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Award Number: DAMD17-99-1-9074

TITLE: Functional Significance of Mutant p53 in Breast Cancer

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 99 -30 Jun 02)

4. TITLE AND SUBTITLE
Functional Significance of Mutant p53 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9074

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Approximately 50% of human cancers have accumulated missense mutations in the gatekeeping tumor suppressor protein p53, usually resulting in genomic instability and a very poor prognosis. The wild-type p53 protein is required for assessing DNA damage in cells and making the decision to either induce cell cycle arrest to facilitate DNA repair, or to induce a suicide response in those cells with irreparable damage. In human tumors, many hot-spot mutations are found within the DNA-binding domain of p53, rendering it incapable of sequence-specific transactivation of target genes such as p21, bax, and mdm2. Some of these mutants, in addition to having dominant-negative functions, also gain novel functions by interacting with proteins differently from the wild-type p53 protein. One such gain-of-function p53 mutant possesses an Arg to His substitution at codon 175 (172 in mice) and has been shown to be involved in the dysregulation of centrosome duplication leading to abnormal mitoses and subsequent aneuploidy. Because centrosome abnormalities and aneuploidy are often seen in high-grade breast tumors, unraveling the mechanism behind the involvement of p53172 R-H in centrosome dysregulation will help us to understand the progression of mammary carcinogenesis. In order to identify potential indirect target genes regulated by this mutant, we employed a suppressive subtractive hybridization technique to generate a cDNA library specific to p53 null mammary epithelial cells (MECs) expressing the 172 R-H mutant. cDNA made from p53 null mammary epithelial cells transiently transfected with wild-type p53 was subtracted from cDNA made from mutant p53 transfected cells. The subtraction procedure generated a pool of cDNAs differentially expressed in the presence of the mutant protein; many interesting genes were revealed to be candidates for regulation by mutant p53. Some of these include developmental, metabolic, transcriptional, translational, and structural genes, as well as genes involved in transformation, signal transduction, chromatin remodeling, DNA repair and apoptosis.

14. SUBJECT TERMS
breast cancer, predoctoral

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Approximately 50% of human cancers have p53 alterations, and patients with these cancers have poorer prognoses. When functional, the p53 protein blocks proliferation of cells that have sustained DNA damage and induces apoptosis in cells too badly damaged to undergo repair. Mutant forms of p53 are often no longer protective, and in many cases have acquired additional functions, which make them more deleterious than the simple absence of wild-type p53. The p53 175R-H mutant is one such gain-of-function mutant. It is a conformational mutant and can no longer bind DNA, although it retains its ability to bind other proteins. It is hypothesized that mutant p53 proteins may gain novel functions by interacting with other proteins that supply a DNA-binding domain. The resulting complex could then use the p53 transactivation domain to modulate a novel set of genes.

p53 has also been implicated in the regulation of the G2/M spindle checkpoint and mitosis, with particularly striking effects upon centrosome duplication. Centrosomal hypertrophy is implicated in at least two processes that adversely affect prognosis in cancer patients: 1) loss of cell polarity and tissue organization, and 2) an increased occurance of multipolar mitoses, which predisposes to the development of aneuploidy. In addition, centrosomes have been shown to be larger and more numerous in high-grade breast adenocarcinomas. Unraveling the mechanism behind the involvement of the 172R-H mutant in the dysregulation of centrosome duplication leading to aneuploidy is of great interest to this project and could help us to better understand tumor progression.

A p53 172R-H transgenic mouse model was generated in the laboratory, and studies of this mouse indicated that the presence of this p53 mutant did not decrease apoptosis or increase proliferation, but did promote the development of aneuploid tumors following carcinogen treatment. Similar results were seen when these mice were crossed with mice carrying other mammary-targeted oncogenes; tumors arising in the p53 mutant-carrying bitransgenic mice were frequently aneuploid. The current studies were initiated in order to address the mechanistic issues: specifically, how the p53 175R-H mutant contributes to mammary tumorigenesis and the development of genomic instability.

Body

Specific Aims:

In order to understand the role of the p53 172R-H mutant in mammary tumorigenesis, the scope of the grant proposal is outlined by the following specific aims :

1) To study the effect of the WAP-p53 172R-H transgene on genomic instability in early stages of mammary tumorigenesis

2) To investigate p53 172R-H as a gain-of-function mutant by identification of potential indirect target genes and/or novel protein-protein interactions

Progress made toward these aims:

Specific Aim 1: To study the effect of the WAP-p53 172R-H transgene on genomic instability in early stages of mammary tumorigenesis.

