression.

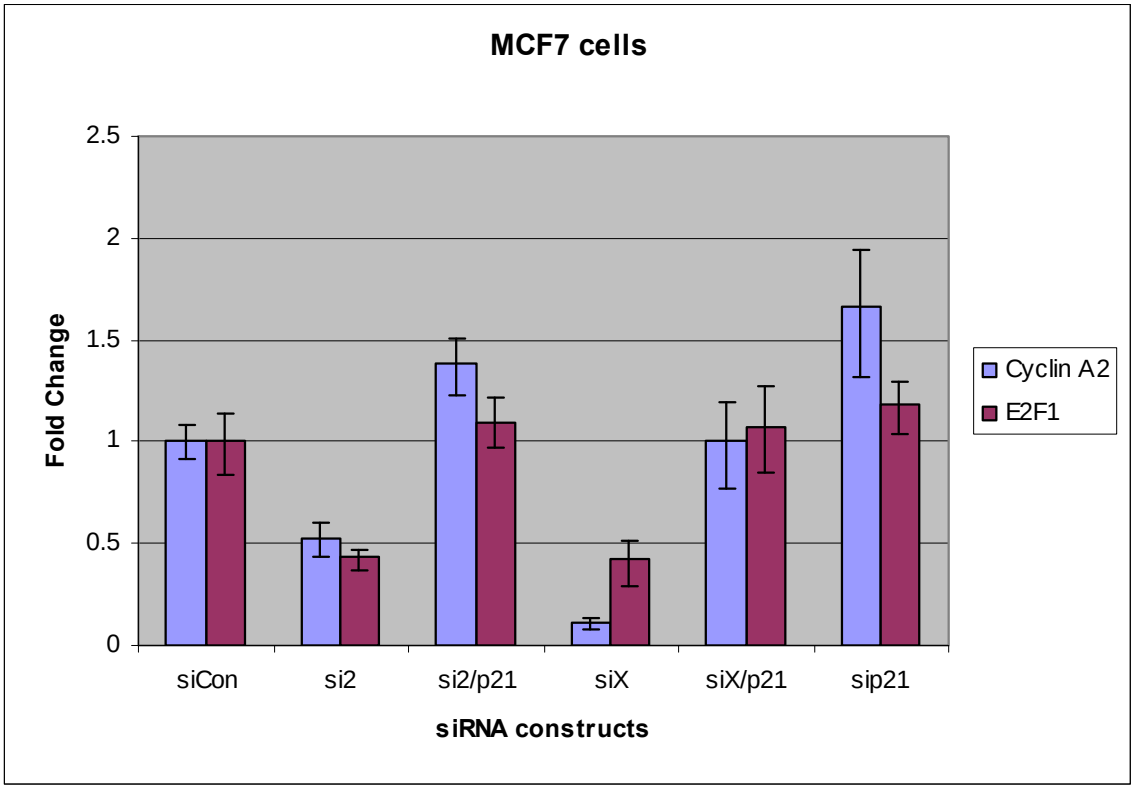


Figure 27: Repression of E2F-1 targets by hdm2 and hdmX knockdown is blocked by concurrent knockdown of p21. MCF7 breast cancer cells were reverse transfected(See Materials and Methods) with the indicated RNAi combinations. Twenty-four hours later, RNA was isolated and subjected to RT-qPCR to quantify expression of Cyclin A2 and E2F-1 after normalization to GAPDH. Expression levels (Y-axis) were relative to siCon and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

