Artemis C-terminal region facilitates V(D)J recombination through its interactions with DNA Ligase IV and DNA-PKcs

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Artemis is an endonuclease that opens coding hairpin ends during V(D)J recombination and has critical roles in postirradiation cell survival. A direct role for the C-terminal region of Artemis in V(D)J recombination has not been defined, despite the presence of immunodeficiency and lymphoma development in patients with deletions in this region. Here, we report that the Artemis C-terminal region directly interacts with the DNA-binding domain of Ligase IV, a DNA Ligase which plays essential roles in DNA repair and V(D)J recombination. The Artemis–Ligase IV interaction is specific and occurs independently of the presence of DNA and DNA–protein kinase catalytic subunit (DNA-PKcs), another protein known to interact with the Artemis C-terminal region. Point mutations in Artemis that disrupt its interaction with Ligase IV or DNA-PKcs reduce V(D)J recombination, and Artemis mutations that affect interactions with Ligase IV and DNA-PKcs show additive detrimental effects on coding joint formation. Signal joint formation remains unaffected. Our data reveal that the C-terminal region of Artemis influences V(D)J recombination through its interaction with both Ligase IV and DNA-PKcs.

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Abbreviations used: DBD, DNA-binding domain; DNA-PKcs, DNA–protein kinase catalytic subunit; EtBr, ethidium bromide; FNT, Flag-NLS-thioredoxin; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; IP, immunoprecipitation; RS-SCID, SCID associated with radiosensitivity; TCE, total cell extract.

Artemis is a member of the metallo-β-lactamase superfamily of proteins (Moshous et al., 2001). Given its homology to enzymes that have nucleic acids as their substrates (Callebaut et al., 2002), Artemis was tested for nuclease function in vitro and was found to possess an endonuclease activity that can open DNA hairpins (intermediates of coding joints formed during V(D)J recombination) when in complex with DNA–protein kinase catalytic subunit (DNA-PKcs; Ma et al., 2002). Autophosphorylation of DNA-PKcs and the presence of Ku70/80 facilitate the endonuclease activity of Artemis (Goodarzi et al., 2006; Weterings et al., 2009).

Artemis was identified as the protein mutated in patients with SCID associated with radiosensitivity (RS-SCID). The majority of Artemis mutations that cause RS-SCID are located within its highly conserved N-terminal domain (Dudásová and Chovanec, 2003; Musio et al., 2005; Evans et al., 2006; Pannicke et al., 2010), termed as the catalytic core of the protein, with its C-terminal region shown to be dispensable for V(D)J recombination on plasmid substrates (Poinsignon et al., 2004). Unexpectedly however, six patients with hypomorphic mutations in Artemis have been identified so far that have

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either partial or complete deletion of the C-terminal region, and two of them present RS-SCID associated with predisposition to B cell lymphoma (Moshous et al., 2003; Musio et al., 2005; van der Burg et al., 2007). The disease phenotype ranges from partial to complete SCID and is correlative to the size of the protein truncated and thus possibly its residual activity, implying an important role of the C-terminal region of Artemis in development of the immune system (Moshous et al., 2003; Musio et al., 2005; van der Burg et al., 2007). The observation that two of the patients developed lethal and aggressive EBV-associated B cell lymphomas (Moshous et al., 2003) argues for the importance of the C-terminal region in Artemis's role as a genomic caretaker. In addition, Artemis-null cells could not be complemented for their increased radiosensitivity phenotype by expression of the N-terminal domain alone and were only partially complemented by one of the patient's truncated form, further suggesting a role for Artemis' C-terminal region in double-strand break repair (Moshous et al., 2003; Poinsignon et al., 2004). The mouse model of one of the Artemis mutations that truncate its C-terminal region showed that this region participates in both V(D)J recombination and DNA repair (Huang et al., 2009). Moreover, recently this mutation was also demonstrated to cause aberrant intra- and interchromosomal V(D)J joining events (Jacobs et al., 2011). So far, DNA-PKcs has been shown to interact with Artemis through the C-terminal region (Soubeyrand et al., 2006). 3 basal and 11 DNA-PKcs–mediated phosphorylation sites have been located in the C-terminal region, although the in vivo relevance of its phosphorylation and DNA-PKcs interaction is unclear (Ma et al., 2005; Goodarzi et al., 2006; Soubeyrand et al., 2006). The C-terminal region (amino acids 385–692) constitutes almost half of the Artemis protein, and it is encoded by single exon 14 (Poinsignon et al., 2004). Its sequence analysis suggests that it represents a novel protein domain within which there are regions of high conservation across numerous species. Nevertheless, the structure and direct function for this C-terminal region still remain elusive.

The protein–protein interaction analyses described in this study show that Artemis interacts with DNA Ligase IV (referred to as Ligase IV in the text). Ligase IV is an ATP-dependent DNA Ligase that plays critical roles in the development of the immune system, stem cell exhaustion, aging (Nijnik et al., 2007), and neural growth and development (Barnes et al., 1998; Gao et al., 1998). Targeted disruption of both the alleles of Ligase IV in a human pre-B cell line led to radiosensitivity and ablation of V(D)J recombination (Grawunder et al., 1998). Together, these studies define a direct and specific role of Ligase IV in both V(D)J recombination and DNA double-strand break repair.

