To the Graduate Council:

I am submitting herewith a thesis written by Walter Guy Wiles IV entitled “Synergistic Effects of BubR1 and p53 Deficiency in Tumor Formation.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Sundar Venkatachalam_____
Major Professor

We have read this thesis
and recommend its acceptance:

Ranjan Ganguly_____

Ana Kitazono_____

Accepted for the Council:

Anne Mayhew____

Vice Chancellor and
Dean of Graduate Studies

(Original signatures are on file with official student records)

Synergistic Effects of BubR1 and p53 Deficiency in Tumor Formation

A Thesis Presented
For the Masters of Science Degree
The University of Tennessee, Knoxville

Walter Guy Wiles IV
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Dedication:

This thesis is dedicated to my wife, April Wiles, and my parents Guy and Paula Wiles and Ron and Gail Williams for their love, their belief in me, and encouraging me to succeed.

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Abstract:

Regulation of cell proliferation within an organism is a necessary and complex process involving several proteins serving as controls at various cell cycle phases known as cell cycle checkpoints. One of these checkpoints, the mitotic spindle checkpoint, controls the advancement from metaphase to anaphase during mitosis and monitors the proper attachment of the microtubules to the kinetochore. The spindle checkpoint protein, BubR1 is a protein kinase that localizes to the kinetochores to monitor proper microtubule attachment. BubR1 is able to inhibit the anaphase promoting complex (APC) and delay the onset of anaphase. Concurrent with its role in regulating cell cycle, mutations in *BubR1* have been observed in various human cancers. In this work, we examine the effects of *BubR1* deficiency at the cellular and organism level using an inbred mouse strain that is deficient for the *BubR1* gene expression. We showed that the complete loss of BubR1 resulted in an embryonic lethal phenotype and this phenotype could not be rescued in a *p53* deficient background. Additionally, mice heterozygous for BubR1 showed a decreased life expectancy and an increased incidence of tumorigenesis. Furthermore, our data indicates that the loss of BubR1 synergizes with *p53* deficiency to increase the susceptibility of cancer formation in mice and alters the tumor spectrum of the *p53* deficient mice. Finally we show that the *BubR1* deficiency increases cellular growth kinetics and transformation potential of MEFs derived from compound *BubR1; p53* mutants. This data provides insight into the importance of *BubR1* in the prevention of tumorigensis and its role as a checkpoint protein.

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Nomenclature

cpm/ml	counts per minute per milliliter
µl	microliter
µM	micromolar
mg	milligrams
mM	millimolar
ng	nanograms

Abbreviations

APC/C	anaphase promoting complex/cyclosome
CDC	cell division cycle/control
CDK	cyclin-dependent kinase
CFU	colony forming unit
CIN	chromosomal instability
DMEM	Dulboc's modified eagle media
DNA	deoxyribonucleic acid
E10.5	embryonic day 10.5
E13.5	embryonic day 13.5
FBS	fetal bovine serum
G1	gap phase 1
G2	gap phase 2
M	mitosis
MCC	mitotic checkpoint complex
MEF	mouse embryonic fibroblast
NLS	nuclear localization signal
PBS	phosphate buffered saline
Pen/Strep	penicillin/ streptomycin
RNA	ribonucleic acid
ROS	reactive oxygen species
S	synthesis
TBP	TATA binding protein
TAF	TBP associated factors

I. Introduction:

Cancer Overview and Statistics:

Cancer is a disease of uncontrolled cell growth and division (1). In this disease, a single cell, through mutation, becomes unable to monitor or control its proliferation. This unchecked proliferation grants a growth advantage to the cell, and with time, allows further mutations to accumulate within the cells created by the original cell, called clones. These accumulated mutations will alter the clones in such a way that they are able to escape regular growth control signals and, in some instances, develop the ability to escape their primary site, referred to as a tumor, and spread throughout the body of the organism (1). Mortality is often due to the fact that the rapidly dividing cells fail to differentiate and perform the function they are designed to. Alternatively, these masses can grow, affecting the normal functioning of adjacent organs or they can usurp the blood supply to the normal tissue causing the organs near the mass to fail (2). Mammals have evolved a variety of mechanisms to control/regulate cellular proliferation. However, over time, these control mechanisms can become compromised due to accumulation of genetic changes that result in uncontrolled cell growth.

Cancer is a disease prevalent world wide, and the loss of life and costs of treatment have remained consistently high over the last 50 years, even though medical advances have lowered the mortality and cost of many other commonly seen health threats. Cancer can effect any organ or tissue type, but for the purposes of this study, we will examine the trends of the disease overall and the statistics of colon/rectal cancer. According to the American Cancer Society, in 2005, cancer was the second highest cause

of death with 22.8% of all deaths being attributed to cancer or its complications. Cancer deaths are second only to heart disease which is the leading cause of death with 28.5% of all deaths. The difference between the two, 5.7%, is relatively small especially when one looks at the separation between the second and third leading cause of death, cerebrovascular diseases, which accounts for only 6.7% of all deaths. This information alone would be enough to promote a desire to understand this disease, but with the actual number of deaths indicated, the need becomes more pressing. It is estimated that in the US alone, 295,280 men and 275,000 women died from cancer in 2005. The leading type of cancer for these people is lung cancer (31%), though cancers of the colon/rectum will cost 10% of both men and women their lives, and are the third leading cause of deaths from cancer. Also, it is estimated that 710, 040 men and 662, 870 women in the US were diagnosed with new cases of cancer in 2005. Again, cancers of the colon/rectum will be the third most common cancer to be diagnosed. According to these statistics, one out of two men and one out of three women will develop cancer over the course of their lifetime with one out of 17 of those, for both genders, being cancers of the colon. Also it has been estimated that the cost of cancer in 2003 was $189.5 billion. Thus, it becomes apparent that cancer is a disease in which discovering effective treatments to prevent or reduce disease occurrence is an important step in promoting people's health (2).

Cancer and the Cell Cycle:

Since cancer is a disease caused by uncontrolled cellular division, it is first necessary to examine the way in which cell division occurs under normal conditions. Cellular division consists of four stages. These stages are G1 (gap phase 1), S

(synthesis), G2 (gap phase 2), and M (mitosis). In G1, the cell has just finished a round of cellular division and requires time to grow and obtain the nutrients and build the proteins necessary to begin the next round of cellular division. Once the required needs are met, the cell will enter S phase where the cell will replicate its DNA. DNA synthesis is followed by a second gap phase where, again, the cell makes sure that it has time to gather the necessary machinery to allow accurate and timely division as well as to assure the integrity of the cell's genetic material. Finally, during the M-phase, the cell undergoes mitosis and completes cellular division. The cell cycle is conserved in all dividing cells and is regulated by proteins known as cyclins and their partners, the cyclin-dependent kinases. (CDK). The CDKs are found constitutively in the cell during all of the cell cycle stages. Cyclins on the other hand, are only expressed during the stage in which they associate with their CDK partners. The cyclins then are ubiquitinated and destroyed by the proteosome to allow progression into the next stage of the cell cycle (1, 3, 4). To facilitate the ubiquitination and subsequent destruction of these products, they contain a conserved amino acid sequence of **R**xx**L**xxxx**S**. This sequence, known as the destruction box sequence, is found in many cyclins as well as several other cell cycle proteins that are regulated in a cell cycle dependent manner, such as BubR1. One way in which normal cells develop a cancerous phenotype is through the improper activation or production of these cyclins controlling the passage of the cell from one stage of the cell cycle to the next (5).

Cell Cycle Checkpoints and Mitosis:

To assure the proper progression through the cell cycle, and to prevent the progression to the next stage before the requirements of the previous stage are met, the cell cycle has safeguards known as checkpoints. These checkpoints are found at critical junctions of the cell cycle and allow the cell cycle to be arrested if there is DNA damage or if the cell cycle progression stalls due to aberrant conditions. Upon activation of these checkpoints, the cell will cease the cycle at whichever point it is in until the event that activated the checkpoint is corrected for, allowing the cell cycle to continue. If the situation is too dire, extended activation of the checkpoint will promote apoptosis. The three commonly described cell cycle checkpoints are the checkpoints found at the G1/S transition, which allows the cell to begin DNA replication, the G2/M transition, which assures that the DNA was replicated without error, and the mitotic spindle checkpoint which helps assure that there is accurate chromosome segregation (4, 6). A fourth cell cycle check point is the intra-S checkpoint, which is activated when the replication fork encounters DNA damage during replication. This checkpoint enhances genomic stability by slowing DNA synthesis to allow time for DNA repair. (7)

The G1/S checkpoint is activated by DNA damage and will stall the progression into S-phase by preventing the firing of the replication origins or progression of the replication fork until the damage is repaired. This G1/S arrest allows for repair of the template DNA and prevents DNA damage from becoming a fixed mutation in the cell for subsequent divisions. The major players of the G1/S checkpoint include the proteins ATM, ATR, p53 and p21 (8). In this checkpoint, DNA damage is sensed by ATM Kinase and p53 protein levels are increased. The p53 tumor suppressor gene protein,

then activates several gene products leading to delay of the cell cycle progression (9). Once the DNA damage is repaired, the checkpoint signal is reduced through the destabilization and degradation of p53, and the cell is able to continue on to and through S-phase.

After DNA replication, there is a second DNA damage checkpoint to assure that both the template and the replicated DNA are undamaged. This occurs after replication but before progression into mitosis and serves to assure that the daughter cells produced both have DNA that is identical to the parent cell (4). The major components of the G2/M checkpoint include Chk1, Chk2, and p53 proteins. If DNA damage is detected during the G2-M transition phase, ATM or ATR will activate Chk1 and Chk2. These, in turn, will phosphorylate and inactivate the phosphatase Cdc25c (10, 11). The role of Cdc25C is to remove an inhibiting phosphorylation on Cdc2 (tyrosine-15). This inhibition of Cdc2 stops the cell cycle from progressing into M-phase until the DNA damage is repaired. The p53 protein is also activated by ATM or ATR in response to DNA damage and serves a role similar here as in the G1/S checkpoint by inducing the expression of p21 that arrests the cells at the G2/M phase. It is only after the progression through these two checkpoints that the cell enters M-phase and subsequent cellular division (6, 12).

Mitosis is a multi-step process in which the parent cell must condense its DNA into paired chromosomes, align the chromosomes along the metaphase plate, accurately separate the sister chromatids, and finally reform the nuclear membrane and de-condense the DNA. The first step or stage of mitosis is known as prophase, and it is here that the nuclear membrane breaks down and the DNA begins to condense. At this time, the sister

chromatids are secured together by proteins called cohesins, serving as molecular glue so that they can eventually be divided equally between the two daughter cells. There is then an intermediate stage known as prometaphase, where the chromosomes have condensed and are free in the cytoplasm and the centrioles have reached the poles and begin building microtubules. These microtubules will extend out into the cytoplasm and, upon making contact, will bind to the kinetochore of the chromosomes. Eventually, at metaphase, the microtubules will align the chromosomes up in the middle of the cell with the microtubules attached to either side of the kinetochores. This places tension across each kinetochore, signaling bipolar attachment. Once aligned across the metaphase plate, the chromosomes then go through anaphase where the sister chromatids separate and move to the poles. Once congregated at the poles, the chromosomes de-condense and the nuclear membrane reforms around each set during telophase (figure 1). The cellular contents then divide in a process known as cytokinesis and the two daughter cells are now formed (1, 4).

