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Genome instability and embryonic developmental defects in RMI1 deficient mice

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Abstract

RMI1 forms an evolutionarily conserved complex with BLM/TOP3 /RMI2 (BTR complex) to prevent and resolve aberrant recombination products, thereby promoting genome stability. Most of our knowledge about RMI1 function has been obtained from biochemical studies *in vitro*. In contrast, the role of RMI1 *in vivo* remains unclear. Previous attempts to generate an *Rmi1* knockout mouse line resulted in pre-implantation embryonic lethality, precluding the use of mouse embryonic fibroblasts (MEFs) and embryonic morphology to assess the role of RMI1 *in vivo*. Here, we report the generation of an *Rmi1* deficient mouse line (hy/hy) that develops until 9.5 days *post coitum* (dpc) with marked defects in development. MEFs derived from *Rmi1*^{hy/hy} are characterized by severely impaired cell proliferation, frequently having elevated DNA content, high numbers of micronuclei and an elevated percentage of partial condensed chromosomes. Our results demonstrate the importance of RMI1 in maintaining genome integrity and normal embryonic development.

Keywords

homologous recombination; DNA repair; genome instability

1. Introduction

Unresolved or excess recombination products pose a significant threat to cell survival, while imprecise resolution of recombination intermediates may result in a number of chromosome rearrangements and genome instability. A highly regulated interplay between mechanisms that promote and suppress recombination is critical for genome stability, as well as suppression of cancer and premature aging. The BLM helicase and its yeast homolog Sgs1 have been implicated in preventing and resolving potentially mutagenic DNA structures that result in deleterious intermediates of recombination in both mitotic and meiotic cells,

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

elevated sister chromatid exchange, and increased chromosomal rearrangements [1-8]. Bloom's syndrome is a human genetic disorder caused by mutations in BLM, which encodes one of the five known human RecQ helicases [9]. This condition is associated with predisposition to a diverse range of cancers and impaired development [10, 11]. Cells isolated from individuals with Bloom's syndrome [5], a *Blm*^{-/-} conditional knockout in lymphocytes [12], and cells treated with siRNA specific for BLM [13, 14] are both characterized by chromosomal abnormalities and elevated levels of sister chromatid exchange, suggesting that the BLM protein is required to prevent and/or resolve mutagenic structures *in vivo*.

Consistent with the ability of BLM/Sgs1 to direct DNA recombination events away from deleterious intermediates, BLM and TOP3 together can dissolve a number of recombination intermediates *in vitro* [15-23]. Two recently described members of the BTR complex, RMI1 and RMI2 [13, 24-26], appear to stimulate its enzymatic functions [20, 22, 27-29]. Indeed, depletion of RMI1 results in increased levels of sister chromatid exchange similar to BLM knockdowns [13, 30]. Stability of the BTR complex is also dependent on RMI1 as depletion of RMI1 disrupts the BTR complex and decreases levels of its protein components, especially TOP3 [13, 24].

In addition to processing intermediates formed by recombination, more general roles for the BTR complex during DNA replication include the processing of stalled replication forks and the activation of the S-phase checkpoint under replication stress [31-33]. The latter may arise when the DNA replication machinery encounters obstructive DNA lesions and/or DNA secondary structures. Again, RMI1 plays an important role in this BTR function by mediating efficient recruitment of the complex to the stalled replication fork [31, 33, 34]. In addition it has recently been suggested that RMI1, independently of its function in the BTR complex, promotes progression of the replication fork [31].

Mouse knockouts for *Blm* and *Top3* have been generated, and it has been reported that complete disruption of either of these genes results in embryonic lethality [14, 35]. *Blm* mutant embryos die at 13.5 days *post coitum* (dpc) and are delayed in development but display no obvious morphological abnormalities [14]. Furthermore, red blood cells and embryonic fibroblasts from *Blm*^{-/-} mouse showed a large number of micronuclei and evidence of chromosome instability [14]. *Top3*^{-/-} embryos died at a pre-implantation stage and recovered blastocysts showed slow growth followed by a complete termination in proliferation [35]. Two previous attempts to generate an *Rmi1* knockout mouse resulted in pre-implantation embryonic lethality [36, 37]. Thus, at present the *in vivo* requirements of mammalian RMI1 have only been studied in knockdowns obtained from siRNA-treated cultured cells. Here we report the generation of an *Rmi1*^{hy/hy} mouse line that develops until 9.5 dpc. This allowed us to determine the requirement of RMI1 in normal embryonic development and, importantly, to obtain mouse embryonic fibroblasts (MEFs) to study the cellular phenotype that results from RMI1 depletion. We observed that cultured *Rmi1*^{hy/hy} MEFs exhibit severely impaired cell proliferation and frequently show elevated DNA content. In addition, high numbers of micronuclei and an elevated percentage of partially condensed chromosomes are characteristic in these cells. These results indicate that RMI1 is important for maintaining genome integrity.

