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HETEROZYGOSITY FOR A BUB1 MUTATION CAUSES FEMALE-SPECIFIC GERM CELL ANEUPLOIDY IN MICE

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ABSTRACT

Aneuploidy, the most common chromosomal abnormality at birth and the main ascertained cause of pregnancy loss in humans, originates primarily from chromosome segregation errors during oogenesis. Here we report that heterozygosity for a mutation in the mitotic checkpoint kinase gene, *Bub1*, induces aneuploidy in female germ cells of mice, and that the effect increases with advancing maternal age. Analysis of Bub1 heterozygous oocytes showed that aneuploidy occurred primarily during the first meiotic division and involved premature sister chromatid separation. Furthermore, aneuploidy was inherited in zygotes and resulted in the loss of embryos after implantation. The incidence of aneuploidy in zygotes was sufficient to explain the reduced litter size in matings with Bub1 heterozygous females. No effects were seen in germ cells from heterozygous males. These findings show that Bub1 dysfunction is linked to inherited aneuploidy in female germ cells and may contribute to the maternal age-related increase in aneuploidy and pregnancy loss.

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INTRODUCTION

Aneuploidy is the main ascertained cause of pregnancy loss in humans and the most common chromosomal abnormality at birth (1, 2). Aneuploidy in germ cells is primarily due to chromosomal segregation errors during the first meiotic division of oogenesis (1, 3). Numerous hypotheses and etiologies have been proposed for human aneuploidy, however, the only consistent finding remains its positive correlation with maternal age (2). Accurate chromosome segregation requires the coordinated interaction of many protein kinases and phosphatases, cellular organelles such as microtubules, motor proteins, centrosomes, kinetochores, and spindle checkpoint proteins. The spindle assembly checkpoint (SAC) is comprised of a set of highly conserved proteins, including Mad (Mitotic arrest deficient) and Bub (Budding uninhibited by benzimidazole) proteins, and arrests cells during the mitotic metaphase to anaphase transition in response to kinetochores that are unattached to spindle microtubules or lack of tension from spindle fibers (3-6). The SAC prevents the precocious separation of sister chromatids and ensures accurate chromosomal segregation at anaphase by affecting the activity of the anaphase promoting complex/Cyclosome (APC/C) (7). The APC/C ubiquitinates the securin component of the securin-separase complex in mitosis and tags it for degradation. Upon degradation of securin, separase is free to cleave the Scc1 subunit of the cohesin complex that holds sister chromatids together and allow their separation. In addition to its well known role during mitosis, the SAC is active during mammalian oogenesis (8-10) and APC/C activity is required for homologous disjunction during the first meiotic division (11). Furthermore, meiosis in vertebrates requires separase-dependent depletion of Rec8 along chromosomal arms for subsequent chiasmata

resolution (12). Lack of a functional spindle checkpoint due to defects in any of the SAC proteins may lead to chromosome missegregation and aneuploidy (8, 9, 13, 14).

The budding uninhibited by benzimidazole protein 1 (Bub1) is a protein kinase that localizes to kinetochores very early during mitosis and is required for the efficient kinetochore localization of other SAC proteins (15) and monitoring microtubule attachment to the kinetochore (16). In response to spindle damage, Bub1 phosphorylates Mad1 leading to the dissociation of the Mad1-Mad2 complex. Unbound Mad2 can then bind and inhibit Cdc20, an activator of APC/C (7, 17). Depletion of Bub1 in mammalian cells leads to misaligned chromosomes during mitosis (18) and to loss of chromatid cohesion (19-21). Conditional deletion of Bub1 in mice leads to developmental and proliferation defects in mouse embryonic fibroblasts (MEFs) and spermatogonial cells (21), while a hypomorphic mouse mutant model that expresses reduced levels of Bub1 shows an increased incidence of tumors (22). In mouse oocytes, Bub1 localizes to kinetochores (10, 23) and a dominant-negative form of Bub1 that perturbs the kinetochore localization activity of the wild type protein leads to the acceleration of meiosis I, as has also been shown for Mad2 (9, 24, 25). It was recently shown that specific depletion of Bub1 in mouse oocytes results in chromosome missegregation at meiosis I and loss of cohesion between sister chromatids (26).

In this study, we analyzed the functional role(s) of Bub1 during embryogenesis and germ cell development in mice using a knock in gene-trap mutation within the Bub1 gene that leads to the generation of a truncated Bub1-βgeo fusion protein containing the first 269 amino acids of Bub1. The mutated Bub1 protein includes the Mad3-Bub1 domain and the kinetochore binding domain fused to β-galactosidase-neomycin (βgeo) reporter protein (Figure 1A-C and Supplementary figure 1). We show that heterozygosity for this mutation results in female-

specific, age-dependent increases in germ cell aneuploidy that is inherited in zygotes and affects the survival of the embryos. Our results suggest that heterozygous mutations in the human BUB1 gene may be an etiological cause of the known maternal-age effect for germ cell aneuploidy.

RESULTS

Generation of Bub1 mutant mice

To generate mutant mice that are homozygous for the *Bub1* mutant allele ($Bub1^{m/m}$), we intercrossed F1 heterozygous offspring ($Bub1^{+/m}$) obtained from Bub1 chimeric males. Intercrosses of the F1 heterozygotes yielded no $Bub1^{m/m}$ offspring, confirming that the *Bub1* homozygous mutation leads to embryonic lethality in mice (22). The essential role for Bub1 in embryonic development is consistent with studies that have shown a similar role for other SAC proteins (27-29). Intercrosses between heterozygous males and wild type (WT) females yielded the expected ratio of heterozygous and WT offspring (60 $Bub1^{+/m}$ and 70 $Bub1^{+/+}$ offspring from 19 matings) indicating that heterozygosity for *Bub1* did not affect embryonic development. Expression analysis of the surrogate marker (β-gal) driven by the Bub1 promoter in E10.5 mutant heterozygous embryos showed that Bub1 was ubiquitously expressed in the embryo (Figure 1D). In adult mouse tissues, Bub1 expression was high in thymus, ovary, testis. However, much lower Bub1 expression levels were noted in tissues with differentiated cells that have a low proliferation potential (Supplementary figure 2). Expression analysis of the Bub1-βgeo fusion protein in metaphase cells showed that the fusion protein localizes to the kinetochores suggesting a putative dominant negative function by competing for kinetochore

binding with the wild type protein with intact kinase activity or preventing interactions with other SAC components (Figure 1E).

Bub1 heterozygous female mice are sub-fertile

During the breedings aimed at generating homozygous mutants we noted that the heterozygous intercrosses yielded abnormally low litter sizes (22 pups from 12 matings) that were not accounted for by the embryonic lethality of $Bub1^{m/m}$ embryos. To further investigate this phenotype, we generated offspring from intercrosses between $Bub1^{+/m}$ and WT mice (Figure 1F). Crosses between WT females and heterozygous males, as well as WT females and WT males, yielded similar litter sizes (6.8 ± 1.2 vs. 7.5 ± 0.5 S.E.). However, intercrosses involving $Bub1^{+/m}$ females (with WT males or heterozygous males) yielded a significantly lower number of offspring (1.7 ± 0.4 and 1.7 ± 0.5, respectively). To confirm that the phenotype was directly related to disruption of the Bub1 gene, we induced deletion of the splice acceptor (SA) sequence from the gene-trap by mating Bub1 mutant mice with transgenic mice that expressed Cre-recombinase under the control of mouse protamine 1 promoter. The deletion of the SA sequence is expected to result in the re-expression of the WT Bub1 protein from the gene-trap allele (Supplementary Figure 1). When females heterozygous for the gene-trap that lacked the splice acceptor (SA- females) were mated with WT males the litter size was comparable to WT crosses indicating that the SA deletion had reversed the sub-fertility defect (Figure 1F).

