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ANALYSIS OF BUB1 DEFICIENCY IN MICE

A Thesis Presented
for the
Master of Science Degree
The University of Tennessee, Knoxville

Shawn Michael Leland
May 2005

Dedication

This thesis is dedicated to my parents, Lee and Lois Leland, for their love and support, and to my beautiful wife, Mindy, whose belief in me, support, and encouragement helped me to achieve my goal. I will be forever grateful to her.

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Abstract

Accurate chromosome segregation is essential for living organisms. Errors in chromosome segregation can lead to the gain or loss of whole chromosomes, a condition known as aneuploidy, which is present in many cancers. One mechanism employed by the cell to monitor chromosome segregation is the spindle checkpoint, a complex signaling pathway which ensures chromosomes are properly aligned on the metaphase plate before the cell proceeds into anaphase. When lagging chromosomes are present, the spindle checkpoint delays the metaphase-to-anaphase transition, which enables the lagging chromosomes to properly orient before the cell proceeds into anaphase. Microtubule attachment to the kinetochore and tension across the kinetochore are both monitored by the spindle checkpoint, and a single unattached kinetochore is sufficient for spindle checkpoint activation. The delay in mitosis is due to the inactivation of the anaphase promoting complex (APC), a ubiquitin ligase required for sister chromatid separation.

Bub1 is a spindle checkpoint protein kinase that localizes to kinetochores lacking attachment to microtubules and is necessary for the localization of other checkpoint proteins, but its role is not clearly understood. We have used *Bub1* knockout mice to study the effects of Bub1 loss and its role in tumorigenesis. Our data suggest *Bub1* homozygous null mutations are lethal ~6.5 d.p.c., and *Bub1* heterozygous females exhibit reduced reproductive capacity. Our data also suggest *Bub1* heterozygous mouse embryonic fibroblasts (MEFs), to a limited extent, exit mitosis prematurely in the presence of the spindle damaging agent nocodazole. In addition, *Bub1* heterozygous cells do not differ in their growth kinetics from wildtype cells, nor do they differ with respect

to colony forming assays. Our *Bub1* heterozygous mice do not show an increased incidence of tumor formation compared to our wildtype mice, even at 1½ years of age. These data suggest other insults, in addition to the loss of one copy of *Bub1*, are needed to drive the tumorigenesis process.

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1. Introduction

The Spindle Checkpoint

In order for cells to remain viable, they must be able to faithfully replicate their chromosomes and must also ensure their replicated genomes are segregated properly in mitosis, enabling each daughter cell to receive the normal complement of chromosomes. Many surveillance mechanisms are employed to achieve this. Among these mechanisms are G1, G2/M, and spindle assembly checkpoints, with each checkpoint monitoring different parts of the cell cycle and different perturbations. Entry of the cell into mitosis is controlled by checkpoints which monitor DNA damage and replication, while exit from mitosis is controlled by checkpoints which monitor spindle assembly and position. Cell cycle checkpoints ensure later events in the cell cycle are initiated only after earlier events have been properly completed. Prior to mitosis, the chromosomes must duplicate and the cell must manufacture a spindle, consisting of microtubules, which transport the separated chromatids into the two daughter cells. Before anaphase, sister chromatids are held together by the cohesin complex to prevent the premature separation of these sister chromatids. During mitosis, spindles attach to the kinetochores of the chromosomes, and once the cohesin complex is cleaved, the two sister chromatids are pulled to opposite poles. The spindles form between the centromeres, enabling bipolar attachment of the chromosomes. This attachment of microtubules occurs after the chromosomes have condensed during prophase and the nuclear envelope has broken down. The chromosomes then align themselves at the metaphase plate. The checkpoint which monitors this is the spindle assembly checkpoint, which ensures sister chromatids are properly attached to microtubules before the cell proceeds into anaphase, preventing large

scale chromosome missegregation and aneuploidy. This signaling complex prevents anaphase onset until all chromosomes have properly attached to spindle microtubules. When kinetochores which are not attached to microtubules are present, the spindle assembly checkpoint arrests the cell in metaphase until all the kinetochores have become attached to microtubules (Figure 1). Amazingly, a single unattached kinetochore is sufficient to activate the spindle assembly checkpoint.

This phenomenon was noticed as far back as 1980, when Zieve et al treated human cells with the microtubule inhibitor nocodazole and found large numbers of cells in mitosis (Zieve, 1980). In the early 1990's, it was known that cells use feedback control to delay the exit from mitosis while awaiting completion of the spindle assembly (Murray, 1992). The three *Bub* (budding uninhibited by benzimidazole) genes (Bub1, Bub2, and Bub3) had recently been cloned, but not much was known about them at this time. This work was expanded upon in 1995 (Li, 1995) when mantid spermatocytes were observed to arrest in metaphase for 4 hours when the kinetochore was not under tension. These spermatocytes proceeded into anaphase in 55 minutes after tension was applied by a microneedle, suggesting a checkpoint which monitored the tension across the kinetochore. Evidence also exists which supports a model where the checkpoint also monitors the microtubule occupancy at the kinetochores. Mad2, a component of the spindle assembly checkpoint, localizes to the kinetochore after chromosome condensation, but is not seen at the kinetochore in late metaphase, when the chromosomes are correctly attached to the spindle (Waters, 1998). Cells treated with taxol, which reduces tension across the kinetochore without destroying the spindle attachment to the kinetochore, showed a lack of staining for Mad2, indicating

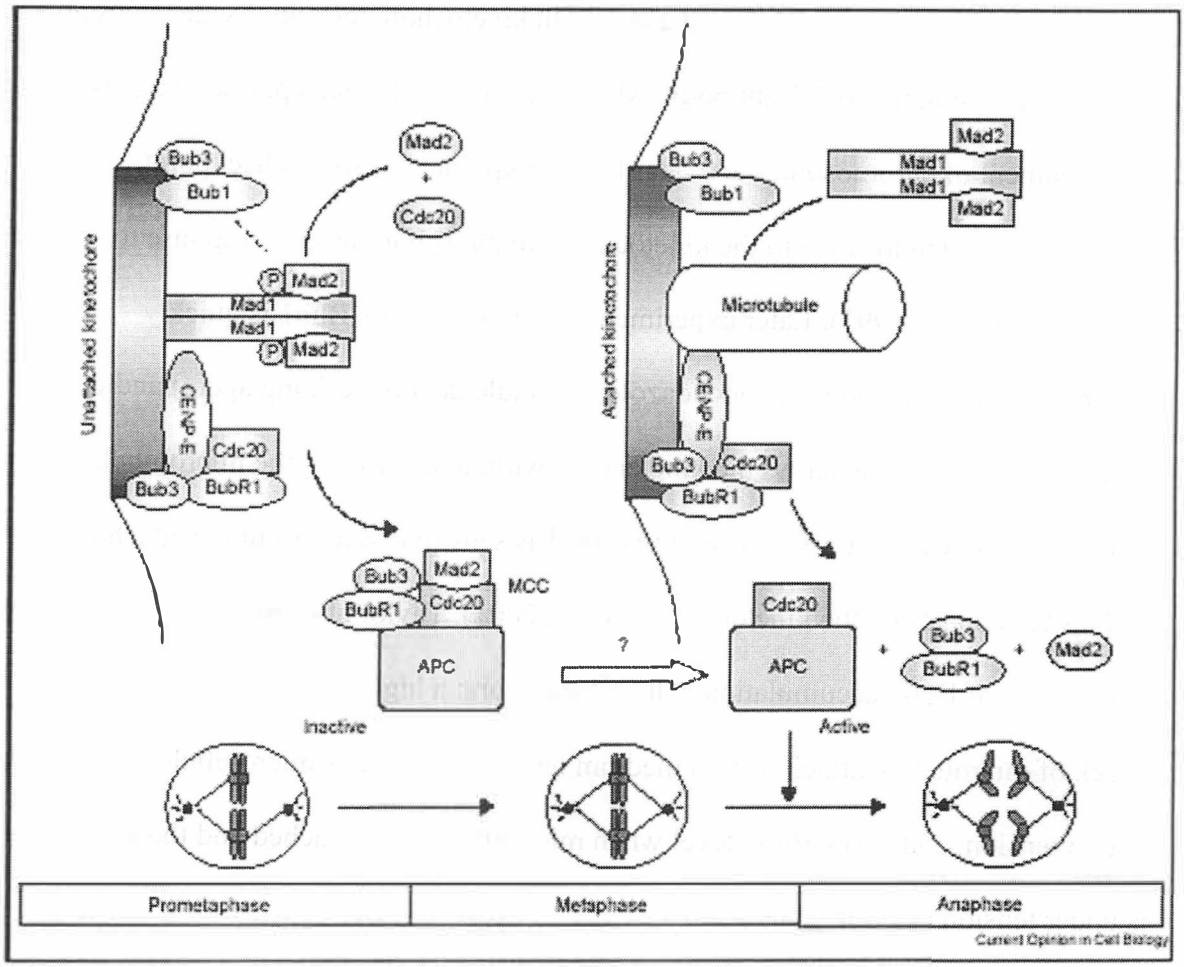


Figure 1. The spindle checkpoint. When unattached kinetochores are present, checkpoint proteins, such as Mad1and Mad2, localize to the kinetochores during prometaphase. Bub1 can phosphorylate Mad1, leading to dissociation of Mad 2. Mad 2, BubR1, Bub3, and Cdc20 can form a complex (the mitotic checkpoint complex, or MCC) which inhibits the activity of the anaphase promoting complex (APC). The inhibition of the APC prevents the cleavage of the SCC1 subunit of the cohesin complex and delays the onset of anaphase. When kinetochores are attached, Mad 1 and Mad2 cannot localize to the kinetochore. No MCC is produced, and Cdc20 can activate the APC. Once activated, the APC ubiquitinates securin, leading to the degradation of SCC1 and anaphase onset. (Yu, H. "Regulation of APC-Cdc20 by the spindle checkpoint", *Current Opinion in Cell Biology*, 14:706-714, 2002.)

microtubule occupancy is monitored along with kinetochore tension. Evidence from early experiments using the 3F3/2 antibody, which recognizes phopshoepitopes, suggested the misalignment of chromosomes activates the checkpoint, and some checkpoint components which localize to the kinetochore are phosphorylated in response to spindle damage (Gorbsky, 1993). Later experiments did indeed show Bub1 is quickly phosphorylated in response to nocodazole (a spindle depolymerizing agent) and taxol (which reduces tension across the kinetochore without destroying the microtubule-kinetochore attachment), which suggests Bub1 is sensitive to microtubule attachment as well as tension across the kinetochore (Taylor, 2001). Taylor also proposed there may be three levels of Bub1 accumulation at the kinetochore: a high level of Bub1 when there is a lack of microtubule attachment, a medium level when there is microtubule attachment but no tension, and a low Bub1 level when microtubules are attached and the kinetochore is under tension (Taylor, 2001).

