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Functional Analysis of Bub 1

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Appendix E - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL


Name: Amanda C Geetry

College: Arts & Sciences Department: BCMB

Faculty Mentor: Dr. Swadesan Venkatchalan

PROJECT TITLE: Functional Analysis of Bub1

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed:  Faculty Mentor

Date: 5/3/2004

General Assessment - please provide a short paragraph that highlights the most significant features of the project.

Comments (Optional):

Functional Analysis of Bub1

Senior Honors Project

Amanda Gentry

May 3, 2004

Functional Analysis of Bub1

The ability of an organism to live and grow is dependent upon cellular division. A cell contains all of the genetic information necessary for cellular function and propagation. To maintain homeostasis, it is essential for an organism to accurately transmit its genetic material during every cell division. The cell division also referred as the “cell cycle” consists of a sequence of distinct events that culminates in cellular division. The cell cycle consists of regulatory steps known as “checkpoints” that ensure the completion of earlier events before the initiation of later events in the cell cycle. One such regulation is the spindle checkpoint. The spindle checkpoint consists of a cascade of protein interactions, including Bub1, that serve to ensure the proper and equal distribution of chromatids, or genetic information, during the process of cell division. Inactivation of cell cycle regulatory proteins via genetic mutations or deletions usually leads to aberrant regulation of the cell cycle. Deregulated cell cycle can in turn lead to uncontrolled cell division cycles that result in pathological conditions like cancer.

The Cell Cycle

An organism’s genetic information is encoded by DNA found on its chromosomes. Correct cellular division requires that each chromosome exactly replicate and segregate its genetic information in order that the two resulting daughter cells might be genetically identical. This process is regulated by the cell cycle.

The cell cycle consists of four phases, G1 (gap 1), S phase (synthetic), G2 (gap 2), and mitosis. A cell enters G1 phase immediately following the end of the previous cell cycle, and can spend anywhere from a few hours to a few years at this stage. G1 phase is

followed by S phase during which DNA replication occurs. The S phase refers to the synthesis of each DNA molecule, or chromosome, resulting in sister chromatids that contain identical genetic information. The sister chromatids are physically linked at a structure known as the centromere. It is at the centromere that a proteinaceous complex called the kinetochore forms. The kinetochore serves as the attachment point for the microtubules that govern chromosome movement during mitosis. However, before mitosis occurs, the cell proceeds through G2 phase, by the end of which the cell's mass has doubled in size.

Mitosis is the process of actual cell division. If all goes well, mitosis results in two daughter cells genetically identical to the parent cell. Mitosis is an elaborate process consisting of five distinct but continuous stages: prophase, prometaphase, metaphase, anaphase, and telophase. Prophase, the initial stage of mitosis, is marked by chromosome condensation, the beginning formation of the mitotic spindle, and the formation of centrosomes, or microtubule organizing centers that are positioned at opposite poles of the cell. The breaking of the nuclear membrane signals the beginning of prometaphase. In this stage, chromosomes attach at their kinetochores to microtubules of the mitotic spindle and begin to migrate to a point midway between the two spindle poles. In metaphase, the chromosomes are aligned at the equatorial plane of the cell. The sister chromatids are attached at their kinetochores to microtubules emanating from opposite poles. It is at this stage that the spindle checkpoint comes into play.

If the chromosomes are properly aligned and attached to opposing microtubules, as regulated by the spindle checkpoint, mitosis proceeds into anaphase. During anaphase, the sister chromatids separate at their centromere, becoming independent daughter

chromosomes. These daughter chromosomes move to opposite poles of the cell. The final stage of mitosis, telophase, is marked by the reformation of the nuclear membrane. Cell division is completed by the cleaving of cytoplasm, or cytokinesis, resulting in two complete, identical daughter cells.

The Spindle Checkpoint

The spindle checkpoint ensures the fidelity of chromosome segregation in mitosis. The correct segregation of chromosomes is of utmost importance to the life of both the cell and the organism. Incorrect segregation of chromosomes can lead to aneuploidy and genomic instability, both of which are hallmarks of cancer.