2. Materials and methods

2.1. Mice

An embryonic stem (ES) cell line (clone Rmi1^{Gt(PST18949)Mfgc}) was purchased from the International Mouse Strain Resource (<http://www.findmice.org/index.jsp>). Injection into

blastocyst and chimeric mouse generation were performed by the Toronto Centre for Phenogenomics (Toronto, Canada). C57BL/6 mice were purchased from Jax laboratories.

2.2. Dissection of embryos and genotyping

Heterozygous mice were bred to obtain wild-type, heterozygote (*Rmi1^{wt/hy}*), and homozygous mutant (*Rmi1^{hy/hy}*) embryos. Mice were kept on a 12 hour light-dark cycle. The morning of the day on which a vaginal plug was detected was designated 0.5 dpc. Embryos were dissected from the uterus in phosphate-buffered saline, and yolk sacs were used for genotyping by polymerase chain reaction (PCR). Yolk sacs were digested for 1 hour at 98°C in 25mM NaOH, 5mM EDTA followed by the same volume of 40mM Tris-HCl pH 5.5. Primers used for genotyping are illustrated on Figure 1A and their sequences shown on supplementary Table 1. PCR cycling conditions consisted of an initial denaturation at 94°C for 3 minutes; followed by 35 cycles of 94°C 30 seconds, 58°C 45 seconds, 72°C 50 seconds with a final extension step of 72°C for 5 minutes. Reactions were performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems) and products were analyzed on 1.5% agarose gel.

2.3. Histological analysis

The uterine horns containing 9.5 dpc embryos were removed and placed in ice cold 1X PBS. Each embryo was separated by cutting between the implantation site and immediately transferred to 10% neutral-buffered formalin (Sigma) and fixed overnight. Fixed embryos were either processed for paraffin or cryo embedding. To access the embryonic morphology, paraffin serial-sections were stained with hematoxylin and eosin. Cryo serial sections were used to identify apoptotic cells by TUNEL assay (Roche) following manufacturer instructions. The detection of mitotic cells was done by immunostaining with the mitotic marker anti-phosphohistone H3 (pHH3) (Upstate). Cryo sections were blocked with 5% normal goat serum in PBS + 0.1% Triton for 2 hours at room temperature, immunostaining with pHH3 at 1:100 in blocking solution overnight at 4°C, washed 5 times in PBS + 0.1% Triton, incubated with a secondary antibody conjugated to Alexa 488 at 1:500 dilution for 45 minutes at room temperature, washed 5 times with PBS + 0.1% Triton. Slides were mounted with VectaShield containing DAPI. For genotyping of histological sections, the embryonic tissues were initially scraped from slides, transferred into DEXPAT reagent (TaKaRa) and genotyped by PCR.

2.4. Quantitative RT-PCR

After dissection, embryos were kept in 10 volumes of RNAlater stabilization reagent (Qiagen) at 4°C until genotyping from yolk sac was performed. Total RNA was extracted from wild-type, *Rmi1^{wt/hy}* and *Rmi1^{hy/hy}* embryos using the RNeasy Mini Kit (Qiagen). 2µg of RNA was used to synthesize cDNA (High Capacity RNA-To-cDNA Kit, Applied Biosystem). SYBR® Green based quantitative PCR was carried out using Power SYBR® Green PCR Master Mix (Applied biosystems). Primers were designed to anneal to the boundaries of exon 2 (qRmi1F) and exon 3 (qRmi1R) of *Rmi1*. For quantitative RT-PCR of BTR complex components, primers were designed to anneal to the boundaries of exon 14 (qBlmF) and exon 15 (qBlmR) of *Blm*, exons 4 (qTop3aF) and 5 (qTop3aR) of *Top3*, exons 1 (qRmi2F) and 2 of *Rmi2* (qRmi2R), exons 7 (qAtmF) and 8 (qAtmR) of *Atm*, exons 2 (qAxin2F) and 3 (qAxin2R) of *Axin2* (supplementary Table 1). A constitutive expression gene, β -actin, was used as endogenous control. Cycling conditions were: 95°C 10 minutes; 95°C 10 seconds, 60°C 30 seconds, for 40 cycles. The melting curve was obtained under conditions of 95°C 10 sec and 65°C to 95°C performed in increments of 0.5°C 5 seconds, in a BioRad CFX96 thermocycler. The comparative threshold cycle method (Ct) was used to determine the relative fold change in gene expression and β -actin was used as internal

control. Four independent experiments were used to compile data and present as a mean with standard deviation. Two-tailed student t-test was used for statistics analysis.