It is during mitosis that we find the third cell cycle checkpoint, known as the mitotic spindle checkpoint. In order to assure proper separation of the sister chromatids to the daughter cells, the chromosomes must be aligned on the metaphase plate and each of the chromatids must be attached to microtubules originating from opposing spindle poles. Since the microtubules employ a "search and capture" method of attachment, progression must be delayed until all of the chromosomes are attached and tension is generated across the kinetochore, signaling bipolar attachment (13, 14). It has been shown that the mitotic

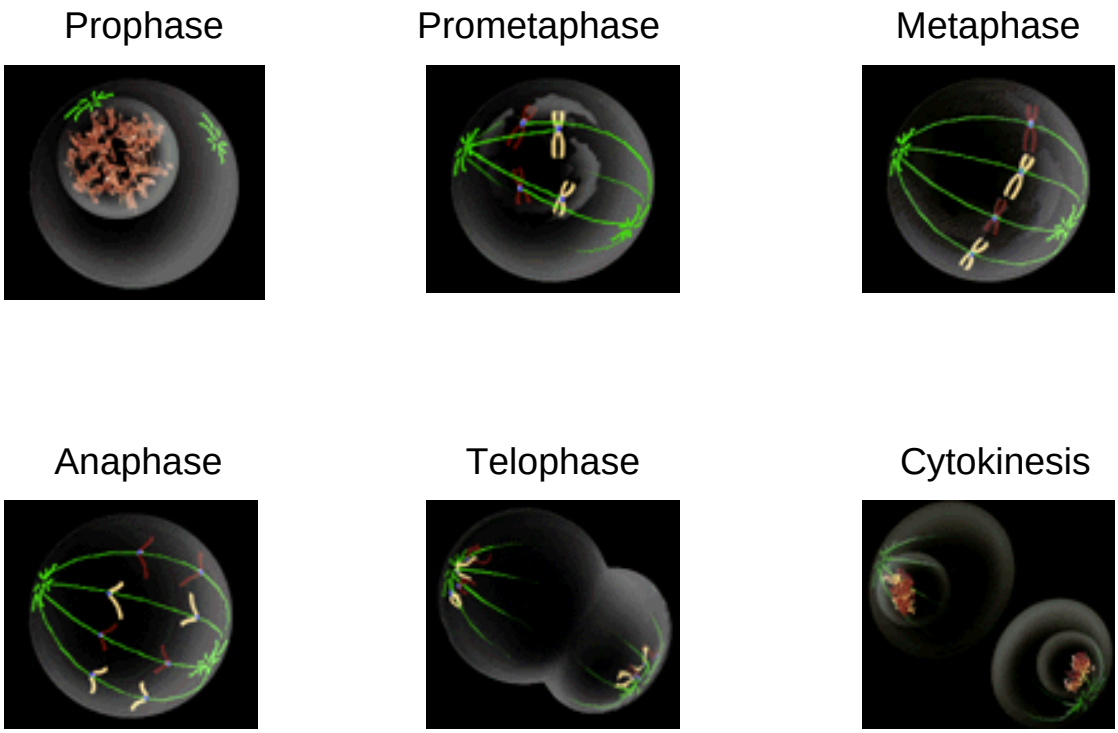

Figure 1: The stages of mitosis. This diagram shows the stages of mitosis. In prophase and prometaphase the nuclear membrane breaks down and the DNA condenses. It is here that many of the mitotic spindle checkpoint proteins will bind to the kinetochores of the chromosomes. Once condensed, microtubules will attach to the chromosomes and line them on the metaphase plate (metaphase) and upon alignment the sister chromatids will be separated and pulled towards the poles (anaphase). Finally the chromosomes will reach the poles and de-condense and the nuclear membrane regenerated and the cell will divide (telophase and cytokinesis). (images from http://www.metu.edu.tr/~e118872/project/index.htm)

spindle checkpoint signal is activated even if only a single kinetochore remains unattached (15). The major components in the mammalian mitotic spindle checkpoint include Mad1 and Mad2, Bub1, BubR1, and Bub3 (16). These proteins all localize to the kinetochores of unattached microtubules during prometaphase and become activated during metaphase, where they create a complex known as the Mitotic checkpoint complex (MCC), along with the binding of Cdc20. Once the MCC is formed and activated, believed to be through various phosphorylation events by the other checkpoint proteins, the MCC then prevents the activation of the anaphase promoting complex or cyclosome (APC/C) (17). The APC complex directs the ubiquitination of several components including the protein securin. This degradation of securin allows its bound partner, separase, to be released and cleave Scc1, a protein involved in keeping the sister chromatids together during the early stages of mitosis. This frees the sister chromatids from each other and allows the separation and movement of the chromatids to the spindle poles. Also, the APC will ubiquitinate several of the mitotic proteins marking them for degradation, allowing mitosis to end and the cell cycle to start anew (18, 19) (figure 2).

Role of p53 as a Checkpoint Protein:

Overview:

As mentioned above, cancer is a disease stemming from uncontrolled cellular proliferation. In order to lose control of proliferation mutation in key cell cycle components or their checkpoints are necessary. It is interesting to note that the most commonly mutated gene in human cancers is p53. As previously stated, p53 serves as a checkpoint in both the G1/S and the G2/M phases. This makes p53 an ideal protein for

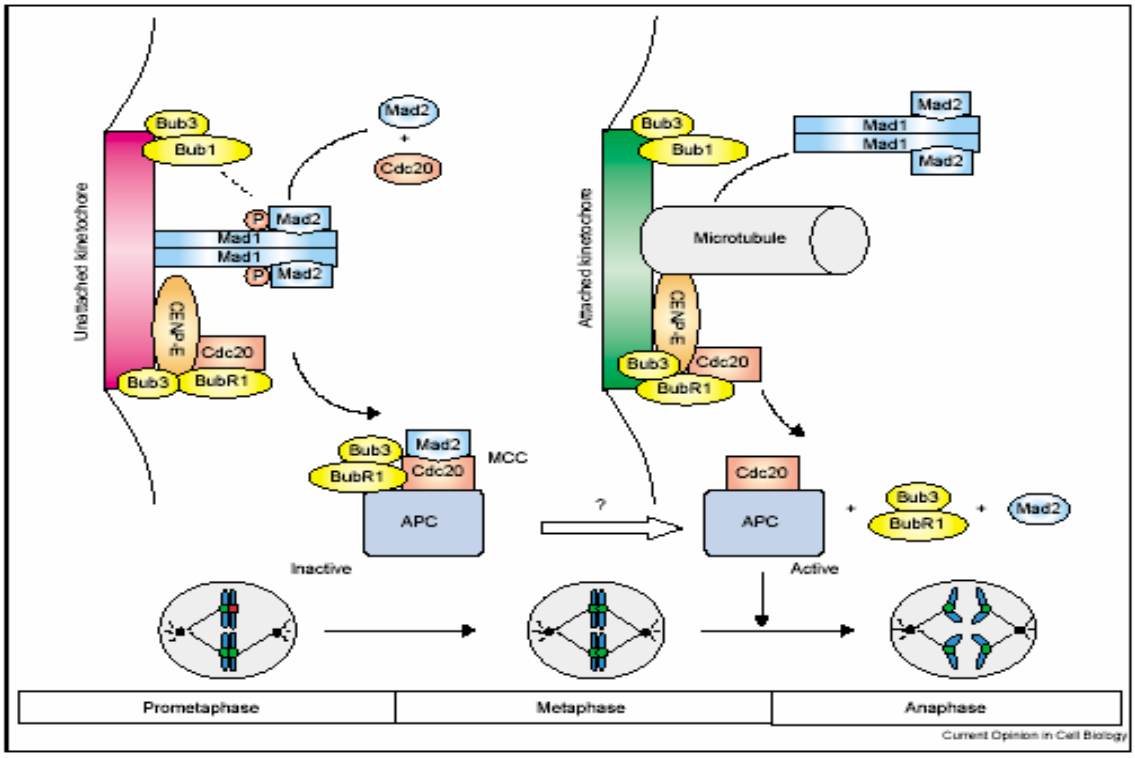

Figure 2: The mitotic spindle checkpoint. This diagram shows the role of BubR1 as well as other spindle checkpoint proteins in the activation of the mitotic spindle checkpoint. In prometaphase, unattached kinetochores will allow the localization of multiple checkpoint proteins including BubR1 and Mad2. Once localized and activated (believe to be through phosphorylation events) these proteins interact with Cdc20 to inhibit the APC. Once attachment and tension is signaled these checkpoint proteins become inactive and the APC is no longer inhibited and anaphase begins (20).

cancer study since insights on the mechanisms of p53 mediated cell cycle control could serve as the foundation for new or more effective cancer treatment strategies.

The gene product of *p53* is a protein of ~53 kDa that is made up of three domains. These domains include an activation domain at the amino-terminal spanning residues 1-42, a central core containing the sequence-specific DNA-binding domain spanning residues 100-300, and finally a carboxyl-terminal, multi-functional domain spanning residues 300-399. Each of these domains connects through a flexible linker sequence (21). In the protein, there are five evolutionarily conserved regions with the first one being in the N-terminal activation domain and the other four found in the central core. Multiple phosphorylation sites are found in both the N-terminal and C-terminal domains, which are used to regulate the activity of p53. (22)

Activation of p53:

After exposure to DNA damaging agents, multiple forms of DNA damage are induced that include double strand breaks and base alterations. This damage leads to the activation of p53 through the actions of multiple kinases that phosphorylate p53 at multiple sites. The kinases involved in this response include JNK, ATM and ATR (10, 11). In addition to the phosphorylating events at serines 15, 20, and 37, there is also a de-phosphorylating event at serine 376 which opens a new site in p53 for interaction with the scaffold protein 14-3-3 (8, 23, 24). Phosphorylation of the above serines, stabilize the protein and inhibit its interaction with the negative regulator protein Mdm-2. Once stable, p53 is transported into the nucleus via phosphorylation of serine 392 by CK II where it tetramerizes and exhibits its transcriptional activity (25-27). Unlike the rapid

response seen to ionizing radiation, DNA damaged induced by UV has a slower response time. Since the damage caused by UV is most often the formation of bulky lesions such as thymidine dimers there is no immediate detection of the lesion until the replicative or transcriptional machinery stalls upon reaching the lesion. Once the damage is detected; the stalling of RNA polymerase II causes serine 33 on p53 is phosphorylated by the CDK Activating Kinase (CAK) from TFIIH. Additionally, ATR activates Chk I and Chk II which phosphorylate serine 20 while ATR directly phosphorylates serine 15 and 37 (28). As seen above in response to ionizing radiation, these phosphorylation events, lead to the stabilization of p53. Once stabilized, p53 is again phosphorylated on serine 392, and transported into the nucleus where it tetramerizes and induces the transcription of the DNA damage response genes (29). The various phosphorylations of p53 lead to the alteration of the protein structure and expose the central core's sequence-specific DNA-binding domain as well as allowing the N-terminal activation domains to interact with other transcriptional factors such as TATA box-binding protein (TBP) and its associated factors (TAFs) (30) . Finally, since p53 accumulation will sequester the cofactors involved in transcription, those genes that do not contain a p53 specific binding site will not be transcriptionally active until the level of p53 is restored to its base (31-34).

Physiological role of p53:

The activation of p53 leads to three outcomes for the cell; cell cycle arrest, cellular senescence, or apoptosis. By keeping with these outcomes, p53 is able to protect the genome from accumulating mutations. However, since these effects prevent cellular growth, p53 must be tightly regulated to prevent wasteful delay, arrest and/or

unnecessary apoptosis. In many organisms, the extensive loss of cells due to up regulation of p53 can lead to problems which can put the entire organism at risk. On the other hand, if p53 is too tightly regulated due to an over-expression of its negative regulators or an under-expression of the gene itself, the stability of the genome can become uncertain due to the lack of p53-dependent checkpoint activation. Once activated, the results of the activation must be decided as to whether the cell undergoes cell cycle arrest or apoptosis. This decision is based on several factors including the cell type, severity of damage, and the time that p53 activity was induced.

Most of the genes induced by p53 lead to several outcomes that include cell cycle arrest, apoptosis, and cellular senescence. An example of p53 induced cell cycle arrest is the induction of the *p21* gene whose product promotes cell cycle arrest in G1/S through binding and inhibition of the CDKs necessary for cell cycle progression. Activated p53 has been shown to bind to the *p21* promoter. This binding increases transcription of the gene product and p21 will then form part of a quaternary complex with PCNA and the cyclin/CDK complex. This quaternary complex will inhibit the cyclin/CDK activity, arresting the cell cycle in G1. In addition to *p21*, after ionizing radiation, p53 will also bind to the promoter of *GADD45* (growth arrest and DNA damage inducible gene) and activate it. This protein will then interact with PCNA, a replication and repair factor and inhibit it. With the inhibition of PCNA the cell will be unable to enter S-phase and the cell cycle comes to a halt. Also, it has been shown that *GADD45* is able to alter the chromatin accessibility which could inhibit progression into S-phase by maintaining the DNA in an inaccessible form to other replication factors until the damage is repaired (22, 26). In some cell types, activation of p21 by p53 leads to prolonged cellular arrest.

Dependent on the damage or the cell type, the activation of p53 may induce apoptosis (35). This pathway begins similar to the cell cycle arrest pathway in that p53 is activated, stabilized, and transported into the cell nucleus. However, from here things become a bit less characterized. While it is known that p53 mediated cell cycle arrest requires the protein's transcriptional activity, initial reports seemed to indicate that the transcriptional activity may not be necessary and it was suggested that p53 may have both a transcription dependent and independent pathway to induce apoptosis. (22) However, a later work showed that the induction of apoptosis through p53 is a three step process which requires p53 to be transcriptionally active. From this study it was determined that upon the activation of p53, a subset of genes known as the *p53 inducible genes* (*Pigs*) are promoted. The transcription of these genes, which code for several proteins involved in regulation of reactive oxygen species (ROS), will lead to an increase in the level of ROS. This increase of the ROS level will then work to decrease the stability of the mitochondrial membrane that leads to Cytochrome-C release. Once released, Cytochrome-C will interact with several other apoptotic proteins ultimately leading to the activation of the caspase cascade that will induce genomic degradation and membrane blebbing, hallmarks of apoptosis.

Regulation of *p53* activity

As seen from the information presented already, the regulation of p53 is tightly controlled and the activating cascade requires several players to properly stabilize, activate and localize the protein so that it may exert its effect. In addition to these activating proteins, p53 is also regulated on its transcriptional and translational levels.

