



Impact of the DNA polymerase Theta on the DNA replication program



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ARTICLE INFO

Article history:

Received 27 November 2014

Accepted 30 November 2014

Available online 5 December 2014

Keywords:

DNA polymerase theta

DNA replication timing

Microarray

ABSTRACT

The physiological function of the human DNA polymerase θ (pol θ) is still unclear despite its *in vitro* translesion synthesis capacity during DNA damage repair process. However this DNA polymerase is always present along the cell cycle in the absence of replication stress and DNA damage. Is there a different molecular function? We present the genomic data of replication timing in depleted pol θ cells (GSE49693) and in cells overexpressing pol θ (GSE53070) indicating that Pol θ holds a novel role in the absence of external stress as a critical determinant of the replication timing program in human cells.

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Specifications

Organism/cell line/tissue	<i>Homo sapiens</i> RKO cell lines for depletion of pol θ MRC5-SV cell lines for overexpression of pol θ
Sex	Male or female if applicable
Sequencer or array type	GPL10123 Agilent-022060 SurePrint G3 Human CGH
Data format	Microarray 4 × 180 K
Experimental factors	Raw data
Experimental features	Early S-phase versus Late S-phase
Experimental features	Two-condition experiments, siRNA control vs. siRNA pol θ cells and overexpressed pol θ cells versus control cells. Biological replicates: 2 control replicates, 2 transfected replicates.

Direct link to deposited data

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49693>.

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53070>.

Experimental design, materials and methods

Cell transfection

Pol θ depleted cells

RKO cells were transfected with siRNA targeting POL θ by two independent pools of siRNA:

- POLQ-1 siRNA: ON-TARGET plus smart pool of 4 POLQ siRNA from Dharmacon (Thermo Fisher Scientific, Waltham, USA): CCUUAAGA

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CUGUAGGUACU, ACACAGUAGGCGAGAGUAU, CGACUAAGAUAGAU CAUUU, CAAACAACCCUUAUCGUAAA

- siRNA POLQ-2: ON-TARGET plus smart pool of 4 POLQ siRNA from Dharmacon: UCAGAGGGAUGGAGCUAAU, GAGAUUACCCUUAUCAC CUA, CAAUUUUACAGUACGGAAA, UGAUAGAUUAGCCUAGUA

RKO cells were transfected with siRNA Luciferase as control (5'-CGUACGCGAAUACUUCGAdTdT-3' from Sigma-Aldrich, Saint-Louis, USA).

Cells overexpressing Pol θ

MRC5-SV fibroblasts were transfected with the pol θ overexpression vector. The design and construction of the pol θ expression vector as well as the overexpressing Pol θ cells and their isogenic controls were previously described [1].

Transfections were performed by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA) at the final concentration of 50 nM following the manufacturer's suggestions. Transfection medium was changed after 8 h to complete medium.

Growth protocol

RKO cells: The human RKO cell line was purchased from ATCC and grown in DMEM with GlutaMAX I, high-glucose, sodium pyruvate (Gibco, Life Technologies), supplemented with 10% fetal bovine serum (Lonza), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹) (Gibco) at 37 °C, 5% CO₂ and 5% O₂ (standard culture conditions).

MRC5-SV: The Human MRC5-SV cell line was purchased from ATCC and grown in Modified Eagle Medium with GlutaMAX™ I, High glucose,

Sodium Pyruvate (Gibco, Life technologies), supplemented with 10% Fetal Bovine Serum (Lonza), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco) at 37 °C, 5% CO₂ and 5% O₂ (standard culture conditions).

BrdU treatment

Cells were incubated with BrdU (50 µM) for 1 hour, collected, washed three times in PBS, then fixed in 75% ethanol, and stored at −20 °C. Fixed cells were first re-suspended in PBS with RNase (0.5 mg/ml) and then with propidium iodide (50 µg/ml) a 30 min incubation at room temperature before the cell sorting.

Cell sorting

80,000 cells were sorted in two fractions S1 and S2 using INFLUX 500 (Cytocopia purchased by BD Biosciences) corresponding to early and late fractions respectively (Fig. 1).

