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

Dimitris N. Papageorgiou [a], Elena Karkoulia [a,1], Alexandra Amaral-Psarris [a], Pavel Burda [b], Katarzyna Kolodziej [c], Jeroen Demmers [d], Jörg Bungert [e], Tomas Stopka [f], John Strouboulis [a,*]

[a] Division of Molecular Oncology, Biomedical Sciences Research Center "Alexander Fleming", Vari, Greece
[b] Institute of Hematology and Blood Transfusion, Prague, Czech Republic
[c] Department of Cell Biology, Erasmus Medical Center, Rotterdam, The Netherlands
[d] Proteomics Center, Erasmus Medical Center, Rotterdam, The Netherlands
[e] Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, USA
[f] Biocev, 1st Medical Faculty, Charles University, Prague, Czech Republic

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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.

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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],

* Corresponding author at: Institute of Molecular Biology and Biotechnology, Foundation for Research & Technology Hellas, Heraklion, Crete, Greece.
E-mail address: john_strouboulis@imbb.forth.gr (J. Strouboulis).
[1] Present address: Institute of Molecular Biology and Biotechnology, Foundation for Research & Technology Hellas, Heraklion, Crete, Greece.

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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.

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2.7. Mass spectrometry

Mass spectrometry was carried out as previously described [34].

2.8. Immunoprecipitations

Immunoprecipitations were carried out as previously described [41]. Briefly, MEL nuclear extract (1 mg) was immunoprecipitated with 50 μl of paramagnetic protein A or G beads (Dynabeads® Life Technologies) and 8 μg of antibody crosslinked to the beads using 20 mM dimethyl pimelimidate (Sigma), by overnight incubation at 4 °C on a rotating platform. Bound material was eluted by boiling in 1× Laemmli buffer. For the GATA-1 immunodepletion a rat monoclonal antibody (sc-265) and a rabbit polyclonal antibody (ab11852) were used sequentially in order to completely deplete GATA-1 protein from the nuclear extract. The GATA-1 depleted nuclear extract was subsequently immunoprecipitated using a rabbit polyclonal DNMT1 antibody (70–201, Bioacademia). Immunoprecipitates, IgG controls and supernatants were all analyzed by Western immunoblotting. DNMT1 immunoprecipitations in K562 nuclear extracts were carried out using the *anti*-DNMT1 Ab13537 antibody (Abcam, UK).

2.9. Superose 6 gel filtration chromatography

Size fractionation of protein complexes was carried out as previously described [37].

2.10. ChIP and re-ChIP assays

Formaldehyde crosslinked chromatin from 10^7 induced MEL or K562 cells was prepared and used in ChIP assays as previously described [42]. ChIP enrichment and student *t*-test for a set of biological replicates assayed in triplicate by qPCR were done as previously described [43]. *Re*-ChIP analysis was done as previously described [44]. Antibodies: *anti*-DNMT1 (BioAcademia 70–201 with MEL chromatin; Abcam ab13537 with K562 chromatin), anti-ZBP89 (Abcam ab69933), anti-ZNF143 (clone 2B4, Abnova H00007702-M01), control IgG (Santa Cruz sc2027; NI01-EMB Biosciences). Primer sequences were as follows:

*mSpi*1-14/15 URE forward TAACCCCTGCACATGAAAGCC

mSpi-14/15 URE reverse TCTGGGCAGGGTCAGAGTGCC
mSpi1 negative forward GCATCTGGTGGGTGGACAAG
mSpi1 negative reverse GCGCGCCATCTTCTGGTA
hSpi1-17.5kb URE forward GGATGGCTGAGGTTGATGGTTGA
hSpi1-17.5kb URE reverse CAGCAGACAGGGATGAAGACAGAAGA
hSpi1 negative forward CCATAGCGACACAGAATAACATGGTGG
hSpi1 negative reverse GGCATGAGCCAACGCACAGG

2.11. Luciferase reporter assay

HEK cells were used in transient transfection assays as previously described [45]. Luciferase assays were done using the Dual-Luciferase Reporter Assay System (Promega) according to the supplier's instructions. The G1HE (124-235) luciferase reporter construct and the ZBP-89 expression vector were kindly provided by Dr. Masayuki Yamamoto (Tohoku University, Japan) [45]. GFP tagged DNMT1 expression vector was provided by Dr. *C. Cardoso* (see above). The GATA-1 cDNA was cloned into the pcDNA3.1 vector (Life Technologies). Plasmid pEGFP1-N1 (Life Technologies) was used as control. Luciferase assays were carried out as three different transfections (biological triplicates) with two technical replicates per transfection for each condition.

3. Results

3.1. DNMT1 co-purifies with hematopoietic transcription factors in MEL cells

In order to identify DNMT1 protein interacting partners in erythroid cells, we made use of the BirA-mediated biotin tagging system that we have previously employed for the characterization of nuclear protein complexes in the proerythroblastic mouse erythroleukemia (MEL) cell model, which can be chemically induced to undergo terminal erythroid differentiation [36,37,41]. A stable MEL cell clone expressing the *E. coli* BirA biotin ligase [36] was transfected with a construct bearing the Avi-tagged Dnmt1 mouse cDNA under the control of the human β-globin promoter and Locus Control Region [38]. Stable transfectants were screened for expression of Avi-tagged DNMT1 and one clone expressing high levels of tagged DNMT1 was selected for further experimentation (Suppl. Fig. 1A). We tested the efficiency of BirA-mediated biotinylation of tagged DNMT1 in this clone and found that the majority of tagged DNMT1 was captured by streptavidin paramagnetic beads (Suppl. Fig. 1A), suggesting very high efficiency of DNMT1 biotinylation tagging in this clone.

We next proceeded to isolate DNMT1 protein complexes from MEL nuclear extracts prepared from the aforementioned Avi-tagged DNMT1 MEL cell clone, induced to undergo terminal erythroid differentiation by treatment with DMSO for 72 h [37]. Nuclear extracts were subjected to streptavidin pulldown and bound material was eluted, resolved by SDS-PAGE and visualized by staining with Colloidal Blue. DNMT1 protein could be clearly seen in the lane of the eluted material, together with co-eluted proteins (Suppl. Fig. 1B). In order to identify the DNMT1 co-eluting proteins, the entire lane was excised, trypsinized and analyzed by mass spectrometry (MS) in three parallel experiments. The MS analysis revealed multiple nuclear factors co-eluting with DNMT1. Amongst them were known DNMT1 protein interactors such as, DMAP1, MeCP2, LSD1 and members of the NuRD complex (Table 1). As a control, interactions with MeCP2 and members of the NuRD complex in MEL cells were verified by streptavidin pulldowns of nuclear extracts expressing biotin tagged DNMT1 and, in addition, by immunoprecipitating endogenous DNMT1 protein in nuclear extracts from untransfected MEL cells (Suppl. Fig. 2). These findings validate our approach of DNMT1 biotinylation tagging in MEL cells for identifying protein interacting partners.

