Genomic Instability, Defective Spermatogenesis, Immunodeficiency, and Cancer in a Mouse Model of the RIDDLE Syndrome

Toshiyuki Bohgaki1,2, Miyuki Bohgaki1,2, Renato Cardoso1, Stephanie Panier2, Dimphy Zeegers3, Li Li1, Grant S. Stewart4, Otto Sanchez5, M. Prakash Hande4, Daniel Durocher2, Anne Hakem1,*, Razqallah Hakem1,†

1 Ontario Cancer Institute, University Health Network and Department of Medical Biophysics, University of Toronto, Toronto, Canada, 2 Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada, 3 Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, 4 Cancer Research UK, Institute for Cancer Studies, Birmingham University, Birmingham, United Kingdom, 5 University of Ontario Institute of Technology, Oshawa, Canada

Abstract

Eukaryotic cells have evolved to use complex pathways for DNA damage signaling and repair to maintain genomic integrity. RNF168 is a novel E3 ligase that functions downstream of ATM, γ-H2A.X, MDC1, and RNF8. It has been shown to ubiquitylate histone H2A and to facilitate the recruitment of other DNA damage response proteins, including 53BP1, to sites of DNA break. In addition, RNF168 mutations have been causally linked to the human RIDDLE syndrome. In this study, we report that Rnf168−/− mice are immunodeficient and exhibit increased radiosensitivity. Rnf168−/− males suffer from impaired spermatogenesis in an age-dependent manner. Interestingly, in contrast to H2a.x−/−, Mdc1−/−, and Rnf8−/− cells, transient recruitment of 53bp1 to DNA double-strand breaks was abolished in Rnf168−/− cells. Remarkably, similar to 53bp1 inactivation, but different from H2a.x deficiency, inactivation of Rnf168 impairs long-range V(D)J recombination in thymocytes and results in long insertions at the class-switch junctions of B-cells. Loss of Rnf168 increases genomic instability and synergizes with p53 inactivation in promoting tumorigenesis. Our data reveal the important physiological functions of Rnf168 and support its role in both γ-H2a.x-Mdc1-Rnf8-dependent and -independent signaling pathways of DNA double-strand breaks. These results highlight a central role for Rnf168 in the hierarchical network of DNA break signaling that maintains genomic integrity and suppresses cancer development in mammals.

Introduction

DNA damage checkpoint signaling and DNA repair pathways are key elements of the DNA damage response (DDR) and are critical for the maintenance of genomic integrity [1–3]. Mammalian cells constantly experience DNA damage as a result of exogenous exposure to ionizing radiation (IR), ultraviolet light (UV), chemical compounds, and radical oxygen species, as well as endogenous insults due to DNA replication errors. In addition, double-strand DNA breaks (DSBs) are also programmed to occur during immune-receptor rearrangements and meiosis.

Mutations of genes involved in DNA damage signaling or repair can lead to many diseases including neurodegenerative diseases, immunodeficiency and cancer, underlining the importance of these processes [1,4,5]. Among the various types of DNA damage, DSBs are the most serious and require elaborated networks of proteins to signal and repair the damage. In mammalian cells, DSBs are initially recognized by the Mre11/Rad50/NBS1 (MRN) complex that induces the activation and recruitment of the ataxia-telangiectasia-mutated (ATM) kinase to the break sites [6–8]. At the flanking sites of DSBs, H2A.X, a variant of the histone H2A, is rapidly phosphorylated at the serine 139 residue (γ-H2A.X). Phosphorylation of H2A.X is mediated by activated ATM, which itself is phosphorylated at serine 1981 (phospho-ATM-S1981), or alternatively by two other phosphoinositide 3-kinase like kinases (PIKKs), namely the ataxia telangiectasia and Rad3 related (ATR) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Active ATM also phosphorylates a number of other proteins including the structural maintenance of chromosomes 1 (SMC1), the Nijmegen breakage syndrome protein 1 (NBS1), the checkpoint kinase 2 (Chk2), the breast cancer 1- early onset (BRCA1) and the mediator of DNA damage checkpoint protein-1 (MDC1). Subsequent to its phosphorylation, MDC1 binds to γ-H2A.X via its tandem BRCA1 C-terminal (BRCT) domains, and recruits additional active ATM to sites flanking the DSBs [7,9,10]. MDC1 also associates with MRN complex through its Ser-Asp-Thr (SDT) repeats and the fork-head associated (FHA) domain of NBS1. Furthermore, through its C-terminus, NBS1 also interacts...
with ATM [11–15]. As such, MDC1 bridges the interaction of MRN to γ-H2AX and ATM. Enrichment of ATM-MDC1 and ATM-MRN at the break sites further amplify the phosphorylation of H2AX and triggers the recruitment of other DNA damage response proteins to the DSB flanking regions [16]. The three conserved T-Q-X-F clusters between 698 to 800 amino acids of MDC1 are also phosphorylated by ATM. The threonine-phosphorylated MDC1 has been shown to interact with the FHA domain of RING finger protein 8 (RNF8), thus recruiting this E3 ligase to sites of DNA damage. Subsequently, RNF8 along with the E2 ubiquitin-conjugating enzyme UBC13, mediate lysine ubiquitylation of histone H2A at the flanking regions of DSBs [17–19]. Such ubiquitylated form of H2A interacts with the MIU2 (motif interacting with ubiquitin 2) domain of RNF168 and recruits this E3 ligase to DSB sites, allowing it to further ubiquitylate surrounding H2A [20–22]. This likely amplifies the modification of chromatin structure at regions adjacent to DSBs, and facilitates the recruitment of tumor suppressor p53 binding protein 1 (53BP1). In addition, RAP80 selectively binds to the K63-polyubiquitin chains on H2A via its tandem ubiquitin interaction motifs (UIMs) [23], and acts as a bridge to recruit BRCA1 to the regions of DSBs. In sum, DSBs signaling is a highly regulated process, in which RNF168 plays a major role through its contribution to the recruitment of various downstream DDR proteins.

**RNF168 is mutated in RIDDLE syndrome, which is characterized by radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties [24].** RNF168 contains a RING finger domain and two MIU domains. The RING finger domain of RNF168 is critical for its ubiquitin E3 ligase activity, whereas its MIU2 domain mediates its binding to ubiquitylated H2A [20–22]. Knockdown of RNF168 in human cells significantly impaired the formation of 53BP1, RAP80 and BRCA1 ionizing radiation induced foci (IRIF). While recent studies have revealed a role for RNF168 in ubiquitylating H2A during DSB signaling [20–22], the full physiological functions of RNF168 are not understood.

Here, we report the generation of a mouse model for RNF168 deficiency. Rnf168−/− mice are viable. Consistent with the clinical features of RIDDLE syndrome, Rnf168−/− mice are immunodeficient, and their cells show increased radiosensitivity. Similar to 53bp1 deficiency, long-range V(D)J recombination of T-cell receptor (TCR)δ is also impaired in Rnf168−/− thymocytes, while aberrant long insertions have been observed at class switch junctions of Rnf168−/− B-lymphocytes. Moreover, in contrast to the transient recruitment of 53bp1 to DSBs in H2a.x-, Mdc1- and Rnf8-deficient mouse embryonic fibroblasts (MEFs), 53bp1 recruitment to DSB sites is completely abolished in Rnf168-deficient MEFs. Our data also demonstrate novel roles of Rnf168 in spermatogenesis, maintenance of genomic integrity and cancer, and therefore, further highlight the other important physiological functions of this molecule.