Initial characterization of mammary tumors arising spontaneously (following pituitary isograft) in mice bitransgenic for WAP-p53 172R-H and WAP-TGF-α indicated that 57% of tumors arising in the mutant p53-expressing bitransgenic mice were aneuploid, whereas no aneuploid tumors were observed in the WAP-TGF-α control mice. Both sets of tumors arise with a latency of 100-110 days post-isograft. Loss of wild-type p53 is known to influence aneuploidy through dysregulation of centrosome duplication. Furthermore, centrosomes from high-grade human breast cancers are abnormal in many respects, indicating that there may be a relationship between centrosome abnormality and aneuploidy, which is a marker of poor prognosis. It has been shown that aberrant centrosome duplication can be detected in early hyperplasias and even in phenotypically normal, but transformed, tissue. We initially hypothesized that the aneuploidy we had observed in bitransgenic tumors might be accompanied by centrosome dysregulation. Furthermore, given the short timeframe in which both of these sets of tumors arose, we could examine mammary glands of mice at defined timepoints within the 110 day window for aberrant centrosome numbers occurring in phenotypically normal tissue and preceding the development of aneuploid tumors.

Although preliminary results indicated that there might be more centrosomes in bitransgenic tumors, the complete study did not bear this out. Neither group of tumors appeared to contain aberrant centrosome numbers, despite the ploidy variance. Furthermore, the pretumor samples from both groups appeared to have normal numbers of centrosomes at the timepoints examined, which were 15, 30, 45, 60, and 90 days post-isograft.

However, this did not discount the possiblity that some transient centrosome abnormality resulting in later ploidy-related consequences might be induced very soon (*i.e.*, less than 15 days) after expression of the p53 172R-H mutant in the mouse mammary gland. In order to address this possibility, we extended this specific aim to include *in vitro* studies. p53-null mammary epithelial cell [MEC] cultures were transfected with either wild-type p53 or p53 172R-H using an adenoviral method, and subsequently immunostained for both p53 and gamma-tubulin, a component of centrosomes. Transfected (*i.e.*, p53-positive) cell centrosome numbers were analyzed and compared to those of untransfected cells. These experiments indicated that cells transfected with the p53 mutant did indeed demonstrate aberrant centrosome numbers by 3 days post-transfection, whereas this was not seen in mock-transfected cells or cells transfected with wild-type p53. However, very few MECs stably-transfected with the mutant p53 had aberrant centrosome numbers, suggesting that the majority of the abnormal cells seen in the transient transfection experiments underwent apoptosis due to failure in mitosis. Additional experiments indicated that MECs stably transfected with the p53 mutant displayed reduced apoptosis, both basal and DNA-damage induced, suggesting a second mechanism by which this p53 mutant might also contribute to mammary tumorigenesis. We hypothesize that the early centrosome abnormalities create a cellular environment in which genomic instability is more frequent than usual, compounded by diminished apoptotic capabilities in the same cells, and that this forms the basis of the tumor predisposition seen in the mouse models.

Specific Aim 2: p53 172R-H as a gain-of-function mutant: the identification of potential indirect target genes and/or novel protein-protein interactions

Previous Observations

It is known that the p53 172R-H mutant cannot bind to p53 consensus binding sites, but there are several papers suggesting that p53 may have indirect transcriptional effects. Furthermore, it is possible that this p53 mutant may mediate its gain-of-function effects partially through aberrant protein-protein interactions. Any genes up- or down-regulated preferentially in the presence of the mutant could be directly involved in the dysregulation of normal centrosome number and maintenance of chromosomal stability. The potential transcriptional effects of the p53 172R-H mutant are currently being addressed. Initially, we proposed to use cDNA obtained from p53-null MECs transiently transfected with mutant p53, wild-type p53, or mock-transfected cells to screen Atlas™ Arrays (Clontech) for differentially expressed genes. However, due to difficulties with data analysis, we were not confident regarding the validity of data obtained from these screens, and so took an alternative approach. We employed CLONTECH's PCR-Select™ cDNA Subtraction Kit to perform a suppressive subtractive hybridization in which cDNA made from p53 null cells transfected with wildtype p53 was subtracted from cDNA made from mutant p53 transfected cells. The subtraction procedure generated a pool of cDNAs differentially expressed in the presence of the mutant protein two days after transfection. These cDNAs were then cloned into pGEM vectors; 900 plasmids were differentially screened with forward and reverse subtracted probes, and 120 clones were chosen to be sequenced following the screening. An example of this