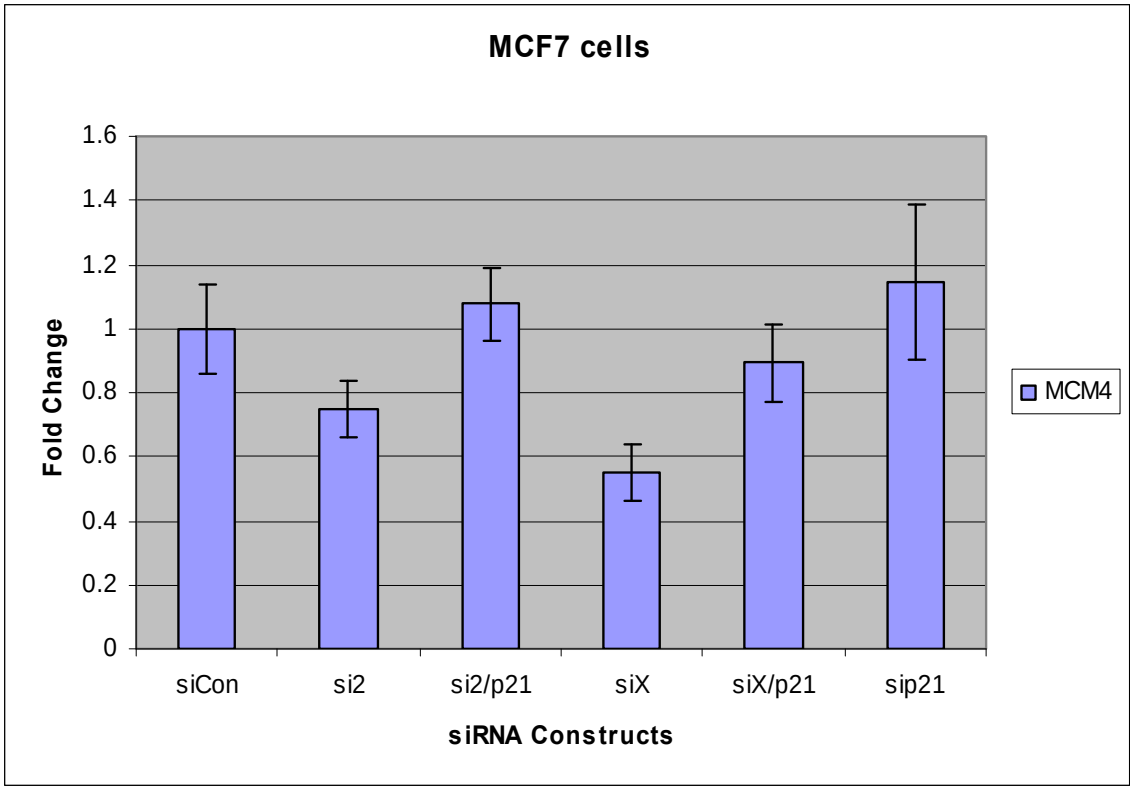


Figure 28: Reduction of MCM4 transcripts by hdm2 and hdmX knockdown is blocked by concurrent knockdown of p21. MCF7 breast cancer cells were reverse transfected(See Materials and Methods) with the indicated RNAi combinations. Twenty-four hours later, RNA was isolated and subjected to RT-qPCR to quantify expression of MCM4 after normalization to GAPDH. Expression levels (Y-axis) were relative to siCon and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