**Results**

Rnf168 is dispensable for embryonic and postnatal development

To investigate the physiological role of Rnf168, we generated Rnf168−/− mice using two different gene trap embryonic stem cell (ES) clones (156B6 and 405F11). Gene trap of Rnf168 in the two ES clones was confirmed by Southern blot, PCR and sequencing of the genomic site of the retrovirus integration (Figure S1A and S1B). RT-PCR revealed the disruption of full length Rnf168 transcript in the two Rnf168 gene trap ES clones (Figure S1C). The loss of Rnf168 protein was also confirmed in Rnf168−/− MEFs and splenocytes by immunoblotting (Figure S1D and S1E).

Intercrossing of Rnf168−/− mice obtained with 156B6 and 405F11 ES clones indicated that Rnf168−/− mice were viable and born at the expected Mendelian ratio (Table S1). Rnf168−/− mice did not exhibit any gross developmental defects and were indistinguishable from their wildtype (WT) littersmates. Collectively, these data indicate that the E3 ligase Rnf168 is not required for embryonic and postnatal development.

Rnf168 deficiency leads to increased radiation sensitivity

Mutation of the RNF168 gene is associated with RIDDLE syndrome [22,24]. To determine whether Rnf168 deficiency in mice confers increased sensitivity to DNA damage, primary lymphocytes isolated from peripheral lymph nodes (LN) of Rnf168−/− mice and WT littersmates were subjected to various doses of IR or UV. Lymphocyte viability was examined using 7-aminoactinomycin D (7AAD) and flow cytometry. These studies demonstrate a significant increase in the sensitivity of Rnf168−/− lymphocytes to IR and UV treatment (Figure 1A).

DNA damage signaling plays essential roles in the activation of checkpoints and cell cycle progression [25]. Examination of the effect of Rnf168 inactivation on cell cycle progression using G1/S synchronized MEFs showed no difference between Rnf168−/− and WT MEFs (Figure S2A).

Atm−/−, H2a.x−/−, Mdc1−/−, Rnf8−/− and 53bp1−/− cells exhibit a defect in the early G2/M checkpoint [10,17–19,26–28]. Atm and 53bp1-deficient cells display late G2/M accumulation and inactivation of 53bp1 results in a mild impairment of the intra-S phase checkpoint [26,28]. Therefore, we examined the effects of Rnf168 deficiency on cell cycle checkpoints. Immunostaining of irradiated Rnf168−/− and WT MEFs with anti-phospho-histone H3 (pHH3), and co-staining with propidium iodide (PI) demonstrated a higher proportion of irradiated Rnf168−/− MEFs (35.2±1.8%) have progressed to M phase compared to WT MEFs (10.7±4.3%, p<0.05) (Figure 1B). Next, the effect of irradiation on cell cycle progression of Rnf168−/− MEFs and WT controls was examined using the Bromodeoxyuridine (BrdU) and PI assay. The percentage of Rnf168−/− cells in S phase 3 hours post-IR (10 Gy) was slightly increased compared to WT cells (Figure 1C). These data suggest that similar to 53bp1−/− MEFs [24,28], Rnf168−/− MEFs also have a mild defect in the intra-S phase checkpoint. In addition, cell cycle analyses of Rnf168−/− and WT MEFs 18 hours post-IR indicated that a higher proportion of the Rnf168−/− MEFs is found at the G2/M phase (Figure 1D). Therefore, similar to H2a.x, Mdc1, Rnf8 and 53bp1, Rnf168 is dispensable for the activation of the G1/S checkpoint, but is important for enforcing the G2/M DNA damage checkpoint.
Rnf168 promotes the recruitment of 53bp1 and Brca1 to DNA breaks

Through ubiquitylation of histone H2A, RNF168 plays an important role in the recruitment of 53BP1 to the sites of DNA breaks downstream of H2A.X, MDC1 and RNF8 [20–22]. Although H2a.x, Mdc1 and Rnf8 are each essential for the stable ‘retention’ of 53bp1 foci at DSB sites, studies of H2a.x−/−, Mdc1−/− and Rnf8−/− MEFs demonstrate that these cells can still transiently recruit 53bp1 to form IRIF [29]. In addition, a partial accumulation of 53bp1 at DSBs has been also observed in activated Rnf8−/− B-cells post-irradiation [30]. To investigate the effect of Rnf168 deficiency on the recruitment of 53bp1 to sites of DSBs, Rnf168−/−,

Figure 1. Impaired DNA damage response in Rnf168 deficient cells. (A) Cells were subjected to IR or UV, and the extent of cell death was determined 24 hours later. Three independent experiments were performed. Data are presented as the mean ± SEM. *p<0.05. (B) Primary MEFs (passage 2) either untreated or irradiated (2 Gy) were stained with anti-phospho-histone H3 (pHH3) antibody and PI at 1 hour post IR. The percentage of mitotic cells was determined by FACS. Three independent experiments were performed. Data are presented as the mean ± SEM. *p<0.05. (C) Cell cycle progression post IR (10 Gy) treatment of WT and Rnf168−/− primary MEFs (Passage 2) was examined using BrdU/PI assay and FACS. Representative data are shown for three independent experiments. (D) Accumulation at the G2 phase of WT and Rnf168−/− primary MEFs (passage 2) post IR (10 Gy). Cell cycle profiles were examined by PI staining and representative data are shown from three independent experiments. doi:10.1371/journal.pgen.1001381.g001
**Rnf168** is dispensable for the activation of ATM signaling pathway

To examine whether Rnf168 deficiency affects the activation of ATM pathway, WT and **Rnf168**/−/− thymocytes were irradiated *ex vivo*. One hour post-IR, the protein levels of total Atm, as well as the expression and phosphorylation levels of its downstream targets, were assessed by immunoblot (IB) analysis. The protein levels of Atm were similar between **Rnf168**/−/− and WT thymocytes in response to IR (Figure 3A). Phosphorylation levels of the Atm substrates Smc1 (serine 966), Nbs1 (serine 343), Chk2 (threonine 68) and p53 (serine 15), were similar or slightly higher of the Atm substrates Smc1 (serine 966), Nbs1 (serine 343), Chk2 (threonine 68) and p53 (serine 15) in **Rnf168**/−/− compared to WT controls (Figure 3A and 3B). IR also induced a slightly higher phosphorylation level of Chk2 (threonine 68) and p53 (serine 15) in **Rnf168**/−/− MEFs compared to WT controls (Figure 3C). We also observed increased γ-H2ax and Mdc1 foci formation in unirradiated and irradiated **Rnf168**/−/− MEFs compared to WT controls (Figure 3D and 3E, Figure S3C and S3D). In addiction, γ-H2ax-x- and Mdc1- IRIF remained visible longer in irradiated **Rnf168**/−/− MEFs compared to WT controls, suggesting defective DSB repair in the absence of Rnf168. As both Atm and DNA-PK can phosphorylate H2a.x at serine 139 in response to IR [31], we examined the effects of inhibition of DNA-PK on γ-H2ax-IRIF in WT and **Rnf168**/−/− MEFS. The levels of γ-H2ax-IRIF in irradiated WT and **Rnf168**/−/− MEFS were not affected by DNA-PK inhibitors (Figure 3F). Collectively, these data indicate that Rnf168 is dispensable for the activation of ATM-Chk2-p53 pathway and that ATM signaling pathway is unaffected in **Rnf168**/−/− cells.

