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Enrichment of *Cxcl12* promoter with TET2: a possible link between promoter demethylation and enhanced gene expression in the absence of PARP-1

Anja Z. Tolić[1], Jovana J. Rajić[1], Marija B. Đorđević[1], Miloš M. Đorđević[1], Svetlana S. Dinić[1], Nevena M. Grdović[1], Jelena D. Arambašić Jovanović[1], Mirjana V. Mihailović[1], Goran Đ. Poznanović[1], Tomasz P. Jurkowski[2,3], Melita S. Vidaković[1] and Aleksandra S. Uskoković[1,*]

[1]*Department of Molecular Biology, Institute for Biological Research "Siniša Stanković" – National Institute of the Republic of Serbia, University of Belgrade*, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

[2]*Institute of Biochemistry, University of Stuttgart*, Pfaffenwaldring 55, 70569 Stuttgart, Germany

[3]*School of Biosciences, Cardiff University, Cardiff, Wales, Sir Martin Evans Building*, Museum Avenue, Cardiff, CF10 3AX, UK

***Corresponding author**: auskokovic@ibiss.bg.ac.rs

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Abstract: Previously, we described the link between C-X-C motif chemokine 12 (Cxcl12) gene induction and DNA hypomethylation in the absence of poly(ADP-ribose) polymerase 1 (PARP-1). We have now firmly established that demethylation is the primary cause of gene induction on the basis of *Cxcl12* gene upregulation upon treatment with the demethylating agent 5-azacytidine (5-aza). Since the demethylation state of *Cxcl12* is favored by PARP-1 absence, we investigated the presence of ten-eleven translocation (TET) proteins on the *Cxcl12* promoter in order to corroborate the relationship between the demethylation process and increased gene expression that occurs in the absence of PARP-1. Analysis was performed on the promoter region within CpG islands of *Cxcl12* from control mouse embryonic fibroblasts (NIH3T3) and PARP-1 knock-out mouse embryonic fibroblasts (PARP1-/-). The lack of PARP-1 increased the abundance of TET2 on the *Cxcl12* promoter, suggesting that TET-mediated demethylation provoked by the absence of PARP-1 could account for the observed increased expression of this chemokine. Deciphering the regulation of DNA (de)methylation factors that control *Cxcl12* expression may provide an additional therapeutic approach in pharmacological interventions where gene switching on or off based on targeted stimulation or inhibition is necessary.

Keywords: DNA demethylation; 5-aza; TET2; PARP-1; CXCL12

INTRODUCTION

DNA methylation is a covalent modification of genomic DNA that occurs predominantly at CG dinucleotides (CpG) where DNA methyltransferase (DNMTs) enzymes mediate the transfer of a methyl group to the 5-position of cytosines, generating 5-methylcytosine (5mC), which is assumed to be a fifth DNA base [1-3]. Ten-eleven translocation 1-3 (TET1-3) proteins, members of the DNA hydroxylase family, affect the methyl group on 5mC by catalyzing the consecutive oxidation of 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [4,5]. Further, 5caC can be replaced by an unmethylated cytosine by thymine-DNA glycosylase (TDG) via the base excision repair (BER) pathway [6]. Hence TET proteins can generate both new epigenetic marks (5hmC, 5fC and 5caC) in genomic DNA and also reverse the 5mC to the unmethylated state which comprises the process of active DNA demethylation [7,8]. Since DNA (de)methylation is closely linked to gene expression, it is important to explore the regulation of these mechanisms when related to various diseases which are characterized by aberrant DNA methylation. Poly(ADP-ribose) polymerase (PARP) enzymes, which catalyze covalent attachment of poly-ADP-ribose (PAR) units to acceptor proteins (PARylation), take part in the regulation of DNA (de)methylation processes [9]. PARP-1 is the founding member of PARP family that participates

in the production of PAR polymers. Besides PARP-1 automodification where the enzyme PARylates itself, it can also PARylate covalently other target proteins, thereby modulating their activities and functions. PAR moieties can also bind non-covalently to target proteins that expand the number of PARP-1 interacting partners subjected to PARylation [9-11]. The link between PARP-1 and TET activity in DNA demethylation is emerging as a newly discovered role of PARP-1 [11].

