Impaired Bub1 Function In vivo Compromises Tension-Dependent Checkpoint Function Leading to Aneuploidy and Tumorigenesis

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Abstract

Bub1 is a serine/threonine kinase originally described as a core component of the spindle assembly checkpoint (SAC) mechanism in yeast. Bub1 binding at kinetochores has been reported to be required for SAC function and localization of other SAC components. A proper SAC is believed to be essential for murine embryonic development, as all previously described null mutations in SAC components in mice cause embryonic lethality. We produced mice harboring a Bub1 mutant allele lacking exons 2 and 3, resulting in a hypomorphic mutant expressed at <5% of wild-type levels. Despite this significant reduction, homozygous mutant animals are viable on a mixed 129P2/B6 or FVB background but display increased tumorigenesis with aging, whereas mice with a C57Bl/6J background die perinatally. Bub1 mutant murine embryonic fibroblasts (MEFs) display defects in chromosome congression to the metaphase plate, severe chromosome missegregation, and aneuploidy accompanied by high levels of premature senescence. Mutant MEFs have a robust SAC in response to nocodazole treatment but an impaired response to Taxol. Mutant MEFs also show reduced kinetochore localization of BubR1, but not of Mad2. The significant reduction in SAC response to Taxol, but not nocodazole, coupled with the reduced binding of BubR1, but not Mad2, indicates that Bub1 is particularly critical for the SAC response to a lack of tension on kinetochores. Thus, Bub1 is essential for proper chromosome segregation, a defect that can lead to severe phenotypes, including perinatal lethality and a predisposition to cancer. [Cancer Res 2009;69(1):45–54]

Introduction

Proper chromosome segregation is critical at every cell division to prevent aneuploidy, a condition associated with aggressive cancer, birth defects, and infertility (reviewed in ref. 1). The spindle assembly checkpoint (SAC) is a major guardian of mitotic fidelity. The SAC works as a checkpoint and timing mechanism to delay sister chromatid separation and anaphase onset until all chromosomes achieve bipolar attachment to microtubules at the metaphase plate. The SAC can be activated by lack of kinetochore-microtubule attachment or loss of tension on the kinetochore microtubules, thus allowing it to respond to the major types of aneuploidy-generating defects that arise during mitotic progression (reviewed in refs. 1–3). The SAC blocks anaphase entry by inhibiting the APC/Cdc20 complex, a multisubunit ubiquitin ligase that induces anaphase by promoting proteosomal degradation of mitotic substrates. The mechanism of APC/Cdc20 inhibition by the SAC is multilayered, including inhibitory binding of SAC components Mad2 and/or BubR1 to Cdc20 and Bub1-mediated inhibitory phosphorylation of APC/Cdc20 (3). Distinct inhibitory pathways may be activated by different conditions (e.g., loss of microtubule attachment versus loss of tension), although it remains unclear whether the different regulatory mechanisms function in separate pathways or as part of an integrated signaling system. The relative contributions of each mechanism to overall mitotic fidelity are also not well understood.

The spindle checkpoint machinery is encoded by a core group of genes originally identified in Saccharomyces cerevisiae, including Mad1–3, Bub1, Bub3, and Mps1. Yeasts lacking core SAC components grow normally under unperturbed conditions yet display increased rates of chromosome missegregation and are unable to grow in the presence of spindle poisons, such as nocodazole (4–6). Studies of SAC function in mammals have been limited by the fact that murine null mutations in core SAC components, including Bub3, Mad1, Mad2, and BubR1, cause very early embryonic lethality, precluding analysis of embryonic fibroblasts or later developmental stages in null mutants (7–10). However, mouse embryonic fibroblasts (MEF) heterozygous for Mad2, Bub3, and BubR1 displayed haploinsufficiency, resulting in higher levels of mitotic abnormalities (9, 11, 12). Furthermore, whereas heterozygous animals were viable in each case, they displayed increased age-related spontaneous (11) or carcinogen-induced (9, 13) tumorigenesis. Mice with a hypomorphic BubR1 allele that reduced BubR1 levels to ~11% of wild type (wt) were viable but displayed cellular senescence, growth retardation, and progeroid features without a significant increase in tumor formation (12). These studies indicate that the SAC is essential for early embryonic development in mammals but that the phenotypes obtained from incomplete SAC impairment may vary across a broad range, depending on the depleted component and the degree of depletion. SAC components may also have SAC-independent functions that contribute to the observed phenotypes in some cases (14–16).

Despite its identification as a core spindle checkpoint component in yeasts, the functions of Bub1 have not been fully elucidated. Bub1 is a serine/threonine kinase that localizes to kinetochores during mitosis and mediates efficient kinetochore localization of other SAC components. Bub1 deletion in yeasts causes high chromosome missegregation rates under normal conditions. Mice harboring a Bub1 mutant allele lacking exons 2 and 3, resulting in a hypomorphic mutant expressed at <5% of wild type, are viable on a mixed 129P2/B6 or FVB background but display increased tumorigenesis with aging, whereas mice with a C57Bl/6J background die perinatally. Bub1 mutant murine embryonic fibroblasts (MEFs) display defects in chromosome congression to the metaphase plate, severe chromosome missegregation, and aneuploidy accompanied by high levels of premature senescence. Mutant MEFs have a robust SAC in response to nocodazole treatment but an impaired response to Taxol. Mutant MEFs also show reduced kinetochore localization of BubR1, but not of Mad2. The significant reduction in SAC response to Taxol, but not nocodazole, coupled with the reduced binding of BubR1, but not Mad2, indicates that Bub1 is particularly critical for the SAC response to a lack of tension on kinetochores. Thus, Bub1 is essential for proper chromosome segregation, a defect that can lead to severe phenotypes, including perinatal lethality and a predisposition to cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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References

