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TITLE: Deciphering the Role of Alternative nonhomologous End Joining (Alt-NHEJ) DNA Repair in Breast Cancer

PRINCIPAL INVESTIGATOR: Pedro A. Mateos-Gomez

CONTRACTING ORGANIZATION: New York University
New York, NY 10016

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Deciphering the Role of Alternative nonhomologous End Joining (Alt-NHEJ) DNA Repair in Breast Cancer

Pedro A. Mateos-Gomez
E-Mail: pedro.mateosgomez@med.nyu.edu

New York University School of Medicine
540 1st Avenue
New York, NY 10016-6402

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Approved for Public Release; Distribution Unlimited

The alternative non-homologous end-joining (NHEJ) machinery facilitates several genomic rearrangements, some of which can lead to cellular transformation. This error-prone repair pathway is triggered upon telomere de-protection to promote the formation of deleterious chromosome end-to-end fusions. We showed that Polq inhibition suppresses alternative NHEJ at dysfunctional telomeres, and hinders chromosomal translocations at non-telomeric loci. In addition, we found that loss of Polq results in increased rates of homology-directed repair (HR), evident by recombination of dysfunctional telomeres and accumulation of RAD51 at double-stranded breaks. Lastly, we showed that depletion of PolQ had a synergistic effect on cell survival in the absence of BRCA genes, suggesting that the inhibition of this mutagenic polymerase represents a valid therapeutic avenue for tumors carrying mutations in homology-directed repair genes. Here we report that PolQ inhibition can be used to increase the efficiency of CRISPR targeting. Function-Structure analysis of PolQ indicated that the helicase and polymerase domains are relevant for its activity, in contrast deletion of the RAD51 interaction motif did no have an impact in translocation frequency, RAD51 foci formation or survival of BRCA1 depleted cells. Finally, PolQ and RPA interplay at DSB to promote A-NHEJ or HR respectively.
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1. **Introduction:**

DNA damage and particularly double strand breaks (DSB) are highly dangerous for cells, improper repair of those lesions contributes to the accumulation of mutations and genomic instability, which can lead to cellular transformation and cancer progression. DSBs are repaired by three different mechanisms, the Homologous Recombination (HR; error-free), the Classical Non-Homologous end-joining (C-NHEJ; minimally error-prone) and the Alternative-NHEJ (A-NHEJ; highly error-prone). In contrast to the former two the last one is poorly studied, only few factors involved in the A-NHEJ are already known. In recent years, a number of evidences for its implication on generating genomic instability in tumor cells have been found, including HR deficient tumors of the breast. Interestingly, it has been shown that tumors deficient in HR are sensitive to PARP1 inhibition, a factor required for the A-NHEJ. The mechanism under this sensitivity is not fully understood and resistance to these inhibitors has been reported. My hypothesis is that HR deficient tumors are dependent on the A-NHEJ DNA repair pathway for its survival. Therefore, cell death due to PARP1 inhibition is at least in part due to blocking of the A-NHEJ, what would drive the accumulation of improperly or unrepaired breaks. Inhibition of other relevant factors for the A-NHEJ could have the same impact on the survival of breast tumors that relies on this mechanism. Altogether, understanding the underlying basis of A-NHEJ and its impact of breast cancer progression should provide new insights that can be translated into the clinic. To that end I will pursue the following three aims: 1- establish the function of the A-NHEJ in inherited and sporadic breast cancers; 2- elucidate the function of PolQ in the A-NHEJ and in breast cancer progression; 3- define the molecular mechanism of alt-NHEJ.

2. **Keywords:**

- DNA double strand break, DSB.
- Alternative non-homologous end-joining, A-NHEJ.
- Classical non-homologous end-joining, C-NHEJ.
- Homologous Recombination, HR.
- DNA Polymerase theta, PolQ.
- Breast cancer.
- Breast cancer associated gene 1 and 2, BRCA1 and BRCA2.
- Telomeres.
- Poly-ADP-ribose polymerase 1, PARP1.
- Chromosomal translocation.
- Chromosomal aberrancies.
- Chromosomal fusions.
- CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats.
- DNA ligase 3 and 4
- Ku80
• Mouse embryonic fibroblast, MEF.
• Mouse embryonic stem cells, mESC.