The spindle checkpoint ensures the correct segregation of sister chromatids during mitosis by monitoring and ascertaining the correct attachment of chromosomes, at their kinetochores, to microtubules during metaphase. In the event of an unattached kinetochore, or lack of tension across a kinetochore, the spindle checkpoint is activated and arrests the cell cycle during the metaphase to anaphase transition.

The presence of a kinetochore is necessary for spindle checkpoint activation. In studies involving yeast it was found the chromosomes whose centromeres lacked kinetochores did not arrest in mitosis, even in the presence of spindle damage. It is clear that if there is no kinetochore present, there will be no attachment of microtubules, or tension across chromosome. That the spindle checkpoint did not cause mitotic arrest in the absence of kinetochore, suggests that either a kinetochore or the components of the kinetochore are required for a functional checkpoint (Lew and Burke, 2003). Though the consequences of a failed checkpoint and incorrect chromosome segregation are quite

detrimental to proper cell division, the biological processes underlying this checkpoint are not completely understood, making the spindle checkpoint a hot topic of current molecular biology research.

The spindle checkpoint is made up of a plethora of proteins that interact to affect the activity of the anaphase promoting complex. Though much has been elucidated about the activity of the spindle checkpoint, the exact workings of the checkpoint are still a matter of study and debate. At the top of this debate is the question, what activates the spindle checkpoint?

Two major models exist as to what causes the activation of the spindle checkpoint. One model holds that the checkpoint is activated in response to an absence of tension across the kinetochore. The other model maintains that the checkpoint is active only in response to unattached kinetochores. The activation of the spindle checkpoint in response to a lack of tension across the kinetochore is supported by research in mantid spermatocytes (Amon, 1999). Mantid spermatocytes are genotypically XXY. In 10% of meioses, one of the X chromosomes is unpaired. These cells become permanently arrested in meiosis. However, if tension is applied on the unpaired X chromosome by external sources, a microneedle for example, the cell will enter anaphase (Lew and Burke, 2003). This data clearly supports the theory that a lack of tension across the kinetochore is sufficient to activate the spindle checkpoint and arrest the cell cycle.

Research in budding yeast supports the second model of checkpoint activation. In budding yeast, if all kinetochores are attached, but tension is missing, anaphase still proceeds (Amon, 1999). In light of research supporting both models, one should not

assume that this is an either/or situation. Perhaps two different pathways of spindle checkpoint activation exist. Or perhaps the spindle checkpoint is activated by either unattached kinetochores, lack of tension, or both.

Though a spindle checkpoint pathway is found in most eukaryotic organisms; however, the components and methods of the pathways, though similar, are not always the same. Nonetheless, research points to a protein signaling cascade being at the basis of the spindle checkpoint.

Spindle Checkpoint Proteins

The spindle checkpoint, and in fact, the entire cell cycle, is made up of a series of phosphorylation and dephosphorylation events. Phosphorylation serves as a signal for either the activation or deactivation of the proteins that regulate the cell cycle (Vanoosthuyse and Hardwick, 2003). Discerning the events involved in the spindle checkpoint requires an understanding of the proteins involved. One must discover the sequence of protein interactions that together achieve arrest of the cell before anaphase. Studies into the order and complexities of spindle protein interactions have not yet elucidated a clear set of events that affect mitotic cell arrest in the presence of unattached chromatids.

Bub1 is one of many proteins that make up the spindle checkpoint cascade. Research in yeast, *Xenopus*, mouse, and human cells has led to the discovery of many proteins that are involved in the spindle checkpoint. These proteins include Mad1, Mad2, Mps1, Bub1, BubR1, Bub3, Zw10, Rod, Cdc2 and Cdc20. The exact role each of these proteins play in the spindle checkpoint has yet to be completely discerned.

While it is known that the activator of the signaling cascade is either an unattached kinetochore or lack of tension across a kinetochore, it is not yet known what protein senses the defect. Similarly, it is known that the target of the signal cascade is the anaphase promoting complex (APC), which exists in a complex with the protein Cdc20 and has ubiquitin ligase activity (Amon, 1999). However, the mechanism by which the signal gets to and inhibits this complex is still under consideration. In recent years, much has been discovered about the workings of the mitotic spindle checkpoint.