Although published findings establish the clear relevance of the C-terminal region of Artemis in V(D)J recombination, they do not inform us on the roles that this region might have during V(D)J recombination and/or what are the relevant functions missing in its absence. The analysis that we present here attempts to fill this gap by demonstrating that the Artemis C-terminal region interacts with Ligase IV and that this interaction and the previously reported interaction of Artemis with DNA-PKcs are important for efficient coding joint formation. Both these interactions thus constitute a major function of the Artemis C-terminal region during V(D)J recombination. With these results, we can also speculate that loss of interaction of Artemis with Ligase IV may be one of the important molecular mechanisms for development of immunodeficiency in patients lacking the C-terminal region of Artemis.

RESULTS AND DISCUSSION

Artemis forms a complex with Ligase IV–XRCC4, and this interaction is independent of both DNA and DNA-PKcs

To investigate novel Artemis functions, we postulated that in addition to its reported interaction with DNA-PKcs, Artemis could interact with additional NHEJ (nonhomologous end joining) factors. Our initial experiments using overexpressed proteins in 293T cells combined with immunoblot analysis showed that Artemis interacted with the Ligase IV–XRCC4 complex. This interaction was further confirmed by mass spectrometric identification of proteins that coimmunoprecipitated with Artemis (not depicted). To investigate whether this association exists between endogenous proteins, anti-Artemis immunoprecipitations (IPs) were performed with the WT Nalm 6 and Ligase IV–deficient N114P2 human pre-B cell lines

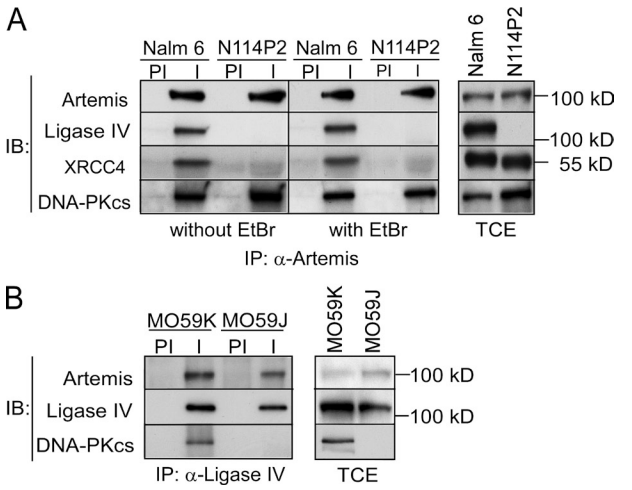


Figure 1. Artemis and Ligase IV form an endogenous complex that is independent of DNA and DNA-PKcs. (A) IPs were performed using Nalm 6 and Ligase IV–deficient N114P2 pre-B cell line lysates and polyclonal anti-Artemis antibody in the absence or presence of 200 µg/ml EtBr. After IP, proteins eluted from protein G beads were analyzed by immunoblot (IB) with anti-Artemis, anti–Ligase IV, anti-XRCC4, and anti–DNA-PKcs. The TCE controls are shown for the corresponding proteins in both the cell lines. PI, preimmune serum; I, anti-Artemis immune serum. (B) IPs were performed using M059K and DNA-PKcs–deficient M059J cell lysates and a mixture of polyclonal anti–Ligase IV antibody against amino acids 1-240 and affinity-purified anti–Ligase IV antibody against amino acids 711–911, in the presence of 200 µg/ml EtBr. After IP, proteins eluted from protein G beads were analyzed by immunoblot with anti-Artemis, anti–Ligase IV, and anti–DNA-PKcs. TCE controls are shown for the corresponding proteins in both cell lines. A representative IP of two independent experiments is shown. PI, preimmune serum; I, anti–Ligase IV immune serum.

