A Dual Interaction between the DNA Damage Response Protein MDC1 and the RAG1 Subunit of the V(D)J Recombinase*

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Background: The molecular link between the cleavage and repair phases of V(D)J recombination is unknown.

Results: The RAG1 subunit of the V(D)J recombinase directly interacts with the repair factor MDC1 via two distinct binding interfaces.

Conclusion: The MDC1-RAG1 interaction may link cleavage and repair during V(D)J recombination.

Significance: The interaction may provide new mechanistic insight into V(D)J recombination.

The first step in V(D)J recombination is the formation of specific DNA double-strand breaks (DSBs) by the RAG1 and RAG2 proteins, which form the RAG recombinase. DSBs activate a complex network of proteins termed the DNA damage response (DDR). A key early event in the DDR is the phosphorylation of histone H2AX around DSBs, which forms a binding site for the tandem BRCA1 C-terminal (tBRCT) domain of MDC1. This event is required for subsequent signal amplification and recruitment of additional DDR proteins to the break site. RAG1 bears a histone H2AX-like motif at its C terminus (R1Ct), making it a putative MDC1-binding protein. In this work we show that the tBRCT domain of MDC1 binds the R1Ct motif of RAG1. Surprisingly, we also observed a second binding interface between the two proteins that involves the Proline-Serine-Threonine-rich (PST) repeats of MDC1 and the N-terminal non-core region of RAG1 (R1Nt). The repeats-R1Nt interaction is constitutive, whereas the tBRCT-R1Ct interaction likely requires phosphorylation of the R1Ct motif of RAG1. As the C terminus of RAG1 has been implicated in inhibition of RAG activity, we propose a model in which phosphorylation of the R1Ct motif of RAG1 functions as a self-initiated regulatory signal.

V(D)J recombination is a somatic recombination process in which the variable region of antigen receptor genes is assembled from component V, D, and J gene segments (1–7). The proteins encoded by the recombination-activating genes 1 and 2 (RAG1 and RAG2) form the RAG recombinase, which is sufficient in vitro and necessary in vivo to perform the cleavage phase of V(D)J recombination. RAG introduces DNA double-strand breaks (DSBs) specifically at the borders between two coding segments and their flanking recombination signal sequences. After cleavage, RAG is retained at DSBs in a post-cleavage complex, where it plays a role in directing repair via the ubiquitous non-homologous end joining DSB repair pathway. It is not yet known how RAG channels DSB repair into the non-homologous end joining pathway.

Loss of RAG1, RAG2, or any of the core non-homologous end joining proteins results in no cleavage or inefficient repair, respectively, of antigen receptor loci, leading to a strong block in lymphocyte development (8–11). Null mutations of either RAG gene in humans lead to a T−B− severe combined immunodeficiency phenotype. Hypomorphic RAG mutations in humans cause a unique severe combined immunodeficiency phenotype known as Omenn syndrome (OS), characterized by lack of B cells and a reduced oligoclonal T cell repertoire. The minimal domains that retain catalytic activity in the RAG proteins, termed “core” RAGs, have been studied extensively because of their attractive biochemical properties. However, it is clear that the non-core regions of the RAG proteins are important for proper V(D)J recombination in vivo (12). This is supported by the fact that mice expressing core-RAG1 or core-RAG2 show impaired lymphocyte development and aberrant V(D)J recombination (13–16). Furthermore, some OS cases are caused by mutations or deletions of the non-core regions of RAG1 or RAG2 (12, 17, 18). The mechanism by which these non-core RAG regions act in the regulation of V(D)J recombination is not well understood.

DNA DSBs lead to the activation of the DNA damage response (DDR) (19, 20). The first step in this response is the

The abbreviations used are: DSB, double-strand break; OS, Omenn syndrome; DDR, DNA damage response; MRN, Mre11-Rad50-NBS1; tBRCT, tandem BRCT; FHA, forhead-associated; RAG, recombination activating gene; ATM, ataxia telangiectasia mutated; BRCT, BRCA1 C-terminal; PST, proline-serine-threonine-rich; XLF, XRCC4-like factor; MBP, maltose-binding protein.

