<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Class switching and meiotic defects in mice lacking the E3 ubiquitin ligase RNF8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Santos, MA; Huen, MSY; Jankovic, M; Chen, HT; LópezContreras, AJ; Klein, IA; Wong, N; Barbancho, JLR; FernandezCapetillo, O; Nussenzweig, MC; Chen, J; Nussenzweig, A</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>Journal Of Experimental Medicine, 2010, v. 207 n. 5, p. 973-981</td>
</tr>
<tr>
<td><strong>Issued Date</strong></td>
<td>2010</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10722/65449">http://hdl.handle.net/10722/65449</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.; The Journal of Experimental Medicine. Copyright © Rockefeller University Press.</td>
</tr>
</tbody>
</table>
Class switching and meiotic defects in mice lacking the E3 ubiquitin ligase RNF8

Margarida Almeida Santos,¹ Michael S.Y. Huen,²,³ Mila Jankovic,⁴ Hua-Tang Chen,¹ Andrés J. López-Contreras,⁵ Isaac A. Klein,⁴ Nancy Wong,¹ Juan L.R. Barbancho,⁵ Oscar Fernandez-Capetillo,⁵ Michel C. Nussenzweig,⁴ Junjie Chen,⁶ and André Nussenzweig¹

Abbreviations used: AID, activation-induced cytidine deaminase; CSR, class switch recombination; delRING, RING deleted; DSB, DNA double-strand break; FISH, fluorescence in situ hybridization; HR, homologous recombination; IgH, immunoglobulin heavy chain; IR, ionizing radiation; shRNA, small hairpin RNA.

53BP1 is a well-known mediator of the cellular response to DNA damage. Two alternative mechanisms have been proposed to explain 53BP1's interaction with DNA double-strand breaks (DSBs), one by binding to methylated histones and the other via an RNF8 E3 ligase–dependent ubiquitylation pathway. The formation of RNF8 and 53BP1 irradiation–induced foci are both dependent on histone H2AX. To evaluate the contribution of the RNF8–dependent pathway to 53BP1 function, we generated RNF8 knockout mice. We report that RNF8 deficiency results in defective class switch recombination (CSR) and accumulation of unresolved immunoglobulin heavy chain–associated DSBs. The CSR DSB repair defect is milder than that observed in the absence of 53BP1 but similar to that found in H2AX−/− mice. Moreover, similar to H2AX but different from 53BP1 deficiency, RNF8−/− males are sterile, and this is associated with defective ubiquitylation of the XY chromatin. Combined loss of H2AX and RNF8 does not cause further impairment in CSR, demonstrating that the two genes function epistatically. Importantly, although 53BP1 foci formation is RNF8 dependent, its binding to chromatin is preserved in the absence of RNF8. This suggests a two-step mechanism for 53BP1 association with chromatin in which constitutive loading is dependent on interactions with methylated histones, whereas DNA damage–inducible RNF8–dependent ubiquitylation allows its accumulation at damaged chromatin.

Protein ubiquitylation is emerging as an important posttranslational modification used to maintain genomic stability (Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007; Alpi and Patel, 2009; Doıl et al., 2009; Panier and Durocher, 2009; Stewart et al., 2009). One component of this pathway is the E3 ubiquitin ligase RNF8, for which RNA interference–induced foci are both dependent on histone H2AX. To evaluate the contribution of the RNF8–dependent pathway to 53BP1 function, we generated RNF8 knockout mice. We report that RNF8 deficiency results in defective class switch recombination (CSR) and accumulation of unresolved immunoglobulin heavy chain–associated DSBs. The CSR DSB repair defect is milder than that observed in the absence of 53BP1 but similar to that found in H2AX−/− mice. Moreover, similar to H2AX but different from 53BP1 deficiency, RNF8−/− males are sterile, and this is associated with defective ubiquitylation of the XY chromatin. Combined loss of H2AX and RNF8 does not cause further impairment in CSR, demonstrating that the two genes function epistatically. Importantly, although 53BP1 foci formation is RNF8 dependent, its binding to chromatin is preserved in the absence of RNF8. This suggests a two-step mechanism for 53BP1 association with chromatin in which constitutive loading is dependent on interactions with methylated histones, whereas DNA damage–inducible RNF8–dependent ubiquitylation allows its accumulation at damaged chromatin.

