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# Functions and Regulation of Human Artemis in Double Strand Break Repair

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**Abstract** Cells, which lacked the activity of the nuclease Artemis, retained approximately 10% of unrepaired double strand breaks (DSBs) at later timepoints after ionizing radiation. Ionizing radiation induced hyperphosphorylation of Artemis mainly by ATM and in ATM deficient cells to a minor extent by DNA PK. After induction of DSBs with modified ends by a high dose of calicheamicin γ1, Artemis was phosphorylated by DNA PK. The type of calicheamicin γ1-induced DSBs is likely to represent a subclass of DSBs induced by ionizing radiation. DNA PK-dependent phosphorylation of Artemis after treatment with DSB inducing agents increased the cellular retention of Artemis, maintained its interaction with DNA ends and activated its endonucleolytic activity. The following model is suggested: ATM-dependent phosphorylation of Artemis after ionizing radiation could prevent DNA PK-dependent phosphorylation and activation of undesired endonucleolytic activity at DSBs, which do not require endonucleolytic processing by Artemis. The Artemis:DNA PK complex could be involved in the repair of DSBs, which carry modified ends and are refractory to repair by otherwise lesion specific enzymes because of the presence of an inhibitory lesion in the opposite strand. J. Cell. Biochem. 100: 1346–1351, 2007. 

**Key words:** Artemis; ATM; DNA PK; calicheamicin γ1 induced DNA damage

Patients suffering from radiosensitive severe combined immunodeficiency (RS-SCID) or Athabascan SCID (SCIDA) carry mutations in the Artemis gene. Bone marrow and fibroblast cells of these patients were sensitive to γ-rays and were deficient in coding joint formation during V(D)J recombination. The latter lead to a premature arrest of T- and B-cell maturation and opportunistic infections due to the lack of acquired immunity [Cavazzana-Calvo et al., 1993; Nicolas et al., 1998; Moshous et al., 2001; Li et al., 2002]. The Artemis protein carries metallo-β-lactamase and βCASP domains at its amino terminus [Moshous et al., 2001]. These domains harbor the nuclease activity of Artemis required for the processing of hairpinned coding end intermediates during V(D)J recombination. In vitro, Artemis had single-strand specific 5′-3′ exonuclease activity. It gained structure specific endonucleolytic activity by binding to and phosphorylation by the kinase subunit DNA PKcs. The Artemis-DNA PKcs complex cleaved hairpin substrates, 5′ and 3′ overhangs and various other substrates containing single to double-strand transitions near the transition region. Based on these in vitro studies, it was suggested that the Artemis-DNA PKcs complex removed secondary DNA structures during the sequential ligation of DNA ends in the course of double strand break (DSB) repair [Ma et al., 2002, 2005]. In vivo, DSB induction and repair can be monitored by determining the number of focal protein aggregates of phosphorylated histone H2AX (γH2AX) by

Abbreviations used: 53BP1, Tumor protein p53 binding protein 1; ATR, Ataxia telangiectasia and RAD3-related protein; ATM, Ataxia telangiectasia mutated protein; βCASP, metallo-β-lactamase associated CPSF Artemis SNM1/PSO2; Chk1; Checkpoint kinase 1; Chk2, Checkpoint kinase 2; DNA PK, DNA-dependent protein kinase; DNA PKcs, catalytic subunit of DNA PK; LIG4, DNA Ligase IV; Mre11, Meiotic recombination 11 protein; Nbs1, Nijmegen breakage syndrome 1 protein; RNAi, RNA interference; UV, ultraviolet; XRCC4, X-ray repair, complementing defective, in Chinese hamster, 4 protein; XLF, XRCC4-like factor