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recruitment of the Mre11/Rad50/Nbs1 (MRN) complex and ATM to the DSBs (21). ATM, a central DDR kinase, is activated, leading to phosphorylation of multiple targets (22). ATM phosphorylates the C terminus of histone variant H2AX in the vicinity of the DSB to yield γ-H2AX (23). The DDR protein MDC1 specifically and directly binds the C terminus of H2AX only in its phosphorylated form. The ability of MDC1 to discriminate between H2AX and γ-H2AX is imparted by its tandem BRCT (tBRCT) domain, which acts as a phosphospecific binding module (24). MDC1 recruits additional MRN complexes (25, 26) and ATM molecules (27), as well as other DDR proteins, to the break. This leads to amplification of the initial signal and formation of a microscopically visible focus. Focus formation plays an important role in DSB repair, activation of cell cycle checkpoints, and apoptosis. MDC1 and H2AX are crucial for the DDR, as knockout mice for either show impaired focus formation by multiple DDR proteins and, as a result, a defective response to DSBs (27–29).

Although much work has been done to characterize both the cleavage and repair phases of V(D)J recombination, the molecular mechanism linking these phases is poorly understood. Evidence for an interaction between RAG1 and Ku70/Ku80 represents the only known link between RAG and a repair factor (30). An early report showed that γ-H2AX and Nbs1 form RAG-dependent foci that correspond to actively rearranging loci in thymocytes (31). This observation suggests that V(D)J recombination involves activation of the entire DDR cascade, which plays a role in repair of RAG-induced breaks. However, single deletion of most key DDR genes in mice does not lead to a strong block in V(D)J recombination (27–29, 32, 33). H2AX and MDC1 knockout mice do not exhibit a strong immunological phenotype, indicating that these proteins are not essential for V(D)J recombination (27–29). Two recent reports show that although H2AX and MDC1 are indeed not required for repair of breaks during V(D)J recombination, they play a role in preventing aberrant repair by holding broken ends together and protecting them from resection (34, 35). ATM and the MRN complex are also not essential for V(D)J recombination. However, ATM, and recently also the MRN complex, were shown to be important for prevention of atypical rearrangement products termed hybrid joints, as well as suppression of genomic instability associated with V(D)J recombination (32, 33). More recently, a surprising phenotype was found in mice deficient in both ATM and XLF, a core non-homologous end joining factor (36). Knockout of either gene does not block repair of RAG-induced breaks, whereas lymphocytes from double knockout mice display a severe block in the repair of such breaks. This demonstrates an important role for both proteins in V(D)J recombination and highlights the complex mode of regulation and redundancy in this process.

In this work, we present evidence for a direct interaction between the V(D)J recombinase and the DDR protein MDC1. Detailed mapping of this interaction shows a surprisingly complex mode of binding that involves two domains within MDC1 and two non-core regions of RAG1. The N terminus of RAG1 binds the PST repeats of MDC1 and the C terminus of RAG1 binds the tBRCT domain of MDC1. The C terminus of RAG1 bears a striking similarity to the C terminus of histone H2AX and might be regulated by recombination-related phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Expression vectors for GST, GST-tBRCT, HA-MDC1, and HA-MDC1ΔBRCT were described previously (37). The GST repeats and GST-Nbs1-FHA-tBRCT were a kind gift from Dr. Zhenkun Lou (38). HA-MDC1 repeats and HA-MDC1ΔΔ were created by cutting the HA-MDC1 vector either with BfuAI alone or with BfuAI and NotI, respectively, followed by filling in with a Klenow fragment and ligation. All other HA-MDC1 vectors were created by PCR amplification of the relevant fragment using human MDC1 cDNA as a template and cloning into a pcDNA3 vector that contains an N-terminal double HA-tag (pcDNA3-HA). pEBB-RAG1, which encodes full-length murine RAG1, was described previously (39). pEBB-RAG1ΔC was created by standard mutagenesis of the pEBB-RAG1 vector to introduce a stop codon at position 1033. Full-length HA-RAG1 was created by cloning a BamHI/NotI fragment from pEBB-RAG1 into pcDNA3-HA. All other HA-RAG1 vectors, as well as MBP-RAG1 vectors, were created by PCR amplification of the relevant fragments of RAG1 using pEBB-RAG1 as a template and cloning into the pcDNA3-HA or pMBP-parallel-2 vectors, respectively. All cloned vectors were sequenced to verify their correct sequence.