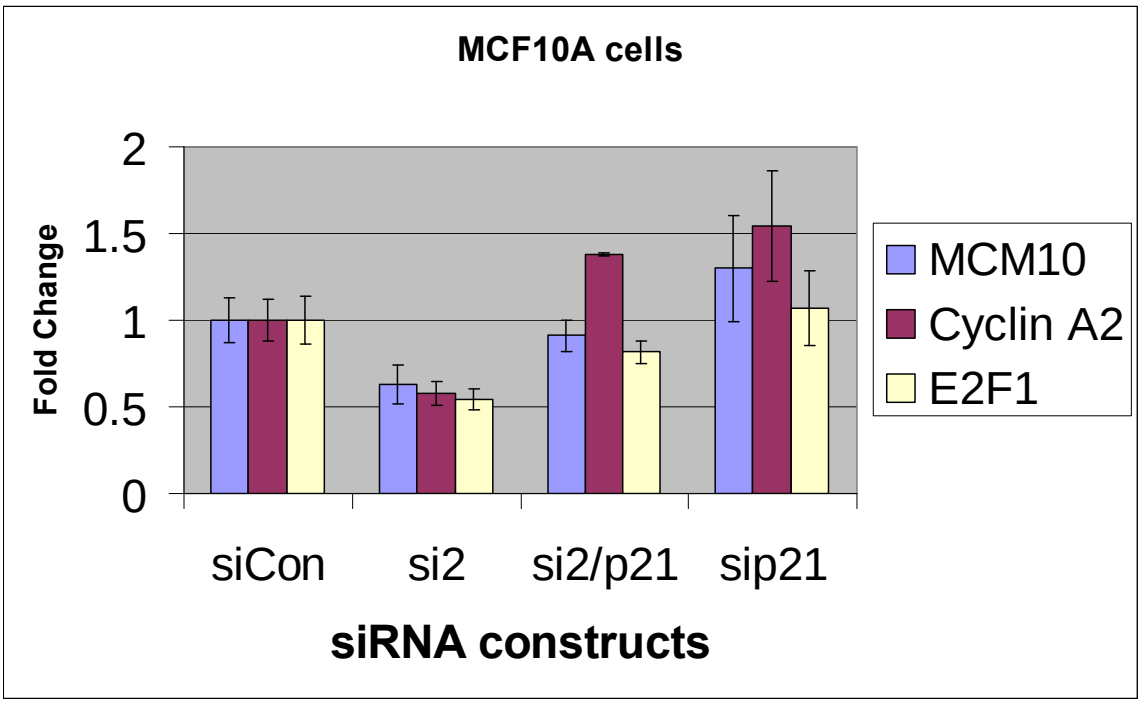


Figure 29: Repression of E2F-1 targets by sihdm2 is blocked by concurrent knockdown of p21 in mammalian mammary epithelial cells. MCF10A mammary epithelial cells were reverse transfected(See Materials and Methods) with the indicated RNAi combinations. Twenty-four hours later, RNA was isolated and subjected to RT-qPCR to quantify expression of MCM10, Cyclin A2 and E2F-1 after normalization to GAPDH. Expression levels (Y-axis) were relative to siCon and reported as fold changes. Error bars represent the 95% confidence interval of the relative expression.

V. Discussion

Human tumors employ a number of different genetic mechanisms to inactivate p53 function. More important to this study are tumor cell lines where one or both of p53's negative regulators, Hdm2 and HdmX, are overexpressed leading to loss of p53 functionality. Using a RNAi approach along with DNA microarrays, we set out to better understand the effect of activation of p53 in cells in which Hdm2 and or HdmX are overexpressed. The DNA microarray experiment was used to first test the effect of knocking-down both Hdm2 and HdmX on known p53 regulated genes, but also provided a means of discovering possible novel p53 regulated genes.

SLC1a1 was a gene that was seen to be increased upon knocking down either Hdm2 and HdmX; these results come directly from the microarray experiment (Figure 2) along with a subsequent experiment in MCF7 where both Hdm2 and HdmX expression were knocked-down (Figure 3). Following these two experiments, a number of DNA damaging experiments were performed to activate wild-type p53 in response to cellular stress. Results from these experiments suggested that SLC1a1 gene expression was either unaltered (Figures 10, 12) or decreased (Figures 5, 14) by genotoxic stress. p21, a well-known p53 target gene, was used as a positive control in which its transcript levels were increased (Figures 5, 10, 12, 14). This suggests that SLC1a1 is not transactivated by cellular stress which is counterintuitive in terms of being a possible p53 target gene.

Another experiment in which p53 was overexpressed provided results that suggest that SLC1a1 gene expression is unaltered by p53 overexpression, while p21 transcript levels were increased as much as 25-fold (Figure 15). Taking all of these results into account, SLC1a1 does not appear to fit the classic model for acting as a p53 transcriptional target gene. My reasoning for last statement is because SLC1a1 expression was not induced due to cellular stress or overexpression of p53. To understanding p53's possible regulation on SLC1a1 gene expression, implementing other mechanisms in which p53 is activated in a non-genotoxic fashion may prove to be a better approach.

Though not a probable direct transcriptional target, SLC1a1 may still be regulated by p53 through an indirect mechanism being cell cycle arrest. Re-activating p53 in MCF7 cells by knocking-down either Hdm2 or HdmX resulted in a growth arrest with no detectable apoptosis (data not shown). So it may be possible that SLC1a1 gene expression is regulated by the cell cycle which in turn is influenced by p53 activity. To test this hypothesis, a cell cycle arrest assay (serum deprivation, aphidicolin treatment, etc) may prove to be helpful in uncovering if the cell cycle indeed has any effect on SLC1a1 gene expression. Another possible explanation for the increased expression of SLC1a1 upon re-activating p53, is that Hdm2 and HdmX could be working independently of p53 and possess a regulatory function on SLC1a1. Though Hdm2 and HdmX are critical to regulating p53, both Hdm2 and HdmX have been found to possess functions independent of p53. In the absence of p53, Mdm2 still plays a role in cell cycle control, DNA repair, and transcriptional regulation (49). MdmX has been found to have a surprising role in suppressing tumorigenesis by promoting centrosome clustering and bipolar mitosis (27).