We next determined whether the reduced litter size in $Bub1^{+/m}$ females was due to implantation defects. We analyzed uterine content in pregnant females between 6.5 and 9.5 days post coitum (d.p.c.) and found a slight reduction (~30%) in the number of implantation sites between WT and $Bub1^{+/m}$ females (Table 1). Analysis of the harvested embryos showed arrested

development of a majority of the embryos at various stages that ranged from E6.5 through E8.0 (Table 1 and Supplementary Figure 3). Furthermore, the data also suggested that the $Bub1^{+/m}$ females were unable to support the normal development of embryos irrespective of their genotype as ~80% of the implanted embryos were either arrested or dead even in crosses between $Bub1^{+/m}$ females and WT males that were expected to yield an equal proportion of WT and heterozygous embryos. To determine if the sub-fertile phenotype of the $Bub1^{+/m}$ females was due to defects in follicular or uterine development, we performed histological analysis of serial sections of ovaries and uteri harvested from age matched mutant and WT mice (ages 7, 13 and 28 weeks). No significant differences in the various stages of follicular or uterine development between the two groups were seen; however $Bub1^{+/m}$ females exhibited slightly increased levels of epithelial apoptosis, multifocal superficial stromal necrosis, and neutrophilic endometrial infiltrates (Supplementary Figure 4 and data not shown). Finally, analyses of cultured blastocysts obtained at E3.5 from Bub1 mutant females crossed to WT males showed that a majority of blastocysts did not grow in culture (Supplementary Figure 5) suggesting that preimplantation development is also impaired in $Bub1^{+/m}$ females.

Bub1 mutation leads to maternal age-dependent germ cell aneuploidy

To determine whether the reduced litter size in crosses with $Bub1^{+/m}$ females was due to the generation of aneuploidy during oogenesis we mated superovulated $Bub1^{+/m}$ females with B6C3F1 males and collected zygotes from the oviducts to analyze the chromosome complements of maternally- and paternally-derived pronuclei. Normal numbers of eggs were recovered from $Bub1^{+/m}$ females (28.1 average per $Bub1^{+/m}$ female versus 26.8 for WT females). As shown in Table 2, 38 of 47 zygotes from 8-14 week-old $Bub1^{+/m}$ females had aneuploid maternal

chromosome complements (15 were hypohaploid, i.e., missing chromosomes; and 23 were hyperhaploid, i.e., had extra chromosomes) (Figures 2B-C). These frequencies were significantly different ($P<0.001$; Chi-square) from those observed in zygotes from WT females (Table 2). To determine whether these chromosomal segregation errors were occurring during meiosis I, we analyzed metaphase II (MII) oocytes from $Bub1^{+/m}$ and WT females. Fifty-nine of 78 MII oocytes from 8-14 week-old $Bub1^{+/m}$ females were aneuploid (25 were hypohaploid and 34 were hyperhaploid), compared to only 3 of 75 in WT females (Table 2). Comparisons of the results obtained in oocytes and zygotes showed good agreement between the incidences of hypohaploid and hyperhaploid maternal chromosome complements (Figure 2D) suggesting that the majority of chromosomal segregation errors were occurring during meiosis I. These results show that Bub1 heterozygosity induced extremely high levels of aneuploidy in female germ cells and that inherited aneuploidy is most likely the cause of the embryonic lethality observed in matings with $Bub1^{+/m}$ females.

An important insight into the event(s) that underlie the generation of aneuploidy during meiosis I in $Bub1^{+/m}$ females was provided by the observation that ~70% of the oocytes from mutant females presented with single chromatids (or half-dyads, Figure 2B) in comparison to less than 7% in WT females. This strongly suggests that the Bub1 mutation affected chromatid cohesion and resulted in premature sister chromatid separation (PSCS). Of the 38 aneuploid oocytes with unpaired chromatids found in $Bub1^{+/m}$ females (Supplementary Table 1), 30 had an odd number of single chromatids suggesting that the majority of PSCS occurred during meiosis I. However, PSCS could not explain all of the aneuploid events observed, but was likely involved in the genesis of ~85% of aneuploidy. Specifically, of the 34 hyperhaploid MII oocytes observed in $Bub1^{+/m}$ females, 19 (56%) originated from PSCS, 5 (15%) by nondisjunction, that

is, had more than 20 dyads and no single chromatids, and 10 (29%) by a combination of PSCS and nondisjunction.

The effects of the Bub1 mutation seem to aggravate with maternal age. As shown in Figure 2E, older $Bub1^{+/m}$ females (14-week-old) had significantly higher ($P<0.002$; Chi square) frequencies of oocytes with PSCS than younger $Bub1^{+/m}$ females (8-week-old). Older females also had higher frequencies of hyperhaploid oocytes and zygotes, although these differences were not statistically significant ($P>0.1$; Chi square). In addition, no litters were obtained from Bub1 heterozygote females that were at least 24 weeks old, further supporting an effect of maternal age on aneuploidy induction.

The dramatic effects of heretozygosity for the Bub1 mutation on meiotic chromosome segregation are restricted to female germ cells. Only 3 out 109 zygotes recovered from WT females mated to $Bub1^{+/m}$ males were aneuploid (2 hypohaploid and one hyperhaploid). These frequencies were not different from those observed in control matings (Supplementary Table 2). To exclude the possibility that the lack of aneuploidy in zygotes fathered by $Bub1^{+/m}$ males was due to selective elimination of aneuploid spermatids during postmeiosis, we analyzed MII spermatocytes isolated from testes from the same males used for the zygote studies. Analysis of 108 MII spermatocytes (Supplementary Table 2) showed no hyperhaploidy and no PSCS.

DISCUSSION

Despite the prevalence of aneuploidy in human female germ cells, its importance to female fertility and the well established maternal age effect, we know little about the causes and factors that predispose to chromosome nondisjunction during oogenesis (2). In recent years, the use of mouse models with mutations in genes involved in meiotic recombination and

chromosome segregation have shed light on factors that assure proper chromosome segregation. It is becoming clear that disturbances in meiotic recombination are important contributors to meiotic nondisjunction. Increased female germ cell aneuploidy rates have been observed in mice lacking the synaptonemal complex protein 3 (Scp3) (30, 31) , the meiosis-specific cohesin Smc1β (32) and the homologous recombination protein Rad51c (33). An important role for histone acetylation in chromosome alignment and meiotic spindle organization has also been demonstrated (34, 35). It has so far been difficult to assess the role of SAC proteins during gametogenesis because their deletion has invariably resulted in embryonic lethality (22, 27-29). However, a recent study that utilized a floxed Bub1 allele and a Zp3-Cre recombinase transgene approach to specifically delete Bub1 in mouse oocytes (26) demonstrated the important role of Bub1 in assuring proper chromosome segregation in mammalian oocytes. We show here that heterozygosity for a Bub1 mutation predisposes female germ cells, but not male germ cells, to chromosome missegregation and produces a phenotype that is indistinguishable from that generated by the complete deletion of Bub1 protein (26). Furthermore, we demonstrate that the Bub1 mutation affects sister chromatid cohesion resulting in the induction of aneuploidy primarily during meiosis I, that aneuploidy increases with advancing maternal age, and that such aneuploidy is inherited in zygotes. More importantly, the incidence of aneuploidy in zygotes is sufficient to explain the reduced litter size observed in matings with Bub1 heterozygous females providing a direct link between heterozygosity for Bub1 in females and aneuploid frequencies in their offspring. Our results suggest that a functional deficiency in the Bub1 protein, either through age-related decline in mRNA levels or age-related accumulation of mutations that inactivate the protein may represent a mechanism that contributes to the known maternal age-related increase in aneuploidy (2) and fertility defects in women with multiple abortions.

