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Posted Date: 30 March 2026

doi: 10.20944/preprints202603.2331.v1

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Article

Processing of Mammalian Episomal Substrates by Hypomorphic Artemis Mutants and Role of DNA-PKcs Phosphorylation

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Abstract

Background: Inherited hypomorphic Artemis alleles have been identified in patients that cause combined immunodeficiency syndromes of varying severity. Characteristically, these are premature translation termination mutants (D451X, T432X, S385X; where X represents stop codon) resulting in either full or partial loss of C terminus. Functional evidence exists, suggesting a role of these hypomorphic mutants in impairing general double-strand break (DSB) repair bringing about genomic instability and protumorigenic chromosomal rearrangements; a discrete function outside of its canonical function in V(D)J recombination. Here, we characterize the effect of these mutants on episomal end-joining substrates in a model system for DSBs induced in a near physiological environment. **Results**: We employed replica plating assay to determine the effect on repair fidelity of end-joining upon overexpression of different Artemis variants. We found, markedly increase nuclease activity of the S385X (βCASP) mutant resulting in increase in number of episomes with truncations. Further, we sought to determine the effect of inhibition of DNA-PKcs phosphorylation (T2609 cluster) in regulation of episomal repair fidelity in cell lines expressing Artemis mutants. Upon inhibition of phosphorylation, we found out reduced number episomes with truncations resulting in increase in repair fidelity. **Conclusions**: Our work provides a novel venue to study the effects of Artemis mutants using an episomal system containing an inducible DSB. Our work indicates that the S385X deletion is more nucleolytically active probably because it is less regulated (lacks C terminal domain). We also provide evidence for an important role of DNA-PKcs in facilitating endonuclease activity of hypomorphic Artemis mutants.

Keywords: episomal model of NHEJ in mammalian cells; hypomorphic artemis mutants; DNA-PKcs T2609 phosphorylation; endonuclease activity, end-processing; repair fidelity

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1. Background

Non-homologous end joining (NHEJ) is the predominant form of DNA repair for double-strand breaks (DSBs) in mammalian cells [1]. Briefly, Ku (Ku70/Ku80 heterodimer) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are required in early stages of NHEJ for tethering and protection of DNA ends after DSB detection. While simple DSBs (blunt ended DSBs) do not require any processing before the ligation by XRCC4-XLF and DNA ligase IV, complex DSBs with non-ligatable ends require processing by specific enzymes before ligation. End processing involves resection of ends, removal of blocking groups, or fill in of gaps to create amenable ends for ligation. Several different proteins are implicated in resecting DNA ends for NHEJ; which includes endonucleases like Artemis and Metnase, WRN (RecQ helicase), PNKP-like factor (APLF) [2]. Artemis, as a nuclease, has been well characterized as a canonical component of mammalian NHEJ pathway. Artemis is composed of 692 amino acids (aa), of which 1- 385 residues constitute the N-terminal catalytic metallo β-lactamsae /β-CASP nuclease domain and 386 - 692 aa residues constitute

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the non-conserved C terminal regulatory domain. Artemis possesses 5′ – 3′ exonuclease activity on single strand DNA (ssDNA) and, in the presence of DNA-PKcs and ATP acquires endonuclease activity towards 5′/3′ overhangs as well as DNA hairpins [3]. Artemis was initially discovered as the gene inactivated in human radiosensitive T^-B^- severe combined immunodeficiency syndrome (RS-SCID), characterized by the absence of B and T lymphocytes and hypersensitivity to ionizing radiation [4]. Traditionally, Artemis function is primarily implicated in programmed DNA rearrangement i.e. V(D)J recombination, responsible for generation of functional diversity in B and T lymphocytes. In the context of general DNA DSB repair, Artemis is known to be required for the repair of only a subset of DNA-damage events *in vivo* [5].

Inherited hypomorphic Artemis alleles have been identified in patients that are shown to cause combined immunodeficiency syndromes of varying severity due to reduced number of B and T lymphocytes [4]. Characteristically, these are premature translation termination mutants (D451X, T432X, S385X; where X represents stop codon) resulting in loss of coding sequence within the C terminus (aa 386-692). Aggressive EBV-associated lymphomas are also observed in these patients along with partial immunodeficiency. To this end, elegant studies by *Huang et al.,* and *Jacobs et al.,* have highlighted the deleterious impact of hypomorphic Artemis allele (D451X/p70 allele) on genome integrity even when the wild type allele is present [6,7]. Intermediate hypersensitivity to DSBs has been observed in mouse embryonic fibroblasts (MEFs) expressing p70 allele with significantly increased genome instability as judged from increased number of chromosome and chromatid breaks. These results provide evidence that expression of hypomorphic Artemis alleles result in aberrant general DSB repair and subsequent chromosomal instability along with impairment of its canonical function in lymphoid-specific V(D)J recombination.

DNA-PKcs, both interacts and heavily phosphorylates Artemis C terminal domain and is found as a stable complex in cells [8–10]. Interestingly, these mutants (except β-CASP/S385X) retain the DNA-PKcs binding site (aa residues 401 and 402) but lack the multiple phosphorylation sites found on Artemis C terminal [7]. Although, *Ma et al.,* first proposed that activation of endonucleolytic activity of Artemis is dependent upon DNA-Pkcs mediated phosphorylation of Artemis C terminal [10,11], subsequently it was shown that at least *in vitro*, mutation of the DNA-PKcs phosphorylation sites in Artemis had no effect on its endonucleolytic activity. Rather, DNA-PKcs autophosphorylation has been proposed to facilitate endonuclease activity of Artemis [12]. So far, at least 60 different sites on DNA-PKcs have been identified to be phosphorylated by the kinase itself [13]. The two most recognized autophosphorylation clusters within the DNA-PKcs are ABCDE (T2609) cluster and PQR (S2056) cluster. Studies from several different groups have indicated the functional necessity and importance of T2609 cluster phosphorylation in regulating different aspects of classical NHEJ under physiological condition *in vivo* (for a review, refer [14]). In the context of NHEJ associated end processing, phosphorylation within the T2609 cluster has been shown to promote DNA end processing, whereas phosphorylation within the S2056 cluster has been shown to be inhibitory [15,16]. So far, effect of hypomorphic Artemis alleles on fidelity or kinetics of general end-joining repair and putative role of DNA-PKcs autophosphorylation in regulating Artemis endonuclease activity is yet to be fully validated in intact cells under physiological conditions.