Transcriptionally, *p53* is induced in response to the stress factors such as AP-1, NF-kB and Myc/Max as well as the products of YY1 and NF1. These transcription factors will bind to the promoter of *p53* and promote transcription of the gene (36). The *p53* gene is transcriptionally repressed by the Pax transcription factor family (37). Also, the viral protein Tax and the over expression of c-Jun have been shown to repress transcription of *p53*(38, 39). During the translation of the p53 mRNA, p53 has been shown to be able to repress its own translation through the 3' untranslated region (UTR) in human and the 5' UTR in murine p53. These UTR sequences can form stable secondary structures which can repress translation through interaction with the RNA binding factors.

Though p53 is regulated both in its transcription and translation, the majority of its regulation is seen post-translationally. In the cell, cytoplasmic p53 has a short lifespan of only a couple minutes. The protein p53 can induce the transcription of proteins which attenuate its activity serving as an auto-regulatory feedback. An example of this is the *mdm-2* gene, whose protein product binds to p53, preventing its nuclear localization and promoting its degradation, is a downstream target of p53 (22, 25, 31). Thus p53 up-regulates its own inhibitory factors, thereby assuring that p53 activity will diminish quickly after the DNA damage is repaired (40). Cytoplasmic p53 is bound to the protein MDM-2 and ubiquitinated, targeting the protein for degradation by the 26s proteosome (41, 42). In addition to this degradative pathway to regulate p53, the localization of p53 is another way of controlling p53 activity. For p53 to function, it must first be able to enter the nucleus of the cell. To do this, p53 has a nuclear localization signal which must be accessible to recognition sites on the nuclear membrane and so one method of regulation is via the binding of other proteins which hide the signal. One example of this

is the binding of p53 to Mdm-2 to prevent access of the localization domain. Also, once imported into the nucleus, p53 may bind to MDM-2 and be subsequently exported. By controlling the degradation or localization of p53, the cell assures that any protein present in the cell remains at sub-active levels until activation and stabilization of the protein is needed (22, 25).

Effects of p53 Inactivation:

With p53 serving such an important role in maintaining genomic stability and providing the means to arrest cell cycle progression or promote apoptosis, it is no small wonder that mutation of p53 or the complete loss of the gene is commonly seen in cancer. The most commonly seen mutations of p53 are found in the DNA binding regions of the protein. These mutations all serve to prevent p53 from inducing the transcription of cell cycle arrest or apoptotic genes (31). Additionally, mutations of the NLS region of the protein have been characterized. It has been observed that mutation of the NLS region or alteration of the residues lysine 305 and arginine 306 leads to the cytoplasmic localization of p53 and its inactivation (25). Finally, mutation of either p53 or MDM-2 to prevent their dissociation will lead to a substantial reduction of inducible p53 which will prevent p53 mediated cell cycle arrest or apoptosis (34). In all of these mutations, the inactivation of p53 leads to increased proliferation, genomic instability, and the accumulation of mutation due to the loss of cell cycle arrest and apoptosis.

Role of BubR1 as a Checkpoint Protein:

Overview

The protein, BubR1, is a part of the mitotic spindle checkpoint which serves to assure anaphase delay until the chromosomes are properly aligned on the metaphase plate with proper microtubule attachment and tension along all the kinotechores. The human *BubR1* gene is located on chromosome 15q15 and its product is considered to be one of the major players in the prevention of chromosomal instability (CIN). While BubR1 mutations are not prevalent in all cancers, studies have shown that mutations of *BubR1* do occur in some colorectal cancers. (43) In addition, chromosomal rearrangements of 15q15 have been observed in some leukemias. These rearrangements may promote the inactivation of BubR1 as a checkpoint control protein and increase the likelihood of tumor progression (44). *BubR1* is a gene found in mammals but not in yeast and has homology to both yeast genes *BUB1* and *MAD3,* hence the names *BubR1* (b*ub1-related*). BubR1 was first identified in a study by Davenport et al. using differential expression of normal and leukemic mouse thymocytes. In this study, a homologue to the yeast checkpoint protein Bub1 was discovered and this novel family member, named mBub1b, was shown to have 40% sequence similarity to murine Bub1a over 4 extended domains. To better understand this novel protein, Davenport et al. used the clone fragment B13 from their differential expression studies to isolate a 3,647 base pair cDNA from the 16 day mouse embryo cDNA library and determined some of the properties of this new protein (44). As mentioned above, the *BubR1* gene transcribes a message of 3,647 base pairs making up 23 exons with a promoter located 1,368 base pairs upstream of exon 1. It was also found that a GC-rich region rests 150 base pairs upstream of exon 1. This

translates to a protein with 1,052 residues across four major domains. These domains are a Ken-box motif, a signaling sequence for degradation, (residues 26-28), a Mad3-like region (residues 52-204), a Bub3 binding domain (residues 392-433) and a kinase domain (residues 749-1018) (45). The kinase domain of BubR1 is atypical in that the kinase domains of Bub1, BubR1 and the yeast Bub1 are more closely related to each other than to other kinase domains. Even with this being the case, BubR1 contains significant alterations which mask its relationship to other kinases outside of the bub family. An example of this is the substitutions of Bub1's Glutamate 127 and Aspartate 170 with BubR1's Asparagine and Arginine (44). These alterations are explained in Davenport's work as proofs that BubR1 has different substrates than Bub1. Along with these domains, BubR1 has a destruction box sequence immediately following its amino-terminal domain, (residues 222-230 in the mouse Bub1b) and two sequences that could serve as nuclear localization signals (residues 229-234 and 412-117) (44). Also, later work showed that BubR1 contained a dileucine motif (residues 958-963) this motif is commonly seen as an adaptor beta chain recognition motif (46). The destruction box sequence, RSSLAELKS, is located between the Mad3-like and the Bub3 binding domains and serves to promote degradation of the protein through ubiquitination of the lysine residues targeting the protein for destruction by the proteosome. This destruction box allows the degradation of BubR1 in a cell cycle-dependent manner (44, 47). Along with these various sequences and within these domains are several phosphorylation sites. These sites are hypothesized to serve as activators and regulators for BubR1 activity in the event of mitotic spindle checkpoint activations (figure 3).

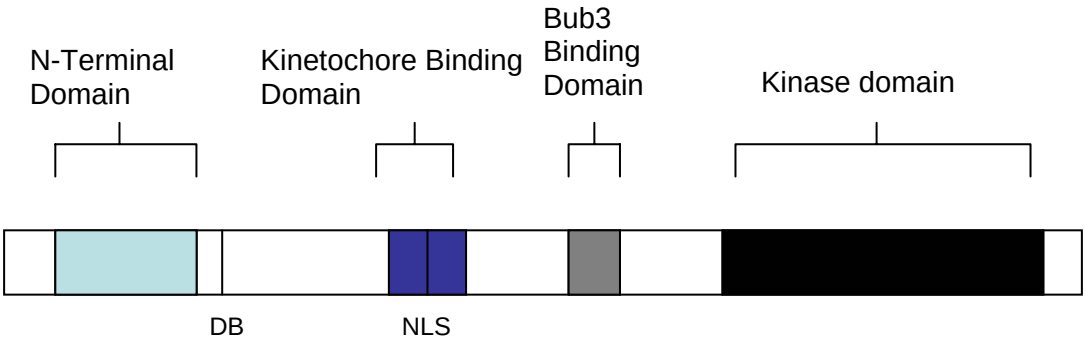

Figure 3: The BubR1 protein. This diagram shows the BubR1 protein and its evolutionarily conserved domains as well as their role in the activity of BubR1. The Destruction box (DB) is located just past the N-terminal domain of the protein (containing its Mad3 like region and Ken box motif). The nuclear localization sequence (NLS) is located within the kinetochore binding domain.

Functional role of BubR1:

As implied by its multiple structural domains and motifs, the role of BubR1 in the cell is complex. The exact function and binding partners as well as the kinase substrates of this protein have been sought out since it was first isolated in 1998 (43, 44). Many researchers have spent years on this gene and while strides have been made and some of its key roles determined, there is still a great deal to learn about this gene and its product.

The isolation and study of BubR1 done by Davenport determined that the product was expressed in all dividing cells and highly expressed in the spleen and thymus. This work showed that expression of this gene peaked around the G2/M phase of the cell cycle and due to its homology to Bub1, it was determined that BubR1 has a distinct role in the mitotic checkpoint. This finding was confirmed in a separate study by Cahill et al. who examined 19 colorectal cancer cell lines that exhibited chromosomal instability (CIN) and found that in two of them there was a mutation of the human b*ub1* homologue *BUBR1*(43, 44). One of the first studies to determine BubR1's binding partners and regulation was done by Chan et al examining the interaction of BubR1 with the centromeric motor protein E (CENP-E). It was found that while BubR1 levels were lowest in G1, it steadily increased towards mitosis. During Mitosis, BubR1 was hyper-phosphorylated and it was found that this hyper-phosphorylation increased the kinase activity of the protein (48, 49). Their studies also showed that BubR1 was capable of binding to CENP-E in the cytoplasm of interphase cells through interactions of 641 residues at its C-terminal. Furthermore it was observed that BubR1 could localize to the kinetochores of unaligned chromosomes during prometaphase. This localization would

occur after the localization of CENP-F but before the localization of CENP-E suggesting that BubR1 may phosphorylate substrates on the kinetochore to allow the binding of CENP-E. Because it was accepted that BubR1 had a distinct role in the mitotic checkpoint and this discovery that BubR1 interacted with CENP-E, a motor protein, it was suggested that the BubR1/CENP-E complex served as a mechanosensor for the unaligned chromosome (49). Later experiments were able to show that BubR1 was an essential component of the mitotic checkpoint though CENP-E was not. Additionally, the kinase activity of BubR1 was examined and it was observed that while BubR1 had no detectable kinase activity in either interphase cells, or in mitotic checkpoint activated cells, through addition of the drug nocodazole, BubR1 showed a high level of kinase ability both for itself as well as a number of exogenous substrates using *in vitro* kinase assays. It was determined that one role of BubR1 was to prevent normal mitotic exit until the chromosomes had aligned properly and this arrest was through specific interactions with the Anaphase Promoting Complex (APC). In addition to this role in the mitotic checkpoint activation, it was confirmed that BubR1 served to monitor functions specified by CENP-E as a tension checkpoint (48). In 2001, in a study by Skoufias et al. it was confirmed that one of the major roles of BubR1 was to sense tension across the chromosome's kinetochore. It was shown that in the event of tension loss while microtubule attachment was maintained, Bub1 and BubR1 returned to the kinotechore to delay anaphase until tension was restored. It was suggested that BubR1 and another mitotic checkpoint protein Mad2 were parts of two distinct checkpoints, one sensing tension and the other sensing microtubule attachment (50). However, this model was later proven to be incorrect through a study by Shannon et al. when it was shown that in

cell lines under hypothermic conditions, the major cause of prometaphase delay due to lack of tension, that the mitotic delay could be attenuated either through the addition of antibodies against Mad2 or BubR1. Additionally, it was observed that the addition of both antibodies did not accelerate the progression into anaphase. This showed that BubR1 and Mad2 were components of the same checkpoint pathway (51). Other researchers were able to provide insight into the function of BubR1 showing that once localized to the outer kinetochore plate, BubR1 binds to CENP-E and Bub3 (20, 52). This localization event is dependent on the previous localization of Bub1 and in the event Bub1 is missing or unable to bind to the kinetochore, the amount of BubR1 localized to the kinetochore is greatly reduced. Finally, it was observed that BubR1 must first interact with Bub3 before it could bind to the kinetochore (53, 54).

BubR1 activation and mitotic spindle checkpoint:

In the event of tension loss or microtubule instability during mitosis, BubR1 is hyper-phosphorylated and becomes active. This phosphorylation event is facilitated by Mad1 on the kinetochore and serves to alter BubR1 in such a way that it is able to interact directly with the APC as part of the mitotic checkpoint complex made up of the other checkpoint proteins Mad2 and Bub3 along with the APC cofactor Cdc20 or its sub-complexes (55). Without the presence of Mad1, while still on the kinetochore, and Mad2, BubR1 would not be able to interact with Cdc20 (53). This interaction inhibits the activity of the APC by preventing meaningful interactions between the APC and its substrates (20, 45, 52, 53). In all of these studies, it was shown that the BubR1 kinase domain was not necessary for the direct stoichiometric binding of BubR1 or the mitotic

checkpoint complex (MCC) to inhibit the APC. In addition to direct binding of the MCC to inhibit the APC, Yoon et al. observed that BubR1 was able to phosphorylate Cdc20 in vitro and that this phosphorylation event prevented the interaction between Cdc20 and the APC, effectively leading to a metaphase/anaphase block (45).