DNA extraction

A proteinase K treatment (0.2 mg/ml) was performed in both fractions in lysis buffer (50 mM Tris pH = 8; 10 mM EDTA; 300 mM NaCl) during 2 h at 65 °C. DNA is protected from light. Then, tRNA was added (50 ng/µl) and a phenol-chloroform extraction followed by an ethanol precipitation was performed. The pellet is resuspended in 100 µl of Tris buffer (10 mM). Then the two fractions were sonicated

in order to obtain fragments between 500 and 1000 bp. DNA is denatured at 95 °C 5 min and then kept on ice during 10 min.

Neo-synthesized DNA was immunoprecipitated with BrdU antibodies (10 µg, Anti BrdU Pure, BD Biosciences, # 347580) by an immunoprecipitated indirect method. Neo-synthesized DNA was incubated overnight at 4 °C with 10 µg of BrdU antibody, then incubated during 2 h with magnetic beads prepared as mentioned by the manufacturer (Adamtech; ChIP adambeads #04342). Beads were washed with different buffers (IP buffer: Tris pH = 8 10 mM; EDTA 1 mM; NaCl 150 mM, Triton × 100 0.5%; BufferB: Tris pH = 8 20 mM; EDTA 2 mM; NaCl 250 mM; Triton × 100 0.25%). Beads are resuspended in Tris buffer (10 mM). The reversion step was then performed with SDS (1%) and 0.5 mg of proteinase K at 65 °C overnight. Neo-synthesized DNA is purified by a pheno-chloroform treatment and an ethanol precipitation. Pellet is resuspended in Tris buffer (pH = 8, 10 mM). To control the quality of enrichment of early and late fractions in S1 and S2, qPCR was performed (Fig. 2A, B, C, D) with CAV2 oligonucleotides as early control (GGCTTGGAGTTTCAACAGGA and CGCAGGTACGAGTCAC AACA) and with bgRM8 oligonucleotides as late control (GGGAAGGA AATGCAAGACAA and AATTTGGCTGCTTAGCATGG) and normalized with amplicon from neo-synthesized mitochondrial DNA (AACCGCTA RGTATTCGTACA and GGTGATTGCTGTACTTGCTTG).

WGA amplification and labeling

Microarray hybridization requires a minimum amount of 500 ng of DNA. To obtain sufficient specific immunoprecipitated neo-synthesized