growth conditions, in addition to lethality under perturbed conditions (4–6). Studies in vertebrate systems have been confined to biochemical and cell culture models and have produced some conflicting results about Bub1 roles in mitotic regulation. Bub1 is essential for spindle checkpoint activity in Xenopus extracts, and the bulk of this activity requires the NH2 terminus, with minor requirements for the COOH terminal kinase functions (17, 18). In cultured mammalian cell lines, Bub1 reduction via RNA interference (RNAi) produced conflicting results. In one case, Bub1 was surprisingly dispensable for the SAC function but was required for chromosome congression (15). An independent study found that Bub1 was required for a functional SAC response to nocodazole while confirming the role in chromosome congression (16). These differences are difficult to reconcile but may stem from distinct levels of Bub1 depletion and/or differences in cell lines used for the studies. More recent studies have revealed a role for Bub1 in meiotic and mitotic sister chromatid cohesion via interaction with shugoshin (19, 20). Finally, compromised Bub1 function has been indirectly associated with tumorigenesis, indicating an important need to understand the molecular links associated with Bub1 function, mitotic regulation, and tumor suppression (21–24).

To better understand Bub1 functions and the consequences of Bub1 loss in vivo, we developed a conditional Bub1 mutation in mice. We report here that this mutation is hypomorphic, causing a severe reduction in Bub1 levels. Mice homozygous for the Bub1 hypomorph mutation are viable on mixed 129P2/B6 and FVB genetic backgrounds but display high rates of tumorigenesis. The mutant phenotype is more severe on a C57Bl/6J background, where genetic backgrounds, but display high rates of tumorigenesis. The hypomorph mutation are viable on mixed 129P2/B6 and FVB severe reduction in Bub1 levels. Mice homozygous for the mice. Wereportherethatthismutationishypomorphic,causinga defects ranging from early lethality to tumorigenesis.

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Materials and Methods

**Bub1 gene targeting and mice.** Bub1 genomic BAC clones were isolated from a 129SvEv library by hybridization screening and subsequent PCR verification. The bacterial strain EL350 (25) was used for ‘‘recombinering’’ to subclone a 13-kb fragment encompassing Bub1 exons 1 to 6 into a pUC19-based plasmid, including a phosphoglycerate kinase–thyminidine kinase gene (a gift of R. Thresher, University of North Carolina Animal Models Core Facility). A loxP site was inserted in intron 1 by recombineering a loxP-flanked zeocin cassette, followed by Cre-mediated cassette removal in EL350. A loxP and FRT-flanked neomycin resistance cassette was subsequently inserted into intron 3. The targeting vector was linearized and used for gene targeting in E14 ES cells (129P2/OlaHsd strain). ES cell targeting was verified by PCR and Southern blot, and correctly targeted clones were injected into C57Bl/6J blastocysts for chimera generation. Chimeric males were bred to C57Bl/6J females, and germline transmission of the Bub1neo allele was verified by PCR. The Bub12/2 and Bub1fl alleles were generated by crossing the Bub1neo allele with an X-linked CMV-Cre strain (26). Bub12/2 viability phenotypes were studied on three genetic backgrounds: 129/B6 (F2-F4), B6N4+, and FVB/NJ. For the FVB study, the Bub12/2 allele was independently derived from the Bub1neo allele by crossing (FVB/N)Bub1neo/J animals to an FVB-2-3- actin-Cre deleter strain.

The Cre transgene was generated by segregation before intercrossing Bub12/2 animals. MEF studies were performed with MEFs derived from embryos on the C57Bl/6J background (B6N+). All mice were maintained according to the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

**Bub1fl allele.** Bub1 gene-trapped ES cell clone XG453 was obtained from Bay Genomics. The gene trap is produced by a splice-acceptor-lacZ cassette within Bub1 intron 7. XG453 ES cells were injected into C57Bl/6J blastocysts, and resulting chimeras were bred to C57Bl/6J females and screened for germline transmission by PCR. Animals used in experiments reported here were backcrossed one to two generations to C57Bl/6J. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Cell culture.** MEF lines were generated from e13.5–e14.5 using standard protocols. MEFs were cultured at 5% CO2 in media containing DMEM–H with 10% fetal bovine serum, 2 mmol/L glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 55 μmol/L 2-mercaptoethanol and were passaged every 2 d by plating 500,000 viable cells. All experiments with MEFs were performed at passages 3 to 4, unless otherwise stated. Growth curves were generated by counting total number of viable cells at each passage. Drug treatments were used at the following concentrations: nocodazole (Sigma-Aldrich), 200 ng/mL; paclitaxel (Sigma-Aldrich), 1 μmol/L; MG132 (Calbiochem), 10 μmol/L.

**Spindle checkpoint analysis.** Asynchronously growing MEFs were treated with 200 ng/mL nocodazole for indicated times, trypsinized, fixed for 20 min in 1% formaldehyde, spun down, and resuspended in 70% cold ethanol. For flow cytometry, cells were stained with anti–phosphorylated histone H3 (Upstate Biotechnology) at 1:500 and Alexafluor 488 (Invitrogen) at 1:1,000, followed by propidium iodide. Cells (30,000) were analyzed on a Becton Dickinson FACScan, and the number of phosphorylated histone H3–positive cells was measured as a percentage of total cells.