Sister chromatids are held together by cohesin proteins. In order for sister chromatids to separate in anaphase, these cohesins must be cleaved. When all chromatids are properly aligned, the cohesin proteins are cleaved by the protease separase. However, until the cell is ready to proceed through this step, separase is inhibited by the protein securin. Securin is ubiquitinated and degraded by the APC-Cdc20, allowing the onset of anaphase (Lew and Burke, 2003).

The spindle checkpoint is activated upon improper chromosome attachment at metaphase by an as yet undetermined method. It is known, however, that the spindle checkpoint delays anaphase by blocking the APC-Cdc20 degradation of securin. It is thought that the action of the APC-Cdc20 is inhibited by the binding of Mad2 to Cdc20. One hypothesis suggests that Mad2 exists in complex with Mad1 and, in response to spindle damage Mad2 dissociates from the Mad1/Mad2 complex to form a new complex with Cdc20, thereby inhibiting the APC and blocking securin degradation and sister chromatid separation. It is also known that APC inhibition is spatially limited. In cells containing two mitotic spindles, a defect in one spindle does not inhibit the second

spindle from proceeding to anaphase. This suggests that the proteins involved in the spindle checkpoint work only within a limited distance (Lew and Burke, 2003).

While it is known that the spindle checkpoint blocks the APC and, therefore, sister chromatid separation, it is not known how the spindle checkpoint is dismantled. As the spindle checkpoint is responsible for inhibiting APC in response to spindle damage, it follows that the spindle checkpoint must be dismantled in order to remove APC inhibition and allow anaphase. One theory suggests that upon correction of chromosome alignment, spindle checkpoint proteins diffuse away from the kinetochore. However, it is just as likely that there is motor-assisted transport of checkpoint proteins away from the kinetochore (Yu, 2002). More research is needed in order to discover the method of checkpoint disassembly.

The Role of Bub1

The role of Bub1 in the spindle checkpoint cascade is not completely understood. Much of what is known about the role of Bub 1 in the spindle checkpoint comes from studies of budding and fission yeast and the frog *Xenopus*. Findings involving Bub1 in these organisms are not always consistent; therefore, the role of Bub1 in higher mammals must be further studied.

Bub1 is known to be a checkpoint kinase that localizes to the kinetochore during mitosis. Bub1 is needed for the localization of other spindle checkpoint components in mitosis as well as for maintaining sister chromatid cohesion during meiosis I. In studying fission yeast, Bub1 was found to have both phosphorylation and kinase domains (Vanoosthuyse and Hardwick, 2003). The kinase activity of Bub1 is on the protein's C-

terminus. Bub1 kinase activity is required for spindle checkpoint function. This was shown by generating point mutations in a critical lysine residue of the Bub1 kinase domain. These mutants did not exhibit kinase activity and showed improper spindle checkpoint function (Yamaguchi, 2003).

The phosphorylation domain of Bub1, found on the protein's N-terminus, contains four phosphorylation sites for the protein Cdc2, a cyclin dependent kinase which, in fission yeast, exists in complex with the APC. In fission yeast, Bub1 must be hyperphosphorylated for full checkpoint response; however, hyperphosphorylation is not necessary for localization of Bub1 to the kinetochore. Interestingly, fission yeast strains that harbor Bub1 mutations at the four consensus Cdc2 phosphorylation sites are still phosphorylated to some extent. This tells us that Bub1 is also a substrate for one, if not several, other kinases and that there are other potential phosphorylation sites within the Bub1 protein (Vanoosthuyse and Hardwick, 2003). In fission yeast as well as in human cells, Bub1 is phosphorylated only in response to spindle damage. Contrastingly, *Xenopus* Bub1 is constitutively phosphorylated, and Bub1 in budding yeast is phosphorylated only during mitosis (Vanoosthuyse and Hardwick, 2003).