differential screening process is illustrated in Figure A-1. Of the102 clones identified in sequence homology databases, many interesting genes were revealed to be candidates for transcriptional regulation by mutant p53. A wide variety of genes were identified – some of these include developmental (EED), metabolic, transcriptional (TRAP100, SRA), translational (Trt, Naca), and structural genes (γ-actin), as well as genes involved in transofmation (MAT1), signal transduction (SDF1, $G\alpha_s$, Pitpn, MRK, calcyclin), chromatin remodeling (Psma1, Hmg1, H2A), DNA repair (Ku70) and apoptosis (Naip1). In addition, 11 ESTs were identified. A summary of the types of genes identified from this screen is described in Table A-1. We were especially interested in the genes involved in transcription, chromatin remodeling, and DNA repair and decided to pursue the involvement of mutant p53 in these processes in an attempt to discover its role in mammary carcinogenesis.

Recent Observations

To initiate the verification process, six clones were chosen to produce radiolabeled probes for Northern analysis – EED, Nm23, SRA, Ku70, HMG1, and EST 4-9B. Some of these were chosen due to the magnitude of the differences in signal intensities, and some were chosen based on hypothetical or established associations with p53. A summary of these genes, their reported functions, and potential relationships with p53 can be found in Table A-2. Both mammary cell culture Northerns and mammary tissue Northerns were performed on the aforementioned six SSH clones.

To obtain the RNA required to verify the up- or down-regulation of the SSH clones, p53 null MECs were retrovirally infected with either mutant p53 or β-gal retroviral constructs and harvested 48, 72, and 96 hours following infection. Following verification of mutant p53 expression in this cell line, total RNA was isolated from these cells and probed with EED, HMG1, SRA, Ku70, Nm23, and EST 4-9B PCR-amplified cDNAs. The results of these hybridizations, as well as normalization by hybridization to cyclophilin, are illustrated in Figures A-2 and A-3.

Expression of the SSH clones was higher in mutant p53 infected cells than β-gal infected cells in all cases (for all clones observed and at all time points recorded). However, changes in expression levels were more dramatic for some genes than others. Relative band intensities were normalized to cyclophilin hybridization intensities and differences in expression were observed to range from 1.6 – 5.1 fold. As predicted by the reverse southern analysis of the PCR-amplified SSH clones, all of the SSH genes observed increased in expression when mutant p53 was expressed.

In order to determine whether the six SSH clones were also up-regulated in the whole mammary gland of a mutant p53 transgenic mouse model, WAP-p53R172H transgenic mice were impregnated. In this model, mutant p53 is driven by the WAP (whey acidic protein) promoter. WAP is a milk protein gene expressed at very high levels during pregnancy; it is turned on at day 10 of pregnancy in the mouse. Therefore, with this model, mutant p53 is expressed at high levels in mid- and late-pregnant animals. Total RNA was extracted from the mammary glands of day 16 pregnant animals and

subjected to northern hybridization with the six different clones in question. Figures 3 and 4 illustrate the relative expression levels of EED, HMG1, SRA, Ku70, Nm23, and EST 4-9B in pregnant versus virgin transgenic animals. Additionally, two p53 positive (340T and 430T) and two p53 negative (443T and 457T) Wnt transgenic mammary tumors (provided by Larry Donehower, Baylor College of Medicine) were used in order to observe changes in expression levels of the SSH clones during mammary tumorigenesis. It is important to note that the RNA was extracted from entire mammary glands for this particular set of northern experiments; therefore, several different cell types are potentially present. Conversely, the Northern blots performed on the aforementioned cell cultures theoretically represent a pure population of mammary epithelial cells. As a result, the observed changes in gene expression were minimal for the mammary tissue Northerns. However, several interesting observations can still be made from these experiments.