**Immunofluorescence.** Coverslips were fixed in 2% formaldehyde, permeabilized in PBS + 0.5% Triton X-100 (PBS-TX), and washed in PBS. After blocking for 30 min in PBS-TX plus 5% nonfat dried milk (NFDM) or bovine serum albumin (BSA), coverslips were incubated overnight with primary antibodies. Antibodies were used at the following concentrations: rabbit anti–phosphorylated histone H3, 1:500 (Upstate Biotechnology); anti–β-tubulin, 1:250 (Tub2.1, Sigma-Aldrich); sheep anti-Bub1, 1:500 (Steve Taylor); rabbit anti-Mad2, 1:500 (E.D. Salmon); rabbit anti-BubR1, 1:500 (BD Biosciences); human anti-centromere, 1:500 (ACA, Antibodies Incorporated). After washes with PBS-TX, coverslips were incubated for 1 h with appropriate secondary antibodies (Alexafluor 488 or 594) at 1:500 in PBS-TX plus 5% NFDM or BSA, washed with PBS-TX, stained with 4,6-diamidino-2-phenylindole (DAPI), and mounted with Fluoromount G (Electron Microscopy Sciences). Analysis was carried out on a Leica DMRXA microscope. See supplementary data for specifics on kinetochore localization methods.

**Karyotyping.** MEFs at passage 5 were treated with Karyomax colcemid (Invitrogen) for 4 h, trypsinized, resuspended in warm 75 mmol/L hypotonic KCl buffer, and incubated for 20 min. Cells were washed at least four times in Carnoy’s buffer before being resuspended in fresh Carnoy’s fix, dropped into cold, ethanol-washed slides, and stained with DAPI. Images of single-cell spreads were acquired on a Leica DMRXA microscope with a 63× objective. Chromosomes from distinct individual cells were counted, and chromosome numbers >70 for a single cell being discarded as endoreduplication.

**Senescence assay.** MEFs grown on coverslips at indicated passages were fixed in 1% formaldehyde and 0.1% glutaraldehyde for 5 min before being treated overnight (14–18 h) in senescence-associated β-galactosidase assay buffer (see ref. 27). Cells were counterstained with DAPI, and cells, staining positive for senescence, were counted as a percentage of total cells.

**Western blotting and immunoprecipitation.** Subconfluent MEFs were treated with 200 ng/mL nocodazole for 6 h, lysed in modified radioimmunoprecipitation assay buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked for 30 to 60 min in PBS + 0.1% Tween 20 (PBS-T) with 5% NFDM, incubated overnight at 4°C with anti-Bub1 at 1:1,000 (a kind gift from Steve Taylor),
Bub1f+/

the clones were used to generate chimeric founders that transmitted A recombination (Supplementary Fig. S1 intron 3, thus allowing deletion of exons 2 and 3 by Cre-mediated

A targeting construct was designed with a loxP

A exons 2 and 3 was predicted to cause a null allele by creating a mutation of the murine Bub1 gene by homologous recombination in ES cells. Based on available sequence information, deletion of a portion of the NH2 terminal conserved domain.

and diluted in PBS-T + 5% NFDM. For immunoprecipitation, plates of subconfluent cells were treated with 200 ng/mL of nocodazole for 6 h and 10 μM of MG132 for 2 h. Cells were collected from plates as above. Total protein (500 μg) was incubated overnight at 4°C with 50 μL anti-Bub1 sera (28).

Results

Generation of the Bub1A2-3 allele. We generated a conditional mutation of the murine Bub1 gene by homologous recombination in ES cells. Based on available sequence information, deletion of exons 2 and 3 was predicted to create a null allele by creating a framsheft early in exon 4, leading to a premature stop codon in exon 6 (Fig. 1A). The targeting construct was designed with a loxP site in intron 1 and a loxP-flanked neomycin resistance cassette in intron 3, thus allowing deletion of exons 2 and 3 by Cre-mediated recombination (Supplementary Fig. S1A). Correctly targeted ES cell clones were used to generate chimeric founders that transmitted the Bub1M allele through the germline (Supplementary Fig. S1B). Bub1mice were crossed to a germline Cre deleter strain (see Materials and Methods) to produce the Bub1flox allele (Bub1F) by removal of the neomycin resistance cassette and the Bub1 allele by removal of the neo cassette and exons 2 and 3. Bub1M, Bub1F, and Bub1A2-3 mice are viable and fertile. Genotypes were confirmed by PCR (Supplementary Fig. S1C).

Bub1A2-3 allele is dependent on genetic background. Homozygous deletion of spindle checkpoint genes Mad2, Bub3, and BubR1 in mice causes embryonic lethality at e4.5-7.5 during rapid expansion of the inner cell mass (7, 8, 10). Bub1A2-3 mice were intercrossed to test whether Bub1 loss also causes early embryonic lethality. Surprisingly, Bub1A2-3 animals were obtained at near Mendelian ratios from intercrosses on a mixed 129P2/B6 genetic background (Table 1). However, intercrosses performed after backcrossing four or more generations to C57Bl/6 yielded live Bub1A2-3 animals at only 20% of expected Mendelian frequency (Table 1), and nearly all live-born (B6)Bub1A2-3 mice died perinatally. (B6)Bub1A2-3 mice seemed to breathe and nurse normally but displayed running and failure to thrive in the early postnatal period. The average weight of Bub1A2-3 animals was 1.19 ± 0.06 g versus 1.41 ± 0.13 g for wt (P < 0.01) on day of birth. To date, only two homozygous animals survived to weaning at 21 days. To determine when the remaining 80% of (B6)Bub1A2-3 mice die, embryos were generated from (B6)Bub1A2-3 intercrosses at E13.5-14.5 and E17.5-18.5. At both E13.5-14.5 and E17.5-18.5, (B6)Bub1A2-3, (B6)Bub1A2-3, and (B6)Bub1A2-3 embryos were recovered at Mendelian ratios (Table 1). Histologic analysis of sections from e14.5 and e18.5 embryos indicated that the Bub1A2-3 allele is modified by the genetic background. The perinatal lethal survival is dependent on genetic background. The perinatal lethal survival is dependent on genetic background. The perinatal lethal survival is dependent on genetic background. The perinatal lethal survival is dependent on genetic background.
with results from inactivation of other spindle checkpoint genes in mice, strongly suggests that the Bub1\textsuperscript{2-3} allele retains partial function.