Bub1's exact position in the spindle checkpoint cascade is a matter of much speculation. Bub1 is thought to act upstream of the Mad proteins and the APC-Cdc20 complex (Amon, 1999). Evidence suggests that at some point in the cascade Bub1 physically associates with and phosphorylates Bub3 and Mad1; however, many theories also support Bub1 being in complex with Bub3 and Mad1, Mad2, and Mad3. And in *Xenopus*, Bub1 is thought to recruit Mad1, Mad2, and Bub3 to the kinetochore (Yamaguchi, 2003). A simplified view of the spindle checkpoint has Bub1

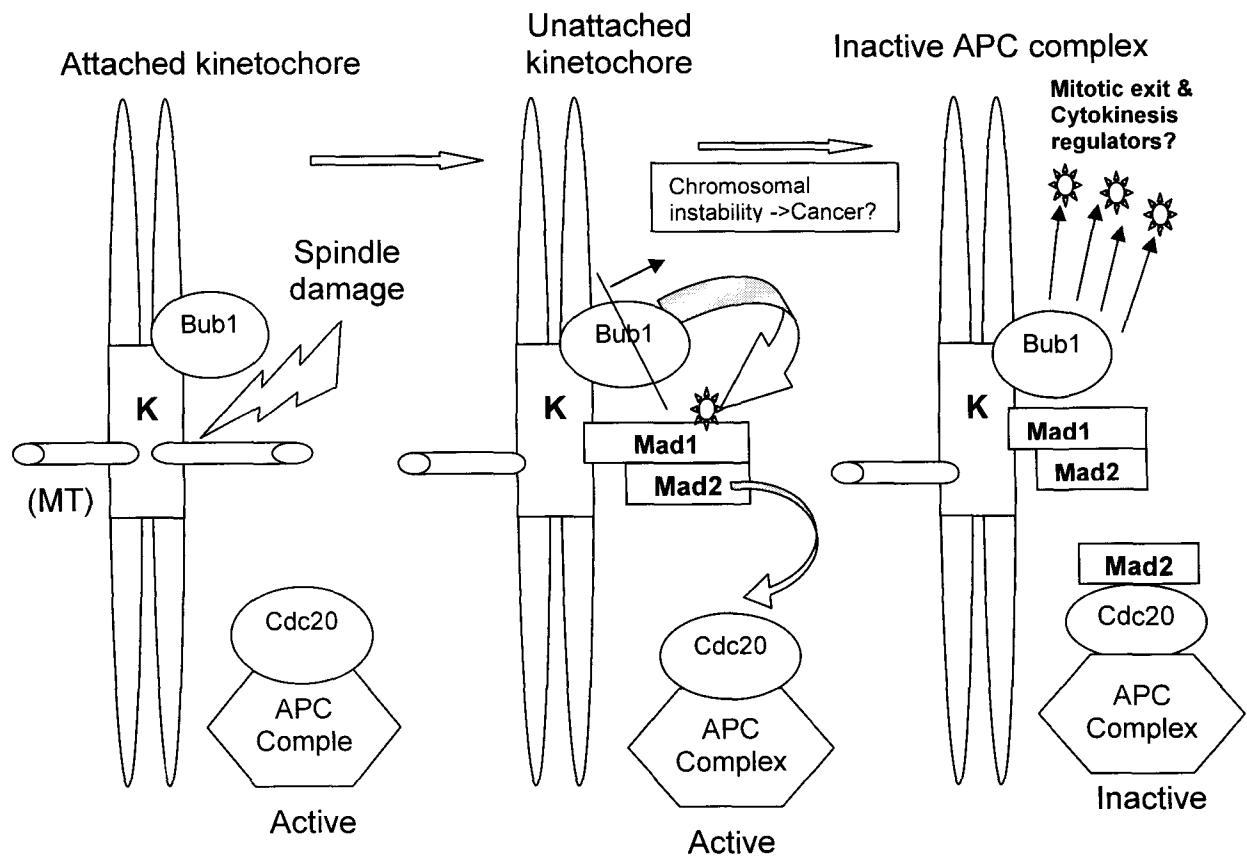
phosphorylating Mad1. The phosphorylation event triggers the separation of Mad1 from the Mad1/Mad2 complex. Mad2 then binds to the protein Cdc20. This binding inactivates the APC, effectively blocking the onset of anaphase.