3. **Accomplishments:**

**Major goals of the project:**

1. Characterize alt-NHEJ in cell lines and patient samples.
   Completion percentage: 10%

2. Elucidate the function of PolQ in A-NHEJ and its relevance to breast cancer progression.
   Completion percentage: 90%

3. Screen for factors involved in the A-NHEJ.
   Completion percentage: 5%

**Accomplished under these goals:**

1. Characterize alt-NHEJ in cell lines and patient samples:

   **Subtask 1:** The first part of this aim was to determine whether several breast cancer cell lines significantly use the A-NHEJ repair pathway. We have already shown (last year report and Mateos-Gomez et al. Nature 2015) the relevance of the A-NHEJ for the proliferation of two breast cancer cell lines, MCF-7 (HR proficient) and HCC1937 (BRCA1 and HR deficient). Our results indicated that the absence of HR was the reason for this cell line to require A-NHEJ, but it was not clear why an HR proficient cell line (MCF-7) was sensitive to PolQ depletion.

2. Elucidate the function of PolQ in A-NHEJ and its relevance to breast cancer progression:

   **Subtask 2.3:** since in subtask 2.2 we showed that HR deficient tumor cell lines are extremely dependent on PolQ for its survival, and that even when they are not treated with DNA damaging agents in combination with PolQ depletion, I decided to explore more deeply the mechanism by which PolQ acts.

   In subtask 2.1 I showed that PolQ promotes A-NHEJ while suppresses HR and I have analyzed the impact of PolQ on CRISPR targeting when HR is required to introduce the desired modification:
**Figure 1.** CRISPR targeting is enhanced with PolQ absence in a locus and Cas9 dependent manner. A) Flow cytometry analysis to quantify ZsGreen expression in mouse CCE embryonic stem cells (mESc) (CCE a mES cell line derived from 129/Sv mouse strain; Robertson E, Bradley A, Kuehn M, Evans M, Nature. 1986 Oct 2-8; 323(6087):445-8. Keller G, Kennedy M, Papayannopoulou T, Wiles MV, Mol Cell Biol. 1993 Jan; 13(1):473-86. Numbers represent the percentage of cells in each quadrant. Three independent populations of CCE mES cells, with distinct ZsGreen intensity were isolated (highlighted in 1, 2 and 3 circles). B) Genotyping PCR for Sox2 on DNA extracted from the three highlighted groups of cells (1A) in POLQ proficient (POLQ+/+ , WT) and PolQ deficient (PolQ-/-, Null) CCE mES cells. Group 1 depicts non-targeted cells, Group 2 represents cells carrying heterozygous Sox2 cells and in group 3 both alleles of Sox 2 were targeted. C) Left, graph depicting results of FACS analysis for ZsGreen positive CCE mES cells (POLQ+/+ and PolQ-/-) treated with the indicated Cas9 nuclease plasmid (also encoding for a gRNA targeting the Sox2 gene) and a donor plasmid with the sequence to be inserted at the end of the gene. DNA-PK inhibitor was used to block repair by classical NHEJ (Non-Homologous End-joining). Right, cells treated with Cas9-nickase containing plasmid that also encodes for two Sox2 gRNAs. Three independent experiments, each performed in duplicate. The control gRNAs were designed to target two independent loci in the cells (H3F3B and Rosa26) to which, the donor plasmid has no homology. D) Left, depicts results of FACS analysis for ZsGreen positive mES cells (POLQ WT and null, derived from B6.Cg-Poqltm1Jcs/J mouse) treated with the indicated Cas9 nuclease and the donor plasmid. The Cas9 plasmid also encodes the gRNA that targets Hsp90ab1. Right, cells treated with a Cas9-nickase containing plasmid that encodes for two Hsp90ab1 gRNAs. This experiment was performed with two different Cas9-nickase plasmids, one encoding two gRNAs that generates 3' overhangs after cleavage and other that generates 5' overhangs. Three independent experiments, each performed in duplicate. E) Depicts results of FACS analysis for ZsGreen positive MEFs cells (POLQ WT and null, derived from B6.Cg-Poqltm1Jcs/J mouse) treated with the indicated Cas9 as in Figure 1D. Three independent experiments, each performed in duplicate.