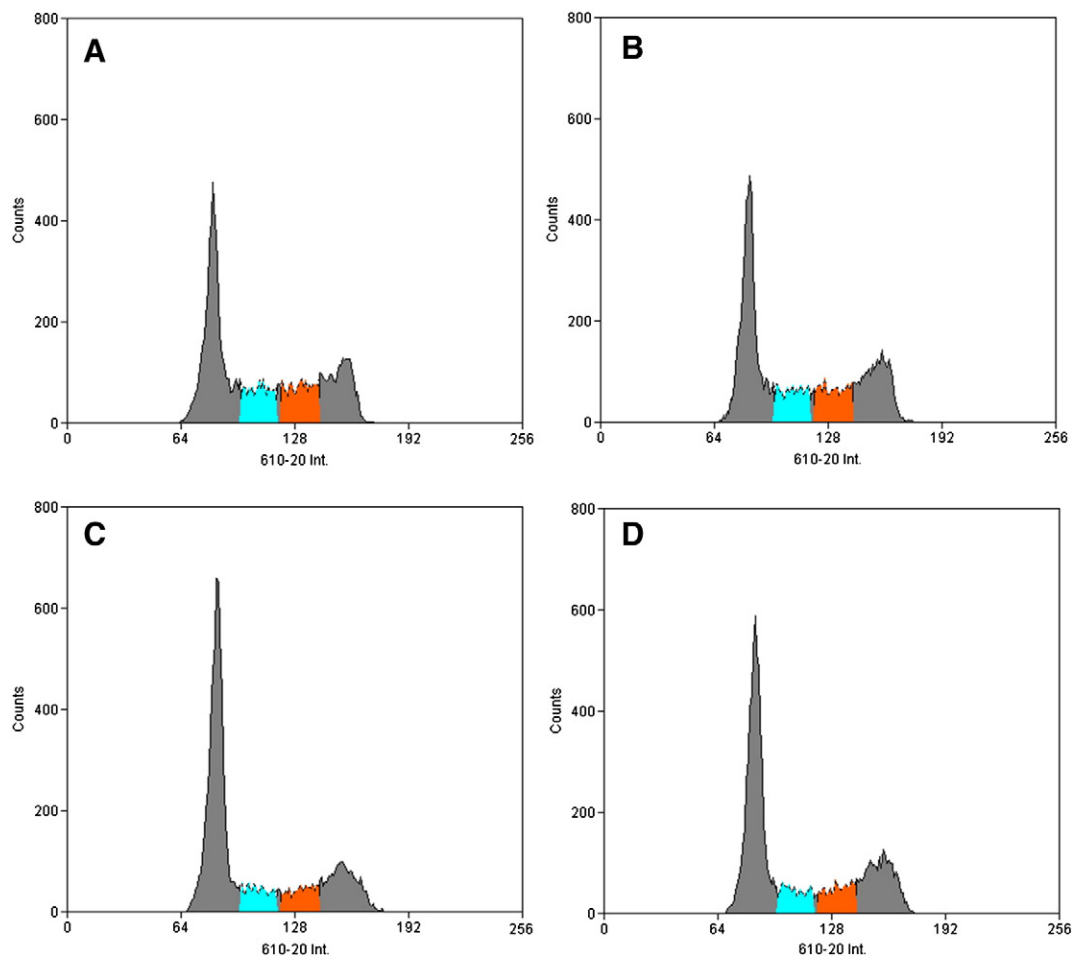


Fig. 1. Different profiles of FACS analysis for each cell lines used in our experiments. A: RKO cell line transfected with the siRNA LUC. B: RKO cell line transfected with siRNA *pol θ*. C: MRC5-SV control cell line. D: MRC5-SV cell line overexpressing *pol θ*. Blue fraction corresponds to early replicated fraction assigned S1. Orange fraction corresponds to late replicated fraction assigned S2.

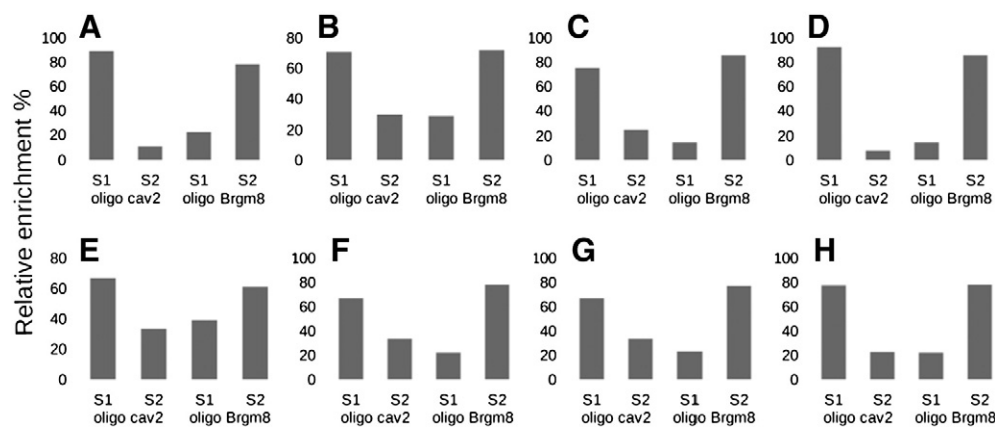


Fig. 2. Quality control of neo-synthesized DNA isolation by quantitative PCR before WGA amplification (A, B, C, D) and after WGA amplification (E, F, G, H). Oligo Cav2 permits to control early replicated enrichment and Brgm8, late replicated enrichment. S1 corresponds to early replicated fraction isolated by cell sorting, S2 to late replicated fraction. A and E assign RKO siRNA LUC, B and F, RKO siRNA *pol θ*. C and G call MRC5-SV control cell line, D and H, MRC5-SV cell line overexpressing *pol θ*.

DNA, whole genome amplification was conducted (WGA, Sigma). To be sure that this step does not introduce bias, a qPCR after WGA was performed to confirm the specific enrichment in each fraction S1 and S2 (Fig. 2E, F, G, H). After amplification, early and late neo-synthesized DNA fractions were labeled with Cy3 and Cy5 ULS molecules (Genomic DNA labeling Kit, Agilent) as recommended by the manufacturer.

Hybridization protocol

The hybridization was performed according to the manufacturer's instructions on 4 × 180 K human microarray (SurePrint G3 Human CGH Microarray Kit, 4 × 180 K, AGILENT Technologies, genome reference Hg18) that covers the whole genome with one probe every 13 Kb (11 KB in RefSeq sequences).