For example, both EED and Nm23 Northerns exhibited extra bands not observed in the cell culture Northerns that can be interpreted as alternative spliceforms of these genes. Due to the absence of these splice forms in the liver RNA samples, it is possible that these additional bands represent mammary-specific splicing isoforms. With regard to EED, a previously unreported, slower-migrating 3.2 Kb transcript was observed in addition to the 1.7 and 2.2 Kb transcripts reported in the literature. Interestingly, this splice form appears to be absent in the mammary glands of pregnant transgenic animals, but was detected at relatively high levels in the mammary glands of pregnant FVB wild-type animals (see Figure 4). There is no apparent difference in the expression of this novel splice form between the virgin transgenic and virgin wild-type mammary RNA samples. Similar to what was observed for EED, Nm23 exhibited an identical pattern of alternate splice form expression within the observed samples. In addition to the previously reported 0.8 Kb primary transcript, a previously unreported, slower-migrating 2.3 Kb transcript was observed which was absent in the pregnant transgenic animals, but expressed at higher levels in the FVB wild-type pregnant animals. Also similar to the EED expression profile, levels of Nm23 in virgin transgenic and virgin FVB wild-type animals were essentially the same. The remaining genes (SRA, HMG-1, Ku70, and EST 4-9B), as well as the primary transcripts of both EED and Nm23, exhibited no significant changes in expression between transgenic pregnant and wild-type pregnant mammary RNA samples.

Two separate and interesting observations can be made from the screening of the two p53 positive and two p53 negative mammary tumor samples from Wnt transgenic animals. First, there was a diverse and somewhat contradictory pattern of Ku70 expression within these tumor samples. Ku70 was up-regulated by approximately two-fold in both samples of p53 negative tumors (443T and 457T) when compared to its expression levels observed in any of the non-tumor samples. However, Ku70 was up-regulated in only one of the p53 positive tumor samples (340T), and this was observed to be approximately five-fold greater when compared to the non-tumor samples. The second interesting observation can be seen with the differences in both SRA and HMG-1 expression between the tumor samples. The relative levels of SRA and HMG-1 expression in both p53 negative tumor samples (443T and 457T) were similar to that

observed for all non-tumor samples; however, levels of SRA and HMG-1 expression decreased (by approximately three-fold) in both p53 positive tumor samples (340T and430T) when compared to all other samples (tumor and non-tumor) assayed.

Previous Research Accomplishments

1. Determined that centrosome amplification is not necessarily coincident with aneuploidy in a bitransgenic mouse mammary tumor model

2. Demonstrated transient centrosome abnormalities in p53 175R-H-transfected MECs

3. Demonstrated that stably-transfected cell populations mimic tumor populations in that they lack significant percentages of cells with centrosome abnormalities

4. Demonstrated that MECs stably transfected with p53 175R-H display diminished basal and DNA damage-induced apoptotic responses

5. Generated a subtractive cDNA library containing cDNAs differentially expressed in p53 null MECs transiently transfected with p53 172 R-H

6. Revealed important and significant candidate genes possibly regulated by mutant p53, such as EED, Hmg1, Ku70, SRA, and Nm23

7. Generation of a retroviral construct containing the p53 R172H mutant cDNA

8. Development of a retrovirally-mediated gene transfer technique using a p53-null mammary epithelial cell line

9.

10. Propagation and re-establishment of the WAP-p53R172H transgenic mouse colony

Key Research Accomplishments Since the Last Status Report

1. Successful transduction of a p53-null mammary epithelial cell (MEC) line with a p53R175H retroviral construct

2. Successful expression of the p53 R175H mutant in this cell line

3. Northern analysis of p53-null MECs expressing the p53 R175H mutant for verification of the up-regulation of the following genes by mutant p53: EED, HMG-1, SRA, Ku70, Nm23, and GRASP

4. Northern analysis of whole mammary glands from transgenic animals expressing p53R172H

Reportable Outcomes

Data from this project was presented at two national meetings:

Molecular Biology and Pathology of Neoplasia Workshop, Keystone, CO, July 1998.

Mouse Models of Mammary Tumorigenesis Meeting, Bar Harbor, ME, October, 1999.

Data from this project has been published in two peer-reviewed journal articles:

Murphy, K.L., and Rosen, J.M. (2000) Mutant p53 and genomic instability in a transgenic mouse model of breast cancer. *Oncogene*, **19**:1045.

Murphy, K.L., Dennis, A.P, and Rosen, J.M. (2000) A gain-of-function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. *FASEB J.*, 14, 2291-2302.

The following degrees will be awarded as a result of the research supported by this grant:
A Ph.D. in Cell and Molecular Biology, Baylor College of Medicine, was awarded to Kristen Murphy in June of 2000.

