Master Thesis

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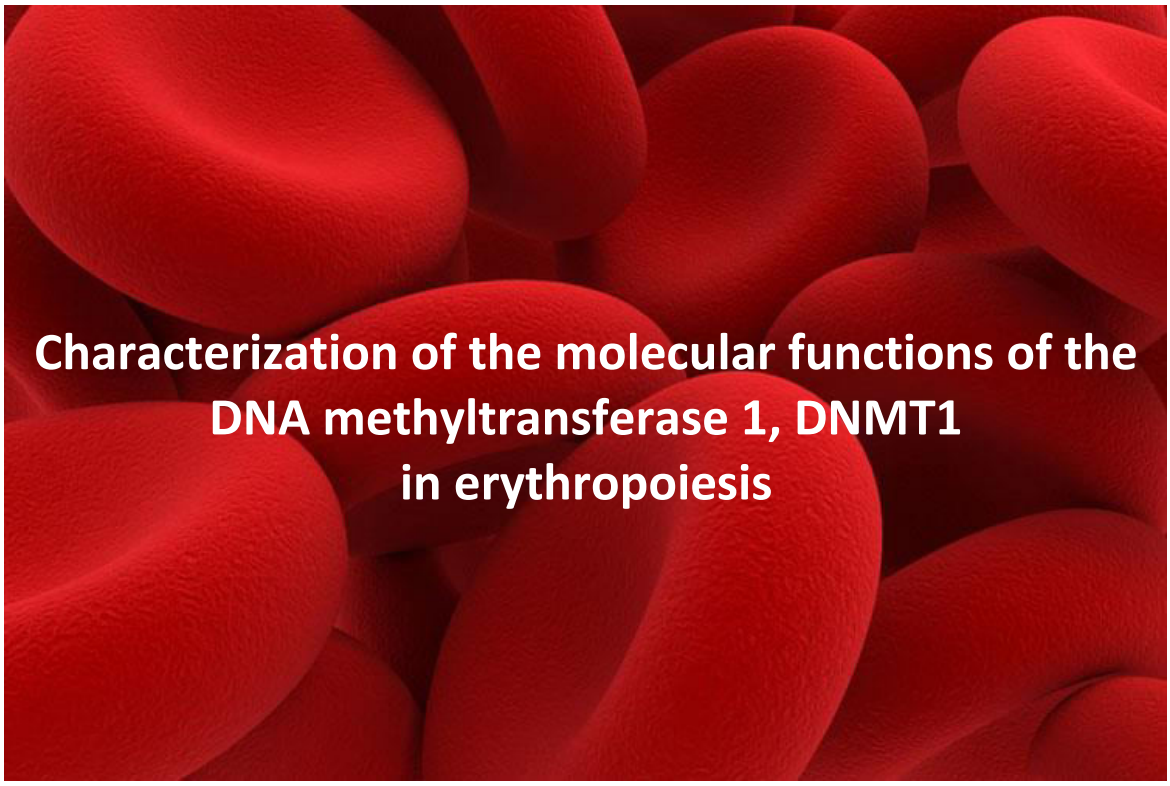

Characterization of the molecular functions of the DNA methyltransferase 1, DNMT1 in erythropoiesis

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1. Summary

DNA methylation is essential for embryonic development as well as in diverse biological processes. The DNA methyltransferase Dnmt1 is responsible for maintaining the methylation patterns on daughter strands after DNA replication, but it is also known for mediating repression. DNMT1 knock out/down (KO/KD) studies in hematopoietic lineages or in mice, revealed that DNMT1 drives HSCs to myeloerythroid fate. Previous publications of our laboratory showed that DNMT1 interacts with several erythroid transcription factors (GATA1, FOG-1, GFI-1b) forming a core complex composed by DNMT1 and transcription factors ZBP89 and ZNF143, which interacts with distinct hematopoietic protein subcomplexes. Additionally, the PCNA Binding domain (PBD) of DNMT1 proved necessary and sufficient for DNMT1 interaction with these transcription factors. Moreover, DNMT1 KD experiments in murine erythroleukemic (MEL) cells revealed defects in cell cycle arrest in erythroid differentiation due to impaired repression of genes responsible for cell proliferation. The main aim of this study is to further elucidate the role of DNMT1 in erythroid cell differentiation. To these ends, I carried out a phenotypic characterization of DNMT1 KO MEL cells previously generated by CRISPR/Cas9. It was found that DNMT1 KO cells display a phenotype similar to the knockdown experiments concerning cell cycle progression and impaired erythroid differentiation.