2.5. EdU incorporation

DNA synthesis was accessed by incorporation of EdU (Click-iT Imaging Kit, Invitrogen). After 17 days in culture, 3×10^4 MEFs were inoculated onto 13cm round glass coverslip and allowed to adhere for 24 hours. Then, cells were incubated with $5 \mu\text{g/ml}$ of EdU (Invitrogen) for 2, 4, 8 and 24 hours and washed in PBS 1X twice. Finally, MEFs were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Detection of EdU was performed following the manufacturer instructions. DNA was visualized with DAPI staining.

2.6. Flow cytometry

Cells from wild-type and *Rmi^{hy/hy}* embryos were fixed in 70% ethanol and kept at -20°C until analysis. Briefly, after centrifugation the cell pellet was washed with PBS 1X and resuspended in 500 μl Krishnan's reagent (0.1 mg/ml propidium iodide, 0.02 mg/ml RNase A, 0.3% NP-40 and 0.1% Na citrate) and incubated in the dark for 20 minutes at 25°C . Data acquisition was carried out using FACSCalibur and analyzed using the FloJo Cell Cycle analysis software. In addition, DNA ploidy (N) and cell cycle phases were determined [38].

2.7. Cell counting

Cell Growth curves were performed by a manual count method to determine the proliferative rate of mouse embryonic fibroblasts. Cells were seeded at 1×10^4 cells/well in a 24-well tissue culture plate (Greiner) and viable cells were counted daily by the Trypan blue dye exclusion method.

2.8. Micronuclei assay and nuclear morphometry

After 17 days in culture, 3×10^4 cells were inoculated onto 13cm round glass coverslips and allowed to adhere for 24 hours. Cells were washed in PBS 1X, fixed with 4% paraformaldehyde for 30 minutes and then permeabilized with 0.5% Triton X-100 in PBS 1X for 5 minutes on ice. After, two washes with 1X PBS, nuclei were stained with DAPI. The number of micronuclei was manually counted and the nuclear area was determined automatically using AxioVision 4.8 software. Micronuclei were identified as those with a diameter of less than 1/3 of normal nuclei.

2.9. Metaphase spreads

Metaphase spreads were done as previously described [39]. Immediately after dissection, embryos were washed briefly with 1X PBS followed by incubation with nocodazol 200ng/ml for 4 hours. Embryos were then transferred to a hypotonic solution (0.56% KCl) for 30 minutes at room temperature followed by overnight fixation in ice cold methanol:acetic acid (3:1). Dissociation of the embryos was carried out in 60% acetic acid at 4°C . Dissociated embryos were centrifuged at 1000Xg for 5 minutes, resuspended in fixative solution (3:1 methanol:acetic acid) and transferred to slides. Metaphase spreads were stained with Giemsa. After washing with Gur's buffer (Invitrogen), the spreads were mounted in Permount.

2.10. Gamma irradiation and γH2AX immunostaining

After 17 days in culture, MEFs derived from both wild-type and *Rmi^{hy/hy}* embryos were transferred to coverslips and allowed to adhere overnight in a 37°C incubator with 5% CO_2 . Coverslips were transferred to a plate containing fresh medium and exposed to 50Gy of gamma irradiation using a gamma cell irradiator at 0.5Gy per minute. After irradiation, coverslips containing cells were returned to the incubator and collected at the time points

indicated. Non irradiated cells were used as a control. Cells were fixed in 4% paraformaldehyde for 20 minutes, permeabilized with 0.5% Triton in PBS 1X for 20 min and washed with PBS 1X. Cells were blocked with 4% BSA in PBS 1X for 30 minutes at room temperature, followed by incubation with anti- H2AX (Millipore) at 1:1000 dilution for 2 hours at room temperature. Secondary antibody conjugated to Alexa 596 was used at 1:500 dilution for 30 minutes at room temperature. Analysis of anti- H2AX image intensity was performed using MetaMorph 6.3 software (Molecular Devices Corporation).

3. Results

3.1. Generation of RMI1 deficient mice

We generated a mouse line with a gene trap-disrupted allele, *Rmi1*^{Gt(PST18949)Mfge} (Figure 1A). The ES cell contained a gene trap vector inserted into intron 1, flanked by exon 1 and 2 of *Rmi1* gene located on chromosome 13. Exons 1 and 2 are non-coding exons, but necessary for the expression of Rmi1. PCR analysis was used to identify animals that were wild-type (346bp PCR product), heterozygous (wt/hy, 346bp and 478bp products) and homozygous (hy/hy, 478bp product) (Figure 1A and B). *Rmi1*^{wt/hy} are fertile, did not display any apparent tissue anomaly, showed no sign of shortened life span and did not display evidence of tumor development up to 20 months of age.