In our previous study we reported that PARP-1 promotes DNA methylation and the opposite, that lack of PARP-1 is connected with the demethylation state of *Cxcl12* gene; this also corresponds with changes in *Cxcl12* expression, which tends to increase in the absence of PARP-1 [12]. CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is a potent CXC chemokine produced by different cell types. It is involved in the migration of a variety of cells, including hematopoietic progenitor and stem cells, endothelial cells and most leukocytes [13]. CXCL12 is involved in physiological and pathological processes, such as development, cell survival, tissue repair and regeneration, as well as cancer [14,15]. The methylation status of the *Cxcl12* gene plays an important role in tumor progression in human carcinogenesis. For example, epigenetically-controlled downregulation of the *Cxcl12* by promoter hypermethylation has been detected in breast tumors [16] and in osteosarcomas [17], and is connected with metastatic potential and poor survival prognosis. Hence, the methylation status of *Cxcl12* could be used as a target for therapeutic interventions.

Since we previously found PARP-1 to be a potential upstream player in methylation events that modulated *Cxcl12* expression, our aim was to examine whether a lack of PARP-1 would induce TET enzymes to demethylate *Cxcl12*.

MATERIALS AND METHODS

Cell culture and treatment

The following cell lines were used: mouse embryonic fibroblasts, NIH3T3 (ATCC- CRL-1658), and PARP-1 knock-out (PARP1-/-) cells [18]. The cells were cultured in high glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin (all cell culture reagents were supplied by Biological Industries, Beit Haemek Ltd., Israel). NIH3T3 cells were treated with 15 μM 5-azacytidine (5-aza) solubilized in dimethyl sulfoxide (DMSO) for 72 h, with the treatment replaced every 24 h. In parallel with the 5-aza treatment, control NIH3T3 cells were treated with DMSO in the same manner.

RNA isolation, reverse transcription and real-time quantitative PCR (RT-qpcr)

Total RNA was isolated from NIH3T3 cells treated with 5-aza or DMSO and PARP1-/- cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA). Reverse transcription was performed on 1 μg of DNase I-treated RNA by the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with a mix of oligo(dT)18 and random hexamer primers. For RT-qPCR we used QuantStudio 3 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). The thermal cycles were as follows: initial denaturation at 95°C for 10 min and 40 cycles of two-step PCR at 95°C for 15 s and at 60°C for 60 s. Expression of *Cxcl12* was estimated relative to GAPDH (as an internal control) by the delta Ct method (2^{dCt}) with previously published primers [12] (Supplementary Table S1). The obtained data was log2 transformed for statistical testing and statistical significance was estimated using ANOVA with Dunnett's *post-hoc* test used to compare NIH3T3 5-aza-treated and PARP-/- cells with control NIH3T3 cells mock-treated with DMSO. For graphs, mean values and error bars were transformed back to the linear scale.

DNA isolation, bisulfite conversion and methylation-specific PCR (MSP)

To isolate high molecular weight DNA we used cell lysis buffer (2 mM EDTA, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.5% sodium dodecyl sulfate (SDS)) supplemented with 0.04 μg/mL proteinase K. After overnight incubation at 55°C, the lysates were ethanol-precipitated with cold 75 mM sodium acetate diluted in absolute ethanol. The isolated high molecular weight DNA was resuspended in water.