An M.S. in Cell and Molecular Biology, Baylor College of Medicine, was awarded to Renee O'Lear in September, 2002

Conclusions

The presence of p53 mutations has been well established as a poor prognostic factor in human breast tumors and other cancers, and is believed to participate in either the progression or initiation of mammary carcinogenesis. Several hotspot mutations,

including p53 R175H, are the most common mutations observed in cancers and are thought to result in the most severe phenotypes. Since p53's transcriptional activity plays such a critical role in normal genome maintenance and stability, it is not surprising that when this activity is impaired due to a particular mutation, such as that found in p53 R175H, abnormal cell division and genomic instability often become unavoidable. In order to better understand the sequence of events leading to genomic instability, it is important to observe cellular changes, including those at the transcriptional level, as early as possible after these critical mutations occur. Using a cell culture model in which the R175H p53 mutation is introduced into a p53 null background allows for the observation of such changes in the earliest stages – only a couple of days following the mutant's introduction. We used the suppressive subtractive hybridization technique in order to detect even the smallest of changes in gene transcription between p53-null mammary epithelial cells (MECs) expressing the R175H mutant and those expressing wild-type p53.

Of the six genes investigated, all were verified to be up-regulated by mutant p53 by Northern analysis. Furthermore, the degree of differential expression observed for these genes ranged from 1.6X to 5.1X, with levels peaking for most genes 48 hours following the introduction of mutant p53 into the p53 null cell line. This demonstrates both the accuracy and sensitivity of the suppressive subtractive hybridization procedure employed. Because we were interested in looking at differences in gene expression during the earliest stages of transformation, we conducted the subtraction procedure 2 days following the introduction of the mutant into our p53 null mammary epithelial cell line. Because the two samples subtracted from each other for this purpose only differed with the expression of a single gene, mutant p53, we did not expect to observe an enormous number of genes to be differentially expressed, nor did we expect the differences in expression levels of these genes to be easily detectable with microarray technology. We, therefore, undertook an experimental procedure that was previously reported to detect both low abundance and novel genes.

This study demonstrates that the gain-of-function R175H p53 mutant protein is indeed capable of altering gene transcription either directly or indirectly. The genes induced by mutant p53 were found to be diverse in both structure and function, indicating that mutant p53 is involved in a wide variety of cellular processes, any or all of which may be contributing to genomic instability and cancer. However, this finding only begins to address the question concerning the mechanism governing the gain-of-function p53 mutants and how they are conferring their novel properties upon the cell. Because the evidence supporting the participation of these mutants in novel protein-protein interactions is strong, such as how the other p53 family members are adversely affected by these p53 mutants, it is most likely that p53 R175H and other gain-of-function mutants are exerting their affects on the cell at both the transcriptional and post-translational levels.

References

Murphy, K.L., and Rosen, J.M. (2000) Mutant p53 and genomic instability in a transgenic mouse model of breast cancer. *Oncogene*, **19**:1045.

Murphy, K.L., Dennis, A.P, and Rosen, J.M. (2000) A gain-of-function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. *FASEB J.*, 14: 2291-2302..

Appendix

Gene Category	Percent of SSH Screen	Genes Identified
Transcription	6%	Y-box transcription factor, TRAP 100, Repeat family 3 gene, Trg, SRA, Lap2 BP1
Translation	5%	Trt, eIF-2β, cysteinyl-tRNA transferase, NACA, EF1-alpha
Signal Transduction	13%	SDF-1α, guanine nucleotide binding protein, cathepsin L, **calcyclin, minopontin, Ndpp1, Pitpn, MRK, p47, PP1 regulatory subunit, MAPK-1
Developmental	2%	EED, Embigen precursor
Chromatin Remodeling	5%	*Hmg1, EED, Hnrnp A2, Histone H2A.z
Oncogenes	1%	MAT1
DNA Repair	1%	Ku 70
Apoptosis	1%	Naip1 apoptotic inhibitory protein
Immunity	8%	MHC Class I heavy chain precursor, T-cell receptor α locus, T complex protein 1, Prothymosin α, **β2-microglobulin, macrophage interferon IP 10
Structural	7%	Cytoskeletal γ-actin, **cytoskeletal β-actin, Syndecan 2, tropomyosin 5, ER p99
Metabolic	14%	ODC antizyme, LDH-A, ADH, thioredoxin, *ferritin heavy chain, ferritin light chain, lysosomal ATPase, alpha enolase, ER p99, *GRASP, *glucosamine-6-sulfatase
Protein Modification	6%	26S proteasome subunits p112, Lmp7, Psma3 and PsmdII, proteasome activator PA28β, UB-specific protease unp
Mitochondrial	6%	Mitochondrial carrier homologue 2, Atpa1, ***mitochondrial DNA
Ribosomal	12%	*RpL26, RpL8, *Rps4x, *****RpL35a, RpL21
Centrosomal Kinases	3%	**Nucleoside diphosphate kinase B (Nm23)
ESTSs	6%	