The Bub1\textsubscript{2-3} allele is hypomorphic. The recovery of viable Bub1\textsubscript{2-3} mice suggested the Bub1\textsuperscript{2-3} allele may be hypomorphic rather than null. To test this hypothesis, we obtained mice harboring a Bub1 gene-trap allele with a β-gal cassette and splice acceptor inserted in intron 7 (see Materials and Methods). Intercrosses of Bub1\textsuperscript{gt8/+} animals revealed a likely maternal defect in breeding, as very few live mice of any genotype were recovered from these crosses and 90% of embryos were resorbed by e14.5. Crosses of Bub1\textsuperscript{2-3/-} females with Bub1\textsuperscript{gt8/+} males showed that compound heterozygous (Bub1\textsuperscript{2-3/-}) animals were absent before e11.5 (Table 1). Because the genetic background in these experiments is mixed 129/B6 (a background that supported survival of Bub1\textsuperscript{2-3/-}) animals, the failure to recover compound heterozygous animals at midgestation is strong genetic evidence that the Bub1\textsuperscript{2-3} allele is hypomorphic, as the Bub1\textsuperscript{gt8} allele is apparently more severe.

To determine the nature of the Bub1\textsuperscript{2-3} mutation, we first analyzed mRNA production in MEFs from E14.5 embryos. Quantitative reverse transcription–PCR (RT-PCR; not shown) and Northern hybridization analysis showed equivalent RNA levels in Bub1\textsuperscript{2-3/-} and Bub1\textsuperscript{2-3/2-3} MEFs (Supplementary Fig. S2A). Bub1\textsuperscript{2-3/2-3} MEFs expressed a single Bub1 mRNA that was smaller than the wt mRNA, consistent with the deletion of exons 2 and 3. cDNA from Bub1\textsuperscript{2-3/2-3} MEFs was cloned and sequenced, demonstrating that the mRNA produced from the mutant allele is spliced from exon 1 to exon 4, as predicted (Supplementary Fig. S2B). No novel alternatively spliced RNAs were detected by Northern or RT-PCR analyses using various primer sets across the Bub1 mRNA sequence (data not shown). Together, these data suggest that the residual function of the Bub1\textsuperscript{2-3} allele, if any, must be derived from the Δ2-3 mutant mRNA.

Analysis of the predicted mRNA from the Bub1\textsuperscript{2-3} allele suggested two alternative possibilities for mutant protein expression: (a) an ATG codon 5′ to the published start codon could produce a protein that is out of frame until reaching the exon 1 to exon 4 splice but in-frame thereafter (aa77–aa1058) and/or (b) translation from in-frame internal ATGs present in exon 4 or beyond producing NH2 terminal truncations (aa103 or aa173–aa1058; Fig. 1A). These ATGs are not in Kozak consensus sequences but could still give rise to protein (30). To distinguish these possibilities, we analyzed protein from Bub1\textsuperscript{2-3/2-3} MEFs by Western blot using an antibody raised against Bub1 aa336–aa489 (31). No Bub1 protein was detected in extracts from Bub1\textsuperscript{2-3/2-3} MEFs, whereas Bub1\textsuperscript{2-3/-} and Bub1\textsuperscript{2-3/+-} expressed a Bub1 protein of expected size (Fig. 1B). Likewise, no protein was detected in 129/B6 Bub1\textsuperscript{2-3/2-3} MEFs or tissues (data not shown). Titration of wt cell lysate showed the sensitivity of Bub1 detection to be 5% of normal endogenous levels (Fig. 1C), indicating that the Bub1\textsuperscript{2-3} allele disrupts production of the wt Bub1 protein and that any protein produced from the mutant allele must be produced at <5% of wt levels. Immunoprecipitation–Western blot analysis using an antibody recognizing residues 1-331 of the Bub1 protein for immunoprecipitation (28) and the antibody recognizing residues 336–489 for Western blot analysis (31) detected a mutant-specific band of ~115 kDa in Bub1\textsuperscript{2-3/2-3} and Bub1\textsuperscript{2-3/-} samples (Fig. 1D). This protein could be produced by translation from either an upstream or downstream ATG and, thus, lacks at least the NH2 terminal 73 aa of Bub1. Thus, the phenotypes observed in Bub1\textsuperscript{2-3/2-3} mice and MEFs could be due to the lack of a critical NH2 terminal Bub1 domain, severely diminished protein levels, or both.