**Antibodies**—The commercial antibodies used in this study were mouse monoclonal anti-CDC27 (BD Transduction Laboratories, catalog no. 610455) and mouse monoclonal MBP-probe (Santa Cruz Biotechnology, catalog no. R29.6 sc-13564). Sheep polyclonal anti-MDC1 was used for immunoprecipitation (40) whereas mouse monoclonal anti-MDC1, clone MDC1–50 (Sigma-Aldrich, catalog no. M2444) was used for Western blotting. Mouse monoclonal anti-HA clone 12CA5 was used for both immunoprecipitations and Western blotting. Rabbit monoclonal antibodies anti-RAG1 (clone no. 23) and anti-RAG2 (clone no. 39) were produced using core-RAG1 and RAG2 residues 1–490 as antigens.

**Recombinant Protein Expression and Purification**—GST or MBP fusion proteins were expressed in *Escherichia coli* strain BL21 and purified using glutathione-Sepharose 4B (GE Healthcare, catalog no. 17-0756-01) or amylose resin (New England Biolabs, catalog no. E8021S), respectively.

**Cell Culture and Cell Lines**—293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. V-abl, Nalm6, and 5B3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, streptomycin, and 50 μM β-mercaptoethanol. 5B3 cells were grown in the presence of 1 mM L-histidinol (Sigma-Aldrich) and 0.5 mg/liter tetracycline (Fluka). Activation of v-abl cells was done by adding STI-571 (Novartis) to a final concentration of 3 μM. WT and Art(−/−) v-abl cells (A70) were obtained from Prof. Barry Sleckman (32, 34). 5B3 cells were obtained from Prof. Eugene Oltz (41). Nalm6 cells were obtained from Dr. Tzipi Shlomai. MDC1(−/−) v-abl cells were established from MDC1(−/−) mice, which were obtained from Prof. Zhenkun Lou (27), after crossing with...
mice that carry the Eμ-Bcl2 transgene, as described previously (32). Analysis of hybrid joint formation was carried out as described previously (32).

**GST Pull-down and Immunoprecipitation Assays**—GST fusion proteins (20 μg) or antibodies were immobilized onto glutathione-Sepharose or protein A/G-agarose, respectively, and incubated with whole cell protein extracts for 2 h at 4 °C. Bound proteins were washed four times with wash buffer (30 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 0.5 mM DTT, 150 mM NaCl, 0.2% Triton X-100), eluted with SDS sample buffer by boiling for 5 min, and analyzed by SDS-PAGE and subsequent Western blotting.

**Peptide Pull-down**—Peptides that correspond to the 12 C-terminal residues of RAG1 (1029–1040) were synthesized with N-terminal biotin and a regular serine or a phospho-serine at position 1037 (Biotin-EDSLESQDSMEF-COOH and Biotin-EDSLESQDSpSMEF-COOH; 21st Century Biochemicals). 1 nmol of each peptide was bound to streptavidin beads, incubated with 20 μg of purified GST fusion proteins, washed four times with wash buffer, and analyzed by SDS-PAGE and Coomassie staining.

**RESULTS**

The C Terminus of RAG1 Contains an H2AX-like Motif—tBRCT domains can act as phospho-binding modules (42–44). Phospho-serine peptide library binding experiments show that the tBRCT domain of MDC1 preferentially binds peptides with glutamic acid at position +2 and an aromatic residue at position +3 (24, 43). Binding is dramatically enhanced if this motif is located at the C terminus, thus producing a consensus sequence of pS-X-E/F-Y-COOH. Two proteins with C termini that conform to this consensus sequence bind the tBRCT domain of MDC1 in a strictly phospho-dependent manner: histone H2AX (pSQEY-COOH) (24) and the anaphase-promoting complex/cyclosome (APC/C) subunit CDC27 (pSDEF-COOH) (37). Sequence analysis of RAG1 reveals that it harbors a similar motif at its C terminus (SMEF-COOH), which we refer to hereafter as R1Ct (Fig. 1A). This motif is conserved in all mammalian orthologs of RAG1, but not in chicken (Gallus gallus) or zebrafish (Danio rerio) (Fig. 1B). Interestingly, clear orthologs of MDC1 have only been identified thus far in mammals, correlating with the appearance of R1Ct. These observations raise the possibility that RAG1 and MDC1
interact via the phosphorylated R1Ct motif of RAG1 and the tBRCT domain of MDC1.