In vitro DNA cleavage assays done with incubating model DNA substrates with several different purified Artemis C terminal truncated proteins have failed to give a clear picture. For example, *Niewolik et al.*, showed that Artemis truncation mutants alone can function in hairpin opening without DNA-PKcs as long as the putative inhibitory tail is removed but they require DNA-PKcs for overhang (5′/3′) cleavage activity [10]. However, *Huang et al.,* did not see any appreciable cleavage in absence of DNA-PKcs for any type of model substrates (hairpin, 5′ or 3′ overhang) [7]. The latter result is in agreement with *Goodarzi et al.,* who showed DNA-PK autophosphorylation rather than Artemis C terminal phosphorylation facilitates Artemis endonuclease activity as mentioned above [12]. When looked at *in vivo* effect of the C terminal truncated proteins on V(D)J recombination, *Huang et al.,* reported impaired V(D)J recombination within the endogenous antigen receptor loci in Artemis homozygous mutant (D451x/p70 protein) lymphocytes, but failed to see any difference in an assay

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based on transfection of V(D)J recombination competent model plasmid substrate [7]. We wanted to characterize the effect of these mutants on end-joining substrates in our novel episomal model system [17] in a physiologically relevant setting. We also aimed to check and see the effect of inhibition of DNA-PKcs phosphorylation (T2609 cluster) in modulation of repair outcome in cell lines stably overexpressing the Artemis C terminal truncated proteins.

2. Materials and Methods

2.1. Preparation of Stable Cell Lines Maintaining Episomal HO-CAT Plasmid

The 34 bp HO endonuclease target sequence (agatcttttagtttcagctttccgcaacagtata) was cloned in to the HindIII site of the pREP4/CAT shuttle vector just before the chloramphenicol acetyl transferase (CAT) coding sequence and the plasmid was renamed as HO-CAT plasmid. Further, HO-CAT plasmid was transfected in to 293T cells which maintain the plasmid episomal due to presence of EBNA-1, under hygromycin selection (Hygromycin B/Calbiochem, Cat # 400052). All the four expression constructs of Artemis with myc-6X His tag (WT, D37NMut, D451X, and S385X) were a kind gift from Dr. JoAnn Sekiguchi; University of Michigan, Ann Arbor. Stable cell lines expressing similar level of proteins for different Artemis variants along with empty vector control were generated in 293T cells under Blasticidin (Mediatech, Cat # 30-100-RB) selection and subsequently HO-CAT plasmid was transfected in to all of them. All the cell lines were cultured using DMEM with 10% FCS as growth medium at 5% CO2 and 37 °C, and selected using both Hygromycin and Blasticidin.

2.2. Assay for DNA Cleavage and Repair

In a typical assay, for each different time point, 150,000 cells were infected with the recombinant adeno virus encoding HO endonuclease (Gift from Dr. Hamish Young, Columbia University, NY, USA) at 10-30 MOI, referred to as pPF446::HO in a plasmid map in [18]. DNA from cells was collected using either Wizard SV genomic DNA purification system (Promega, Cat # A2360) or Wizard plus SV miniprep DNA purification (Promega,Cat # A1460) protocol. Equivalent amount of DNA was used in PCR and qPCR reactions for CAT and AMP regions using GoTaq DNA polymerase kit (Promega, Cat # M3001) and DynAmo Flash SYBR Green qPCR kit (Thermo Scientific, Cat # F-415S) respectively. 5′-CTACAACAAGGCAAGGCTTGACC-3′ and 5′-TCTAGTTGTGGTTTGTCCAAACTCATC-3′ were used as forward and reverse primers respectively for CAT amplicon. 5′-TTCCGTGTCGCCCTTATTCCC-3′ and 5′-GGCACCTATCTCAGCGATCTG-3′ were used as forward and reverse primers respectively for AMP region in PCR reactions. PCR conditions: 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 39 s with a final extension of 10 minutes at 72 °C. The CAT signal was normalized to AMP signal. For qPCR, 5′-GTACCAGCTGCTAGCAAGCT-3′ and 5′-TCAACGGTGGTATATCCAGTGAT-3′ were used as forward and reverse primers respectively for the CAT region (amplicon size 133bp). 5′-CATCGAACTGGATCTCAACAGCG-3′ and 5′-GTCATGCCATCCGTAAGATGCT-3′ were used as forward and reverse primers respectively for AMP region.