### Table 1. Viability of Bub1 mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Background</th>
<th>Live births</th>
<th>E18.5</th>
<th>E14.5</th>
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<tbody>
<tr>
<td>Bub1\textsuperscript{12-3/+} × Bub1\textsuperscript{2-3/+}</td>
<td>B6</td>
<td>40</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>B6</td>
<td>162</td>
<td>15</td>
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<tr>
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<td>FVB</td>
<td>35</td>
<td></td>
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<tr>
<td>Δ2-3/Δ2-3</td>
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<td>+/+</td>
<td>B6/129</td>
<td>21</td>
<td>10</td>
<td></td>
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<td>26</td>
<td>17</td>
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<tr>
<td>Δ2-3/Δ2-3</td>
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*P < 0.005. † Two mice survived to weaning at 21 d.
**Bub1**<sup>Δ2-3/Δ2-3</sup> cells when treated with nocodazole (44% of cells had clear kinetochore localization versus 99% of wt; *P* < 0.05) or Taxol (15% versus 83%, respectively, *P* < 0.05; Fig. 2D). These results indicate that Bub1 is necessary for proper BubR1 kinetochore localization in response to lack of kinetochore-microtubule attachment and, particularly, to the lack of microtubule tension.

**Bub1**<sup>Δ2-3/Δ2-3</sup> MEFs exhibit a defective spindle checkpoint, chromosome segregation errors, and increased aneuploidy. Kinetochore localization of SAC proteins Mad2 and BubR1 has previously been speculated to be necessary for SAC function. The reduced BubR1 kinetochore localization in **Bub1**<sup>Δ2-3/Δ2-3</sup> MEFs suggested that these cells may have a defective SAC response. We tested spindle checkpoint responses of **Bub1**<sup>Δ2-3/Δ2-3</sup>, **Bub1**<sup>Δ2-3/Δ2-3</sup>, and wt MEFs by measuring mitotic indices (percentage of positive phosphorylated histone H3) after treatment with nocodazole or Taxol. **Bub1**<sup>+/+</sup>, **Bub1**<sup>Δ2-3/+</sup>, and **Bub1**<sup>Δ2-3/Δ2-3</sup> MEFs all showed an increased mitotic index by 12 hours after nocodazole addition compared with untreated cultures, indicating that each genotype retains at least partial SAC function in response to spindle ablation (Fig. 3A). Nocodazole-treated **Bub1**<sup>Δ2-3/Δ2-3</sup> and **Bub1**<sup>Δ2-3/Δ2-3</sup> cultures contained only modestly lower indices (5.8% and 7%, respectively) than the **Bub1**<sup>+/+</sup> cultures (8.9%). This result contrasts with reported results from MEFs heterozygous for a null mutation in Bub3 or a hypomorphic mutation of BubR1, which show a severely impaired arrest in response to nocodazole treatment (9, 12). Likewise, there was no increase in premature sister chromatid separation after

![Figure 2](attachment:image.png)

**Figure 2.** Bub1 and BubR1 are reduced at kinetochores in **Bub1**<sup>Δ2-3/Δ2-3</sup> MEFs. A, left, quantitation of Bub1 levels at kinetochores normalized to ACA (centromere marker); right, representative Bub1, ACA, and merged images from wt and Bub1 mutant MEFs. B, representative images illustrating staining level classifications used in quantitations for C-D. C, Mad2 kinetochore localization after treatment with nocodazole (left) and Taxol (right). Bars, percentage of cells of each class depicted in B. D, BubR1 kinetochore localization after treatment with nocodazole (left) and Taxol (right). *n* = 50 cells each from three individual experiments for C and D.
treatment with colcemid (2.5% in \(Bub1^{Δ2-3/Δ2-3}\) versus 1.5% in \(Bub1^{++}\)), another microtubule depolymerizing agent. In contrast to nocodazole treatment, \(Bub1^{Δ2-3/Δ2-3}\) MEFs did display a severe SAC defect in response to Taxol treatment (mitotic index, 3.5% for \(Bub1^{Δ2-3/Δ2-3}\) versus 8% for \(Bub1^{++}\); Fig. 3A). These data suggest that reduced protein levels and/or NH2 terminal deletion of Bub1 is more detrimental to the response to the loss of tension than the loss of attachment.

The primary role of the spindle checkpoint is to aid proper segregation of the chromosomes by delaying anaphase until all chromosomes are attached and under tension by spindle fibers. Loss of the spindle checkpoint has been linked to increased aneuploidy in several models of spindle checkpoint loss (9, 12). To test whether relaxation, but not complete abrogation of the spindle checkpoint, can increase aneuploidy under normal growth conditions, we determined the chromosome counts in metaphase spreads from \(Bub1\) mutant and wt MEFs. Seventy-six percent of metaphases from \(Bub1^{Δ2-3/Δ2-3}\) MEFs were aneuploid, compared with 9% of wt and 23% of \(Bub1^{Δ2-3/+}\) metaphases (Fig. 3B). Most of the aneuploid cells showed gain or loss of only one to two chromosomes. These high levels of aneuploidy represent an extreme level of chromosome instability, indicating that Bub1 is essential for proper chromosome segregation under unperturbed growth conditions.

Whereas the aneuploidy in \(Bub1^{Δ2-3/Δ2-3}\) MEFs could be due to a relaxed spindle checkpoint, Bub1 has also been implicated in proper chromosome congression (i.e., the movement of chromosomes to the metaphase plate; refs. 15, 16). To test whether \(Bub1\)
mutant MEFs congress chromosomes properly, synchronized MEFs were treated with the proteasome inhibitor MG132 as cells were entering mitosis. Inhibiting the proteasome blocks securin and cyclin B degradation, preventing the separation of sister chromatids at the metaphase plate and stopping mitosis in metaphase. Defects in chromosome congression are detected by an increase in chromosomes that have not aligned at the metaphase plate. Consistent with previous RNAi studies (15, 16), MG132-treated Bub1<sup>122/122-3/122-3</sup> MEFs showed an increase in chromosome congression defects, with 19.6% of mutant versus 5.7% of wt MEFs displaying uncongressed chromosomes (Fig. 3C). This result supports the conclusion that Bub1 plays a role in chromosome congression and suggests that defective congression may contribute to extreme aneuploidy of Bub1<sup>122/122-3/122-3</sup> MEFs.