**Age-dependent requirement for Rnf168 in spermatogenesis**

H2a.x, Mdc1 and Rnf8 are required for DDR upstream to Rnf168, and are essential for male fertility [10,27,30,32–35], whereas 53bp1 homozygous mutant males are fertile [28]. We therefore investigated the fertility of **Rnf168**/−/− mice, **Rnf168**/−/− males and females were fertile at 8 weeks of age, albeit their intercrosses produced smaller litters (6.0 pups±0.6) compared to WT littersmates (9.8 pups±0.7, p<0.05) (Figure 4A). In contrast, 12-month-old **Rnf168**/−/− males showed either a significantly reduced fertility compared to WT littermates or were completely infertile (Figure 4B). Testicular sizes were comparable between 8-week-old **Rnf168**/−/− and WT littermates, whereas at 12 months of age, **Rnf168**/−/− males showed reduced testicular size compared to WT littermates (Figure 4C). Histological examination of testes from 8-week-old **Rnf168**/−/− males indicated no abnormalities compared to WT littermates (Figure 4D). However, testes from aged **Rnf168**/−/− mice displayed signs of testicular degeneration and atrophy as evidenced by the reduced number or lack of spermatids in seminiferous tubules, the increased vacuolization of germ cells and the prominent Leydig cells compared to WT littermates (Figure 4D). The amount of sperm was also drastically decreased in the epididymis of 12-month-old **Rnf168**/−/− males compared to controls (Figure 4E). All together, these data indicate that Rnf168 is required for spermatogenesis in an age-dependent manner.

**Inactivation of Rnf168 impairs immunoglobulin class switch recombination and results in immunodeficiency**

The proportion of B and T lymphocytes in one RIDDLE syndrome patient was reported to be within the normal range; however, this patient was immunodeficient and exhibited low levels of serum immunoglobulin G (IgG) [24]. To investigate the effect of **Rnf168** deficiency on lymphocyte maturation, we first examined bone marrow (BM) from 6–8-week-old **Rnf168**/−/− mice and their WT littermates. BM cellularity and the number of Pro- and Pre-B-cells were not affected by **Rnf168** deletion (Figure S4A). Similarly, examination of the number of splenocytes, LN cells and their subpopulations also showed no significant difference between **Rnf168**/−/− mice and WT controls (Figure S4B and S4C).

The repair of programmed DSBs is essential for class switch recombination (CSR) of immunoglobulins [36,37]. Failure to initiate, signal, or repair these programmed DSBs will lead to defective CSR and immunodeficiency. To assess the role of Rnf168 in Ig heavy chain (Igh) class switching in vivo, we evaluated the concentrations of serum Ig isotypes in **Rnf168**/−/− and WT littermates. Total serum IgG concentrations were significantly lower in 6–8-week-old **Rnf168**/−/− mice and in 9–12-month-old **Rnf168**/−/− mice compared to age-matched WT littermates (Figure 5A). To further examine the in vivo role of Rnf168 in CSR, we determined the portion of IgG1- and IgG3-expressing B-cells in Peyer’s patches from **Rnf168**/−/− mice and WT controls. B-cells in the Peyer’s patches constantly encounter the gut flora, and, therefore, they exhibit elevated levels of CSR [38]. Peyer’s patches from **Rnf168**/−/− mice showed decreased representation of IgG1- and IgG3-expressing B-cells compared to WT littermates (p<0.05, Figure 5B and 5C).

We further examined the role **Rnf168** plays in CSR for various Ig heavy chain isotypes using purified splenic B-cells from **Rnf168**/−/− mice and WT littermates. When B-cells were cultivated in culture with LPS±IL4, **Rnf168**-deficient B-cells displayed a significantly reduced secretion of IgG1, IgG2a and IgG3 compared to WT controls (Figure 5D). We also stimulated *in vitro* purified splenic B-cells with anti-CD40 or LPS in combination with IL-4, and examined the levels of CSR to IgG1. To analyze the efficiency of CSR in each cell division, we used FACs analysis to assess the expression of surface IgG1 on CFSE-labeled stimulated B-cells (Figure 5E and 5F, Figure S4D and S4E). The portion of B-cells expressing surface IgG1 post activation was significantly reduced in **Rnf168**/−/− mice (13.2±1.1%) compared to WT controls (28.3±0.8%, p<0.05) (Figure 5E). Examination of the CFSE dilution profiles indicated similar proliferation levels of mature B-cells from WT and **Rnf168**/−/− mice (Figure 5F and Figure S4E). In addition, the efficiency of CSR in each cell division was decreased in activated **Rnf168**/−/− B-cells compared to WT B-cells (Figure 5F). These data indicate that CSR defects in **Rnf168**/−/− B-cells were directly due to the loss of Rnf168 function, rather than a result of proliferation defects of the mutant B-cells.

The RING finger domain of RNF168 is critical for its E3 ligase activity, while its MIU2 domain is important for its recruitment to DSB sites [20,22]. To further explore the role of **Rnf168** in CSR, we generated deletion or point mutant **Rnf168** constructs using the pMSCV retroviral vector. Purified B-cells from WT or **Rnf168**/−/− mice were activated with LPS and IL-4 and then retrovirally transduced with the various Rnf168-expression constructs. These
Figure 2. Rnf168 is required for the recruitment of 53bp1 to DNA damage sites. (A) Representative micrographs of MEFs stained with anti-53bp1 antibody and DAPI. Rnf168<sup>−/−</sup> and WT MEFs were untreated or exposed to 5 Gy of IR and fixed at the indicated time post IR. Bars, 20 μm. (B) Representative micrographs of MEFs stained with anti-53bp1 antibody and DAPI are shown. Rnf168<sup>−/−</sup>, Rnf8<sup>−/−</sup> and WT MEFs were untreated or exposed to 5 Gy of IR and fixed at the indicated time post IR. Three independent experiments were performed. Bars, 20 μm. (C) Recovery of 53bp1 IRIF formation in Rnf168<sup>−/−</sup> MEFs complemented with full length RNF168. Immortalized Rnf168<sup>−/−</sup> MEFs were mock-transfected or transfected with GFP-tagged RNF168 expression vectors and cultured for 24 hours. Cells were fixed at 1 hour post-IR (5 Gy) and processed for immunofluorescence staining with anti-53bp1 antibodies. Three independent experiments were performed. Bars, 20 μm. (D) Clonogenic assay was performed to examine radiosensitivity of Rnf168<sup>−/−</sup> MEFs complemented with exogenous Rnf168 (left panel). Expression level of Rnf168 is shown for the MEFs used for the clonogenic assay (right panel). Data shown is representative of three independent experiments and is presented as the mean ± SEM. *p<0.05. (E) Representative micrographs of MEFs stained with anti-Breca1 antibody and DAPI. Rnf168<sup>−/−</sup> and WT MEFs were either untreated or exposed to 5 Gy of IR and fixed at the indicated time after IR. Bars, 2 μm.

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cells were then examined for functional rescue of the impaired CSR by WT or mutants Rnf168 (Figure 5G, 5H and Figure S4F). Rnf168<sup>−/−</sup> B-cells, infected with pMSCV expressing either WT Rnf168 or Rnf168 with its MIU1 domain deleted (ΔMIU1), rescued IgG1 CSR to the level of WT B-cells. However, Rnf168<sup>−/−</sup> B-cells infected with retroviruses expressing Rnf168 with mutated RING finger domain (C21S; cysteine 21 residue to serine), deleted MIU2 domain (ΔMIU2) or deleted MIU1 and MIU2 domains (ΔMIU1/2), showed no rescue of their CSR defects.

We next examined the switch recombination directly by using a quantitative digestion-circularization PCR (DC-PCR) assay [39]. These analyses demonstrated lower frequency of S<sub>m</sub>-S<sub>c</sub>1 switch recombination in activated Rnf168<sup>−/−</sup> B-cells compared to WT B-cells (Figure 5I and Figure S4G).