2.3. Immunoblotting

Protein samples from different time points were collected by RIPA lysis buffer (50mM Tris pH 8.0, 0.1% SDS, 1% Triton-X, 1mM EDTA, 150mM NaCl, and with 1X protease inhibitors – SIGMAFAST protease inhibitor cocktail tablets, Cat # S8820) and quantitated using BCA protein assay kit (Pierce, Cat # 23223). Equal amounts were run on a 6% polyacrylamide gel, blotted on to Immobilon-P (Millipore, Cat # IPVH08100), and subsequently probed. Either anti-myc (Santa Cruz Biotech., Cat # sc-40), anti-His (Thermo Scientific, Cat # MA1-21315), or anti-Artemis (Abcam, Cat # ab83309) was used to detect Artemis variants. Other antibodies used are anti-DNA-PKcs (Santa Cruz Biotech., Cat # sc-5282), anti-phospho T2609 DNA-PKcs (GenScript, Cat # A00494), anti–Ku70 (Santa Cruz Biotech., Cat # sc-5309). Stripping was done using Re-Blot plus mild (Millipore, Cat # 2502). The

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blot was either developed using Pierce ECL reagent (Thermo Scientific, Cat # 32106) or Opti-4CN (Bio-Rad, Cat # 170-8235).

2.4. NheI Screening and Bacterial Transformation

Plasmids recovered from each different time points were subjected to overnight NheI digestion (200ng in 20μL reaction, 5U of NheI) (Promega, Cat # R6501) which has its recognition sequence very close to HO induced cleavage site. Subsequently, the enzyme was heat inactivated and the digested miniprep DNA (all 20μL) was used to transform XL-1 Blue supercompetent cells (Agilent, Cat # 200236) and plated on an Ampicillin plate (100μg/mL). Random colonies were picked from each plate, minipreps were prepared, individual clones were linearized again with BstBI (ThermoScientific, Cat # ER0121-site near the pUC ori; distant site from NheI digestion site or HO cleavage site) and run on 1% agarose gel. Approximately 50 colonies were analyzed for each different cell lines in multiple independent experiments.

2.5. Replica Plating Assay

Plasmid DNA isolated from different cell lines at different time points was used to transform XL-1 Blue supercompetent cells and plated on Ampicillin plate (100μg/mL). The plate was incubated for 16h at 37 °C and subsequently replica plated on to a chloramphenicol plate (50μg/mL). Colonies were counted with Bio-Rad ChemiDoc (Cat # 170-8265) machine using Quantity-One software.

2.6. Preparation of Cell Extracts

1 million cells were used to prepare the whole cell extract. Cells were collected off the plate and washed once with cold 1X PBS. Subsequently, the cells were washed once with hypotonic buffer (25mM Tris pH 7.9, 1mM MgCl2, 0.4mM CaCl2, and 0.5mM DTT), spun down, and re-suspended again in 200μL of hypotonic buffer and kept in ice for 20 minutes. The cells were homogenized in a tight fitting Dounce homogenizer (30 strokes) and the debris was spun down by spinning at 13000 rpm for 10 minutes at 4 °C. The supernatant was collected, protein concentration was measured, and used in the DNA cleavage reaction.

2.7. Preparation of Radiolabeled Substrate

A ~ 320 bp of PCR product was generated from HO-CAT plasmid (CAT amplicon) with one of the primers containing a BamHI site. Subsequently, the PCR product was purified using Wizard SV Gel and PCR Clean-Up System (Promega, Cat # A9281) and digested with BamHI. Further, end-labeling was carried out with Klenow polymerase (Promega, M220A) in a 25μL reaction with 1μg of digested PCR product for 30 minutes at room temperature and dGTP-α^{32}P (Perkin Elmer, Cat # BLU514H250UC). Subsequently, the reaction was stopped by heating the mixture at 70 °C for 5 minutes. This substrate was used in *in vitro* DNA cleavage reactions.

2.8. In Vitro DNA Cleavage Assay

The reaction was carried out in a 10μL reaction mixture with 150ng of radiolabeled substrate, 10mM MgCl2, 50mM NaCl, 0.25mM ATP, and 1μg cell extract. The mixture was incubated at 37 °C for 1h. Then, the reactions were stopped by adding 5μL of stop solution (100mM EDTA, 1% SDS, 1mg/mL Proteinase K) and 3μL of 6x loading dye and incubated at 55 °C for 30min-1h. Then, the mixture was run in a 1% agarose gel.

2.9. siRNA Knock Down

DNA-PKcs was transiently knocked down in different cell lines using DNA-PKcs specific siRNA (Santa Cruz Biotech., Cat # sc-35200) using Lipofectamine-2000 (Life Technologies, Cat # 11668-019) as the transfection reagent (used according to manufacturer's instruction). Briefly, 200,000 cells were plated in a single well of a six well plate of the all the cell lines (293T, WT, D451X, and S385X). 24 hrs

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later, cells were transfected with either 100nM of DNA-PKcs siRNA or control siRNA. DNA-PKcs expression was checked after 48 hrs by immunoblotting.

3. Results and Discussion

3.1. Effect of Overexpression of Hypomorphic Artemis Mutants on Episomal Repair Fidelity in Mammalian Cells

We have recently characterized a novel episomal model system to accurately represent repair activities on extrachromosomal substrates in intact mammalian cells [17]. Briefly, in a typical cleavage and repair assay, a single site specific DSB was generated in the episomal population by infecting the cells with recombinant adeno-virus (Ad-HO) encoding HO endonuclease; which recognizes a 34 base pair (bp) sequence on episomes and produces a DSB with a 4 bp 3′ overhang (detailed description in [17]). The HO recognition site has been cloned into HO-CAT plasmid (maintained by the cells episomally) which was used as the end-joining substrate. Supp. Figure S1A (reproduced from [17]) depicts a map of the HO-CAT episome with important restriction sites. Episomes can be recovered from cells during a time course of cleavage and repair and can be analyzed using various assays (chloramphenicol replica plating assay/NheI digestion assay, see description in methods and [17]) to determine the repair fidelity. Cleavage kinetics of the episomes can also be assayed using either end point PCR or qPCR post infection, with a primer pair across the break site (CAT amplicon) (Supp. Figure S1B & S1C). Hence, to study effect of Artemis variants on episomal end-joining, we created four different stable cell lines overexpressing wild type Artemis (WT), endonuclease deficient Artemis (D37N), and two hypomorphic mutants namely D451X and S385X (β-CASP). All of these cell lines harbor HO-CAT episomes. For the sake of simplicity, we will refer to them as 293T (with empty vector), WT, D37N, D451X, and S385X highlighting the type of Artemis protein they express.