How does a mutation for Bub1 result in chromosome missegregation and why are the effects limited to female germ cells? Unlike a recent Bub1 mouse model with a hypomorphic allele that expressed ~20% of the WT protein and did not exhibit fertility defects (22), our mutational strategy generated a mutant Bub1 protein that lacks the kinase domain but that still contains the kinetochore binding domain. The mutant protein is able to bind to kinetochores (Figure 1E) and may compete with the WT protein resulting in a dominant negative mutation. Support for this hypothesis comes from another study which showed that a truncated mutant for Bub1 (amino acids 1-331) disrupted the SAC in mitotic cells by competing with the endogenous Bub1 kinase for kinetochore localization (24). Alternatively, the mutated protein may be unable to recruit other SAC proteins to the kinetochore or to properly interact with the cohesin protein complex resulting in premature centromeric separation of sister chromatids (19, 20). It was recently demonstrated that deletion of the Bub1 gene in mouse oocytes causes premature activation of the APC/C and separase and that the precocious activation of these two SAC components was responsible for many of the phenotypes caused by Bub1 depletion (26). Interestingly, these authors also demonstrated that a mutated Bub1 protein that lacked the kinase domain, as the mutated version of Bub1 in our heterozygous mouse model, prevented the precocious activation of the APC/C (26). Earlier studies have shown that Bub1 is necessary for the centromeric localization of the shugoshin proteins (Sgol in meiosis and Sgo2 in mitosis of fission yeast (36)), as well as Rec8 in fission yeast (37). Furthermore, recruitment of PP2A that regulates Rec8 cleavage also depends on Sgo1 in fission and budding yeast (38). Rec8 localization to centromeres in mouse oocytes depends on Sgo2 and deletion of Sgo2 leads to fertility defects in mice (23, 39). Together with these findings, our data suggest that the Bub1 function affected in our heterozygous model is its role in protecting centromere cohesion through

recruitment of shugoshin, PP2A and Rec8 proteins and prevention of separase-mediated removal of cohesin (19, 20). Further evidence for the role of Bub1 in sister chromatid cohesion is strengthened by earlier observations that have shown a dual role for Bub1 in chromosomal congression (involving the regulation of kinetochore-microtubule attachments) in addition to a role in SAC signaling (18). Our data show that abnormal Bub1 function results in the premature separation of chromosomes into separated sister chromatids, a mechanism that has been proposed to be involved in the genesis of the majority of human germ cell aneuploidy (40).

Maternal age is a well-established etiological factor in the genesis of human aneuploidy (2). Therefore, we investigated whether the effects of heterozygosity for the Bub1 mutation increased with maternal age. The results showed that 14-week old $Bub1^{+/m}$ females had significantly ($P<0.05$) higher levels of PSCS than 8-week old $Bub1^{+/m}$ females (Figure 2E). The frequencies of hyperhaploid oocytes and zygotes, although higher in older females, did not show significant age-related increases. However, this was more likely due to the already high frequencies of these events in the 8-week old females, than to a lack of an age effect. To further substantiate age-dependent increase in aneuploidy, there was complete loss of fertility in $Bub1^{+/m}$ females that were older than 24 weeks (6 months). Interestingly, decline of *Bub1* mRNA levels has been reported in human oocytes of older women, particularly during meiosis I (41). This suggests an additive, or possibly synergistic, effect between the presence of the Bub1 mutation and the age-dependent reduction of Bub1 mRNA levels that results in the progressive reduction in the amount of the wild type Bub1 protein with augmented loss of chromatid cohesion and possibly SAC dysfunction. Indeed, weakening of sister chromatid cohesion has been also suggested as an important component of the maternal age effect (42).

Sexually dimorphic phenotypes in mice have been reported for several mutations involving genes with roles in meiotic chromosome segregation (31, 33, 43). However, the lack of an effect in male germ cells was unexpected given the recently demonstrated importance of Bub1 in spermatogenesis (21). It is important to note that the spermatogenesis defects reported in the conditional deletion of Bub1 in males resulted from mitotic defects that lead to the loss of proliferation of spermatogonial cells (21) whereas our data implicate a specific role for Bub1 in meiosis that affects female fertility. Bub1 is expressed in testis (44) and a recent conditional Bub1 nullizygous model clearly demonstrated impaired chromosome segregation and spermatogenic progression in the absence of Bub1 (21). Our results strongly suggest that Bub1 has different functions during oogenesis and spermatogenesis or that a yet unidentified testis-specific protein compensates for the reduced level of normal Bub1 protein in heterozygote males. Genomic studies have shown the presence of a testis-specific mRNA species, in addition to the full length Bub1 mRNA found in testis and other tissues, that encodes for a protein that lacks the first 421 amino acids of the Bub1 protein (44). This testis-specific transcript derived from an alternative start site or alternative splicing event would generate a testis-specific protein that would not be affected by our mutational scheme and may be sufficient to prevent chromosome missegregation during spermatogenesis. Furthermore, it is interesting to note that heterozygosity for another SAC component, Mad2, affects chromosome segregation in oocytes (8) and differences in spindle association of Mad2 and BubR1 have been reported between spermatogenesis and oogenesis (45, 46). Therefore, there may be qualitative and quantitative differences in the SAC machinery between oogenesis and spermatogenesis.

The generation of various mutant mouse models have led to major advances in dissecting the molecular mechanisms and a better understanding of causes of infertility during the last

decade (47). The mouse model described here should be valuable for elucidating the mechanisms responsible for the known sex differences in the susceptibility to chromosome missegregation during meiosis and understanding the basis of the maternal age-dependent increase in germ cell aneuploidy. Our results suggest that heterozygous mutations in the human *BUB1* gene that lead to truncated versions of the protein and mutations interfering with recruitment/binding to other SAC components could be etiological causes for fertility defects in women with multiple abortions and be involved in the known maternal-age effect for germ cell aneuploidy.

Materials and Methods

Generation of Bub1 mutant mice

We generated Bub1 deficient mice using the Baygenomics gene-trap embryonic stem (ES) cell resource (48). The Baygenomics insertional mutagenesis strategy utilizes a gene-trap cassette consisting of a splice-acceptor-βgeo cassette (beta-galactosidase-neomycin fusion gene). The Bub1 trapped ES cells were obtained and characterized further. *Bub1*disruption was confirmed by using primers specific for *Bub1* exon 8 (5'- AAGAAGCATGAGCAGTGGGTT-3') and the gene-trap sequences (5'- ACC TGG CTC CTA TGG GAT AG -3'). A schematic of the Bub1 gene-trap insertion, its effect on the Bub1 gene product and genotype analyses of the mutant and WT alleles are shown in supplementary figure 1. Sequencing of the PCR product indicated that the gene trap was integrated within intron 8 of the *Bub1* gene. Bub1 targeted ES cells were used for blastocyst injections to generate founder mice and F1 progeny. The insertion of the gene trap leads to the loss of expression of the 17 downstream exons. At the protein level, the gene-trap insertion was determined to be downstream of the kinetochore localization domain and upstream of the C-terminal kinase domain. The gene-trap strategy employed to generate the Bub1 mutant ES cells also leads to the generation of a putative truncated Bub1-βgeo fusion protein containing the first 269 amino acids (lacking the kinase domain) of the 1,057 amino-acid long wild-type protein (16). The use of animals in this study was approved by the Institutional Animal Care and Use Committee at both the University of Tennessee and Lawrence Berkeley National Laboratory.