Several spindle checkpoint components have been identified; among them are Bub1, BubR1, Bub3, Mad1, and Mad2. This complex signaling cascade works to inactivate the anaphase promoting complex (APC), which is a multi-subunit ubiquitin ligase needed for the degradation of securin and cyclin B, both of which are degraded before the onset of anaphase. Two models are proposed to explain the inhibition of the APC, the Sequestration Model and the Direct Inhibition Model (Chan, 2003). The Sequestration Model posits that Mad2 directly binds Cdc20 and inhibits it from activating the APC. This model also suggests the unattached kinetochore converts Mad2 to a conformation which can bind Cdc20. In the Direct Inhibition Model, a mitotic checkpoint complex (MCC) consisting of Mad2, BubR1, Bub3, and Cdc20 binds directly to the

APC, inhibiting it from performing its ubiquitin ligase function, thereby preventing the onset of anaphase. In this model, unattached kinetochores generate a "wait anaphase" signal which arrests the cell in metaphase. The current model proposes Mad1, in complex with Mad2, binds to unattached kinetochores, along with Bub1, BubR1, and Bub3. In response to the lack of attachment of microtubules, Bub1 is autophosphorylated and also phosphorylated by Cdc2. Bub1 is then surmised to phosphorylate Mad1, which leads to the dissociation of Mad2. Some evidence suggests unphosphorylated Mad2 interacts with Mad1 and the APC (Wassmann, 2003). Theoretically, Mad2 could be directly phosphorylated (although evidence suggests Bub1 is not the kinase which does this (Seeley, 1999)) leading to its dissociation. Alternatively, the phosphorylation of Mad1 by Bub1 could induce a conformational change in Mad1, which could expose a buried serine residue on Mad2, enabling this residue to be phosphorylated and leading to the dissociation of Mad2. Wassmann, et al, (2004) have shown Mad2 dissociates from Mad1 upon phosphorylation, but the exact mechanism has yet to be fully elucidated. If Mad2 must be phosphorylated in order to dissociate from Mad1, a phosphatase would be needed to dephosphorylate Mad2 to allow it to bind and inhibit the APC. Mad2 is not thought to inhibit the APC alone, however. The evidence suggests Mad2, along with BubR1, Bub3, and Cdc20 form a mitotic checkpoint complex which binds and inhibits the APC. The target of the spindle assembly checkpoint, the APC, ubiquitinates securin and tags it for degradation by the proteasome. The degradation of securin enables separase to cleave the SCC1 subunit of the cohesin complex, thereby allowing sister chromatids to separate and the cell to proceed into anaphase.

Bub1

Bub1 is a ~120 kD protein kinase which localizes, during prophase and prometaphase, to kinetochores and is recruited to kinetochores of chromosomes which lack attachment to microtubules. Bub1 contains 1085 amino acids and 25 exons, with the kinase domain located in the C-terminal region, and the kinetochore localization domain in the N-terminal region. Cdc2 phosphorylates Bub1 at the CDK (cyclin dependent kinase) consensus sites in response to spindle damage in mitotic cells (Yamaguchi, 2003). Bub1 becomes hyperphosphorylated during spindle damage. Bub1 yeast mutants which cannot be phosphorylated are checkpoint defective, indicating the need for Bub1 phosphorylation in a normal checkpoint. Complete spindle checkpoint function requires Bub1 kinase activity, but this activity is not sufficient for checkpoint function. Association of Bub1 with Bub3 is needed for the kinase activity of Bub1, which phosphorylates itself, Bub3, Mad1, and Cdc20. Experiments in which the kinase domain of Bub1 was deleted or Bub1 was unable to be phosphorylated showed these mutants were more sensitive to thiabendazole (TBZ – a mitotic spindle assembly inhibitor) as single mutants and double mutants than were widtype cells, but were still more resistant than Bub1 null mutants. This would seem to indicate that the phosphorylation of Bub1 and its kinase activity are important, but some other aspects of Bub1 are needed for a full checkpoint response (Yamaguchi, 2003).

Bub1 is part of a complex network of checkpoint components which acts to generate a diffusible signal (a "wait anaphase" signal) at the kinetochore. The function of this signal is to block the action of the APC. The APC mediates the ubiquitination of securin, which inhibits anaphase by blocking sister chromatid segregation (Alexandru,

1999). Bub1 has been shown to be a central player in triggering the spindle checkpoint signal from the kinetochore (Sharp-Baker, 2001). Bub1 must first form a complex with Bub3 before it can localize to the kinetochore (Taylor, 1998). Using an anti-Bub1 antibody, Sharp-Baker, et al were able to show a failure of mitotic arrest in *Xenopus* egg extracts treated with nocodazole when Bub1 function was removed. Bub1 localization to the kinetochore upon treatment with nocodazole was also seen (Sharp-Baker, 2001). Microtubule attachment alone was not sufficient for Bub1 dissociation from the kinetochores, indicating Bub1 is also involved in the tension sensing mechanism. Sharp-Baker (2001) also showed Bub1 is needed for localization of other checkpoint proteins to the kinetochore. Immunodepletion of Bub1 from *Xenopus* egg extracts revealed diminished kinetochore staining of Bub1 along with Bub3, Mad1, Mad2, and Cenp-E, suggesting Bub1 is needed for the localization of these proteins to the kinetochore (Sharp-Baker, 2001). Johnson et al (2004) also show Bub1 is needed for efficient localization of BubR1, Cenp-F, Cenp-E, and Mad2 in human cells. They propose an ordered pathway where Bub1 acts as a regulator of assembly of numerous checkpoint proteins. Interestingly, when Aurora B is repressed using RNAi, the level of Bub1 bound to the kinetochore is reduced to ~50%. The effect on BubR1 and Cenp-E was greater (reduced to ~40% and ~20% respectively), which they suggest could mean a dual function for Aurora B: targeting Bub1 to the kinetochore along with increasing the affinity of BubR1 and Cenp-E for kinetochores which are bound by Bub1 (Johnson, 2004). Alternatively, the reduced levels of BubR1 and Cenp-E could result from the reduced Bub1 level. If Bub1 is required for efficient localization of these checkpoint

proteins, then it would stand to reason that a lower level of kinetochore-bound Bub1 would lead to lower levels of BubR1 and Cenp-E.

Bub1 has also been shown to be an important component for chromosome congression (Johnson, 2004). Cells in which Bub1 had been repressed by means of RNAi showed congression defects when compared to control cells. Most chromosomes in Bub1-repressed cells could align, but the number of cells which had one or more unaligned chromosomes went from 10% in controls to 78% in Bub1-repressed cells (Johnson, 2004). Confirmation of this finding came from experiments performed with the proteasome inhibitor MG132. Johnson et al (2004) reasoned that if chromosomes could bi-orient but had congression defects, inhibiting the metaphase-to-anaphase transition would allow more time for the chromosomes to align properly at the metaphase plate, thereby increasing the metaphase index. This is, in fact, what occurred, suggesting that Bub1 plays a role in chromosome congression (Johnson, 2004). One interesting result from this study was the finding by Johnson et al that Bub1 repression had no effect on the spindle checkpoint in nocodazole-treated cells. This is contrary to findings using *Xenopus* extracts in which Bub1 had been inactivated with anti-Bub1 antibodies. Bub1-depleted *Xenopus* extracts exposed to nocodazole failed to arrest in mitosis, while extracts which retained Bub1 function arrested at mitosis in response to nocodazole (Sharp-Baker, 2001). Also, HeLa cells expressing a Bub1 dominant-negative mutant had a compromised spindle checkpoint (Taylor, 1997). Johnson et al used RNAi to repress several checkpoint proteins. RNAi, however, is not 100% efficient, so residual Bub1 may be adequate for spindle checkpoint function. Even though Bub1 was reduced by more than 80%, kinetochore-bound BubR1 and Mad2 were reduced to approximately 25% of

the control (Johnson, 2004). BubR1 and Mad2 comprise part of the mitotic checkpoint complex (MCC) which inhibits the APC. It is entirely possible that 25% of the checkpoint signal is adequate for checkpoint maintenance, while at the same time this level may not be sufficient for proper chromosome congression. Two possibilities for this result have been proposed (Ditchfield, 2003, Johnson, 2004). One possibility is that more than one pathway is involved in mitotic arrest following damage to the mitotic spindle, and Bub1 is only required for one pathway. In this case, a Bub1-independent pathway could compensate for the loss of Bub1. This possibility seems to contradict the previous observations of Sharp-Baker (2001) and Taylor (1997). The other possibility is the spindle checkpoint consists of one pathway, but losing Bub1 only partially blocks this pathway. Bub1 seems to be required for efficient kinetochore localization of BubR1 and Mad2, but as was seen earlier, the levels of these two checkpoint proteins were still ~25% even when the Bub1 level was reduced by more than 80% (Johnson, 2004). It seems possible that complete loss of Bub1 would reduce the localization of these even further, but some BubR1 and Mad2 may still localize to the kinetochore, allowing for minimal checkpoint function. The levels of BubR1 and Mad2 required for the prevention of mitotic exit in response to spindle damage have yet to be determined. The dynamics of Bub1 at the kinetochore are such that a large pool may not be needed. FRAP studies have shown Bub1 has a half-life of ~57 seconds at unattached kinetochores (Howell, 2004), while other checkpoint proteins, such as Mad2, have shorter half-lives [Mad2 has a half-life of approximately 19 seconds (Howell, 2004); Cdc20, the activator of the APC, has a half-life of approximately 5.1 seconds (Kallio, 2002)]. Bub1 appears to be a fairly stable component of unattached kinetochores, which could indicate another role, perhaps

serving as a scaffold to aid in the binding of other checkpoint proteins like BubR1 and Bub3 (not bound to Bub1), rather than Bub1 as a component of the mitotic checkpoint complex (MCC). Indeed, Bub1 has not been found to be part of the MCC (Musacchio, 2002). Shah et al (2004) achieve results similar to Howell in their FRAP studies. Bub1 cycling through the kinetochore was slow, and upon photobleaching, Bub1-YFP did not significantly recover at the kinetochore during normal mitosis (Shah, 2004). After attachment, Bub1 remained localized at the kinetochore, whereas Mad1 and Mad2 relocalized to the spindle poles. Again, this would seem to indicate Bub1 is a stable component of the kinetochore and serves a scaffolding and/or recruitment role in the spindle assembly checkpoint. The turnover of Bub1 at attached kinetochores increases, with a half-life of ~25 seconds, still considerably slower than other checkpoint proteins (Howell, 2004). FRAP data also indicate only a small fraction of checkpoint proteins localize to the kinetochores at one point in time, meaning the vast majority of these proteins, including Bub1, are located in the cytoplasmic pool (Howell, 2004). Howell's (2004) FRAP data indicate only 0.4% of Bub1 was localized to unattached kinetochores in prometaphase. The FRAP data would seem to support a catalytic model for the spindle checkpoint, with Bub1, along with Cenp-C, localizing to the kinetochore and remaining stably bound there (along with Mad1 and a stable form of Mad2), while components of the wait-anaphase signal cycle quickly through the kinetochore.

Aurora B is required for localization of Bub1 (and other checkpoint proteins) to the kinetochore in *Xenopus* extracts (Vigneron, 2004). Aurora B-depleted extracts demonstrated a loss of kinetochore binding of Bub1, as well as Mps1, Cenp-E, Bub3, Mad1, and Mad2, indicating Aurora B is far upstream in the spindle checkpoint. These

studies also showed the interdependence of Bub1, Mps1, and Cenp-E for their efficient localization to the kinetochore, which is somewhat contradictory to the work of Sharp-Baker (2001) and Johnson (2004). These two studies suggest Bub1 is needed for kinetochore localization of Cenp-E, but Cenp-E was not needed for kinetochore localization of Bub1.

Localization studies also indicate the kinetochore localization of Cdc20 and the APC are regulated by checkpoint proteins. Depletion of Aurora B, Mps1, Bub1, and Cenp-E from *Xenopus* extracts led to a loss of kinetochore localization of Cdc20, along with Cdc27 and Cdc23, two subunits of the APC (Vigneron, 2004). Cdc20 and the APC were also shown to localize independently of each other (Vigneron, 2004). Interestingly, the APC has been shown to be present throughout the cell cycle, as well as the inhibitory mitotic checkpoint complex (MCC), but inhibition only occurs in APC purified from mitotic cells, which would indicate the APC is somehow modified during mitosis so it can be recognized and inhibited by the MCC. Perhaps recruitment to the kinetochore induces a modification of the APC which allows it to be inhibited. The modification to the APC is unknown.