Additional functions of BubR1:

Another checkpoint protein phosphorylated by BubR1 is the breast cancer susceptibility gene BRCA2. The downstream effects of this phosphorylation event are still not clear but it is suspected that this allows interaction of BRCA2 with its substrates leading to cell cycle arrest (52). In a study by Shin et al., it was suggested that the role of BubR1 activation may not just lie in its activities during spindle checkpoint activation but also that the prolonged activation of the spindle checkpoint through BubR1 could promote apoptosis in those cells that eventually adapted to the arrest and exited mitosis, effectively executing a "fail safe" mechanism to prevent the propagation of cells breaching the mitotic checkpoint. This induction of apoptosis was found to be through the intrinsic pathway and the activation of caspase-9 leading to the subsequent activation of caspase-3 and apoptosis. A study by Vogel et al. further showed that cells need a functional mitotic checkpoint to activate post mitotic G1 arrest (56). Finally, Lens et al. found that for BubR1 to sustain its activity during prolonged checkpoint activation, the protein Survivin was necessary. It was also suggested that BubR1 was a possible substrate for Aurora B Kinase (47, 57). In addition to its role in the Spindle checkpoint, BubR1 has been found to interact with Beta2-adaptin, a subunit of AP2, on its N-terminal "trunk" domain and the C-terminal kinase domain of BubR1. AP2 is a member of the

assembly protein family involved in vesicular transport through the cell. AP2 mediates rapid endocytosis of the plasma membrane. It was found that BubR1 is able to interact with B2-adaptin through out the cell cycle suggesting that BubR1 might have a novel role in the regulation of vesicular intracellular traffic by regulating the soluble pools of B2-adaptin (46). Finally, in recent work by Fang et al. it was observed that MEF cell lines deficient for BubR1 exhibited compromised mitotic arrest and DNA repair after DNA damage by UV or the drug doxorubicin. DNA repair was found to be compromised in these cell lines through the down-regulation of p53, p21, phospho-H2AX and the enhanced degradation of PARP-1. Taken together this strongly suggests that a deficiency of BubR1 leads to continued cell cycling even after exposure to DNA damaging agents and through the down-regulation of key components of the DNA damage repair pathways, allows for the increased possibility of DNA damage and tumorigenesis (58)

Effects of BubR1 inactivation:

As it has been shown, BubR1 is an important component of the spindle checkpoint and has shown promise to play a role in several other checkpoints and cellular functions as well. BubR1 became a gene of interest due to the fact that mutant forms of the gene were observed in two colorectal cancer cell lines and that these cell lines exhibited chromosomal instability. From this it was suggested that the aneuploidy seen in cancer was due to the loss of chromosomal stability and that this loss was reflected through the mutation or loss of BubR1 (43). The idea that mutation in BubR1 caused chromosomal instability and its mutation was a factor in tumorigenesis was supported by

the work of Oshima et al who found that in four T-cell leukemia/lymphoma cell lines, BubR1 was found to have two missense mutations, one nonsense mutation, and an internal deletion (59). Furthermore, in breast cancer patients the aberrant expression of the protein encoded by the breast cancer specific gene 1 (BCSG1) has been shown to target BubR1 for degradation (60).

With the ability to create knock-out mice using the gene trapping method, BubR1 haplo-insufficient mice were able to be bred to examine the effects of the loss of one *BubR1* allele. By pairing mice both missing one functional allele of *BubR1,* it was determined that *BubR1* was an essential gene and that embryos nullizygous for *BubR1* begin to die 6.5 days after conception and that by 8.5 days, the nullizygous embryos are all reabsorbed. It was also determined that this embryonic death was due to extensive apoptosis (61, 62). The heterozygous mice lived well into adulthood but still exhibited problems and increased susceptibility to carcinogens. It was found that the spleens of heterozygous mice were enlarged with an increase in the number of mature megakaryocytes. These mice exhibit anemia and defects in platelet formation. It was hypothesized that this increase in spleenic megakaryocytes was due to the loss of proper cell division that in turn resulted in the formation of multinucleated megakaryocytes (62). It was also observed that mice heterozygous for the BubR1 gene trap were prone to tumors after exposure to azoxymethane, a known colon carcinogen. Heterozygous mice exposed to carcinogens develop colon masses two months after exposure while those with both functional alleles did not develop masses until six to eight months after treatment. It was also found that the heterozygous mice also developed tumors of the lung and liver, that was not observed in the wild-type mice (61). Later, Baker et al were

able to produce a hypomorphic mouse that expressed only 11% of the wild-type BubR1 level (BubR1 heterozygous mice express ~25% of wild-type levels). These mice were able to survive to adulthood but showed severe phenotypes including cachexia and lordokyphosis from three to six months of age and had a median lifespan of only six months, compared to the approximate two years observed in wild-type mice. These mice were also found to be infertile. Embryonic fibroblasts of BubR1 hypomorphic mice showed a severely compromised spindle checkpoint and a high level of aneuploidy. It was suggested in this study that BubR1 had a role in aging as those mice hypomorphic for BubR1 exhibited symptoms of advanced age (63). In work done by Rao et al. it was observed that BubR1 insufficiency had a synergistic effect with other proliferative control genes in the progression of tumorigenesis. It was observed that in a mouse strain exhibiting a mutation of the *adenomatous polyposis coli* gene (Apc), the introduction of BubR1 insufficiency led to a ten fold increase in the incidence of tumors in the colon. It was also seen that these tumors were in advanced stages of development. It was hypothesized that this increased tumor incidence was due to the role BubR1 played to prevent chromosomal instability. (64)

Rational for Generating *BubR1/p53* Compound Mutants:

In this study, we have analyzed the synergistic effects of p53 deletion in the tumorigenesis in mice deficient for BubR1. Studies have shown that cancer is a multi-step process that requires the inactivation of a variety of gene products involved in proliferation control. Furthermore, Fearon and Vogelstein and colleagues have shown the necessity for p53 inactivation in human colon cancer patients (65). Based on these

observations we hypothesized that the additional inactivation of the p53 tumor suppressor gene will either enhance the tumor susceptibility, indicated by a decrease in the lifespan, of the BubR1 mutant mice and/or induce the formation of colon cancer. To measure tumor susceptibility we generate a compound mutant for BubR1 and p53 and monitored the colony for a period of two and a half years.

II. Materials and Methods:

Generation of *BubR1* Mutant Mice in a *p53* Deficient Background:

In order to study the role of BubR1 in the spindle assembly checkpoint and cancer formation, *BubR1* deficient mice were generated using embryonic stem (ES) cells obtained from the Mutant Mouse Resource Center at UC Davis. A gene trap approach was used to achieve disruption of the *BubR1* gene as described earlier (66). The gene trap, containing a splice acceptor and an ATG-less betagalactosidase-neomycin fusion cassette, was determined to have inserted into intron 2 of the *BubR1* gene. The gene-trapped ES cells, from the 129P2/ OlaHsd strain, were analyzed by PCR and Southern blotting to confirm the presence of the trap within the *BubR1* gene. Upon confirmation, the mutant ES cells were used for injection into 3.5 day old blastocysts and implanted into a pseudo-pregnant c57 bl/6 mouse. Nine chimeric mice were generated from these blastocyst injections and of these, four founder males produced germ line litters for the *BubR1* deletion as detected by the agouti coat color. Tail clips were taken from the first two litters for genotype analysis. These analyses indicated equal numbers of wild type and heterozygous offspring consistent with the expected Mendelian ratio. Additionally, the heterozygous mice had no recognizable developmental defects. Heterozygous F1 offspring from the chimera males were then crossed in an effort to obtain *BubR1* nullizygotes. To generate compound heterozygotes, the BubR1 mutant mice were crossed to our *p53* deficient mouse strains (67).

DNA Extraction:

To obtain DNA from our mice for Southern blotting and PCR based genotyping, we first took tail clippings from the pups during weaning and incubated each tail separately in 500 μl of tail lysis buffer (50 mM Tris ph 7.5, 50 mM EDTA, 100 mM NaCl, 1% SDS) along with 10 μl of Proteinase K and 1.25 μl 2M DTT. These tail clippings were then incubated overnight at 55-60 degrees Celsius. After incubation, 500 μl of phenol: chloroform (24:1) was added and the contents were mixed well. The lysates were then spun in a microcentrifuge for three minutes at 13,000 rpm. Approximately 400 μl of the top layer (the aqueous phase) are removed into a second tube. After collecting the aqueous phase, twice the volume (800 μl) of 100% ethanol is added to each sample. Genomic DNA was precipitated out of solution and spooled out and placed into a third tube containing 50-100 μl of 5 mM Tris (10mM Tris, 1mM EDTA) depending on the amount of DNA recovered. DNA was solubilized overnight and concentrations determined for use in southern blotting and PCR genotyping.

BubR1 Genotyping:

To determine the *BubR1* genotype, we employed a PCR based strategy. For each sample, 200 ng of DNA was added to 23 μl of a PCR master mix (containing 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (each), 0.2 μM of the forward and reverse primers, 1.0 unit of Platinum *Taq* DNA Polymerase). The PCR conditions for the insertion and wild-type PCR were as follows: an initial two minute denaturation step at 94°C, followed by another denaturation step for 30 seconds at 94°C, annealing for 30

seconds at 54°C followed by an extension step for 135 seconds at 72°C. The second denaturation, annealing, and extension steps were repeated for 32 cycles and then a final five minute extension step at 72°C. For the Trap PCR the conditions were essentially the same with a decrease in extension time (75 seconds). The primers used to determine if the trap was located in the DNA were TR2 (5'CAACACTTGTATGGCCTTGGCG-3') and TR3 (5'GTGAGCGAGTAACAACCCGTC-3'). This created a PCR product of 665 bp and verified that the trap was located in the sample. To determine the proper insertion of the gene trap, the primers GS1 (5'TTGGCAAAGCAAGAGTCAGC-3') and TR1 (5'CCCAACTGACCTTGGGCAAGAACATA-3') were used, creating a PCR product of 2.4 kb. These two products together verified the presence and proper insertion of the gene trap in our *BubR1* gene. To determine if the wild-type allele was there we used the primers GS1 and GS2 (5'CCAGCCTAGGATACTTGGAGA-3'). This produced a PCR product of 2.2 kb and indicated the presence of the undisturbed intron.

Generation of Mouse Embryonic Fibroblasts (MEF):

Compound heterozygous crosses were performed and the pregnant female sacrificed at embryonic day 13.5 and the embryos removed and placed into separate 15 ml test tubes. Using a two ml syringe with a small bore needle, the embryo was placed into the syringe and pressed through the needle into cell media (DMEM, 15% FBS, 10 units of Pen/Strep). The cell suspension was then added to 2 large plates and incubated for two to three days. Once confluent, the plates were split 1:3 and allowed to grow until confluent and then split again at a ratio 1:3. A portion of the cells were isolated and used for experiments, while the rest of the cells were frozen in liquid nitrogen.

Cell Proliferation Assay:

Cell lines at the same passage were removed from liquid nitrogen storage and plated on 60 mm culture plates in media (DMEM, 15% FBS, 10 units of PenStrep). At 90% confluence, cell were trypsinized, and re-plated, onto a 100 mm plate, and allowed to grow to 80% confluence. Once confluent, these plates are then trypsinized and counted (to count the cells, 50 ul of the collected cell suspension was added to 100 ul of PBS/EDTA and from that mixture 50 ul of the solution was placed onto a hemocytometer and the four blocks counted, divided by four and multiplied first by the dilution factor and then by 10,000 to get the number of cells per milliliter of cell suspension) and 125,000 cells are seeded onto two 6-well plates and allowed to grow for one day. On day two and each of the following five days, two wells from each cell line are trypsinized and counted. These numbers are then plotted.

Colony Forming Unit Assay (CFU):

Cell lines were removed from liquid nitrogen and seeded onto 60 mm plates with media (DMEM, 15% FBS, 10 units of PenStrep) and then passage on a three-day/four-day cycle until passage six, seeding 400,000 cells each time. At passage six, the plates were trypsinized and counted and 400,000 cells were passaged on to passage 12 where the second part of this study was done.

At passages six and twelve, 2,500 cells were seeded onto each of four small plates and allowed to grow for nine days with media changes every three days. After this growth period, the media was removed from each plate, rinsed with PBS, and 100%

methanol was added to each plate to fix the cells for nine minutes. After fixing the cells, Giemsa stain (1.25 ml Giemsa stain, 1.5 ml 100% Methanol, QS to 50 ml) was added to cover the cells and allowed to incubate for 9 minutes. After this incubation, the stain was removed and each plate was rinsed twice with distilled water to remove any access stain remaining in the plate. After drying, colonies consisting of 40 cells or more were considered in the colony count.

Cell Cycle Analysis:

Low passage MEFs cell lines were seeded on to 35 mm tissue culture plates (300,000 cells/plate) and were allowed to grow for ~24 hours before the addition of the spindle poison, nocodazole (125ng/ml). Cells were trypsinized at various time points (8, 24 and 48 hours) and collected by centrifugation. Single cell suspensions were prepared from the cell pellets using a vortex, fixed in 70% ethanol, and stored at -20°C until the DNA content could be examined via flow cytometry for each genotype.

Animal Husbandry:

Mice were housed in small sterilizable cages with isolation lids and fed *ad lib*. At 21 days, pups were weaned and a tail clipping was taken for DNA extraction. In this controlled environment, mice were examined 3 times weekly for signs of illness or tumor formation. In the event a mouse was found to exhibit morbidity or have developed a palpable mass, it was euthanized using carbon dioxide and dissected. During autopsy, tumor tissues and organs exhibiting abnormal morphology were removed and a sample of

it was formalin-fixed and another sample was removed and stored at -80°C to be used for genotyping.