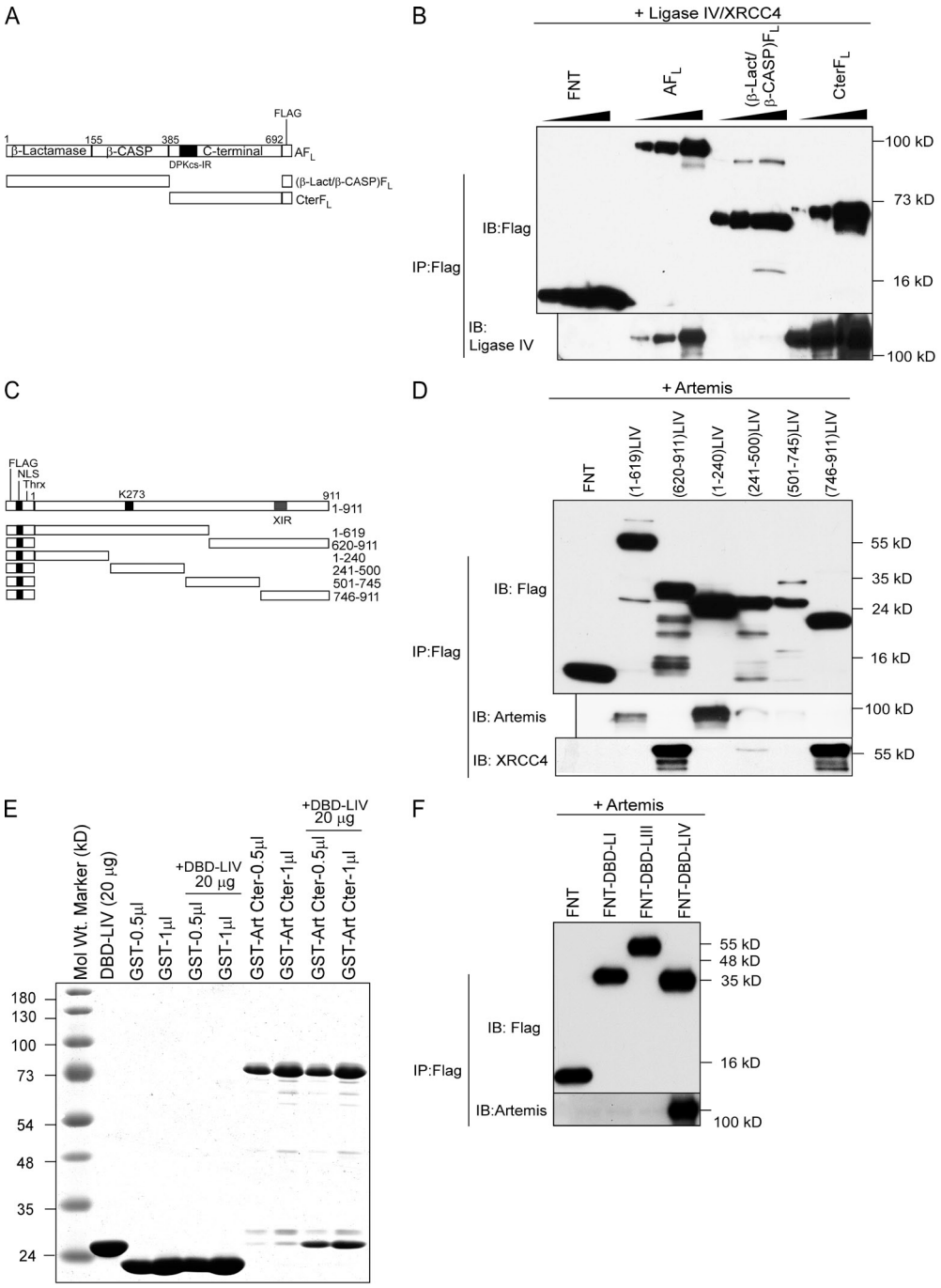


Figure 2. The C-terminal region of Artemis directly and specifically interacts with the DBD of Ligase IV. (A) Schematic representation of protein domains of Artemis. The DNA-PKcs interaction region (DPKcs-IR) is indicated. (B) 293T cells were transfected with FNT, full-length Artemis Flag (AF_L), N-terminal Artemis Flag (amino acids 1–385; (β-Lact/β-CASP)F_L), and C-terminal Artemis Flag (amino acids 386–692; Cter-F_L) with untagged full-length Ligase IV and XRCC4. Immunoblot (IB) analysis of Flag IP using anti-Flag and anti–Ligase IV is shown. (C) Schematic representation of FNT-tagged Ligase IV deletions. The XRCC4 interaction region (XIR) is indicated. (D) 293T cells were transfected with FNT or the FNT-tagged truncations of Ligase IV with nontagged full-length Artemis. Immunoblot analysis of Flag IP using anti-Flag, anti-Artemis, and anti-XRCC4 is shown. (E) GST or Gst–Art Cter was immobilized on glutathione beads and incubated with buffer alone or with the DBD of Ligase IV (DBD-LIV). Samples were washed thoroughly, eluted in denaturing sample buffer, separated on 10–15% SDS-PAGE, and visualized by Coomassie blue stain. The amount of DBD–Ligase IV used as input is shown. This analysis was repeated at least three times with freshly purified proteins. A representative experiment is shown. (F) 293T cells were transfected with FNT or the FNT-tagged DBD–Ligase I (amino acids 262–535), DBD–Ligase III (amino acids 1–390), and DBD–Ligase IV (amino acids 1–240) with nontagged full-length Artemis. Immunoblot analysis of Flag IP using anti-Flag and anti-Artemis is shown. Immunoblot analysis for B, D, and F was performed at least two times from independent transfections, and representative results are shown.