Table A-1. Categories of genes identified in SSH screen. Asterisks denote the additional number of times that particular gene appeared during the screening process.

Genes Pursued for Verification	Approx Fold Change	Reported Function	Possible Relationships with p53
1. *SRA*	↓ 2X	RNA co-activator involved in the formation of general transcriptional machinery; aberrant levels observed in human breast tumors	Both SRA and p53 shown to associate directly with SRC-1; all may potentially form a multi-protein complex
2. *Ku 70*	↑ 2X	Involved in DNA double-strand break repair; heterodimerizes with Ku80 to form a complex which recognizes and binds to double-strand breaks	The Ku70/Ku80 heterodimer activates DNA-PK, which has been reported to indirectly phosphorylate p53
3. *EED*	↑ 10 X	Polycomb-group gene expressed early in development and throughout adulthood that suppresses a wide variety of developmentally-regulated genes; involved in X-inactivation in females	No reported link to p53; however, may possibly be related to EED at the transcriptional level
4. *Nm23*	↑ 10 X	Nucleoside diphosphate kinase (GDP→GTP); involved in tubulin dynamicity; shown to associate with centrosomes	No reported link to p53; however, mutant p53 also associates with centrosomes and induces both centrosome hyperamplification and genomic instability
5. *HMG 1*	↑ 3 X	General transcriptional activator; bind DNA and converts it to an open transformation so that transcription factors may have access to the DNA	Shown to associate directly with p53 and increase its transcriptional activation
6. *EST 4-9B (GRASP-like protein)*	↑ 15 X	Contains a similar ATP-binding domain to glutathione-S-transferase; not well characterized	No reported link to p53; originally pursued from this screen as an EST due to its dramatic fold change

Table A-2. Six genes chosen from SSH screening results to be pursued for verification of differential regulation by mutant p53.