A RAG1 C-terminal Peptide Binds the tBRCT Domain in a Phospho-dependent Manner—To test more directly whether R1Ct can bind the tBRCT domain of MDC1, we performed a peptide pull-down assay. A short peptide that corresponds to R1Ct displayed very weak binding to purified GST-tBRCT, whereas an identical peptide phosphorylated on Ser-1037 gave robust binding (Fig. 1C, lanes 6 and 10). A point mutation in the tBRCT domain (K1936M-tBRCTm), which has been shown to disrupt phosphospecific interactions (24, 37), virtually abolished binding to the phosphopeptide (Fig. 1C, lane 11). GST alone did not show any binding to either peptide, suggesting that the very weak binding of the tBRCT domain to the unmodified peptide is mediated by the tBRCT portion of the fusion protein (Fig. 1C, compare lane 5 to lanes 6 and 7). As another control for specificity, we tested the ability of the two peptides to pull down the tBRCT domain of NBS1. We expressed GST fused to an N-terminal fragment of NBS1, which spans both its forkhead-associated (FHA) domain and its tBRCT domain. These domains were demonstrated to form one compact supramodular domain (45, 46) that mediates binding to the phospho-Ser-Asp-Thr (SDT) repeats of MDC1 (25, 26, 47). The FHA-tBRCT domain of NBS1 did not exhibit any binding to either RAG1 peptide (Fig. 1C, lanes 8 and 12), strengthening the notion that the binding of the phospho-RAG1 peptide is specific to the tBRCT domain of MDC1. Overall, our data show that efficient R1Ct-tBRCT binding requires phosphorylation of R1Ct as well as the phospho-binding capability of the tBRCT domain of MDC1. Accordingly, our results raise the possibility that RAG1 phosphorylation on Ser-1037 occurs in vivo.

MDC1 Binds RAG1—To test whether RAG1 interacts with MDC1, we performed immunoprecipitation (IP) experiments using antibodies directed against MDC1. As RAG1 is normally expressed only in developing lymphocytes, we examined this question in the RAG-positive human lymphoblastic cell line Nalm6 and in 293T cells overexpressing RAG1. Immunoprecipitation with an antibody specific for MDC1 revealed that RAG1 associates with MDC1 in Nalm6 whole cell extracts (Fig. 2A). This was also true with RAG1-expressing 293T cells (Fig. 2B, lane 5). As 293T cells do not express RAG2, we conclude that binding of RAG1 to MDC1 does not require RAG2.

The C Terminus of RAG1 Is Required for Binding to the tBRCT Domain—We next sought to test whether the interaction between MDC1 and RAG1 requires the tBRCT domain of MDC1 and the R1Ct motif of RAG1. Purified recombinant GST-tBRCT was used in pull-down experiments and could successfully retrieve RAG1, which was overexpressed in 293T cells (Fig. 2B, lane 9). Most importantly, R1Ct was absolutely required for binding by the tBRCT domain, as a RAG1 mutant lacking its eight most C-terminal residues did not bind to the tBRCT domain (Fig. 2B, lane 10). Endogenous CDC27 served as an internal control for this experiment, as it bound the tBRCT domain in both cases. When the same extracts were used in an immunoprecipitation experiment, we were surprised to see that endogenous MDC1 could bind full-length RAG1 and the
C-terminal truncated mutant equally well (Fig. 2B, lanes 5 and 6). This suggests that MDC1 and RAG1 each contain an additional site of interaction. If this were the case, deletion of the tBRCT domain of MDC1 would still allow binding of RAG1. Indeed, a tBRCT-deleted form of MDC1 bound RAG1 much like full-length MDC1 (Fig. 2C, lanes 5 and 6). CDC27 again served as an important internal control as it binds MDC1 exclusively via the tBRCT domain (37) and, as expected, did not interact substantially with the tBRCT-deleted form of MDC1 (Fig. 2C, lane 6). Overall, these results confirm our initial hypothesis of an interaction between MDC1 and RAG1, but unexpectedly reveal that these proteins can bind each other through two distinct interfaces.

The PST Repeats of MDC1 bind RAG1—To map the second binding site in MDC1, we performed coimmunoprecipitation experiments using 293T cells coexpressing RAG1 and deletion mutants of MDC1. We found that a C-terminal fragment of MDC1, which includes the PST repeats (hereafter referred to as the repeats) and the tBRCT domain, was capable of binding RAG1 (Fig. 2C, lanes 5 and 6). CDC27 again served as an important internal control as it binds MDC1 exclusively via the tBRCT domain (37) and, as expected, did not interact substantially with the tBRCT-deleted form of MDC1 (Fig. 2C, lane 6). Overall, these results confirm our initial hypothesis of an interaction between MDC1 and RAG1, but unexpectedly reveal that these proteins can bind each other through two distinct interfaces.