A striking observation from the MS analysis was the co-purification with DNMT1 of a number of transcription factors (Table 1). Amongst them are transcription factors that have been previously linked to hematopoiesis, such as Rbpj [46], Vav [47], NFI-A [48], NFI-X and YY1 [49], and factors that have been specifically linked to erythropoiesis, including GATA1 [50], GFI1-B [51], MYEF2 [52] and FOG-1 [53]. GATA1 and its close interacting partner, Friend of GATA1 (FOG-1) are essential for erythroid differentiation [54–56] [57] and together act as transcriptional repressors or activators, depending on target gene context [3]. The transcriptional repressor GFI1B has been previously shown to be essential for erythroid differentiation [58] and an interacting partner of GATA1 [41]. The MS analysis also identified the broadly expressed transcription factors ZBP-89 (also known as ZFP148) and ZNF143 (also known as ZFP143) as co-purifying with DNMT1. Interestingly, the *Zbp-89* gene trap in chimeric mice results in impaired erythropoiesis in the fetal liver [59], whereas morpholino knockdowns for *znf143* in zebrafish result in hematopoietic phenotypes as evidenced by a severe reduction in *gata1* expression [60]. Notably, both ZBP-89 and ZNF143 were previously associated with the GATA1/FOG-1 protein complex in erythroid and megakaryocytic cells ([59,61]). Thus, it appears that DNMT1 co-purifies with a number of transcription factors previously shown to be essential for hemato/erythropoiesis and which are known to physically interact with the erythroid master regulator GATA1. We therefore focused our subsequent analysis on DNMT1 interactions with the GATA1-related transcription factors. The complete MS analysis of

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Table 1
Selected proteins co-purifying with DNMT1 identified by mass spectrometry.

	Mass spec 1			*Mass spec 2*			*Mass spec 3*		
Protein identity	Mascot score	Unique peptides	Total peptides	Mascot score	Unique peptides	Total peptides	Mascot score	Unique peptides	Total peptides
DNMT1	9650	106	2690	8912	108	2579	7696	91	3261
PCNA	1326	16	128	883	12	60	785	10	50
MeCP2	169	3	3	505	6	6	–	–	–
DMAP1	222	5	5	–	–	–	1020	15	26
Lsd1	–	–	–	–	–	–	797	13	22
NuRD complex									
HDAC1	381	6	8	307	6	7	570	10	23
CHD4	278	4	4	1383	24	25	2471	40	110
Rbbp4	251	4	7	474	9	9	720	11	15
Rbbp7	251	4	7	425	7	7	463	8	12
Mta1	226	4	4	328	4	5	672	12	25
Mta2	58	2	2	199	3	4	691	10	15
HDAC2	–	–	–	249	5	6	630	10	17
Transcription factors									
Zbp-89	1341	15	38	889	12	29	1145	15	49
YY1	497	6	8	–	–	–	754	10	25
Gfi1-b	427	6	12	399	7	13	49	2	2
Rbpj	423	5	8	355	5	6	268	4	6
NFI-A	221	5	5	161	4	4	290	6	6
NFI-X	188	4	5	303	7	8	64	2	2
ZNF143	132	2	2	243	3	3	317	5	11
GATA-1	88	2	4	277	5	8	42	1	1
Vav1	–	–	–	162	3	3	278	5	6
FOG-1	–	–	–	–	–	–	185	2	3
Myef2	–	–	–	320	4	4	1101	14	15

DNMT1 co-purifying nuclear factors will be published elsewhere (in preparation).

3.2. DNMT1 interacts with hematopoietic transcription factors

To validate the interactions between DNMT1 and the hematopoietic transcription factors described above, we utilized streptavidin pulldowns of nuclear extracts from differentiated MEL cells expressing biotin-tagged DNMT1 (Fig. 1A) and co-immunoprecipitations in nuclear extracts from differentiated, non-transfected MEL cells (Fig. 1B). We were able to verify DNMT1 interactions with GATA1, ZBP-89, ZNF143 and GFI1B, whereas DNMT1 interaction with FOG-1 could only be confirmed by immunoprecipitation (Fig. 1B). Of the non-GATA1 interacting transcription factors tested, we were able to validate DNMT1 interaction with YY1, both by streptavidin pulldown and immunoprecipitation, but not for Vav, NFI-A, NFI-X or RBP-J (data not shown), which thus appear to co-purify with DNMT1 non-specifically. It is of interest that the endogenous DNMT1 protein appears to interact primarily with the long isoforms of FOG-1 and GFI1B which contain N-terminal repressor domains not present in their shorter isoforms [62,63]. As further validation, we carried out reverse immunoprecipitations using *anti*-ZNF143 and anti ZBP-89 antibodies (Fig. 1C,D). Indeed, we observed that ZNF143 interacts with DNMT1, ZBP-89, GATA1 and FOG-1 (the long isoform) but not with GFI1B. Similarly, ZBP-89 co-immunoprecipitated with GATA1 and the FOG-1 long isoform, as previously reported [59] and, additionally, with ZNF143 and DNMT1, but not with GFI1B (Fig. 1D). GATA1 immunoprecipitations shown in Fig. 3B further confirmed these findings (see also Fig. 4B). In conclusion, streptavidin pulldowns and immunoprecipitations combined

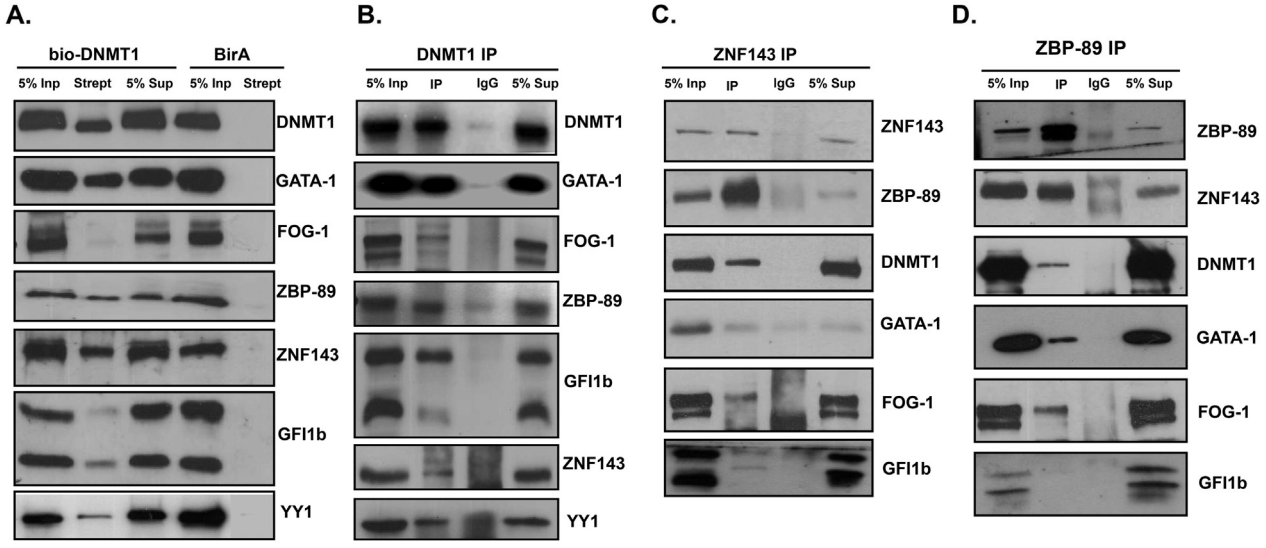

Fig. 1. DNMT1 interacts with several hematopoietic transcription factors. (A) Streptavidin pulldowns from nuclear extracts of DMSO induced MEL cells expressing biotinylated Avi-tagged DNMT1 or control cells expressing only BirA. Immunoprecipitations (IP) using DNMT1 (B), ZNF143 (C), or ZBP-89 (D) antibodies in nuclear extracts of DMSO induced non-transfected MEL cells. IgG lanes: normal isotype matched IgG control immunoprecipitations.