Southern Blotting:

To determine the *p53* genotype of the mice, southern blot assays were performed. Genomic DNA (3 μg) from the tail clipping was digested with Bam HI overnight at 37°C. The next day the samples were subjected to electrophoresis, buffered in tris-acetate-EDTA (40mM tris base, 20 mM acetate, 2mM EDTA) and imaged. After imaging, the membrane was washed in 0.25 M HCl for 5 minutes, rinsed 4 times with distilled water and transferred onto a nylon membrane overnight using 0.4 N NaOH. Following DNA transfer, the membrane containing the blotted DNA was rinsed with 2X SSC, dried at 37°C and blocked with pre-hybridization buffer (1.3% SDS, 2X SSPE, 1% milk, and 2 mg denatured salmon sperm DNA) for 5 hours at 68°C. After blocking, the pre-hybridization buffer was removed and 20 ml of hybridization buffer was added (10% dextran sulfate, 1.5X SSPE, 1% SDS, 0.5% milk) along with the radio-labeled probe. ($1x10^{6}$ cpm/ml). After incubating overnight, the hybridization buffer was removed and the membrane was rinsed with 2X SSC followed by a series of solutions (Solution 1: 2X SSC, 0.1% SDS. Solution 2: 0.5X SSC, 0.1% SDS. Solution 3: 0.2X SSC, 0.2% SDS). Once the membrane activity is between 2,000 and 3,000 counts per minute, the membrane was exposed on x-ray film overnight at -80°C. This was to reduce the amount of nonspecific binding of the radioprobe to the membrane, allowing for clearer results. The radio-labeled probe was generated using 100ng of DNA specific to exons 2-6 (a 600 bp fragment from the Kpn I digested plasmid (LR10) containing murine p53 cDNA) of

the *p53* gene. This probe was then labeled with dCTP[α-^{32}P] using random primers. The specific activity of the radiolabel was 3,000 Ci/mmol. In addition to the two possible bands that represent the wild type and mutant alleles, the p53 probe also binds to pseudogene regardless of the p53 genotype (figure 4).

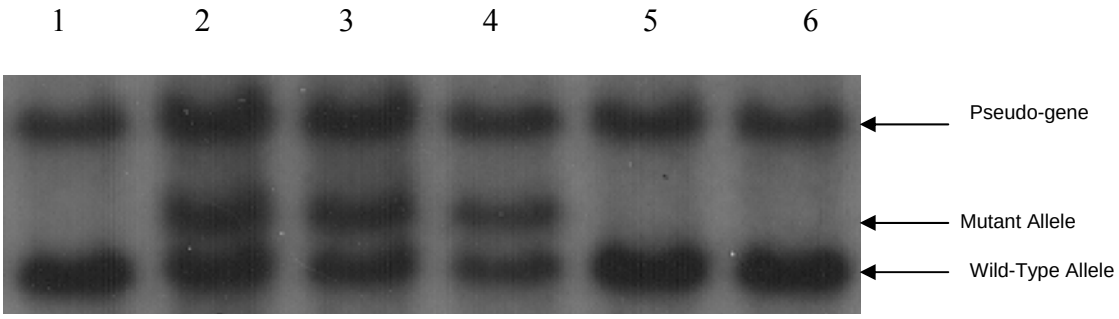

Figure 4: Southern blot results indicate the p53 genotype. An image of the southern blot with the bands labeled for 6 samples (from genomic DNA extracted from the tails of mice). The top band is a pseudogene, the middle band indicating the mutant allele (in which a small portion of exon 5 was deleted and a neomycin fusion cassette inserted) and the bottom band indicates the presence of the wild type allele.

III. Results:

Characterization of the *BubR1* gene Trap Insertion Site:

The 5' RACE data obtained from the Mutant Mouse Regional Resource Center (MMRRC) indicated that the trap had inserted into intron 2 of the gene. Using this data, we designed primers specific for exon 2 of *BubR1* (GS1) and the gene trap (TR1). PCR amplification produced a 2.4 Kb fragment that was specific only to the trapped allele (as determined by the trap-specific PCR primers TR2 and TR3). Sequence analysis of the PCR product revealed that the trap had inserted into intron 2 of the *BubR1* gene at base 2,170. Integration of the trap also resulted in the loss of the first 880 base pairs within the trap. However, this did not affect the function of the trap since the splice acceptor lies outside these lost bases (~1,276). Based on this information, we designed primers adjacent to the insertion site to amplify a 2.2 Kb fragment specific for the wild-type allele. A schematic representation of the primer sites with respect to the gene trap are shown in figure 5. An example of the PCR products obtained for various combinations of primer pairs is shown in figure 6.

BubR1 Deficiency Affects Embryogenesis:

We generated F1 heterozygotes using male chimeras and wild type females. The BubR1 heterozygotes developed normally and were capable of producing progeny. In order to generate mice that were completely deficient for the BubR1 allele we

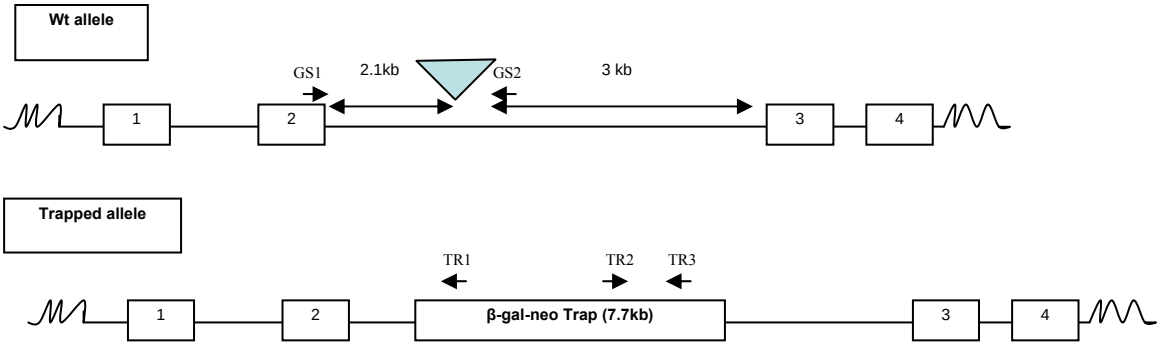

Figure 5: The *BubR1* gene and the location of the PCR primers. This schematic representation of the wild-type and trapped alleles show the positions of the PCR primers used for genotyping. In addition to this the blue triangle indicates the location where the gene trap inserts into the *BubR1* gene.

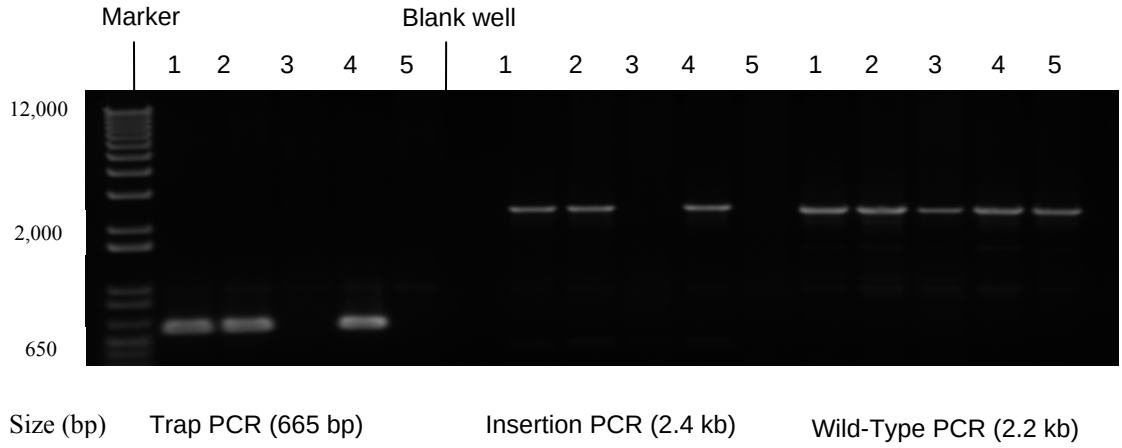

Figure 6: PCR indicates the *BubR1* genotype. This gel image provides *BubR1* genotype data for 5 samples. The marker here is a 1 kb+ ladder. The trap PCR used the primers TR2 and TR3 from the schematic above while the Insertion and wild type PCRs used the primer GS1 and either TR1 (the insertion) of GS2 (the wild type). The trap and insertion PCR indicate the presence of the trapped allele (samples 1, 2 and 4). Absence of a band indicates a wild type genotype (see samples 3 and 5). The wild-type PCR indicates the presence of the wild-type allele. Absence of a band would signify the loss of the wild-type allele.

intercrossed the F1 heterozygotes. Using the PCR primers specific for exon 2 and intron 2 in the *BubR1* gene, we found that the BubR1 homozygous mutation led to embryonic lethality (table 1). It was during this work that other groups published similar results and showed that in these nullizygous embryos, lethality was due to mitotic catastrophe and an up-regulation of apoptosis (62).

Generation of *BubR1/p53* Compound Mutants:

Since the embryonic lethality observed in BubR1 null animals was due to apoptosis and a possible up-regulation of the tumor suppressor p53, a transcription factor for several apoptotic genes, we hypothesized that the BubR1 nullizygotes could be rescued in a p53 deficient background. To test this, we generated BubR1/p53 compound mutants to determine if the loss of p53 would rescue this lethal phenotype. The BubR1 heterozygous offspring were crossed with p53 mutant mice to generate our compound heterozygous mice. To determine if p53 deficiency rescued the embryonic lethality of BubR1 nullizygotes, various crosses were done in which both mice were heterozygous for BubR1 and deficient for p53 (tables 2a-c). Overall, during the course of 39 crosses, 243 mice were produced. Using the PCR genotyping strategy described above, it was found that the progeny exhibited all the expected genotypes in their approximate ratios with the exception of the BubR1 nullizygotes. This indicated that p53 deficiency does not rescue the embryonic lethal phenotype observed in a BubR1 nullizygous animals. Since p53 deficiency failed to rescue the lethality observed in the BubR1 nullizygotes, we wanted to learn if the deficiency was able to delay the lethal phenotype during embryogenesis. Since we ere unable to produce mice nullizygous for *BubR1*, and since

Table 1: Embryonic lethality of BubR1 nullizygotes.

BubR1$^{+/-}$ X *BubR1*$^{+/-}$ [n=7]		
BubR1 WT	BubR1 Het	BubR1 Null
11	16	0

This table shows the results of multiple BubR1 heterozygous pairings. Mice were genotyped for BubR1 using the PCR strategies mentioned earlier. From these pairings no BubR1 nullizygous progeny were generated. Other groups produced similar results and found that this lack of nullizygous progeny was due to apoptosis and reabsorption of the nullizygous embryos at embryonic day 6.5 (62).

Table 2: p53 deficiency does not rescue the lethality of the BubR1 nullizygotes.

a.

BubR1$^{+/-}$ *p53*$^{+/-}$ X *BubR1*$^{+/-}$ *p53*$^{+/-}$ [n=4]						
BubR1Het/ p53 Wt	BubR1 Wt/ p53 Het	BubR1 Wt/ p53 Null	BubR1 Het/ p53 Wt	BubR1 Het/ p53 Het	BubR1 Het/ p53 Null	BubR1 Null / p53 Wt, Het, Null
2	4	1	1	12	4	0

b.

BubR1$^{+/-}$ *p53*$^{+/-}$ X *BubR1*$^{+/-}$ *p53*$^{-/-}$ [n=18]				
BubR1 Wt/ p53 Het	BubR1 Wt/ p53 Null	BubR1 Het/ p53 Het	BubR1 Het/ p53 Null	BubR1 Null / p53 Het, Null
14	12	32	17	0

c.

BubR1$^{+/-}$ *p53* $^{-/-}$ X *BubR1*$^{+/-}$ *p53*$^{-/-}$ [n=2]		
BubR1 Wt/ p53 Null	BubR1 Het/ p53 Null	BubR1 Null / p53 Null
6	4	0

A table showing the genotype of the crossed mice as well as the number of crosses [n] of each type and the number of offspring produced for each possible genotype. Mice were genotyped using the PCR and Southern blotting strategies mentioned previously. P53 deficiency was unable to rescue the lethality of the BubR1 nullizygotes even if both parents were heterozygous (a), or if one (b) or both parents (c) were nullizygous for p53.

earlier studies have shown that p53 deficiency can delay embryonic lethality of DNA damage response gene mutants (68, 69), we attempted to generate MEF lines nullizygous for *BubR1*. Various crosses were set up and MEFs were generated from E10.5 and E13.5 day embryos as explained above. BubR1 nullizygous cell lines were not generated from any of the crosses, indicating that p53 deficiency was unable to delay the lethal phenotype at least up to E10.5 (table 3 and data not shown).