This article is distributed under the terms of an Attribution–Noncommercial–Share Alike license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
of class switch recombination (CSR)–associated breaks (Stewart et al., 2007). Recent studies demonstrated that RNF168 acts to amplify RNF8-initiated protein ubiquitylation at sites of DNA double-strand breaks (DSBs; Doil et al., 2009; Stewart et al., 2009). Collectively, these results suggest that beyond phosphorylation, ubiquitylation might play an active role in the signaling of DSBs. However, the physiological relevance of such a response and the extent to which it modulates 53BP1 functions in vivo remain unexplored. In this paper, we report the effects of RNF8 deletion in mice. Our results reveal 53BP1-independent functions of RNF8 in meiotic recombination and, conversely, RNF8-independent functions of 53BP1 in CSR.

RESULTS AND DISCUSSION

To determine whether the ubiquitin-dependent arm of the DSB response affects CSR, we disrupted RNF8 in mice (Fig. S1 A). RNF8−/− mice were born at Mendelian frequencies, and the absence of RNF8 protein was confirmed by Western blotting with antibodies raised against full-length human RNF8 (Fig. S1 B).

53BP1−/− and H2AX−/− mice exhibit a reduction in the number of mature lymphocytes (Celeste et al., 2002; Difilippantonio et al., 2008). Similarly, there was a 40–50% reduction in the number of thymocytes and B cells in the spleens of RNF8−/− mice (Fig. 1 A). 53BP1−/− thymocytes express low levels of TCRβ, which is associated with defective V(D)J recombination (Difilippantonio et al., 2008). Despite the decreased cellularity, RNF8−/−, like H2AX−/−, thymocytes showed normal TCRβ expression (Fig. 1 A; Celeste et al., 2002).

To further compare 53BP1 and RNF8 deficiencies, we examined the efficiency of CSR in the mutant mice. RNF8 deficiency caused a significant reduction in the frequency of IgG1 or IgG3 surface expression in response to activation with LPS/IL-4 or LPS alone, respectively (Fig. 1, B and C). However, this reduction in CSR was less severe than the one observed in 53BP1-deficient B cells (Fig. 1 B).

To assess whether loss of RNF8 affects cellular proliferation, we labeled LPS/IL-4–activated B cells with CFSE. In contrast to the normal proliferation of 53BP1 mutant B cells (Ward et al., 2004), CFSE dye dilution histograms revealed that RNF8−/− B cells underwent fewer cell divisions than WT cells (Fig. 1 D). One potential explanation for the proliferation defect (Fig. 1 D) and lymphopenia (Fig. 1 A) is that RNF8 regulates HR (Huang et al., 2009), which is required for DNA replication. For example, loss of RNF8 leads to a defect in the ability to relocalize Brca1 and Rad51 to sites of DNA damage (Huen et al., 2007; Kolas et al., 2007; Maitland et al., 2007; Wang and Elledge, 2007). Consistent with this notion, we found that IR-induced Rad51 focus formation was partially impaired in RNF8−/− B cells (Fig. S2 A). Impaired Rad51 focus formation was not caused by alterations in cell-cycle distribution, as determined by cellular DNA content (Fig. S2 B). Because proliferation defects might affect CSR, we measured surface IgG1 and IgG3 levels as a function of cell division (Fig. 1 E). Even in cells that had undergone several rounds of cell division, CSR was significantly reduced in the absence of RNF8 (Fig. 1 E).

To determine at which level RNF8 deficiency affects CSR, we measured activation-induced cytidine deaminase (AID) expression and determined whether AID targeting to the switch region was affected by analysis of Sμ mutation in B cells stimulated with LPS and IL-4. The level of AID mRNA in stimulated B cells was not affected by RNF8 deletion (Fig. 2 A). Moreover, we found similar levels of Sμ mutation in IgM+ RNF8−/− and RNF8−/− B cells (Fig. 2 B). These results indicate that RNF8 is dispensable for AID accessibility to the Sμ switch region.

Sμ–Sγ1 switch region junctions cloned from 53BP1−/− B cells show a high frequency of DNA insertions because of defective processing of CSR DSBs (Reina-San-Martin et al., 2007). To ascertain whether switch junctions are also abnormal in the absence of RNF8, we cloned and sequenced switch region junctions from B cells stimulated with LPS and IL-4. Microhomology at junctions was mildly increased in the absence of RNF8 (Fig. 2 C). More strikingly, 24% of the total RNF8−/− CSR junctions analyzed showed showed insertions >2 nt compared with only 7% in WT mice (Fig. 2 C). Thus, RNF8, like 53BP1, prevents insertions at switch junctions.