**Figure 3. Atm signaling pathway is not affected in the absence of Rnf168.** (A–C) IB analysis of untreated or irradiated WT and Rnf168<sup>−/−</sup> thymocytes (A and B) or MEFs (C). Cells were irradiated as indicated and examined 1 hour post-IR by immunoblotting using the indicated antibodies. Representative data are shown from three independent experiments. (D) Representative micrographs of MEFs stained with anti γ-H2a.x antibody and DAPI. Rnf168<sup>−/−</sup> and WT MEFs were untreated or exposed to 5 Gy of IR and fixed at the indicated time after IR. Bars, 20 μm. Three independent experiments were performed. (E) Representative micrographs of MEFs stained with anti-Mdc1 antibody and DAPI. Rnf168<sup>−/−</sup> and WT MEFs were untreated or exposed to 5 Gy of IR and fixed at the indicated times post IR. Three independent experiments were performed. Bars, 20 μm. (F) Representative micrographs of MEFs treated with DNA-PK inhibitors and stained with anti γ-H2a.x antibody and DAPI. Rnf168<sup>−/−</sup> and WT MEFs were treated with DNA-PK inhibitors (NU7026 or NU7441) and cultured for 1 hour. MEFs were left untreated or exposed to 5 Gy of IR and fixed 2 hours post-IR. Three independent experiments were performed. Bars, 20 μm.

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The DNA sequences of CSR junctions can offer additional insights into the mechanistic defects of the joining process [40]. For instance, B-cells with homozygous mutations in Xrcc4 or DNA Ligase IV undergo impaired CSR and form CSR junctions with increased sequence microhomology, indicating the use of an alternative joining pathway in these mutant cells [41,42]. Previous studies reported that B-cells deficient for H2a.x or 53bp1 show no significant differences in the extent of microhomology at the switch junctions [40,43–45]. To examine the effects of Rnf168 deficiency on the nucleotide composition of the switch junction regions, we cloned and sequenced the Sm-Sc1 junctions from Rnf168−/− and WT B-cells stimulated in vitro with LPS and IL-4. Such analysis revealed no significant differences in the extent of donor/acceptor homology (Figure 6A and 6B), in the frequency of mutations (Figure 6C) nor in the average length of overlaps (Table S2). Interestingly, in contrast to WT controls, 5.1% of the examined
Figure 5. Impaired class switch recombination in Rnf168−/− mice. (A) Serum immunoglobulin levels in 6–8-week-old and 9–12-month-old Rnf168−/− mice. The data are presented as the mean ± SEM (n = 3–5). (B and C) FACS analysis of IgG1 (B) or IgG3 (C) and IgM expression on B220+ B-cells (left panels) and average percentages of IgG1+ or IgG3+ B-cells (right panels) from Peyer’s patches. Three independent experiments were performed. (D) Secreted immunoglobulins were analyzed in the supernatants of B-cell cultures after 4 days of in vitro stimulation with LPS with or without IL-4. The data are presented as the mean ± SEM from three independent experiments. (E) FACS analysis of IgG1 expression on CFSE labeled B-cells stimulated with anti-CD40 plus IL-4 for 4 days (left panels) and average percentages of IgG1 switched cells (right panel). Five independent experiments were performed. (F) CFSE staining profiles of B-cells stimulated with anti-CD40 antibody plus IL-4 for 4 days (upper panel) and percentages of IgG1+ cells that had undergone the indicated number of cell divisions (lower panel). Representative data are shown from three independent experiments. (G) Schematic representations of WT and mutants Rnf168 cloned into the MSCV-IRES-GFP vector. (H) B-cells infected with the indicated ecotropic retroviruses [MSCV-mutated or full-length (FL) Rnf168-IRES-GFP] were stimulated with LPS plus IL-4 for 4 days, and the level of CSR to IgG1 was examined by FACS. Data are presented as the mean ± SEM of four independent experiments. * indicates p<0.05 compared to WT. (I) Representative DC-PCR for Ss−Sy1 recombination is shown from three independent experiments. nAchR served to normalize for the amount of input DNA. Fivefold serial dilutions were used as templates. H2O: no input DNA.

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Figure 6. Analysis of the effect of Rnf168 inactivation on Sm-Sc1CSR junctions. (A) Analysis of Sm-Sc1 CSR junctions. Overlap was determined by identifying the longest region at the switch junction of perfect uninterrupted donor/acceptor identity. No significant differences were observed. Representative data are shown from more than thirty clones in three independent experiments. (B) Purified splenic B cells were stimulated with LPS and IL-4 for 4 days. Genomic DNA was amplified by PCR and Sm-Sc1 junctions were sequenced. The percentage of junctions with the indicated nucleotide overlap is indicated (37 sequences from three WT mice and 39 sequences from three Rnf168^-/- mice were analyzed). (C) Mutations in B-cells stimulated with LPS plus IL-4 for 4 days. Mutations near the Sm-Sc1 junctions (±50 bp) and frequencies of mutations are shown (37 sequences from three WT mice and 39 sequences from three Rnf168^-/- mice were analyzed). The numbers of observed mutations are indicated in the periphery of the circular charts. (D) Sm/Sc1 junctions with unusual insertions obtained from Rnf168^-/- B-cells. Sm/Sc1 sequences are shown in bold. The Sm and Sc1 [NT_114985.2 (strain 129/SvJ)] germline sequences are shown above or below each junction sequence. Lower-case letters indicate insertions. (l) indicates identity between nucleotides. Homology at the junctions is boxed. Two clones, 26L10.7 and 24L4.1, were obtained from independent experiments. 37 sequences from three WT mice and 39 sequences from three Rnf168^-/- mice were analyzed.

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CSR junctions in Rnf168<sup>−/−</sup> B-cells were found to harbor long insertions (Figure 6D). Similar long insertions of nucleotides were observed in 2 out of 40 (5%) CSR junctions of 33bp<sup>1−/−</sup> B-cells but not in A<sup>m</sup>−/−, H<sub>2</sub>a.x<sup>−/−</sup>, N<sub>b</sub>6<sup>−/−</sup> or WT B-cells [40,43,44,46]. These data therefore suggest that Rnf168 contributes to the 53bp1 function in the synopsis of DNA ends.

Collectively, these data demonstrate that Rnf168 is required for in vivo and in vitro CSR, and its inactivation in mutant mice leads to immunodeficiency, which parallels the symptoms of the RIDDLE syndrome. Furthermore, we demonstrate that the RING finger and MIU2 domains of Rnf168 are dispensable for efficient CSR.

Finally, our data also indicate that, similar to 33bp1<sup>−/−</sup> B-cells, Rnf168<sup>−/−</sup> B-cells display increased frequency of long nucleotide insertions at the CSR junctions.

A role for Rnf168 in long-range V(D)J recombination

33bp1<sup>−/−</sup> mice exhibit impaired early thymocyte development, an accumulation of early CD4<sup>+</sup>CD8<sup>+</sup> double negative (DN) thymocytes at the DNIII stage (CD4<sup>+</sup>CD25<sup>+</sup>), reduced TCRβ expression and lower frequency of TCRγδ cells compared to WT littermates [28,47,48]. In contrast to the pronounced decrease of TCRβ expression in 33bp1<sup>−/−</sup> thymocytes, expression of TCRβ was not significantly affected in thymocytes deficient for H<sub>2</sub>a.x, M<sub>d</sub>c1 or R<sub<f>β</sub>0 [30,34,47].