3.2. RMI1 deficiency leads to embryonic lethality in mice

The genotyping of 235 offspring from 39 different litters yielded no viable *Rmi1*^{hy/hy} (Figure 1C). The absence of *Rmi1*^{hy/hy} among the litters indicates that the homozygous disruption of *Rmi1* is embryonic lethal in mice. To investigate the developmental stage at which lethality occurs we carried out timed matings. Pregnant mice were euthanized at 8.5, 9.5, 10.5 and 11.5 dpc. After embryonic dissection, the genotype of each embryo was determined by PCR analysis of the yolk sac. Live *Rmi1*^{hy/hy} embryos were detected from 8.5 dpc to 9.5 dpc with a genotype distribution of the expected Mendelian ratios. At 10.5 dpc only *Rmi1*^{hy/hy} embryos undergoing degeneration or almost absorbed were detected, suggesting that they died at 9.5 dpc (Table 1). Analysis of *Rmi1* mRNA expression in 9.5 dpc embryos was assessed by quantitative real time PCR. Compared to wild-type littermates, we observed approximately 5% of mRNA expression in *Rmi1*^{hy/hy} (a characteristic of hypomorph mutants). Heterozygous embryos expressed approximately half the mRNA of that observed for wild-type (Figure 1D).

We used RT-PCR and mRNA obtained from *Rmi1*^{hy/hy} and wild-type littermates to determine the level of expression of *Blm*, *Topo3*, *Rmi1* and *Rmi2*. Compared to the wild-type, *Rmi1*^{hy/hy} cells showed significantly decreased expression of *Blm*, (0.46 ± 0.02 , n=16), *Topo3* (0.39 ± 0.13 , n=16), and *Rmi2* (0.60 ± 0.07 , n=16). This is in agreement with previous work showing that depletion of RMI1 results in a decrease in the components of the BTR protein complex [13, 24]. As expected, we detected marginal expression of *Rmi1*, and observed no changes in the level of expression of genes non-related to the BTR complex (*Atm*, 0.91 ± 0.12 , n=16; *Axin2*, 1.19 ± 0.05 , n=3 and *Irf8*, 0.98 ± 0.121 , n=6).

3.3. RMI1 deficiency leads to severe embryonic development delay, low mitotic index and extensive apoptosis

Macroscopic analysis of 8.5 dpc *Rmi1*^{hy/hy} embryos and wild-type littermates showed no developmental differences (data not shown). However, gross growth and morphological characteristics became overt by 9.5 dpc. In sharp contrast to the wild-type, 9.5 dpc *Rmi1*^{hy/hy} embryos were distinctly smaller, most of them had not undergone axial rotation, showed a severe development delay (Figure 2A), and yolk sacs did not show a normal pattern of vascularization (data not shown). Histological analysis of 9.5 dpc *Rmi1*^{hy/hy}

embryos showed lack of limb bud formation, opened neural tube and a defined tail-bud structure suggesting that the development of *Rmi1^{hy/hy}* embryos is interrupted at 8.5 dpc (Figure 2B).

We performed TUNEL assays on sections of 9.5 dpc wild-type and *Rmi1^{hy/hy}* embryos to determine the presence of apoptotic cells. The number of apoptotic cells in *Rmi1^{hy/hy}* was notably higher than that of wild-type embryos, indicating that at 9.5 dpc, *Rmi1^{hy/hy}* embryos suffer massive cell death (Figure 2C).

3.4. RMI1 deficiency results in severely impaired cell proliferation and elevated DNA content

RMI1, in the context of the BTR complex, promotes processing of some intermediates of DNA recombination repair (i.e., Holliday junctions) and promotes re-initiation of collapsed replication forks. Elimination of excess recombination, which is essential for the maintenance of genome stability, and the completion of DNA replication are required in the normal cell cycle. We therefore reasoned that deletion of RMI1 might result in alteration of cell proliferation. To test this hypothesis we obtained MEFs from *Rmi1^{hy/hy}* embryos at 8.5 and 9.5 dpc. During a seven day-growth period wild-type MEFs proliferated normally, however cell growth was severely impaired in *Rmi1^{hy/hy}* MEFs of both 9.5 dpc (Figure 3A) and 8.5dpc embryos (data not shown). We did not observe signal of cell proliferation in *Rmi1^{hy/hy}* cells cultured for periods of up to six months (results not shown). We also tested the effect of the protein phosphatase inhibitor okadaic acid on cultured MEFs. Okadaic acid has been described to act as a stimulator of proliferation with effects on cell cycle checkpoints, i.e. accelerate G2/Mitosis I progression [40, 41]. Cell arrest in *Rmi1^{hy/hy}* is apparently irreversible, as cultured MEFs do not undergo mitosis even after treatment with okadaic acid (data not shown). This precluded further analysis of sister chromatid exchange to assess whether, as previously observed for BLM deficient cells [11], loss of RMI1 may lead to chromosome instability.