The murine homolog of Bub1 was shown to be necessary for proper progression through mitosis. Cells which expressed a dominant-negative murine Bub1 (mBub1) escaped cell death after release from nocodazole. Cells were exposed to nocodazole for 18 hours. Wild type cells accumulated in mitosis, but after 18 hours of nocodazole exposure, these cells were able to exit from mitosis. A significant portion of these cells, however, had DNA contents less than 2N, indicative of cell death (Taylor, 1997). These cells also had morphological features consistent with apoptosis (Taylor, 1997). Cells

which expressed dominant-negative Bub1 also accumulated in mitosis, but the mitotic index was significantly reduced compared to cells with wildtype Bub1 (Taylor, 1997). This would suggest many cells had already left mitosis. Upon exit from mitosis, these cells had DNA contents greater than 4N (Taylor, 1997), which would seem to indicate progress through the cell cycle even in the presence of spindle damage. Many of these cells accumulated 8N DNA content, indicating these cells completed S phase. The fact that cells with wildtype Bub1 underwent apoptosis after prolonged spindle damage seems to indicate the presence of a checkpoint in G1. Evidence exists of just such a checkpoint which is dependent on functional p53. A prolonged mitotic arrest is vital for this postmitotic G1 arrest (Vogel, 2004).

Bub1 also seems to be required for the timing of normal mitosis. Wildtype cells and cells expressing a dominant-negative murine Bub1 both proceed through the cell cycle, returning to G1 18 hours after release from G1/S (Taylor, 1997). This would seem to suggest the dominant-negative Bub1 did not affect chromosome segregation (these experiments were done in the absence of nocodazole, hence the spindle was functional). At the 12-hour time point, though, more cells in the dominant-negative population returned to G1 than cells in the wildtype population. Since both populations of cells entered mitosis at the same time, the difference, as suggested by the evidence, must be due to accelerated exit from mitosis (Taylor, 1997). Results from later experiments indicated the dominant-negative cells exited mitosis 25 minutes faster than cells with wildtype Bub1 (Taylor, 1997). Alignment of the cells did not seem to be affected, so the delay before anaphase seemed to be sufficient. The dominant-negative Bub1 may compete with endogenous Bub1 for binding sites on the kinetochore (Taylor, 1997). This

would lower the amount of functional Bub1 at the kinetochore, which, in turn, would probably lower the strength of the emitted "wait anaphase" signal toward a threshold. When the signal falls below this threshold, the anaphase promoting complex would be activated, and the cell would proceed into anaphase. The nature of this putative threshold has not been determined. Over the course of several mitoses, a decrease in the amount of time spent in prometaphase (when the chromosomes are aligning along the metaphase plate) could lead to missegregation, which could simply be due to the cell not having enough time to properly align its chromosomes before progressing into anaphase.

Bub1 and Cancer

The spindle assembly checkpoint monitors the attachment of microtubules to kinetochores in cells, and blocks anaphase onset until all chromosomes are properly attached. If the spindle assembly checkpoint fails, the potential for aneuploidy exists due to progression of the cell into anaphase even when unattached or mono-oriented chromosomes are still present. These unattached or mono-oriented chromosomes fail to align properly along the metaphase plate, and hence are referred to as lagging chromosomes. Lagging chromosomes at anaphase can lead to an abnormal chromosome number (aneuploidy). Many human cancers do exhibit gains or losses of whole chromosomes (Lengauer, 1998), possibly due to chromosomal instability (CIN) which persists throughout the lifetime of the tumor. The molecular basis of CIN is not understood. It would seem to follow, though, that mutations in genes involved in the spindle assembly checkpoint could lead to cancer. Evidence does exist which implicates spindle assembly checkpoint genes in CIN. First, aneuploid cell lines which exhibited

CIN had an aberrant response to the spindle-damaging agents nocodazole and colcemid. Instead of arresting in metaphase for a relatively long period of time, the CIN cells exited mitosis and started another round of DNA synthesis (Cahill, 1998). Second, changes in the sequence of human mitotic checkpoint genes have been identified in some human cancers. One human colorectal cancer cell line showed a deletion of 197 bp, which was predicted to remove codons 76 to 141, and also create a frameshift (Cahill, 1998). Another human colorectal cancer cell line had a mutation at codon 492, which changed a conserved serine residue to tyrosine (Cahill, 1998). Both of these mutations were somatic, and were seen in their primary tumors but not in normal tissue. These mutations also had dominant-negative properties. Expression of either of these mutant *Bub1* genes in MIN cells (cells with microsatellite instability – these cells have a normal spindle checkpoint) altered the mitotic indices of transfected cells after these cells were exposed to the spindle damaging drug nocodazole (Cahill, 1998). The mitotic indices of the cells expressing the mutant *Bub1* genes was significantly reduced compared to cells transfected with wildtype *Bub1* or control vector. The reduction in mitotic index suggests cells exited mitosis even in the presence of spindle damage. These cells also entered into S-phase and duplicated their DNA, as evidenced by a lower reduction in BrdU incorporation compared to wildtype cells (Cahill, 1998). Somatic mutations of *Bub1* were also observed in primary lung cancer (Gemma, 2000). In addition to spindle checkpoint genes, yeast studies have revealed many genes which, when altered, can lead to CIN, including genes involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function, and centrosome formation (Lengauer, 1998). The basis for CIN is still not understood, but given that genetic defects of many genes can

lead to CIN, the evidence suggests many genes could play a role in CIN. CIN may be prevalent in many cancers because there are many genes which could contribute to this phenotype when these genes are mutated. This could provide a cell with the instability needed for the development of multiple genetic alterations leading to malignancy (Lengauer, 1998). It would seem that multiple mutations would be needed for the development of CIN.

2. Results

Generation of *Bub1* Deficient Mice

In order to study the role of *Bub1* in the spindle checkpoint and cancer formation, *Bub1* deficient mice were generated. A gene trap approach was used to achieve disruption of the *Bub1* gene (Figure 2). Embryonic stem (ES) cells which had been characterized to have a gene trap insertion within the *Bub1* gene were obtained from Bay Genomics. The gene trap was determined to have inserted in intron 8 of the *Bub1* gene. These trapped ES cells were analyzed by PCR and Southern blotting to confirm the presence of the trap within the *Bub1* gene. Upon confirmation, the trapped ES cells were used for injection into 3.5 day old blastocysts. Seven chimeric mice were generated from these blastocyst injections, and of these seven, three founder males produced germ line litters for *Bub1* 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 wildtype and heterozygous offspring. Additionally, the heterozygous mice had no recognizable developmental defects. Heterozygous F1 offspring from the chimeric males were then crossed in an effort to obtain *Bub1* nullizygous mice.

Genotyping *Bub1* Deficient Mice

A strategy employing PCR and Southern blotting was used to determine the genotypes of offspring generated from our crosses. Before genotypes could be obtained, DNA was extracted from tail clips using the phenol:chloroform extraction technique described in the Material and Methods section. Primers which generated a nested set of products were used for genotyping. Our PCR protocol made use of several sets of primers

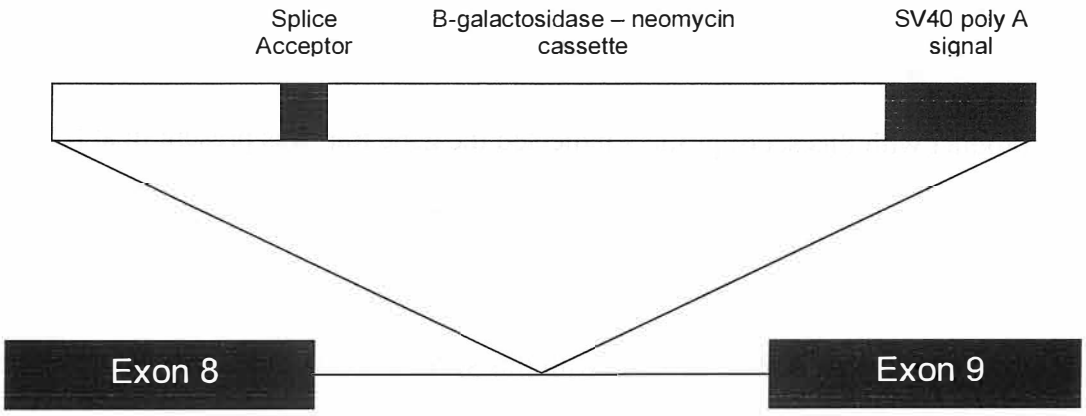


Figure 2. The gene trap used to disrupt the *Bub1* gene. The gene trap inserted into intron 8 of the *Bub1* gene. The β-galactosidase - neomycin cassette allows for selection as well as visualization of gene expression when gene-trapped cells are stained with X-gal.

to determine the genotype of the offspring. One set consisted of a forward primer specific for exon 8 of the *Bub1* gene and a reverse primer specific for the gene trap. The presence of a PCR product with this set of primers indicated the gene trap was present in at least one allele of the *Bub1* gene. A second set of primers consisted of the same forward primer specific for exon 8 of the *Bub1* gene, along with a reverse primer specific for intron 8 of the *Bub1* gene. A PCR product with this set of primers indicated the presence of at least one wildtype allele. The presence of the gene trap would, however, preclude the production of any PCR product with this set of primers, due to the limited length of time for the extension step in our PCR protocol. The presence of the trap added approximately 8800 bp between the exon 8 primer sequence and the intron 8 primer sequence, thus preventing the synthesis of complementary strands of sufficient length to hybridize together and produce a visible product. The third set of primers consisted of forward and reverse primers with sequences specific for the gene trap. These primers should only produce a product in heterozygous or null offspring. One additional benefit of using primers specific for the gene trap is the ability to confirm that the trap is not present in any other gene. Our heterozygous mice should have a product, while wildtype mice should not. Using this combination of primers, our PCRs should provide confirmation that our gene trap is not present in any gene except the *Bub1* gene. Representative agarose gels from genotyping experiments performed on offspring of our crosses are presented (Figures 3, 4, and 5).

While PCR is a powerful tool for genotyping, it does have some drawbacks. Non-specific bands become problematic, especially when faint, yet detectable, bands are present at the expected size based on the primers used. In such cases, the genotype is

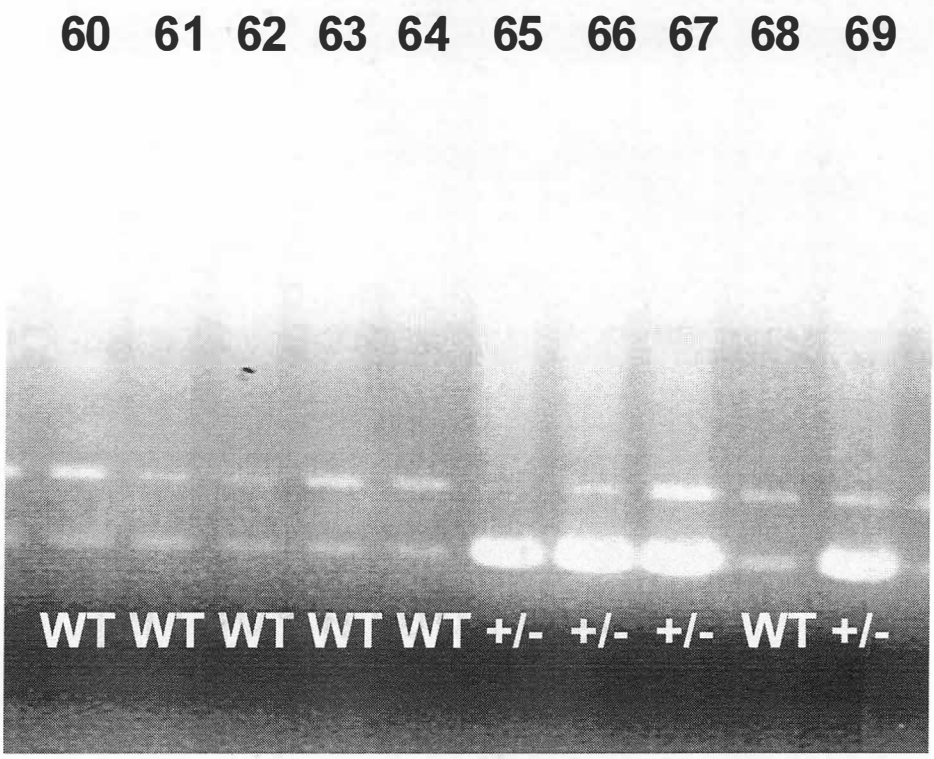


Figure 3. PCR genotyping of mice. This is a representative gel from a PCR genotyping experiment using the forward primer specific for intron 8 and a reverse primer specific for the gene trap. The presence of bright bands in samples 65, 66, 67, and 69 indicate the gene trap is present in a least one allele. The other lanes, based on this experiment, are wildtype. Faint bands such as those seen here present a problem when determining the genotype. For this reason, several sets of primers are used, along with Southern blotting, to determine with certainty the genotype of the mice.