Scan protocol

Microarrays were scanned with an Agilent's High-Resolution C Scanner using a resolution of 2 μm and the autofocus option.

Data processing

Feature extraction was performed with the Feature Extraction 9.1 software (Agilent technologies). For each experiment, the raw data sets were automatically normalized by the Feature extraction software using the CGH_1105_oct12 FE protocol browser. Analysis was performed with the Agilent Genomic Workbench 5.0 software. The \log^2 -ratio timing profiles (Fig. 3) were smoothed using the Triangular Moving Average option of the Agilent Genomic Workbench 5.0 software with the Triangular algorithm and 500-kb windows.

The algorithm from CGH applications of the Agilent Genomic Workbench 5.0 software was used in order to determine the replication domains (early and late domains) in *Pol θ*-depleted RKO cells and in *Pol θ*-overexpressing MRC5-SV cells. The aberration detection algorithms associated with the Z-score and with a threshold of 1.8 define the boundaries and magnitudes of the regions of DNA loss or gain corresponding to the late and early replicating domains respectively (Regions with a positive log-ratio is replicated early and late when the log-ratio is negative).

Basic analysis

A comparison was conducted between early and late domains between each cell lines. As mentioned by Hiratani et al. [2], two major changes can be characterized. The intersection between *CTRL* siRNA late and *POLQ-1* siRNA early intervals will reveal segments that are replicated earlier due to the depletion of *Pol θ*. Conversely with *CTRL* siRNA early versus *POLQ-1* siRNA late domains, intervals that are replicated later will be characterized. Similarly, the intersection between MRC5-*CTRL* late and MRC5-overexpressing *Pol θ* early intervals will reveal segments that have become earlier by the overexpression of *Pol θ*, and conversely with MRC5-*CTRL* early versus MRC5-overexpressing *Pol θ* late domains, intervals that are replicated late will be characterized. The intersection was performed with the Intersection GALAXY tools (Operate on genomic intervals, <https://main.g2.bx.psu.edu/>).

Discussion

Here we put in evidence a new function of *Pol θ* because a change in expression of this protein disturbs the replication timing program.

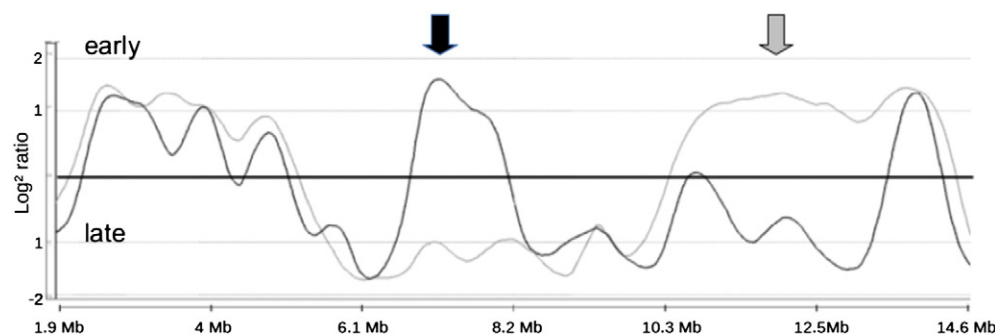


Fig. 3. Microarray profiles of the timing of replication on chromosome 6: 1 997 799–14 527 487 (12.5 Mb, hg18); Two replicates for each cell line. Continuous lines: RKO siRNA control. Dashed lines: RKO siRNA *polQ*. Arrows indicate the main changes in the timing of replication. Black arrows: Early to late (EtoL), gray arrows: late to early (LtoE).

These data and other evidences are published recently and demonstrate the molecular role of Pol θ in DNA replication [3]. This novel Pol θ function could be particularly important within the context of cancer because in colon, breast and lung cancers, a high *pol* θ expression was observed and associated with poor clinical outcome of patients [1,4–7].

Acknowledgments

We gratefully acknowledge Marcel Méchali (IGH—Montpellier), leader of the French 'Replication of eukaryotic chromosomes and its checkpoint' GDR 2915—CNRS Network, which allowed the adequate collaborations to develop this project. We thank Nicole Bogetto (IJM—Paris) for their technical support. This work was supported by La Ligue contre le cancer (Equipe Labellisée 2013 to J.-S.H.; Comité Ile-de-France to G.B.) and a GEFLUC grant (to J.-C.C).

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