In order to understand better how PolQ works I decided to use CRISPR to mutate the different motives of the protein that has been already identified:
**Figure 2.** PolQ lacking polymerase activity (ΔPol) or ATPase activity (ΔATPase) does not promote A-NHEJ, favors HR and reduce the viability in the absence of BRCA1. **A)** Scheme depicting Polθ domains. CRISPR/Cas9 gene targeting was employed to create ΔPol (two aminoacids substitutions at D2494G and E2495S), ΔATPase (one aminoacid substitution K120G), ΔRad51 (deletion of aminoacids D844-M890) and KO (deletion of the exon 3 that introduces a STOP codon after exon 2). Two independently clonal cell lines for each mutant were analyzed. **B)** Frequency of chromosomal translocations (Der-6). Bars represent mean of four independent experiments ± SD (two experiments per clonal cell line). ** represents p=0.006 (two-tailed student’s t test). PCR products were sequenced to confirm translocation and identify possible insertions. **C)** Quantitative analyses of colony formation in embryonic stem cells carrying the indicated mutations after BRCA1 depletion. The number of colonies in control shRNA-treated cells was set to 100%. The knockdown efficiency for BRCA1 was 80%. Bars represent mean of two independent experiments ± s.e.m. **D)** Graph representing Rad51 accumulation following IR treatment (2Gy). Cell were fixed 3 hours after irradiation. Bars represent mean of two independent experiments ± s.e.m.

In our previous studies I characterized the interplay between the A-NHEJ and the Homologous Recombination. I used the TRF1/2^{F/F} p53^-/- Lig4^-/- mouse embryonic fibroblast (MEFs), where in the spread of metaphasic chromosomes from individual cells we can observe fusions of chromosomes due to the A-NHEJ, and exchanges of sequence between telomere sister chromatids (T-SCE) due to the HR. I showed that depletion by shRNA of two components of the A-NHEJ, PolQ and DNA ligase 3 (that would act downstream of PolQ), led to a reduction of the number of chromosome fusions (A-NHEJ), and that only PolQ depletion increased the number of T-SCEs (HR). These results indicate that PolQ promotes the A-NHEJ while is counteracting the HR. It has been described that RPA1, a single stranded DNA binding protein that is involved in HR, counteracts annealing of resected DNA ends and promotes further resection. It is also known that PolQ binds short over hangs. In order to explore a possible role of RPA in counteracting the A-NHEJ I analyzed metaphasic chromosomes from TRF1/2^{F/F} p53^-/- Lig4^-/- MEFs after RPA1 depletion.
Figure 3. RPA1 counteracts A-NHEJ. A) To test if RPA1 represses A-NHEJ at telomeres, I depleted RPA1 in TRF1/2F/F p53-/- Lig4-/- MEFs and monitored the fusion of telomeres. Western blot showing RPA1 levels after shRNA treatment. B) Quantification of telomere fusion (alt-NHEJ) by FISH. C) and D) Quantification of telomere fusion (alt-NHEJ) and T-SCE (HDR), C and D respectively, using CO-FISF in cells transduced with RPA1, or control shRNA. Cells were previously transduced with empty vector (EV), Myc-RPA1 and Myc-RPA-D258Y (that has reduced DNA binding activity). Myc-RPA1 versions were resistant to the shRNA. Mean ± SD, n=3, *p<0.05, **p<0.01; two-tailed student’s t-test. E) Representative western blot for what shown in C) and D).
3- Screen for factors involved in the A-NHEJ:

Subtask 1: Validation of the clonal cell line obtained to perform the screen.
On the preliminary data of the application I showed that in order to achieve reversible, complete, and rapid TRF1/2 depletion, I implemented the auxin-inducible degron (AID) system. AID-tagged proteins are rapidly degraded from auxin treated mammalian cells expressing TIR1 protein. Protein degradation is reversible, and previous protein levels are re-instanted after Auxin withdrawal. I adapted this robust system by expressing AID-tagged TRF1/2 as well as TIR1 in TRF1F/FTRF2F/FKu80−/−p53−/− MEFs from which the endogenous TRF1/2 were deleted with Cre. The screen is based on the transient de-protection of telomeres (AID-TRF1/2 degradation) by the addition of auxin, what activates the DNA damage signaling and allows chromosome fusion by the A-NHEJ to happen. Removal of the auxin should quench the DNA damage signaling but maintain the covalent fusions of telomeres, reducing the cellular proliferation due to the presence of fusions. If siRNAs in the screen target the A-NHEJ, the fusions would be blocked and cellular proliferation would be higher after auxin withdrawal.
In last year report, I showed that the system worked efficiently for the transient degradation of AID-TRF1/2, but also that AID-TRF1/2 did not fully replace the function of endogenous TRF1/2 reducing the proliferation of the clonal cell lines. I conclude that this cell lines were not suitable for the purpose of the screen.
Then I started the generation of a cell line with endogenous TRF1 and TRF2 tagged with HA-AID and Myc-AID minimal versions in mESC, using CRISPR genome editing tool. I did not obtain a clone that properly expressed the tagged TRF1 and TRF2 with the TIR1.

Opportunities for training and professional development that the project has provided:

- One to one weekly meeting with my mentor Agnel Sfeir.
- Weekly lab meeting or journal club.
- I have presented my work twice in the lab meeting since last year.
- Several meetings with my co-mentor Dr. Robert Schneider and members of his lab to discuss experiments, future perspectives and learn methods of how to characterize tumors \textit{in-vivo}.
- Poster presentation at the annual retreat of the Skirball Institute of Biomolecular Medicine. October 2015.
- \textbf{Oral presentation}. Department weekly meeting. April 2015.
- Poster presentation for the Genome Integrity discussion group at the New York Academy of Sciences. June 2016.
- Poster Presenter at the Gordon Research Seminar on Mutagenesis (GRS) held 06/04/2016 - 06/05/2016 at PGA Catalunya Business and Convention Centre in Gerona, Spain. Presented poster titled: Investigating the Interplay
between Polymerase theta and RPA1 during DNA double-strand break repair.

• Poster Presenter at the Gordon Research Conference on Mutagenesis held 06/05/2016 - 06/10/2016 at PGA Catalunya Business and Convention Centre in Gerona, Spain. Presented poster titled: Investigating the Interplay between Polymerase theta and RPA1 during DNA double-strand break repair.

Dissemination of result to communities of interest:

Nothing to report.

Plan to do during the next reporting period to accomplish the goals:

Aim 1: I will follow the established plan on the SOW.

Aim 2: This part of the project can be considered completed. We have already shown that HR defective breast cancer cell lines are dependent on the PolQ mediated DSB repair for its survival, and that a cell line like MCF-7 that is HR proficient reduces its proliferation after PolQ depletion. With regards to subtask 2.3 on the SOW we will perform cell proliferation and colony assays with transient PolQ depletion in combination with DNA damage inducer agents.

Aim 3: I have decided to go back to the original strategy of expressing exogenous tagged TRF1 and TRF2 that are fully functional. In the preliminary data that I showed, TRF1 was tagged N-ter and TRF2 was tagged C-ter. I will tag N-ter and C-ter both proteins with full and shorter versions of the AID, then I will explore the possible combinations. After validating which one of the new cell lines is suitable for the screen, I will proceed as planned.

4. Impact:

Impact on the development of the principal discipline of the project:

Our findings about the relevance of the A-NHEJ repair pathway, and particularly PolQ, for the survival of breast cancer cell lines is opening a new avenue for the treatment of this kind of tumors, and some others where the A-NHEJ or PolQ could also have a relevant role. Our data indicate that PolQ mediated A-NHEJ repair is not simply a backup pathway with a minor role, only being activated when other pathways are blocked, but is instead a constitutive part of the DNA damage response with a significant importance for the cancer cells survival and tumor progression.

Impact on other disciplines:
Nothing to report.

**Impact on technology transfer:**
Nothing to report.

**Impact on society beyond science and technology:**
Nothing to report.

5. **Changes/Problems:**
Nothing to report.

6. **Products:**
Nothing to report.

7. **Participants & other collaborating organizations:**

   **Individuals that have worked on the project:**

   No change.

   **Changes in the active or other support of the PI or senior/key personnel since last reporting period:**

   Nothing to report.

   **Other organizations that were involved as partners:**

   Nothing to report.

8. **Special reporting requirements:**

   Not applicable.

9. **Appendices:**

   Nothing to report.