Taylor *et al.* (2001) studied Bub1 in human cell lines. They found that Bub1 localizes to the kinetochore during prometaphase. However, this localization can be asymmetrical depending on microtubule attachment. If, of a pair of sister chromatids, one is properly attached to microtubules and the other is not, Bub1 stains weaker at the kinetochore closer to the spindle, suggesting that Bub1 localizes in higher concentrations at the unattached chromatid. After a 30 minute treatment with the microtubule depolymerizing drug nocodazole, the amount of Bub1 localizing at kinetochores increases. This is expected, as Bub1 is known to localize to kinetochores in response to spindle damage. Interestingly, levels of Bub1 at kinetochores also increased after treatment with Taxol, a drug which causes loss of microtubule tension. This data supports the idea that the spindle checkpoint is activated in response to either, or both, improper microtubule attachment or lack of tension across the kinetochore.

Taylor *et al.* (2001) also examined the localization of Bub1 at various stages of the cell cycle. They found that Bub1 localized to kinetochores during prophase, prometaphase, and metaphase, but not during anaphase. This suggests that, in humans, Bub1 dissociates from the kinetochore once chromosomes are properly aligned.

Though Bub1's role in the cell cycle is not completely understood, its importance is unquestionable. Bub1 is necessary for an active spindle checkpoint. Without an active checkpoint, cells are susceptible to chromosomal instability and aneuploidy which can lead to cancer. In light of this, the role that Bub1 plays in cancer formation and

proliferation is currently under investigation. A drastically simplified view of the spindle checkpoint and the role of Bub1 is shown.



Bub1 and Cancer

Cancer is, by definition, uncontrolled growth of abnormal cells. Many cancers spring from improper cell division. Inappropriate segregation of chromosomes during mitosis can lead to a loss or gain of chromosomes in the daughter cells. This promotes

genomic instability, a well known phenotype of cancer. Therefore, the role of the spindle checkpoint in ensuring the fidelity of genetic transmission is imperative.

Genomic instability is often the result of chromosomal instability, a phenomenon that leads to aneuploidy, or abnormal chromosome number in a cell (Cahill et al., 1998). Aneuploidy can result from many different chromosomal aberrations including gains or losses of entire chromosomes or chromosomal parts, rearrangements of chromosomes or parts of chromosome, or translocations, deletions, or amplifications of the nucleotide bases that make up chromosomes (Jallepalli and Lengauer, 2001). Chromosomal instability is often the result of a non-functioning mitotic checkpoint (Cahill, 1998).

Cancer cells often show mutations in, or irregular expression of, mitotic checkpoint proteins. Checkpoint proteins that have been implicated in cancers include p53, ATM, CHK2, MAD1, MAD2, securin, and, of course, Bub1 (Jallepalli and Lengauer, 2001). Recent research has explored the prevalence and role of Bub1 in colorectal cancers. In one study, human colorectal cancer cell lines were treated with nocodazole, a drug that disrupts microtubules. Treatment of normal cells with nocodazole leads to activation of the spindle checkpoint and mitotic arrest at metaphase. However, in colorectal cell lines that exhibit chromosomal instability, mitotic arrest did not occur. Cells continued through the cell cycle, leading to aneuploidy. The same results occurred when the cell lines were treated with colcemid, another microtubule blocking drug (Cahill et al., 1998).

The abovementioned study showed that the colon cancer cell lines deficient for mitotic arrest were also mutant for the *Bub1* gene. One cell line contained a 197 base pair deletion that spanned codons 76-141 of the *Bub1* gene. This deletion further led to a

frameshift mutation of the following codons (Cahill et al., 1998). This mutation affected codons of the kinetochore localization domain of Bub1, shedding light on the chromosomal instability that is characteristic of these colon cancers. As previously discussed, Bub1 localization to the kinetochore is necessary not only for localization of other checkpoint proteins, but also for an active mitotic checkpoint.

Another study into the role of Bub1 in human colorectal cancers found that cell lines containing a Bub1 mutation were heterozygous. In other words, these cancer cells contained one wild-type, or normal, copy of Bub1, and one mutated form of the gene. The Bub1 mutations were isolated and cloned. Later, the cloned mutations were introduced into mitotic checkpoint proficient cells. In this situation, the mutated form was able to inactivate the spindle checkpoint. As two wild-type copies of Bub1 were also present in the cell, this data suggests that Bub1 mutations have a dominant negative effect (Jallepalli and Lengauer, 2001).