Cytogenetic analyses of oocytes, spermatocytes, and zygotes

$Bub1^{+/m}$ and WT littermates were generated by mating $Bub1^{+/m}$ males and WT females at the University of Tennessee and then shipped to Lawrence Berkeley National Laboratory.

B6C3F1 mice were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA). Mice 8-14 weeks of age were maintained under a 12 hr light/dark photoperiod at room temperature of 21-23° C and relative humidity of 50 ± 5%. Female mice were superovulated by an i.p. injection of 7.5 I.U. of pregnant mare's serum (PMS, Sigma Co.) followed 48 h later by an i.p. injection of 5.0 I.U. of human chorionic gonadotrophin (hCG, Sigma Co.). Females were caged with males (1:1) ~4 hr after HCG injection and checked for vaginal plugs the next morning. Unmated females were used for isolation of MII oocytes (~20 hr after HCG). Mated females received an i.p. injection of 0.08 mg/kg colchicine (CAS No. 64-86-8, Sigma Co.) dissolved in 0.2 ml of distilled water 24 hr after HCG to prevent the union of the two parental pronuclei and arrest zygotic development at the metaphase stage of the first cleavage division (49) and were euthanized by CO_2 inhalation 6 hr after colchicine injection, zygotes were flushed out from isolated oviducts and processed according to the mass harvest procedure (50). The prepared slides were air-dried for at least 24 hr at room temperature and stored in nitrogen atmosphere at -20°C until use. DAPI at 0.25 µg/ml diluted in Vectashield mounting medium was used as counterstaining. An oocyte was considered aneuploid if it contained a chromosomal number different from 20 when both dyads and single chromatids (or half-dyads) were counted. For example, oocytes with 19 dyads plus 2 single chromatids (or 18 dyads plus 4 monads) were considered euploid, while oocytes with 19 dyads plus 3 single chromatids were considered hyperhaploid.

Testis preparations were made according to a standard method (51). Slides from 4 $Bub1^{+/m}$ animals were scored. They were stained with 0.25 µg/ml 4,6-diamidino-2-phenylindole (DAPI) diluted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Each slide was examined using a 40x objective for localizing MII spermatocytes and then with a 100x

objective for identifying chromosomal abnormalities. At least 25 MII spermatocytes per mouse were analyzed.

Immuno-fluorescence analysis of Bub1 mutant MEFs:

$Bub1^{+/m}$ mouse embryonic fibroblasts were grown on glass cover slides and fixed with 4% formaldehyde at 25^{o}C (10 min) and blocked (10% donkey serum, 0.1% Triton-X-100 in PBS) for 1 hour. Slides were incubated with anti-bodies against beta-galactosidase (1:200 dilution in blocking solution overnight at 4^{o}C) followed with Texas-red conjugated secondary antibodies (1:100 dilution in blocking solution at 25^{o}C). Slides were then incubated with FITC-conjugated anti-α tubulin antibodies (1:200 dilution) overnight and counterstained with DAPI (0.1 μg/ml) to visualize mitotic cells during immuno-fluorescence analysis.

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FIGURE LEGENDS

Figure 1 Bub1 mutant females are subfertile. (**A**) Gene structure of the murine Bub1. The relative insertion site is indicated by an arrow. (**B**) Bub1 protein organization. The functional domains within the Bub1 protein are shown and the relative trap insertion site is indicated by an arrow. (**C**) Bub1-βgeo mutant protein organization. The replacement of the C-terminal amino acids by the β-galactosidase-neomycin (β-geo) fusion peptide is shown. (**D**) Expression of *Bub1* in 10.5 d.p.c. embryos using the β-geo reporter mediated conversion of X-gal substrate. Photograph shows a Bub1 heterozygote embryo (stained blue on the left) and a control WT littermate embryo obtained from a WT female mouse crossed to a $Bub^{+/m}$ male. (**E**) Localization of the mutant Bub1-β-geo fusion peptide to kinetochores in $Bub^{+/m}$ mouse embryonic fibroblasts (MEFs). Immuno-fluorescence analysis of a metaphase cell stained with FITC-conjugated α-tubulin antibodies to visualize microtubules (green), anti-β-galactosidase antibodies bound to Texas-red conjugated secondary antibodies (red) and 4,6-diamidino-2-phenylindole (DAPI, stained as blue to visualize DNA) is shown above. Note the dotted appearance of the Bub1-β-geo fusion protein signals at the chromosome-microtubule junctions that indicate kinetochore localization. Inset shows the same image without the DAPI stained DNA for better visualization of the kinetochore localization of the Bub1-β-galactosidase fusion protein. (**F**) The average litter size of various intercrosses obtained from $Bub1^{+/m}$ and WT mice are shown. The bars indicate the standard error within the data set obtained from each intercross and n refers to the total number of crosses from each intercross. The differences between the litter sizes obtained from the heterozygous females were significantly different from the intercrosses obtained from the WT females (*$P < 0.0001$, student t-test).

Figure 2. Metaphase II oocytes and zygotes from Bub1$^{+/m}$ females have abnormal chromosome numbers. 8-16-week-old WT or Bub1$^{+/m}$ females were mated with B6C3F1 males and zygotes collected ~30 hr after the induction of superovulation. Oocytes were collected from females that did not mate ~20 hr after the induction of superovulation. (**A**) Normal metaphase II oocyte with 20 dyads from a wild type female. (**B**) Metaphase II oocyte from a Bub1$^{+/m}$ female with a total of 21 chromosomes. Arrowheads indicate single chromatids. (**C**) Zygote from a Bub$^{+/m}$ female with 20 paternal chromosomes on the left and 23 maternal chromosomes on the right. (**D**) Comparisons of aneuploidy frequencies in metaphase II oocytes and zygotes from Bub$^{+/m}$ females showing similar levels of hypohaploid and hyperhaploid maternal complements before and after fertilization. (**E**) Age-dependent increase in the incidences of premature sister chromatid separation (PSCS) and hyperploid oocytes and zygotes in Bub1$^{+/m}$ female of different ages. *P<0.002 (Chisquare).

Table 1. Comparison of implantation rates and embryo phenotypes in Bub1 $^{+/m}$ and wild type females between 6.5 to 9.5 days post coitus.

Parent genotype (Total implantation sites analyzed)	Normal embryos	Arrested or abnormal embryos	Complete loss of embryo	Implantation sites (Average)
Het ♀ X Het ♂ (n=24)	29%	25%	46%	6.0
Het ♀ X WT ♂ (n=23)	22%	65%	13%	7.6
WT ♀ X Het ♂ (n=19)	95%	0	5%	9.5
WT ♀ X WT ♂ (n=10)	90%	0	10%	10

Table 2. Aneuploidy frequencies in germ cells of Bub1 heterozygous female mice.

Female strain	Male strain	Total cells	Maternal chromosome number (%)			
			<20	20	21-25	40
Metaphase II oocytes						
Bub1 WT		75	2 (2.7)	72 (96.0)	1 (1.3)	0 (0.0)
$Bub1^{+/m}$		78	25 (32.1)*	19 (24.3)	34 (43.6)*	0 (0.0)
Zygotes						
Bub1 WT	B6C3F1	70	3 (4.3)	60 (85.7)	4 (5.7)	3 (4.3)
$Bub1^{+/m}$	B6C3F1	47	15 (31.9)*	9 (19.1)	23 (48.9)*	0 (0.0)

*P<0.001 vs Bub1 WT females (Chi-square).