The EZ-DNA methylation kit (D5002; Zymo Research, Orange, CA, USA) was used for bisulfite conversion of genomic DNA, as per the provided instructions. This bisulfite-converted DNA served as a template for MSP analysis of DNA methylation changes. The promoter (1MU), the exon-intron boundary (2MU) and part of the intron (3MU) of *Cxcl12* were analyzed (Fig. 2) with three sets of MSP primers (Supplementary Table S1) that were previously published [12]. Each set was comprised of two pairs of primers targeted to the same location yet differentiating between the methylation states, as one pair was complementary to the methylated (M) and the other to the unmethylated (U) bisulfite-converted target DNA sequence. Separate reactions were set up for each primer pair, ensuring that reactions for one sample with primers from the same set were always run in parallel, within the same PCR run. Each reaction had a volume of 10 μL and contained 2 μM primers, 60 ng of bisulfite-converted DNA and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). The QuantStudio 3 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) was used for the runs with the following temperature cycling profile: initial denaturation at 95°C for 10 min and 40 cycles of two-step PCR at 95°C for 15 s and 58°C for 60 s. Finally, to assess the relative level of methylation, threshold cycle values (Ct) for M and U primer pairs from the same set were used to calculate the methylation index [19] using the following equation:

$$2^{(\text{Ct for U primer pair reaction})-(\text{Ct for M primer pair reaction})}.$$

The obtained data was log2 transformed for statistical testing and statistical significance was estimated using ANOVA with Dunnett's *post-hoc* test to compare the NIH3T3 5-aza-treated and PARP-/- cells with control NIH3T3 cells mock-treated with DMSO. For graphs, mean values and error bars were transformed back to the linear scale.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed using the Pierce™ Magnetic ChIP Kit (Thermo Fisher Scientific, USA) according to the protocol. In brief, NIH3T3 and PARP-/- chromatin were cross-linked with 1% formaldehyde (Zorka Pharma, Serbia) and the process was stopped with a glycine solution after 10 min at room temperature. After extraction, the chromatin was digested with 0.005 U/μL micrococcal nuclease and sheared on ice by sonication (7 rounds of 20 s sonication and 30 s rest). Immunoprecipitation was performed using Protein A/G Magnetic Beads (USA) and the following antibodies: anti-RNA polymerase II (RNA pol II) (supplied with the ChIP kit), normal rabbit IgG (supplied with the ChIP kit), 5 μg of anti-TET1 (Millipore Merck, USA) and 5 μg of anti-TET2 (Millipore Merck, USA). The cross-linking was reversed at 65°C and the immunoprecipitated samples and samples containing 10% of total input were treated with 40 μg proteinase at 65°C for 1.5 h. The abundance of the target sequence was assessed by quantitative PCR (qPCR) using QuantStudio 3 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). Primers (Supplementary Table S1) were designed to amplify part of the promotor region directly adjacent to the transcription start site (TSS) of *Cxcl12* (Fig. 2). To quantify the enrichment, first the quantity of ChIP and IgG samples was determined from a standard curve generated with qPCR data of 10-fold dilution series of the 10% total input samples. Enrichment was calculated by normalizing to IgG sample quantity and fold enrichment was reported relative to the corresponding NIH3T3 samples (immunoprecipitated by the same antibody) from the same experiment. The experiment was repeated twice.

Co-immunoprecipitation (IP)

NIH3T3 and PARP-/- cells were lysed in ice-cold ProteoJET™ Mammalian Cell Lysis Reagent (Thermo Fisher Scientific, USA), supplemented with a protease inhibitor cocktail, for 30 min at 4°C. Cell lysates (300 μg) were precleared by incubation with Protein A/G agarose-coupled beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The precleared lysate was incubated overnight at 4°C on a tube rotator with 2 μg of anti-TET2 antibody (Millipore Merck, USA), which was followed by the addition of protein A/G agarose-coupled beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and further incubation at 4°C for 4 h. The agarose beads were pelleted and washed 5 times with a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2 mM phenyl methyl sulfo-

nyl fluoride (PMSF) and a protease inhibitor cocktail. Proteins were eluted from the beads by boiling in a sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 100 mM dithiothreitol, 0.02% bromophenol blue), separated by 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) alongside 70 μg of proteins from the NIH3T3 lysate. The proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes and analyzed by immunoblotting.

