KAP-1 promotes resection of broken DNA ends not protected by \(\gamma\)-H2AX and 53BP1 in G1-phase lymphocytes

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KAP-1 Promotes Resection of Broken DNA Ends Not Protected by γ-H2AX and 53BP1 in G1-Phase Lymphocytes

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The resection of broken DNA ends is required for DNA double-strand break (DSB) repair by homologous recombination (HR) but can inhibit normal repair by nonhomologous end joining (NHEJ), the main DSB repair pathway in G1-phase cells. Antigen receptor gene assembly proceeds through DNA DSB intermediates generated in G1-phase lymphocytes by the RAG endonuclease. These DSBs activate ATM, which phosphorylates H2AX, forming γ-H2AX in flanking chromatin. γ-H2AX prevents CtIP from initiating resection of RAG DSBs. Whether there are additional proteins required to promote resection of these DNA ends is not known. KRAB-associated protein 1 (KAP-1) (TRIM28) is a transcriptional repressor that modulates chromatin structure and has been implicated in the repair of DNA DSBs in heterochromatin. Here, we show that in murine G1-phase lymphocytes, KAP-1 promotes resection of DSBs that are not protected by H2AX and its downstream effector 53BP1. In these murine cells, KAP-1 activity in DNA end resection is attenuated by a single-amino-acid change that reflects a KAP-1 polymorphism between primates and other mammalian species. These findings establish KAP-1 as a component of the machinery that can resect DNA ends in G1-phase cells and suggest that there may be species-specific features to this activity.

The repair of DNA double-strand breaks (DSBs) is carried out by either homologous recombination (HR) or nonhomologous end joining (NHEJ) (1, 2). HR functions to repair DSBs generated in the S and G2 phases of the cell cycle. In contrast, NHEJ functions to repair DSBs at all phases of the cell cycle, and in G1-phase cells, it is the primary DSB repair pathway. DNA end resection and the formation of single-strand overhangs are critical steps in repair pathway choice (3, 4). During HR, CtIP promotes the processing of DNA ends, forming 3’ single-strand overhangs that bind RPA, initiating HR (3, 4). In contrast, NHEJ most efficiently repairs DNA ends that are blunt or that have short single-strand overhangs (4).

Lymphocyte antigen receptor gene assembly occurs through the process of V(D)J recombination (5). This reaction is initiated by RAG-1 and RAG-2, which form the RAG endonuclease (6). RAG introduces DNA DSBs at the border of two recombining gene segments, forming a hairpin-sealed coding end and a blunt signal end at each DSB (6). NHEJ joins the coding ends, forming a coding joint, and the signal ends, forming a signal joint (7, 8). The NHEJ proteins required for V(D)J recombination include Ku70, Ku80, and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which together form the DNA-PK complex (7, 8). The Artemis endonuclease is required to open hairpin-sealed coding ends, and DNA ligase IV ligates the signal and coding ends (7, 8). XRCC4 functions with DNA ligase IV to promote joining (7, 8). XLF also functions in the repair of RAG DSBs, but this function is redundant with other factors (9–11). V(D)J recombination occurs only in the G1 phase of the cell cycle, due in part to the degradation of RAG-2 during S phase (12).

RAG DSBs activate the ATM kinase and DNA damage responses (DDRs) (7). This includes the phosphorylation of the histone variant H2AX (forming γ-H2AX) in chromatin for several hundred kilobases flanking RAG DSBs (13). In G1-phase cells, the formation of γ-H2AX prevents CtIP from initiating the resection of RAG DSBs (11). Indeed, in H2AX-deficient lymphocytes, CtIP promotes hairpin opening and resection of coding ends in the absence of Artemis (11). Whether additional proteins also function in this recombination process is not known.

KRAB-associated protein 1 (KAP-1) (also known as TRIM28, TIF1β, or KRIP-1) has been implicated in DNA DSB repair (14–16). KAP-1 is a member of the tripartite motif (TRIM) family of proteins, which includes approximately 100 members in humans (17–20). These proteins have N-terminal tripartite motifs composed of RING, B-box, and coiled-coil domains that promote homo- and hetero-oligomerization (17–19). This oligomerization is important for the stability and function of many TRIM proteins. The C termini of the TRIM proteins are diverse and composed of domains that mediate the many different functions of these proteins, including transcriptional regulation, modulation of signaling pathways, alteration of chromatin structure, pathogen recognition, and DNA DSB repair. TRIM proteins can exhibit significant polymorphisms over short evolutionary distances that may be important for some of their species-specific functions, such as pathogen recognition and defense (18–20).