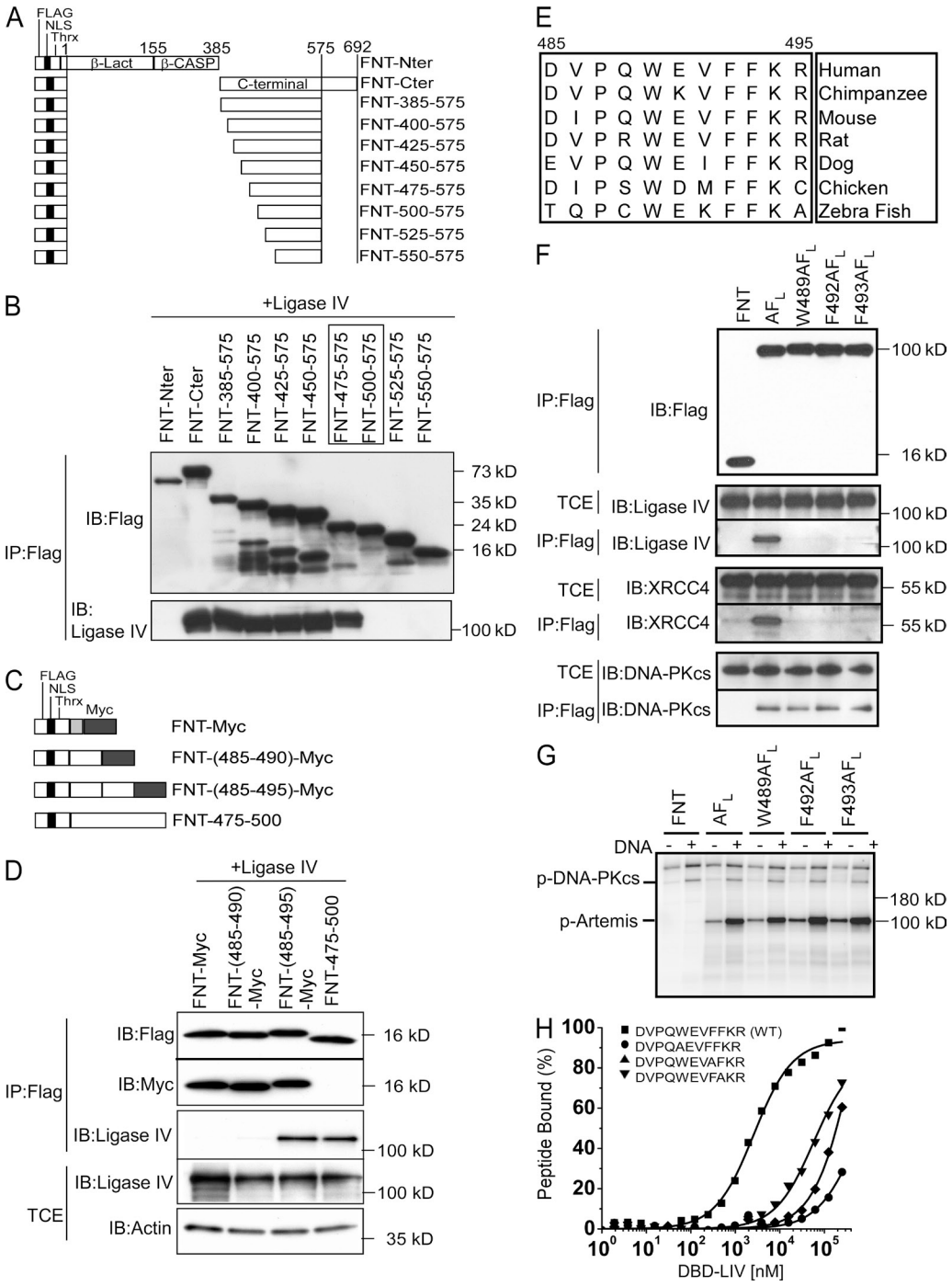


Figure 3. Amino acids W489, F492, and F493 within the Artemis C-terminal region are critical for its interaction with Ligase IV. (A) Schematic representation of FNT-tagged deletions of Artemis. (B) 293T cells were transfected with FNT-tagged N-terminal Artemis (FNT-Nter; amino acids 1–385), FNT-tagged C-terminal Artemis (FNT-Cter; amino acids 386–692), or FNT-tagged truncations of Artemis with untagged full-length Ligase IV. Immunoblot (IB) analysis of Flag IP using anti-Flag and anti–Ligase IV is shown. The region within Artemis important for interaction with Ligase IV is boxed. (C) Schematic representation of Artemis peptide constructs cloned between two tags: FNT and Myc to enable detection of expression. (D) 293T cells were transfected with FNT-Myc or Artemis peptide expression constructs with untagged full-length Ligase IV. Immunoblot analysis of Flag IP using anti-Flag, anti-Myc, and anti–Ligase IV is shown. (E) Amino acid sequence alignment of Artemis amino acids 485–495 (the minimal region required for interaction with Ligase IV) from seven different species. (F) Flag IP was performed on cell lysates of 293T cells transfected with FNT, Artemis Flag (AF_L), or its point mutants $W489AF_L$, $F492AF_L$, and $F493AF_L$. Immunoblot analysis for Flag, endogenous Ligase IV, XRCC4, and DNA-PKcs was performed. Levels of Ligase IV, XRCC4, and DNA-PKcs in TCEs are shown as controls. (G) In vitro phosphorylation assay was performed with equal amounts of Flag immunoprecipitated Artemis protein in the absence (−) or presence (+) of exogenous 35 bp DNA. 0.34 pmol DNA-PKcs was added to all the reactions. Phosphorylated