BubR1 Deficiency Enhances Tumor Formation in p53 Mutant Mice:

To determine the effect of the *BubR1* and *p53* gene deficiency on overall survival and tumor formation we monitored the mutants and wild type littermates for a period of two and a half years. Mice exhibiting morbidity and signs of tumor formation were euthanized and autopsies were performed. The ages of the mice were recorded on a spreadsheet and used to create survival curves for each genotype (figure 7). As shown in the graph, the loss of one allele for either gene decreases the survivability of the mice and the loss of one allele in both genes further decreases the survivability implying a cumulative effect for the compound mutant. The wild-type mice survived the longest with a median age of 104.8 weeks. With the loss of one allele for either BubR1 or p53, survival decreased (63.1 weeks for the *p53* heterozygous mutant and 70 weeks for the *BubR1* heterozygous mutant). For both of these single mutants, when compared to the wild type, the survival was significantly different (P value= 0.0077 for the wild type versus the *BubR1* heterozygous mutant and P value= <0.0001 for the wild type versus the *p53* heterozygous mutant). When the compound heterozygous mutant was analyzed, it was found that the survival was decreased further (50 weeks) and that this was also

Table 3: p53 deficiency is unable to delay the lethal phenotype observed in *BubR1* nullizygotes.

MEF Crosses: *BubR1*$^{+/-}$ *p53*$^{+/-}$ X *BubR1*$^{+/-}$ *p53*$^{+/-}$ [n=2]						
BubR1 Wt/ p53 Wt	BubR1 Wt/ p53 Het	BubR1 Wt/ p53 Null	BubR1 Het/ p53 Wt	BubR1 Het/ p53 Het	BubR1 Het/ p53 Null	BubR1 Null / p53 Wt, Het. Null
1	*	*	2	2	6	0

A table of the MEF cell lines produced from two double heterozygous mutants. While no p53 single mutants were produced from these two crosses, these two cell lines were available through previous work by the investigator. (*) Later crosses using mice from the colony produced MEF lines including both the Wt/Het and Wt/Null genotypes. At both E10.5 and E13.5 there is no generationof BubR1 nullizygous cell lines.

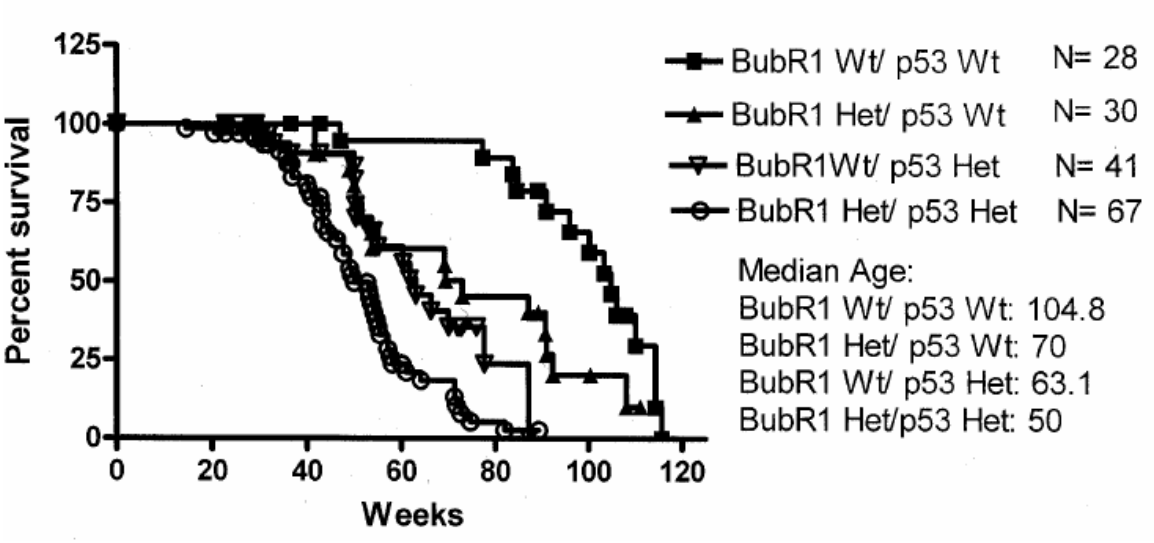

Figure 7: Survival of wild type, heterozygous and compound heterozygous mutant mice. All mice were of mixed inbred (C57Bl/6 X 129P2/OlaHsd) background. The percentages of tumor-free survivors are plotted as function of age in weeks (Prism 4 statistical software, GraphPad, Software Inc. San Diego, CA). Animals were monitored for tumors, morbidity or spontaneous death over a period of two and a half years (168 weeks). Moribund or tumor bearing mice were sacrificed and necroposied. Tumors or tissues with abnormal morphology were subjected to histopathological analysis to confirm their status as tumors.

significant when compared to the wild-type (P value= <0.0001 for the compound mutant versus the wild-type). However, it was also determined the survival curves of the single mutation genotypes (*BubR1*$^{+/+}$ *p53*$^{+/-}$, and *BubR1*$^{+/-}$ *p53*$^{+/+}$) were not significantly different when compared to each other (P value= 0.1418). With this information it is then important to make sure that the survival curve of the double mutation is significantly different when compared to both of the single mutation genotypes. It was found that for comparison to both of the single mutants, the survival curve of the double mutant was significantly different (P value= 0.005 versus the *BubR1*$^{+/+}$ *p53*$^{+/-}$ and P value= 0.0002 for the *BubR1*$^{+/-}$ *p53*$^{+/+}$). The two p53 nullizygous survival curves were also compared and though not significantly different to one another (P value= 0.9755), they were significant when compared to the other genotypes. However, with the complete loss of p53, the genotype of BubR1 has no additive effect on the survivability due to the rapid induction of lymphatic tumors in the p53 nullizygous background (figure 8).

The formalin-fixed samples mentioned above were then paraffin embedded and made into slides for the pathologist to examine and verify the tumor presence and type. The majority of the tumors observed were of lymphomas of the spleen and thymus. While spleenic and thymic lymphomas are commonly seen in p53 deficient models, mice deficient for BubR1 also showed the involvement of liver and lung tumors possibly due to the metastasis of the primary lymphomas, something not seen in the p53 single mutants (figures 9 and 10)

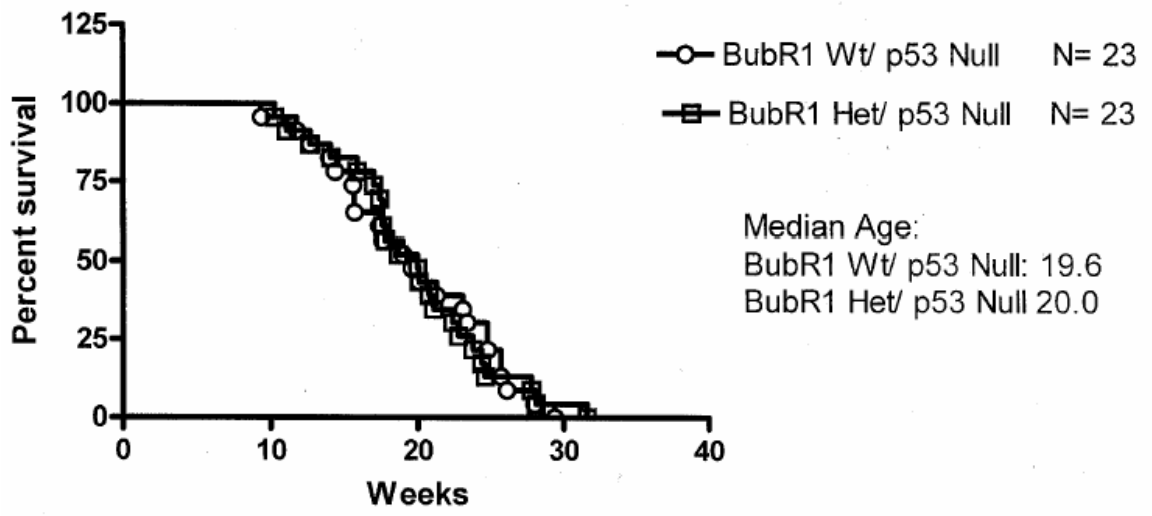

Figure 8: Survival of BubR1 mutants in p53 nullizygous background. All mice were of mixed inbred (C58BL/6 X 129SP2/OlaHsd) background. The percentages of tumor-free survivors are plotted as function of age in weeks. Animals were monitored for tumors, morbidity or spontaneous death over a period of 2.5 years (168 weeks). Moribund or tumor bearing mice were sacrificed and necroposied. Tumors or enlarged tissues were subjected to histopathological analysis to confirm their status as tumors.

Tumor Spectrum of the Single and Compound Heterozygous Mice

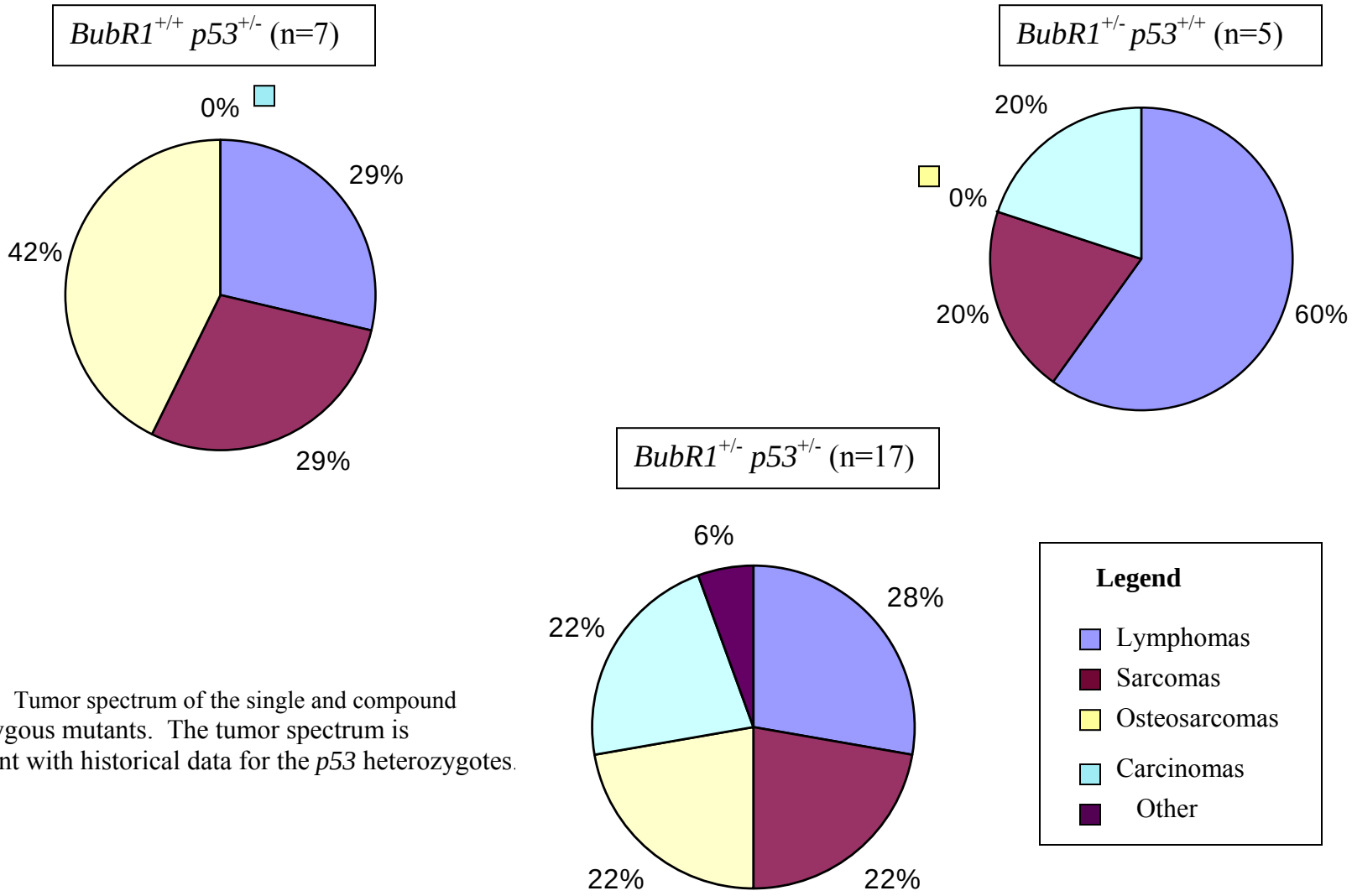

Figure 9: Tumor spectrum of the single and compound heterozygous mutants. The tumor spectrum is consistent with historical data for the *p53* heterozygotes.

Tumor Spectrum of the Single and Compound Nullizygous Mice



Figure 10: Tumor Spectrum of the single and compound nullizygous mice. The tumor spectrum is consistent for the *p53* nullizygotes. There is no observed shift in the tumor spectrum possibly due to the rapid induction of spleenic and thymic lymphomas in the *p53* nullizygous background.

Gene Dosage Effect of BubR1 During Tumorigenesis

To determine if there was a loss of heterozygosity for *BubR1* in the compound heterozygotes, we performed PCR analysis of the DNA isolated from the tumors. Of the16 *BubR1/p53* heterozygous mutants examined, none of the tumors had lost the wild type allele. This indicates that the loss of a single copy of *BubR1* gene was sufficient for the tumorigenic phenotype observed in the compound mutants.