KAP-1 is involved in the repair of DSBs generated in heterochromatic regions of the genome in both mouse and human cells (15, 21). KAP-1 has a C-terminal plant homeodomain (PHD)
with E3 SUMO ligase activity, required for binding of the nucleosome remodeler CHD3 to the adjacent bromodomain (22). The C terminus of KAP-1 also contains a serine residue (serine 824) that is phosphorylated by ATM in response to DNA DSBs (14). Phosphorylation of this residue disrupts the interaction of KAP-1 and CHD3, which is required for efficient repair of heterochromatic DSBs (21–23). The DNA repair factor 53BP1, which localizes to chromatin containing γ-H2AX, is required for optimal heterochromatic DSB repair mediated by KAP-1 phosphorylation and chromatin relaxation (23, 24). 53BP1 has also been implicated in preventing DNA end resection, antagonizing HR and promoting NHEJ (24–28).

Here, we show that mouse KAP-1 (mKAP-1) functions to promote the resection of DNA DSBs in murine G1-phase lymphocytes when these DNA ends are not protected by H2AX and 53BP1. A single-amino-acid change that reflects a KAP-1 polymorphism between primates and other mammalian species disrupts its ability to promote DNA end resection in murine cells. These findings establish a novel function for KAP-1 in DNA DSB repair. The potential species-specific aspects of this KAP-1 function are discussed.

### MATERIALS AND METHODS

#### Cell culture.

Abelson-transformed pre-B cells that express a Bcl2 transgene (abl pre-B cells) were generated, and the pMX-DELCI retroviral recombination substrate was introduced as previously described (29). The generation of $L_{tg}$V$^+$ abl pre-B cells from $L_{tg}$V$^{+\text{abl pre-B}}$ abl pre-B cells was carried out as previously described (11). For RAG induction, abl pre-B cells were treated with 3 μM imatinib for the indicated number of days at 10^6 cells/ml. The ATM kinase inhibitor U015933 (Tocris) was used at 15 μM.

#### Southern blotting.

Native and denaturing (1:2 ratio of native and denaturing) Southern blots analyses of V(D)J recombination of the pMX-DELCI retroviral recombination substrate were carried out as previously described (11). Southern blot analyses of Eb/ZFN (zinc finger nucleases) DSBs were performed as previously described (30). The Eb probe was generated by PCR amplification using the oligonucleotides 5’-GGTAAACGGGCACTGAGGAC-3’ and 5’-CCATGTGTCATACCTGAAGCCC-3’. The Erag probe was generated by PCR amplification using the oligonucleotides 5’-AACCTCCCTCAGGAGAGCATC-3’ and 5’-TGTACTGTTGACAGCAAA-3’.

#### Immunofluorescence.

abl pre-B cells were treated with imatinib for 2 days. A total of 10^6 cells were washed in 1× phosphate-buffered saline (PBS) and attached to glass slides coated with Cell-Tak (BD). Cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 5 min, and then washed with PBS. Immunostaining with primary and secondary antibodies was performed with a blocking solution of 3% bovine serum albumin (BSA) in PBS at 37°C for 1 h, and cells were washed with PBS. Immunofluorescence in situ hybridization (immunofluo- Femur) was performed with a masscap microphone with optical sections separated by 0.12 μm. Three-dimensional distances between the centers of mass of two foci were measured by using the Sync Measure 3D Image plug-in. Igk alleles were scored as 53BP1 positive (53BP1^+ ) when the center-to-center distance between the Igk and 53BP1 foci was 1 μm or less.

### Lentiviral knockdown and retroviral cDNA expression.

Knockdown using lentiviral U6 short hairpin RNAs (shRNAs) was performed with a pFLRU/Thy1.1 lentiviral vector derived from pFLRU/YFP, which was described previously (11). We replaced the yellow fluorescent protein (YFP) cDNA in the original vector with Thy1.1 cDNA and then cloned it into U6 shRNAs to generate pFLRU/shRNA:Thy1.1 vectors. The shRNA sense sequences used were 5’-GGGATATGGCTTTGGGTCA-3’ for KAP-1, 5’-G AGCAGACCCTTCTAGTA-3’ for ChIP, and 5’-GGTTCACTGTGACCA ATTCTG-3’ as a nontargeting control (ctrl). Lentiviruses were generated and abl pre-B cells were transduced as previously described (11). Cells expressing the pFLRU-shRNA vectors were obtained by magnetic cell sorting using CD90.1 (Thy1.1) MicroBeads and MS columns (Miltenyi).