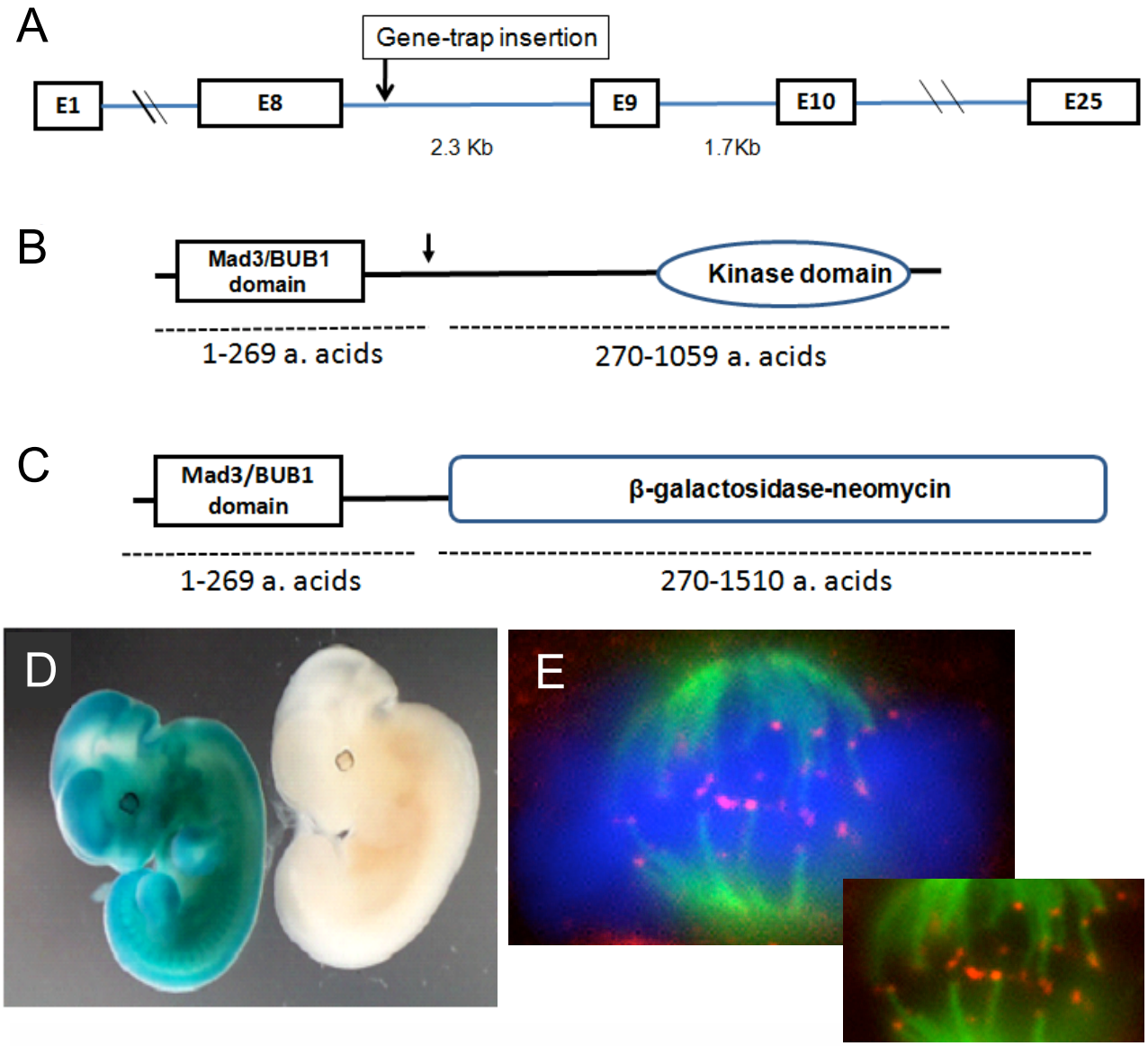
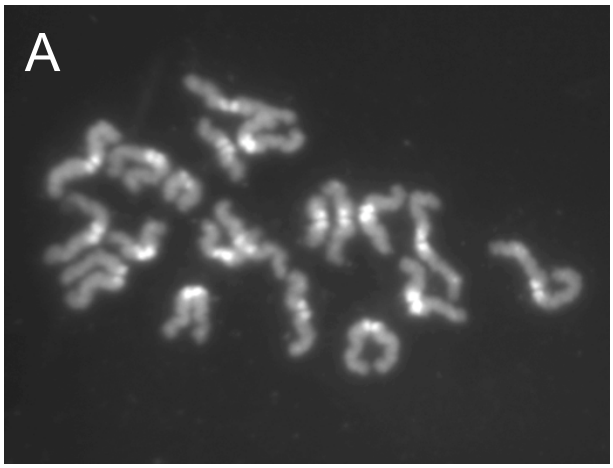
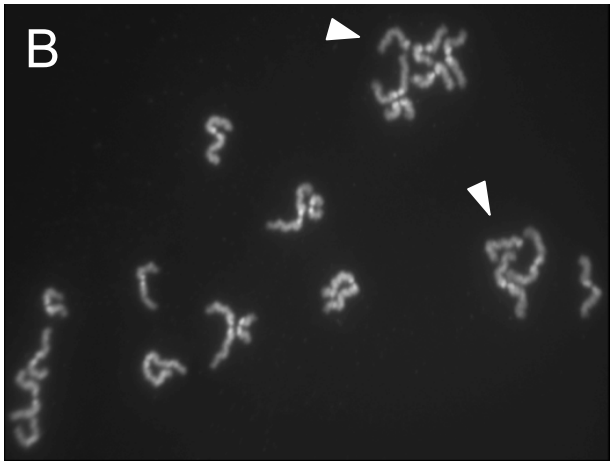

A
Gene-trap insertion
E1
E8
E9
E10
E25
2.3 Kb
1.7Kb
B
Mad3/BUB1 domain
Kinase domain
1-269 a. acids
270-1059 a. acids
C
Mad3/BUB1 domain
β-galactosidase-neomycin
1-269 a. acids
270-1510 a. acids
D
E
F
Average Litter Size
12
10
8
6
4
2
0
*
*
(n = 4)
WT ♀ x WT ♂
(n = 19)
WT ♀ x Het ♂
(n = 7)
Het ♀ x WT ♂
(n =11)
Het ♀ x Het ♂
(n = 3)
SA - ♀ x WT ♂

Figure 1


A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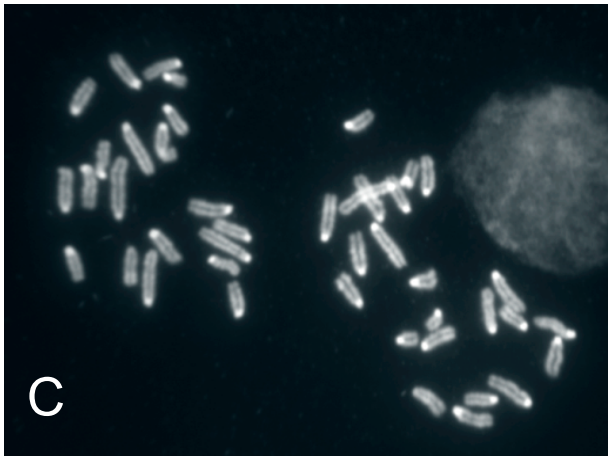
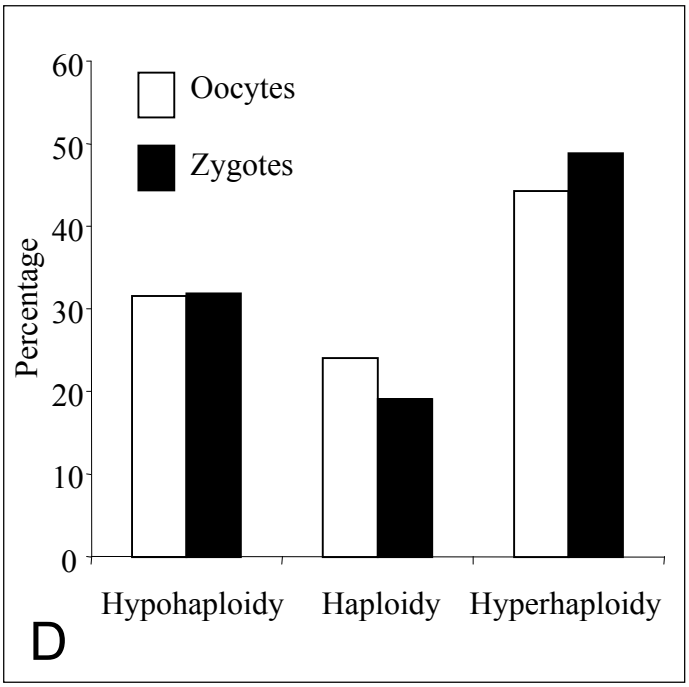