Chromosomal instability is also typical of breast, prostate oropharynx, lung, and pancreatic cancers (Jallipalli and Lengauer, 2001). Hempen *et al.* (2003) found Bub1 mutations in pancreatic cancer cells that did not have an active mitotic checkpoint. These mutations included two separate amino acid substitutions in exon 8 of the same allele. Exon 8 of Bub1 is involved in the region of the gene that serves as a nuclear-localization signal. As before, these mutations were heterozygous, providing further support for Bub1 mutations' behaving in a dominant negative fashion.

Cancer rarely results from a single genetic mutation. Instead, a cancer phenotype usually involves several gene mutations or the abrogation of several genetic pathways. Hence, these studies, while implicating Bub1 in cancer, must be taken in context. The

studies were done on cancer lines which may include various other gene mutations in addition to mutations in Bub1. Therefore, more research into the role of Bub1 in cancer is necessary.

A Murine Bub1 Model

In order to further study the role of Bub1 in the spindle checkpoint and cancer formation in higher organisms, a Bub1 knockout mouse model was generated by the laboratory of Sundaresan Venkatachalm here at the University of Tennessee.

Generation of Bub1 deficient mice:

In order to study the role of Bub1 in cancer formation, we have generated Bub1 deficient mice using the Baygenomics genetrapp embryonic stem (ES) cell resource (Stryke *et al.*, 2003). One of the ES cell clones that had been characterized to have a gene trap insertion within the Bub1 gene was represented in the Baygenomics ES cell library. The Bub1 trapped ES cells were obtained from Baygenomics and analyzed by PCR to confirm Bub1 disruption by using primers that were specific for *bub1* and the gene-trap sequences. Figure 2 shows the insertion site of the gene-trap vector within the *bub1* gene and the primer positions. The validated ES cells were used for blastocyst injections and chimeric founder mice were generated by microinjection services provided under a subcontract from the University of Massachusetts Medical School transgenic core. Out of the seven high degree chimeras obtained from the blastocyst injections, our initial analysis of the first two litters from founder males indicate that three of the

chimeric founders have produced agouti germ line litters for Bub1 deletion. Genotype analyses of tail clips from the first two litters of F1 agouti mice indicate an equal distribution of wild type and heterozygous offspring with no obvious developmental defects in the heterozygous mice. The PCR based genotype analysis of five F1 heterozygous and wildtype offspring is also shown below the targeting scheme.

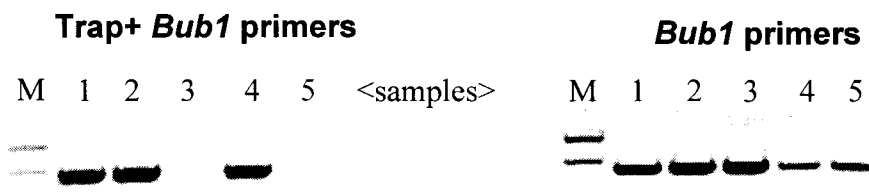
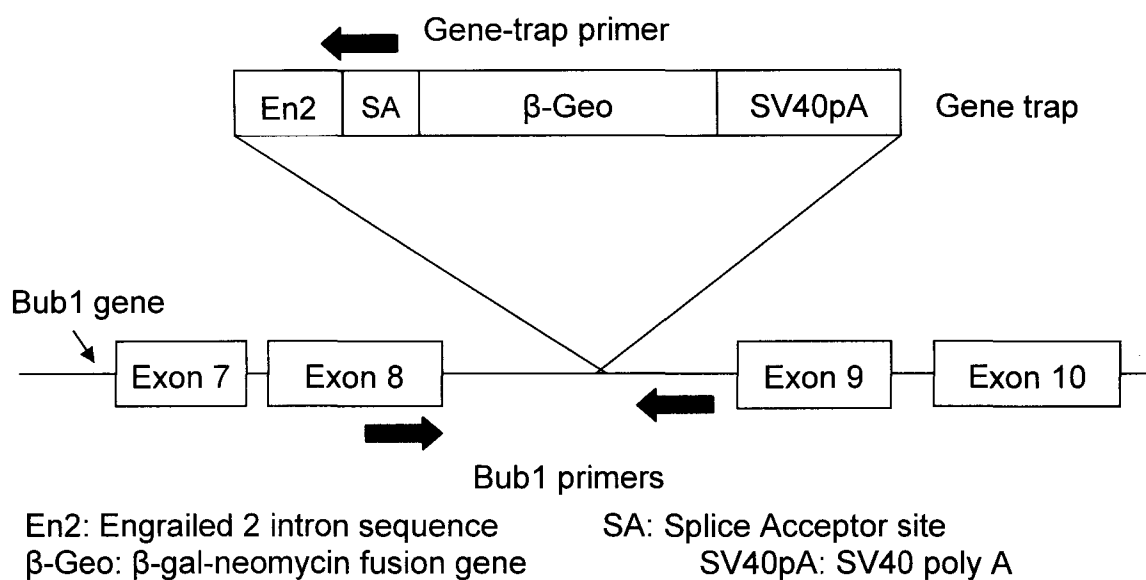