Plasmids for expression of human KAP-1 (hKAP-1) cDNAs were derived from pOZ-FH-N-KAP1 (32). The mouse KAP-1 cDNA was generated by using RNA from a wild-type C57BL/6J mouse. Mouse KAP-1 was cloned downstream of the Flag-hemagglutinin (Flag-HA) tag in pOZ-FH-N by using Xhol/NotI, replacing the human KAP-1 cDNA. Flag-tagged KAP-1 hybrids were generated by PCR amplification of mouse or human cDNA. An shRNA-resistant mouse KAP-1 cDNA was generated by changing 3 nucleotides in the mouse KAP-1 cDNA complementary to the KAP-1 shRNA. Flag-tagged KAP-1 hybrids and point mutants were cloned into pOZ-FH-N. Retroviruses were generated and transduced into abl pre-B cells as previously described (11). Cells expressing the pOZ vectors were obtained by magnetic cell sorting using human CD25 MicroBeads and MS columns (Miltenyi).

#### Immunoblot analysis.

Whole-cell lysates were generated by using LDS sample buffer (Invitrogen) supplemented with dithiothreitol (DTT). Standard immunoblotting techniques were used, as previously described (11). Primary antibodies used include anti-KAP-1 (rabbit polyclonal, recognizing mouse and human KAP-1; GeneTex), anti-Flag M2 (Sigma), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma).

#### Analysis of protein structure order.

Protein disorder for mKAP-1 and hKAP-1 was predicted by PrDOS (http://prdos.bgc.jp/cgi-bin/top .cg), using protein sequences for mKAP-1 (NCBI accession number NP_053518.2) and hKAP-1 (accession number NP_005753.1) (33). A 5% false-positive rate was used.

#### Induction of DSBs using zinc finger nucleases.

A pair of custom zinc finger nucleases (Eb/ZFN, Eb1/ZFN and Eb2/ZFN, specific to a region of the mouse T cell receptor beta (Tcrb) loci near the enhancer) (Eb), were previously described (30). Eb1/ZFN and Eb2/ZFN were expressed under a tetracycline-responsive element with a constitutively expressed Thy1.1 or Thy1.1 marker, generating the lentiviral vectors TRE-Eb1:ZFN-Thy1.2 and TRE-Eb2:ZFN-Thy1.1. The lentivirus was generated and abl pre-B cells were transduced as previously described (11). Cells expressing both Thy1.1 and Thy1.2 were selected by magnetically activated cell sorting (MACS) (Miltenyi) in consecutive sorts for individual markers. Cells with Eb/ZFN were treated with imatinib for 1 day to arrest cells in G1 phase and 4 μg/ml doxycycline (Sigma) for either 1 or 2 days.

#### RPA-binding assay.

The RPA-binding assay was performed as previously described, with the following modifications (34). abl pre-B cells treated with imatinib for 2 days were subjected to 5 μg/ml bleocin treatment for 24 h before preextraction with 0.2% Triton X-100. Fixation and permeabilization were performed with Fix/Perm solution (BD Biosciences). RPA32 staining was performed with Perm Wash (BD Biosciences) with anti-RPA32 antibody (rat monoclonal antibody [MAb], clone 4E4; Cell Signaling) (1:1,000 dilution). Secondary staining was performed by using Alexa Fluor 488–goat anti-rat IgG (Invitrogen). DNA content was assayed by using 7-aminoactinomycin D (7AAD), and stained cells were analyzed by using a FACSCalibur instrument.
RESULTS

53BP1 inhibits resection of RAG DSBs in G1-phase lymphocytes. The cellular responses to RAG DSBs can be assessed in Abelson-transformed pre-B cells that express a Bcl2 transgene, here referred to as abl pre-B cells (7, 29, 35). Treatment of these cells with the abl kinase inhibitor imatinib leads to G1 cell cycle arrest, RAG induction, and initiation of V(D)J recombination at the endogenous immunoglobulin kappa (Igk) light chain locus and at chromosomally integrated retroviral recombination substrates, such as pMX-DELCJ (see the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf) (29). In abl pre-B cells with a single copy of pMX-DELCJ and deficient in Artemis (Art−/−:DELCJ) or DNA ligase IV (LigIV−/−:DELCJ), imatinib treatment leads to the accumulation of unrepaird coding ends at the Igk locus (see the data posted at the URL mentioned above) and pMX-DELCJ (Fig. 1A; see also the data posted at the URL mentioned above).

53BP1 foci form at RAG DSBs in LigIV−/− abl pre-B cells. This is evidenced by immuno-FISH analyses revealing that 66% (94/142) of 53BP1 foci colocalize with an Igk locus probe in LigIV−/− abl pre-B cells treated with imatinib (Fig. 1B). 53BP1 foci do not form in RAG-deficient (Rag−/−) abl pre-B cells (Fig. 1C). Moreover, 53BP1 foci do not form at RAG DSBs in LigIV−/−:H2AX−/− abl pre-B cells, in agreement with previous studies showing that 53BP1 retention at genotoxic DSBs depends on the formation of γ-H2AX (Fig. 1C) (24).