C


60
50
40
30
20
10
0
Percentage
Oocytes
Zygotes
Hypohaploidy
Haploidy
Hyperhaploidy
D

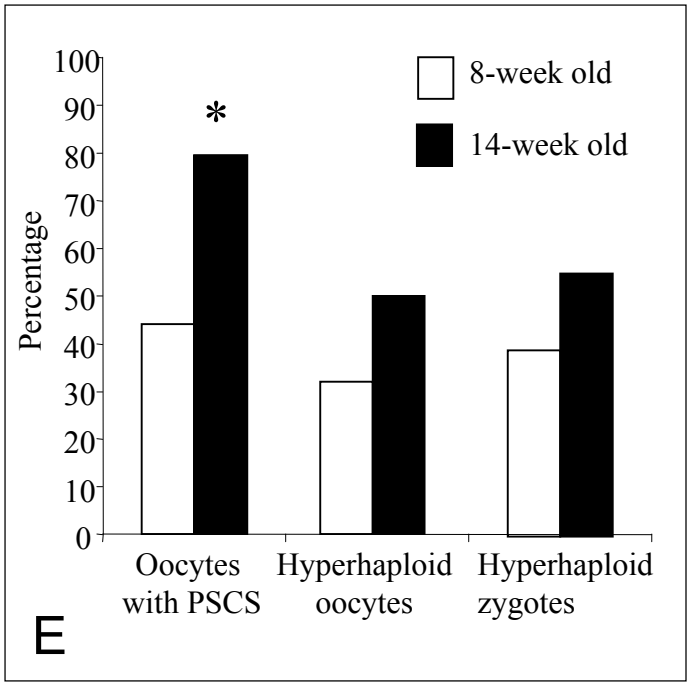

100
90
80
70
60
50
40
30
20
10
0
Percentage
*
8-week old
14-week old
Oocytes with PSCS
Hyperhaploid oocytes
Hyperhaploid zygotes
E

Figure 2

SUPPLEMENTARY INFORMATION

HETEROZYGOSITY FOR A BUB1 MUTATION CAUSES FEMALE-SPECIFIC GERM CELL ANEUPLOIDY IN MICE

Leland et al.

Supplementary Table 1. Chromosome number distribution in oocytes and zygotes from Bub1 wild type (WT) and ($^{+/m}$) heterozygous females.

Chromosome number[a]	Chromosome kyrotype[b]	Bub1 WT oocytes	Bub1$^{+/m}$ oocytes	Bub1 WT zygotes	Bub1$^{+/m}$ zygotes
14	12c + 4m		1		
14.5					
15					
15.5	15c + 1m		1		
16	16c				1
16.5					
17	17c		1	2	1
17.5	17c + 1m		3		
18	18c		6		7
18.5	18c + 1m		3		
19	18c + 2m 19c	 2	1 6	 1	 6
19.5	19c + 1m		3		
20	18c + 4m 19c + 2m 20c	 4 68	1 1 17	 60	 9
20.5	19c + 3m 20c + 1m	 1	2 7		
21	18c + 6m 20c + 2m 21c		1 1 6		 13
21.5	19c + 5m 20c + 3m 21c + 1m		1 2 3		
22	21c + 2m 22c		2 2	 4	 5
22.5	22c + 1m		2		
23	23c				2
23.5	23c + 1m		1		
24	23c + 2m 24c		1		 2
24.5	20c + 9m 22c + 5m		1 1		
25	24c + 2m 25c		1		 1
40				3	
Total		75	78	70	47

[a]For the maternal complement.
bWhole chromosomes or dyads are indicated by ‘c’; while single chromatids or half chromosomes (or monads) are indicated by ‘m’.

Supplementary Table 2. Aneuploidy frequencies in germ cells of Bub1 heterozygote male mice. Bub1 +/- males were mated with B6C3F1 females and zygotes collected ~30 hr after the induction of superovulation. Bub1 WT males from the same litters of the heterozygotes males were used as controls. Metaphase II spermatocytes were collected from testis from 4 of the same males that had been used for the mating experiments.

Female strain	Male strain	Total cells	Paternal chromosome number			
			<20	20	21-25	40
Metaphase II spermatocytes						
	Bub1 WT	127	7 (5.5)	120 (94.5)	0 (0.0)	0 (0.0)
	$Bub1^{+/m}$	109	4 (3.7)	105 (96.3)	0 (0.0)	0 (0.0)
Zygotes						
B6C3F1	Bub1 WT	157	8 (5.1)	148 (93.0)	1 (0.6)	0 (0.0)
B6C3F1	$Bub1^{+/m}$	109	2 (1.8)	106 (96.3)	1 (0.9)	0 (0.0)

RNA isolation and RT-PCR protocol

Total RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen) from various mouse tissues from adult mice. First strand cDNA synthesis was performed with 2.5µg of total RNA, random hexamers, and M-MLV reverse transcriptase (Promega, Madison, WI). A similar amount (2.5µg) of total RNA was subjected to the above mentioned conditions in the absence of M-MLV reverse transcriptase. For PCR assays, 2µl of the reaction mixture (from a total of 40 µl) obtained from the first strand cDNA synthesis reaction was used. The following primer pairs were used for the RT-PCR analyses:

<u>WT Bub1:</u>

Bub-1 (Forward): 5'-AAT GCT CTG TCA GCT CAT CTG TGG-3'

Bub-1 (Reverse): 5'-AGA AGC AGG AAG GTC CTT GTG TGA-3'

<u>Bub1-β-geo:</u>

Bub-1 (Forward): 5'-AAT GCT CTG TCA GCT CAT CTG TGG-3'

1665r (Reverse): 5'-TTT CCC AGT CAC GAC GTT GT-3'

<u>Ribosomal protein L38</u>:

RPL38 (forward): 5'-TTC GGT TCT CAT CGC TGT GAG TGT-3'

RPL38 (reverse): 5'-TCT TGA CAG ACT TGG CAT CCT TCC-3'.

Reversal of gene-trap

The presence of a floxed splice acceptor (SA with lox-P sites on either side) allowed us to generate mice that were lacking the SA sequence (indicated as SA-) within the gene-trap. To generate SA- mice, we crossed the Bub1 mutants to transgenic mice that expressed Cre-recombinase under the control of mouse

protamine 1 promoter for Cre-mediated excision of the splice acceptor sequences and re-expression of Bub1 in spermatocytes (1). Female mice lacking the splice acceptor sequence were generated from germline compound mutants using this strategy. As shown in supplementary figure 1 (last three lanes), loss of the splice acceptor results in a smaller PCR product (that was further confirmed by sequencing) with primers that are specific for the gene-trap.