The N-terminal Non-core Region of RAG1 Binds the PST Repeats of MDC1 Directly—To map the second binding site in RAG1, we performed GST pull-down experiments with purified GST repeats fragment and protein extracts of 293T cells expressing HA-tagged fragments of RAG1. Full-length RAG1 and the entire N-terminal non-core portion of RAG1 bound the repeats, whereas the core region of RAG1 or the zinc dimerization domain did not (Fig. 4A). To support this result, we purified MBP-tagged fragments of the N terminus of RAG1 from bacteria and tested their ability to interact with purified GST repeats. Again, the N-terminal non-core region of RAG1 was able to bind the repeats, whereas the zinc dimerization domain could not (Fig. 4B, lanes 10 and 12). Furthermore, deletion of the zinc dimerization domain from the RAG1 N-terminal region impaired binding to the repeats. These data point to the repeats as the second RAG1-binding site in MDC1.
Because this experiment was done using recombinant proteins that lies within the N-terminal portion of residues 1–264.

Repeats-binding interface in RAG1 is a slightly smaller region that did not affect binding (Fig. 4B, lane 11). These data indicate that an N-terminal portion of RAG1 spanning residues 1–264, which we refer to as R1Nt, represents a second binding site for MDC1. Intriguingly, a shorter MBP-tagged fragment of RAG1 was detected in both samples that cover R1Nt (Fig. 4B, arrowheads). This shorter fragment, most probably a spontaneous breakdown product, was also retrieved by the repeats of MDC1 (Fig. 4B, lanes 10 and 11), suggesting that the minimal repeats-binding interface in RAG1 is a slightly smaller region that lies within the N-terminal portion of residues 1–264. Because this experiment was done using recombinant proteins purified from bacteria, it supports the conclusion that the repeats-R1Nt interaction is direct and does not require post-translational modifications.

Binding of Endogenous RAG1 to the PST Repeats and tBRCT Domain of MDC1—To extend our work to endogenous RAG1, we performed experiments using Abelson murine leukemia virus transformed progenitor B cell lines (hereafter referred to as v-abl cells). These cells proliferate in culture but do not progress beyond the pro- to pre-B cell stage. However, treatment of these cells with the c-Abi kinase inhibitor, STI-571 (also known as Gleevec or imatinib), induces cell cycle arrest accompanied by high expression levels of RAG (48). As a result, these cells start to differentiate and actively and efficiently rearrange their endogenous loci and artificial V(D)J recombination substrates. Endogenous RAG1 accumulates rapidly after induction of WT v-abl cells with STI-571, and its levels remain constant for several days (Fig. 5A, lanes 1–5). We used whole cell extracts from v-abl cells in a GST pull-down experiment and could detect robust binding of RAG1 to the repeats of MDC1, regardless of the duration of activation (Fig. 5A, lanes 11–15). Although RAG1 binding to the repeats was consistent, binding to the tBRCT was not. We could detect weak binding to the tBRCT in some experiments but not others, with no obvious explanation for the variability (data not shown). Why is binding of RAG1 to the tBRCT domain difficult to detect? One possibility is that only a small fraction of RAG1 undergoes phosphorylation.

Given the relatively low level of expression of endogenous RAG1, it is plausible that detection of the interaction is at or below the threshold of this assay. For higher levels of RAG expression we employed another well established inducible system for analysis of V(D)J recombination: the mature B cell line 5B3 (41). These cells express high levels of RAG proteins upon tetracycline withdrawal (Tet-Off). Induction of 5B3 cells resulted in high levels of RAG1 expression (Fig. 5B, lanes 1–4). Whole cell extracts from these cells were used in a GST pull-down assay. Similar to what we saw with extracts from v-abl cells, RAG1 was retrieved by the repeats of MDC1 regardless of the duration of activation (Fig. 5B, lanes 11–13). Notably, we could also detect binding of RAG1 to the tBRCT domain of MDC1. Although binding was weaker than that seen with the repeats, it was clearly above background (Fig. 5B, compare lanes 5–7 and 8–10). This binding pattern was also seen with overexpressed RAG1 in 293T cells (Fig. 5C) as well as with endogenous RAG1 in Nalm6 cells (Fig. 5D). Importantly, endogenous RAG2 followed the same binding pattern as RAG1 (Fig. 5D), supporting the notion that the repeat-R1Nt interaction does not interfere with the formation of the RAG complex.