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with reverse immunoprecipitations confirm the initial observations that DNMT1 interacts with a number of GATA1-related hematopoietic transcription factors in MEL cells. These observations were further strengthened by the fractionation profiles of DNMT1, GATA1, ZNF143 and ZBP-89 proteins in differentiated MEL nuclear extracts size-fractionated by gel filtration using a Superose 6 column, which appear to overlap in high molecular weight fractions 21–25 (Suppl. Fig. 3). Of note also is the identical fractionation profile of biotin-tagged DNMT1, as detected by streptavidin-HRP, to that of the endogenous DNMT1 protein in non-transfected MEL nuclear extracts (Suppl. Fig. 3). Taken together, the co-immunoprecipitation experiments along with the overlapping gel filtration fractionation profiles confirm the capacity of these proteins to interact in stable protein complexes in MEL cells.

3.3. DNMT1 forms subcomplexes with hematopoietic transcription factors

The reverse immunoprecipitations in Fig. 1C-D suggested that DNMT1 can undergo non-overlapping interactions with transcription factors ZBP-89/ZNF143 and GFI1B, despite the fact that these transcription factors also interact with GATA1 in MEL cells. Consequently, we wished to investigate further the role of GATA1 in the formation of DNMT1 complexes with other transcription factors. To this end, we first immunodepleted GATA1 from the nuclear extracts of differentiated MEL cells, followed by DNMT1 immunoprecipitation and assaying for co-immunoprecipitation of GATA1 and DNMT1 interacting transcription factors (Fig. 2A). In order to completely immunodeplete GATA1, we carried out two sequential immunoprecipitations using two different *anti*-GATA1 antibodies which, together, removed practically all of the GATA1 protein from the nuclear extract (lanes 5 and 6, Fig. 2B). Analysis of the GATA1 immunoprecipitates confirmed the GATA1 interactions with DNMT1 and also with ZNF143, ZBP-89, GFI1B, FOG-1 and members of the NuRD complex CHD4 and HDAC1 (Fig. 2B, lanes 2 and 3), as also shown previously [41,59]. Interestingly, immunoprecipitation of the DNMT1 protein in the GATA1-immunodepleted nuclear extract (lane 4, Fig. 2B) showed that DNMT1 could still co-immunoprecipitate with ZBP-89, ZNF143 and trace amounts of CHD4 and HDAC1, but not with GATA1, GFI1B or FOG-1, presumably because the fraction of DNMT1 protein complexes containing these factors had been removed in the GATA1 immunodepletion steps. Taken together, these data suggest that DNMT1 can form two distinct subcomplexes with ZBP-89 and ZNF143 transcription factors, with or without GATA1 and FOG-1. In addition, as ZBP-89 and ZNF143 do not appear to interact with GFI1B (Fig. 1C,D) and given that it has been previously shown in MEL cells that GATA1 interacts with GFI1B independently of FOG-1 [41], we propose that DNMT1 forms two additional subcomplexes with GATA1/FOG-1/ZBP89/ZNF143 and with GATA1/GFI1B (see Discussion and Fig. 7). These DNMT1 subcomplexes may be dynamic during MEL cell differentiation as ZBP-89 protein levels decline markedly, whereas ZNF143 levels appear to increase and DNMT1 protein levels remain constant in MEL cells induced to undergo terminal differentiation (Suppl. Fig. 4). It is also of interest that the majority of the NuRD components CHD4 and HDAC1 interacting with DNMT1, were immunoprecipitated in the GATA1 immunodepletion step (lanes 2–4, Fig. 2B), suggesting that GATA1 interactions with DNMT1, presumably including FOG-1 [41,64], may also involve the NuRD complex.

3.4. DNMT1 interacts with ZBP-89 and ZNF143 in non-hematopoietic cells

ZBP-89 and ZNF143 are broadly expressed transcription factors with conserved functions extending beyond the hematopoietic system [65,