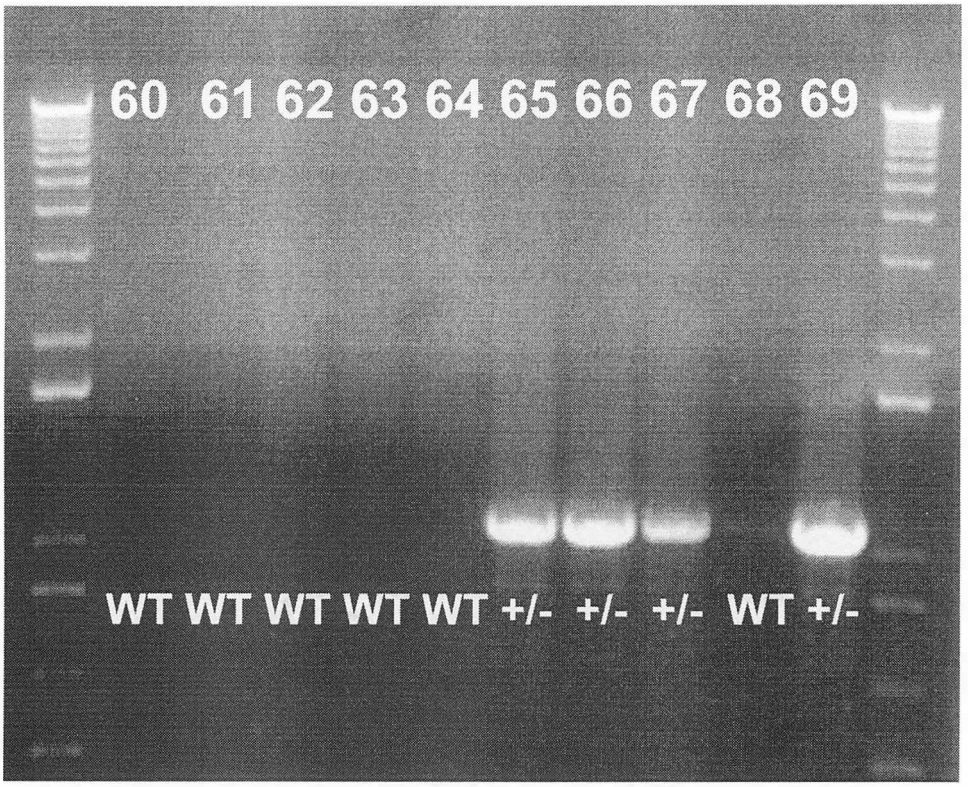


Figure 4. PCR genotyping of mice using gene trap primers. This is a gel from a PCR genotyping experiment using primers specific for the gene trap. PCR using these primers allows confirmation of the genotype found using the exon 8 and gene trap primers.

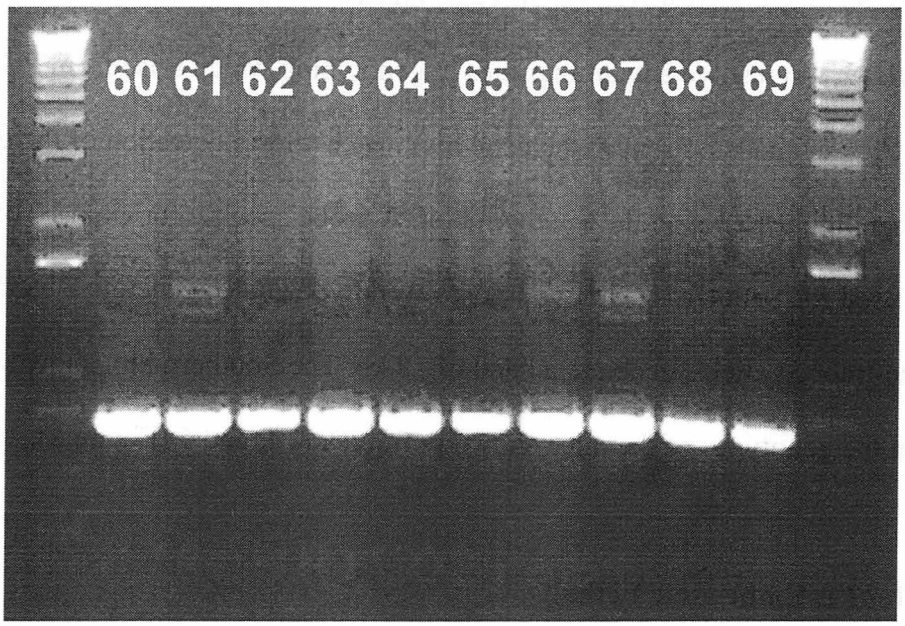


Figure 5. PCR genotyping of mice using exon 8 and intron 8 primers. The primers used for this PCR are specific for exon 8 and intron 8 of Bub1. A PCR product with these primers indicates the presence of at least 1 wildtype allele.

ambiguous, and must be resolved by different means. We have employed a Southern blotting strategy to resolve any ambiguities in genotyping which arise from PCR. An *EcoRI* restriction site is present in the gene trap which allows detection of the trap by different banding patterns on the Southern blot. A probe specific for exon 10 of the *Bub1* gene was used for Southern blotting purposes. Wildtype alleles generate a band of ~10 kb while gene-trapped alleles generate a band of ~8 kb. The Southern blot shown was used to confirm the genotypes of the mice shown in figures 3, 4, and 5 (Figure 6).

Loss of *Bub1* is Embryonic Lethal

In order to generate mutant mice nullizygous for *Bub1* (in a mixed c57BL/6 X SV129 background), we intercrossed the F1 heterozygous offspring obtained from our *Bub1* chimeric males. The data indicate *Bub1* nullizygosity leads to embryonic lethality in mice. Our intercrosses of F1 heterozygous mice produced a ratio of *Bub1*+/- and wildtype offspring of ~2:1. No *Bub1* -/- offspring were produced from our crosses, indicating loss of *Bub1* is embryonic lethal, and *Bub1* is essential for embryonic development. Eleven different intercrosses produced a total of 22 pups, and these pups were genotyped by PCR and Southern blotting as described before. The genotypes of the pups obtained from our intercrosses are presented (Table 1).

Timed matings with *Bub1*+/- mice were performed to determine the day of embryonic lethality in *Bub1* -/- offspring. Analysis of embryos obtained from four females sacrificed between 6.5 and 9.5 d.p.c. suggests lethality occurs ~6.5 d.p.c. These data are consistent with the data obtained from mice in which other spindle checkpoint genes, such as *Bub3* and *Mad2*, have been deleted. *Bub3* and *Mad2* nullizygosity

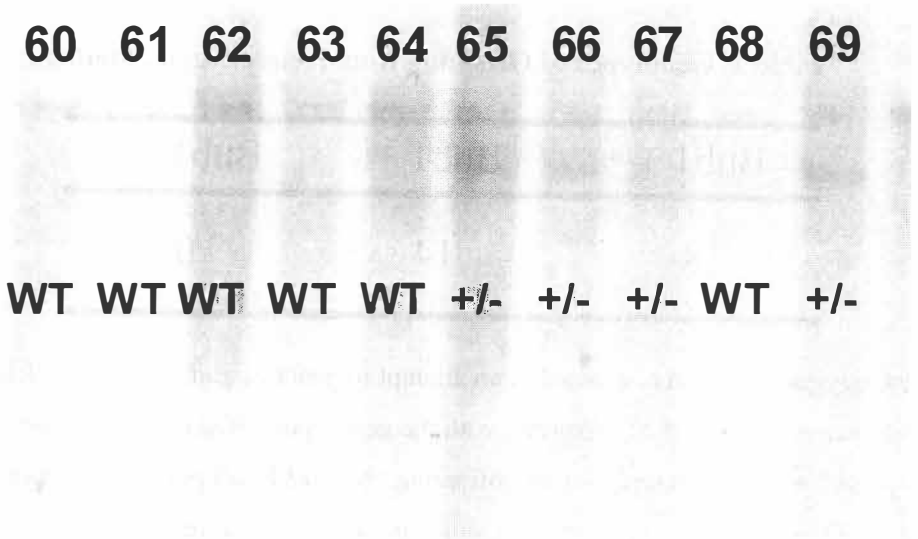


Figure 6. Southern blot for genotype confirmation. This is a Southern blot used to confirm the genotypes of the mice in figures 3, 4, and 5. The 10 kb band indicates the presence of a wildtype allele, whereas the 8 kb band is indicative of the gene trap. Samples 65, 66, 67, and 69 have 2 bands, indicating they are heterozygous.

Table 1. Genotypes of Offspring from Heterozygous Matings

Bub1 +/+	Bub1 +/-	Bub1 -/-
8	14	0

Bub 1 heterozygous mice were crossed in an attempt to generate *Bub1* null mice. Eleven different intercrosses yielded 22 offspring, with the genotypes noted above. A ratio of ~2:1 (*Bub1*+/- : *Bub1* +/+) was observed in our offspring. No *Bub1* null pups were obtained, indicating *Bub1* nullizygosity is embryonic lethal and *Bub1* is required for development.

also lead to embryonic lethality ~6.5 – 7.5 d.p.c. (Kalitsis, 2000 and Dobles, 2000).

Analysis of Bub1 Expression During Embryogenesis

The embryonic lethality of *Bub1* indicates *Bub1* is essential for normal development in mice. The expression pattern of Bub1 was examined in an attempt to understand the role of *Bub1* during embryogenesis. The gene trap contains a promoterless β-galactosidase – neomycin fusion gene which enabled us to analyze the expression of the trapped *Bub1* gene. Embryos (10.5 d.p.c.) were obtained from timed matings between *Bub1* heterozygous males and wildtype females and stained with X-gal to visualize Bub1 expression. Bub1 is ubiquitously expressed throughout the embryo at 10.5 d.p.c. (Figure 7.) The role of *Bub1* is to help ensure proper segregation of sister chromatids during cell division, thus ensuring each daughter cell receives the normal complement of chromosomes, so it follows that Bub1 would be ubiquitously expressed during mouse embryogenesis. Analysis of Bub1 expression patterns at other stages of development may also shed light on the role of *Bub1* in embryogenesis.