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immunofluorescence. Artemis deficient cells retained approximately 10% of unrepaired DSBs at later timepoints after ionizing radiation (IR). This subpathway of Non-homologous end joining (NHEJ) additionally involved ATM, H2AX, 53BP1, Nbs1, Mre11, and DNA PK. Core NHEJ was normal in Artemis deficient cells and depended on LIG4-XRCC4-XLF/Cernunnos and Ku. DNA PKcs played a non-essential, facilitating role in this process [Riballo et al., 2004; Ahnesorg et al., 2006; Buck et al., 2006]. Based on these in vivo studies it was suggested that Artemis might be involved in the processing of a subset of non-ligatable ends in the chromatin context of DSBs [Riballo et al., 2004]. These could represent complex breaks comprising IR-induced single strand breaks (SSBs), DSBs, sugar damage, and base damage in close vicinity and/or breaks with modified ends.

## WHAT IS THE IN VIVO SUBSTRATE OF ARTEMIS?

Artemis deficient cells showed increased sensitivity to the DSB inducing agents calicheamicin $\gamma$1, bleomycin, neocarzinostatin or IR and all agents caused increased cellular retention of Artemis [Nicolas et al., 1998; Rooney et al., 2002, 2003; Drouet et al., 2006]. The latter suggests that Artemis is recruited to the DSB surrounding chromatin after DSB induction. Whereas IR damaged DNA randomly, calicheamicin $\gamma$1 induced DNA strand breaks in a sequence specific manner with preference for the sequence TCCT:ACCA [Henner et al., 1982; Zein et al., 1988]. Both types of treatments caused clustered DNA damage, which consisted of two or more closely spaced strand breaks, abasic sites, and oxidized bases [Dedon and Goldberg, 1992; Sutherland et al., 2000, 2002]. However, calicheamicin $\gamma$1 generated DSB in cellular DNA with a DSB/SSB ratio of 1:3, whereas X-rays caused DSBs at a DSB/SSB ratio of 1:20 [Elmroth et al., 2003]. Calicheamicin $\gamma$1 induced DSBs with 3′ protruding overhangs, most of which carried 3′ phosphoglycolate and 5′ nucleoside 5′ aldehyde residues (Fig. 1). Bifunctional reagents like calichamicin $\gamma$1 also caused protein–DNA and DNA–DNA crosslinks [Dedon and Goldberg, 1992; Friedberg et al., 2006]. IR induced a multitude of lesions including base damage, sugar damage, SSBs, DSBs, DNA–protein, and DNA–DNA crosslinks. The majority of IR-induced strand breaks had modified termini, which required further processing to become ligatable. These included 3′ phosphate and 3′ phosphoglycolate termini, fragmented sugars, and ends lacking the terminal base residue. Most of the 5′ ends retained phosphate groups [Friedberg et al., 2006]. Human apurinic/apyrimidinic endonuclease 1 (APE1) is the major 3′ phosphoglycolate processing enzyme in human cells [Parsons et al., 2004]. Approximately 80% of calicheamicin $\gamma$1-induced DSBs and 20–30% of breaks induced by $\gamma$ irradiation were not repaired by APE1 in vitro [Chaudhry et al., 1999]. It is likely that repair resistant, IR-induced breaks comprise clustered lesions consisting of an apurinic/apyrimidinic (AP) site and a closely opposed SSB like calicheamicin $\gamma$1-induced DSBs. The DSB inducing drugs bleomycin and neocarzinostatin also generated DSBs with 3′ phosphoglycolate residues on blunt and 3′ recessed or 3′ protruding termini, respectively [Dedon and Goldberg, 1992]. In vitro studies showed that phosphoglycolate residues at blunt or recessed 3′ termini were poor substrates for APE1, whereas phosphoglycolate residues at protruding 3′ termini were not repaired at all by APE1 [Suh et al., 1997]. Therefore, Artemis could be involved in the repair of DSBs, which carry modified ends and are refractory to repair by otherwise lesion specific enzymes because of the presence of an inhibitory lesion in the opposite strand. The fact that Artemis deficient cells retain approximately 10% of unrepaired DSBs after IR, suggests that Artemis is either involved in the processing of the lesions on both strands of the DNA duplex, or that repair of one lesion in an Artemis-dependent process is the prerequisite for repair of the lesion on the other strand (Fig. 1). Insights about the exact role of Artemis in the repair of the calicheamicin $\gamma$1 type of DSBs could be gained by the following assays: (i) in vitro analysis of the activity of Artemis on DNA cut by calicheamicin $\gamma$1 under physiological conditions, (ii) analysis of repair products generated in calicheamicin $\gamma$1 treated Artemis proficient and deficient cells, which carry a genome integrated calicheamicin $\gamma$1 substrate.