To detect phospho-Ser-1037-RAG1 more directly, we raised phosphospecific antibodies in two rabbits using the peptides described in Fig. 1C for immunization and affinity purification. Unfortunately, weak signal and high background precluded conclusive detection of RAG1 phosphorylated at Ser-1037, despite extensive analyses (data not shown). Our difficulty in detecting phospho-Ser-1037-RAG1 might be due to phosphorylation levels that are below the detection limit of these antibodies. Although our data are suggestive of phosphorylation of RAG1 on Ser-1037, we have not been able to confirm this. To fully understand the importance and potential regulatory role

Dual Interaction between MDC1 and RAG1

FIGURE 4. An N-terminal non-core region of RAG1 binds the repeats of MDC1 directly. A, HA-tagged full-length RAG1 or fragments of RAG1, as depicted in the diagram in the bottom panel, were overexpressed in 293T cells. Protein extracts from these cells were used in a GST pull-down assay with GST or a GST-repeats (residues 1090–1700 of human MDC1) fusion protein. B, MBP alone or MBP-tagged RAG1 fragments, as depicted in the diagram in the bottom panel, were expressed in bacteria, purified, and used in a GST pull-down assay with GST or a GST-repeat fusion protein. Bound proteins and 5% of the input were analyzed by SDS-PAGE and blotted as indicated on the left. Data in all panels are representative of at least three independent experiments. ZDD, Zinc Dimerization Domain; N, N-terminal fragment; NΔZ, N-terminal Δ ZDD; Z, ZDD.

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of this modification in V(D)J recombination, it will be important to establish its pattern in vivo.

DISCUSSION

In this work we describe an interaction between MDC1 and RAG1. Mapping of this interaction reveals that the PST repeats of MDC1 bind an N-terminal non-core region of RAG1 (R1Nt) whereas the tBRCT domain of MDC1 binds the H2AX-like C-terminal motif of RAG1 (R1Ct) (depicted schematically in Fig. 5E).

The two binding interfaces identified show distinct properties. The repeats-R1Nt interaction shows robust binding, which does not require posttranslational modifications. In contrast, the tBRCT-R1Ct interaction is likely phospho-dependent, on the basis of the much stronger binding of the tBRCT domain of MDC1 to a RAG1 C-terminal peptide phosphorylated on Ser-1037 (Fig. 1C). Although the tBRCT domain is an established protein-protein interaction module that appears in other proteins and has been studied extensively (42, 44, 49), the PST repeats are unique to MDC1 and bear no clear sequence similarity to other proteins. The PST repeats occupy almost a third of human MDC1 and are composed of 19 imperfect repeats of ~41 residues. The only report that describes a role for this domain suggests that it acts as a protein-protein interaction motif (50). That work shows that MDC1 binds DNA-PK via the PST repeats, and that this interaction is required for autophosphorylation of DNA-PKcs and DSB repair. Interestingly, this interaction occurs even in the absence of DSBs, suggesting a constitutive mode of binding, similar to that observed with RAG1. It is not clear how MDC1 exerts its effect on DNA-PK. It is also not known if the MDC1-DNA-PK interaction is direct, and if so, if it is mediated by the catalytic subunit of DNA-PK (DNA-PKcs) or by the Ku70 and/or Ku80 subunits.

MDC1 contains additional protein-protein interaction modules which are repetitive in their nature - the SDT repeats and the TQXF repeats, which mediate binding to NBS1 (25, 26, 47) and RNF8 (51, 52), respectively. In both cases, a single repeat can bind its target in vitro. However, multiple repeats allow stronger binding and are required for a functional interaction in vivo. Does this requirement for multiple repeats hold true for the PST repeats? To address this issue we asked whether a single PST repeat can bind RAG1. To avoid bias for any one specific PST repeat, we generated a synthetic consensus sequence based on all PST repeats and expressed it as a GST fusion. This fusion protein failed to bind RAG1, suggesting...
that multiple repeats are required for this interaction (data not shown).