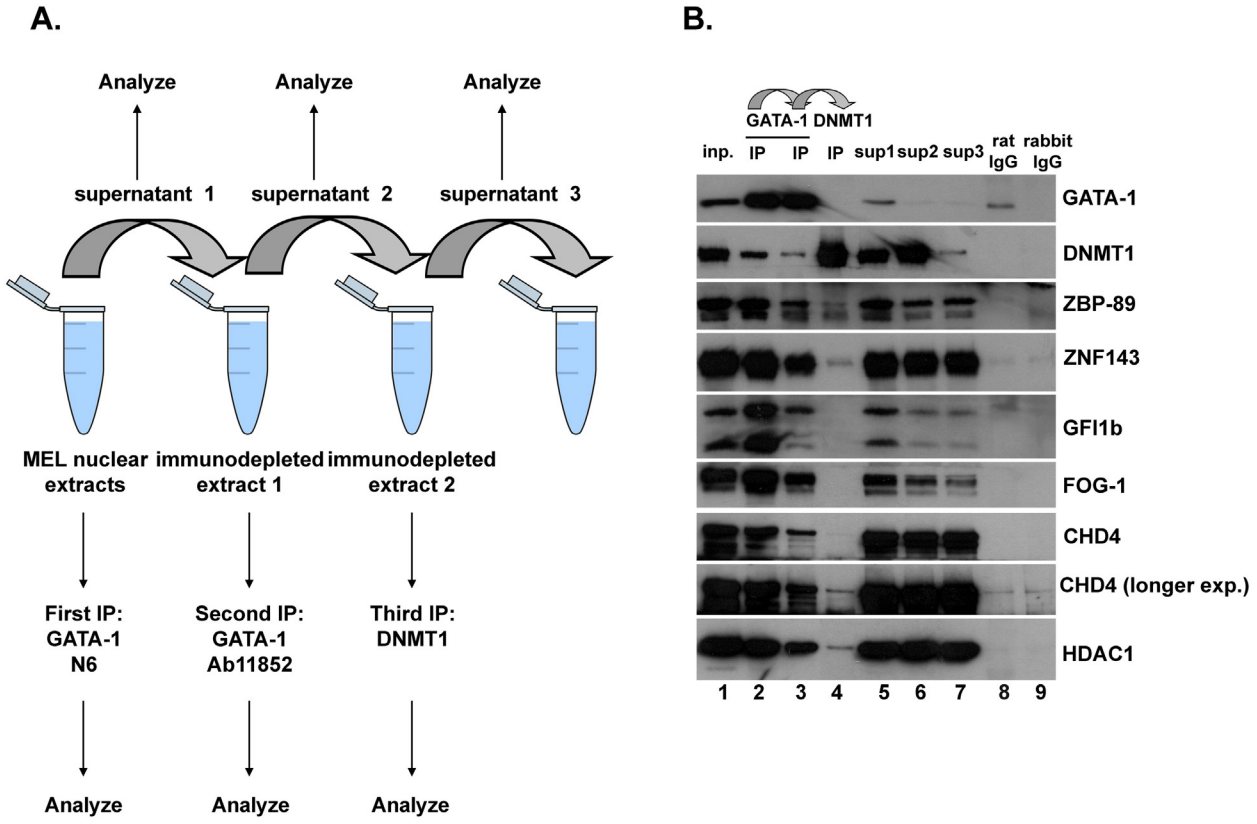

Fig. 2. Sequential immunodepletion and immunoprecipitation experiments show that DNMT1 forms distinct complexes with hematopoietic transcription factors in MEL cells. (A) Schematic representation of the GATA-1 immunodepletion/DNMT1 immunoprecipitation experiment. (B) Sequential immunodepletions of GATA1 from nuclear extracts of DMSO induced MEL cells using a monoclonal and a polyclonal GATA-1 antibody followed by *anti*-DNMT1 immunoprecipitation. All immunoprecipitates were assayed for the presence of the proteins indicated to the right of the panel. In addition, all immunodepleted supernatants (sup1–3) were assayed for the efficiency of immunoprecipitations. MEL nuclear extracts were also incubated with isotype matched normal IgGs as control.

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66]. Given also that DNMT1 is ubiquitously expressed, we tested whether the DNMT1 interactions with ZBP-89 and ZNF143 also existed in non-hematopoietic cells. To this end, we used the HEK293 human embryonic kidney cell line that expresses DNMT1, ZBP-89 and ZNF143 (Fig. 3). Immunoprecipitation of HEK293 nuclear extracts with an *anti*-DNMT1 antibody co-precipitated endogenous ZBP-89 and ZNF143, as well as the NuRD members CHD4 and HDAC1 (Fig. 3A). These data show that the DNMT1/ZBP-89/ZNF143 complex also exists in non-hematopoietic cells of human origin, thus suggesting broad, conserved functions for this complex. Interestingly, when HEK293 cells were co-transfected with Avi-tagged GATA1 and BirA biotin ligase followed by streptavidin pulldown of biotinylated GATA1, we observed that exogenously expressed GATA1 was also capable of interacting with the endogenous DNMT1/ZBP-89/ZNF143 complex (Fig. 3B), suggesting that it is recruited to this complex in the absence of other erythroid specific co-factors. By contrast, transfected FOG-1 or GFI1b failed to interact with the DNMT1/ZBP-89/ZNF143 complex in HKE293 cells, even in the presence of co-transfected GATA1 (data not shown) suggesting that additional, as yet unidentified, hematopoietic specific co-factors and/or tissue-specific post-translational modifications may be needed for establishing interactions with DNMT1.

3.5. The 17 amino acid PCNA binding domain (PBD) of DNMT1 mediates interactions with ZBP-89, ZNF143 and GATA1

The DNMT1 protein consists of a number of subdomains reported to mediate multiple interactions with several protein partners (see Introduction and recently reviewed in [67]). In order to identify which of the DNMT1 protein subdomain(s) is responsible for mediating the interactions described here with transcription factors ZBP-89, ZNF143 and GATA1, we utilized several DNMT1 deletion constructs tagged by fusion with GFP [20] (Fig. 4A). Each of these deletion mutants was co-transfected with a GATA1 expression vector in HEK293 cells. Nuclear extracts isolated from these transient transfectants were subjected to GATA1 immunoprecipitations followed by Western blot analysis using *anti*-GFP antibody to detect the DNMT1 deletion mutants (Fig. 4B). From these experiments, it can be seen that, unlike all other DNMT1 mutants tested, the DNMT1 deletion mutant lacking the short (17 amino acid) PCNA binding domain (PBD; construct GMTΔPBD) could no longer interact with GATA1 (Fig. 4B), thus suggesting that is essential for mediating DNMT1 interactions with GATA1. In order to exclude the possibility that the GATA1-DNMT1 interaction mediated by the PBD is somehow related to the DNA replication machinery, we co-transfected GATA1 with a construct that carries a mutant PBD domain bearing a single amino acid substitution at Q162E, which abrogates interaction of DNMT1 with PCNA (Fig. 4A, construct PBDQ162E–GFP) [68]. Fig. 4B shows that transfected GATA1 can clearly immunoprecipitate co-transfected PBDQ162EGFP protein, thus confirming that thePBD is the DNMT1 domain responsible for the interaction with GATA1. It should be noted that transfected GATA1 can also interact with endogenous DNMT1, ZBP-89 and ZNF143 proteins in HEK293 cells (Fig. 4A, lower panels).

We also tested in transiently transfected HEK293 cells whether the DNMT1 deletion mutants could interact with the endogenous ZBP-89 and ZNF143 proteins (Fig. 4C-D). As for GATA1, we also found using ZBP-89 or ZNF143 immunoprecipitations that the PBD domain of DNMT1 is necessary for interactions with these factors (Fig. 4C,D). In conclusion, our data demonstrate that the relatively short PBD domain, in addition to its function in mediating DNMT1 interactions with PCNA, is also responsible for mediating interactions with specific transcription factors such as GATA1, ZBP-89 and ZNF143. Furthermore, the interactions of DNMT1 with ZBP-89 and ZNF143 were not dependent on GATA1.

3.6. The DNMT1/ZBP-89/GATA1 complex binds to the repressed PU.1 locus in human erythroleukemic cells

We next assessed whether DNMT1 interactions with GATA1 and ZBP-89 and ZNF143 are also observed in chromatin in a specific gene context. We recently demonstrated that GATA1 recruits DNMT1 to the gene locus for the myeloid transcription factor PU.1 (SPI1) to repress its transcription in human erythroleukemic K562 cells [69]. Specifically, it was shown that the Upstream Regulatory Element (URE) located approximately 17 kb upstream of the PU.1 transcription start site (TSS) was chromatin immunoprecipitated (ChIP) using GATA1 and DNMT1 antibodies [69]. We thus tested whether GATA1 and DNMT1 bound to the PU.1 URE in the context of the DNMT1/ZBP-89/ZNF143 protein subcomplex we describe here. We first established by immunoprecipitation that DNMT1 interacts with ZBP-89 and ZNF143 in nuclear extracts from K562 cells (Fig. 5A-B). We next used ChIP to demonstrate interactions for DNMT1, ZBP-89 and ZNF143 on DNA (Fig. 5C) followed by ChIP-reCHIP to show co-occupancy of the PU.1 URE by ZBP-89 and DNMT1 (Fig. 5D), thus confirming that DNMT1 (and presumably GATA1 [69]) and ZNF143) bind to the PU.1 URE in K562 cells as a complex with ZBP-89.

It was recently shown in another study that ZBP-89 binds to the URE in the mouse PU.1 gene locus in MEL cells [70]. Given our findings with the PU.1 URE in human K562 erythroleukemic cells, we tested whether DNMT1 binding was also present at the murine URE in MEL cells and, indeed, ChIP assays using chromatin from differentiated MEL cells confirmed DNMT1 binding to the murine PU.1 URE (Fig. 5E). In addition, we were able to detect ZNF143 binding to the same region in the murine PU.1 URE (Fig. 5E).