FACS analysis of thymocytes from Rnf168<sup>−/−</sup> mice showed increased frequency of DN thymocytes compared to WT littermates (Figure 5A). Examination of DN subpopulations indicated a significantly impaired transition of Rnf168<sup>−/−</sup> early thymocytes at the DNIII stage (Figure 7A, left panel). In addition, total number of DNIII thymocytes in Rnf168<sup>−/−</sup> mice (1.5×10<sup>5</sup>±2.8×10<sup>4</sup>) was significantly increased compared to WT littermates (1.1×10<sup>5</sup>±1.9×10<sup>4</sup>, p = 0.025) [Figure 7A, right panel]. Rnf168<sup>−/−</sup> thymocytes also displayed a mild decrease in their expression of TCRβ compared to WT controls (Figure 7B) and the frequency of TCRγδ thymocytes in Rnf168<sup>−/−</sup> mice was significantly reduced compared to WT controls (Figure 5B; WT 0.24±0.21%, Rnf168<sup>−/−</sup> 0.20±0.01%, p<0.005). Productive rearrangement of the TCRβ locus takes place during the DNIII to DNIV transition and leads to pre-TCR expression [49–51]. Only cells expressing functional pre-TCR undergo exponential expansion, a process referred to as β-selection [49–51]. To assess the proliferation level of each DN thymocyte subpopulations, we examined in vivo thymocyte BrdU uptake in WT and Rnf168<sup>−/−</sup> mice. The number of BrdU<sup>+</sup> cells was not affected in Rnf168<sup>−/−</sup> mice compared to WT littermates (Figure 7C). Therefore, the increased number of DNIII thymocytes, the decreased expression of TCRβ and the reduced number of TCRγδ cells in Rnf168<sup>−/−</sup> thymocytes were not due to proliferative defects. However, it remains possible that developmental defects can also contribute to these differences.

Loss of function of 53bp1 results in impaired TcR locus integrity due to dysfunctional long-range V(D)J rearrangement [47]. To examine whether Rnf168 facilitates joining of distal gene segments during V(D)J recombination, we performed quantitative PCR assays of the partial (D<sub>E2</sub>361 and D<sub>δ1</sub>-D<sub>E2</sub>) and complete (V<sub>δ3</sub>-D<sub>δ1</sub> and V<sub>δ4</sub>-D<sub>δ1</sub>) rearrangements. We observed that short-range rearrangements were more abundant in Rnf168<sup>−/−</sup> thymocytes compared to WT controls (Figure 7D, left panel, Figure 7E, and Figure S5C). On the other hand, complete V6-to-D8β<sub>δ1</sub> recombination was significantly reduced in Rnf168<sup>−/−</sup> thymocytes compared to WT controls (Figure 7D, right panel, Figure 7E and Figure S5C).

These data support a role for Rnf168 in early thymocyte development and indicate that long-range V(D)J recombination is impaired in the absence of Rnf168.

Role of Rnf168 in maintaining genomic integrity and suppressing cancer

Defective signaling or repair of DSBs impairs genomic integrity [4,52]. For instance, inactivation of Atm, H<sub>2</sub>a.x, M<sub>d</sub>c1, Rn<sub>f</sub>β1 or Brca1 results in increased genomic instability [10,27,28,30,48,53–56]. To evaluate the effect of Rnf168 inactivation on genomic integrity, we examined for chromosomal aberrations in metaphase spreads of LPS activated Rnf168<sup>−/−</sup> and WT B-cells (Figure 8A and 8B). The level of spontaneous chromosomal aberrations was elevated in Rnf168<sup>−/−</sup> cells (25.2±9.0%) compared to WT cells (2.5±0.8%, p<0.05). Interestingly, Rnf168<sup>−/−</sup> cells demonstrated increased frequencies of DNA breaks (15.2±6.0%) and structural chromosomal aberrations (9.2±3.0%) compared to WT controls (DNA breaks: 2.5±0.8%, p<0.05, structural chromosomal aberrations: 0%, p<0.05). Increased spontaneous genomic instability in Rnf168<sup>−/−</sup> cells was consistent with the increased frequency of spontaneous γH<sub>2</sub>a.x- and M<sub>d</sub>c1-IRF in Rnf168<sup>−/−</sup> cells (Figure 3D and 3E). In response to IR (2 Gy), the level of genomic instability was further elevated in Rnf168<sup>−/−</sup> cells (65±2.2%) compared to WT controls (33.8±1%, p<0.05). The frequencies of IR-induced breaks (50.8±1.7%) and structural chromosomal aberrations (17.5±2.4%) were greater in Rnf168<sup>−/−</sup> cells compared to WT cells (DNA breaks: 23.3±8.4%, p<0.05; structural chromosomal aberrations: 12.5±1.4%, p<0.05). Therefore, Rnf168 deficiency leads to increased spontaneous and IR-induced genomic instability.

Mice deficient for M<sub>d</sub>c1, Rn<sub>f</sub>β1, 53bp1 or Brca1 have increased cancer susceptibility likely due to their elevated levels of genomic instability [28,30,54–57]. To examine whether inactivation of Rnf168 predisposes for tumor development, we monitored cohorts of Rnf168<sup>−/−</sup> mice (n = 56) and Rnf168<sup>−/−</sup> (n = 52) mice for a period of 12 months; however, none of the monitored Rnf168<sup>−/−</sup> mice developed tumors during the 1 year period (Figure 3C).

Activated Rnf168<sup>−/−</sup> B cells displayed increased genomic instability, but the activation of Atm-Chk2-p53 pathway remained intact in the absence of Rnf168. These findings prompted us to examine whether this pathway has any role in preventing tumorigenesis in Rnf168<sup>−/−</sup> mice. To do so, we generated mice lacking both Rnf168 and p53, Rnf168<sup>−/−</sup> p53<sup>−/−</sup> mice showed no gross overall developmental defects compared to their single mutant littermates. Cohorts of Rnf168<sup>−/−</sup> p53<sup>−/−</sup> mice (n = 11) and p53<sup>−/−</sup> mice (n = 18) were monitored for survival. Interestingly, we observed a significant decrease in the tumor free survival of Rnf168<sup>−/−</sup> p53<sup>−/−</sup> mice compared to p53<sup>−/−</sup> controls (p = 0.0096, log-rank test).

Consistent with previous studies [58–60], the majority of tumors developed by p53<sup>−/−</sup> mice were thymomas (Table S3). However, a different spectrum of tumors was observed in Rnf168<sup>−/−</sup> p53<sup>−/−</sup> mice, including thymomas (Figure 8D and 8E), B-cell lymphomas (Figure 8F and 8G), hemangiosarcoma (Figure S6A and S6B) and sarcoma (Figure S6C and S6D). Rnf168<sup>−/−</sup> p53<sup>−/−</sup> thymomas and B-cell lymphomas were found to infiltrate various non-lymphoid organs including lung, liver and salivary glands (Figure S6E–S6H).