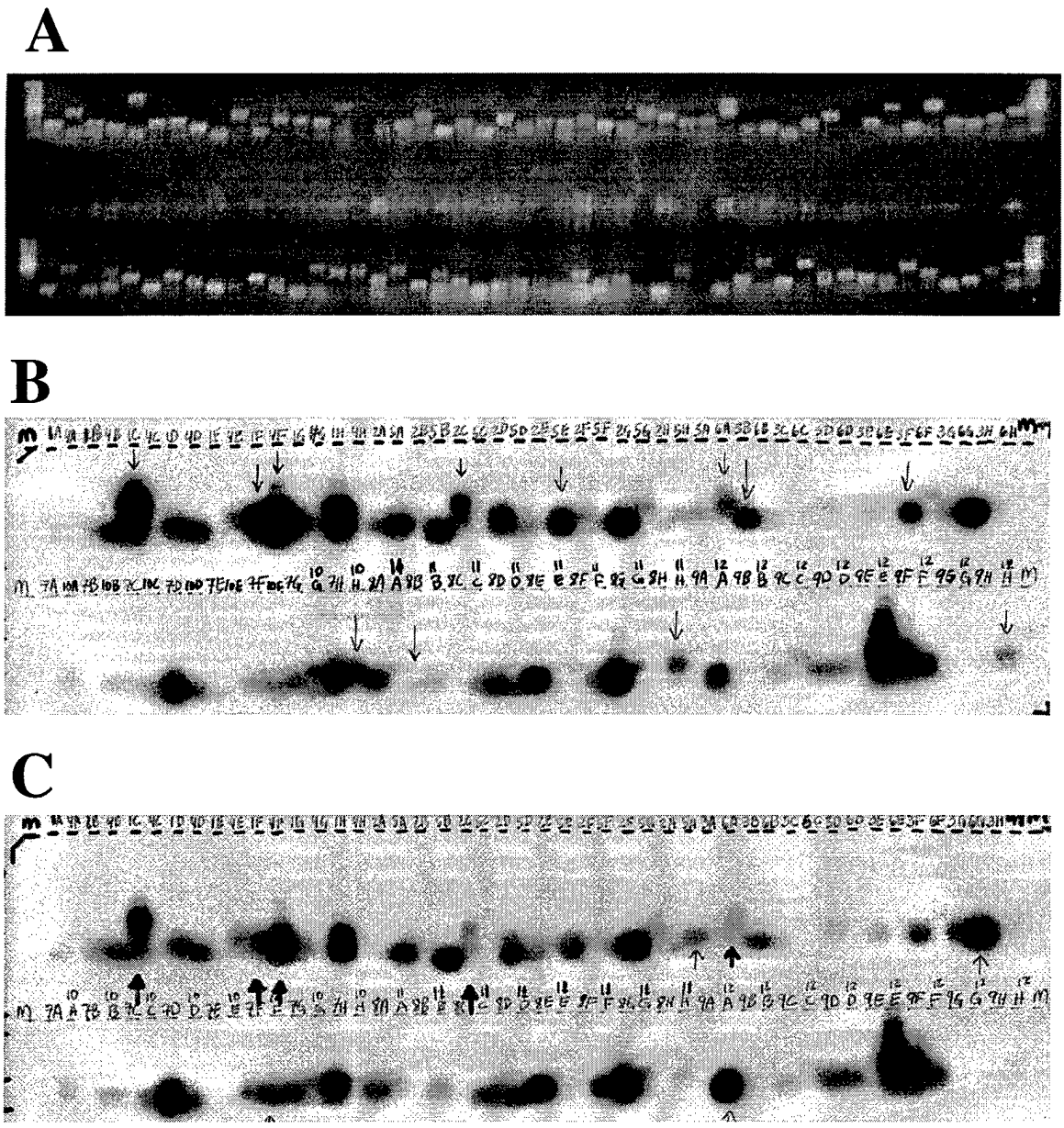

Figure A-1. Differential screening of SSH clone-set #1. Forty-eight insert-positive clones were arrayed by high-density agarose gel electrophoresis (A). Reverse Southern analysis was performed on these clones using forward (B) and reverse (C) subtracted radiolabeled cDNA probes.