***BubR1* Deficiency Enhances Cell Proliferation and Cell Transformation Potential:**

To better understand the effects of BubR1 deficiency on cellular proliferation we compared the cell growth kinetics of wild type and mutant MEFs. Single mutant and compound mutant MEFs were used in cellular growth assays. Analysis of the growth curves indicated that the loss of either the p53 or the BubR1 alleles resulted in an increase in the proliferation capacity (figure 11). Furthermore, compound heterozygous cell lines had an increased proliferation rate when compared to both the wild type and the single heterozygous lines. Interestingly, in the case of the *p53* nullizygous mutants, *BubR1* deficiency had an additive effect on the cellular growth rate indicating that deficiency of *BubR1* allowed a growth advantage in the *p53* nullizygous cell line.

Loss of tumor suppressor genes usually provides a growth advantage that can be correlated to the ability of the cells to grow in sub-optimal conditions. To determine if BubR1 deficiency synergized with the ability of p53 mutant cells, MEFs, we measured the colony forming ability of these cells. Colony forming assays were performed using the MEF cell lines generated. Consistent with the cell growth kinetic assays, we found that the loss of *BubR1* and *p53* either alone or in

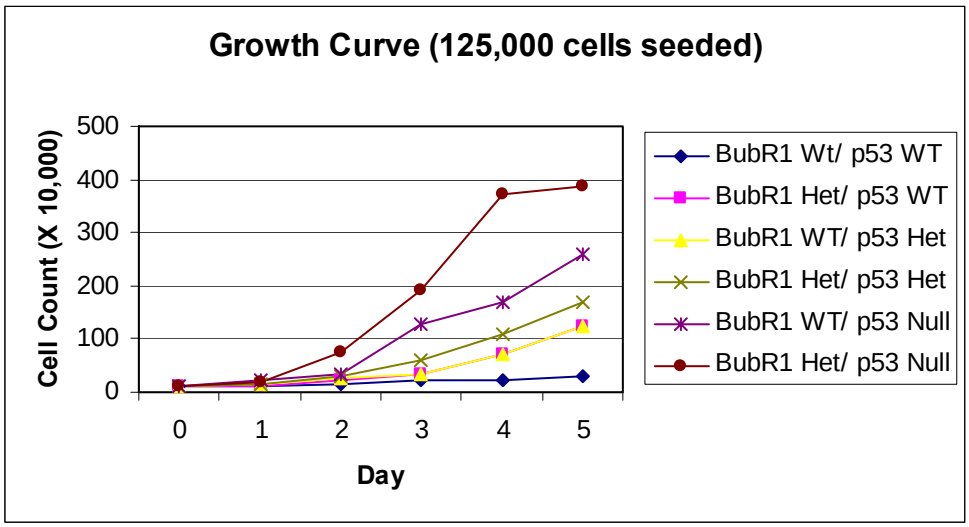

Figure 11: BubR1 and p53 Deficiency has a synergistic effect on cellular proliferation. This representative graph shows the growth kinetics of the various *BubR1/p53* genotypes and their growth over 5 days. In each experiment (repeated 3 times) 125,000 cells were seeded onto each plate.

combination increased the colony forming potential (figure 12). Furthermore, our preliminary analysis of *BubR1* deficiency in *p53* nullizygous MEFs indicated that the BubR1 compound mutants (*BubR1*$^{+/-}$ *p53*$^{-/-}$) were more susceptible to mitotic slippage when treated with the spindle poison, nocodazole (data not shown).

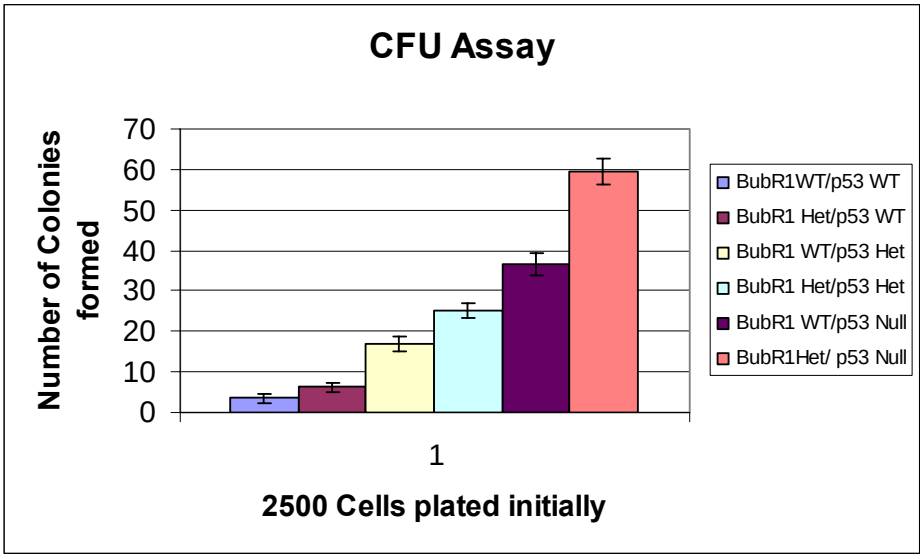

Figure 12: BubR1 and p53 deficiency has a synergistic effect in the transformation potential of MEFs. This is a representative graph showing the CFU assay results, after nine days of growth, based on the genotype of each cell line. In all the three experiments a similar trend was observed.

IV. Discussion

Tumorigenesis is a multi-step process in which the mutation or loss of one gene is usually insufficient to promote a cancer phenotype. However this single loss can provide a growth advantage to cells and with time, start a domino effect of genetic loss in the organism until a mutant cell line loses cell cycle controls resulting in uncontrolled proliferation. This proliferation can then feedback on itself and enhance the risk of genetic mutation that can ultimately lead to the formation of a tumor mass. In a subset of colonic cancers, it was found that the mitotic spindle checkpoint gene *BubR1* was mutated and it was proposed that this mutation could lead to chromosomal instability and an increased risk of tumorigenesis (43, 44). Following experiments showed that mouse models deficient for BubR1 showed an increased susceptibility to carcinogen induced tumor formation, anemia, and splenomegaly (61, 62). Additional studies have shown that BubR1 deficiency increased the cancer formation susceptibility in a mouse background known to be prone for colonic tumors (through a mutation of the *Adenomatous polyposis coli* gene) (64).

The purpose of our project was to examine the effects of a combined *BubR1/p53* deficiency as human cancers show a preponderance of *p53* mutations along with a subset showing mutations in the *BUBR1* gene. Our work confirmed the results from others in showing that the complete loss of *BubR1* was embryonic lethal (61, 62). Additionally, we showed that the loss of p53 was not able to rescue or significantly delay this lethality during embryogenesis. It is possible that the loss of p53 may slightly delay the lethality

of the BubR1 nullizygous embryos past the observed lethality from E6.5-E8.5 to E9.5, but this will require further work to determine the embryonic lethality at that stage.

The tumor incidence and survival data of BubR1 mutants in the presence or absence of p53 provide evidence that the loss of BubR1 leads to an increased mortality as well as an alteration in the type of tumors observed. In previous work examining the tumor incidence and lifespan of *p53* deficient mouse models, it was found that the loss of one allele leads to an increased incidence of tumorigenesis with a specificity for osteosarcomas and soft tissue sarcomas by 15 months while a complete loss of the gene leads to the formation of lymphomas within 6 months (70, 71). In the compound mutants for *BubR1* and *p53*, the earliest incidence of tumorigenesis was observed around 36 weeks with the mice having a median lifespan of 50 weeks. The tumor spectrum of these mice was also different with the involvement of spleen, thymus, liver and lung tumors. The induction of lung and liver tumors are rarely observed in the *p53* mutants. We also compared the survival of the *p53* heterozygous and the *BubR1* heterozygous and found that there were no significant differences even though the median age of the *p53* heterozygotes were 7 weeks earlier than the *BubR1* heterozygotes. However comparison of the survival data between the heterozygotes and the compound heterozygotes ($p53^{+/-}$ $BubR1^{+/-}$) showed a significant decrease in the median life span and an increase in the tumor incidence of the compound mutants. A likely explanation of these results is that with the additional mutation of BubR1 there is an increased incidence of chromosomal instability and mitotic slippage and these two events lead to other genetic alterations and an increase in tumorigenesis. This difference was not apparent in a *p53* nullizygous background due to the fact that a complete deficiency of *p53* induces a rapid

tumorigenesis phenotype that prevents the analysis of any additional effects of *BubR1* deficiency.

To better understand the synergistic effects of *BubR1* on *p53* deficiency induced tumorigenesis, we studied the phenotypes of the mutants at the cellular level. Analysis of cell growth kinetics showed that while the single heterozygous mutants had similar kinetics, the compound mutant cell lines had an increased growth rate. This is consistent with the checkpoint role of both of the proteins during the cell cycle, which would confer a growth advantage to the compound mutant. Furthermore, the increased cellular proliferation rate of the compound heterozygotes translates to an enhanced tumorigenesis phenotype at the organismal level. These results also verify that while p53 and BubR1 are primarily involved in separate checkpoint systems, the loss of both the checkpoints leads to increased cell proliferation. If these two proteins worked together only in a single checkpoint then the effects of the double mutant would not be additive but rather would show the same growth curve to the single mutants. Interestingly, we observed significant differences in the growth rates of compound mutants that were nullizygous for p53 ($p53^{-/-}$ $BubR1^{+/-}$ and $p53^{-/-}$ $BubR1^{+/+}$). This observation provides additional evidence that the loss of BubR1 protein intensifies the effects of p53 loss during tumorigenesis. We observed a similar trend in the colony forming assays in which the loss of BubR1 resulted in a higher transformation potential of the p53 heterozygotes and nullizygotes ($p53^{-/+}$ $BubR1^{+/-}$ and $p53^{-/-}$ $BubR1^{+/-}$).

In our work we were able to generate a mouse model defective for two genes involved in the regulation of various phases of the cell cycle. This work has confirmed the results of previous studies in determining the effect of the *BubR1* nullizygosity during

embryogenesis and extended these observations to show that p53 deficiency can not rescue the embryonic lethal phenotype in the *BubR1* nullizygotes. Our results indicate the importance of the BubR1 protein at earlier stages of mammalian development. In this work, we have also shown for the first time, that the mutation of BubR1 alters the life span and increases the incidence of tumorigenesis and these effects are amplified in a p53 deficient background. The synergistic effects of BubR1 deficiency on a p53 deficient background are consistent with the recent finding that showed a role for the BubR1 protein in effecting a DNA damage response after treatment with DNA damaging agents (58). The initial purpose of this study was to generate a mouse model for colon cancer formation. However, our data suggests that the mutant mice generated so far are not susceptible to colon cancers. The lack of colon cancers is possibly due to strain specific differences of tumor susceptibilities seen in various inbred mouse strains (72). Based on our experimental observations, we plan to back cross our *BubR1* deficient mouse model from the C57 BL/6 strain to the Balb/C strain. In addition, the progression of human colon cancer requires additional mutations that involve the activation of the *RAS* oncogene and the inactivation of the tumor suppressor genes *p16* and *APC*. Therefore an inherent disadvantage of our mouse model would be the requirement of additional mutations. Nonetheless, our results suggest that the loss of the BubR1 gene can increase the susceptibility of tumorigenesis in mice.