The inability to complete CSR is frequently associated with accumulation of immunoglobulin heavy chain (IgH)–associated breaks (Franco et al., 2006, 2008; Ramiro et al., 2006). To examine IgH instability, we used an IgH locus–specific/telomere fluorescence in situ hybridization (FISH) assay in which the loss of telomeric signals near the IgH locus on chromosome 12 is indicative of unresolved DSBs (Fig. 2 D; Ramiro et al., 2006). Surprisingly, distinct from 53BP1, H2AX, or ataxia telangiectasia mutated deficiency (Celeste et al., 2003; Ramiro et al., 2006), we failed to detect IgH breaks in RNF8−/− B cells (Fig. 2 E). We considered the possibility that RNF8-deficient cells with aberrations might be eliminated by a p53-dependent pathway before they reach metaphase, as previously observed in LPS-activated DNA-Pkcs−/− cells (Franco et al., 2008). To test this we performed small hairpin RNA (shRNA)–mediated knockdown of p53 in LPS-activated B cells. In two independent experiments, we observed an increase in the accumulation of IgH-associated breaks in p53-depleted RNF8−/− cells relative to controls (Fig. 2 E). These results suggest that RNF8 is necessary for joining a subset of breaks during CSR, but that RNF8−/− cells with unresolved DSBs are normally eliminated in a p53-dependent manner.

53BP1 and RNF8 localization to DSBs is dependent on H2AX phosphorylation (Celeste et al., 2002; Huen et al., 2007; Kolas et al., 2007; Maitland et al., 2007; Wang and Elledge, 2007), and H2AX/H2A are ubiquitylated by RNF8 (Huen et al., 2007; Maitland et al., 2007). Nevertheless, RNF8 and H2AX deficiency leads to a milder CSR defect than 53BP1−/− (Fig. 1 B; Ward et al., 2004) and no detectable defect in thymocyte TCRβ expression (Fig. 1 A; Difilippantonio et al., 2008), suggestive of H2AX–independent functions for 53BP1.
In addition to the regulation of focus formation, H2AX has an essential role in male spermatogenesis. Specifically, H2AX−/− mice exhibit impaired XY synapsis caused by defective synapsis of the XY chromosomes during meiotic prophase (Celeste et al., 2002; Fernandez-Capetillo et al., 2003b). In contrast, 53BP1-deficient mice are fertile, illustrating that H2AX also displays 53BP1-independent functions in the context of meiosis. Similar to H2AX−/− males, RNF8-deficient males failed to breed, had reduced testes size (Fig. 3 B),

To determine whether H2AX and RNF8 function epistatically, we produced mice deficient for both H2AX (Celeste et al., 2002) and RNF8 by breeding. RNF8−/− H2AX−/− mice showed impairment of CSR at levels similar to those found in H2AX−/− B cells (Fig. 3 A and Fig. S3), which is still less severe than 53BP1 deficiency (Fig. 1 B). These results indicate that H2AX and RNF8 function in the same pathway during CSR, and that 53BP1 has a unique role that is not entirely regulated by H2AX and RNF8.

In addition to the regulation of focus formation, H2AX has an essential role in male spermatogenesis. Specifically, H2AX−/− mice exhibit impaired XY synapsis caused by defective synapsis of the XY chromosomes during meiotic prophase (Celeste et al., 2002; Fernandez-Capetillo et al., 2003b). In contrast, 53BP1-deficient mice are fertile, illustrating that H2AX also displays 53BP1-independent functions in the context of meiosis. Similar to H2AX−/− males, RNF8-deficient males failed to breed, had reduced testes size (Fig. 3 B),
and showed a marked reduction in sperm (Fig. 3 C). Furthermore, histological analysis revealed that there was considerable degeneration in seminiferous tubules in RNF8−/− mice, indicated by an increase in the number of vacuoles and Leydig cells (Fig. 3 C, top). Given that H2AX phosphorylation coats the XY chromatin of male meiotic cells, we explored whether a similar phenomenon was occurring with respect to ubiquitylation. To this end, we stained spermatocytes with antibodies against FK2, which marks ubiquitinated proteins. Like phosphorylated H2AX, ubiquitylated conjugates coated the sex body in pachytene-stage cells (Fig. 3 C, bottom left). Strikingly, despite normal H2AX phosphorylation of the sex body, FK2 staining was lost in RNF8 knockout spermatocytes (Fig. 3 C, bottom right). Collectively, these results suggest that RNF8 acts downstream of H2AX but distinct from 53BP1 in the regulation of CSR and male meiosis.