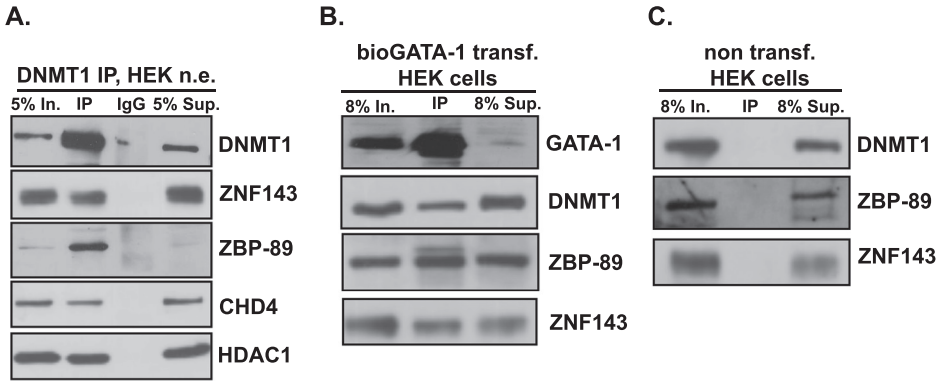

Fig. 3. DNMT1 interacts with ZBP-89 and ZNF143 in HEK 293 cells. (A) DNMT1 immunoprecipitation along with isotype matched IgG from nuclear extracts of HEK 293 cells shows co-immunoprecipitation of ZNF143, ZBP-89 and members of the NuRD complex (HDAC1 and CHD4) as positive control. (B) Nuclear extracts from HEK 293 cells co-transfected with constructs expressing Avi-tagged GATA-1 protein and BirA were used for streptavidin pulldowns to show GATA1 interactions with endogenous DNMT1, ZNF143 and ZBP-89 proteins. (C). Control streptavidin pulldowns from non-transfected HEK293.

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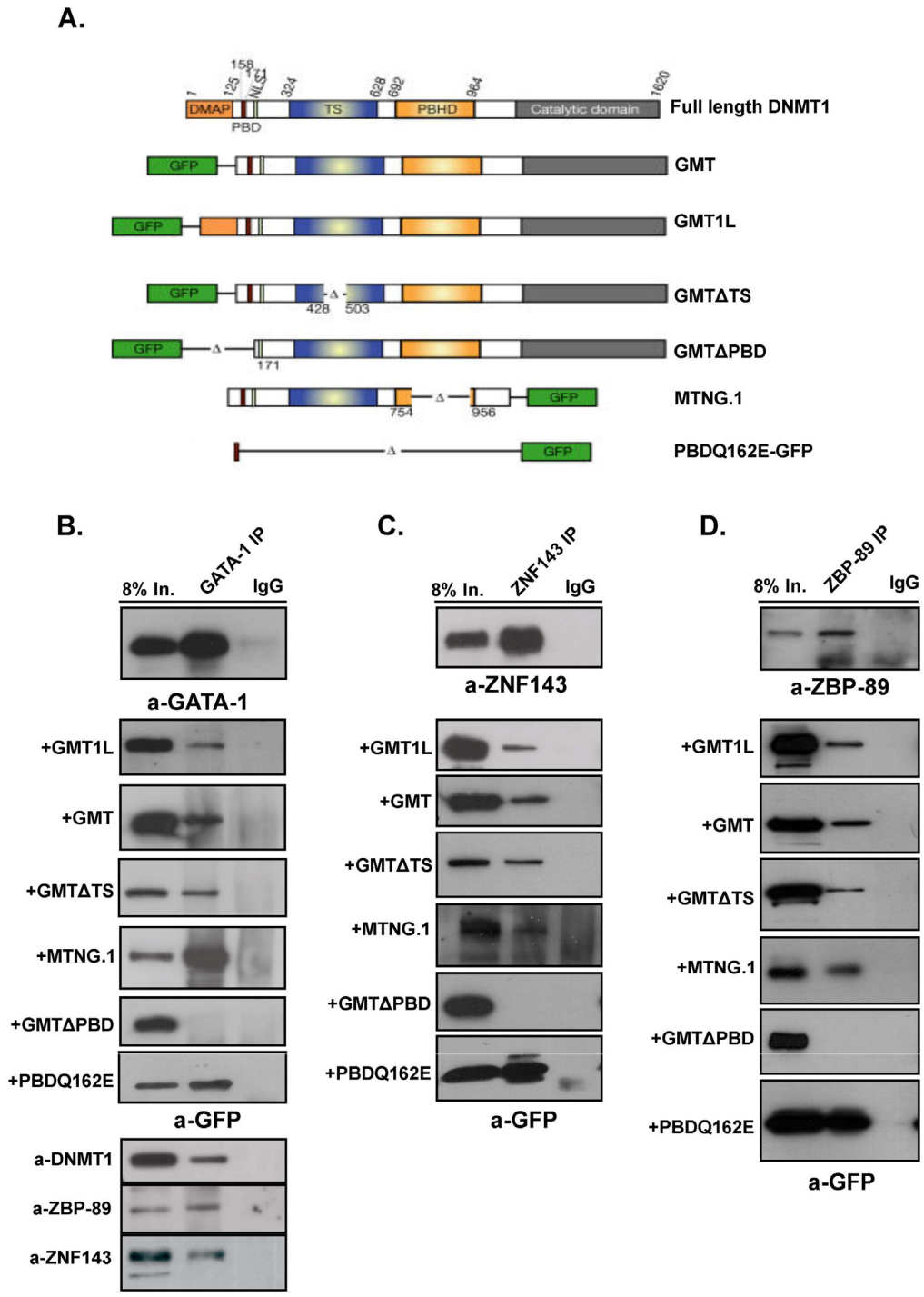

Fig. 4. The 17 amino acid PCNA binding domain (PBD) of DNMT1 mediates interactions with ZBP-89, ZNF143 and GATA1 in HEK 293 cells. (A) Schematic representation of the DNMT1 deletion mutants used. DMAP: DMAP1 transcriptional repressor interacting domain; PBD: PCNA binding domain; NLS: Nuclear localization signal; TS: Targeting sequence; PBHD: Polybromo homology domain. (B) Immunoprecipitation of GATA1 from nuclear extracts of HEK 293 cells transiently co-transfected with vectors expressing GATA1 and each one of the GFP tagged DNMT1 deletion mutants shown in panel A. Immunoprecipitates were detected with anti-GATA1 (top panel), anti-GFP (middle panels) and anti-DNMT1, anti-ZBP-89 and anti-ZNF143 antibodies (lower panels). (C) Immunoprecipitation of endogenous ZNF143 and (D) ZBP-89 from nuclear extracts of HEK 293 cells transfected with vectors expressing the GFP tagged DNMT-1 deletion mutants shown in panel A.