Immunoblot analysis

NIH3T3 and PARP-/- cells were lysed in ice-cold RIPA buffer (Sigma Aldrich, USA) supplemented with a protease inhibitor cocktail for 30 min at 4°C. Equal amounts of cell lysates were separated by 12% SDS-PAGE and electrotransferred onto PVDF membranes. Immunoblotting for lysates or IP samples was performed by overnight incubation at 4°C with anti-PARP-1 (H-250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-TET2 primary antibodies (Millipore Merck, USA) and anti-GAPDH (FL-335, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as loading control for lysates. This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody at room temperature for 1 h. Staining was performed by the enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions.

RESULTS

Cxcl12 expression was increased in 5-aza-treated NIH3T3 and PARP-/- cells

Treatment of NIH3T3 cells with the demethylating agent 5-aza induced a higher level of *Cxcl12* expression as measured by real-time PCR. Treated NIH3T3 cells showed on average a 5.5-fold increase compared

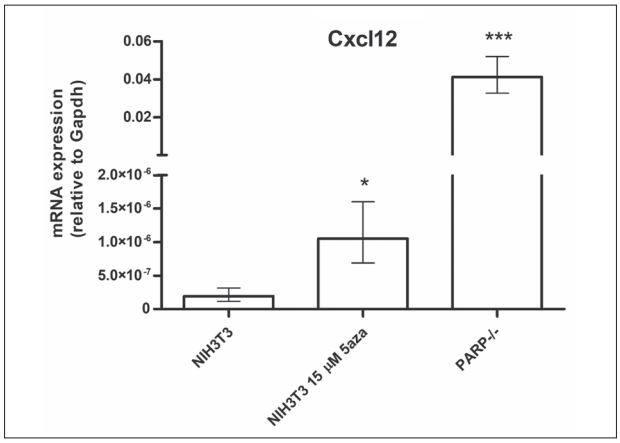

Fig. 1. Relative expression level of *Cxcl12* in control NIH3T3 cells (mock-treated with DMSO), NIH3T3 cells treated with 15 μM 5-aza and PARP−/− cells (n = 5) measured by RT-qPCR. Statistical significance is reported for the comparison of NIH3T3 5-aza-treated and PARP-/- cells to the control NIH3T3 cells. Results are presented as the mean±standard error of the mean, *p≤0.05, ***p ≤ 0.001, n-number of independent experiments.

to control NIH3T3 cells, while PARP-/- cells showed an even greater increase of $2x10^5$-fold compared to the control (Fig. 1). Both observed increases were statistically significant (p_{5aza}=0.02 and $p_{PARP-/-}$=$1.9x10^{-8}$).

Methylation changes of *Cxcl12* in 5-aza-treated NIH3T3 and PARP-/- cells

As previously established [12], murine Cxcl12 gene has a CpG island that spans a part of its promoter, first exon and part of the first intron (Fig. 2). The methylation changes of *Cxcl12* on these regions were examined as follows: amplicons analyzed by MSP covered 8 CpGs in the promoter, 23 CpGs at the exon-intron boundary and 8 CpGs in the intron region. In accordance with the changes in expression, we observed that both the treatment of NIH3T3 cells with 5-aza and the absence of PARP-1 resulted in demethylation of *Cxcl12*. Cells treated with 5-aza exhibited a statistically significant decrease in methylation, on average 45.4-

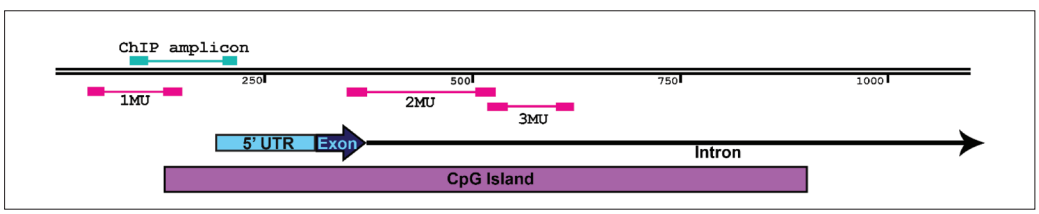

Fig. 2. Schematic representation of murine *Cxcl12* and positions of primers used for MSP and ChIP qPCR.