Uncongressed chromosomes should trigger a SAC response, delaying anaphase onset until congression is complete and kinetochores are under tension. We reasoned that the reduced sensitivity of the SAC response in Bub1<sup>122/122-3/122-3</sup> MEFs may allow cells to enter anaphase despite the presence of one or more unattached or mono-oriented kinetochores, resulting in daughter cells with gain or loss of one to two chromosomes. This model predicts that unperturbed cells should have high rates of anaphase lagging chromosomes. Indeed, analysis of unperturbed Bub1<sup>122/122-3/122-3</sup> MEFs during anaphase revealed that 47% of Bub1<sup>122/122-3/122-3</sup> MEFs in anaphase had one or more lagging chromosomes compared with only 9% of wt and 8% of heterozygotes (Fig. 3D). Taken together, these results suggest that the extreme aneuploidy of Bub1<sup>122/122-3/122-3</sup> MEFs is caused by a combination of defects in chromosome congression and SAC activation.

**Bub1<sup>122/122-3/122-3</sup> MEFs are growth impaired with high rates of senescence.** High levels of aneuploidy in BubR1 hypomorphic animals and MEFs correlated with growth impairment and high levels of senescence (12). Furthermore, a recent study indicated that RNAi knockdown of Bub1 in primary human fibroblasts resulted in increased senescence (34). We determined growth and senescence rates in Bub1<sup>122/122-3/122-3</sup> MEFs to assess whether similar phenotypes occur in this system. Wt and heterozygous cultures grew at similar rates, with expected decreases in cell accumulation at higher passages due to senescence (35), whereas Bub1<sup>122/122-3/122-3</sup> MEFs grew much more slowly (Fig. 4A). To ascertain the cause of the growth differences, we assessed levels of apoptosis and senescence. Bub1<sup>122/122-3/122-3</sup> MEFs did not show increased apoptosis relative to wt and heterozygous cells, as measured by Annexin V staining (data not shown). However, senescence-associated β-galactosidase assays revealed that Bub1<sup>122/122-3/122-3</sup> MEFs had a significant increase in senescence compared with wt MEFs at passage 3 (9.1% versus 3.1) and passage 5 (15.4% versus 4.4%) examined (Fig. 4B and C). The percentage of BrdUrd incorporating cells was also lower in Bub1<sup>122/122-3/122-3</sup> MEFs, consistent with the increase in senescent cells (not shown). Bub1<sup>122/122-3/122-3</sup> MEFs did not show marked increases in the DNA damage marker phosphorylated H2AX (3.1% versus 2.6% in wt MEFs; Fig. 4D), indicating that DNA breaks did not contribute significantly to the senescent phenotype. These results indicate that the high levels of chromosome missegregation and aneuploidy in Bub1<sup>122/122-3/122-3</sup> cells trigger growth impairment via premature senescence.

**Impaired Bub1 function predisposes to increased tumorigenesis.** Aneuploidy arising from spindle checkpoint impairment or other defects has long been hypothesized to contribute to tumor formation. In support of this hypothesis, most heterozygous mouse spindle checkpoint mutants generated to date have shown modest increases in spontaneous and/or carcinogen-induced tumor formation (11–13, 36). However, some evidence suggests that extreme aneuploidy may inhibit tumorigenesis (12, 37). To determine if Bub1 impairment contributes to tumor formation, cohorts of (129/B6)Bub1<sup>122/122-3/122-3</sup> and controls were of ages of 2 years. Approximately, 76% of (129/B6)Bub1<sup>122/122-3/122-3</sup> mice developed tumors by 23 to 25 months compared with 42% of (129/B6)Bub1<sup>122/122-3/122-3</sup> mice (P < 0.05) and ~28% of (129/B6)Bub1<sup>+/+</sup> animals (P < 0.05; Fig. 5A) As in aging wt mice, the majority of (129/B6)Bub1<sup>122/122-3/122-3</sup> tumors were of liver origin and occurred at an increased frequency. Importantly, Bub1 mutation also

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**Figure 4.** Bub1<sup>122/122-3/122-3</sup> MEF cultures have reduced growth and increased senescence. A, total cell numbers accumulated at indicated passages. Cells (500,000) were plated at each passage (lower markers) and total cells counted upon passage 2 d later (top markers). B, representative acidic β-galactosidase staining (blue) of a Bub1<sup>122/122-3/122-3</sup> culture. C, senescent cells as a percentage of total cells at passages 3, 5, and 7. n = 3 independent experiments per genotype. D, Bub1<sup>122/122-3/122-3</sup> MEFs do not show increased DNA damage. Percentage of cells positive for phosphorylated H2AX immunostaining. Teal bars indicate duplicate control samples treated with doxorubicin to induce DNA damage. *, P < 0.05. Bars, SE.
broadened the tumor spectrum. Lung tumors appeared in 16% (4 of 25) of (129/B6)Bub1<sup>12-3/12-3</sup> mice, whereas no lung tumors were found in wt littermate controls (Fig. 5B). Whereas a significant increase in spontaneous tumors was not observed in the (129/B6)Bub1<sup>12-3/12-3</sup> cohort, a lung tumor and a brain tumor were observed, suggesting a possible change in tumor spectrum. These data indicate that Bub1 is a low penetrance tumor suppressor, likely with significant quantitative effects based on the genetic modification of phenotypes in distinct genetic backgrounds. Together with the results from Bub1<sup>12-3/12-3</sup> MEFs, these data indicate that chromosome missegregation defects leading to aneuploidy in cells of Bub1 mutant mice drive postnatal developmental defects ranging from lethality to tumor formation, depending on the associated genetic context.