To examine chromosomal translocations in these tumors, we performed multicolor fluorescence in situ hybridization (mFISH) experiments using three B-cell lymphomas and one thymoma from Rnf168<sup>−/−</sup> mice (Table S4). Two of the examined Rnf168<sup>−/−</sup> p53<sup>−/−</sup> B-cell lymphomas carried clonal reciprocal translocations between chromosomes 12 and 15 (t(12;15) and t(15;12)) (Table S4 and Figure 8H). A clonal non-reciprocal translocation, t(9;11), was also observed in one of the examined Rnf168<sup>−/−</sup> p53<sup>−/−</sup> B-cell lymphomas (Table S4). Finally, complex chromosomal abnormalities were observed in the examined Rnf168<sup>−/−</sup> p53<sup>−/−</sup> thymoma (Table S4).
Figure 7. Effect of Rnf168 deficiency on thymocyte development, TCRβ expression, and long-range V(D)J recombination. (A) Flow cytometric analyses of DN thymocytes from WT and Rnf168<sup>−/−</sup> mice and average number of CD4<sup>+</sup>CD8<sup>−</sup>CD44<sup>+</sup>CD25<sup>−</sup>(DNIII) cells. Data are presented as the mean ± SEM (n = 13 for each genotype). *p < 0.05. (B) Expression levels of TCRβ in total thymocytes and CD4<sup>+</sup>CD8<sup>+</sup> (DP) cells. (C) Histograms of the mean number of BrdU<sup>+</sup> cells in each subpopulation of DN thymocytes from WT (n = 3) or Rnf168<sup>−/−</sup> (n = 3) mice. Data are presented as the mean ± SEM. (D) Relative frequency of Tcrβ locus rearrangements in total thymocytes. Quantitative assessment of genomic DNA rearrangements of Dβ1 to Dβ2, Dβ2 to Jβ1, and Vβ4 and Vβ6 to (D)Jβ1 genes were performed by real-time quantitative PCR and normalized to the signal of the non-rearranging DNA 3′ of Jβ2. Data are presented as the mean ± SEM (WT n = 9; Rnf168<sup>−/−</sup> n = 7). **p < 0.005. (E) Representative primary PCR data for genomic DNA rearrangements of Dβ1 to Dβ2, Dβ2 to Jβ1, and Vβ4 and Vβ5 to (D)Jβ1.

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Collectively, these data demonstrate that Rnf168 is important for maintaining genomic integrity, and it cooperates with p53 in suppressing tumorigenesis.

**Discussion**

The RNF168 gene is located on human chromosome 3q29, and encodes a novel E3 ligase that plays an important role in the signaling of DSBs [20–22]. Interestingly, mutations of RNF168 have recently been identified as the genetic defects leading to RIDDLE syndrome in humans [22,24]. In order to investigate the *in vivo* functions of this E3 ligase, we have generated a mouse model for Rnf168 mutation.

Similar to mice mutant for its upstream DSBs signaling proteins, including H2a.x, Mdc1 and Rnf8, *Rnf168*<sup>−/−</sup> mice are viable [10,27,30,34,35]. In contrast to *Rag2*<sup>−/−</sup> mice that display reduced number of lymphocytes [30,34], no defect in T- or B-cell numbers was observed in *Rnf168*<sup>−/−</sup> mice. The normal number of lymphocytes in *Rnf168*<sup>−/−</sup> mice is in accordance with the lack of lymphopenia in the patient with RIDDLE syndrome [24].

Initial recruitment of 53bp1 to DSB sites is not affected in the absence of H2a.x, Mdc1 or Rnf8 [10,29,30,63–65]. However, inactivation of Rnf168 in MEFs completely abolishes both the transient and retained 53bp1 IRIFs. These data highlight the importance of Rnf168 for the initial recruitment of 53bp1 to DSB sites, and indicate that this function of Rnf168 is independent of H2a.x, Mdc1 and Rnf8.

Immunodeficiency, a hallmark for RIDDLE syndrome, is characterized by normal ratio of T and B cells, but low levels of...
In Vivo Functions of the E3 Ligase Rnf168

Materials and Methods

Generation of Rnf168-deficient mice

For the generation of Rnf168-deficient mice, two gene-trap ES clones, 136B6 and 405F11, were obtained from The Center for Modeling Human Disease (Toronto, Canada). In both ES clones, Rnf168 gene was disrupted by the integration of the gene trap vectors between exons 5 and 6 of Rnf168. Both ES cell clones were successfully used to generate Rnf168 mutant mice. Southern blotting and PCR analysis confirmed the disruption of Rnf168 locus and were used to genotype the animals. The following primers were used for PCR genotyping: Rnf168 mutant allele (forward 5′-ATGCGGCTCT-TATGCCTTTCTTCT-3′ and reverse 5′-GCCGAAGACTCGAA-CCTTGG-3′), Rnf168 WT allele (forward 5′-GCCCAAGACTG- TGCGCTATTAGCTT-3′ and reverse 5′-GCCGAAGACTCGC-GAACCTTG-3′). Southern blot analysis was performed using standard procedures. Rnf168 probe was generated by PCR using the following primers: 5′-TATTCCGTGCCTGCTGCTGCT-3′ and 5′-CTCAACCCCTCTGGCCCTGAG-3′. Rnf168−/− mice were generated by intercrossing Rnf168−/− mice obtained from each gene-trap clone. All mice in this study were in a mixed 129/J×C57BL/6 genetic background, and were maintained in a specific-pathogen free environment.

Cell culture and generation of MEFs

MEFs were generated from day 12.5 embryos using standard procedures. MEFs were cultured in DMEM (Gibco Invitrogen Corporation) supplemented with 10% FCS. Splenocytes, thymocytes and lymphocytes were cultured under the same conditions in RPMI1640 (Gibco) with 10% FCS. 3T3 immortalized MEFs were cultured under the same conditions in RPMI1640 (Gibco) with 10% FCS.

Clonogenic assay

Mouse Rnf168 cDNA amplified by PCR from WT MEF total cDNA was cloned into pBABE-puro retroviral vector. Virus supernatant was collected 48 hour post transfection of phoenix cells with pBABE-puro-Rnf168. 3T3 immortalized MEFs were infected with Mock or Rnf168 retroviruses and were subjected to puromycin selection. Infected cells (1×104) were seeded to 6 cm dishes, irradiated (2, 4 and 6 Gy) and cultured for 11 days. Number of colonies was counted with crystal violet staining.

Flow cytometry

Single cell suspensions were stained with antibodies at 4°C in PBS with 1% FCS (Wincent, Inc.). The following antibodies conjugated to allophycocyanin, PE, FITC or peridinin chlorophyll protein were used for staining: anti-CD4, anti-CD8, anti-TCRβ, anti-TCRγδ, anti-Thy1.2, anti-B220, anti-CD25, anti-CD44, anti-IgG1, anti-IgG3 and anti-IgM (BD Biosciences, eBiosciences). Stained cells were analyzed by FACS (FACSCalibur, BD Biosciences) using the CellQuest software (BD Biosciences) or FlowJo analysis software (Tree star).

Cell death assay

LN cells (2×106) were either treated with IR (0–4 Gy) or UV (0–80 J/m2). After 24 hours, cell death was examined using 7-aminoactinomycin D (Sigma).

Cell cycle and checkpoints analysis

MEFs (passage 2–5) were synchronized using aphidicolin (Merck-Calbiochem) and either left untreated or treated with 10 Gy of IR. Subsequently, BrdU (Roche) was added to the cultures at various time points. MEFs were harvested, fixed using 70% ethanol and stained with FTC conjugated anti-BrdU.
Western blot and immunoprecipitation analysis

Total protein extracts from cells were prepared using modified RIPA buffer (2 mM Tris-HCl (pH 7.5), 5 mM EDTA, 130 mM NaCl, 1% NP-40, 1% deoxycholate, 0.025% SDS, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail tablets (Roche, Branchburg). Proteins were separated on 10% homemade or 4–20% Tris-Glycine gradient polyacrylamide gels (Novex, Invitrogen). The following antibodies were used in 5% powdered milk (Carnation, Nestle) in TBS-T: affinity purified anti-Rnf168 antibodies (raised against either a murine GST-Rnf1681–518 and a murine Rnf168 N-terminal or against a murine GST-Rnf168); anti-Chk2 antibody (raised against murine Chk2 [30–45]); anti-phospho Chk2 threonine 68 antibody (Cell Signaling Technology), anti-p53 antibody (FL393, SantaCruz), anti-phospho p53 serine 15 antibody (Cell Signaling Technology), anti-Nbs1 antibody (Novus Biologicals), anti-phospho Nbs1 serine 343 antibody (Novus Biologicals), anti-Smc1 antibody (Abcam), anti-phospho Smc1 (Abcam) and anti-Atn antibody (Cell Signaling Technology).