3.7. DNMT1 serves as a co-repressor to ZBP-89 and GATA-1

Previous work by Ohneda et al. on the functional dissection of a hematopoietic specific enhancer known as G1HE located upstream of the mouse GATA1 gene locus, identified a small fragment in the 5′ region of the G1HE (nucleotides 124–135) which included binding sites for ZBP-89 and GATA1 (Fig. 6A) [45]. The G1HE (124–235) fragment was capable of mediating transcriptional upregulation of a linked luciferase reporter when co-transfected with ZBP-89 [45]. Interestingly, co-transfection of GATA1 together with ZBP-89 led to significant inhibition of the transactivation of the luciferase reporter compared to ZBP-89 alone [45]. Given that we identified DNMT1 interactions with ZBP-89 and GATA1 in MEL cells, we wanted to test whether DNMT1 was implicated in the repression of the G1HE (124–235) luciferase reporter when co-transfected with ZBP-89 and GATA1. We thus repeated the transfection of the G1HE (124–235) luciferase reporter in HEK293 cells and

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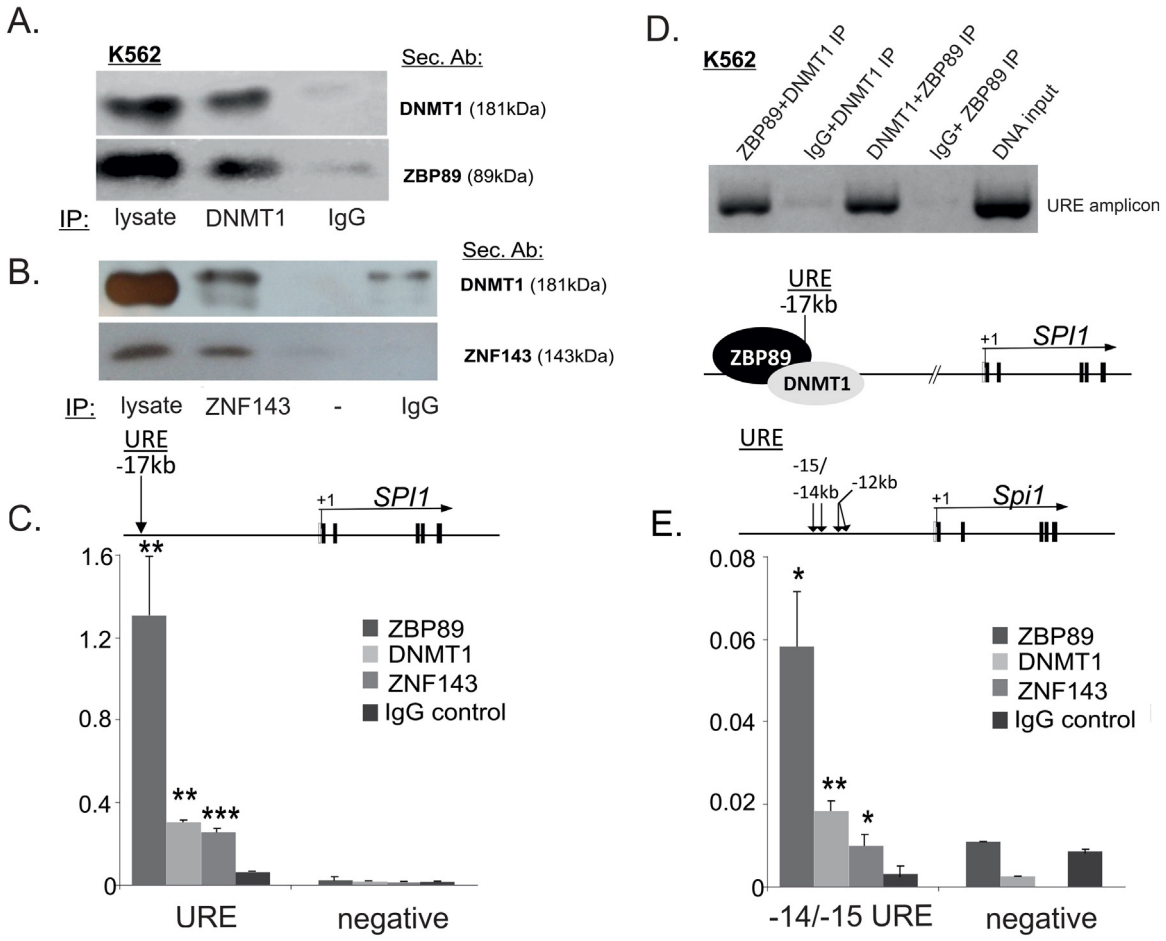

Fig. 5. (A) DNMT1 immunoprecipitates ZBP-89 in nuclear extracts from human erythroleukemic K562 cells (middle lane). (B) ZNF143 immunoprecipitates DNMT1 in nuclear extracts from human erythroleukemic K562 cells (second lane). Lysate: input nuclear extract; IgG lane: control immunoprecipitation using isotype-matched immunoglobulins. (C) ChIP assay showing DNMT1 and ZBP-89 binding to the -17 kb upstream regulatory element (URE) of the human PU.1 (*SPI1*) gene locus in crosslinked chromatin from K562 cells. An unrelated sequence from elsewhere within the locus was used as negative control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as obtained by *t*-test compared to the negative control ChIP. (D) ChIP-reChIP assays showing co-binding of DNMT1 and ZBP-89 to the -17 kb URE of the PU.1 (*SPI1*) gene locus in K562 cells. ChIP-reChIP was done both ways using DNMT1 or ZBP-89 antibody in the first ChIP experiment. (E) ChIP assayshowing binding of DNMT1 and ZBP-89 to the murine −14/−15 kb URE of the PU.1 (*Spi1*) gene locus in crosslinked chromatin from mouse erythroleukemic MEL cells. Negative control represents ChIP using an unrelated sequence from the *Spi1* gene locus. * $p < 0.05$, ** $p < 0.01$ as obtained by t-test compared to the negative control ChIP.

indeed showed, as before [45], that transfecting ZBP-89 with the reporter plasmid led to an approximately 4-fold transcriptional activation compared to the reporter vector alone (Fig. 6B). Co-transfection of GATA1 with ZBP-89 leads to down regulation of ZBP-89 transactivation of the reporter construct and this downregulation is further enhanced by co-transfection of DNMT1 together with ZBP-89 and GATA1 (Fig. 6B). These observations support the notion that ZBP-89 and GATA1 recruit DNMT1 to the reporter plasmid to repress transcriptional activity even further, an effect that appears to be specific and not due to the GFP fusion (Fig. 6B).

4. Discussion

Previous work in mouse erythroleukemic (MEL) cells identified DNMT1 as a protein interacting partner of the TR2/TR4 nuclear receptor complex implicated in human γ-globin gene repression in the adult stage or erythropoiesis [34]. In addition, several lines of evidence have implicated DNA methylation in the regulation of globin gene expression and of erythroid differentiation in general [33]. In an effort to shed more light as to potential DNMT1 functions in erythropoiesis, we used a biotinylation tagging approach to characterize DNMT1 protein complexes in MEL cells. We identified by mass spectrometry a large number of potential DNMT1 interacting nuclear proteins involved in chromatin structure and modification, in chromosome structure, or in nuclear functions such as DNA replication (manuscript in preparation). Here, we focused on a number of transcription factors with established roles in hemato/erythropoiesis, which we identified as co-purifying with DNMT1. These include GATA1, a critical transcription factor for erythroid differentiation and transcription factors ZBP-89, ZNF143, GFI1b and FOG-1, all previously reported to interact with GATA1 and to fulfill important functions in erythroid differentiation [41,51,53,57,59–61]. We did not identify by mass spectrometry TR2/TR4 or BCL11A co-purifying with DNMT1. We suggest that is due to a lower abundance of the subcomplexes that DNMT1 forms with these factors which, in the case of TR2/TR4, has previously necessitated enrichment by fractionation using size exclusion chromatography [34].