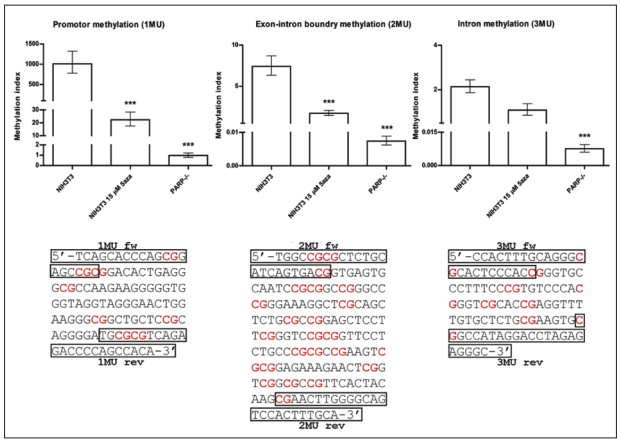

Fig. 3. Methylation levels of different parts of *Cxcl12* of control NIH3T3 cells (mock-treated with DMSO), NIH3T3 cells treated with 15 μM 5-aza and PARP–/– cells measured by methylation-specific PCR (MSP) (n = 4). Under each graph, the amplicon sequence is represented with highlighted CG dinucleotides. Statistical significance is reported for the comparison of NIH3T3 5-aza-treated and PARP-/- cells to the control NIH3T3 cells. Results are presented as the mean±standard error of the mean, *p≤0.05, ***p≤0.001, n-number of independent experiments.

fold in the promoter region ($p=1x10^{-6}$) and 4.3-fold at the exon-intron boundary ($p=3.9x10^{-4}$), while the intron region was not significantly affected (Fig. 3). PARP-/- cells showed a significantly higher level of demethylation (Fig. 3). Compared to NIH3T3 cells, methylation in PARP-/- cells was decreased 1032.4-fold in the promoter ($p=3.6x10^{-8}$), 1002.4-fold in the exon-intron boundary ($p=3.1x10^{-8}$) and even 285.3-fold in the intron region ($p=5.2x10^{-8}$).

The level of TET2 at the *Cxcl12* promoter is higher in the absence of PARP-1

ChIP analysis showed that TET proteins interact with the *Cxcl12* promoter. Control ChIP experiments performed with an anti- RNA pol II antibody confirmed that RNA pol II was more abundantly present (on average 5.7-fold more) on the *Cxcl12* promoter of PARP-/- than of NIH3T3 cells (Fig. 4). This result was in agreement with the *Cxcl12* expression profile in these cells. The results for TET1 were deemed inconclusive as no consistent change was observed (data not shown). We therefore focused on TET2, as it was consistently present in higher abundance (on average 1.32-fold) on the analyzed region of the *Cxcl12* promoter in PARP-/- when compared to NIH3T3 cells (Fig. 4).

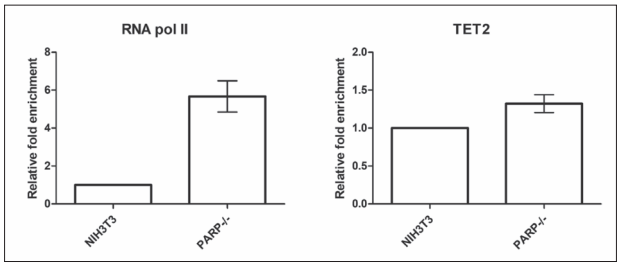

Fig. 4. ChIP-qPCR analysis of RNA pol II and TET2 occupancy at the *Cxcl12* promoter in NIH3T3 and PARP-/- cell lines (n=2). Results are presented as the mean±standard error of the mean.