Bub1 Expression in Tissues

The ubiquitous expression of Bub1 in 10 d.p.c. embryos prompted us to investigate the expression of Bub1 in adult mice. Wildtype and *Bub1* heterozygous mice of the same age (littermates) were sacrificed and their organs were removed in order to obtain total protein lysates for western blotting. Our results indicate only minimal difference in Bub1 levels in tissues from heterozygous and wildtype mice (Figures 8 and 9). Expression of *Bub1* was markedly higher in the lungs compared to the other tissues

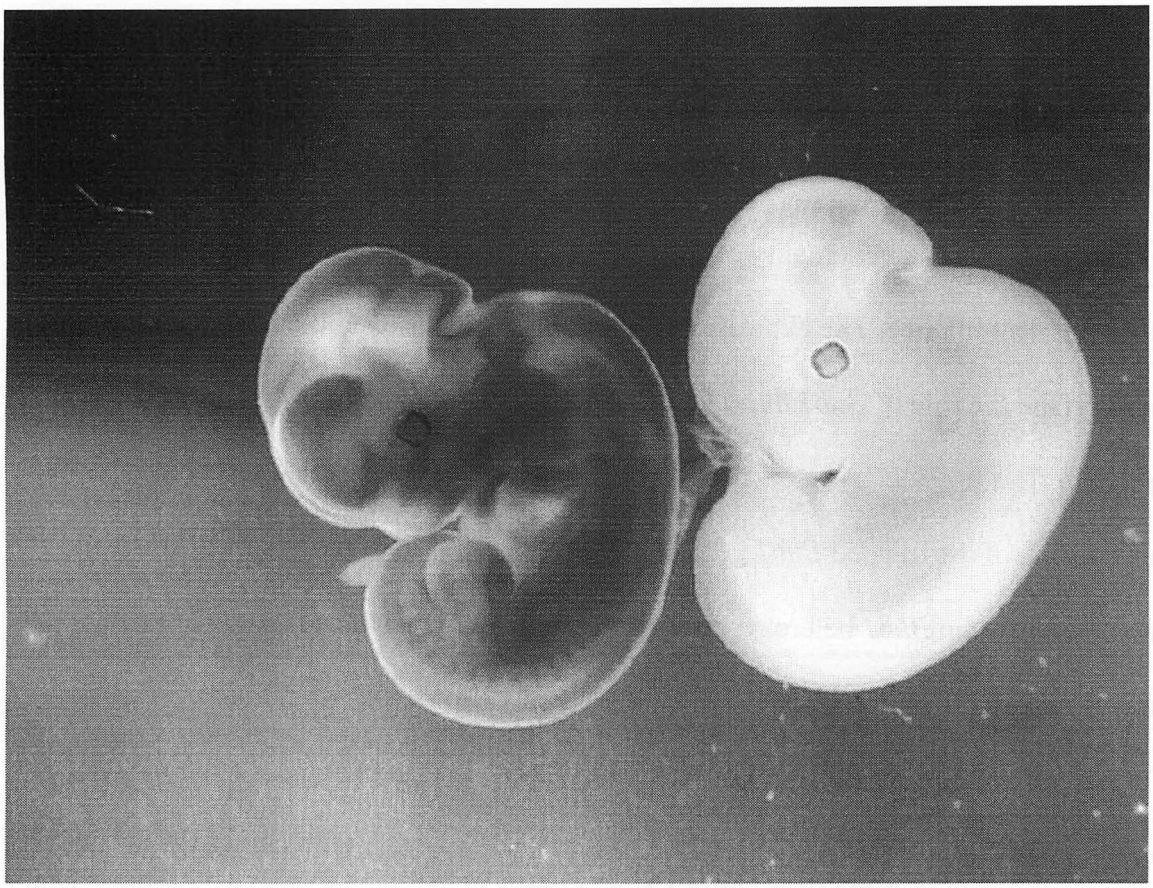


Figure 7. Bub1 is ubiquitously expressed in 10.5 d.p.c. embryos. The gene trap contains a promoterless β-galactosidase – neomycin fusion gene which enabled the visualization of Bub1 expression when the embryo was stained with X-gal.

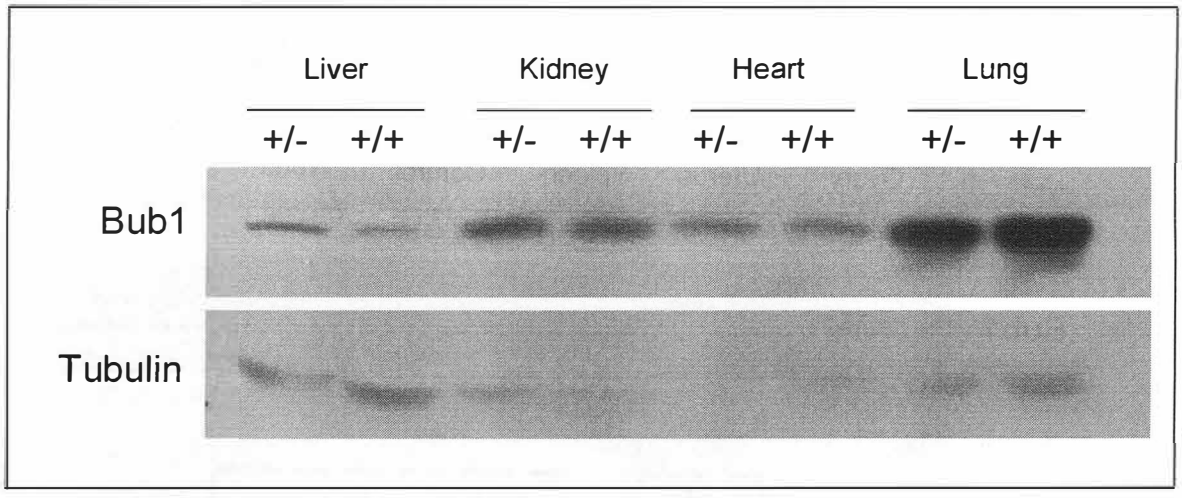


Figure 8. Bub1 expression in tissues. Total cell lysates from various tissues were used to examine Bub1 levels in wildtype and heyterozygous animals. Minimal difference in Bub1 levels is observed in the tissues of wildtype and heterozygous mice. Bub1 appears to be heavily expressed in the lungs.

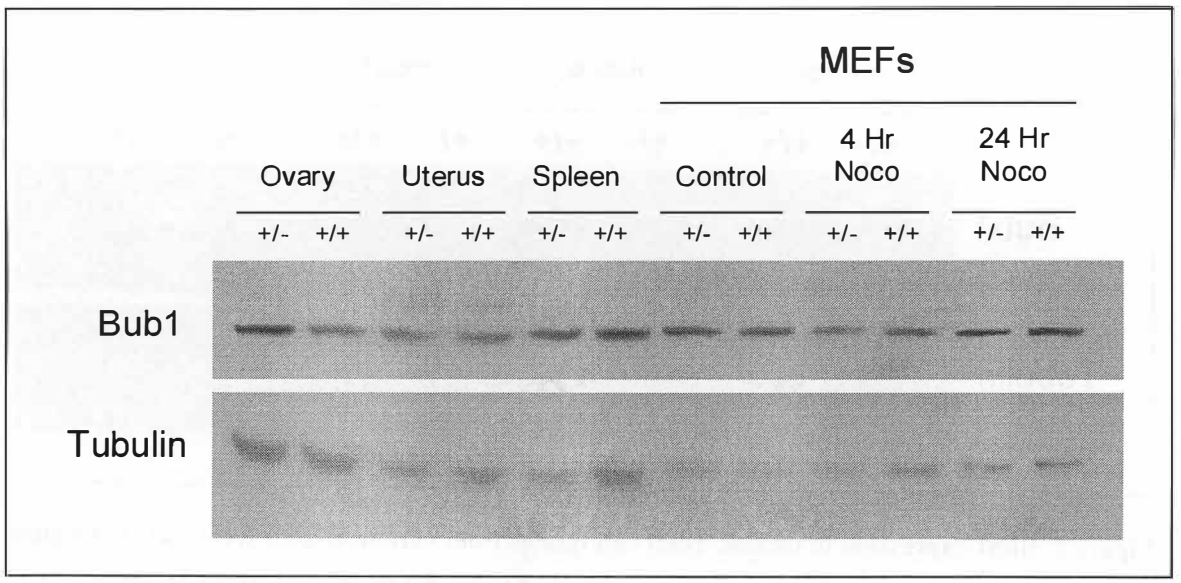


Figure 9. Bub1 expression in tissues and MEFs. Bub1 expression was examined in other tissues as well as MEFs. Again, minimal difference is observed in the tissues of wildtype and heterozygous mice as well as the wildtype and heterozygous MEFs. Interestingly, a second band is observed in the uterus of both wildtype and heterozygous mice.

examined (Figure 8). One interesting observation is a second band in the uterus (Figure 9). Whether this is a second isoform of Bub1 or another protein which cross reacts with our Bub1 antibody remains to be seen. We also looked at Bub1 expression in wildtype and heterozygous MEFs in the presence of spindle damage. Again, only a minimal difference is observed (Figure 9). Perhaps Bub1 is degraded after a threshold level is reached, since a large pool may not be necessary for checkpoint function. One copy of *Bub1* may be sufficient to maintain this threshold level or at least approach this level, resulting in minimal differences in expression between the wildtype and heterozygous tissues and MEFs. Checkpoint function may be partially defective if one copy of *Bub1* is not quite enough to maintain this threshold level, but enough Bub1 is present for a moderate checkpoint response to spindle damage.

Bub1 Heterozygous Females Have Reduced Reproductive Capacity

In order to generate *Bub1* null mice, crosses of *Bub1* heterozygous mice were performed. The litter sizes from these heterozygous intercrosses appeared abnormally low (~2 pups/litter), and the embryonic lethality of *Bub1* -/- embryos did not account for these decreased litter sizes. The low litter sizes pointed to the possibility of fertility defects in *Bub1* +/- mice, so intercrosses with *Bub1* +/- and wildtype mice were performed to determine if the fertility defects were specific to males or females. *Bub1* heterozygous males were crossed to wildtype females, *Bub1* heterozygous females were crossed to wildtype males, and wildtype mice were crossed to serve as controls. The average litter size obtained from wildtype crosses (control) was not significantly different from the average litter size obtained from crosses with *Bub1* heterozygous males and

wildtype females (7.5 ± 0.5 vs. 6.6 ± 0.6, Figure 10). Conversely, the average litter size from crosses of *Bub1* heterozygous females (with wildtype males and *Bub1* +/- males) was significantly lower than control crosses (1.75 ± 0.5 and 2.0 ± 0.4 respectively). These data suggest *Bub1* heterozygous females have a reduced reproductive capacity.

We next analyzed the embryos harvested from pregnant females between 6.5 and 9.5 d.p.c. in an effort to further study the reduced reproductive capacity. The *Bub1* heterozygous females yielded an average of ~6 embryos, which indicated the decreased litter sizes were most likely due to post-implantation defects as opposed to oogenesis defects. Interestingly, the analysis of embryos harvested at 8.5 and 9.5 d.p.c. showed the arrested development of embryos at different stages from 6.5 to 8.5 d.p.c. This suggests *Bub1* heterozygous females have difficulty supporting the normal development of embryos, regardless of the genotype of the embryo, since *Bub1* null embryos die ~6.5 d.p.c. Coordinated cell division cycles of the uterus and embryo are involved in embryo implantation, so it stands to reason that a partially defective spindle checkpoint would have deleterious effects on the ability of the uterus to support pregnancies. Such effects are seen in *Bub3* null embryos, which also die ~6.5 d.p.c., in the form of micronuclei, lagging chromosomes, and irregular nuclear morphology (Kalitsis, 2000). Similar effects are likely to occur in our *Bub1* null embryos.