We validated the DNMT1 interactions with the GATA1-related transcription factors by co-immunoprecipitations of the endogenous proteins in MEL cells and, further, we identified the 17 amino acid PCNA binding domain (PBD) as being responsible for mediating DNMT1 interactions with these factors. In addition, we show that the DNMT1/GATA-1/ZBP-89 interactions are also present in human K562 erythroleukemic cells (Fig. 5A and Burda et al. [69]) and, furthermore, we show that DNMT1 also interacts with the broadly expressed ZBP-89 and ZNF143 transcription factors in non-hematopoietic human cells. Lastly, sequential immunodepletion/immunoprecipitation experiments suggested that DNMT1 forms distinct protein subcomplexes with these transcription factors in MEL cells. Based on these observations, we suggest that

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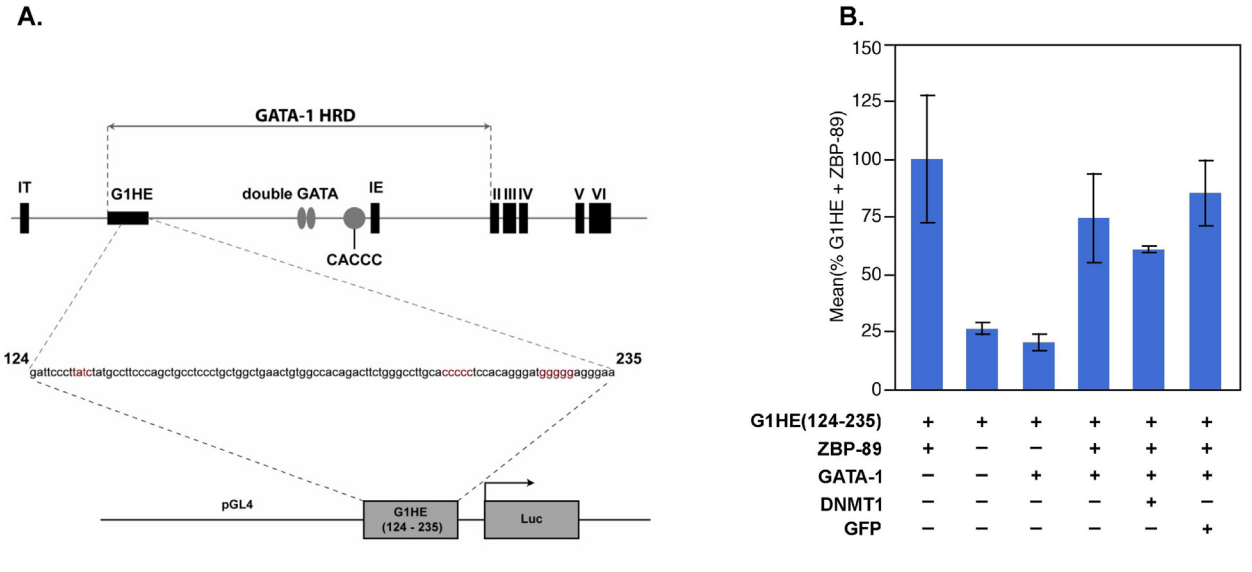

C.

	G1HE(124-235) + ZBP-89 + GATA1 (lane 4)	G1HE(124-235) + ZBP-89 + GATA1 +DNMT1 (lane 5)	G1HE(124-235) + ZBP-89 + GATA1 +GFP (lane 6)
G1HE(124-235) + ZBP-89 (lane 1)	p = 0.0002	p < 0.0001	p = 0.0114
G1HE(124-235) + ZBP-89 + GATA1 +GFP (lane 6)	p = 0.0510	p = 0.0003	n.a.

Fig. 6. Luciferase assays showing that DNMT1 co-expression with ZBP-89 and GATA-1 leads to greater repression of the G1HE (124–235) reporter activity. (A) Schematic representation of the luciferase reporter construct. The GATA1 gene locus is shown, indicating the G1HE hematopoietic enhancer. Shown below are nucleotides 124–235 of the G1HE, which were cloned into a luciferase reporter plasmid. The ZBP-89 and GATA1 binding motifs in the 124–235 fragment are highlighted in red. (B) Luciferase reporter assays of cell lysates of HEK 293 cells co-transfected with the G1HE (124–235) luciferase reporter and various expression constructs expressing ZBP-89, GATA-1, GFP-DNMT1, GFP and Renilla luciferase as a control plasmid. Luciferase activity is shown as a percentage of expression of the luciferase reporter co-transfected with ZBP-89. (C) Table showing pairwise statistical significance between selected luciferase assays shown in panel B, as obtained by t-test. The pairwise comparison of the luciferase reporter alone (lane 2) to the luciferase reporter with GATA1 (lane 3) was not statistically significant ($p = 0.26$).

DNMT1, ZBP-89 and ZNF143 form a "core" complex that exists in different cell types, for example in HEK293 cells as we show here and in hepatoma cells, as previously reported [71]. ZBP-89 was consistently identified by MS as being the most abundant factor that co-purifies with DNMT1 from MEL cells (Table 1), leading us to suggest that it is ZBP-89 that contacts DNMT1 in the core complex, pending further experimental validation.