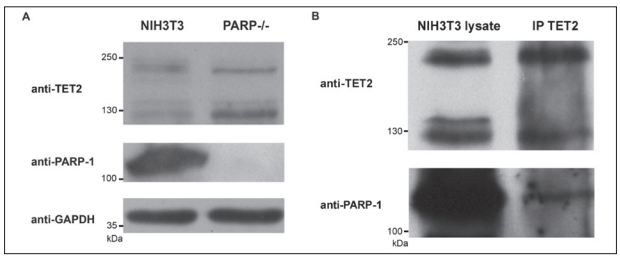

Fig. 5. A – Immunoblot analysis performed with anti-TET2, anti-PARP-1 and anti-GAPDH (loading control) antibodies, on lysates isolated from NIH3T3 and PARP-/- cell lines. **B** - Immunoprecipitation of TET2 from NIH3T3 cells analyzed by immunoblotting with anti-TET2 and anti-PARP-1. Successful immunoprecipitation of TET2 was confirmed as well as coprecipitation of PARP-1 with TET2. IP – immunoprecipitation.

TET2 interacts with PARP-1

Western-blot analysis confirmed the absence of PARP-1 in PARP-/- cells and showed higher levels of TET2 isoforms (223 kDa and 130 kDa) in PARP-/- as compared to NIH3T3 cells (Fig. 5A). Results of IP analysis showed that TET2 was successfully immunoprecipitated from NIH3T3 cell lysates and that it co-precipitated PARP-1, confirming their interaction *in cellulo* (Fig. 5B).

DISCUSSION

In the past decade, much attention has been focused on the role of epigenetic alterations in cancer progression [20]. The increased metastatic potential in some types of cancers, such as colon, mammary and breast carcinomas, was shown to be associated with epigenetic silencing of *Cxcl12* [16,21,22]. Furthermore, considering the ability of CXCL12 to stimulate the growth and survival of pancreatic β-cells, the tran-

scriptional regulation of this chemokine could provide an approach for diabetes treatment [23]. Hence, therapies that target CXCL12 expression via de/methylation possess the potential for therapeutic interventions, and identification of factors that affect the methylation status of this chemokine is of great importance.

In this study we verified that PARP-1 affected the methylation status of *Cxcl12* whereby the absence of PARP-1 promoted TET2 involvement on the *Cxcl12* promoter, linking TET-dependent demethylation and increased gene expression in the absence of PARP-1. Restored expression of *Cxcl12* after treatment with 5-aza, one of the most frequently used demethylating agents that inhibits DNMTs [24,25], confirmed the epigenetic transcriptional silencing of *Cxcl12*. While treatment with 5-aza is presumably based on inactivation of methylation due to rapid loss of DNMT activity, our previous results indicated that in PARP-/- cells the promotion of TET-dependent active demethylation occurs rather than DNMT-related suppressed methylation [12]. We assumed that this scenario also occurs in the *Cxcl12* gene; however, a direct link between TET-mediated DNA demethylation and the transcriptional output is difficult to establish because of the existence of global and locus-specific effects that are difficult to discern [26]. Demethylation of the *Cxcl12* promoter region was expected as it was already established that the *Cxcl12* gene is exposed to epigenetic regulation by methylation of cytosine in CpG dinucleotides located in the promoter sequence [27-29]. In the intron region there was no statistically significant decrease in methylation in NIH3T3 cells treated with 5-aza, whereas a significant decrease was observed in PARP-/- cells. This points to the stronger potential for demethylation of *Cxcl12* in PARP-/- compared to NIH3T3 cells treated with 5-aza, which could have also contributed to the higher increase in *Cxcl12* expression.

Although the absence of PARP-1 in the demethylation event is clear, it remains to be elucidated whether the TETs are responsible for the loss of methylation. Our previous results suggested that PARP-1 could be involved in demethylation as significant increases in both TET1 and TET2 expression were detected in the absence of PARP-1 [12]; herein we showed that the lack of PARP-1 increased TET2 occupancy on the *Cxcl12* promoter. Furthermore, according to the obtained result there was no clear increase in TET1 on the *Cxcl12* promoter (result not shown); however, this does not rule out the possibility that TET1 was also affected by the absence of PARP-1 for recruitment and demethylation events on the *Cxcl12* promoter. This assumption needs further validation.