Figure Schematic representation of Bub1 disruption in ES cells and genotype analysis of F1 agouti germline offspring. An example of the genotype analysis of F1 offspring by PCR is shown below the scheme.

The Effect of Bub1 mutation on Cell Cycle Progression

To study the effect of Bub1 deficiency on cell cycle progression, experiments were performed on mouse embryonic fibroblasts (MEFs) that were wild-type (+/+) and heterozygous (+/-) for Bub1.

Hypothesis: *Bub1* plays a principal role in regulating the spindle checkpoint pathway.

Deletion of *Bub1* will lead to an improper mitotic spindle checkpoint.

The reasoning behind this hypothesis has its basis in the role that Bub1 has been assigned in the spindle checkpoint. From previous studies in yeast, Bub1 was characterized to be necessary for proper mitotic checkpoint functioning. Heterozygous MEF cells are haplo-insufficient; they contain only one functioning copy of the Bub1 protein whereas wild-type MEFs contain two functional copies. It follows that the cells with only one functioning copy of Bub1 will be less proficient at arresting the cell at the metaphase-anaphase transition in response to spindle damage than will wild-type cells with two functioning *Bub1* genes.

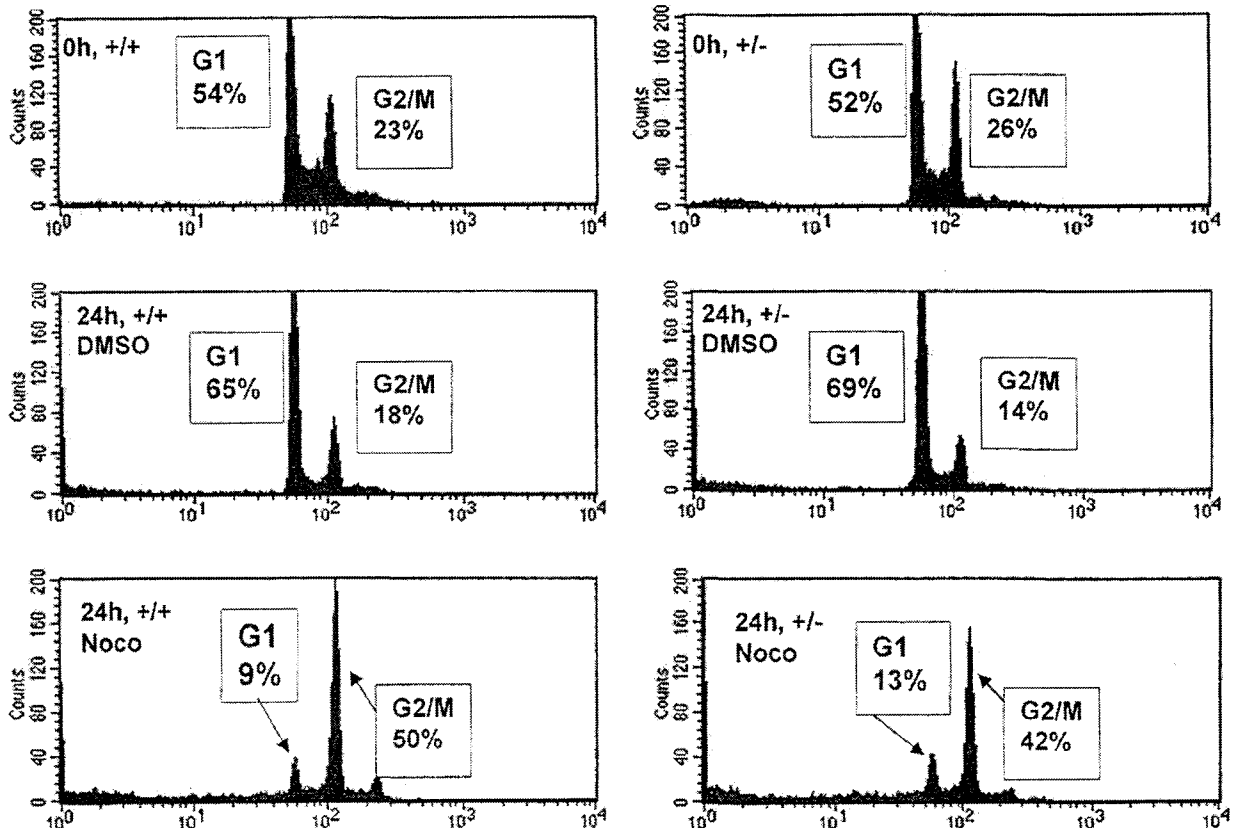
To test this hypothesis, wild-type and heterozygous MEFs were generated by mating a founder knockout male to a wild-type female. The pregnant female was sacrificed 13.5 days post coitus and her embryos cultured to generate Bub1+/+ and Bub1+/- MEFs.