We measured cell incorporation of EdU to determine whether the severely impaired cell proliferation in *Rmi1^{hy/hy}* MEFs is accompanied by deficient DNA replication. After 18 days in culture, wild-type and *Rmi1^{hy/hy}* MEFs were incubated with EdU for 2, 4, 8 and 24 hours (Figure 3B and C). In sharp contrast to wild-type cells, in which incorporation of EdU was detected in 35% of the cells, only a maximum of 1.6% of *Rmi1^{hy/hy}* MEFs were EdU positive after 24-hour incubation (Figure 3C and Table 2).

We also performed immunostaining of 9.5 dpc embryo frozen sections using anti-pHH3, a marker of mitotic cells. Our results revealed that cell proliferate actively in wild-type embryos, however only few mitotic cells can be observed in *Rmi1^{hy/hy}* embryos (Figure 3D).

Flow cytometry analysis of asynchronous MEFs after 18 days of culture (approximately 20,000 cells per condition assayed) revealed that in contrast to wild-type ($14.8\% \pm 4.0$, n=5 number of tested embryos) a higher proportion of *Rmi1^{hy/hy}* cells show apparent 4N DNA content ($31.6\% \pm 7.6$, n=7) ($p=0.0012$, unpaired t-test). Remarkably, the number of polyploidy in *Rmi1^{hy/hy}* MEFs ($>4N$; $13\% \pm 4.1$, n=7) is higher than in wild-type ($2.2\% \pm 0.3$, n=5) ($p=0.0002$, unpaired t-test) (Figure 4A and B). Wild-type and *Rmi1^{hy/hy}* cells from freshly dissociated 9.5 dpc embryos did not show significant difference in ploidy (Supplementary Figure 2A and 2B). We also observed that the average nuclear area of *Rmi1^{hy/hy}* MEFs was significantly higher $576.9\mu\text{m}^2 \pm 272.6$ (n=485) than those of wild-type $261.2\mu\text{m}^2 \pm 85$ (n=459) ($p<0.0001$, Mann-Whitney test) (Figure 4C and D). Taken together our results indicate that RMI1 depletion results in general DNA replication arrest. However, a portion of *Rmi1^{hy/hy}* cells are able to at least partially replicate DNA without entering

mitosis, which results in high DNA content per nucleus. This is a potential explanation for the severe deficiency in cell proliferation observed in *Rmi1^{hy/hy}* embryos.

3.5. Genome instability and incomplete chromosome condensation in RMI1 deficient MEFs

The severely impaired cell proliferation in *Rmi1^{hy/hy}* cultured MEFs may be also caused by genome instability. To test this possibility we determined the number of micronuclei in MEFs, which represent whole or fragmented chromosomes separated from the main nucleus [42, 43]. Cultured *Rmi1^{hy/hy}* MEFs show a higher proportion of micronuclei ($56\% \pm 6.4$, $n=485$) compared to wild-type cells ($12\% \pm 4.7$, $n=459$) ($p=0.0007$, t-test) (Figure 5A-C). The estimated number of micronuclei per cell averaged 1.1 in *Rmi1^{hy/hy}* cells in contrast to 0.14 in wild-type cells. We also observed significant differences in the distribution of the number of micronuclei per cell when comparing the population of wild-type and *Rmi1^{hy/hy}* ($p=0.0776$, Mann-Whitney test). Significant differences were also observed when we compared fractions of cell populations in wild-type versus *Rmi1^{hy/hy}* containing zero micronuclei ($p=0.0013$, paired two tailed t-test analysis), 1 micronuclei ($p=0.0352$), 2 micronuclei ($p=0.0045$) and 3 micronuclei ($p=0.0040$) (Figure 5C). Cytochalasin B treatment did not significantly change the number of counted micronuclei per cell. A high percentage of *Rmi1^{hy/hy}* cells obtained from freshly dissociated embryos contained micronuclei ($43.1\% \pm 7.1$, $n=160$) compared to wild-type ($18.6\% \pm 4.1$, $n=160$) ($p=0.0068$, t-test). As per the distribution of micronuclei in fresh dissociated cells, significant differences were observed only in cells containing zero micronuclei ($p=0.0474$, paired two tailed t-test), 2 micronuclei ($p=0.0374$) and 3 micronuclei ($p=0.0374$) (Supplementary Figure 2A-C).