RAG1 and RAG2 were discovered more than two decades ago (53, 54), yet little is known about their interacting partners. Two very early reports describe an interaction between RAG1 and importin subunits α1 and α2 (originally termed hSRP1 and Rch1, also known as karyopherins α1 and α2) (55, 56). A clear role for these proteins in V(D)J recombination has not been shown. Interestingly, the mode of binding between these importins and RAG1 reveals striking similarity to the interaction between RAG1 and the PST repeats of MDC1. Mapping experiments revealed that an N-terminal fragment of RAG1, spanning residues 1–288, mediated binding to importin α1 (55). This region closely matches our mapping of R1Nt to residues 1–264 (Fig. 4B). Furthermore, both importins also bind RAG1 via a repetitive motif termed armadillo repeats. These are ~42 residue-long protein-protein interaction motifs that typically appear in multiple tandem copies (57). At least four of eight armadillo repeats were required for binding of importin α1 to RAG1 (55). Armadillo repeats show very weak sequence homology between various proteins but adopt a very distinct and conserved structure. In fact, several proteins were classified as containing armadillo repeats on the basis of their structure and not their sequence (57). The PST repeats of MDC1 therefore resemble the armadillo repeats of importins α1 and α2 in two respects: both bind the N terminus of RAG1, and both are composed of similarly sized repetitive motifs. On the basis of these observations we propose that the PST repeats of MDC1 adopt an armadillo or armadillo-like fold. This idea can be tested by the important and informative challenge of solving the crystal structure of the PST repeats.

A more recent report provides evidence for an interaction between RAG1 and Ku70/Ku80 (30). This interaction is also mediated by the N-terminal non-core region of RAG1 and affects signal joint formation. This interaction was not demonstrated via direct biochemical approaches but was rather detected as a super-shift when Ku-specific antibodies were added to mildly extracted and purified RAG1-combination signal sequences complexes. Importantly, addition of purified Ku proteins to purified RAG1 failed to result in such a shift, arguing against a direct interaction. The authors proposed that a bridging factor might be limiting. Given that the PST repeats of MDC1 bind both RAG1 (Figs. 3 and 4) and DNA-PK (50), it is possible that MDC1 bridges between Ku and RAG1.

As noted above, in contrast to the PST repeats, the tBRCT domain of MDC1 is a well-studied domain with known binding targets. The similarity between the C-terminal motif of RAG1 and the C termini of histone H2AX and CDC27 suggests that RAG1 binds the tBRCT domain via a similar mechanism. It is important to stress that phosphorylation of the C termini of H2AX and CDC27 is strictly required for their binding to the tBRCT domain (24, 37). Quantitative isothermal titration calorimetry binding experiments show that a phospho-H2AX peptide binds the tBRCT domain of MDC1 with a \( K_d \) of ~2 μM, whereas binding of the non-phosphorylated peptide cannot even be detected (24). These observations suggest that phosphorylation of RAG1 is similarly crucial for its interaction with the tBRCT domain of MDC1.