So, it is also possible that SLC1a1's gene expression may be regulated by Hdm2 or HdmX irrespective of p53 through a cellular process listed above (cell cycle control, DNA repair, bipolar mitosis). There are many possibilities to test this hypothesis experimentally, one might to use RNAi targeting Hdm2 or HdmX in a cell line which does not possess p53 and then study SLC1a1 gene expression.

Another interesting finding within the microarray data was a subgroup of genes that were repressed upon Hdm2 and Hdm2 knockdown that could be classified as known E2F-1 regulated genes. Using one of these known E2F-1 targets, MCM10, I set out to uncover p53's regulation on E2F-1 targets and the global impact of this effect. Through performing a number of experiments in which p53 was activated, by RNAi targeting Hdm2 and HdmX or DNA damaging experiments, it was shown that activation of p53 leads to de-regulation of the E2F-1 pathway involved in cell cycle progression. I was also able to discover that p53-mediated activation of p21 was responsible for this down-regulation. Other groups have noted that p53 activation of p21 could lead to repression of Chk2 (8) but little is known about the global impact of this effect. p21 has the ability to inhibit CDKs (11) causing cell cycle arrest by limiting the ability of E2F-1 to transactivate its G1-S cell cycle progression target genes. I would like to uncover the biological significance of this observation. That is, what is the importance of E2F-1 deregulation on the arrest of the cell cycle mediated by p53? Another interesting aim would be to fully understand whether the induction of p21 has any effect on p53-mediated apoptosis. These findings would enable us to fully understand the importance of induction of p21 on p53-mediated processes (cell cycle arrest vs apoptosis).

Re-activation of p53 by RNAi targeting its two negative regulators provided insight both on known p53 targets but also opened the possibility for finding possible novel p53 regulated genes. Through microarray findings, SLC1a1 was deemed a possible transcriptional target of p53. Experimental data showed that SLC1a1 gene expression was not induced under cellular stress or by p53 overexpression. These results suggest that SLC1a1 does not appear to be a direct target of p53. p53 regulation may be direct through p53's ability to act as a transcription factor but p53 regulation can also be indirect. Upon activation of p53 by loss of Hdm2 and HdmX indirect regulation leads to a p53-dependent decrease of E2F-1 targets through induction of p21. Taking all these findings into considerations, elucidating possible novel p53 regulated genes may lead to groundbreaking results in cancer research.

VI. References

1. Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA. Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nat Neurosci 2006 Jan;9(1):119-26.

2. Bains JS, Shaw CA. Neurodegenerative disorders in humans: The role of glutathione in oxidative stress-mediated neuronal death. Brain Res Brain Res Rev 1997 Dec;25(3):335-58.

3. Carpentieri F, De Felice M, De Falco M, Rossi M, Pisani FM. Physical and functional interaction between the mini-chromosome maintenance-like DNA helicase and the single-stranded DNA binding protein from the crenarchaeon sulfolobus solfataricus. J Biol Chem 2002 Apr 5;277(14):12118-27.

4. Delavaine L, La Thangue NB. Control of E2F activity by p21Waf1/Cip1. Oncogene 1999 Sep 23;18(39):5381-92.

5. Dringen R. Glutathione metabolism and oxidative stress in neurodegeneration. Eur J Biochem 2000 Aug;267(16):4903.

6. Du T, Ciccotosto GD, Cranston GA, Kocak G, Masters CL, Crouch PJ, Cappai R, White AR. Neurotoxicity from glutathione depletion is mediated by cu-dependent p53 activation. Free Radic Biol Med 2008 Jan 1;44(1):44-55.

7. Efeyan A, Serrano M. P53: Guardian of the genome and policeman of the oncogenes. Cell Cycle 2007 May 2;6(9):1006-10.

8. Gottifredi V, Karni-Schmidt O, Shieh SS, Prives C. p53 down-regulates CHK1 through p21 and the retinoblastoma protein. Mol Cell Biol 2001 Feb;21(4):1066-76.

9. Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H, Bruford EA. The ABCs of solute carriers: Physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. Pflugers Arch 2004 Feb;447(5):465-8.

10. Hiebert SW, Chellappan SP, Horowitz JM, Nevins JR. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev 1992 Feb;6(2):177-85.

11. Hitomi M, Shu J, Agarwal M, Agarwal A, Stacey DW. p21Waf1 inhibits the activity of cyclin dependent kinase 2 by preventing its activating phosphorylation. Oncogene 1998 Aug 27;17(8):959-69.

12. Homesley L, Lei M, Kawasaki Y, Sawyer S, Christensen T, Tye BK. Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. Genes Dev 2000 Apr 15;14(8):913-26.

13. Izumi M, Yanagi K, Mizuno T, Yokoi M, Kawasaki Y, Moon KY, Hurwitz J, Yatagai F, Hanaoka F. The human homolog of saccharomyces cerevisiae Mcm10 interacts with replication factors and dissociates from nuclease-resistant nuclear structures in G(2) phase. Nucleic Acids Res 2000 Dec 1;28(23):4769-77.

14. Kachnic LA, Wu B, Wunsch H, Mekeel KL, DeFrank JS, Tang W, Powell SN. The ability of p53 to activate downstream genes p21(WAF1/cip1) and MDM2, and cell cycle arrest following DNA damage is delayed and attenuated in scid cells deficient in the DNA-dependent protein kinase. J Biol Chem 1999 May 7;274(19):13111-7.

15. Kanai Y, Hediger MA. The glutamate/neutral amino acid transporter family SLC1: Molecular, physiological and pharmacological aspects. Pflugers Arch 2004 Feb;447(5):469-79.

16. Kiryu-Seo S, Gamo K, Tachibana T, Tanaka K, Kiyama H. Unique anti-apoptotic activity of EAAC1 in injured motor neurons. EMBO J 2006 Jul 26;25(14):3411-21.

17. Kiyama H, Kiryu-Seo S. Multiple functions of glutamate transporter EAAC1 in motor neurons. Brain Nerve 2007 Dec;59(12):1325-32.

18. Klein C, Vassilev LT. Targeting the p53-MDM2 interaction to treat cancer. Br J Cancer 2004 Oct 18;91(8):1415-9.

19. Korenjak M, Brehm A. E2F-rb complexes regulating transcription of genes important for differentiation and development. Curr Opin Genet Dev 2005 Oct;15(5):520-7.

20. Kuribayashi K, El-Deiry WS. Regulation of programmed cell death by the p53 pathway. Adv Exp Med Biol 2008;615:201-21.

21. Lam EW, Watson RJ. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J 1993 Jul;12(7):2705-13.

22. Lee TK, Lau TC, Ng IO. Doxorubicin-induced apoptosis and chemosensitivity in hepatoma cell lines. Cancer Chemother Pharmacol 2002 Jan;49(1):78-86.

23. Lei M, Tye BK. Initiating DNA synthesis: From recruiting to activating the MCM complex. J Cell Sci 2001 Apr;114(Pt 8):1447-54.

24. Leone G, DeGregori J, Yan Z, Jakoi L, Ishida S, Williams RS, Nevins JR. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev 1998 Jul 15;12(14):2120-30.

25. Liu Y, Lagowski JP, Vanderbeek GE, Kulesz-Martin MF. Facilitated search for specific genomic targets by p53 C-terminal basic DNA binding domain. Cancer Biol Ther 2004 Nov;3(11):1102-8.

26. Marine JC, Dyer MA, Jochemsen AG. MDMX: From bench to bedside. J Cell Sci 2007 Feb 1;120(Pt 3):371-8.

27. Matijasevic Z, Steinman HA, Hoover K, Jones SN. MdmX promotes bipolar mitosis to suppress transformation and tumorigenesis in p53-deficient cells and mice. Mol Cell Biol 2008 Feb;28(4):1265-73.

28. Mayol X, Grana X. pRB, p107 and p130 as transcriptional regulators: Role in cell growth and differentiation. Prog Cell Cycle Res 1997;3:157-69.