The MEF cells were cultured through two passages. Both wild-type and heterozygous passage two cells were then treated with 100ng/mL of the microtubule

depolymerizing drug nocodazole for a period of 24 hours. Nocodazole was diluted in the chemical DMSO to arrive at a concentration of 100ng/mL. Therefore, a set of control cell plates were treated with an equal volume of DMSO (0.1%) to standardize the experiment. Cells from plates not treated with nocodazole were also cultured to serve as a control.

Untreated cells (0hr) and cells treated for 24 hours with DMSO and nocodazole were collected and fixed with 70% ethanol. Then, cells were stained with propidium iodide and analyzed for DNA content using a Coulter EPICS flow cytometer. 10,000 cells of each treatment and cell type were analyzed.

Flow Cytometry Results



Results

Flow cytometry provides data on the percentage of cells that are in the different phases of the cell cycle. Both untreated and DMSO treated wild-type and heterozygous cells showed the majority of their cells in the G1 phase. This data is consistent of a normal cell cycle that does not encounter spindle damage.

Contrastingly, cells treated with nocodazole show the majority of their cells in the G2/M phase. This data is consistent with a functioning spindle checkpoint. Upon damage of microtubules, as would occur in the presence of nocodazole, cells with a proper spindle checkpoint should arrest in mitosis at the metaphase-anaphase transition to ensure that improper chromosome segregation does not occur.

However, while both wild-type and heterozygous cells treated with nocodazole tended to accumulate in G2/M, wild-type cells did so to a greater extent than did heterozygous cells (50% as compared to 42%). Put another way, wild-type cells had a lower percentage of cells in the G1 phase than did heterozygous cells (9%-13%). These data indicate a limited ability of Bub1 heterozygous cells to evade the spindle checkpoint and proceed to G1 phase of the cell cycle and provides preliminary support for the hypothesis that Bub1 plays a pivotal role in the spindle checkpoint.

The Future

The information presented here provides only a basic background into the research that has been done thus far on the spindle checkpoint in general, and, more specifically, the role of Bub1 in the spindle checkpoint. Future research in murine *Bub1* knockouts should provide further insight into *Bub1*'s role in regulating mitosis in higher

vertebrates. We plan to generate cell lines that are completely lacking the Bub1 protein to further unravel the effects of Bub1 deficiency on cell cycle progression. In addition, heterozygous mice will also be monitored for cancer susceptibility over a period of two years.

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