**Discussion**

We have probed the functions of the mammalian mitotic regulator Bub1 by generating a hypomorphic mutation (Bub1<sup>12-3</sup>) in mice that lacks part of an NH<sub>2</sub> terminal conserved domain implicated in spindle checkpoint function in yeast. In addition, the protein is expressed at severely diminished levels, yet retains sufficient function to support development to birth or adulthood, depending on genetic context. In the C57Bl6/J background, homozygous mutant mice die perinatally with a "failure-to-thrive" phenotype, whereas FVB and mixed 129P2/C57Bl6/J backgrounds support survival to late adulthood. However, these mice develop a high incidence of cancers compared with wt littermates solidifying a role for impaired Bub1 function in tumorigenesis.

Bub1<sup>12-3</sup> was characterized as a hypomorphic mutant by demonstrating that compound heterozygotes with a gene-trapped allele, Bub1<sup>gt8</sup>, die by E11.5. Indeed, two contemporary reports show that Bub1<sup>12-3</sup> mutants arrest by E7.5 (38, 39), consistent with the early embryonic lethality previously described for homozygous null mutants of the SAC genes Mad2, BubR1, and Bub3 (7, 8, 10). Based on the proposed Bub1 ATG translation start site, the Bub1<sup>12-3</sup> mutation was designed to produce a severely truncated protein lacking the kinase domain and expected to be nonfunctional. Analyses of the mRNA expressed in mutant MEFs indicate that the transcript lacking exons 2 and 3 is expressed at normal levels. Immunoprecipitation–Western blot analysis of nocodazole-arrested MEFs identified a unique protein in Bub1<sup>12-3</sup> and Bub1<sup>12-3/12-3</sup> MEFs, which is ~5 kDa smaller than wt Bub1 and present at <5% of wt levels. Thus, the mutant Bub1 protein seems to be produced by translation from an alternative ATG, the most likely of which would give rise to an NH<sub>2</sub> terminal truncation starting at exon 4, which retains the COOH terminal kinase domain and the kinetochore binding domain. The NH<sub>2</sub> terminal domain of Bub1 is highly conserved in nearly all species, but its function is still largely unknown. In fission yeast, checkpoint activity and kinetochore localization were mapped to residues 28-160, whereas mammalian Bub1 residues 200-300 were found to be required for SAC function and kinetochore localization (6, 32). Because the mutant protein is expressed at such low levels, the current studies cannot distinguish whether impaired function results from deletion of a critical NH<sub>2</sub> terminal function, insufficient protein, or both. Future studies will help to distinguish these possibilities, as well as determine the reason for the background-dependent phenotypic differences found in C57Bl6/J and FVB or mixed background mice. Nonetheless, this mutant provides a tool for probing the effects of reduced Bub1 functions in cells and during embryonic and adult development.

**Bub1 in mitotic checkpoint regulation.** MEFs derived from mutant and wt littermate embryos were used to assess the cellular phenotypes associated with impaired Bub1 function. A recent RNAi study in cell lines questioned a role for mammalian Bub1 in SAC function (15), whereas a distinct study found that Bub1 depletion ablated the SAC (16). We show here that, despite undetectable levels of Bub1 binding at kinetochores, Mad2 is able to localize at kinetochores in Bub1<sup>12-3/12-3</sup> MEFs, which arrest in mitosis when treated with nocodazole. These surprising results indicate that very little Bub1 is required for SAC activity in response to microtubule ablation in MEFs. Perera and colleagues (39) recently found that Bub1<sup>12-3</sup> MEFs do not show a sustained arrest after treatment with nocodazole, whereas Jeganathan and colleagues (38) showed that MEFs with ~20% of wt Bub1 activity arrest for a shorter duration than wt MEFs in nocodazole. The distinct methodologies used (duration of mitotic arrest versus mitotic index) make it difficult to directly compare these results to ours and could be responsible for the discrepancy. However, this inconsistency raises the possibility of a scenario where we have underrepresented the amount of Bub1<sup>12-3</sup> protein expressed and are primarily observing the effects of the NH<sub>2</sub> terminal deletion of the allele. Nevertheless, our results are supported by previous findings with Bub1 small interfering RNA that only 5% of endogenous levels could support a mitotic arrest with spindle ablation (15, 16). These results together are consistent with a model in which a small amount of Bub1 is sufficient to arrest cells in response to spindle ablation. This contrasts with the haploinsufficiency of the SAC function in MEFs heterozygous for Bub3 or hypomorphic for BubR1, which show very little mitotic arrest after nocodazole treatment (9, 12).

