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Distinct and overlapping DNMT1 interactions with multiple transcription factors in erythroid cells: Evidence for co-repressor functions

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ABSTRACT

DNMT1 is the maintenance DNA methyltransferase shown to be essential for embryonic development and cellular growth and differentiation in many somatic tissues in mammals. Increasing evidence has also suggested a role for DNMT1 in repressing gene expression through interactions with specific transcription factors. Previously, we identified DNMT1 as an interacting partner of the TR2/TR4 nuclear receptor heterodimer in erythroid cells, implicated in the developmental silencing of fetal β-type globin genes in the adult stage of human erythropoiesis. Here, we extended this work by using a biotinylation tagging approach to characterize DNMT1 protein complexes in mouse erythroleukemic cells. We identified novel DNMT1 interactions with several hematopoietic transcription factors with essential roles in erythroid differentiation, including GATA1, GFI-1b and FOG-1. We provide evidence for DNMT1 forming distinct protein subcomplexes with specific transcription factors and propose the existence of a "core" DNMT1 complex with the transcription factors ZBP-89 and ZNF143, which is also present in non-hematopoietic cells. Furthermore, we identified the short (17a.a.) PCNA Binding Domain (PBD) located near the N-terminus of DNMT1 as being necessary for mediating interactions with the transcription factors described herein. Lastly, we provide evidence for DNMT1 serving as a co-repressor of ZBP-89 and GATA1 acting through upstream regulatory elements of the PU.1 and GATA1 gene loci.

1. Introduction

DNA methylation is a major epigenetic modification that occurs through the covalent addition of a methyl group to the C5 position of a cytosine residue. DNA methylation is most prevalent at CpG sites although it may also occur, to a lesser extent, at non CpG-sites [1]. In mammals, the enzymes responsible for carrying out this process are the DNA methyltransferases DNMT1, DNMT3a and DNMT3b [2]. DNMT1 is the methyltransferase responsible for the maintenance of CpG methylation following DNA replication. It has a strong preference for hemimethylated DNA as a substrate over unmethylated DNA [3–5]. During S-phase, DNMT1 is localized at DNA replication foci whereas in interphase it becomes diffuse [6]. Although DNMT1 acts predominantly as a maintenance DNA methyltransferase, several lines of evidence have shown that it may also have a *de novo* methylation function *in vitro* [5,7,8] and also in knockout cells for the *de novo* methyltransferases DNMT3a and DNMT3b [9,10].

DNMT1 is expressed ubiquitously and is essential for cellular growth and differentiation in many tissues and cell types in mammals [11]. Targeted disruption of the *Dnmt1* gene in mice leads to delayed development and embryonic lethality after midgestation [12]. Mouse embryonic stem cells (ESCs) are viable after targeted disruption of *Dnmt1*, however they die shortly after differentiation is induced [12,13]. Many observations have linked DNMT1 function to cell growth regulation. For example, complete inactivation of DNMT1 in a human cancer cell line results in G2 phase arrest causing mitotic catastrophe [14], whereas conditional inactivation of *Dnmt1* in primary mouse embryonic fibroblasts led to p53-dependent cell death [15]. In human ESCs, the null allele of Dnmt1 results in increased DNA damage and G1 arrest [16],

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whereas mice carrying a hypomorphic *Dnmt1* allele are prone to tumour development due to chromosomal instability [17].

The DNMT1 protein has a molecular weight of 183 kDa and is highly conserved between mouse and human with an almost 77% identity at the protein level. The C-terminal end of the protein contains the catalytic methyltransferase domain [4], whereas the N-terminal and central parts contain a number of additional regulatory domains, namely, the DMAP1 interacting domain (DMAP1), the PCNA binding domain (PBD), the nuclear localization signal (NLS), the targeting sequence (TS) and the polybromo homology domain (PBHD) (see Fig. 4A). The DMAP1 domain of DNMT1 binds the transcriptional co-repressor DMAP1 [18], whereas the PBD mediates interaction with PCNA thus recruiting DNMT1 to replication foci [19]. The TS domain mediates targeting of DNMT1 to centromeric heterochromatin [20] and also facilitates DNMT1 dimerization [21]. The PBHD consists of the BAH1 and BAH2 (Bromo-adjacent homology 1 and 2) subdomains implicated in protein interactions [22]. Besides interactions with the aforementioned co-factors, DNMT1 has been shown to interact with, amongst others, SET7/9 [23], the NuRD complex [24], the G9a [25] and EZH2 [26] histone lysine methyltransferases, the Lsd1 histone demethylase [27] and the Rb and E2F1 transcription factors [28]. Thus, the complex DNMT1 protein structure underlies the multiple protein interactions that it undergoes in the nucleus in fulfilling functions that extend beyond its methyltransferase catalytic activity.

Even though DNA methylation has been studied extensively in hematopoiesis [29,30] the role of DNMT1 is still under investigation. Conditional knockout of the *Dnmt1* gene using GATA1-Cre mice results in embryonic lethality [31]. Conditional DNMT1 knockout in hematopoietic stem cells (HSCs) restricts HSC differentiation to the myeloid progeny as they cannot differentiate into lymphoid cells [32]. In erythroid cells, DNA methylation has been studied extensively as chemical inhibition of DNMT1 activity results in human γ-globin gene reactivation in the adult stage, thus offering a potential therapeutic route to treating hemoglobinopathies [33]. This was further strengthened by recent work by us and others, showing that transcription factors, such as TR2/TR4 and BCL11A, implicated in the repression of human γ-globin expression in adult erythroid cells, interacted with DNMT1 [34,35]. Specifically, we showed previously that DNMT1 co-purifies with the TR2/TR4 nuclear receptors, which bind to the embryonic β-type globin promoters [34], whereas more recently DNMT1 was found to associate with the BCL11A transcription factor in the silencing of γ-globin expression in primary human adult erythroid cells [35].