As has been observed for abl pre-B cells deficient in H2AX and either Artemis (Art−/−:H2AX−/−:DELCJ) or DNA ligase IV (LigIV−/−:H2AX−/−:DELCJ), pMX-DELCJ coding ends are also significantly resected in abl pre-B cells deficient in 53BP1 and either Artemis (Art−/−:53BP1−/−:DELCJ) or DNA ligase IV (LigIV−/−:53BP1−/−:DELCJ) (Fig. 1A; see also the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf) (11). Compared to H2AX-deficient cells, many of the pMX-DELCJ coding ends generated in 53BP1−/− deficient cells were undetectable, as they were resected past the C4b probe (Fig. 1A; see also the data posted at the URL mentioned above). Similar results were observed for coding ends generated at the Igk locus (data not shown). Thus, like H2AX, 53BP1 prevents resection of RAG DSBs in G1-phase lymphocytes.

KAP-1 promotes resection of DNA breaks in G1-phase cells. Because 53BP1 and KAP-1 are functionally connected in the repair of DSBs in heterochromatin, we hypothesized that CtIP-mediated resection, and initiation of V(D)J recombination at the endogenous immunoglobulin kappa (Igk) light chain locus and at chromosomally integrated retroviral recombination substrates, such as pMX-DELCJ (see the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf) (29). In abl pre-B cells with a single copy of pMX-DELCJ and deficient in Artemis (Art−/−:DELCJ) or DNA ligase IV (LigIV−/−:DELCJ), imatinib treatment leads to the accumulation of unrepaird coding ends at the Igk locus (see the data posted at the URL mentioned above) and pMX-DELCJ (Fig. 1A; see also the data posted at the URL mentioned above).

and LigIV−/−:53BP1−/−:DELCJ-58 abl pre-B cells. Similar results were obtained for LigIV−/−:DELCJ-1, LigIV−/−:H2AX−/−:DELCJ-148, and LigIV−/−:53BP1−/−:DELCJ-5 abl pre-B cells. (B) Confocal immuno-FISH of Rag1−/− and LigIV−/− abl pre-B cells treated with imatinib for 2 days. Nuclei were stained by using a BAC for Igk (red) and DAPI (blue). Composite three-dimensional images are displayed as z-projections. Data shown were generated from LigIV−/−:DELCJ-7, LigIV−/−:H2AX−/−:DELCJ-5, and LigIV−/−:53BP1−/−:DELCJ-58 abl pre-B cells. Similar results were obtained for LigIV−/−:DELCJ-1, LigIV−/−:H2AX−/−:DELCJ-148, and LigIV−/−:53BP1−/−:DELCJ-5 abl pre-B cells. (C) Immunofluorescence assay for 53BP1 and γ-H2AX in abl pre-B cells treated with imatinib for 2 days. Nuclei were stained by using a BAC for Igk (red) and DAPI (blue). Composite three-dimensional images are displayed as z-projections. Data shown were generated from LigIV−/−:DELCJ-7, LigIV−/−:H2AX−/−:DELCJ-5, and LigIV−/−:53BP1−/−:DELCJ-58 abl pre-B cells.
down of KAP-1 leads to reduced resection of coding ends at the Igk locus and pMX-DELCl in LigIV−/−:H2AX−/−:DELCl and LigIV−/−:53BP1−/−:DELCl abl pre-B cells (Fig. 2A to C; see also the data posted at the URL mentioned above). This was evidenced by an increase in full-length coding ends at pMX-DELCl and Igk in LigIV−/−:H2AX−/−:DELCl and LigIV−/−:53BP1−/−:DELCl abl pre-B cells expressing a KAP-1 shRNA compared to those expressing a control shRNA (Fig. 2B and C; see also the data posted at the URL mentioned above).

In LigIV−/−:53BP1−/−:DELCl abl pre-B cells, many pMX-DELCl coding ends were extensively resected and thus not detected by the C4b probe (Fig. 2B). However, knockdown of KAP-1 led to diminished resection of these DNA ends, which became detectable as full-length or partially resected ends (Fig. 2B). Knockdown of CtIP using a CtIP shRNA in LigIV−/−:H2AX−/−:DELCl and LigIV−/−:53BP1−/−:DELCl abl pre-B cells severely limited resection at pMX-DELCl and Igk (Fig. 2B and C; see also the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf). Thus, nearly all of the resection in this setting is dependent on CtIP, consistent with previous studies (11). These data suggest that KAP-1 supports CtIP-mediated resection of RAG DSBs in the absence of H2AX and 53BP1.