It has been suggested that RNF8-dependent ubiquitylation at DSBs exposes methylated histones, which in turn regulate 53BP1 binding to DNA damage. Indeed, loss of RNF8 impaired the ability of 53BP1 to form IR-induced
RING-deleted (delRING) form of RNF8 in RNF8−/− B cells that were stimulated to undergo CSR. As expected, 53BP1 foci were visible in RNF8-deficient B cells reconstituted with WT RNF8 but not in the delRING RNF8 (Fig. 4 C). In addition, WT RNF8 reconstituted CSR, whereas the delRING failed to do so (Fig. 4 D). Thus, the RING domain of RNF8 is critical for CSR.

To determine directly whether RNF8 affects the association of 53BP1 with chromatin, we examined the subcellular distribution of 53BP1 in untreated and irradiated cells by cell fractionation. We found that a considerable fraction of cellular 53BP1 was bound to chromatin independently of IR, and that this pool increased only slightly, if at all, after IR (Fig. 5). These results are consistent with photobleaching experiments that indicate that in the absence of DNA damage, 53BP1 interacts with chromatin (Bekker-Jensen et al., 2005). Strikingly, loss of RNF8 did not affect the chromatin binding of 53BP1 (Fig. 5). Thus, although RNF8-dependent ubiquitylation may increase the affinity of 53BP1 for damaged chromatin, the initial loading to chromatin clearly occurs by mechanisms independent of RNF8. As previously proposed in the context of V(D)J recombination, constitutive (DNA damage–independent) 53BP1 chromatin binding might promote long-range CSR, via 53BP1 homooligomerization-mediated synapsis of distal switch regions (Disippaniantonio et al., 2008). In this model, RNF8 would increase the local concentration of 53BP1 or induce contraction of the switch regions in response to AID DSBs, thereby further promoting the pairing of switch regions.
H2AX regulates foci formation of numerous factors that carry out diverse functions in nonhomologous end joining, HR, and DNA damage signaling. Nevertheless, many of these factors have essential roles at sites of DSB independent of their ability to form foci (Celeste et al., 2003). For example, Brca1 is essential for viability and HR, whereas H2AX and RNF8 knockout mice are viable and show only a mild defect in HR, despite the fact that BRCA1 foci are impaired in their absence. Although assays using recombination substrates failed to detect impairment in nonhomologous end joining in H2AX knockout mice (Bassing et al., 2002), H2AX functions in CSR and regulates foci formation of numerous factors, including 53BP1 and Nbs1, which are themselves implicated in DNA end joining and CSR (Celeste et al., 2003). RNF8, acting downstream of H2AX, regulates focus formation of 53BP1 but not of Nbs1. We speculate that because RNF8 regulates only a subset of repair proteins that form H2AX-dependent foci, this may explain why the CSR defect in RNF8 knockout mice is slightly milder than that observed in H2AX−/− and H2AX−/− RNF8−/− mice (Fig. 3 A and Fig. S3). The idea that RNF8 acts epistatically with H2AX in vivo is bolstered by the finding that RNF8−/− males are infertile, with a similar meiotic arrest as in H2AX knockouts.

In light of the role of H2AX in XY body formation, it is tempting to propose that RNF8-dependent ubiquitylation might play an active role in transcriptional silencing and/or chromatin compaction. Moreover, because RNF168 acts together with RNF8 in promoting efficient histone ubiquitylation, we would predict that patients with RIDDLE syndrome should exhibit male sterility. In conclusion, our results indicate that a significant contribution of the CSR and meiotic defects observed in H2AX knockout mice is likely to be the inability to ubiquitylate proteins at sites of CSR DSBs and throughout the male sex body. Consistent with our findings, a recent report showed that CSR to IgA was impaired in mouse CF12F3-3 cells treated with shRNAs that depleted RNF8 and RNF168 (Ramachandran et al., 2010).

MATERIALS AND METHODS

Mice. For generation of RNF8−/− mice, the embryonic stem (ES) cell line RRR260 was used (Bay Genomics). The RNF8 gene was disrupted by a neo gene selection cassette inserted between transcribed exons 4 and 5. The exact insertion sites were mapped by genomic PCR and DNA sequencing. These ES cells were injected into C57BL/6 blastocysts to generate chimeric RNF8−/− mice.
Cells were sorted for five cell divisions and surface IgM expression, and mutant analysis was performed as previously described (Reina-San-Martin et al., 2003).