Figure 1A shows similar level of expression of different Artemis variants in their respective stable cell lines. Of note, the endogenous level of Artemis in 293T cells was found to be very low (Figure 1A inset), as has been reported for its parent cell line HEK293 cells [19], and hence it is safe to assume that the endogenous protein should not interfere with overexpression analysis in this study. To determine impact of overexpression of hypomorphic Artemis mutants on end-joining repair fidelity, we infected the cell lines (293T, WT, D37N, D451X, S385X) with Ad-HO virus to induce a site specific DSB in episomal populations. Episomes were collected at different time points post-infection (PI). qPCR assay was carried out to determine cleavage kinetics in different cell lines. As shown in Figure 1B, kinetics and efficiency of cleavage was very similar across different cell lines under consideration. Subsequently, chloramphenicol replica plating assay was carried out in different cell lines to analyze repair fidelity by determining the fraction of accurately repaired plasmids vs. plasmids with deletions. This assay was used as a screen to detect accurately repaired clones. Recovered plasmids at different time points PI were back transformed into supercompetent bacteria and plated on LB-Ampicillin. The HO site is positioned just before the chloramphenicol resistance marker (CAT) (approx. 35 bp away from the ATG start site) gene in the plasmid. So, inaccurate repair (deletions or insertions) would affect expression of CAT and result in loss of chloramphenicol resistance of that particular clone. This can be detected by replica plating the Ampicillin-bacterial plate onto chloramphenicol plate and looking for missing colonies. As expected, we observed more than 90% replica plating efficiency for episomes recovered from 293T cells and D37N (endonuclease deficient) cells confirming our previous observations [17]. Surprisingly, while the replica plating efficiency was around 75-80% for cells overexpressing WT Artemis, it was approximately 65% and 55% for cells expressing D451X mutant and S385X mutant respectively (Figure 1C). Further, to independently determine the number and nature of nucleotide rearrangements (deletions/insertions) in the inaccurately repaired clones, we digested the rescued plasmid population post-infection, with NheI. NheI has its recognition site located very close to the HO recognition sequence (refer plasmid map in Supp. Figure S1A). Accurately repaired plasmids would maintain the NheI site and get linearized upon NheI digestion and thus could not transform bacteria. NheI digestion assay was

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employed as a complementary assay to detect inaccurately repaired clones. As shown in Figure 2A, selection of rearranged (deleted) plasmids in this fashion, revealed distinct population of truncated plasmids from different cell lines upon transformation into bacteria. Again, the number of inaccurately repaired (deleted) clones obtained from D451X and S385X cell lines (suggested by difference in size upon linearization with BstBI, see methods) was higher in comparison to the ones expressing either WT or D37N Artemis variants, independently confirming results obtained in Figure 1B. Of note, some of the clones where the BstBI site was also lost migrated as multiple topoisomeric forms on the agarose gel. These results suggest a greater impact of hypomorphic Artemis mutants in reducing episomal end-joining repair fidelity for a DSB producing a 4 bp 3′ overhang, which primarily seemed to be happening by increased resection of DNA ends resulting in increased number of deleted plasmids. The effect is more pronounced with the S385X mutant in comparison to WT or D451X variant.