Analysis of Cell Cycle Defects in *Bub1* Heterozygous Cells

Since *Bub1* is a spindle checkpoint gene, we hypothesized that losing one copy would have a detrimental effect on progression through the cell cycle. *Bub1* heterozygous males were bred to wildtype females, and the females were sacrificed 13.5 d.p.c. The

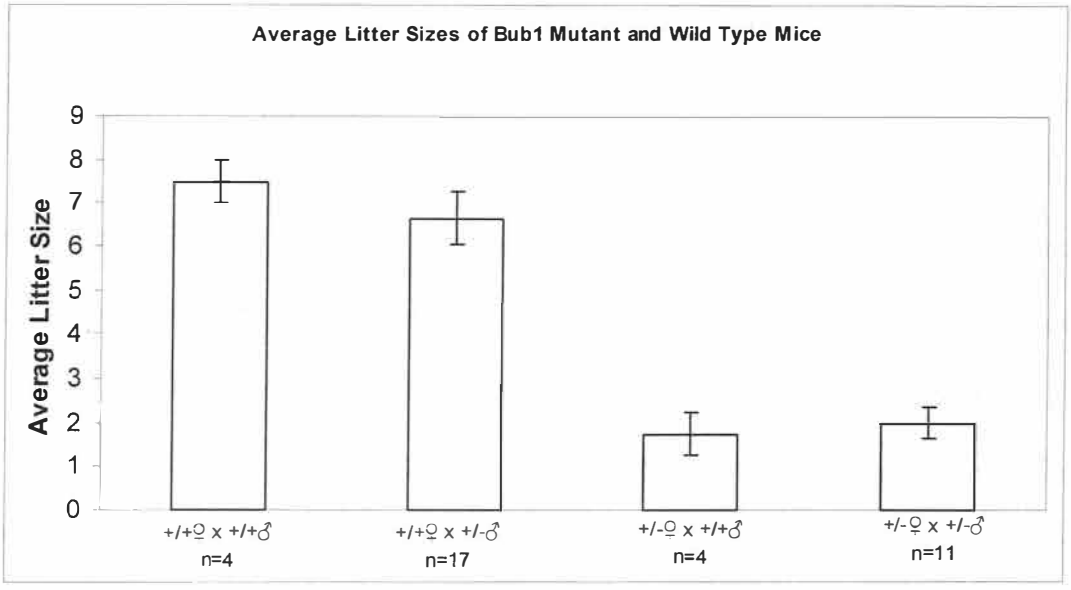


Figure 10. *Bub1* heterozygous females have a reduced reproductive capacity. Various intercrosses were performed to determine if the lower litter sizes from heterozygous matings were due to fertility defects specific to males or females. The average litter size from crosses of wiltype mice were not significantly different from the average litter size obtained from crosses between *Bub1* heterozygous males and wildtype females. The average litter size from crosses of *Bub1* heterozygous females (crossed to wildtype and *Bub1* heterozygous males) were significantly lower compared to wildtype controls. Bars indicate standard error and n represents the total number of crosses performed.

embryos were removed and placed in culture to generate *Bub1* +/+ and *Bub1* +/- mouse embryonic fibroblasts (MEFs). In order to study the effects of losing one copy of *Bub1*, asynchronous cells (from passage 3) were treated with the spindle damaging agent nocodazole (100 ng/mL or 50 ng/mL) for 24 hours, fixed in ice cold 70% ethanol, and stained with propidium iodide for flow cytometry. The data indicate *Bub1* +/- cells are partially defective in their response to spindle damage. The results from 2 different studies performed on 2 different cell lines are shown (Figures 11 and 12). Figure 11 depicts one experiment performed with nocodazole (100 ng/mL) while figure 12 depicts another study performed using a lower nocodazole dose (50 ng/mL). Both experiments yielded similar results. The difference at 24 hours in the nocodazole treated cells in Figure 11 (50% of wildtype cells were arrested in G2/M compared to 42% of *Bub1* +/- cells) indicates more *Bub1* +/- cells are exiting mitosis and continuing through the cell cycle even in the presence of spindle damage. A high rate of apoptosis was observed at this nocodazole dose, so a lower dose was used (Figure 12). Similar results were achieved with the lower dose of nocodazole. The majority of wildtype and heterozygous cells accumulate at G2/M, but a higher percentage of wildtype cells than heterozygous cells are observed at G2/M (43.1% vs. 30.8%). Also, a lower percentage of wildtype cells are seen in G1 compared to *Bub1* +/- cells (13.6% vs. 16.6%), which seems to indicate *Bub1* +/- cells, at least to some extent, are able to escape the spindle checkpoint and progress into G1. We also noticed low levels of polyploid cells (>4N DNA) in the nocodazole-treated cells, with a lower amount in the *Bub1* heterozygous cells than in wildtype (4.4% vs. 7.8%). One other interesting observation was a higher percentage of *Bub1* +/- apoptotic cells compared to wildtype (6.3% vs. 2.3%). Perhaps *Bub1* is involved in

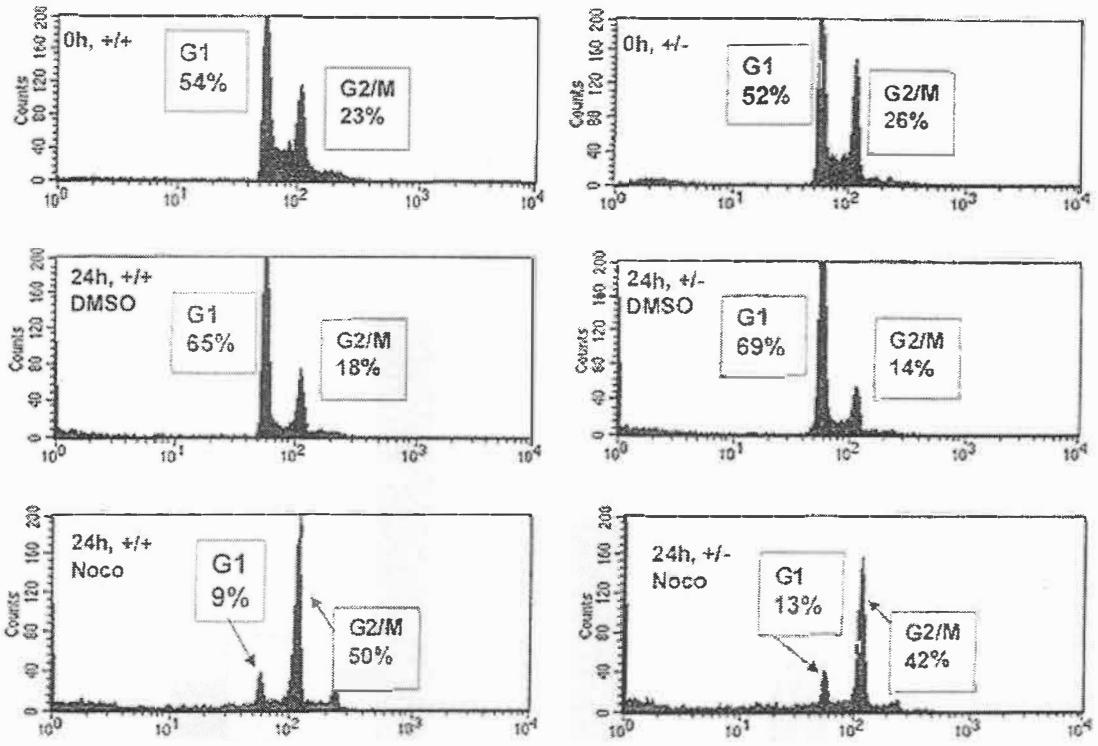


Figure 11. Cell cycle analysis using flow cytometry. *Bub1* heterozygous cells are partially defective in their spindle checkpoint response to nocodazole. Asynchronous cells were treated with 100 ng/mL of nocodazole or DMSO (0.1%) for 24 hours and fixed in 70% ethanol. The cells were stained with propidium iodide and analyzed using a Coulter EPICS flow cytometer.

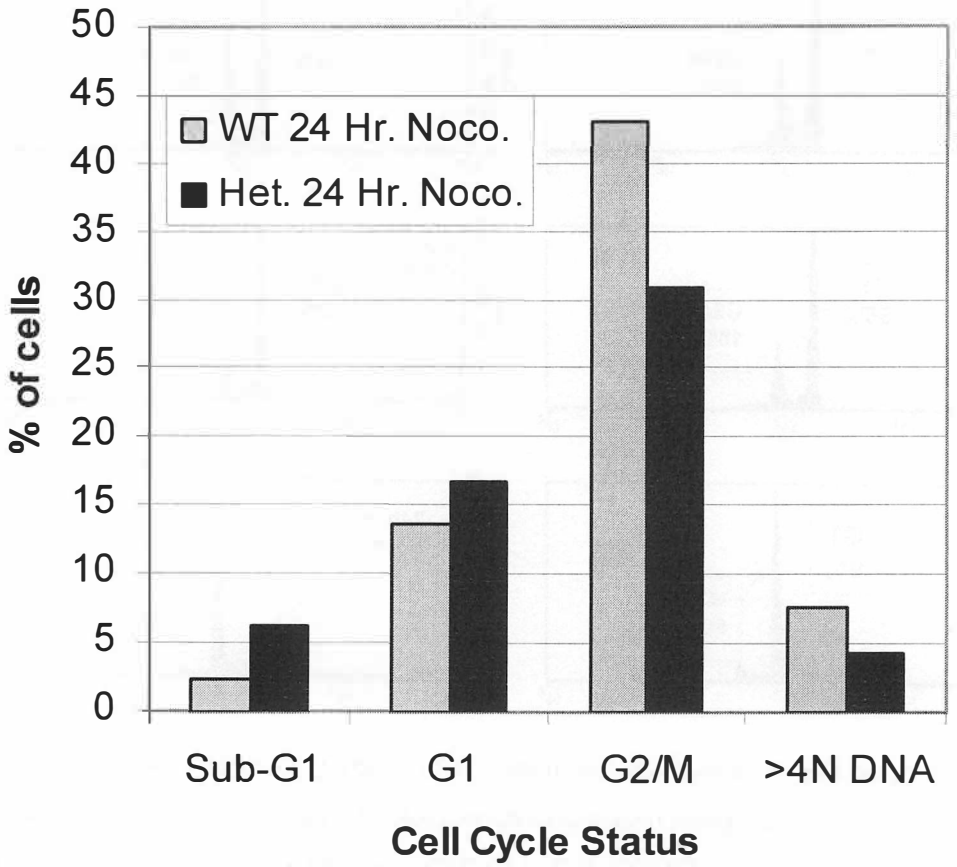


Figure 12. Additional cell cycle analysis using flow cytometry. A lower dose of nocodazole was used as well as a different cell line in this experiment to observe cell cycle defects in *Bub1* +/- cells in the presence of spindle damage. A lower percentage of *Bub1* +/- cells arrest in G2/M than wildtype cells in the presence of spindle damage, indicating more heterozygous cells exit mitosis and continue through the cell cycle.

regulating an apoptotic pathway, hence the difference in apoptosis between wildtype and heterozygous cells.