(Grawunder et al., 1998). Immunoblot analysis showed that Artemis coimmunoprecipitated with endogenous Ligase IV, XRCC4, and DNA-PKcs (Fig. 1 A). In the absence of Ligase IV (in N114P2 cells), Artemis lost its ability to interact with XRCC4 but maintained its previously described interaction with DNA-PKcs (Fig. 1 A). Furthermore, formation of the Artemis–Ligase IV complex was not mediated through DNA because co-IP of Ligase IV through Artemis remained unchanged in the presence of high levels of ethidium bromide (EtBr; 200 μg/ml; Fig. 1 A). Analysis of total cell extracts (TCEs) demonstrates that the levels of Artemis, DNA-PKcs, and XRCC4 were comparable in both the cell lines, and only Ligase IV was absent from N114P2 cells (Fig. 1 A, right). To test whether interaction of Artemis with Ligase IV is dependent on the presence of DNA-PKcs, we used the cell line M059K and its DNA-PKcs–deficient sister cell line M059J (Lees-Miller et al., 1995). In these cell lines, the endogenous Artemis–Ligase IV complex was observed upon IP through anti–Ligase IV antibody. The presence of the Artemis–Ligase IV complex was not affected by the absence of DNA-PKcs in the M059J cell line (Fig. 1 B). Altogether, the data presented in Fig. 1 demonstrate the formation of an endogenous complex of Artemis and Ligase IV and that Artemis's interactions with Ligase IV and DNA-PKcs are independent.

The C-terminal region of Artemis interacts with the DNA-binding domain (DBD) of Ligase IV in a direct and specific manner

The regions of Artemis and Ligase IV required for their interaction were mapped to the Artemis C terminus and the putative DBD of Ligase IV (by sequence homology with Ligase I; Fig. 2, A–D; Pascal et al., 2004). Direct interaction between the Artemis C terminus and DBD–Ligase IV was investigated by performing in vitro binding analysis using purified proteins. Glutathione *S*-transferase (GST) and GST-tagged Artemis C-terminal region purified from bacteria were immobilized on glutathione beads. The beads were then incubated with purified His-tagged DBD–Ligase IV, washed extensively, eluted, and visualized on a Coomassie-stained gel. Fig. 2 E shows that the Artemis C terminus directly interacts with the DBD–Ligase IV. No interaction was observed when the Ligase IV fragment was incubated with control GST beads, demonstrating that the interaction between the Artemis C-terminal region and DBD–Ligase IV is direct and specific. The DBDs of mammalian Ligases I, III, and IV are relatively conserved (Pascal et al., 2004). Therefore, it is possible that Artemis might also interact with the DBD of other Ligases. To explore this possibility, Flag-NLS-thioredoxin (FNT)–tagged DBDs of Ligase I (Pascal et al., 2004), Ligase III (Cotner-Gohara et al., 2008), and Ligase IV were investigated for their interaction with Artemis. These proteins were expressed with untagged Artemis in 293T cells, followed by anti-Flag IP and immunoblot analysis of the elutions for both Flag and Artemis (Fig. 2 F). Artemis coimmunoprecipitated only with DBD–Ligase IV and not with the DBDs of Ligase I or Ligase III. These data suggest that the interaction between Artemis and Ligase IV is highly specific.

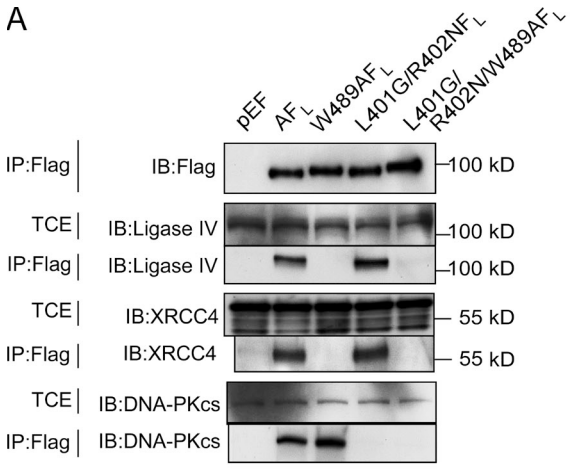
Amino acids W489, F492, and F493 of Artemis are critical for its interaction with Ligase IV

To delineate the relevance of Artemis and Ligase IV interaction in V(D)J recombination, we identified point mutations of Artemis that have lost their ability to interact with Ligase IV. Deletion analysis of Artemis' C-terminal region revealed amino acids 485–495 as the minimal region critical for this interaction (Fig. 3, A–E). Further mutagenesis identified three amino acids, W489, F492, and F493, that when mutated to alanine disrupted formation of the Artemis–Ligase IV complex. Flag-tagged mutants of these highly conserved amino acids, with WT Artemis as the positive and FNT as the negative control, were expressed in 293T cells; tagged proteins were immunoprecipitated, and the associated proteins were analyzed (Fig. 3 F). The three mutants interacted with DNA-PKcs equally well but failed to interact with Ligase IV and thus XRCC4. Control immunoblot on TCE shows equal levels of Ligase IV, XRCC4, and DNA-PKcs. Flag IPs were also analyzed for Artemis' ability to undergo phosphorylation by DNA-PKcs. Similar to WT Artemis, all of the three mutants underwent equal levels of phosphorylation in the presence of 35 bp DNA and exogenous DNA-PKcs (Fig. 3 G). To quantitatively evaluate the effect of Artemis mutagenesis on binding to DBD–Ligase IV in vitro, we examined the binding of DBD–Ligase IV with the Ligase IV–interacting human Artemis peptide (depicted in Fig. 3 E), both WT and point mutations, using fluorescence anisotropy, and the dissociation constant (K_d) was determined (Fig. 3 H). The WT peptide binds to the DBD–Ligase IV with a K_d of ~2.6 μM, whereas the extreme rightward shift of the binding curve with mutant peptides shows the significant abrogation in interaction when W489, F492, and F493 are mutated to alanines. This quantitative analysis confirmed our data that these single point mutations of Artemis disrupt interaction with Ligase IV but are not affected in their interaction or phosphorylation by DNA-PKcs (Fig. 3, F and G).