Beta-galactosidase staining of embryos

Wild type and $Bub1^{+/m}$ embryos were fixed with 1% paraformaldehyde and stained in a solution containing X-gal (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.1 M phosphate buffer, pH 7.3). Genotypes of embryos were determined from genomic DNA isolated from yolk sacs.

Immuno-fluorescence analysis of Bub1 mutant MEFs

$Bub1^{+/m}$ mouse embryonic fibroblasts were grown on glass cover slides and fixed with 4% formaldehyde at 25^{o}C (10 min) and blocked (10% donkey serum, 0.1% Triton-X-100 in PBS) for 1 hour. Slides were incubated with anti-bodies against beta-galactosidase (1:200 dilution in blocking solution overnight at 4^{o}C) followed with Texas-red conjugated secondary antibodies (1:100 dilution in blocking solution at 25^{o}C). Slides were then incubated with FITC-conjugated anti-α tubulin antibodies (1:200 dilution) overnight and counterstained with DAPI (0.1 μg/ml) to visualize mitotic cells during immuno-fluorescence analysis.

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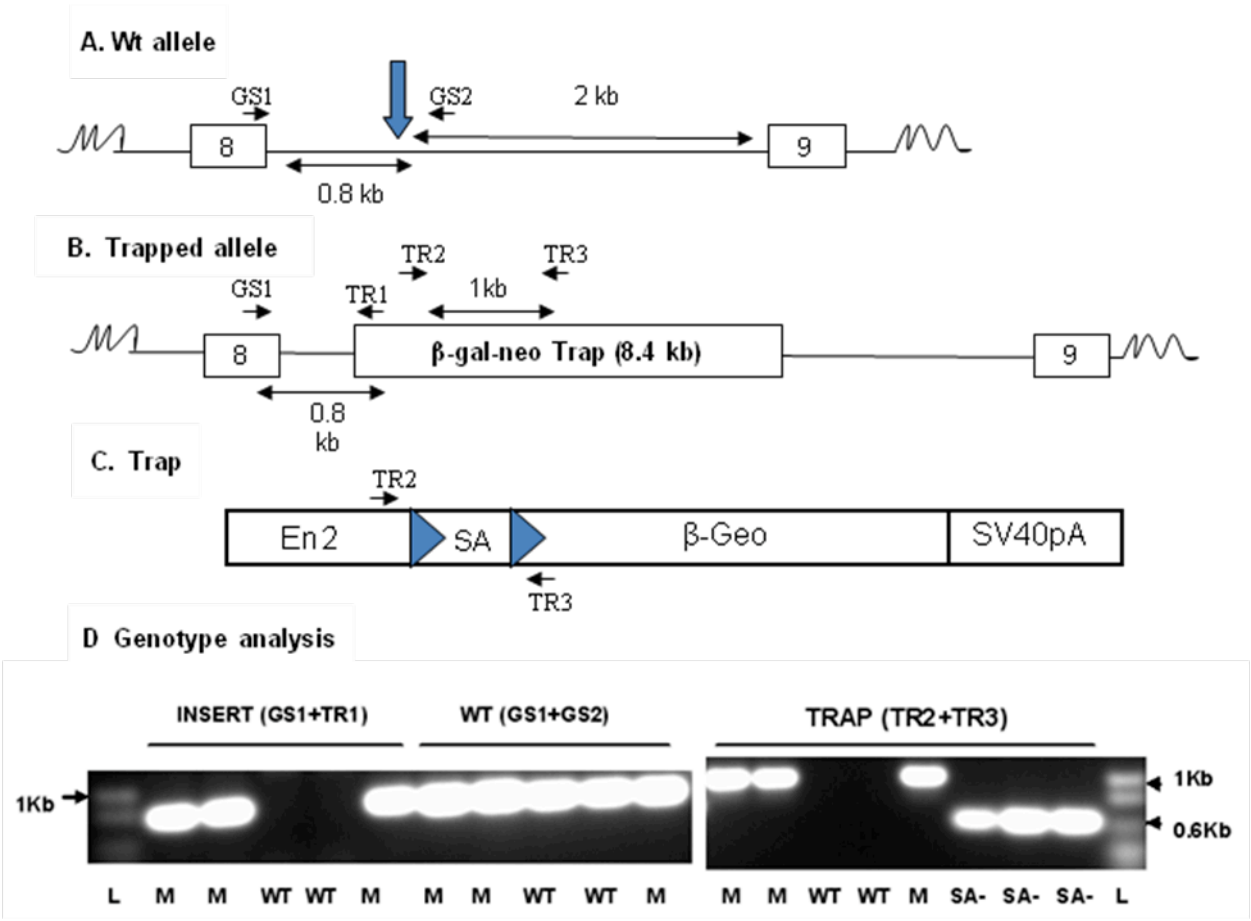
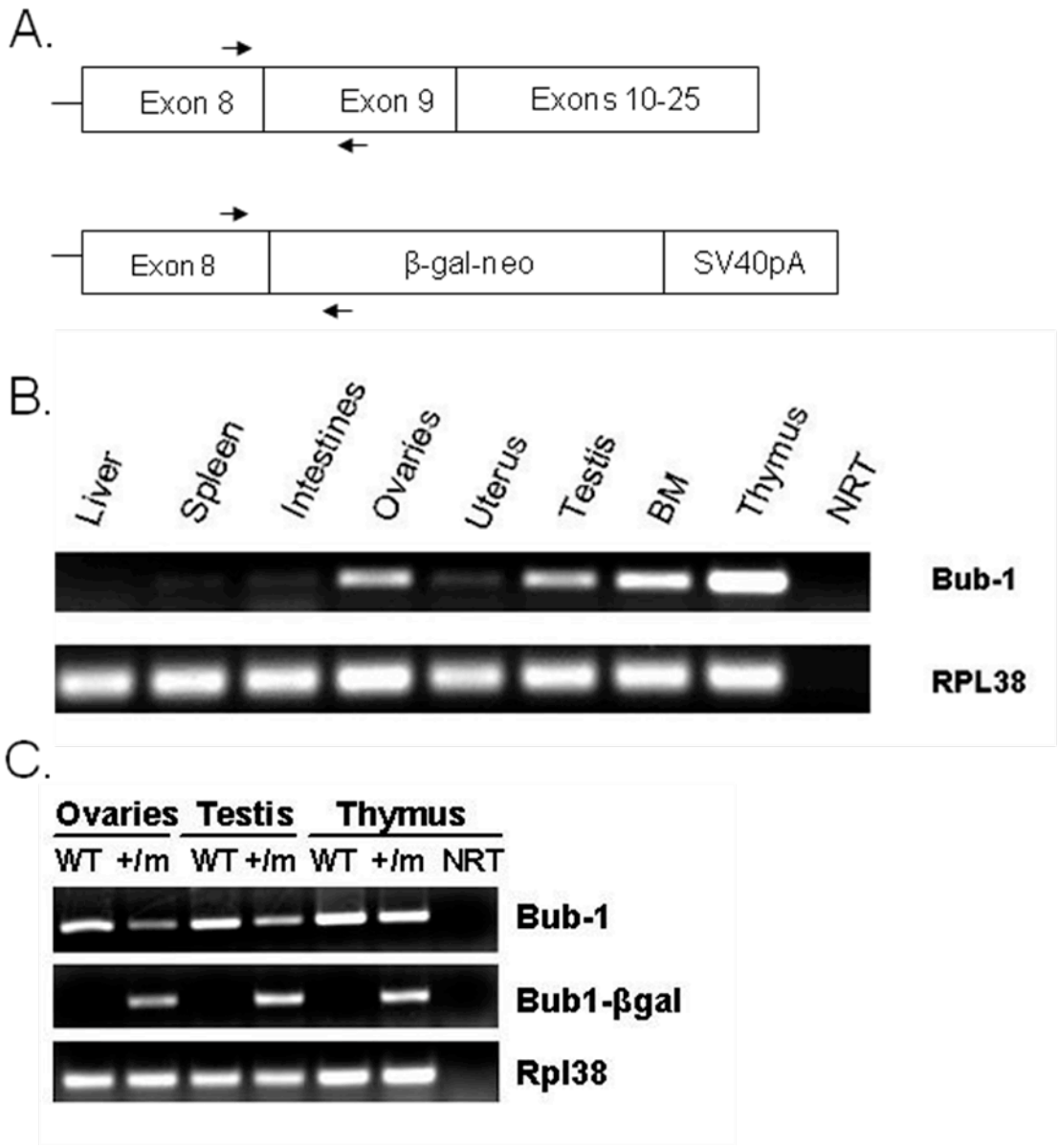
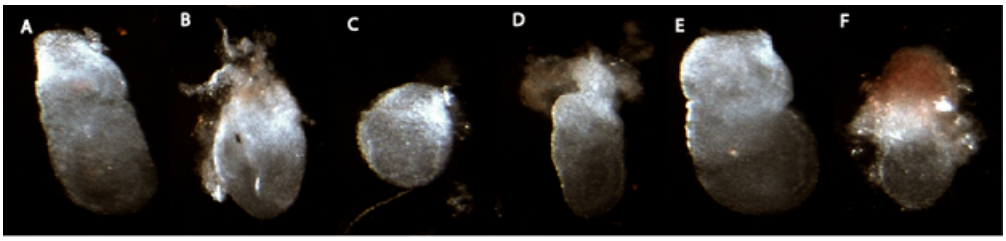