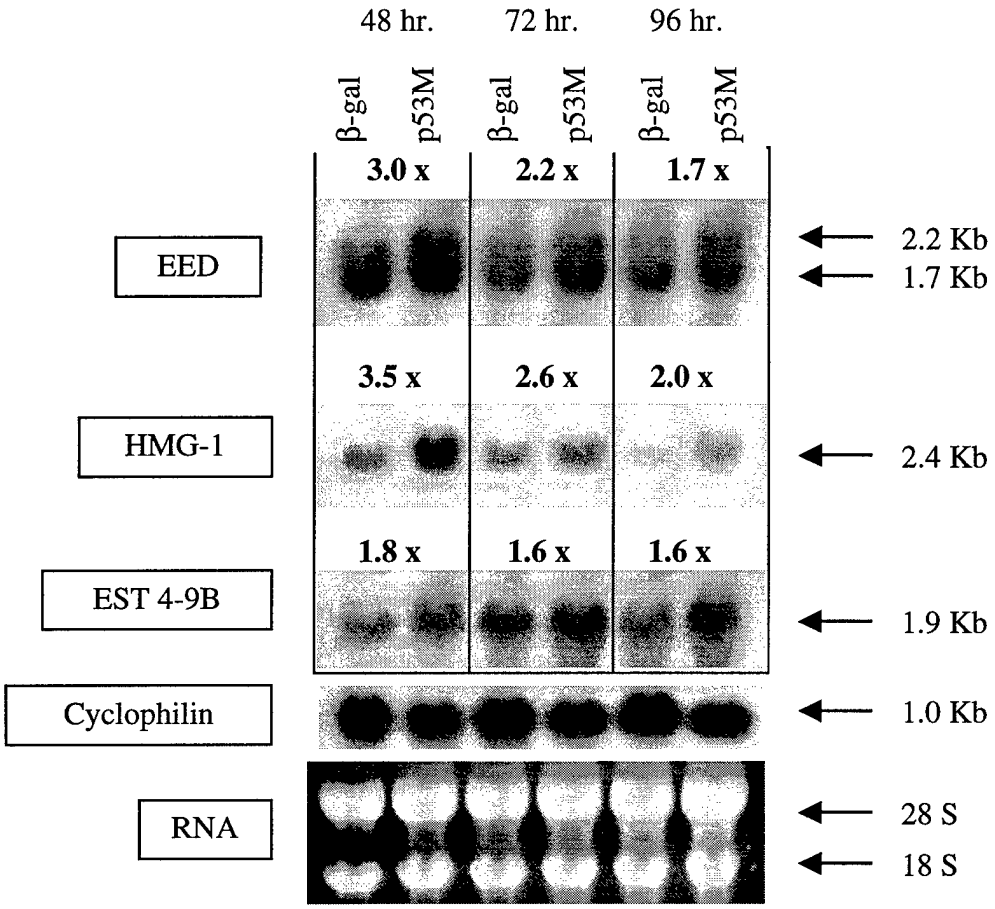

Figure A-2. Gene expression profiles of the SSH clones EED, HMG1, and EST 4-9B in p53 null MECs retrovirally infected with mutant p53 or β-gal, 48, 72, or 96 hrs after infection. Numbers represent the relative differences in fold change between β-gal and mutant p53-expressing cells, and have been normalized to cyclophilin mRNA expression.

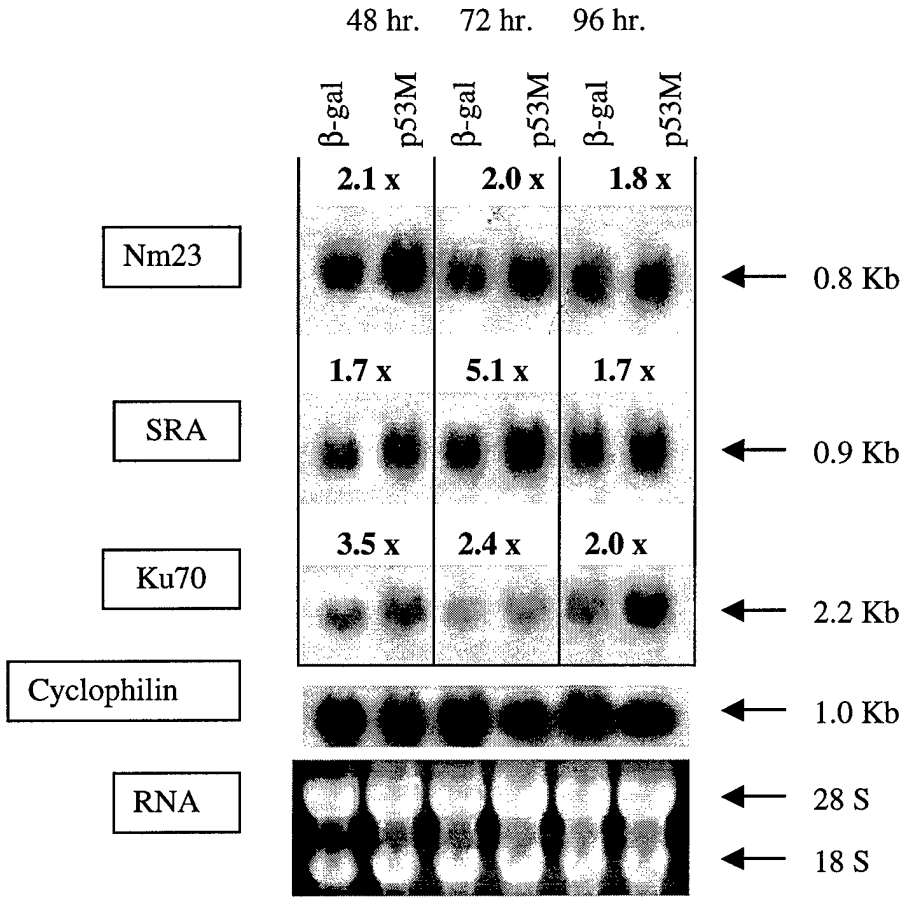

Figure A-3. Gene expression profiles of the SSH clones Nm23, SRA, and Ku70 in p53 null MECs retrovirally infected with mutant p53 or β-gal, 48, 72, or 96 hrs after infection. Numbers represent the relative differences in fold change between β-gal and mutant p53-expressing cells, and have been normalized to cyclophilin mRNA expression.