In addition, previous studies showed that DNMT1 is involved in the repression of γ-globin gene expression in adult-stage erythroid cells by methylating its promoter. Furthermore, several studies have suggested that DNMT1 associates directly or indirectly with BCL11A, a factor known to be responsible for silencing the expression of mouse embryonic b-like globins (εγ and βh1) as well as of the human γ-globin gene. Following these data, we investigated the DNMT1 interactions with BCL11A, our hypothesis being that DNMT1 acts as a co-repressor of these genes during erythropoiesis.

1. Περίληψη

Η μεθυλίωση του DNA είναι κρίσιμη για την εμβρυϊκή ανάπτυξη, καθώς και για ποικίλες βιολογικές διαδικασίες. Η DNA μεθυλτρανσφεράση DNMT1 είναι υπεύθυνη για τη διατήρηση των μοτίβων μεθυλίωσης στις θυγατρικές αλυσίδες του DNA μετά την αντιγραφή, αλλά είναι επίσης γνωστό ότι ρυθμίζει τη γονιδιακή έκφραση ως καταστολέας. Μελέτες με DNMT1 knock out/down (KO/KD) σε αιμοποιητικές σειρές ή σε ποντίκια, αποκάλυψαν ότι η DNMT1 οδηγεί τα αιμοποιητικά βλαστοκύτταρα σε μυελοερυθροειδική μοίρα. Προηγούμενες δημοσιεύσεις του εργαστηρίου μας έδειξαν ότι η DNMT1 αλληλεπιδρά με ποικίλους ερυθροειδικούς παράγοντες(GATA1, FOG-1, GFI-1b), σχηματίζοντας ένα κομβικό σύμπλοκο αποτελούμενο από την DNMT1 και τους μεταγραφικούς παράγοντες ZBP89 και ZNF143, το οποίο αλληλεπιδρά με διακριτά αιμοποιητικά πρωτεϊνικά υποσύμπλοκα. Επιπροσθέτως, η επικράτεια πρόσδεσης του PCNA στην DNMT1 (PCNA Binding domain - PBD), αποδείχθηκε αναγκαίο και ικανό για την αλληλεπίδραση της DNMT1 με αυτούς τους μεταγραφικούς παράγοντες. Επιπλέον, πειράματα DNMT1 KD σε ερυθρολευχαιμικά κύτταρα ποντικού (murine erythroleukemic (MEL) cells), αποκάλυψαν έντονες διαταραχές στην διακοπή του κυτταρικού κύκλου, λόγω προβληματικής καταστολής των γονιδίων που ευθύνονται για τον κυτταρικό πολλαπλασιασμό. Ο βασικός σκοπός αυτής της μελέτης είναι να αποσαφηνιστεί ο ρόλος της DNMT1 στην ερυθροειδική διαφοροποίηση. Προκειμένου να επιτευχθεί αυτός ο σκοπός, δημιουργήθηκαν DNMT1 KO MEL κύτταρα με την μέθοδο CRISPR/Cas9 και επιχειρήθηκε πλήρης χαρακτηρισμός του φαινοτύπου. Τα DNMT1 KO κύτταρα παρουσίασαν φαινότυπο παρόμοιο με αυτόν των DNMT1 knockdown όσον αφορά τον κυτταρικό κύκλο και την προβληματική ερυθροειδική διαφοροποίηση.