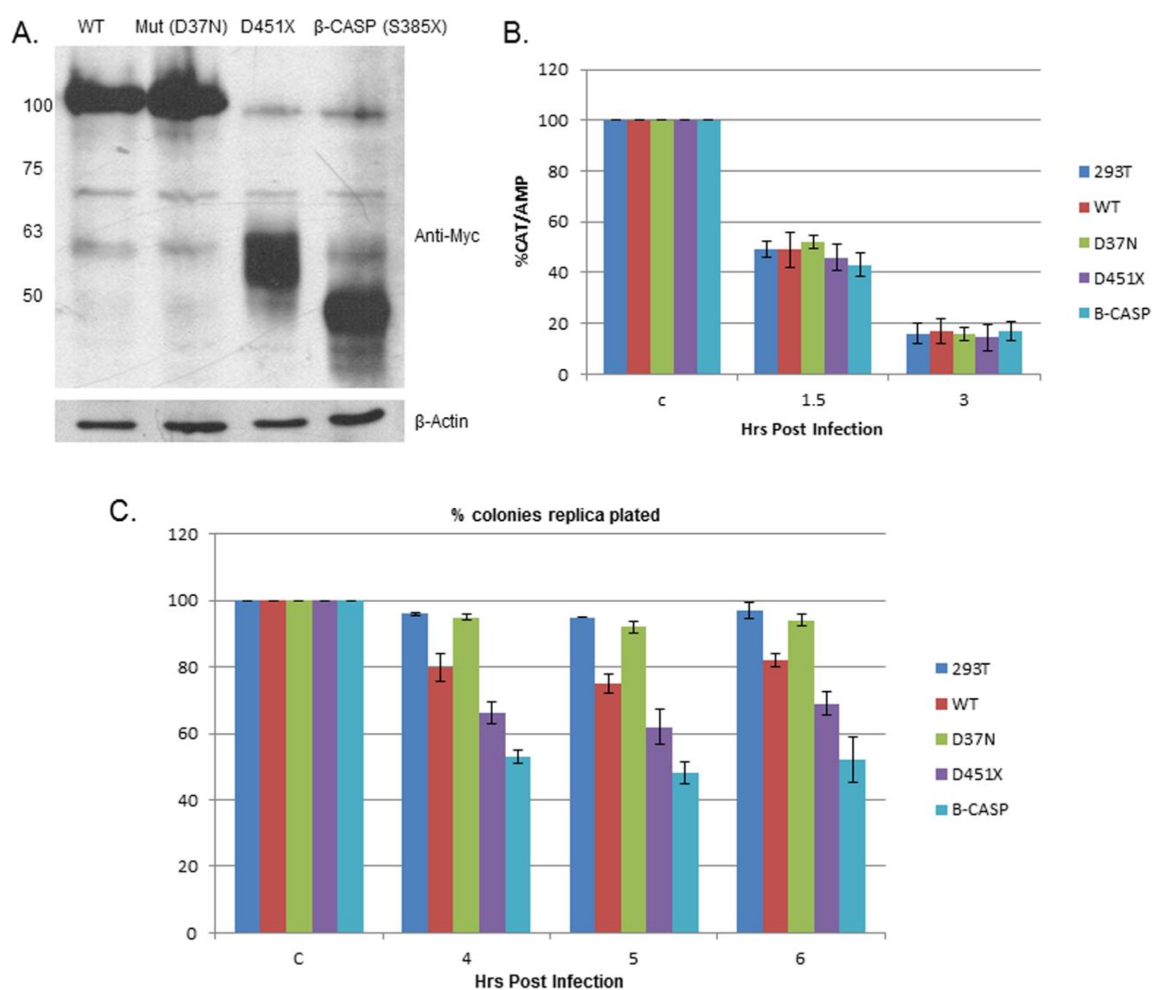

Figure 1. Effect of Artemis variant overexpression on episomal repair fidelity *ex vivo*. **(A)** Immunoblot depicting similar level of expression of different Artemis variants in their respective stable cell lines using α-myc antibody. Inset shows endogenous level of Artemis in 293T cells comparing the expression to stable WT-Artemis expressing cells using α-Artemis antibody. Approximate molecular weight (KDa) is shown on the left. **(B)** qPCR analysis of kinetics of episomal cleavage at the HO site DSB in different Artemis expressing cell lines (Details of plasmid map in Supp. Figure S1 and in [17]). Briefly, cells were infected with adeno-HO (Ad-HO) virus, episomes were recovered at different time points, and qPCR was performed by putting primers across the break site (CAT amplicon). AMP (Ampicillin) region on the episome acts as the positive internal control and used for normalization. In addition, a genomic product was also used to ensure equal total DNA recovery from the samples. qPCR values are represented as (CAT/AMP)% over control. **(C)** Figure showing replica plating

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efficiency (on to chloramphenicol plate) for episomes recovered at indicated time points post Ad-HO infection in cell lines expressing different Artemis variants.