It is also possible that the proliferation defect observed in *Rmi1^{hy/hy}* MEFs may have its origin in chromosome abnormalities. Chromosome spreads prepared from freshly dissociated 9.5 dpc embryos treated with nocodazole showed that, in sharp contrast to wild-type ($4.6\% \pm 0.6\%$ $n=768$ metaphase), approximately $30\% \pm 4.4\%$ of *Rmi1^{hy/hy}* cells ($n=835$) ($p=0.0002$, unpaired t-test) contained partially condensed chromosomes (Figure 5D and E). These cells are negative for TUNEL assay (not shown), indicating that apoptosis may not be the cause of partial chromosome condensation.

3.6. Double-strand break repair in Rmi1^{hy/hy} MEFs

Double-strand break repair can be used as a tool to investigate the ability of cells to repair damaged DNA. We induced double-strand breaks by gamma irradiation and followed their repair by monitoring H2AX immunostaining. We observed a background signal for

H2AX immunostaining, perhaps resulting from replication and/or spontaneous damage in non-irradiated wild-type and *Rmi1^{hy/hy}* MEFs. After irradiation (50 Gy), wild-type and *Rmi1^{hy/hy}* cells showed a peak H2AX signal at 1 hour and displayed similar kinetics of

H2AX signal disappearance (Figure 6). Our results indicate that depletion of RMI1 does not affect the repair of the bulk of double-strand breaks introduced by gamma irradiation. However, the outcome of double-strand break repair, i.e. higher number of crossovers in *Rmi1^{hy/hy}*, remains to be determined.

4. Discussion

Most of our knowledge about RMI1 function(s) has been obtained from *in vitro* biochemical studies. The *in vivo* role of RMI1, in contrast, remains understudied. Previous attempts to generate *Rmi1^{hy/hy}* mouse resulted in pre-implantation embryonic lethality. This impeded the use of MEFs and embryonic morphology to assess the *in vivo* role of RMI1. The mouse model constructed in this study contains a gene trap cassette inserted in the intron flanked by exons 1 and 2, which are non-coding exons. However, the stop codon present on the trap

cassette disrupts the transcription of *Rmi1*. The generated mouse allowed us to analyze initial stages of embryonic development and the cellular phenotype of *Rmi1^{hy/hy}* MEFs. We conclude that RMI1 is essential for normal embryonic development, cell viability and genome stability.

We have shown that our *Rmi1^{hy/hy}* mice died at 9.5 dpc. This phenotype is significantly less severe than two *Rmi1* mutant mice reported previously, which die before implantation [36, 37]. A number of possible reasons could account for this difference. First, genetic backgrounds and distinct target mutations may have an effect on the phenotypes of all three *Rmi1^{hy/hy}* strains. For example, Chen's knockout [37] was created by deleting the third exon of *Rmi1*, as opposed to the disruptive gene trap insertion between exon 1 and 2 in our mice. An alternative explanation for the longer embryonic life of our mice could be attributed to low levels of *Rmi1* expression, as indicated by quantitative PCR analysis (5% of expression with respect to wild-type). This could be an indicator of an *Rmi1* mutation that is not null but hypomorphic.

Knockout mice for other members of the BTR complex have also been generated. Complete inactivation of the BLM loci resulted in embryonic growth retardation with no morphological abnormalities, and lethality at 13.5 dpc [14]. Compared with *Blm^{-/-}* mice, embryonic lethality in our *Rmi1^{hy/hy}* mice occurred at a much earlier stage and was accompanied by a development arrest. A possible explanation for these differences is that RMI1 might be playing an additional role that is independent from its role together with BLM. Supporting this argument is the fact that RMI1 but not BLM is required for normal replication progression [31], and RMI1 can form BLM-independent complexes with TOP3 and RMI2 [24, 44, 45]. Indeed, the ability of RMI1 to interact and regulate TOP3 function may partially explain the earlier embryonic lethality observed for *Rmi1^{hy/hy}* with respect to *Blm^{-/-}*. In agreement with this, the *Top3^{-/-}* exhibits a pre-implantation embryonic lethality [35]. Finally, the fact that RMI1 is required for the stability of other components of the BTR complex should also be considered. Loss of RMI1 resulted in decreased expression of all members of the BTR complex (Figure 1E), which lead to reduced levels of BLM, TOP3 and RMI2 proteins [13, 24, 26]. Therefore it is possible that the phenotype of *Rmi1^{hy/hy}* may result from a compounded deficit of RMI1, TOP3 and BLM.