DNA end resection can lead to the formation of single-strand overhangs that bind the RPA complex. RPA binding to single-strand DNA can be detected by retention of the RPA32 subunit in overhangs that bind the RPA complex. RPA binding to single-strand DNA was assessed and quantified as the fraction of RPA-positive cells either treated or not treated with bleocin. Data shown were generated from KAP-1, or CtIP shRNA treated with imatinib for the indicated numbers of days indicated. (B) Southern blot analysis of pMX-DELCJ as described in the legend of Fig. 1A. LigIV−/−:53BP1−/−:DELCJ abl pre-B cells expressing either ctr, KAP-1, or CtIP shRNA treated with imatinib for the indicated numbers of days were analyzed. Data shown were generated from LigIV−/−:53BP1−/−:DELCJ-6 abl pre-B cells, and similar results were obtained for LigIV−/−:53BP1−/−:DELCJ-8 abl pre-B cells. (C) Density plots comparing lane signals from definitive pMX-DELCJ in abl pre-B cells, and similar results were obtained for LigIV−/−:DELCJ-7 and LigIV−/−:53BP1−/−:DELCJ-5 abl pre-B cells. Similar results were obtained for LigIV−/−:DELCJ-10 and LigIV−/−:53BP1−/−:DELCJ-6 abl pre-B cells.

Human KAP-1 does not promote resection of RAG DSBs in murine lymphocytes. A broad variety of human KAP-1 mutants have been generated, which could be used to define KAP-1 function during DNA end resection (36). To this end, we knocked out KAP-1 or control (ctr) shRNA. GAPDH expression is shown as a protein loading control. Molecular mass markers, in kDa, are indicated. (B) Southern blot analysis of pMX-DELCJ as described in the legend of Fig. 1A. LigIV−/−:53BP1−/−:DELCJ abl pre-B cells expressing either ctr, KAP-1, or CtIP shRNA treated with imatinib for the indicated numbers of days were analyzed. Data shown were generated from LigIV−/−:53BP1−/−:DELCJ-6 abl pre-B cells, and similar results were obtained for LigIV−/−:53BP1−/−:DELCJ-8 abl pre-B cells. (C) Density plots comparing lane signals from definitive pMX-DELCJ in abl pre-B cells, and similar results were obtained for LigIV−/−:53BP1−/−:DELCJ-7 and LigIV−/−:53BP1−/−:DELCJ-5 abl pre-B cells. Similar results were obtained for LigIV−/−:DELCJ-10 and LigIV−/−:53BP1−/−:DELCJ-6 abl pre-B cells.
KAP-1 Promotes Resection in G1-Phase Lymphocytes

FIG 3 Expression of human KAP-1 blocks resection of RAG DSBs. (A) Immunoblot analysis of ectopic KAP-1 expression in LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴⁻ abl pre-B cells and expressed shRNA-resistant cDNAs encoding either mouse KAP-1 (mKAP-1) or human KAP-1 (hKAP-1). The expression levels of these two proteins are similar to that of endogenous KAP-1 (see the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf). As expected, robust resection of pMX-DELC⁴ coding ends was observed for LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴ abl pre-B cells expressing mKAP-1 (see the data posted at the URL mentioned above). However, cells expressing hKAP-1 had reduced coding end resection at pMX-DELC⁴ (see the data posted at the URL mentioned above), which, surprisingly, suggests that hKAP-1 may be defective in mediating resection in murine cells. In addition, ectopic expression of hKAP-1 but not mKAP-1 in LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴ abl pre-B cells resulted in a significant reduction of coding end resection at pMX-DELC⁴, even without knockdown of endogenous KAP-1 (Fig. 3). We conclude that hKAP-1 is not able to promote coding end resection in murine lymphocytes. Moreover, expression of hKAP-1 prevents endogenous mKAP-1 from functioning in DNA end resection.

DNA end resection activity localizes to an uncharacterized region of KAP-1. mKAP-1 and hKAP-1 share 93% identity, with a total of 57 amino acid differences distributed along the length of the protein (see the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf). To determine which differences compromise KAP-1 DNA end resection activity in mouse cells, we generated and analyzed a series of hybrids between mKAP-1 and hKAP-1. mKAP-1h₁–411 has amino acids 1 to 411 replaced by the corresponding amino acids from hKAP-1. Similarly, mKAP-1h₄₁₂–₈₃₅ has amino acids 412 to 835 replaced by the corresponding hKAP-1 amino acids (Fig. 4A). Thus, mKAP-1h₁–₄₁₁ is composed of the N-terminal half of hKAP-1 and the C-terminal half of mKAP-1, whereas mKAP-1₁₉₄₁₂–₈₃₅ is composed of the N-terminal half of mKAP-1 and the C-terminal half of hKAP-1.

LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴ abl pre-B cells expressing mKAP-1h₁–₄₁₁ exhibit robust pMX-DELC⁴ coding end resection, whereas coding ends are not resected in cells expressing mKAP-1₁₉₄₁₂–₈₃₅ (Fig. 4A; see also the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_a_MCB_2014_Supplement.pdf). Thus, the C-terminal half of mKAP-1 appears to be critical for promoting resection. Two additional KAP-1 hybrids that further divide this region were generated. mKAP-1₁₉₄₁₂–₆₂₃ contains the HP1-binding domain and flanking region from hKAP-1, whereas mKAP-₁₉₆₂₄–₈₃₅ contains the C-terminal PHD, bromodomain, and flanking regions of hKAP-1. mKAP-₁₉₆₂₄–₈₃₅, but not mKAP-₁₉₄₁₂–₆₂₃, is able to promote pMX-DELC⁴ coding end resection in LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴ abl pre-B cells (Fig. 4B; see also the data posted at the URL mentioned above). The region between amino acids 412 and 623 was further divided, generating mKAP-₁₉₄₁₂–₅₃₀ and mKAP-₁₅₃₃₁–₆₂₃. While pMX-DELC⁴ coding end resection was robust in LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴ abl pre-B cells expressing mKAP-₁₉₄₁₂–₅₃₀, minimal resection was observed in cells expressing mKAP-₁₅₃₃₁–₆₂₃ (Fig. 4C; see also the data posted at the URL mentioned above).

Thus, replacing amino acids 531 to 623 of mKAP-1 with the panel B at 4 days of imatinib treatment, as described in the legend of Fig. 2C. Data shown were generated from LigIV⁻/⁻:53BP1⁻/⁻:DELC⁴⁻6 abl pre-B cells, and similar results were obtained for LigIV⁻/⁻:53BP1⁻/⁻:DELC⁻⁵ abl pre-B cells.
corresponding amino acids from hKAP-1 renders mKAP-1 unable to promote coding end resection in LigIV−/−:53BP1−/−; DELC abl pre-B cells. This region of KAP-1 does not contain known functional domains, but it is proline rich, a general feature of regions with intrinsically disordered regions (37). Indeed, analyses of mKAP-1 and hKAP-1 using the PrDOS algorithm predicts that this region would be disordered, implying that this region serves as a flexible tether between ordered domains (Fig. 4E) (33).

A single-amino-acid polymorphism defines KAP-1 function in DNA end resection. mKAP-1 and mKAP-1b531–548 differ by 19 amino acids. A subset of KAP-1 hybrid proteins showed that mKAP-1b531–548 is able to promote coding end resection, whereas mKAP-1b531–548 is not (Fig. 4D; see also the data posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_al_MCB_2014_Supplement.pdf). mKAP-1b531–548 differs from mKAP-1 by only 6 amino acids, which were each individually changed to the corresponding amino acid in hKAP-1 and expressed in LigIV−/−:53BP1−/−; DELC abl pre-B cells (Fig. 5A and B). All of these mKAP-1 proteins are able to promote robust coding end resection, except for mKAP-1P548A (Fig. 5C; see also the data posted at the URL mentioned above), which has a single-amino-acid change at position 548 from a proline, normally present in mKAP-1, to an alanine, normally present in hKAP-1. Thus, a single-amino-acid change renders mKAP-1 unable to promote coding end resection in murine lymphocytes.

Analysis of KAP-1 sequences across species revealed that the alanine at position 548 is highly conserved in primates, whereas mKAP-1P548A (Fig. 6Band C). Thus, in abl pre-B cells that express mKAP-1 or hKAP-1A548P but not in those that express hKAP-1 or mKAP-1P548A (Fig. 6B and C). Thus, in abl pre-B cells deficient in H2AX and Artemis, KAP-1 is required to promote opening of hairpin-sealed coding ends.

KAP-1 promotes resection of non-RAG DSBs in G1-phase lymphocytes. We wished to determine whether KAP-1 influences the processing of other types of DNA ends in G1-phase lymphocytes. To this end, we made use of a previously described pair of zinc finger endonuclease fusions, Eb:ZFN1 and Eb:ZFN2, collectively referred to as Eb:ZFN, that generate a DNA DSB at the Tcrb locus (30). Eb:ZFN can be expressed in abl pre-B cells under the control of a tetracycline-inducible promoter. Induction of Eb:ZFN in LigIV−/− abl pre-B cells arrested in G1 phase with imatinib results in a DSB at the Tcrb locus that is not repaired, due to the deficiency in DNA ligase IV (Fig. 7A). Eb:ZFN induction in LigIV−/−:53BP1−/− abl pre-B cells leads to the generation of DSBs at the Tcrb locus that are resected, similar to coding ends generated by RAG cleavage in these cells (Fig. 7A). Moreover, this resection is dependent on ATM, as it is significantly diminished by the addition of the ATM inhibitor KU55933 (Fig. 7B). LigIV−/−:53BP1−/− abl pre-B cells that express mKAP-1P548A are unable to resect DNA ends generated by Eb:ZFN (Fig. 7B). Thus, like RAG DSBs, in G1-phase lymphocytes deficient in 53BP1, KAP-1 promotes the resection of DNA DSBs generated by a zinc finger endonuclease.