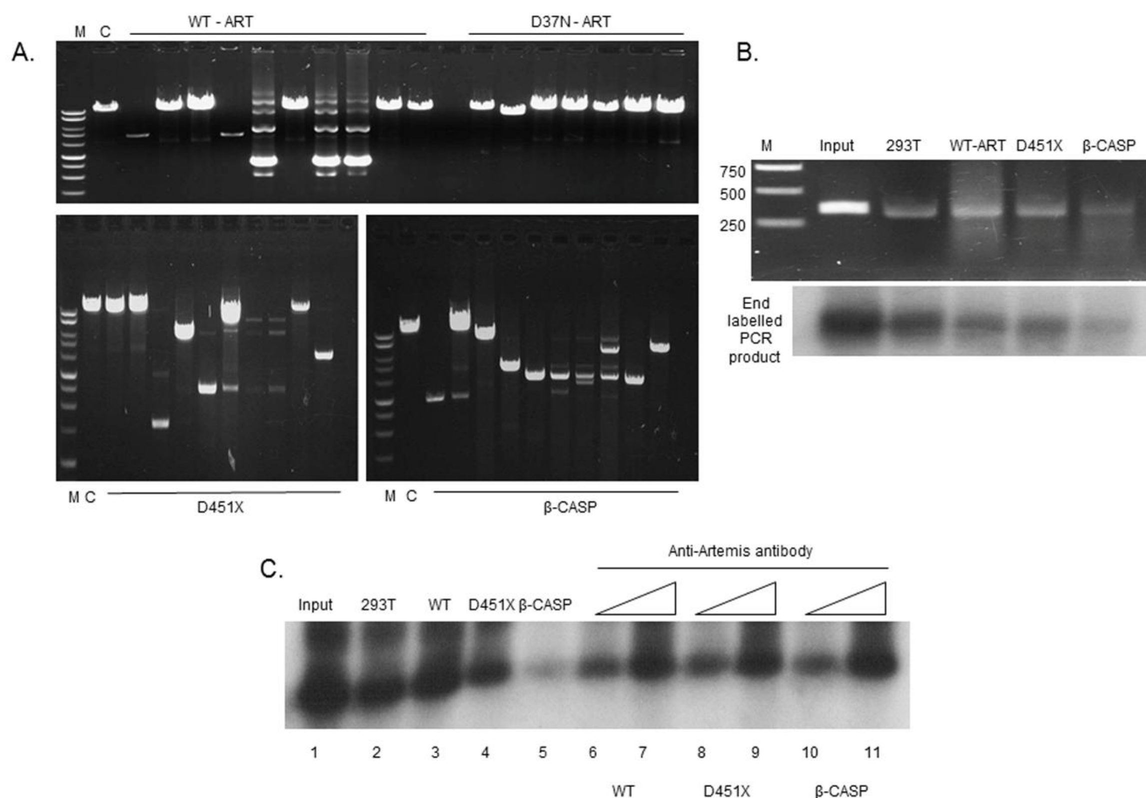

Figure 2. Analysis of nuclease activity of different Artemis variants. (A) Representation of randomly picked clones obtained from indicated cell lines after overnight NheI digestion of rescued plasmid population at 6h time point post Ad-HO infection. Accurately repaired clones were removed after NheI digestion thus enriching for inaccurately repaired clones (see methods for a detailed description). M denotes Promega 1 Kb DNA ladder, C denotes linearized HO-CAT plasmid. **(B)** *In vitro* DNA cleavage assay depicting loss of end-labeled (dGTP- α^{32}P) PCR product with 5′ overhang upon incubation with equal amount of whole cell extract (1µg) of indicated cell lines at 37 °C for 1h. Upper panel is the EtBr stained products running in 1% agarose gel. Lower panel depicts the image obtained after autoradiography exposure. **(C)** Lanes 1-5 depict cleavage of radio-labeled DNA substrate in whole cell extracts as shown in (B). Lanes 6-11 show cleavage in presence of anti-His antibody (against Artemis variants) at two different concentrations (Lanes 7, 9, 11; 2µL, Lanes 6, 8, 10; 0.5µL of 1µg/µL anti-His antibody).

3.2. Analysis of Nuclease Activity of Artemis Variants In Vitro

In vitro studies done with incubating model DNA substrates with purified proteins from different labs [7,11,12] have validated the requirement of DNA-PKcs and ATP for endonuclease function of Artemis. We wanted to validate our in vivo observations of increased end-resection in hypomorphic Artemis mutants in an in vitro assay employing a model substrate and cell extracts from cell lines overexpressing different Artemis variants. To that end, an end-labelled DNA substrate (with 5′ overhang) was incubated with equal amount of cell extracts (in presence of ATP) from all the cell lines. Disappearance of the radio-labelled substrate was measured as an indicator of nuclease activity. As shown in Figure 2B, we clearly observed a loss of the radioactive substrate after 1h incubation with S385X extract in comparison to input and other cell lines. To make sure that the observed effect is due to the activity of Artemis protein and not due to some contaminating exo/endo-nucleases, we performed a similar reaction including Anti-Artemis antibody. Figure 2C shows that,

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we could successfully inhibit the cleaving of the DNA substrate by including competing antibody against Artemis in the reaction [20]. This result further supports our observation that the loss of the radio-labelled substrate is due to the nuclease activity of the Artemis proteins and the activity of hypomorphic Artemis mutants (D451X and S385X) seemed to be markedly higher in comparison to WT Artemis.

3.3. Impact of DNA-PKcs Phosphorylation on Nuclease Function of Hypomorphic Artemis Variants

The autophosphorylation of DNA-PKcs has been shown to facilitate end-processing by full-length Artemis [12]. We wanted to determine whether phosphorylation of DNA-PKcs at T2609 plays any functional role in regulating the observed nuclease activity of these C terminal truncated Artemis mutants upon induction of DSB in our episomal model system. Of note, it has been shown previously that the mutants used in our study (D451X and S385X) and very similar ones (ARM 37 and ARM 23) used by *Niewolik et al*., have lost nearly all of the DNA-PKcs phosphorylation sites [7,10]. This presents us with a unique opportunity to gauge any putative effect of DNA-PKcs phosphorylation upon hypomorphic Artemis mutants' nuclease activity *in vivo*. Single strand breaks (SSBs) along with DSBs induced on plasmids have been shown to activate DNA-PKcs [21,22]. We wanted to determine whether a similar activation of DNA-PKcs occurred upon induction of a physiological DSB in episomal population in our system. 293T cells harboring the HO-CAT plasmid were infected with Ad-HO virus to induce the DSB. DNA-PKcs T2609 phosphorylation status was measured at different time points post-infection. As shown in Figure 3A, there is a basal level of T2609 phosphorylation in 293T cells even in absence of any induced episomal DSBs. A similar status for the S2056 phosphorylation on DNA-PKcs in 293T cells has been previously reported [23]. However, there is marked increase in the T2609 phosphorylation status at time points after Ad-HO infection which subsides upon treatment with wortmannin. We could not detect any appreciable change in DNA-PKcs T2609 phosphorylation upon Ad-HO infection of 293T cells lacking the episomes (data not shown), similar to our previous observation for ATM activation [24]. Wortmannin has been shown to be a potent DNA-PKcs kinase inhibitor at higher concentrations [25–27]. While NU7441 is a much more specific DNA-PKcs inhibitor, the inhibitory effect on T2609 phosphorylation was not optimum for our studies [26]. Hence, we used wortmannin instead and further verified our results with DNA-PKcs knockdown. We wanted to look at the effect of inhibition of DNA-PKcs T2609 phosphorylation upon episomal repair fidelity in presence of different Artemis overexpressing variants. To that end, we carried out a replica plating assay in presence of wortmannin at different time points post Ad-HO infection, as described before. The kinetics and efficiency of HO induced cleavage in different cell lines was very similar to the one presented in Figure 1B (data not shown). We observed a marked increase in replica plating efficiency (Figure 3B), particularly for the two hypomorphic mutants (D451X and S385X) upon wortmannin treatment in comparison to the untreated D451X and S385X cells, as shown previously (refer Figure 1C). NheI digestion assay was carried out to independently look at rearranged clones in rescued plasmid population. Figure 3C shows that the number of rearranged plasmids decreased dramatically for both D451X and S385X mutants upon wortmannin treatment in comparison to the vehicle treated control. Figure 3D represents the average replica plating percentages observed across different time points from multiple independent experiments carried out in presence or absence of wortmannin. Taken together, these results suggest strongly a major role of DNA-PKcs autophosphorylation (rather than DNA-PKcs mediated phosphorylation of Artemis) in determining the episomal repair fidelity. We cannot of course exclude that other phosphorylation substrates of DNA-PK mediated this result.