## REGULATION OF THE ACTIVITY OF ARTEMIS

Several recent publications addressed the question, how the activity of Artemis is regulated in vivo. Artemis was rapidly

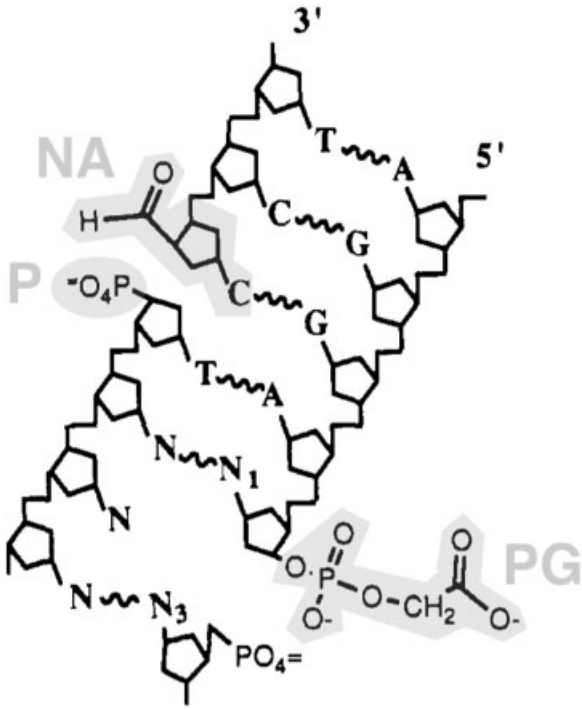


**Fig. 1.** DSBs caused by calicheamicin γ1 are a putative Artemis substrate. Calicheamicin γ1 induces DSBs comprising an AP site and a closely opposed SSB. DSBs have 3′ protruding overhangs and carry modified ends. The latter are shown in gray: 3′ phosphoglycolate (PG), 5′ nucleoside 5′ aldehyde (NA) and 3′ phosphate (P) [Dedon and Goldberg, 1992]. This type of lesion is refractory to repair by the 3′ phosphoglycolate removing enzyme APE1 [Suh et al., 1997; Chaudhry et al., 1999; Parsons et al., 2004]. After phosphorylation by DNA PK, Artemis could be either involved in the processing of the lesions on both strands of the DNA duplex, or repair of one lesion in an Artemis-dependent process could be the prerequisite for repair of the lesion on the other strand. After repair of the lesions on the left hand side in an Artemis-dependent process, the lesion on the right hand side could serve as substrate for APE1 [Suh et al., 1997]. However, in vitro assays by Povirk et al. [2006] suggest that the repair of DSBs induced by calicheamicin γ1, neocarzinostatin or bleomycin could involve trimming of 3′ phosphoglycolate ends by Artemis with low efficiency. Figure after Dedon and Goldberg [1992], modified. Part of figure was reprinted from Dedon and Goldberg [1992] with permission of the American Chemical Society (copyright 2006).