DISCUSSION

53BP1 and γ-H2AX function to protect DNA ends from nucleolytic resection initiated by CIP in G1-phase cells. Retention of 53BP1 at DSBs depends on γ-H2AX (24). Thus, promoting 53BP1 retention at DSBs could be the primary function of γ-H2AX in modulating DNA end processing in G1-phase cells. However, coding ends generally exhibit greater resection in 53BP1-deficient than in H2AX-deficient cells. This could be due to the transient association of 53BP1 with DSBs in H2AX-deficient cells (24). Alternatively, in addition to retaining 53BP1 at broken DNA ends, γ-H2AX may also promote retention of DNA ends in the absence of 53BP1. The possibility that γ-H2AX promotes resection is consistent with its function in HR (38). Thus, similar to ATM, H2AX may have multiple roles in DNA end resection (11).

Here, we show that 53BP1 and H2AX protect DNA ends, at least in part, by suppressing the activity of the TRIM family protein KAP-1, which promotes the aberrant resection of DSBs in G1-phase cells. At RAG DSBs in murine lymphocytes, proper KAP-1 activity is required for efficient opening of hairpin-sealed coding ends when cells are deficient in Artemis and H2AX, dem-

FIG 4 Effect of KAP-1 mouse-human hybrids on resection of RAG DSBs. (A to D, left) Linear diagrams of mKAP-1, hKAP-1, and human-mouse hybrids, as described in the text. mKAP-1 domains are shown in gray, while hKAP-1 domains are shown in red. All KAP-1 hybrid proteins are Flag tagged at the N terminus. (Right) Density plots comparing lane signals from Southern blot analyses of pMX-DELC1, as described in the legend of Fig. 2C, in LigIV−/−:53BP1−/−; DELC abl pre-B cells expressing a retrovirus encoding the indicated KAP-1 hybrid proteins treated with imatinib for 4 days. Primary data are posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_al_MCB_2014_Supplement.pdf. Dotted lines represent KAP-1 hybrids that function similarly to mKAP-1 to promote resection. Solid lines represent KAP-1 hybrids that function to inhibit resection. Data shown were generated from LigIV−/−:53BP1−/−; DELC abl pre-B cells. (E) Probability of disorder, as determined by PrDOS, along the linear amino acid sequences of hKAP-1 and mKAP-1. The gray box indicates the region between amino acids 531 and 548, which is predicted to be disordered.
onstrating that KAP-1 functions at the earliest step of the resection process. In addition to RAG DSBs, mouse KAP-1 promotes the resection of DNA ends generated by a ZFN and by DNA-damaging agents, such as bleocin. Based on our data, CtIP and KAP-1 may be part of the same pathway that generates resection at DNA DSBs in G1-phase cells. It is possible that these two proteins normally interact at DNA DSBs to promote resection, either directly or in a sequential manner, where KAP-1 is required for optimal

FIG 5 A single-amino-acid change at position 548 determines KAP-1 functionality during resection of RAG DSBs. (A) Comparison of amino acid discrepancies between mKAP-1 and mKAP-1h531-548. Human-specific residues are highlighted in red. (B) Immunoblot analysis of LigIV+/−:53BP1+/−:DELCJ abl pre-B cells expressing either an empty retrovirus (Empty) or a retrovirus encoding the indicated mKAP-1 point mutants. (C) Density plots comparing lane signals from Southern blot analyses of PMX-DELCJ, as described in the legend of Fig. 2C, in LigIV+/−:53BP1+/−:DELCJ abl pre-B cells expressing a retrovirus encoding the indicated mKAP-1 point mutants treated with imatinib for 4 days. Primary data are posted at http://pathology.wustl.edu/labs/sleckman/Tubbs_et_al_MCB_2014_Supplement.pdf. (D) Amino acid sequence surrounding position 548 in mKAP-1, mKAP-1P548A, hKAP-1, and hKAP-1A548P. Human-specific residues are highlighted in red. (E) Immunoblot analysis of LigIV+/−:53BP1+/−:DELCJ abl pre-B cells expressing either a ctr shRNA or KAP-1 shRNA in addition to an empty retrovirus or a retrovirus encoding Flag-HA-tagged mKAP-1, mKAP-1P548A, hKAP-1, or hKAP-1A548P. (F) Southern blot analysis of pMX-DELCJ, as described in the legend of Fig. 1A. LigIV+/−:53BP1+/−:DELCJ abl pre-B cells expressing KAP-1 shRNA in addition to a retrovirus encoding mKAP-1, mKAP-1P548A, hKAP-1, or hKAP-1A548P were treated with imatinib for the indicated numbers of days. Data shown were generated from LigIV+/−:53BP1+/−:DELCJ abl pre-B cells, and similar results were obtained for LigIV+/−:53BP1+/−:DELCJ-6 abl pre-B cells.
pre-B cells treated with imatinib for 2 days were either denatured (D) or left
northern virus or a retrovirus encoding Flag-HA-tagged mKAP-1, mKAP-1 P548A,
open coding ends (oCE) are labeled. (B) Denaturing Southern blot analysis of
and
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EcoRV-digested genomic DNA from
similar results were obtained with
shown were generated from
and
Art
H2AX:
DELCJ:
abl pre-B cells, and
-124 abl pre-B cells.
the KAP-1 function in promoting DNA end resection in
53BP1-deficient cells depends on a proline-rich region that is pre-
dicted to be intrinsically disordered. In this region, the integrity of
a single proline residue at position 548 is critical, and changing this
residue to an alanine, found in human KAP-1, compromises
mouse KAP-1 resection activity. Moreover, converting the alanine
to a proline at this position in human KAP-1 enables resection
activity in murine cells.