Bub1: Cell Growth and Transformation

The lack of a functional spindle checkpoint could lead to chromosome missegregation and aneuploidy, which, in turn, could predispose a cell to transformation. Since *Bub1* is a spindle checkpoint gene, we wanted to investigate *Bub1* haploinsufficiency and cell growth kinetics. Cell growth curves were initiated for this purpose. Additionally, we wanted to investigate whether *Bub1* haploinsufficiency alone could lead to transformation, so colony formation assays were undertaken. Wildtype and *Bub1* heterozygous MEFs showed no significant difference in growth over the 5 day period of the growth curve (Figure 13). The colony formation assays also showed no difference between wildtype and heterozygous cells. These data suggest losing one copy of *Bub1* is not sufficient for transformation of cells or increased cell growth. Also, our *Bub1* heterozygous mice have not shown an increased incidence of tumors compared to wildtype mice, even at 1½ years of age. Additional insults, in conjunction with loss of one copy of *Bub1*, may be necessary for transformation of cells.

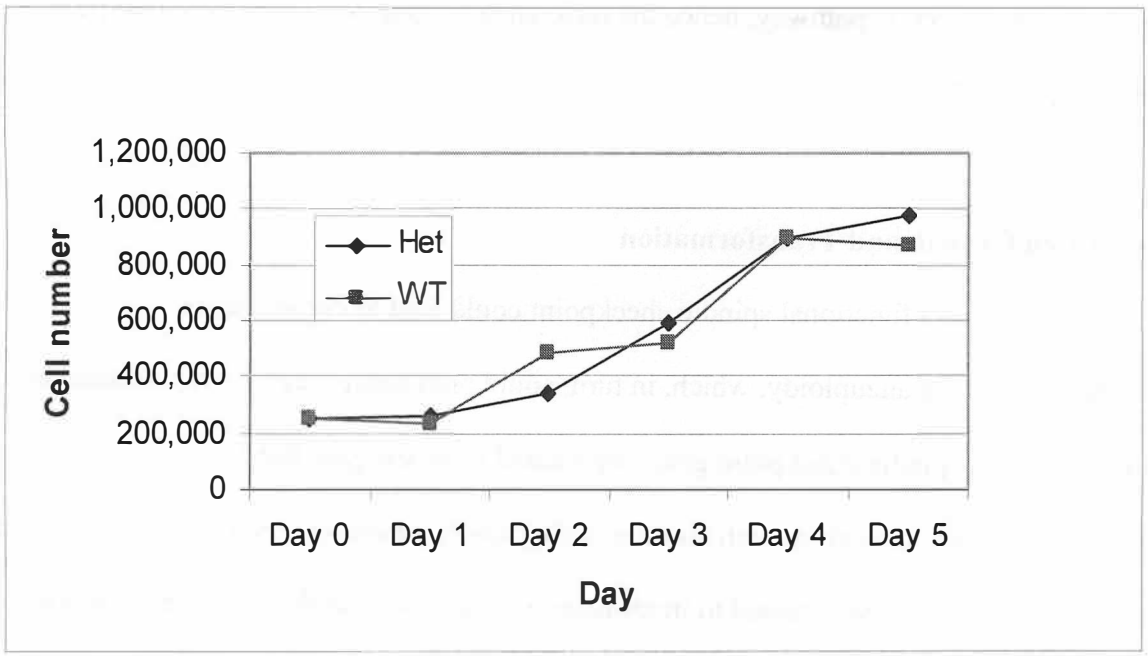


Figure 13. Growth curve for wildtype and *Bub1* heterozygous MEFs over 5 days. Both genotypes began with 250,000 cells. No significant difference is seen between wildtype and heterozygous MEFs over 5 days, suggesting onen copy of *Bub1* is sufficient for checkpoint function.

3. Conclusions

The spindle checkpoint helps to ensure that daughter cells receive the normal complement of chromosomes after each cell division, thus preventing aneuploidy and predisposition to transformation. Such a function is vital to the survival of an organism, and the components of the spindle checkpoint each play an important role in maintaining proper checkpoint function. *Bub1* is an integral constituent of this checkpoint, but its role is still not well understood. To our knowledge, we are the first group to knock out *Bub1* in mice in order to study the effect of *Bub1* loss in a mammalian system. Our chimeric founder males were mated to wildtype females in order to generate *Bub1* heterozygous mice. The heterozygous mice had no recognizable outward developmental defects.

Upon establishment of a reliable and accurate method of genotyping our mice via PCR and Southern blotting, we began mating the *Bub1* heterozygous offspring (F1 generation) from our chimeric founders in an attempt to generate *Bub1* null mice. Our data indicate the loss of *Bub1* is embryonic lethal. Our heterozygous intercrosses yielded 22 offspring, with 8 wildtype and 14 heterozygous pups. No *Bub1* null pups were generated from our 11 crosses, suggesting *Bub1* nullizygosity is embryonic lethal.

Timed matings were initiated in order to determine the day of lethality. Analysis of embryos obtained from females sacrificed between ~6.5 – 9.5 d.p.c. indicate lethality occurs ~6.5 d.p.c. This is consistent with other spindle checkpoint gene knockouts. Loss of *Bub3* and *Mad2* results in embryonic lethality ~6.5 – 7.5 d.p.c. due to mitotic errors such as micronuclei and lagging chromosomes in the case of *Bub3* and chromosome missegregation in the case of *Mad2* (Kalitsis, 2000 and Dobles, 2000). *Bub1* null embryos probably die ~6.5 d.p.c. due to similar mitotic errors.

The lethality of *Bub1* indicates it is an essential gene for embryo development. In order to understand the role of *Bub1* during embryogenesis, the expression of *Bub1* was examined. The presence of a β-galactosidase-neomycin cassette in the gene trap allows for examination of expression of gene-trapped cells by staining with X-gal. Embryos obtained from 10.5 d.p.c. females demonstrate ubiquitous expression of *Bub1*. Since *Bub1* is involved in maintaining proper chromosome number, it stands to reason it would be found throughout the embryo. *Bub1* could also have an unknown role in another pathway which could account for its ubiquitous expression.

We also examined Bub1 expression in various tissues of adult mice as well as MEFs. Only minimal difference is observed in the tissues of wildtype and *Bub1* heterozygous mice. The expression of Bub1 is markedly higher in the lungs compared to other tissues examined. At this point, it is unclear why this is the case. Perhaps Bub1 has an unknown function in a pathway crucial for lung function. One interesting observation on our western blot is the presence of a second band in the uterus of both wildtype and heterozygous mice. We have found no evidence for a second isoform of Bub1 in the literature, so it is unclear what this second band represents. The expression of Bub1 in the MEFs was similar to the tissues, with minimal difference observed between the wildtype and heterozygous MEFs in the presence of spindle damage. Perhaps a threshold level of Bub1 is necessary, and when the level of Bub1 exceeds this threshold, it is degraded. If one copy of Bub1 is sufficient for this threshold level, similar expression patterns in wildtype and heterozygous mice could be observed. A large pool of Bub1 does not seem to be required for checkpoint function, so one copy of *Bub1* may be sufficient to maintain this threshold level, or at least approach this level. If one copy of *Bub1* is not quite

enough to maintain this level, the checkpoint may be slightly defective, but the difference may not be apparent in our western blots.

While performing our crosses to generate *Bub1* null mice, we noticed abnormally small litter sizes, with our heterozygous crosses yielding ~2 pups/litter, compared to ~8 pups/litter for wildtype matings. The embryonic lethality did not account for the small litter sizes. One-fourth of the offspring should be null according to Mendelian inheritance, therefore 2 pups should be lost due to nullizygosity, yielding litter sizes of ~6 pups/litter. Our litter sizes were considerably lower, which led us to believe our heterozygous mice may have fertility defects. Intercrosses were initiated with wildtype and *Bub1* heterozygous mice to determine if the fertility defects were specific to male or female mice. Significantly lower litter sizes were obtained when the female was heterozygous, regardless of the genotype of the male, indicating *Bub1* heterozygous females have a reduced reproductive capacity. Our analysis of embryos obtained from heterozygous females between 6.5 and 9.5 d.p.c. showed arrested development of embryos ranging from 6.5 – 8.5 d.p.c., indicating *Bub1* heterozygous females have difficulty supporting normal embryo development, regardless of the genotype. The embryos arrested at 6.5 d.p.c were most likely null, but the embryos arrested at 8.5 d.p.c probably were not, so heterozygous or wildtype embryos were arresting in their development. Embryo implantation and development requires a complex series of cell divisions, coordinated between the developing embryo and the uterus. Even a partially defective checkpoint could have serious negative consequences, most likely in the form of chromosome missegregation. Similar effects are observed in *Bub3* null embryos, which die ~6.5 d.p.c. These embryos display irregular nuclear morphology and lagging

chromosomes (Kalitsis, 2000). If the checkpoint is not functioning correctly and the cell cycle is not arrested to enable the lagging chromosomes to orient properly along the metaphase plate, the daughter cells may not receive the normal complement of chromosomes, possibly eliciting an apoptotic response. Our data, along with data from other groups, shows loss of *Bub1* can lead to increased apoptosis, and the increased apoptosis could lead to the arrested development of the embryos at various stages of development.

Our analysis of the cell cycle using wildtype and *Bub1* heterozygous MEFs revealed a slightly defective spindle checkpoint when spindle damage is present. A higher percentage of wildtype cells arrested in metaphase after 24 hours in nocodazole than heterozygous cells (50% vs 42% in experiment 1 (Figure 11), 43.1% vs. 30.8% in experiment 2 (Figure 12)). The wildtype cells also had a lower amount of cells in G1 compared to the *Bub1* heterozygous cells (9% vs 13% in experiment 1 (Figure 11), and 13.6% vs 16.6% in experiment 2 (Figure 12)). These numbers indicate the heterozygous cells, to a limited extent, were able to elude the spindle checkpoint, exit mitosis, and continue to cycle even in the presence of spindle damage. Low levels of polyploid cells were observed in both wildtype and heterozygous cells (7.8% in wildtype vs. 4.4% in *Bub1* +/- , Figure 12). The lower percentage of polyploid cells in the *Bub1* heterozygous line may indicate a role for *Bub1* in regulating mitotic exit or cytokinesis, events which occur later in the cell cycle. Lower levels of Bub1 may delay mitotic exit or cytokinesis, thus resulting in the lower level of polyploid cells observed in the heterozygous cell line. Our analysis also revealed that the *Bub1* heterozygous cell line had a higher percentage of apoptotic cells than wildtype (6.3% vs. 2.3%, Figure 12). Our results are in agreement

with earlier work performed in *Drosophila*. The nuclei in larval brains of *Drosophila* mutants which do not express *Bub1* also showed elevated levels of apoptosis (Basu, 1999). Their examination of brains from mutant flies revealed many chromosomes which were extremely fragmented. Basu et al. note that chromosome breakage in *Drosophila* is normally cell lethal and prevents entry into the following round of mitosis (Basu, 1999). Our data, along with the data from Basu et al, suggest *Bub1* has a role in regulating the apoptotic pathway. Perhaps Bub1 phosphorylates and activates a repressor of apoptosis, and when Bub1 levels fall below a threshold, repression of apoptosis is lost, resulting in increased apoptosis. Interestingly, embryos which lack *Mad2* also exhibit increased apoptosis (Dobles, 2000). It could be that lack of a functional checkpoint could result in increased apoptosis, or the increase in apoptosis could be specifically due to the lack of either *Bub1* or *Mad2*. More work is needed to determine if *Bub1* is involved in regulating apoptosis.

Although the cell cycle differences between wildtype and heterozygous cell lines may not be striking, one copy of *Bub1* does not appear to be completely sufficient for checkpoint activation. *Mad2* heterozygous mice developed lung tumors after a long latency period (Michel, 2001), indicating the loss of one copy of this spindle checkpoint gene is capable of promoting tumor formation. Tumorigenesis is a multiple step process which results from the additive effects of the heterozygous state of genes involved in the cell cycle.