Loss of interaction of Artemis with Ligase IV and DNA-PKcs results in impaired V(D)J recombination

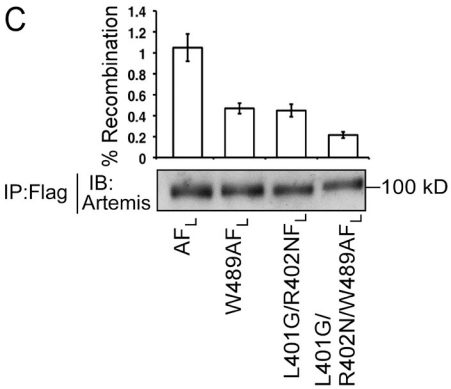
Before performing functional analysis, we confirmed that the three point mutants were expressed at levels comparable with or higher than WT Artemis (unpublished data). When analyzed

DNA-PKcs (p–DNA-PKcs) and Artemis (p-Artemis) were detected on PhosphorImager Storm 860 (Molecular Dynamics). (H) Artemis WT and mutant peptide binding to the DBD of Ligase IV (DBD-LIV) was determined by fluorescence anisotropy. The percentage of peptide bound is plotted versus DBD–Ligase IV concentration to determine the dissociation constants (K_d). The K_d value for binding of WT peptide is 2.6 μM. The mutant peptides did not reach saturation; thus, their K_d values could not be determined. The IPs and assays were repeated at least three times with proteins from independent transfections and/or preparations, and representative results are shown.



B

Conditions	Total Amp	Total Amp/ Cam	%R (CJ)	Avg %R	% of WT
pEF	83300	0	0		
pEF	74500	0	0	0	0
pEF	84000	0	0		
AF_L	85500	860	1.00		
AF_L	84600	800	0.94	1.05	100
AF_L	47000	565	1.20		
$W489AF_L$	79700	350	0.44		
$W489AF_L$	72800	335	0.46	0.48	45.5
$W489AF_L$	70000	375	0.53		
$L401G/R402NF_L$	66300	295	0.54		
$L401G/R402NF_L$	65000	260	0.44	0.45	43.4
$L401G/R402NF_L$	59200	310	0.40		
$L401G/R402N/W489AF_L$	46100	85	0.18		
$L401G/R402N/W489AF_L$	46000	100	0.22	0.21	20.5
$L401G/R402N/W489AF_L$	44600	110	0.25		



D

Conditions	Total Amp	Total Amp/ Cam	%R (SJ)	Avg %R	% of WT
pEF	214000	9467	4.4		
pEF	246000	17900	7.3	5.8	100
AF_L	261000	21600	8.3		
AF_L	190000	13600	7.1	7.7	132
$W489AF_L$	208000	17333	8.33		
$W489AF_L$	278000	14833	5.33	6.8	117
$L401G/R402NF_L$	214000	16900	7.9		
$L401G/R402NF_L$	337000	18533	5.5	6.7	115
$L401G/R402N/W489AF_L$	159000	12067	7.6		
$L401G/R402N/W489AF_L$	225000	14000	6.2	6.9	119

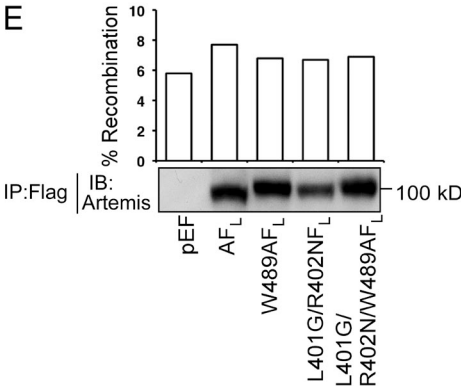


Figure 4. The C-terminal region of Artemis is crucial for efficient V(D)J recombination. (A) Flag IP was performed on cell lysates of 293T cells transfected with pEF, Artemis Flag (AF_L), or its point mutations $W489AF_L$, $L401G/R402NF_L$, and $L401G/R402N/W489AF_L$. Immunoblot (IB) analysis for Flag, endogenous Ligase IV, XRCC4, and DNA-PKcs was performed. Levels of Ligase IV, XRCC4, and DNA-PKcs in TCEs are shown as controls. Immunoblot analysis was performed at least two times from independent transfections, and representative results are shown. (B) The Artemis-deficient Nalm 6 pre-B cell line was transiently transfected with expression vectors encoding Artemis and its mutations along with the coding joint substrate pGG51 in triplicates, followed by plasmid DNA extraction. Coding joint (CJ) formation was determined by a colony formation assay in which the total number of Ampicillin-resistant (Amp) and Ampicillin- and Chloramphenicol-resistant (Amp/Cam) colonies were determined, followed by calculation of their ratio that is presented in the percent recombination (%R) column. (C) Histogram depicting the mean percent recombination achieved in the presence of WT Artemis and its mutations. The bottom panel shows Flag IP performed with one of the replicates followed by immunoblot analysis for Artemis. The error bars are for the triplicates shown in B. (D) The Artemis-deficient Nalm 6 pre-B cell line was transiently transfected with expression vectors for Artemis and its mutations along with the signal joint substrate pGG49 in duplicates, followed by plasmid DNA extraction. Signal joint (SJ) formation was determined by a colony formation assay in which total number of Ampicillin-resistant and Ampicillin- and Chloramphenicol-resistant colonies were determined followed by calculation of their ratio that is presented in the percent recombination column. (E) Histogram depicting the mean percent recombination achieved in presence of WT Artemis and its mutations. The bottom panel depicts Flag IP performed with one of the replicates followed by immunoblot analysis for Artemis. The IP and assays were repeated at least three times from independent transfections, and representative results are shown.