DNMT1 immunoprecipitations in MEL cells show that the DNMT1/ZBP-89/ZNF143 core complex interacts with the hematopoietic lineage-restricted GATA1 and FOG-1 transcription factors (Fig. 1). Moreover, immunodepletion experiments show that the DNMT1 interactions with ZBP-89 and ZNF143 cannot be completely depleted by GATA1 antibodies (Fig. 3B, lanes 2–4) suggesting that only a fraction of the DNMT1/ZBP-89/ZNF143 core complex forms also a complex with GATA1/FOG-1. In addition, GFI1B forms a complex with DNMT1 independently of the core complex with ZBP-89 and ZNF143, since GFI1B does not appear to be immunoprecipitated by antibodies against the latter two factors (Fig. 1C-D). Since the GFI1B/DNMT1 subcomplex is depleted by GATA1 immunoprecipitation (Fig. 3B, lanes 2–4), we surmise that the GFI1B interaction with DNMT1 includes GATA1. Taking into account previous observations showing that GATA1 interactions with FOG-1 and GFI1B are non-overlapping [41], we propose a model whereby DNMT1 forms at least three partially overlapping subcomplexes with specific transcription factors in MEL cells: a core complex with ZBP-89 and ZNF143, which also exists in non-hematopoietic cells, a second complex with ZBP-89, ZNF143, GATA-1 and FOG-1 and a third complex with GATA1 and GFI1B (Fig. 7). It is possible that an additional complex of DNMT1 with ZBP-89 and YY1 may also exist in MEL cells, as ZBP-89 and YY1 have been previously shown to interact in myoblasts [72]. Our data on DNMT1 interactions with hematopoietic transcription factor interactions significantly expand the list of transcription factors reported to interact with DNMT1 which previously included Sp1, E2F1, SNAIL1 and others (reviewed in [73]). We show here that DNMT1 serves as co-repressor of ZBP-89 and GATA1 in repressing reporter gene expression mediated by the G1HE, an upstream regulatory element of the murine GATA1 gene locus which includes two binding sites for ZBP-89 and one for GATA1 (Fig. 6A). It is of interest that in HEK 293 cells, and despite the presence of an endogenous ZBP-89/DNMT1 complex (Fig. 3), transiently transfected ZBP-89 acts as an activator leading to an approximately 4-fold transactivation of the G1HE luciferase reporter plasmid (Fig. 6B). By contrast, co-transfection of GATA1 with ZBP-89 leads to the down regulation of ZBP-89 mediated transactivation of the reporter construct, which is further enhanced by co-transfection of DNMT1 together with ZBP-89 and GATA1 (Fig. 6B). These data suggest that in the presence of GATA1 there is a shift from activating to repressive protein complex(es) recruited by ZBP-89 to the reporter construct, also involving DNMT1 as a co-repressor. In addition, we recently showed that GATA1 recruits DNMT1 to the URE to repress PU.1 expression in human erythroleukemic cells [69]. Our observations add to previous studies describing repressive functions for DNMT1 in cooperation with specific transcription factors in different cellular contexts (for example: [28,74–76]) and further support a role of DNMT1 as a co-repressor in transcriptional regulation, in addition to its functions in maintaining DNA methylation in replication or DNA repair.

In this study, we showed that ZBP-89 is also part of the DNMT1/GATA1 complex previously reported by Burda et al. [69] and is recruited to the URE in the *PU.1 (SPI1)* gene locus in human erythroleukemic K562 cells. In addition, we showed DNMT1 and ZBP-89 co-occupancy of the URE in the murine *Pu.1 (Spi1)* gene locus in MEL cells. Interestingly, DNMT1 and ZBP-89 binding to the mouse *Pu.1* URE in murine erythroid cells does not appear to involve GATA1, in contrast to what was seen in the human *PU.1* URE in K562 cells [69]. We interpret this as

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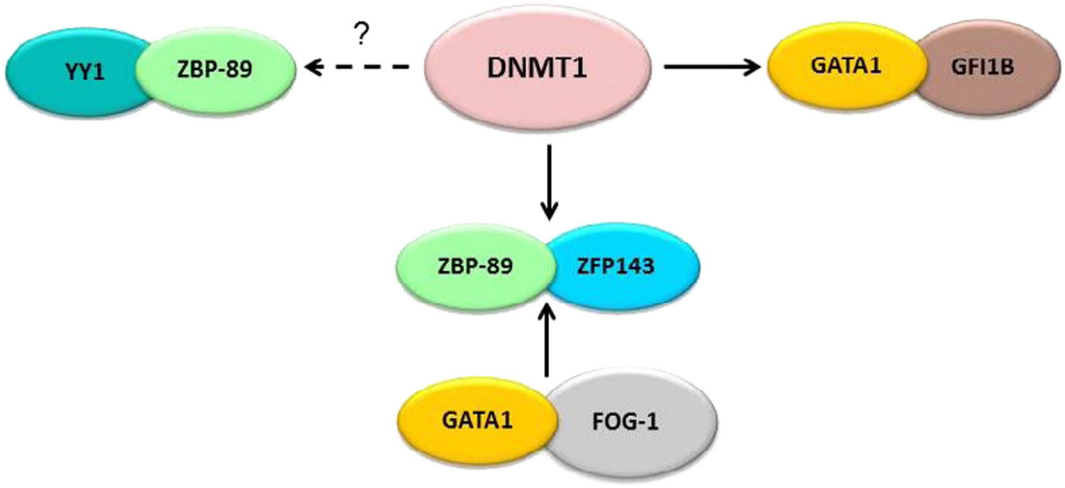

Fig. 7. A model for the DNMT1 protein subcomplexes with transcription factors described here. See main text for more details.

evidence for the "core" DNMT1/ZBP-89 complex binding to the mouse *Pu.1* URE in MEL cells and for the DNMT1/ZBP-89/GATA1 subcomplex binding to the human *PU.1* URE in K562 cells, suggesting differences in the DNMT1/ZBP-89 mediated repression in human *versus* murine erythroid cells.

An intriguing finding of our study was the identification of the small (~17 amino acid) PCNA binding domain (PBD) as being necessary for mediating the interactions between DNMT1 and the transcription factors described here. The PBD is located near the N-terminus of the DNMT1 protein and is required for direct interaction of DNMT1 with PCNA and recruitment to replication foci early during S phase [20,68,77]. It is also required for the recruitment of DNMT1 by PCNA to DNA damage sites in the nucleus [78]. The PBD-mediated DNMT1 interaction with PCNA does not appear to be critical for the maintenance of methylation patterns of newly replicated DNA [68], nor is the PBD essential for DNMT1 methyltransferase activity, though it does appear to augment it [79]. The PBD includes the conserved PIP Box, a short (8 amino acids) PCNA-interacting peptide motif which is present in many PCNA interacting proteins [80,81]. Interestingly, we showed that a single amino acid change within the PIP Box that abolishes DNMT1 interactions with PCNA, did not affect DNMT1 interactions with the transcription factors described here. Thus, our data suggest additional, important functions for the PBD in transcriptional regulation that are independent of the PCNA-mediated DNMT1 functions in DNA replication and repair. It is plausible, for example, that PBD-mediated interactions with transcription factors could serve to recruit DNMT1 to specific gene targets, leading to their transcriptional repression.

The interactions of DNMT1 with transcription factors with established roles in erythropoiesis, hint at potentially important erythroid functions for DNMT1 itself. This is in line with the observation that DNMT1 is the only DNA methyltransferase that is expressed throughout murine fetal liver derived erythroid differentiation [82]. Importantly, it has been reported that the conditional knockout of the murine Dnmt1 gene in the erythroid lineage using GATA1-Cre or EpoR-Cre deleter mice resulted in embryonic lethality [31,35]. In addition, shRNA knockdown experiments have shown DNMT1 to be implicated in fetal γ-globin silencing in adult CD34+ derived human erythroid cells [35] and in mouse embryonic βh1 globin silencing in MEL cells [76]. DNMT1 knockdown experiments in mouse fetal liver-derived erythroid cells showed an acceleration of erythroid specific gene activation [82], whereas our own unpublished work using DNMT1 knockdown in MEL cells also hints at important DNMT1 functions in regulating cell cycle exit in terminal erythroid differentiation (Karkoulia et al., in preparation). Future work will dissect the contribution of the DNMT1/transcription factor complexes described here in regulating specific aspects of DNMT1 functions in erythroid differentiation and gene expression.

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Conflict of interest

The authors have no conflicts of interest to declare.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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