The data obtained from tissue culture experiments suggest loss of one copy of *Bub1* is not sufficient for transformation. No difference was observed in growth curves and colony formation assays with wildtype and *Bub1* heterozygous MEFs. Our *Bub1*

heterozygous mice do not have an increased incidence of tumors compared to wildtype mice, even at 1½ years of age. Our data suggest that while one copy of *Bub1* may not be sufficient for complete checkpoint activation, *Bub1* heterozygosity does not appear to drive the tumorigenesis process. Additional insults may be required, with the net result of these effects leading to tumorigenesis.

Bub1 does not cycle through the kinetochore quickly (Bub1 has a half-life of ~57 seconds at the kinetochore, (Howell, 2004)), unlike some other checkpoint proteins such Mad2 and Cdc20 (with half-lives of ~19 seconds (Howell, 2004) and ~5.1 seconds (Kallio, 2002), respectively). These two proteins are components of the mitotic checkpoint complex (MCC) involved in inactivation of the APC, therefore it would stand to reason that Mad2 and Cdc20 would cycle quickly through the kinetochore so a sufficient amount of the MCC could be produced for APC inactivation. Bub1, on the other hand, seems to fulfill more of a scaffolding role, as it is necessary for the kinetochore localization of other checkpoint proteins. A lower amount of Bub1 may be needed for checkpoint function since it is more stably bound to the kinetochore. A smaller pool of Bub1 could be sufficient, since the turnover at the kinetochore is lower than other checkpoint proteins. Also, most Bub1 resides in the cytoplasm, with approximately 0.4% of Bub1 localized to the kinetochore at prometaphase (Howell, 2004). One copy of *Bub1* may be sufficient for some checkpoint function, but there are some slight checkpoint defects when only one copy is present, as seen in our flow cytometry data. These slight defects, coupled with other insults, such as loss of p53 or BRCA2, could, over time, lead to tumorigenesis. At this point, however, it appears the loss of one copy of *Bub1* is not sufficient to promote tumorigenesis.

The role of *Bub1* in cancer still is not clear. *Bub1* mutations have been observed in some colorectal cancers, as well as lung and breast cancers, but the percentage of these cancers with *Bub1* mutations is small. More work is needed to further elucidate *Bub1*'s role in these pathologies.

4. Materials and Methods

DNA Extraction

A small piece of mouse tail was excised and DNA was extracted using standard techniques. The tail was digested overnight at 55° - 60° C in tail lysis buffer (50 mM Tris pH 7.5, 50 mM EDTA, 100 mM NaCl, 1% SDS), 5 mM DTT, and proteinase K (0.159 μg/μl). The next day phenol:chloroform (pH 8.0) was added to the tail lysate and thoroughly mixed, and centrifuged at 13,000 rpm for 2 minutes. The top aqueous layer was removed and placed in a new microcentrifuge tube, and 100% ethanol was added to precipitate the DNA. The DNA was resuspended in 5 mM Tris (pH 6.8).

Genotyping of Mice

A nested PCR strategy was used to genotype the tail DNA of mice. One set of primers included a forward exon 8 primer and a reverse primer from the gene trap. These primers produced an ~800 bp PCR product. Another set of primers included the forward exon 8 primer and a reverse intronic primer. These primers also produced an ~800 bp PCR product, which necessitated separate PCR reactions for these two sets of primers. A third set of primers consisted entirely of gene trap primers and produced a ~1100 bp PCR product. The PCR reaction mixture consisted of 10X PCR buffer (2.5 μl/sample), 50 mM $MgCl_2$ (1.5 μl/sample), dNTP (1.0 μl/sample), forward and reverse primers (1.0 μl/sample), Platinum Taq (0.25 μl/sample), and ddH_20 to a final reaction volume of 25 μl. The PCR conditions were as follows: an initial denaturation step of 5 minutes at 94°C, followed by another denaturation step of 30 seconds, annealing for 30 seconds at 57°C, and extension for 45 seconds at 72°C. This was repeated for a total of 34 cycles. A final

cycle of 72°C for 2 minutes was also included. The PCR products were run on a 1.5% agarose gel at 85 V for approximately 1.5 – 2 hours. Ethidium bromide was used to visualize the bands, and the gel was photographed on a Kodak apparatus. The presence of a band with the exon 8 and gene trap primers and the exon 8 and intron 8 primers indicates one gene trapped allele (heterozygous). The presence of a band with only the exon 8 and intron 8 primers indicates wildtype alleles are present. The gene trap primers were used to confirm the findings of the exon 8 and gene trap primers. All genotypes were confirmed by Southern blotting using an exon 10 probe.

Generation of Murine Embryonic Fibroblasts (MEFs)

A female mouse 13.5 – 14.5 dpc was sacrificed and the embryos removed. The embryos were then removed from the yolk sac and placed in a 1 cc syringe with an 18 gauge needle. The embryos were then passed repeatedly through the needle into 2 ml of DMEM with 15%FBS in order to break up the embryo. One ml of this mixture was then placed into a tissue culture plate (Nunc 100 mm) with 7 ml of DMEM containing 15% FBS. The MEFs were incubated at 37°C (5% CO_2) until approximately 90% confluent and then passaged. The 2 original plates were passaged into 6 plates, and when these plates were 90% confluent, 5 plates were stored overnight at -80°C, and transferred the next day into liquid nitrogen for storage. One plate was passaged for further experimentation.

Generation of Growth Curves

In order to asses the growth of MEFs, 250,000 cells were placed in 60 mm tissue culture plates with 4 ml of DMEM (with 15% FBS) and incubated at 37°C (5% CO_2). Counting was started the next day and continued until day 5. In order to count the cells, plates were trypsinized (1 ml of trypsin) for 5 minutes. After 5 minutes, 1 ml of DMEM (15% FBS) was added to the trypsin, and the trypsin/DMEM was pipetted several times to dislodge cells, and the cells were placed in a 15 ml Falcon tube. 50 µl were removed and placed on a hemocytometer or diluted when necessary and then placed on a hemocytometer. Ten counts were recorded and plotted on Excel.

Colony Formation Assay

One assay for transformation is the colony formation assay. 3,000 cells were placed in a 60 mm tissue culture plate in 4 ml of DMEM (with 15% FBS) and incubated approximately 10 - 16 days at 37°C (5% CO_2). Media was replaced every 3 days. After the appropriate amount of time had elapsed, the media was removed and the cells were fixed in 95% ethanol for 10 minutes. After removal of the ethanol, the cells were stained with 0.4% crystal violet for 10 minutes, the stain was removed, and the cells were allowed to air dry. Colonies with more than 50 cells were counted. This procedure was done in triplicate, and the results are the averages of the 3 experiments.

Analysis of the Cell Cycle Using Flow Cytometry

0.4×10^6 cells were plated on 60 mm tissue culture plates and nocodazole (25 ng/ml) was added. Samples were taken at various time points (0, 24, 48, and 72 hours)

using the following procedure: the media was removed and the cells were washed once with PBS. Trypsin (1 ml) was added and the plate was incubated at 37°C (in 5% CO_2) for 5 minutes. 1ml PBS was added to the trypsin after incubation, and the trypsin/PBS mixture was pipetted several times over the plate to ensure the removal of all cells. The cells were placed in a 15 ml tube and centrifuged for 5 minutes at 800 rpm. The supernatant was discarded and the pellet was broken apart, and 4 ml of 70% ice cold ethanol was added to fix the cells, which were then stored at -20°C. For flow cytometry, the cells were centrifuged for 5 minutes at 1500 rpm, the supernatant was discarded, and the pellet was resuspended in 400 µl of PBS (with 1mM EDTA). 50 µl of RNase (1 mg/ml) and 50 µl of propidium iodide (400 µg/ml) were added and the cells were incubated at 37°C for 30 minutes. Flow cytometry was performed on a Coulter FACScan flow cytometer. 10,000 cells were analyzed for each treatment.

Western Blots

Total lysates were obtained (by NP-40 lysis) from tissues of sacrificed mice and used for Bub1 immunoblots. The protein concentration of samples was determined using the BCA assay and equal amounts of protein were loaded in each well. Samples were run on 8% SDS-PAGE gels. Samples were prepared as follows: an appropriate amount of 2X loading dye (900 µl 2X dye, 100 µl 2M DTT) was added to the sample, along with NP-40 lysis buffer (150 mM NaCl, 0.75% NP-40, 50 mM Tris HCL pH 7.5). Samples were then boiled for 3 minutes prior to loading on the gel. TriChrome Ranger marker was used for standards. The gel was run at 100V for ~1 hour, then run at 180V for ~2½ - 3 hours, then transferred (transfer buffer – 72 g glycine, 15 g Tris, 1 L methanol, and distilled water to

a final volume of 5 L) onto an Immobilon membrane for 3 hours at 60V. The membrane was blocked for 3 hours in 4% blotto (4% dried milk in 0.05X TBS-Tween), incubated overnight in primary antibody (murine α-Bub 1, 1:500 dilution, murine α-tubulin 1:2000), washed for 5 minutes in 0.05X TBS-Tween, 10 minutes in 2% blotto (2% dried milk), then 5 minutes in 0.05X TBS-Tween. After washing, the membrane was incubated in secondary antibody (rabbit α-mouse, 1:10,000 dilution) for 1 hour, and washed as before. The protein bands were visualized via chemiluminescent reaction.

Southern Blots

Southern blotting was used to confirm PCR genotypes. Extracted DNA was digested with EcoRI overnight at 37°C. The digest was run on a 1.5% agarose gel along with 1 kb+ DNA ladder (Invitrogen). The gel was photographed for reference purposes, and soaked in 0.25 N HCl for 5 minutes, then washed 4 times for 10 minutes each in distilled water. The DNA was transferred onto a nitrocellulose membrane overnight in 0.4 N NaOH. After transfer was complete, the membrane was soaked in 2X SSC for 30 seconds and incubated at 37°C for 1 hour, then incubated in pre-hybridization buffer (4 ml 10% SDS, 3 ml 20X SSPE, 1 ml (2 mg/ml) salmon sperm DNA boiled for 10 minutes, 0.3 g milk powder, and 21 ml distilled water) for 4 – 24 hours at 68°C. After incubation in pre-hybridization buffer, the membrane was incubated overnight in hybridization buffer (16 ml of 12.5% dextran sulfate, 1.5 ml 20X SSPE, 2 ml 10% SDS, 0.1 g milk powder, and 500 µl distilled water) containing radioactive probe. The probe used for blotting consisted of a sequence from exon 10 of Bub1 and was prepared with ^{32}P. After incubation with the hybridization buffer was complete, the membrane was

rinsed briefly in 2X SSC, then rinsed in solution 1 (100 ml 20X SSC, 10 ml 10% SDS, distilled water to a final volume of 1 L) for 10 minutes, then rinsed in solution 2 (25 ml 20X SSC, 10 ml 10% SDS, distilled water to a final volume of 1 L) and the radioactivity of the membrane was assayed. The membrane was then placed under X-ray film in an X-ray cassette and allowed to develop for 24 – 72 hours at -80°C.

Embryo Histology and Staining

Female mice at various stages of pregnancy were sacrificed and the embryos were removed. The embryos were rinsed in PBS and placed in 4% paraformaldehyde for 15 – 20 minutes. The embryos were then rinsed 3 times in PBS, placed in a 15 mL Falcon tube with 1 mL of X-gal stain and 25 μL of X-gal, and incubated in a 35°C water bath for 4 – 48 hours. The tube was wrapped in aluminum foil since X-gal is light sensitive. After incubation, the embryo was removed from the stain, rinsed in PBS, and photographed.

List of References

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Vita

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