What is the cause of embryonic lethality in *Rmi1^{hy/hy}* embryos? Our data reveal several major defects that may contribute to embryonic lethality. First, MEFs from *Rmi1^{hy/hy}* embryos exhibited severely impaired cell proliferation. This agrees with previous reports showing a prominent decrease in cell proliferation in HeLa and HCT116 cells treated with siRNA specific for *Rmi1* [13, 37]. This cell proliferation defect is also observed in mammalian and yeast cells after knockdown or deletion of other components of the BTR complex such as BLM or TOP3 [12, 14, 27]. The severely impaired proliferation in *Rmi1^{hy/hy}* cells may result from a requirement for RMI1 in DNA replication. Indeed, it was recently reported that RMI1 plays an important role in DNA replication progression, and when associated with BLM, and TOP3 mediates the recovery of replication fork stress and fork progression [31, 32, 46]. Furthermore, yeast depleted of RMI1 accumulate in G2/M [30] and persistent activation of this checkpoint probably accounts for the G2/M delay observed in cells treated with DNA replication inhibitors [47]. Interestingly, after 18 days in culture *Rmi1^{hy/hy}* cells show abnormally high DNA content. These ploidy problems could arise from mitotic chromosome missegregation. However, these cells are severely impaired in proliferation and do not undergo mitosis. Therefore, we favor the view in which *Rmi1^{hy/hy}* cells replicate DNA without entering mitosis. An alternative possible cause of the proliferation defect in *Rmi1^{hy/hy}* cells is genome instability. Indeed, we observed that *Rmi1^{hy/hy}* MEFs show an increase in the number of micronuclei, a classical indication of

genome instability [42, 43, 48]. This extensive genetic instability may also serve as a primary reason for the death of mutant embryos.

We also note a high proportion of loosely compacted chromosomes in *Rmi1^{hy/hy}* metaphase cells. Poor chromosome condensation may interfere with normal progression of the remaining phases of mitosis and consequently lead to cell arrest. We observed that a major fraction of cells containing partially condensed chromosomes were negative for TUNEL staining, and the ability of *Rmi1* deficient cells to repair DSBs introduced by gamma irradiation is apparently indistinguishable from wild-type (Figure 6). These results indicate that apoptosis and/or inability to repair DNA damage may not be the cause of the failure of *Rmi1* deficient cells to condense chromosomes. We favor an alternative possible scenario in which deficient DNA replication may be the cause of poor chromosome condensation. Indeed, recent reports show that RMI1 plays an important role in DNA replication progression and when associated with BLM and TOP3 mediates the recovery of replication fork stress and fork progression [31, 32, 46].

In summary, our data shows that RMI1 is required for embryonic development, and provides insight into the poorly understood cellular functions of RMI1 on cell proliferation and in maintaining genome stability. Future studies of RMI1 function will focus on determining BTR-dependent and independent roles of RMI1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Deletion of *Rmi1* in mice results in embryonic developmental defects.
- *Rmi1*^{hy/hy} MEFs exhibit genome instability and severely impaired cell proliferation.
- RMI1 action is crucial for mechanisms that maintain genome stability.

Fig. 1.

Design and generation of the *Rmi1^{hy/hy}* mice. (A) Scheme showing the gene trap strategy used to disrupt the *Rmi1* gene. Exons (E) 1 through 3 are shown by filled boxes. The trapping cassette shows the splice acceptor (SA) the neomycin sequence (Neo) and the polyadenylation sequence (pA). Primers used for genotyping are indicated by arrows. (B) Agarose gel showing PCR products of the genotyping strategy. (C) Percentages of one month old wild-type (n=89), *Rmi1^{wt/hy}* (n=146) and *Rmi1^{hy/hy}* (n=0) adult mice obtained from *Rmi1^{wt/hy}* intercrosses (235 total offspring analyzed). (D) Quantitative RT-PCR of *Rmi1* expression in 9.5 dpc wild-type, *Rmi1^{wt/hy}* and *Rmi1^{hy/hy}* embryos. Primers used (qRmi1f and qRmi1r) are indicated by arrowheads in A. (E) Quantitative RT-PCR of expression of components of the BTR complex and control genes in 9.5 dpc wild-type and *Rmi1^{hy/hy}* embryos. Primers used are described in supplementary Table 1.

Fig. 2. *Rmi1^{hy/hy}* embryos exhibit development delay and elevated numbers of apoptotic cells. (A) Macroscopic analysis of wild-type, *Rmi1^{wt/hy}* and *Rmi1^{hy/hy}* 9.5 dpc embryos. (B) Histological analysis of hematoxylin & eosin-stained wild-type and *Rmi1^{hy/hy}* embryos. Hind limb buds (arrows) and unclosed neural tube (arrow head) are indicated. (C) Note increased numbers of TUNEL positive cells (an indication of apoptosis. Green cells) in *Rmi1^{hy/hy}* with respect to wild-type. Insets show higher magnification of areas with apoptotic cells.