Analysis of splenic B-cells and in vitro CSR

B-cells were purified from spleen by negative selection using Dynal Mouse B-cell Negative Isolation kit (Dynal, Invitrogen). All B-cell experiments were performed using B-cells with a purity of 95.7±0.31%. B-cells (1×10^6) were stimulated with mouse anti-CD40 antibody (10 μg/mL, eBioscience) or LPS (20 μg/mL, Sigma) plus recombinant mouse IL-4 (1000 U/mL, eBioscience) for 4 days, and switching of Ig to IgG1 and IgG2a isotypes was examined. LPS (15 μg/mL) was used to induce IgG2b and IgG3 switching.

Analysis of CSR by FACS

Purified B-cells were labeled with CFSE (Molecular Probes, Invitrogen) and cultured to induce IgG1 class switching for 4 days. Cells were stained with anti-IgG1 antibody and analyzed by FACS using the CellQuest software (BD Biosciences) or FlowJo analysis software (Tree star).

Purification of splenic B-cells and in vitro CSR

Western blot and immunoprecipitation analysis

Analysis of splenic B-cells and in vitro CSR

MSCV infection of primary B-cells

Mutants for Rnf168 cDNA were generated by PCR. Wild-type and mutant Rnf168 cDNAs were cloned into the MSCV-RES-GFP vector. The ecotropic retroviruses expressing WT or mutated Rnf168 were generated by transient transfection of Phoenix cells with the appropriate retrovirus constructs. Purified splenic B-cells were activated with LPS and infected with virus supernatants containing polybrene (Sigma). Infected B-cells were cultured with LPS plus IL-4 for 4 days, and stained with anti-IgG1 for FACS analysis.

Analysis of CSR by FACS

Purified B-cells were labeled with CFSE (Molecular Probes, Invitrogen) and cultured to induce IgG1 class switching for 4 days. Cells were stained with anti-IgG1 antibody and analyzed by FACS using the CellQuest software (BD Biosciences) or FlowJo analysis software (Tree star).

Digestion-circularization PCR (DC-PCR)

Genomic DNA from B-cells stimulated with LPS plus IL-4 for 4 days was digested with EcoRI (New England Biolabs) overnight and ligated for 16 hours with T4 DNA ligase (New England Biolabs). Two rounds of PCR were performed using nested primer pairs for Sα-Sγ1 and nAchR. Primer sequences for the first round of PCR are as follows: Sα-Sγ1, 5′-GAGCAGCTACCAAGGATCAGGGA-3′ and 5′-CTCTACGCCACTGATGAG-3′; and nAchR, 5′-GCAAACCGGCTGGATGAGGCTG-3′ and 5′-GTCCCACTTTAGAACCACCCGCG-3′. Primer sequences for the second round of PCR are as follows: 5′-GAGAGCACAATTCAGAGGAAAG-3′ and 5′-GAGACAGGGTCTCTCAGGTTAGG-3′; and nAchR, 5′-GAGACTGCTGTTGTTTACCCAG-3′ and 5′-GCTTTTCGTGTTAGACCG-3′.

Analysis of Sα-Sγ1 switch recombination junctions

Genomic DNA isolated from stimulated B-cells was amplified by PCR using Pfu ultra polymerase (Stratagene) and the following primers (5μ3, 5′-AATGGTACATCTCAGGTTTATTTAATGG-3′) and γ1-R, 5′-CAATAGTCCTGCTGTTCCTGTTG-3′. PCR products (500–1000 bp) were cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). Sequence analysis of the cloned PCR products was performed using Sequencher software (GeneCode) and NCBI-BLAST.

ELISA

B-cells (1×10^6) were stimulated with mouse anti-CD40 antibody or LPS (20 μg/mL) and IL-4 (1000 U/mL) for 4 days and the levels of IgG1, IgG2a, and IgM isotypes in the culture supernatants were determined. Isotype switching to IgG2b and IgG3 was similarly examined using supernatants from B-cells stimulated with LPS (15 μg/mL). The levels of immunoglobulin in cell culture supernatants and serum from young (6–8 weeks) and old (9–12 months) mice were determined using SBA Clonotyping System/HRP and Mouse Immunoglobulin Isotype Panel (SouthernBiotech).

Real-time PCR

Total RNA was isolated from MEFs using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed using Superscript II (Invitrogen) and oligo(dT) primers according to the manufacturer’s instructions. We performed RT-PCR using the following primers (Rnf168: forward 5′-GAATGTCAGTGCGGGATCTGTA-3′, reverse 5′-GAATGTCAGTGCGGGATCTGTA-3′; AchR: forward 5′-GAGCAGCTGTTGTTTACCCAG-3′, reverse 5′-GAGACTGCTGTTGTTTACCCAG-3′; B-cell: forward 5′-CTCTACGCCACTGATGAG-3′, reverse 5′-GCTTTTCGTGTTAGACCG-3′).

BrdU uptake experiments

Rnf168−/− and WT littermates received two intraperitoneal injections of BrdU (1 mg each) with 2 hours interval. Thymocytes collected from these mice, 2 hours after the second injection, were examined for BrdU incorporation using a BrdU Flow kit (Becton Dickinson) and cytofluorometry.

Immunofluorescence microscopy

MEFs (passage 2–5) grown on glass slides were IR treated (5 Gy), and fixed with 2% paraformaldehyde for 5 min at room temperature. Fixed MEFs were blocked with antibody dilution buffer (10% FCS and 0.05% Triton X-100 in PBS) and incubated with rabbit anti-53bp1 antibody (Bethyl Laboratories), rabbit anti-phospho-H2a.x antibody (Merck), rabbit anti-53bp1 antibody (Bethyl Laboratories), or a home made rabbit anti-Brc1 antibody (raised against a murine Brc11–45) overnight at 4°C. Labeling was detected using Alexa Fluor 488-labeled goat-anti-rabbit immunoglobulin or Alexa Fluor 555-labeled goat-anti-mouse immunoglobulin secondary antibodies (Molecular Probes).
Cells were stained with DAPI for 5 min and then mounted with Mowiol mount solution (Calbiochem). The slides were observed under a Leica DMIRB fluorescence microscope (Germany) equipped with digital camera (Leica DC 300RF). Images were acquired under 100× magnification using Leica Image Manager software. Foci-positive cells were quantified by manual counting.

Immortalized Rgfl68−/− MEFs were either mock-transfected or transfected with wildtype RNF168 GFP-tagged expression vectors. 24 hours post transfection, cells were irradiated (5 Gy), fixed 1 hour later and stained with anti-53bp1 as described earlier.

Immortalized WT and Rgfl68−/− MEFs were also treated with DMSO or DNA-PK inhibitors (NU7026 10 μM) was used for comparisons of survival curves. 2 hours later, and stained with anti-53bp1 as described earlier.

Histology

Paraffin sections of testes, epididymides, tumors and organs were stained with hematoxylin-eosin for histological analyses. The slides were observed under a Leica DM4000B microscope (Germany) equipped with digital camera (Leica DC 300RF). Images were acquired under 100× magnification using Leica Image Manager software.