References

1. Sherr, C. J. 1996. Cancer cell cycles. *Science 274:1672*.
2. ACS. 2005. ACS: 2005 Report. American Cancer Society.
3. Sherr, C. J. 1995. Mammalian G1 cyclins and cell cycle progression. *Proc Assoc Am Physicians 107:181*.
4. Elledge, S. J. 1996. Cell cycle checkpoints: preventing an identity crisis. *Science 274:1664*.
5. Jiang, W., S. M. Kahn, P. Zhou, Y. J. Zhang, A. M. Cacace, A. S. Infante, S. Doi, R. M. Santella, and I. B. Weinstein. 1993. Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene 8:3447*.
6. Hartwell, L. H., and M. B. Kastan. 1994. Cell cycle control and cancer. *Science 266:1821*.
7. Marchetti, M. A., S. Kumar, E. Hartsuiker, M. Maftahi, A. M. Carr, G. A. Freyer, W. C. Burhans, and J. A. Huberman. 2002. A single unbranched S-phase DNA damage and replication fork blockage checkpoint pathway. *Proc Natl Acad Sci U S A 99:7472*.
8. Banin, S., L. Moyal, S. Shieh, Y. Taya, C. W. Anderson, L. Chessa, N. I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh, and Y. Ziv. 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science 281:1674*.
9. Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci U S A 89:7491*.
10. Chehab, N. H., A. Malikzay, M. Appel, and T. D. Halazonetis. 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev 14:278*.
11. Shieh, S. Y., J. Ahn, K. Tamai, Y. Taya, and C. Prives. 2000. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev 14:289*.
12. Paulovich, A. G., D. P. Toczyski, and L. H. Hartwell. 1997. When checkpoints fail. *Cell 88:315*.
13. Li, X., and R. B. Nicklas. 1995. Mitotic forces control a cell-cycle checkpoint. *Nature 373:630*.
14. Kline-Smith, S. L., S. Sandall, and A. Desai. 2005. Kinetochore-spindle microtubule interactions during mitosis. *Curr Opin Cell Biol 17:35*.
15. Rieder, C. L., R. W. Cole, A. Khodjakov, and G. Sluder. 1995. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol 130:941*.
16. Lampson, M. A., and T. M. Kapoor. 2005. The human mitotic checkpoint protein BubR1 regulates chromosome-spindle attachments. *Nat Cell Biol 7:93*.
17. Bharadwaj, R., and H. Yu. 2004. The spindle checkpoint, aneuploidy, and cancer. *Oncogene 23:2016*.
18. Taylor, S. S., D. Hussein, Y. Wang, S. Elderkin, and C. J. Morrow. 2001. Kinetochore localisation and phosphorylation of the mitotic checkpoint

components Bub1 and BubR1 are differentially regulated by spindle events in human cells. *J Cell Sci 114:4385*.
19. Zachariae, W., and K. Nasmyth. 1999. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev 13:2039*.
20. Yu, H. 2002. Regulation of APC-Cdc20 by the spindle checkpoint. *Curr Opin Cell Biol 14:706*.
21. Unger, T., J. A. Mietz, M. Scheffner, C. L. Yee, and P. M. Howley. 1993. Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. *Mol Cell Biol 13:5186*.
22. Ko, L. J., and C. Prives. 1996. p53: puzzle and paradigm. *Genes Dev 10:1054*.
23. Canman, C. E., D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan, and J. D. Siliciano. 1998. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science 281:1677*.
24. Waterman, M. J., E. S. Stavridi, J. L. Waterman, and T. D. Halazonetis. 1998. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nat Genet 19:175*.
25. Ljungman, M. 2000. Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. *Neoplasia 2:208*.
26. Carrier, F., P. T. Georgel, P. Pourquier, M. Blake, H. U. Kontny, M. J. Antinore, M. Gariboldi, T. G. Myers, J. N. Weinstein, Y. Pommier, and A. J. Fornace, Jr. 1999. Gadd45, a p53-responsive stress protein, modifies DNA accessibility on damaged chromatin. *Mol Cell Biol 19:1673*.
27. Dasika, G. K., S. C. Lin, S. Zhao, P. Sung, A. Tomkinson, and E. Y. Lee. 1999. DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. *Oncogene 18:7883*.
28. Unger, T., R. V. Sionov, E. Moallem, C. L. Yee, P. M. Howley, M. Oren, and Y. Haupt. 1999. Mutations in serines 15 and 20 of human p53 impair its apoptotic activity. *Oncogene 18:3205*.
29. Bulavin, D. V., S. Saito, M. C. Hollander, K. Sakaguchi, C. W. Anderson, E. Appella, and A. J. Fornace, Jr. 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *Embo J 18:6845*.
30. Martin, D. W., R. M. Munoz, M. A. Subler, and S. Deb. 1993. p53 binds to the TATA-binding protein-TATA complex. *J Biol Chem 268:13062*.
31. Goodsell, D. S. 1999. The molecular perspective: p53 tumor suppressor. *Stem Cells 17:189*.
32. Ginsberg, D., F. Mechta, M. Yaniv, and M. Oren. 1991. Wild-type p53 can down-modulate the activity of various promoters. *Proc Natl Acad Sci U S A 88:9979*.
33. Budde, A., and I. Grummt. 1999. p53 represses ribosomal gene transcription. *Oncogene 18:1119*.
34. Moshe Oren, J. B., G. Blander, A. Damalas, Y. Daniely, A. David, D. Michael, N. Minsky, N. Russek, O. Shifman, J. Taplick, X. Wang, S. Wilder, A. Zalcenstein. 2002. Understanding the role of p53 in cancer. *Cancer Research and Therapy:174*.

35. Polyak, K., Y. Xia, J. L. Zweier, K. W. Kinzler, and B. Vogelstein. 1997. A model for p53-induced apoptosis. *Nature 389:300*.
36. Furlong, E. E., T. Rein, and F. Martin. 1996. YY1 and NF1 both activate the human p53 promoter by alternatively binding to a composite element, and YY1 and E1A cooperate to amplify p53 promoter activity. *Mol Cell Biol 16:5933*.
37. Stuart, E. T., R. Haffner, M. Oren, and P. Gruss. 1995. Loss of p53 function through PAX-mediated transcriptional repression. *Embo J 14:5638*.
38. Schreiber, M., A. Kolbus, F. Piu, A. Szabowski, U. Mohle-Steinlein, J. Tian, M. Karin, P. Angel, and E. F. Wagner. 1999. Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev 13:607*.
39. Uittenbogaard, M. N., H. A. Giebler, D. Reisman, and J. K. Nyborg. 1995. Transcriptional repression of p53 by human T-cell leukemia virus type I Tax protein. *J Biol Chem 270:28503*.
40. Hudson, J. M., R. Frade, and M. Bar-Eli. 1995. Wild-type p53 regulates its own transcription in a cell-type specific manner. *DNA Cell Biol 14:759*.
41. Fuchs, S. Y., V. Adler, T. Buschmann, X. Wu, and Z. Ronai. 1998. Mdm2 association with p53 targets its ubiquitination. *Oncogene 17:2543*.
42. Fuchs, S. Y., V. Adler, T. Buschmann, Z. Yin, X. Wu, S. N. Jones, and Z. Ronai. 1998. JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes Dev 12:2658*.
43. Cahill, D. P., C. Lengauer, J. Yu, G. J. Riggins, J. K. Willson, S. D. Markowitz, K. W. Kinzler, and B. Vogelstein. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature 392:300*.
44. Davenport, J. W., E. R. Fernandes, L. D. Harris, G. A. Neale, and R. Goorha. 1999. The mouse mitotic checkpoint gene bub1b, a novel bub1 family member, is expressed in a cell cycle-dependent manner. *Genomics 55:113*.
45. Yoon, J., Y. Kang, K. Kim, J. Park, and Y. Kim. 2005. Identification and purification of a soluble region of BubR1: a critical component of the mitotic checkpoint complex. *Protein Expr Purif 44:1*.
46. Cayrol, C., C. Cougoule, and M. Wright. 2002. The beta2-adaptin clathrin adaptor interacts with the mitotic checkpoint kinase BubR1. *Biochem Biophys Res Commun 298:720*.
47. Shin, H. J., K. H. Baek, A. H. Jeon, M. T. Park, S. J. Lee, C. M. Kang, H. S. Lee, S. H. Yoo, D. H. Chung, Y. C. Sung, F. McKeon, and C. W. Lee. 2003. Dual roles of human BubR1, a mitotic checkpoint kinase, in the monitoring of chromosomal instability. *Cancer Cell 4:483*.
48. Chan, G. K., S. A. Jablonski, V. Sudakin, J. C. Hittle, and T. J. Yen. 1999. Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC. *J Cell Biol 146:941*.
49. Chan, G. K., B. T. Schaar, and T. J. Yen. 1998. Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1. *J Cell Biol 143:49*.
50. Skoufias, D. A., P. R. Andreassen, F. B. Lacroix, L. Wilson, and R. L. Margolis. 2001. Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. *Proc Natl Acad Sci U S A 98:4492*.

51. Shannon, K. B., J. C. Canman, and E. D. Salmon. 2002. Mad2 and BubR1 function in a single checkpoint pathway that responds to a loss of tension. *Mol Biol Cell 13:3706*.
52. Seike, M., A. Gemma, Y. Hosoya, Y. Hosomi, T. Okano, F. Kurimoto, K. Uematsu, K. Takenaka, A. Yoshimura, M. Shibuya, K. Ui-Tei, and S. Kudoh. 2002. The promoter region of the human BUBR1 gene and its expression analysis in lung cancer. *Lung Cancer 38:229*.
53. Chen, R. H. 2002. BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. *J Cell Biol 158:487*.
54. Howell, B. J., B. Moree, E. M. Farrar, S. Stewart, G. Fang, and E. D. Salmon. 2004. Spindle checkpoint protein dynamics at kinetochores in living cells. *Curr Biol 14:953*.
55. Li, W., Z. Lan, H. Wu, S. Wu, J. Meadows, J. Chen, V. Zhu, and W. Dai. 1999. BUBR1 phosphorylation is regulated during mitotic checkpoint activation. *Cell Growth Differ 10:769*.
56. Vogel, C., A. Kienitz, I. Hofmann, R. Muller, and H. Bastians. 2004. Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy. *Oncogene 23:6845*.
57. Lens, S. M., R. M. Wolthuis, R. Klompmaker, J. Kauw, R. Agami, T. Brummelkamp, G. Kops, and R. H. Medema. 2003. Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *Embo J 22:2934*.
58. Fang, Y., T. Liu, X. Wang, Y. M. Yang, H. Deng, J. Kunicki, F. Traganos, Z. Darzynkiewicz, L. Lu, and W. Dai. 2006. BubR1 is involved in regulation of DNA damage responses. *Oncogene*.
59. Ohshima, K., S. Haraoka, S. Yoshioka, M. Hamasaki, T. Fujiki, J. Suzumiya, C. Kawasaki, M. Kanda, and M. Kikuchi. 2000. Mutation analysis of mitotic checkpoint genes (hBUB1 and hBUBR1) and microsatellite instability in adult T-cell leukemia/lymphoma. *Cancer Lett 158:141*.
60. Gupta, A., S. Inaba, O. K. Wong, G. Fang, and J. Liu. 2003. Breast cancer-specific gene 1 interacts with the mitotic checkpoint kinase BubR1. *Oncogene 22:7593*.
61. Dai, W., Q. Wang, T. Liu, M. Swamy, Y. Fang, S. Xie, R. Mahmood, Y. M. Yang, M. Xu, and C. V. Rao. 2004. Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. *Cancer Res 64:440*.
62. Wang, Q., T. Liu, Y. Fang, S. Xie, X. Huang, R. Mahmood, G. Ramaswamy, K. M. Sakamoto, Z. Darzynkiewicz, M. Xu, and W. Dai. 2004. BUBR1 deficiency results in abnormal megakaryopoiesis. *Blood 103:1278*.
63. Baker, D. J., K. B. Jeganathan, J. D. Cameron, M. Thompson, S. Juneja, A. Kopecka, R. Kumar, R. B. Jenkins, P. C. de Groen, P. Roche, and J. M. van Deursen. 2004. BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. *Nat Genet 36:744*.
64. Rao, C. V., Y. M. Yang, M. V. Swamy, T. Liu, Y. Fang, R. Mahmood, M. Jhanwar-Uniyal, and W. Dai. 2005. Colonic tumorigenesis in BubR1+/-ApcMin/+

compound mutant mice is linked to premature separation of sister chromatids and enhanced genomic instability. *Proc Natl Acad Sci U S A 102:4365*.

65. Fearon, E. R., and B. Vogelstein. 1990. A genetic model for colorectal tumorigenesis. *Cell 61:759*.
66. Skarnes, W. C. 2000. Gene trapping methods for the identification and functional analysis of cell surface proteins in mice. *Methods Enzymol 328:592*.
67. Venkatachalam, S., Y. P. Shi, S. N. Jones, H. Vogel, A. Bradley, D. Pinkel, and L. A. Donehower. 1998. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *Embo J 17:4657*.
68. Brugarolas, J., and T. Jacks. 1997. Double indemnity: p53, BRCA and cancer. p53 mutation partially rescues developmental arrest in Brca1 and Brca2 null mice, suggesting a role for familial breast cancer genes in DNA damage repair. *Nat Med 3:721*.
69. Cao, L., W. Li, S. Kim, S. G. Brodie, and C. X. Deng. 2003. Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. *Genes Dev 17:201*.
70. Tyner, S. D., S. Venkatachalam, J. Choi, S. Jones, N. Ghebranious, H. Igelmann, X. Lu, G. Soron, B. Cooper, C. Brayton, S. Hee Park, T. Thompson, G. Karsenty, A. Bradley, and L. A. Donehower. 2002. p53 mutant mice that display early ageing-associated phenotypes. *Nature 415:45*.
71. Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature 356:215*.
72. Blackburn, A. C., S. C. McLary, R. Naeem, J. Luszcz, D. W. Stockton, L. A. Donehower, M. Mohammed, J. B. Mailhes, T. Soferr, S. P. Naber, C. N. Otis, and D. J. Jerry. 2004. Loss of heterozygosity occurs via mitotic recombination in Trp53+/- mice and associates with mammary tumor susceptibility of the BALB/c strain. *Cancer Res 64:5140*.

Vita

Walter Guy Wiles IV was born in Beckley, WV on April 10, 1980. He was raised in Bluff City, TN, and attended Bluff City Elementary and Middle School and graduated from Sullivan East High School in May 1998. He graduated from the University of Tennessee, Knoxville in May 2002 with a BS in biology and a concentration in biochemistry, cellular, and molecular Biology. Walter completed his Master of Science degree in Biochemistry, Cellular, and Molecular Biology at the University of Tennessee, Knoxville in May 2006. He currently resides in Knoxville, TN with his wife April.