for V(D)J recombination with plasmid substrates (Gauss and Lieber, 1993), the three Artemis mutants (W489A, F492A, and F493A) showed a mild decrease (30–50%) in coding joint formation, but no effect on signal joint formation was observed (unpublished data). As discussed in the introduction, Artemis–DNA-PKcs interaction through the Artemis C-terminal region has been well established. A double point mutant of Artemis, L401G/R402N, was described that disrupts the Artemis–DNA-PKcs interaction (Soubeyrand et al., 2006). However, when this mutation was analyzed in Artemis-deficient fibroblasts, it showed WT levels of coding joint formation. We explored the possibility that an Artemis protein defective in interaction with both Ligase IV and DNA-PKcs could exhibit defects in coding joint formation in Artemis-deficient human pre-B cells. Artemis mutants L401G/R402N and W489A/L401G/R402N were generated and tested for their DNA-PKcs and Ligase IV interaction in 293T cells. As previously reported (Soubeyrand et al., 2006), L401G/R402N mutation showed no detectable DNA-PKcs interaction.

In contrast, the triple mutation of W489A/L401G/R402N lost interaction with both DNA-PKcs and Ligase IV, as predicted by our results and the published observations (Fig. 4 A; Soubeyrand et al., 2006). Analysis of the mutations W489A, L401G/R402N, and W489A/L401G/R402N in Artemis-deficient human pre-B cells (Kurosawa et al., 2008) showed that although the mutations W489A and L401G/R402N had a moderate but reproducible decrease in coding joint formation, the triple mutation exhibited a significant decrease in coding joint formation, showing only 20% of WT activity (Fig. 4, B and C). The mutations were expressed at levels comparable with Artemis WT (Fig. 4 C). Signal joint analysis of these Artemis mutations in pre-B cells revealed normal signal joint formation (Fig. 4, D and E). Furthermore, sequence analyses of the coding and signal joints did not show any significant differences between the WT Artemis and its mutants (not depicted). Together, the data reveal that the effect of loss of Artemis interaction with Ligase IV and DNA-PKcs is additive. The mutation of Artemis that failed to interact with both Ligase IV and DNA-PKcs showed a stronger defect in coding joint formation, suggesting that the roles of both these interactions, which are independent as shown in Fig. 1, may be different with respect to Artemis's function. Our results also suggest that Artemis-deficient lymphocytes represent a better cellular system when compared with fibroblasts to analyze the multiple roles of the Artemis C-terminal region. Altogether, these findings support the hypothesis that the Artemis C terminus contributes to efficient coding joint formation by interacting with both Ligase IV (in the context of the Ligase IV–XRCC4 complex) and DNA-PKcs. Through these interactions, Artemis might play a key role in bridging the processing and repair steps of V(D)J recombination and facilitating optimal processing and repair activities.

MATERIALS AND METHODS

Antibodies. Rabbit antibodies used are as follows: anti-Artemis against purified full-length recombinant Artemis, anti–Ligase IV raised against amino acids 1–240 and affinity-purified anti–Ligase IV raised against amino acids 711–911 (Przewloka et al., 2003), anti-XRCC4 (Serotec), and anti–DNA-PKcs (Santa Cruz Biotechnology, Inc.). Mouse monoclonal antibodies used are as follows: anti–DNA-PKcs (Santa Cruz Biotechnology, Inc.), anti–Flag M2 (Sigma-Aldrich), anti–c-Myc (Santa Cruz Biotechnology, Inc.), anti–β-Actin (Sigma-Aldrich), and secondary horseradish peroxidase (HRP)–conjugated monoclonal anti–rabbit RG-16 (Sigma-Aldrich). Secondary antibodies used are as follows: HRP-conjugated anti–mouse and anti–rabbit IgG (Thermo Fisher Scientific).

Cells and cell culture. Human pre-B cell lines Nalm 6 and N114P2 were obtained from M.R. Lieber (University of Southern California, Los Angeles, CA; Grawunder et al., 1998), and Artemis-deficient Nalm 6 cells were described previously (Kurosawa et al., 2008). Growth medium and conditions for 293T cells and human pre-B cells were as described previously (West et al., 2005). M059K and M059J human glioblastoma cell lines (Lees-Miller et al., 1995) were obtained from American Type Culture Collection (ATCC) and were cultured according to ATCC guidelines.