29. Meulmeester E, Jochemsen AG. P53: A guide to apoptosis. Curr Cancer Drug Targets 2008 Mar;8(2):87-97.

30. O'Connor PM, Fan S. DNA damage checkpoints: Implications for cancer therapy. Prog Cell Cycle Res 1996;2:165-73.

31. Ohtani K, Iwanaga R, Nakamura M, Ikeda M, Yabuta N, Tsuruga H, Nojima H. Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. Oncogene 1999 Apr 8;18(14):2299-309.

32. Park JH, Bang SW, Jeon Y, Kang S, Hwang DS. Knockdown of human MCM10 exhibits delayed and incomplete chromosome replication. Biochem Biophys Res Commun 2008 Jan 18;365(3):575-82.

33. Radhakrishnan SK, Gierut J, Gartel AL. Multiple alternate p21 transcripts are regulated by p53 in human cells. Oncogene 2006 Mar 16;25(12):1812-5.

34. Rallapalli R, Strachan G, Cho B, Mercer WE, Hall DJ. A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity. J Biol Chem 1999 Mar 19;274(12):8299-308.

35. Ricke RM, Bielinsky AK. Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. Mol Cell 2004 Oct 22;16(2):173-85.

36. Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol 2008 May;9(5):402-12.

37. Sbisa E, Catalano D, Grillo G, Licciulli F, Turi A, Liuni S, Pesole G, De Grassi A, Caratozzolo MF, D'Erchia AM, Navarro B, Tullo A, Saccone C, Gisel A. p53FamTaG: A database resource of human p53, p63 and p73 direct target genes combining in silico prediction and microarray data. BMC Bioinformatics 2007 Mar 8;8 Suppl 1:S20.

38. Schwarz JK, Devoto SH, Smith EJ, Chellappan SP, Jakoi L, Nevins JR. Interactions of the p107 and rb proteins with E2F during the cell proliferation response. EMBO J 1993 Mar;12(3):1013-20.

39. Sopta M, Gallie BL, Gill RM, Hamel PA, Muncaster M, Zacksenhaus E, Phillips RA. The retinoblastoma protein and the cell cycle. Semin Cancer Biol 1992 Jun;3(3):107-13.

40. Toledo F, Wahl GM. MDM2 and MDM4: P53 regulators as targets in anticancer therapy. Int J Biochem Cell Biol 2007;39(7-8):1476-82.

41. Vaghefi H, Hughes AL, Neet KE. Nerve growth factor withdrawal-mediated apoptosis in naive and differentiated PC12 cells through p53/caspase-3-dependent and -independent pathways. J Biol Chem 2004 Apr 9;279(15):15604-14.

42. Vousden KH, Lu X. Live or let die: The cell's response to p53. Nat Rev Cancer 2002 Aug;2(8):594-604.

43. Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu Y, Weng Z, Liu J, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD,

Lim B, Liu ET, Yu Q, Ng HH, Ruan Y. A global map of p53 transcription-factor binding sites in the human genome. Cell 2006 Jan 13;124(1):207-19.

44. Wheeler LW, Lents NH, Baldassare JJ. Cyclin A-CDK activity during G1 phase impairs MCM chromatin loading and inhibits DNA synthesis in mammalian cells. Cell Cycle 2008 Jul;7(14):2179-88.

45. Yamasaki L. Role of the RB tumor suppressor in cancer. Cancer Treat Res 2003;115:209-39.

46. Yoshida K, Inoue I. Expression of MCM10 and TopBP1 is regulated by cell proliferation and UV irradiation via the E2F transcription factor. Oncogene 2004 Aug 19;23(37):6250-60.

47. Yoshida K, Inoue I. Regulation of geminin and Cdt1 expression by E2F transcription factors. Oncogene 2004 May 6;23(21):3802-12.

48. Zerangue N, Kavanaugh MP. Interaction of L-cysteine with a human excitatory amino acid transporter. J Physiol 1996 Jun 1;493 (Pt 2)(Pt 2):419-23.

49. Zhang Z, Zhang R. p53-independent activities of MDM2 and their relevance to cancer therapy. Curr Cancer Drug Targets 2005 Feb;5(1):9-20.

50. Zhou R, Frum R, Deb S, Deb SP. The growth arrest function of the human oncoprotein mouse double minute-2 is disabled by downstream mutation in cancer cells. Cancer Res 2005 Mar 1;65(5):1839-48.