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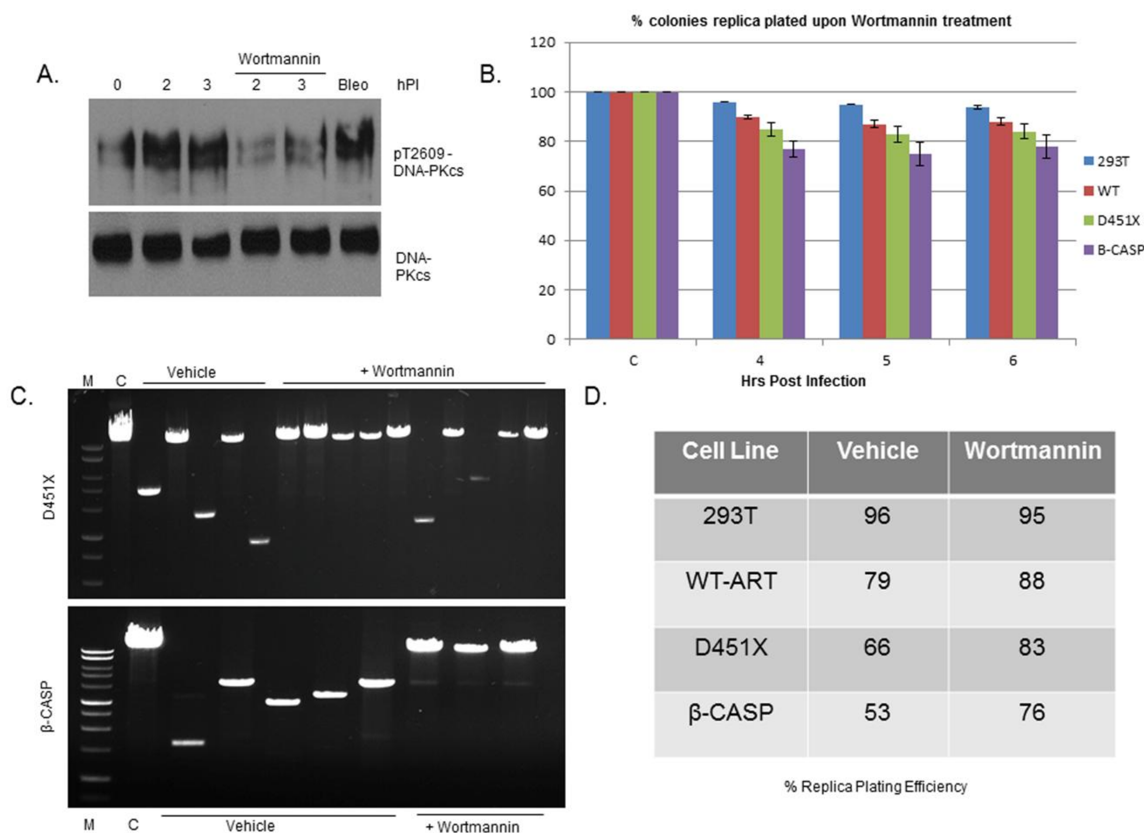

Cell Line	Vehicle	Wortmannin
293T	96	95
WT-ART	79	88
D451X	66	83
β-CASP	53	76

Figure 3. Effect of inhibition of DNA-PKcs autophosphorylation on nuclease activity of Artemis variants. (A) Immunoblot showing phosphorylation status of T2609 of DNA-PKcs upon Ad-HO infection in presence/absence of Wortmannin (30μM). Wortmannin was dissolved in 100% ethanol. 293T cells were infected with Ad-HO virus and cell lysates were prepared at indicated time points. Vehicle/wortmannin was added 1h before the Ad-HO infection and was not removed during the course of the experiment. **(B)** Figure showing replica plating efficiency of recovered plasmid population at indicated time points post Ad-Ho infection in different cell lines in presence of Wortmannin. **(C)** Representation of randomly picked clones obtained after NheI digestion (as described in Figure 2A) in presence of vehicle/wortmannin (6h post infection). **(D)** Table showing average replica plating efficiency in the mentioned cell lines obtained from three independent experiments.

The observation of heightened nuclease activity in case of the S385X mutant can be explained in light of the putative auto inhibitory role of Artemis C terminal towards Artemis function as an endonuclease, first proposed by *Ma et al.*, [11]. However, *NIewolik et al.*, and *Huang et al.*, both did not observe any significant difference between the cleaving pattern of the C-terminal truncated mutants D451X and S385X [7] or ARM 37 and ARM 23 [10] in absence of DNA-PKcs. Only when DNA-PKcs was added to the reaction, both groups have reported an increase in endonuclease activity for the shorter Artemis construct (S385X/ARM 23) at least *in vitro*. Taken together, these results clearly hint towards a role of DNA-PKcs in facilitating access of the nuclease for the DNA ends, as suggested by *Goodarzi et al*, [12]. Our *in vivo* observation of increased replica plating efficiency upon wortmannin treatment (Figure 3B) may be an outcome of inhibition of access of Artemis to DNA ends because of lack of DNA-PKcs phosphorylation in ABCDE cluster resulting in significantly reduced end resection. This is in agreement with a previous report, where in a transient V(D)J recombination assay, there was remarkably reduced loss of nucleotides at the site of coding joints in reactions involving a DNA-PKcs ABCDE cluster mutant (six phosphorylation sites were mutated to alanine) [12,28]. ABCDE mutant has also been shown to block access to a variety of other enzymes [29,30]. Recently, *Lee et al.*, have shown that a functional T2609 cluster along with ATM was important to

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promote coding end joining during V(D)J recombination *in vivo*, further highlighting the functional role of DNA-PKcs T2609 phosphorylation, in context of regulating Artemis activity [31].