hyperphosphorylated to varying extents after IR and other DNA damaging agents like UV, camptothecin, etoposide, and nitrogen mustard [Zhang et al., 2004; Wang et al., 2005]. Artemis interacted in vivo with the phosphatidylinositol-3 kinase-like protein kinases (PIKKs) DNA PK and ATM and was basally phosphorylated in cultured cells in a PIKK-dependent manner [Ma et al., 2002; Poinsignon et al., 2004; Riballo et al., 2004; Zhang et al., 2004; Chen et al., 2005; Wang et al., 2005; Soubeyrand et al., 2006]. In response to treatment with IR Artemis was hyperphosphorylated mainly by ATM in a Nbs1-dependent manner. In ATM deficient cells hyperphosphorylation of Artemis by other kinases including DNA PK was observed to a small extent after higher doses of IR [Poinsignon et al., 2004; Riballo et al., 2004; Zhang et al., 2004; Chen et al., 2005; Goodarzi et al., 2006]. In ATM proficient cells ATR, DNA PK, Chk1, Chk2, and 53BP1 were not involved in the hyperphosphorylation of Artemis after IR cells as far as measurable by differences in electrophoretic mobility [Riballo et al., 2004; Zhang et al., 2004; Chen et al., 2005]. ATR mediated the hyperphosphorylation of Artemis after UV irradiation [Zhang et al., 2004].

In unirradiated cells, Artemis was basally phosphorylated at S516 and S645. After treatment with the DSB inducing drug bleomycin phosphorylation of Artemis at S516 and S645 was enhanced 10- to15-fold [Soubeyrand et al., 2006]. After IR Artemis was hyperphosphorylated at S645 and at additional sites in an ATM-dependent manner. In vitro studies showed that ATM phosphorylated Artemis at S503, S516, and S645 [Poinsignon et al., 2004; Chen et al., 2005; Goodarzi et al., 2006]. Co-immunoprecipitation experiments by Chen et al. [2005] showed an irradiation-dependent, dose-dependent, and ATM-dependent interaction of endogeneous Artemis with endogeneous Nbs1. Additionally, with tagged overexpressed Artemis, the authors could co-immunoprecipitate Nbs1 from unirradiated cells and increasing amounts of Nbs1 after increasing doses of IR [Chen et al., 2005]. This suggests a direct or indirect interaction of Artemis with Nbs1, which is enhanced by DNA damage. In vitro, the Mre11-Rad50-Nbs1 (MRN) complex stimulated the activity of ATM towards its substrates by enhancing ATM-substrate interactions [Paull and Lee, 2005]. In vivo, the hyperphosphorylation of Artemis after IR was dependent on Nbs1 [Chen et al., 2005]. Nbs1 is also an ATM phosphorylation target after IR [Gatei et al., 2000]. In vitro, the phosphorylation of Artemis at S645 by ATM was dependent on the presence of purified MRN complex, but was not further enhanced by the presence of DNA substrates [Goodarzi et al., 2006]. In vivo, Nbs1 formed irradiation-induced foci (IRIF) at the DSB surrounding chromatin and recruited activated ATM to sites of DSBs [Falck et al., 2005]. Therefore, Nbs1 could

function as a recruitment factor for Artemis to the DSB surrounding chromatin and enhance its IR-induced hyperphosphorylation by ATM. However, analysis of multisite phosphorylation mutants showed that Artemis hyperphosphorylation by ATM was dispensable for its endonuclease activity in vitro and for DSB repair and V(D)J recombination in vivo [Goodarzi et al., 2006]. The latter in vivo studies were performed with Artemis deficient cells complemented with non-phosphorylatable or phosphorylatable overexpressed, tagged Artemis. Overexpression of a tagged Artemis protein might override the requirement for ATM-dependent phosphorylation of Artemis in order to achieve recruitment of the protein to the DSB surrounding chromatin, as was already suggested by the co-immunoprecipitation of Nbs1 with overexpressed tagged Artemis from extracts of unirradiated cells [Chen et al., 2005]. Additionally, γH2AX analysis gives insights into the kinetics of DSB repair, but not into its quality. Although ATM-dependent phosphorylation of Artemis was not required for normal DSB repair kinetics, it might influence the quality and accuracy of DSB repair. In ATM deficient cells, DNA PK phosphorylated only a small fraction of the cellular Artemis pool after IR [Goodarzi et al., 2006]. ATM-dependent phosphorylation of Artemis after IR could prevent DNA PK-dependent phosphorylation and activation of undesired endonucleolytic activity at DSBs, which do not require endonucleolytic processing by Artemis.