In the absence of 53BP1, G1-phase lymphocytes expressing
mKAP-1P548A are unable to efficiently resect DSBs generated by
RAG or a ZFN, even when wild-type mouse KAP-1 is expressed at
normal levels. KAP-1 exists as a stable homotrimer in the cell,
raising the possibility that the incorporation of one or more
mKAP-1P548A proteins into this trimer disrupts KAP-1 activity in
a dominant manner. Knockdown of murine KAP-1 leads to a
defect in resection that is less severe than that observed with ecto-
pic expression of mKAP-1P548A. It is possible that the residual
mouse KAP-1 homotrimers remaining after knockdown are suf-
cient to promote DNA end resection albeit at a reduced level.

The proline at position 548 in mouse KAP-1 is part of a PxP
motif, which could serve as a ligand to bind proteins with an SH3
domain (40). Thus, the conversion of proline to alanine could
disrupt the mouse KAP-1 association with a protein containing an
SH3 domain that is required for DNA end resection. Disordered
domains, especially those that encompass proline-rich regions,
can also mediate protein-protein interactions through proline-
rich motifs. The interactions through these motifs might be dis-
rupted by the alanine substitution (41, 42). Finally, this substi-
tution could influence the ability of distinct mouse KAP-1 domains
to cooperatively function with each other in the setting of a single
mouse KAP-1 molecule or homotrimer.

The inability of human KAP-1, a TRIM family protein mem-
ber, to promote resection of broken DNA ends in murine cells was
surprising, as was the modulation of this activity by a single-amino-
acid difference at position 548. The proline residue at position
548 is highly conserved across all mammals except primates,
where there has been a conversion to alanine. In this regard, inte-
grating TRIM proteins as components of generally conserved
DNA damage responses may pose unique evolutionary challenges,
given the diverse activities that TRIM proteins mediate. Some of
these activities, such as responses to pathogens, necessitate signif-
ificant TRIM protein sequence divergence over short evolutionary
distances. For example, TRIM5
rhesus monkey TRIM5α proteins have this activity, mouse
TRIM5α does not (18).

Human KAP-1 (TRIM28) has also recently been shown to have
important functions in preventing HIV integration into the ge-
nome (43). Thus, primate KAP-1 could have acquired evolution-
ment.
FIG 7 KAP-1 promotes resection of non-RAG DSBs in G1-phase lymphocytes. (A) Southern blot analysis of the endogenous Tcrb locus. LigIV^-/-:Eb:ZFNF and LigIV^-/-:53BP1^-/-:Eb:ZFNF abl pre-B cells were treated with imatinib for 1 day and then treated with doxycycline for 1 day in presence or absence of the ATM inhibitor (ATMi) KU55933. Genomic DNA was digested with HindIII, and blots were hybridized with an Eb probe. An Erag probe was used as a DNA loading control. Uncut Tcrb (UN) and un repaired ZFN DSBs are indicated, as are as resected ZFN DSBs. (B) Southern blot analysis of the endogenous Tcrb locus, as described above for panel A. LigIV^-/-:53BP1^-/-:Eb:ZFNF abl pre-B cells were infected with either an empty retrovirus or a retrovirus expressing Flag–HA–mKAP-1 or Flag–HA–mKAP-1P548A. Cells were pretreated with imatinib for 1 day, and doxycycline was then added for the indicated numbers of days.

**REFERENCES**

KAP-1 Promotes Resection in G1-Phase Lymphocytes