TET proteins preferentially bind to DNA containing unmodified cytosine [30-32]. While TET1 is recruited to genomic target sites through direct binding of CXXC domains to CpG-rich oligonucleotides [3,33,34], TET2 lacks the CXXC DNA-binding domain, thus it is more likely that its recruitment on a specific gene is mediated by interactions with other DNA-binding factors [35]. It was found that TET2 interacts with IDAX protein whose CXXC domain binds to unmethylated CpG dinucleotides in the promoter but in a sequence-nonspecific manner [31]. A very recent report described the mechanism for targeting TET2 to specific promoters [36]. It was proposed that transcriptional co-activator SMAD nuclear interacting protein 1 (SNIP1) bridges TET2 to bind to a sequence-specific DNA-binding factor c-MYC, thus facilitating the recruitment of TET2 to regulate c-MYC target genes by promoting DNA demethylation at the promoters [36]. In addition, it is well established that the posttranslational modification, monoubiquitylation, which is catalyzed by CRL4VprBP E3 ligase, promotes the DNA binding of TET2 by stabilizing the conformation of TET2 complexed with DNA (where the monoubiquitylation site directly contacts the DNA) [32,37].

The activity of TETs can be modulated by metabolites, cofactors and several post-translational modifications [35]. Our previous results marked TETs as potentially important factors in *Cxcl12* expression in the absence of PARP-1. This was supported by the detected tendency of *Cxcl12* expression to increase in the presence of TET activator (vitamin C), and to decrease upon treatment with the TET inhibitor dimethyloxaloylglycine (DMOG) in PARP-/- cells [12]. The findings presented herein encourage us to assume that the lack of PARP-1 was responsible for the increased recruitment of TET2 to the *Cxcl12* promoter, which is a prerequisite step that leads to augmented activity and demethylation events.

As interactions between TET2 and PARP-1 in human embryonic kidney 293T (HEK293T) cells were reported [38], it was expected that these proteins establish interactions in NIH3T3 cells, as we have showed. We assumed that these interactions could have an in-

hibitory role on the catalytic activity of TETs, since our previous results showed a global increase in TET expression/activity in the absence of PARP-1 [12]. Hence the catalytic activation of TET enzymes together with the increased recruitment of TET2 in the absence of PARP-1 could have contributed to the local demethylation event on the *Cxcl12* promoter, accounting for its induced expression. This is in collision with some literature data showing that inhibition of PARylation suppresses the production of 5hmC by TET proteins and consequently region-specific demethylation [39]. Nevertheless, the role of PARP-1 in demethylation and its impact on TET activity was recently reviewed and it was pointed out that data implicating PARP/TET interplay in the regulation of (de)methylation that can produce opposite effects, resulting in either increased or decreased 5mC-hydroxylase activity, is still emerging [11]. Consistent with this view is the observation that after inhibition of PARylation, aside from a number of hypermethylated genes, several genes were also found to be hypomethylated [40].

CONCLUSION

Enrichment of the *Cxcl12* promoter with TET2, together with the previously described overall increased expression and activity of TETs in the absence of PARP-1, highlight the connection of PARP-1 and gene expression through (de)methylation events, and support the inhibitory role of PARP-1 in demethylation, which explains the increased expression of *Cxcl12*. Future research should focus on exploring the effect of PARP activity (not only its presence) on *Cxcl12* expression and on epigenetic events as this could enhance the efficacy of PARP inhibitors in their potential clinical translation.

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Author contributions: AU and MV designed the study; AT, JR and MS performed the experiments; MĐ, MM and JAJ analyzed the data statistically; AT and AU wrote the manuscript; TJ, SD, NG, and GP critically reviewed the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

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Supplementary Data

Supplementary Table S1.

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