Vectors. Untagged human Ligase IV was a gift from T. Lindahl (London Research Institute, Cancer Research UK, London, England, UK), and untagged human XRCC4 was obtained from M.R. Lieber. Full-length Artemis cDNA was amplified from the HeLa cDNA library and cloned into pEF vector (Mizushima and Nagata, 1990). Artemis cDNA with Flag tag at its C-terminal end (AF_L) was generated using the following primers: forward, 5′-CGCGGATCCAGTTCTTTCGAGGGGCAGATGGCCGAGTATCCAACTATCTCC-3′; and reverse, 5′-TTTTCCTTTTGCGGCCGCTTATTTATCGTCATCGTCTTTGTAGTCGGCGGCGGTATCTAAGAGTGAGCATTTTC-3′. Artemis point mutants were generated in the AF_L vector with the QuikChange XL site-directed mutagenesis kit (Agilent Technologies) and were confirmed by DNA sequencing. For bacterial expression, the C-terminal region of Artemis (amino acids 386–692) was subcloned in pGex6p1 (GE Healthcare) to generate GST–Artemis C terminus (GST–Art Cter). DBD–Ligase IV (amino acids 1–240) was subcloned in pET15b (EMD) to generate His-DBD–Ligase IV.

Transfections and IPs. 293T cells were transfected using the calcium phosphate method. 48 h after transfection, the cells were harvested and extracted in Buffer A (25 mM Tris-HCl, pH 8.0, 150 mM KCl, 10% glycerol, 0.1% Triton X-100, 0.5 mM EDTA, and 1 mM DTT) with protease inhibitors as described previously, with modifications (West et al., 2005). Cellular extracts were incubated with 200 μg/ml EtBr at 4°C. The cell lysate (supernatant) obtained after centrifugation was used for Flag IP using anti–Flag M2–agarose beads (Sigma-Aldrich), and proteins were eluted with 0.2 mg/ml Flag peptide (Sigma-Aldrich) in Buffer C (25 mM Tris-Cl, pH 8.0, 150 mM KCl, and 20% glycerol; Ma et al., 2002). The immunoprecipitated proteins were separated on 4–20% SDS-PAGE followed by immunoblotting. IP from human pre-B cells was performed as follows: cell lysate from 2×10^9 cells was prepared as described for Flag IP above and divided into four samples (with or without 200 μg/ml EtBr), incubated overnight with 10 μl antibody (preimmune or polyclonal anti-Artemis serum) at 4°C. After incubation with protein G beads (GE Healthcare), proteins were eluted by denaturation in SDS-loading buffer. IP from M059K and M059J cells was performed as for human pre-B cells (using 30×10^6 cells per IP), except that a preclearing step with preimmune serum and protein G beads was used for all samples and the endogenous Artemis–Ligase IV complex was immunoprecipitated with a combination of two anti–Ligase IV antibodies (indicated under Antibodies). HRP-conjugated monoclonal anti–rabbit RG-16 was used as the secondary antibody for immunoblotting for samples from endogenous IPs.

Protein expression and purification. DNA-PKcs was purified from HeLa nuclear extracts using published protocols (Lees-Miller et al., 1992). For bacterial expression, BL21 Codon Plus cells (Agilent Technologies) were transformed with expression vectors described above (see Vectors). Purification of GST-tagged protein was performed with glutathione Sepharose 4B beads (GE Healthcare), and His-tagged protein was isolated with Ni^{2+} beads (QIAGEN). For His-DBD–Ligase IV, His tag was cleaved off from Ni^{2+} beads by thrombin protease (Thermo Fisher Scientific).

In vitro phosphorylation assay. DNA-PKcs–mediated phosphorylation was performed as described previously (Ma et al., 2002) using 0.34 pmol DNA-PKcs per reaction.

In vitro binding assay. Equal amounts of GST and GST–Art Cter, immobilized on glutathione beads, were incubated either alone or with 20 μg His-DBD–Ligase IV in a 500-μl reaction containing Buffer A (100 mM KCl), overnight at 4°C. Beads were washed five times and resuspended in 1× SDS loading buffer, separated on 10–15% SDS-PAGE, and visualized by Coomassie blue stain.

Determination of K_d by fluorescence anisotropy. The dissociation constant (K_d) was determined as described in Lone et al. (2007), with modifications. In brief, 7 nM 6-Carboxyfluorescein (FAM) N-terminal modified peptides were added to increasing concentrations of DBD–Ligase IV (0.1 nM to 250 μM), and the samples were left to equilibrate at 20°C for 30 min before

anisotropy values were measured. K_d value was calculated by fitting a model of single site binding to the data using the program Origin 6.1 (OriginLab). Fluorescently labeled peptides were purchased from Biosynthesis.

V(D)J recombination assay in pre-B cell lines. Human pre-B cell lines (Artemis-deficient Nalm 6; 20×10^6) were electroporated with 1 µg pGG51 or pGG49 plasmid (Gauss and Lieber, 1993) and 1 µg of the indicated control or Artemis expression vectors. 48 h after transfection, cells were harvested, and plasmid DNA was purified and digested with DpnI. Colony formation assay was performed as described previously (Hesse et al., 1987).

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