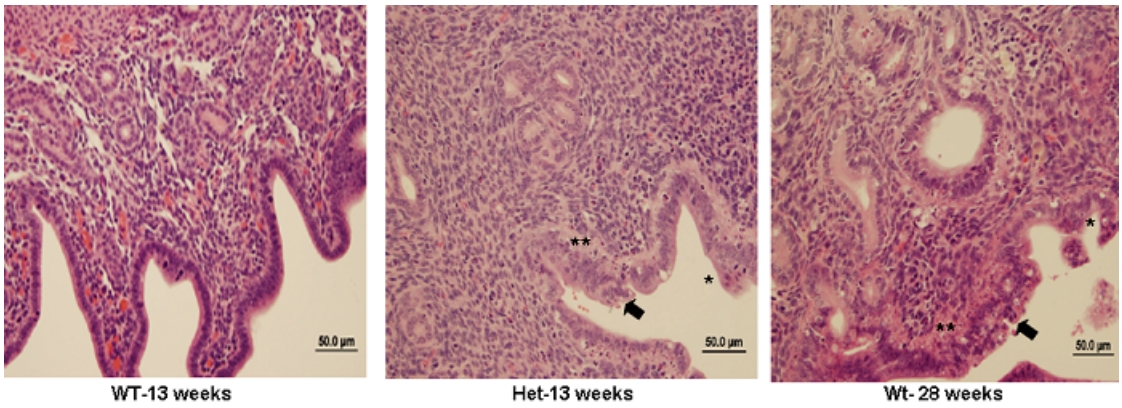
Supplementary Figure 1: Schematic representation of the Bub1 gene disruption and genotype analysis of mutant offspring. (A and B) Structure of the wt and mutant alleles at the gene-trap insertion site. (C) Organization of the genetrap containing a portion of *engrailed 2* intron (En2) and its floxed (represented by triangles) splice acceptor (SA), the beta-galactosidase-neomycin fusion gene, and a SV40 poly-adenylation signal. The location of the various primers used for the genotype analysis are represented by arrows (GS1, GS2: Bub1 specific primers; TR1-TR3, genetrap specific primers). The approximate length of the insertion site within the intron and PCR products are indicated by lines with arrow heads. (D) Genotype analysis of Bub1 mutant mice. Representative results of the PCR based genotype analysis are shown for the indicated primer pair shown within brackets. The last three lanes refer to the splice acceptor negative (SA-) offspring obtained from intercrosses between Cre-transgenic-Bub1 mutants. The loss of splice acceptor leads to a smaller PCR product in the SA- offspring obtained from Cre-recombinase positive heterozygotes (D, last three lanes).



Supplementary Figure 2: Expression analysis of Bub1 and Bub1-βgal fusion mRNA in WT and mutant mouse tissues. (A) Structure of the WT (top) and mutant mRNA (bottom) species are shown. The relative positions of primers used for RT-PCR analysis are represented by arrows. (B) Expression of Bub1 mRNA in various mouse tissues. Rpl38, ribosomal protein 38. (C) Relative expression of WT and mutant Bub1 mRNAs in Bub1 +/m mice in specific tissues. The primers used for the RT-PCR analyses are indicated in panel A.

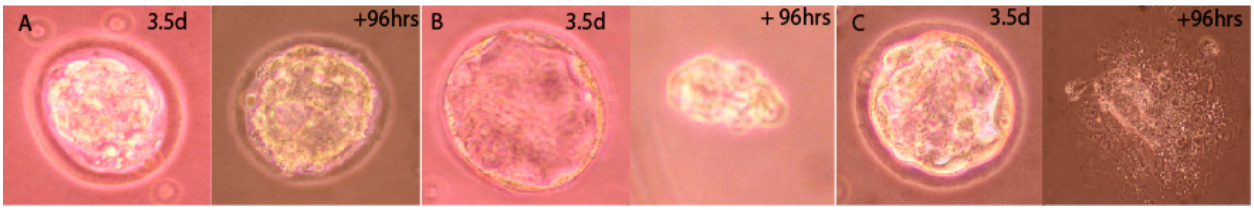


Supplementary Figure 3: Bub1 mutation leads to embryonic arrest. A representative example of embryonic arrest phenotypes obtained from a cross between a mutant female and a wild type male at 7.5 d.p.c. is shown above. A total of eight implantation sites were noted of which two were empty indicating loss of embryos after implantation. In the above example embryos in panels A and E were scored as normal with developmental features appropriate for E7.5 and B-D and F were scored as arrested.



WT-13 weeks Het-13 weeks Wt- 28 weeks

Supplementary Figure 4: Comparison of uteri from diestral Bub1 mutant and WT females. H&E stained sections of uteri from age matched mutant and wild type females are shown above. Note the similarities between the younger mutants and older wild type female that show a mild disarray of luminal epithelial cells (**à**), rare luminal epithelial proliferation, multifocal luminal epithelial apoptosis(*), multifocal superficial stromal necrosis (**), mild neutrophilic endometrial stromal infiltrates, moderate endometrial stromal cellular density, mild deep glandular dilation and fluid retention. In the wild type section the epithelial apoptosis, multifocal superficial stromal necrosis, and neutrophilic endometrial infiltrates are not as prominent.



Supplementary Figure 5: Bub1 mutation leads to embryonic arrest in pre-implantation embryos. A representative example of embryonic arrest phenotypes of blastocysts obtained from Bub1 mutant females crossed to WT males is shown above. A total of six blastocysts were cultured in ES cell medium and only one of the blastocysts (shown in C) grew in culture while others (A, B and data not shown) were arrested.