Fig. 3.

Proliferation defects in MEFs from *Rmi1^{hy/hy}* embryos. (A) Growth curve showing proliferation of mouse embryonic fibroblasts derived from wild-type (n=6, number of embryos), *Rmi1^{wt/hy}* (n=8) and *Rmi1^{hy/hy}* (n=5) 9.5 dpc embryos. The number of cells is plotted relative to the initial number of cells counted (1×10^4 cells for wild-type, *Rmi1^{wt/hy}* and *Rmi1^{hy/hy}*). (B) Image of wild-type and *Rmi1^{hy/hy}* MEFs showing EdU incorporation. Cells that incorporated EdU are green. DAPI staining is in grey. (C) Incorporation of EdU by wild-type and *Rmi1^{hy/hy}* MEFs is plotted as a percentage of cells containing EdU in their nuclei after various times of incubation. Error bars show the standard deviation of the mean. (D) Frozen sections of 9.5 dpc embryos immunostained with anti phospho-histone H3 (green cells). DAPI staining is in green.

Fig. 4. Elevated DNA content and nuclear area in *Rmi1^{hy/hy}* MEFs. (A) A representative result from flow cytometry analysis of DNA content in asynchronous wild-type and *Rmi1^{hy/hy}* cultured MEFs. (B) DNA content analysis of asynchronous cultured MEFs shown as a percentage of cells with different DNA content. MEFs obtained from different embryos (wild-type embryos n=5, *Rmi1^{hy/hy}* MEFs n=7) were used to replicate the results obtained. Error bars show the standard deviation of the mean. (C) Examples of wild-type and *Rmi1^{hy/hy}* MEFs stained with DAPI showing differences in nuclear area. (D) Distribution of nuclear area in wild-type and *Rmi1^{hy/hy}* MEFs.

Fig. 5. Genome instability in *Rmi1^{hy/hy}* deficient cells. (A) Representative wild-type and *Rmi1^{hy/hy}* MEFs stained with DAPI. Note the presence of micronuclei (arrows) in mutant cells. (B) Quantitation of wild-type and *Rmi1^{hy/hy}* cells containing at least one micronucleus. (C) Distribution of the number of micronuclei per cell in wild-type and *Rmi1^{hy/hy}* MEFs populations. (D) Examples of metaphase spreads showing normally condensed chromosomes in wild-type and incompletely condensed chromosomes in *Rmi1^{hy/hy}* MEFs from 9.5 dpc embryos. (E) Quantitation of normal and partially condensed chromosomes from wild-type and *Rmi1^{hy/hy}* metaphase spreads.

Fig. 6. Kinetics of irradiation induced double-strand break repair in wild-type and *Rmi1^{hy/hy}* MEFs. Intensity of immunosignal for γ H2AX is plotted at the indicated times in irradiated and non-irradiated (NI) MEFs.

Table 1

Proportion of wild-type, *Rmi1^{wt/hy}* and *Rmi1^{hy/hy}* embryos obtained from time mating *Rmi1^{wt/hy}* intercrosses.

Number of embryos obtained from <i>Rmi1^{+/-}</i> intercrosses					
dpc	Wild-type	<i>Rmi1^{wt/hy}</i>	<i>Rmi1^{hy/hy}</i>	Resorption	Total
11.5	16 (36.3%)±1.1	25 (56.8%)±1.2	0	3 (6.8%)±0.8	44
10.5	24 (30.7%)±0.7	43 (55.1%)±0.9	1 (1.2%)±0.3	10 (12.8%)±0.8	78
9.5	36 (23.2%)±1.2	74 (47.7%)±1.9	33 (21.2%)±1.2	12 (7.7%)±0.9	155
8.5	14 (29.1%) ±0.8	21 (43.7%) ±2.2	9 (18.7) ±0.8	4 (8.3%) ±0.8	48

Table 2

The mean and standard deviation of wild-type and *Rmi1^{hy/hy}* cells incorporating EdU.

EdU incorporation (hours)	Genotype			
	Wild-type (% EdU positive cells)	N	<i>Rmi1^{hy/hy}</i> (% EdU positive cells)	N
2	15.1±4.2	600	1.7±0.4 ^b	400
4	22.9±3.0	600	1.5±1.4 ^b	400
8	27.2±3.2	600	1.7±1.3 ^b	400
24	35.1±1.4	600	1.6±0.6 ^a	400

^aSignificantly different from wild-type(p 0.0001, t test).

^bSignificantly different from wild-type(p 0.0053, t test).