In contrast to Mad2, BubR1 kinetochore localization is reduced in Bub1<sup>12-3/12-3</sup> MEFs in response to nocodazole and is further reduced after Taxol treatment. A similar reduction in BubR1...
kinetochore localization was recently observed in Bab1-null and hypomorph MEFs, providing a strong consensus that Bab1 is critical for Bub1 localization (38, 39). Previous kinetochore localization studies suggest that Bub1 is responsive to changes in tension, whereas Mad2 binds kinetochores in response to attachment failure (40). The inhibition of APC/Cdc20 in response to nocodazole may be due to integration of signals from both pathways (as evidenced by strong localization of both Bab1 and Mad2 with nocodazole treatment), whereas Taxol may only trigger the Bub1-dependent pathway. While we see a reduction in Bub1 kinetochore localization after nocodazole treatment, the presence of substantial Mad2 at kinetochores in addition to reduced amounts of Bub1 is apparently sufficient for responding to loss of kinetochore-microtubule attachment. Although Bab1 was previously shown to be necessary for efficient localization of both Mad2 and Bub1 to kinetochores, our data suggest that Bab1 is more critical in the tension-sensitive Bub1 signaling pathway than in attachment sensing.

We show here that Bab1<sup>2-3/2-3</sup> MEFs rapidly develop a high frequency of aneuploidy that is likely explained by a weakened SAC response. Nocodazole-treated and Taxol-treated Bab1<sup>2-3/2-3</sup> MEFs have a reduced mitotic index relative to WT cells; yet, these assays are relatively crude and may not accurately measure weakened signaling capacity. A more sensitive test of SAC function comes when most chromosomes achieve bipolar attachment and tension, as the remaining signal from even a single unattached kinetochore must be sufficient to block anaphase onset until attachment and tension are achieved (41, 42). Under these conditions, insufficiency of SAC components may impair the SAC enough to allow anaphase onset, resulting in lagging chromosome(s). The high frequency of Bab1<sup>2-3/2-3</sup> cells with lagging chromosomes, coupled with the fact that only one or two such chromosomes are observed in most cells, suggests that chromosome missegregation in Bab1<sup>2-3/2-3</sup> cells is triggered by a defect in sensing minor, rather than catastrophic, mitotic aberrations. Bab1 has also been reported to play a role in chromosome congression and regulation of sister chromatid cohesion (15, 16, 19, 20). We observed an increase in chromosome congression errors in Bab1<sup>2-3/2-3</sup> MEFs, suggesting that this mechanism may contribute to the mitotic errors in these cells. However, the percentage of cells with obvious congression errors was low relative to the percentage with lagging chromosomes, suggesting that congression errors are unlikely to account for the full extent of missegregation observed. Moreover, noncongressed chromosomes should invoke a SAC arrest under normal conditions. Therefore, we argue that the mitotic defects in Bab1<sup>2-3/2-3</sup> MEFs are a consequence of impairment in both chromosome congression and SAC signaling.

A large body of evidence indicates that cellular senescence suppresses cellular proliferation in response to DNA damage and/or oncogene activation. Here, we show that Bab1<sup>2-3/2-3</sup> MEFs are significantly growth impaired and produce high levels of premature senescence. This effect was less severe in Bab1<sup>2-3</sup> MEFs, which also displayed lower levels of aneuploidy. Thus, a major consequence of impaired Bab1 function and resulting chromosome segregation errors seems to be permanent cell cycle arrest. Increased senescence was also observed in Bab1 hypomorph mice (12), which also display high rates of aneuploidy. Furthermore, aneuploid yeasts were recently shown to display impaired growth and metabolic changes accompanied by evidence of protein degradation and folding distress (43). Thus, it is possible that the increased metabolic demand of extra chromosomes taxes cellular machinery, causing a stress response that triggers senescence. Aneuploidy may also trigger stress responses by causing imbalances in signaling pathways.

**Bab1 and tumor suppression.** Aneuploidy has long been associated with cancers, raising the hypothesis that spindle checkpoint impairment may predispose to tumorigenesis (44–46). Indeed, the presence of Bab1 mutations in human colon cancer cell lines (21) and murine lymphomas (22) fueled this hypothesis. Here, we show a causal relationship between impaired Bab1 function and predisposition to cancer. When analyzed in a background permissive to survival, impaired Bab1 function predisposed to a higher frequency and expanded spectrum of tumors relative to set littermates. Tumors arose in aging mice, indicative of a low penetrance effect, consistent with the notion that noncatastrophic rates of aneuploidy can produce tumorigenic chromosomal imbalances. Mice heterozygous for a null mutation of other SAC genes also resulted in increased tumor incidence, either spontaneously [Mad1 (36), Mad2 (11), Cenp-E (37)] or after carcinogen treatment [BubR1 (13) and Bab3 (9)]. The recent work of Jeganathan and colleagues (38) showed an increasing tumor incidence with progressive loss of Bab1, reaching 55% in mice with ~20% of endogenous Bab1 levels. The higher tumor incidence (76%) in our model correlates with the further reduction in Bab1 levels to <5% and the corresponding increase in mitotic abnormalities. A severe hypomorphic allele of BabR1 caused only a modest increase in tumorigenesis; however, animals aged prematurely, potentially confounding the quantitative assessment of tumor incidence (12). CENPE haploinsufficiency, which also causes severe aneuploidy, was recently shown to have either protective or tumorigenic effects, depending on the genetic and environmental context (37). Thus, the influence of mitotic infidelity on tumor formation seems to be dependent on the cellular and genetic context and likely reflects a quantitative trait. The level of mitotic disruption may be critical for tumor formation, with high levels of chromosome missegregation incompatible with cell survival or activating tumor suppression pathways, such as senescence. Future study of the hypomorphic Bab1<sup>2-3</sup> mutation in the context of an allelic series with other available Bab1 mutations, in addition to alterations in senescence pathway factors, such as p53, should provide valuable insight into the mechanistic relationship between mitotic aberration, chromosome instability, and cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


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