IR treatment was found to enhance the interaction between DNA PKcs and Artemis [Zhang et al., 2004; Chen et al., 2005]. Zhang et al. [2004] observed a consistent decrease of Artemis protein levels upon depletion of DNA PKcs by RNAi. This suggests that Artemis protein stability is increased by the interaction of Artemis with DNA PKcs. However, Drouet et al. [2006] did not observe decreased levels of Artemis in DNA PKcs deficient MO59J glioblastoma cells. These authors also reported that the kinase activity of DNA PKcs is required for the hyperphosphorylation and retention of Artemis in a less extractable cellular protein fraction after treatment of cells with a high dose of the DSB inducing agent calicheamicin γ1 (10 nM). Treatment of cells with the DNA PKcs inhibitor NU7026 abolished the hyperphosphorylation and cellular retention of Artemis after DSB induction. In vitro assays showed that the kinase activity of DNA PKcs stabilized the interaction of Artemis with DNA PKcs and DNA ends in the presence of Ku [Drouet et al., 2006]. This suggests that after DSB induction by calicheamicin γ1 Artemis was hyperphosphorylated by DNA PK, which is a prerequisite for its endonucleolytic activity. The cell's choice of the Artemis hyperphosphorylating kinase might be influenced not only by the nature of the induced DSBs, but also by the calicheamicin γ1 dose used. Calicheamicin γ1 was an efficient activator of DNA PK and ATM and induced high levels of H2AX phosphorylation [Elmroth et al., 2003; Mårtensson et al., 2003; Ismail et al., 2005]. γH2AX foci numbers in human osteosarcoma cells 6 h after treatment with 5–10 pM calicheamicin γ1 for 1 h were comparable to γH2AX foci numbers after 4 Gy γ irradiation. Cells treated in this way also showed comparable cell viability [Farkash et al., 2006]. High doses of 1–10 nM calicheamicin γ1 as used by Drouet et al. [2006] lead to efficient phosphorylation of ATM substrates in ATM deficient cells by other kinases [Ismail et al., 2005]. Similarly, hyperphosphorylation of Artemis by other kinases including DNA PK was detectable in ATM deficient cells after higher doses of IR [Poinsignon et al., 2004; Riballo et al., 2004; Zhang et al., 2004; Chen et al., 2005; Goodarzi et al., 2006]. Autophosphorylation of DNA PKcs at T2609-2647 in the presence of Ku and target DNA was a prerequisite for Artemis endonuclease activity, but it was not required during the endonuclease reaction itself in vitro. Autophosphorylation of DNA PKcs is thought to cause a conformational change in DNA PK, which promotes access of Artemis to DSB break ends [Goodarzi et al., 2006]. Activated DNA PKcs could optimally configure 5′ and 3′ overhangs for the endonucleolytic function of Artemis [Niewolik et al., 2006].

In naive and irradiated cells, Artemis phosphorylated at S516 and S645 formed a similar pattern of nuclear foci, which were excluded from the nucleoli. Artemis foci in irradiated cells did not overlap with DSB associated γH2AX or 53BP1 foci [Soubeyrand et al., 2006]. This suggests that only a small subpool of Artemis is recruited to DSBs at a given time point and that the interaction might be transient.

The following model is suggested: ATM-dependent phosphorylation of Artemis after ionizing irradiation could prevent DNA PK-dependent phosphorylation and activation of

undesired endonucleolytic activity at DSBs, which do not require endonucleolytic processing by Artemis. The Artemis:DNA PK complex could be involved in the repair of DSBs, which carry modified ends and are refractory to repair by otherwise lesion specific enzymes because of the presence of an inhibitory lesion in the opposite strand.

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