Are both domains of MDC1 equally important for binding to RAG1? Although deletion of the tBRCT domain in the context of full-length MDC1 did not detectably diminish binding of MDC1 to RAG1 (Figs. 2C and 3B), deletion of the repeats abolished detectable binding to RAG1 (Fig. 3B). It was surprising that the repeat-deleted MDC1 was not able to bind RAG1, as it still contains the tBRCT domain. Importantly, the tBRCT domain in this fragment is functional, as is evident from the fact that binding of CDC27 was intact (Fig. 3B, lane 11). We also noticed that clear binding by the tBRCT domain of MDC1 typically required strong expression of RAG1 (compare the relatively higher level of RAG1 in Fig. 2B, lanes 1–2 versus Fig. 3B, lanes 1–4). One possibility for these observations is that binding of RAG1 to the tBRCT domain is weaker than its binding to the repeats. Although we cannot rule out this option, binding of the tBRCT domain to a RAG1 phosphopeptide was robust (Fig. 1C). Another option is that only a small fraction of RAG1 is phosphorylated on Ser-1037 in vivo. An emerging theme is that V(D)J recombination occurs within defined chromatin regions, termed recombination centers (58), which are likely to contain only a small fraction of the total RAG protein in the cell. It is possible that RAG1 phosphorylation is restricted to these confined regions and, hence, to a limited number of RAG1 molecules. Another possibility is that the cellular model systems we employed do not faithfully recapitulate the physiologically relevant event that triggers this modification. Our ability to detect some binding of RAG1 to the tBRCT domain using 293T and 5B3 cells might be due to the substantial overexpression of RAG1, perhaps coupled with unregulated RAG1 phosphorylation. However, the fact that we could detect binding of endogenous RAG1 to the tBRCT domain using Nalm6 cells (Fig. 5D) suggests that this phosphorylation may be a relevant physiological event. As histone H2AX becomes phosphorylated in response to DSBs (59), we reasoned that the H2AX-like R1Ct motif may be similarly modified, possibly by RAG-induced DSBs. However, binding of RAG1 to the tBRCT domain was difficult to detect when we used activated v-abl cells, which are known to induce RAG-dependent DSBs efficiently (32). If RAG1 phosphorylation occurs in response to RAG-induced breaks, it may be a transient modification, as such breaks are rapidly repaired. We therefore employed Artemis null v-abl cells, which fail to repair RAG-induced breaks. Similar to WT v-abl cells, we could only occasionally detect weak binding of RAG1 to the tBRCT domain (data not shown). We thus conclude that the accumulation of unrepaired RAG-induced breaks in Art(−/−) v-abl cells does not lead to substantial phosphorylation of the R1Ct motif of RAG1. Another possibility we examined is that general DSBs may lead to R1Ct phosphorylation. However, treatment of RAG1-expressing 293T cells with radiomimetic drugs to induce exogenous DSBs also failed to induce a substantial increase in binding of RAG1 to the tBRCT domain (data not shown).

What are the possible roles of the MDC1-RAG1 interaction? ATM and the MRN complex were recently shown to play a role in preventing formation of hybrid joints (32, 33). As MDC1 regulates recruitment and retention of both ATM (27, 60) and the MRN complex (25, 26, 38, 47) to DSBs, we reasoned that MDC1 may also be important in preventing hybrid joint forma-
tion. To test this possibility we established MDC1 null v-abl pre-B cell lines and analyzed the formation of hybrid joints in comparison to WT and ATM null cells. Extensive analysis of genomic DNA by PCR and Southern blotting did not yield a detectable hybrid joint signal in MDC1 null cells (of either an artificial integrated substrate or the endogenous Igκ locus), whereas genomic DNA from ATM null cells readily produced hybrid joint signals (supplemental Fig. 1 and data not shown). We therefore conclude that MDC1 is not required for prevention of hybrid joint formation.

The DSBs created during V(D)J recombination pose a serious threat to genomic stability. It is therefore not surprising that this process is tightly regulated by multiple and redundant layers (61). It is often the case that a clear phenotype is not seen when a single gene is knocked out. A role for H2AX and MDC1 in end protection in V(D)J recombination was only evident when these genes were knocked out on an Artemis null background, which allowed for aberrant processing of hairpin coding ends to be detected (34). Similarly, a role in repair of RAG-induced breaks in lymphocytes was clearly detected for XLF only when XLF was deleted in cells lacking ATM function (36, 62). It is thus possible that loss of the MDC1-RAG1 interaction is compensated in MDC1 null mice.

When RAG1 and RAG2 were discovered, initial efforts were directed at finding the minimal regions in both proteins that still support V(D)J recombination, thus defining their core regions. Close inspection of these papers revealed three independent reports in which small C-terminal deletions in RAG1 gave a surprising phenotype: ~2-fold higher levels of V(D)J recombination, thus suggesting that the C terminus of RAG1 may be important for inhibiting recombination (39, 63, 64). A very recent report supports these findings and extends them by showing that the C terminus of RAG1 cooperates with the C terminus of RAG2 to inhibit hairpin formation (65). It is thus possible that binding of the tBRCT domain of MDC1 to the C terminus of RAG1 modulates RAG activity. It is tempting to suggest the following model: MDC1 and RAG1 reside in a complex because of the constitutive repeat–R1Nt interaction. RAG1 and RAG2 bind their target DNA and perform cleavage. This leads to local phosphorylation of the C terminus of RAG1 in recombination centers, possibly by DNA-PK or ATM, which in turn leads to binding by the tBRCT domain of MDC1. This presumably modulates the activity of RAG. One attractive option is that this event inhibits further cleavage, thus minimizing the risk of nearly simultaneous RAG-induced breaks. On this model, RAG-dependent cleavage creates a signal that modulates its own activity.

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