ATM kinase has been largely implicated in DNA-damage induced phosphorylation of Artemis *in vivo* [32,33]. We wanted to see whether ATM can substitute for DNA-PKcs in facilitating access to DNA ends in our episomal model system. We have previously shown ATM activation on our model system upon induction of DSB (Supp. Figure S1 in [17]). To this end, in a transient transfection assay, we knocked-down DNA-PKcs in all the respective cell lines to a similar level (Supp. Figure S2) and looked at the replica plating efficiency of rescued plasmid population recovered after 6h post Ad-HO infection. We observed a very similar replica plating efficiency as was observed upon wortmannin treatment (Figure 3B). This result suggests that ATM/ATR cannot substitute for a role of DNA-PKcs in modulating access of Artemis nuclease to DNA ends. Our observation is also in agreement with similar findings by *Niewolik et al.*, [10]. It was not possible for us to determine whether Artemis recruitment to DNA ends was affected upon DNA-PKcs knock-down in our system. However, we presume it not to be the case because of the following observations reported by different labs. Different C terminal Artemis truncations were found to be localized in nucleus in DNA-PKcs deficient CHO-V-3 cells indicating that DNA-PKcs may not be important in nuclear recruitment of Artemis [10], even if it is largely believed that the functional state of Artemis is as a complex with DNA-PKcs in cells [8,9]. Also, *in vitro* assays done with purified Artemis C terminal truncated proteins showed, *albeit* reduced level of cleavage of DNA substrate in absence of DNA-PKcs [7,11]. Similarly, ARM 23 mutant used by *Niewolik et al.*, which does not bind DNA-PKcs, was able to restore cell survival in Artemis-deficient human primary skin fibroblasts after γ-irradiation similar to the level of wild type Artemis [10]. Taken together, these results hint towards existence of a back-up mechanism for recruitment of Artemis protein to DNA damage sites independent of a complex with DNA-PKcs. To this end, it is worthy to mention that in a previous cellular study, DNA-PKcs-Artemis association appeared to be dispensable. Mutation of DNA-PKcs binding site on Artemis did not affect V(D)J recombination of an integrated substrate in comparison to WT Artemis [34].

Conclusions

In this study, we characterized the cleavage activity of hypomorphic Artemis mutants in our episomal model system *ex vivo* and presented evidence for a functional role of DNA-PKcs T2609 phosphorylation in facilitating cleavage of episomal substrates by hypomorphic Artemis mutants**.** Our study explored the consequence of inhibition of DNA-PKcs phosphorylation upon cleaving activity of Artemis C terminal truncation mutants in a physiological setting where the chromatinized DNA is in its near natural state unlike previous studies where non-physiological level of damage was introduced by use of DNA damaging drugs or ionizing radiations.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Figure S1. Typical episomal cleavage and repair assay (Reproduced as described in [17]). (A) Map of the pRep4-HO-CAT plasmid showing key features. HO recognition cassette is cloned in just before CAT gene. Arrows indicate location of the primers. (B) Episomal cleavage-and-repair assay at the HO site DSB during a time course of Ad-HO infection. T-HO-CAT cells were infected with adeno-HO (Ad-HO) virus, cells were collected at different time points, and episomes were recovered. PCR was performed by putting primers across the break site (CAT amplicon). AMP (Ampicillin) region on the episome acts as the positive internal control and used for normalization. In addition, a genomic product was also used to ensure equal total DNA recovery from the samples. (C) qPCR analysis for kinetics of cleavage and repair of epsiomes as explained in (B). qPCR values are represented as (CAT/AMP)% over control. (D) Same as in (C) but in presence of the ATM inhibitior-KU55933 (10μM). Supplementary Figure S2. Effect of DNA-PKcs knock down on nuclease activity of Artemis variants. (A) Immunoblot showing knock down of DNA-PKcs in the respective cell lines. Ku 70 was used as the loading control. (B) Figure showing replica plating efficiency of recovered episomes 6h post infection in different Artemis variant expressing cell lines with transiently knocked down DNA-PKcs.

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Competing interests: The authors declare that there are none with publication of this study.

Authors' contributions: AR and ADB conceived the study and wrote the manuscript. AR carried out the experiments. All authors have approved the final manuscript.

Acknowledgements: This work was supported by Feist-Weiller cancer center grant award to ADB and Carroll Feist pre-doctoral fellowship award to AR. The sponsor had no involvement in the conduct of this research. We thank JoAnn Sekiguchi; University of Michigan, Ann Arbor for her generous gift of Artemis expression constructs.

List of Abbreviations and Acronyms

AMP - Ampicillin
APLF – Aprataxin and PNK like factor
ATM - Ataxia telangiectasia mutated
ATR – Ataxia Telangiectasia and Rad3 related
CAT – Chloramphenicol Acetyltransferase
DDR – DNA damage response
DNA - PKcs- DNA dependent protein kinase catalytic subunit
DSB- Double-Strand Break
HO – Homothallic Endonuclease
h – Hour(s)
NHEJ – Non-homologous end Joining
PCR – Polymerase chain reaction
PI - Post Infection

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