Taken together, DNMT1 appears to play important, yet poorly defined, roles in globin gene regulation and potentially also in erythropoiesis. Thus, deciphering DNMT1 functions may help understand the mechanism of fetal hemoglobin reactivation in adults, which could lead to novel treatments of β-thalassemia. To this end, we utilized herein a biotin-tagging approach coupled to mass spectrometry [36] to identify novel DNMT1 interactions with a number of transcription factors with established roles in erythropoiesis. Furthermore, we provide evidence for DNMT1 acting as a co-repressor to these factors in repressing target genes with important functions in hematopoietic lineage selection and differentiation.

2. Materials and methods

2.1. Plasmid constructs

Full length mouse Dnmt1 cDNA was PCR amplified and cloned into plasmid pTRE-AviTEV [37] and biotin (Avi)-tagged Dnmt1 was recloned in the erythroid specific expression vector pEV-Neo [38] and verified by sequencing. DNMT1 deletion mutants were kindly provided by Dr. *C. Cardoso* (TU, Darmstadt) [20]. The PBDQ162E DNMT1 mutant sequence was synthesized by Geneart® (Life Technologies) and cloned into the pMA-T vector. It was subsequently re-cloned as an *Eco*RI/*Bam*HI fragment in the pEMTPBDGFP plasmid [20].

2.2. Cell culture and cell transfections

MEL cells were cultured and induced to differentiate with DMSO as previously described [39]. A MEL cell clone expressing the BirA biotin ligase [36] was electroporated with linearized Avi-tagged DNMT1 pEV Neo vector and stable clones were double selected using G418 and puromycin (for BirA expression). Large scale cultures for nuclear extract preparation were carried out as previously described [37]. HEK 293 cells were cultured in DMEM High glucose medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (all from Life Technologies) and were transiently transfected using the calcium phosphate method.

2.3. Nuclear extracts

Nuclei from MEL cells and HEK 293 were prepared using the NP-40 lysis method, as previously described [40]. High salt extraction of purified nuclei from MEL cells was carried out as described previously [2]. Nuclei from HEK 293 cells were resuspended in RIPA/no SDS solution (50 mM Tris-HCl pH 7.5, 1%NP-40, 0.25% Na-Deoxycholate, 150 mM NaCl, 1 mM EDTA, 10% Glycerol with protease inhibitors or 1 mM PMSF) and nuclear proteins were extracted for 1 h at 4 °C on a rotating platform. The soluble nuclear extracts were separated from the insoluble pellets by centrifugation at 18,000 ×*g* for 30 min at 4 °C.

2.4. Western blotting

SDS-PAGE and Western immunoblotting were carried out as previously described [37]. Membranes were subjected to enhanced chemiluminescence (ECL prime, GE Healthcare). Streptavidin-HRP (NEL 750, Perkin Elmer) was used for the detection of biotin-tagged DNMT1 in nuclear extracts.

2.5. Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, *CA*): N6 GATA-1 (sc-265), M-20 GATA-1 (sc-1234), M-20 FOG-1 (sc-9361), A-20 FOG-1 (sc-9362), D-19 Gfi-1b (sc-8559), B-7 Gfi-1b (sc-28,356), H-242 Mi2-b (sc-11,378), 10E2 HDAC1 (sc-81,598), C-8 HDAC2 (sc-9959), B-2 GFP(sc-9996), N-19 RbAp48 (sc-8270), A-11 MTA1 (sc-17,773). Rabbit polyclonal DNMT1 antibody (1–248) was purchased from BioAcademia (Osaka, Japan). ZBP-89 rabbit polyclonal antibody was a generous gift from Dr. Alan Cantor (Boston's Children Hospital, MA, USA). Mouse monoclonal ZNF143 (M01) clone 2B4 (H00007702-M01) was purchased from Abnova (Taiwan), rabbit polyclonal ZNF143 was kindly provided by Gary R. Kunkel (Texas A&M University, TX, USA). GATA-1 rabbit polyclonal antibody (ab11852) was purchased from Abcam (Cambridge, UK). MTA2 and MBD2/3 rabbit polyclonal antibodies were kindly donated by Dr. Paul A. Wade (NIH/NIEHS, NC, USA). p66 (07–365) and MeCP2 (07–013) antibodies were purchased from Merck–Millipore (USA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Dako (DakoCytomation, Denmark) and Santa Cruz Biotechnology (Santa Cruz, *CA*).

2.6. Streptavidin pulldowns

Streptavidin pulldowns were done as previously described [37] using 50 μl of resuspended beads (Dynabeads® M-280, Invitrogen) per 1 mg of nuclear extract. Bound material was eluted by boiling for 10 min in 1× Laemmli sample loading buffer and analyzed by Western immunoblotting. For mass spectrometric (MS) analysis, 10-20 mg of nuclear extract were used per streptavidin pulldown. Prior to streptavidin pulldown, nuclear extracts were subjected to benzonase treatment for the removal of nucleic acids.