Cytogenetic analysis

Purified B-cells were activated with LPS for 48 hours, and either left untreated or irradiated as indicated. Cells were collected following 4 hours of colcemid treatment and processed by standard cytogenetic procedures. Number of chromosomes and gross chromosomal rearrangements were determined in 60 metaphase cells per sample from each genotype. The slides were observed under a Leica DMIRB fluorescence microscope (Germany) equipped with digital camera (Leica DC 300RF). Images were acquired under 100× magnification using Leica Image Manager software.

Multicolor fluorescence in situ hybridization (mFISH)

Mouse mFISH probe kit was obtained from MetaSystems GmbH, Germany. In mFISH, all 21 chromosomes are each painted in a different color using combinatorial labeling. The stained metaphases were captured using the Axio Imager Z1 microscope (Carl Zeiss) with filter sets for FITC, Cy3.5, Texas Red, Cy5, Aqua, and DAPI. Images were captured, processed, and analyzed using ISIS mBAND/mFISH imaging software (MetaSystems). Detailed experimental procedures were outlined in earlier publications [74,75].

Statistical analysis

Data are presented as the mean ± SEM. The statistical significance of experimental data (p-values; Values≤0.05) was determined using the Wilcoxon test. Log-rank (Mantel-Cox) test was used for comparisons of survival curves.

Ethics statement

All experiments were performed in compliance with Ontario Cancer Institute animal care committee guidelines.

Supporting Information

Figure S1 Generation of Rgfl68 mutant mice. (A) Schematic representation of wildtype (WT) and mutants alleles of Rgfl68. (B) Heterozygous and homozygous Rgfl68 mutant mice from 156B6 and 405F11 lines were identified by Southern blotting using EcoRV digested tail genomic DNA for 156B6, and EcoRV and Ksal for 405F11 and 5′-flanking probe. Representative data from at least independent five experiments are shown. (C) Representative data of three independent RT-PCR experiments showing the expression levels of Rgfl68 transcripts in WT and Rgfl68−/− MEFs from 156B6 and 405F11 lines. Actin is used as a control. (D, E) Expression of Rnfl68 protein in MEFs and splenocytes from WT and Rgfl68−/− mice. (D) MEF lysates were blotted with anti-Rnfl68 antibody raised against C-terminal Rnfl68 recombinant proteins. (E) Splenocyte whole cell lysates (WCL) were blotted with anti-Rnfl68 antibody raised against recombinant full length Rnfl68. Splenocyte lysates were IP using anti-Rnfl68 antibody against the N-terminal Rnfl68 and were blotted with anti-full length Rnfl68 antibody. In Rgfl68−/− splenocytes from 405F11 ES clone, gene trap construct derived YFP fused Rnfl68 truncated proteins (IR-256 amino acid) were detected. Representative data are shown from three independent experiments. * indicates non specific bands.

Figure S2 Cell cycle analysis of WT and Rgfl68−/− MEFs. (A) Cell cycle analysis of aphidicolin synchronized WT and Rgfl68−/− passage 2 MEFs. BrdU/PI assay and FACS analysis were used. Representative data are shown from three independent experiments.

Figure S3 Quantification of the effect of Rnfl68 inactivation on IRIF for DDR proteins. (A) Quantitative analyses of 53bp1 nuclear foci are shown. Rgfl68−/−, Rgfl68+/− and WT MEFs were either untreated or exposed to 5 Gy of IR and fixed at the indicated times after IR. Three independent experiments were performed. (B) Quantitative analyses of the formation of Brca1 nuclear foci are shown. Rgfl68−/− and WT MEFs were either untreated or exposed to 5 Gy of IR and were fixed at the indicated times after IR. Three independent experiments were performed. (C) Quantitative analyses of the formation of γH2a.x nuclear foci 6 hours post-IR. Rgfl68−/− and WT MEFs were untreated or exposed to 5 Gy of IR. Three independent experiments were performed. (D) Two independent DC-PCR experiments showing the effect of Rnfl68 inactivation on S recombination. (A) Absolute numbers of LN cells are shown. Data are presented as the mean ± SEM. (B) Absolute number of total, Pro-B (B220 IgM CD43+) and Pre-B (B220 IgM CD43+) BM cells from 6–8-week-old mice. Data are presented as the mean ± SEM. (n = 3). (C) Absolute numbers of splenocytes are shown. Data are presented as the mean ± SEM. (n = 12–28). (D) Absolute numbers of LN cells are shown. Data are presented as the mean ± SEM. (n = 12–28). (E) Representative two-color FACS analysis showing IgG1 expression on CFSE stained B-cells stimulated with LPS plus IL-4 for 4 days (left panels) and average percentages of IgG1 switched cells (right panel). Three independent experiments were performed. (F) CFSE staining profiles of WT and Rgfl68−/− B-cells stimulated with LPS plus IL-4 for 4 days. (F) Expression levels of WT or mutated Rnfl68 in B-cells infected with ecotropic retroviruses [MSCV-mutated or full-length (FL) Rnfl68-IRESGFP]. (G) Two independent DC-PCR experiments showing the effect of Rnfl68 inactivation on Sµ-Sγ1 recombination. nkdrr served to normalize for the amount of input DNA. Fivefold serial dilutions were used as templates. H2O: no input DNA.
Figure S5 Effect of Rnf168 deficiency on thymocytes. (A) Increased representation of CD4+CD8− [DN] thymocytes in Rnf168−/− mice (n = 13) compared to WT controls (n = 14), 6–8-week-old mice were analyzed. Data are presented as the mean ± SEM. *p<0.05. (B) Reduced TCRβ+ T-cells in Rnf168−/− (0.2±0.01%, n = 20) compared to WT controls (0.24±0.2%, n = 18), 6–8-week-old mice were analyzed. Data are presented as the mean ± SEM. *p<0.005. (C) Representative primary PCR data for genomic DNA rearrangements of D61 to D62, D62 to Jκ1, and Vκ4 and Vκ5 to (D)Jκ1. Found at: doi:10.1371/journal.pgen.1001381.s006 (2.70 MB TIF)

Figure S6 Tumors in Rnf168−/−/p53−/− mice. (A and B) H&E staining of an hemangiosarcoma from an Rnf168−/−/p53−/− mouse. (C and D) H&E staining of a sarcoma from an Rnf168−/−/p53−/− thymoma invading lung (E) and salivary gland (F). (G and H) H&E staining showing Rnf168−/−/p53−/− lymphoma cells invading lung (G) and liver (H). Scale Bars: 50 μm; (B), 100 μm; (D), 200 μm; (A, E, F, G and H), 500 μm; (C). Found at: doi:10.1371/journal.pgen.1001381.s007 (0.03 MB DOC)

Table S1 Genotypes of pups from intercrosses of Rnf168 heterozygotes. Rnf168−/− mice were viable and were born at the expected Mendelian ratio. Found at: doi:10.1371/journal.pgen.1001381.s008 (0.03 MB DOC)

Table S2 Sequence analysis of Spj-Syl CSR junctions from Rnf168−/− and WT B-cells. In contrast to WT controls, a subset of CSR junctions in Rnf168−/− B-cells displays long nucleotide insertions. Found at: doi:10.1371/journal.pgen.1001381.s009 (0.05 MB DOC)

Table S3 Distribution of tumors developed by Rnf168−/−/p53−/− or p53−/− mice. Rnf168−/−/ p53−/− mice developed a different spectrum of tumors compared to p53−/− mice. Found at: doi:10.1371/journal.pgen.1001381.s010 (0.04 MB DOC)

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Author Contributions
Conceived and designed the experiments: TB MB AH RH. Performed the experiments: TB MB RC DZ MPH AH. Analyzed the data: TB MB OS MPH AH RH. Contributed reagents/materials/analysis tools: SP LJ GSS DD. Wrote the paper: TB MB AH RH.

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