**FISH analysis, immunofluorescence, and immunohistochemistry.** B cells cultured for 3 d with LPS plus IL-4 were arrested at mitosis with colcemid (Invitrogen) treatment, swollen in prewarmed 0.075 M KCl for 15 min at 37°C, and fixed by methanol/acetic acid treatment, and dropped in microscope slides using a humidity chamber. Slides were denatured at 75°C/70% formamide for 7 min; hybridized with the following probes overnight at 37°C; washed, dehydrated, and stained with DAPI; and mounted with VECTASHIELD (Vector Laboratories). We used bacterial artificial chromosome (BAC) probes containing IgH Cα (from C57 3r’ of Ca; Difilippantonio et al., 2002) and telomere repeat–specific peptide nucleic acid probes (Applied Biosystems). For 53BP1 and Rad51 foci analysis, B cells were spun onto coverslips after 72 h of stimulation and immunofluorescence was performed as previously described (Peterson et al., 2001). For coupled immunofluorescence and FISH analysis, day 3 stimulated B cells were spun onto coverslips and processed as described previously (Chen et al., 2000). The IgH-specific BAC probe spanned from C57 to 3r’ of Ca. Rabbit polyclonal anti-53BP1 (Novus Biologicals), rabbit polyclonal anti-Rad51 (H-92; Santa Cruz Biotechnology, Inc.), and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen) were used. Metaphase spread images were captured with a microscope (Axio Imager M2mSystem; Carl Zeiss, Inc.) and foci images were captured with a confocal microscope (LSM 510 MetaII; Carl Zeiss, Inc.). Tests were fixed in formalin and embedded in paraffin for subsequent processing. 2.5-mm sections were treated with citrate for antigenic recovery and processed for immunohistochemistry with FK2 (Invitrogen) antibodies. Hematoxilin was used to counterstain. Sections were scanned with a digitalized system (MIRAX; Carl Zeiss, Inc.). For tissue immunofluorescence, sections were previously deparaffinized and γH2AX (Millipore) antibodies were also used. Immunofluorescence of isolated mouse spermatocytes has been previously described (Fernandez-Capetillo et al., 2003a). Combined SCP3 (gift from M. Tarsounas, Gray Institute for Radiation Oncology and Biology, Oxford, England, UK) and FK2 antibody staining was processed with Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen) secondary antibodies.

**Chromatin fractionation and Western blotting.** B cells were isolated and cultured with LPS and IL-4 for 3 d as described. A portion of the cultures was subjected to 10 Gy of γ irradiation and allowed to recover at 37°C for 1.5 h. Nuclei from 5 million cells were isolated with the ProteoJet Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) and lysed; the manufacturer’s nuclei lysate buffer was supplemented with 30 mM EDTA, 2 mM EGTA, and 10 mM dithiothreitol. The nuclear extract was centrifuged at 1,700 g for 20 min at 4°C; the supernatant was saved at −80°C as the “nuclear-soluble fraction,” and the chromatin pellet was washed twice in 250 µl of 0.1× lys buffer supplement. Chromatin was resuspended in 30 µl of 10 mM Hepes, 10 mM KCl, 1 mM MgCl2, 10% glycerol, 1 mM CaCl2, 1 mM EDTA, 1× protease inhibitors (Roche), and 5 U of micrococcal nuclease (New England Biolabs, Inc.) and then incubated for 45 min at 37°C. The reaction was stopped by the addition of EGTA to 1 mM, and the digested pellet was stored at −80°C as the “chromatin-bound fraction.”

Extracts were run on 3–8% Tris-acetate gels and analyzed by Western blotting with antibodies against 53BP1 (Bethyl Laboratories, Inc.) and H2AX (Novus Biologicals). To examine RNF8 expression, total cell extracts derived from RNF8 WT and −/− deficient mouse embryonic fibroblasts were incubated with anti-RNF8 antibodies and protein A agarose beads for 2 h at 4°C with gentle agitation under denaturing conditions. Beads were washed twice with denaturing buffer and bound proteins were eluted by boiling in sampling buffer. Thereafter, immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies generated against full-length human RNF8.

**Online supplemental material.** Fig. S1 shows the strategy used to disrupt the mouse RNF8 gene. Fig. S2 shows that Rad51 foci formation is impaired in the absence of RNF8. Fig. S3 shows two more independent experiments.
for the data shown in Fig. 3 A. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092308/DC1.

We thank M. Kruhlak, L. Granger, and A. Adams for technical assistance, and members of the Nussenzweig laboratory for helpful discussions.

I.A. Klein was supported by Medical Scientist Training Program grant GM07739 and is a predoctoral fellow of the Cancer Research Institute. This work was supported by the Intramural Research program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

The authors have no conflicting financial interests.

Submitted: 26 October 2009
Accepted: 9 March 2010

REFERENCES


RNFB is required for CSR | Santos et al.