Επιπλέον μελέτες δείχνουν ότι η DNMT1 καταστέλλει την γ-σφαιρίνη σε ερυθροειδικά κύτταρα ενηλίκων μεθυλιώνοντας τον υποκινητή της. Επίσης, αρκετές μελέτες προτείνουν ότι η DNMT1 συνεργάζεται άμεσα ή έμμεσα με τον BCL11A, έναν παράγοντα που είναι γνωστό ότι ευθύνεται για την αποσιώπηση της έκφρασης των εμβρυϊκών β-σφαιρινών στο ποντίκι (εy and βh1), καθώς και της γ-σφαιρίνης στον άνθρωπο. Με βάση αυτά τα δεδομένα, διερευνήσαμε τις αλληλεπιδράσεις της DNMT1 με τον BCL11A, υποθέτοντας ότι η DNMT1 δρα ως συγκαταστολέας αυτών των γονιδίων κατά την ερυθροποίηση.

2. Introduction

Hematopoiesis-erythropoiesis

Hematopoiesis is the process that concerns the generation of all mature blood cells from a pool of primary Hematopoietic Stem Cells (HSCs). These rare cells reside in bone marrow of adult mammals and are characterized by self-renewal and pluripotency, meaning they can either give rise to more HSCs or commit into progenitor cells that differentiate along various pathways to generate the mature blood populations [1]. Given the fact that mature blood cells have a finite life span, HSCs are to replenish all hematopoietic lineages constantly. Hematopoietic progenitors give birth to precursors that eventually lead to the formation of erythrocytes, megakaryocytes, granulocytes, monocytes, macrophages and lymphocytes via distinct differentiation pathways.

Commitment and cell fate are defined by gene expression profiles regulated by transcription factors, as shown in Figure 1 [2].

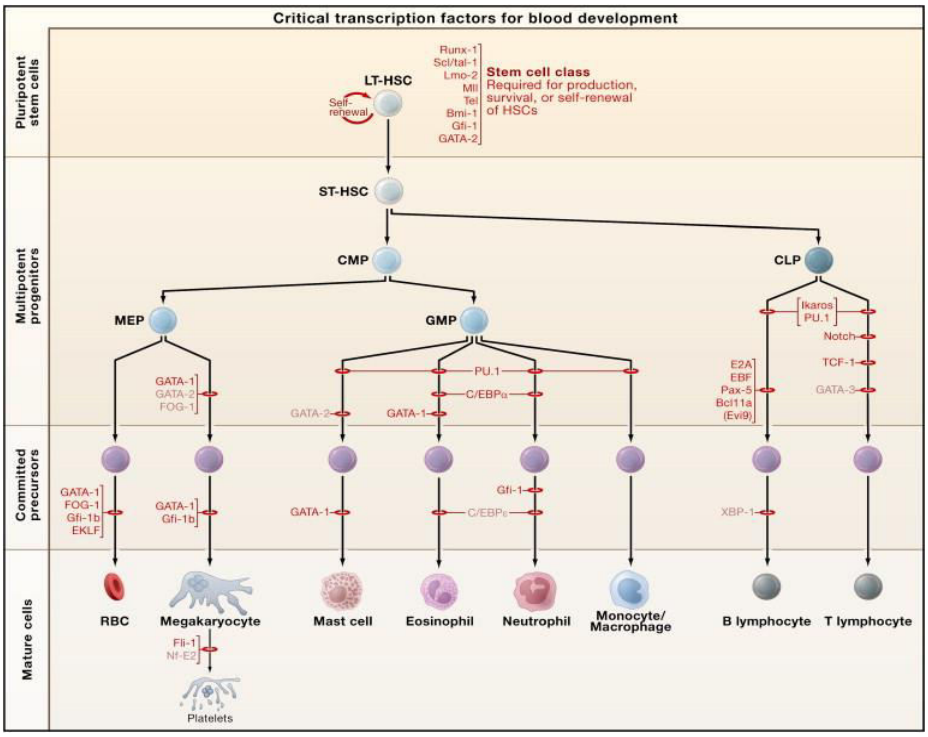


Figure 1 – Hematopoieis (Orkin and Zon, 2008)
Scheme of hematopoietic cell lineages, as determined by transcription factors
(abbreviations: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells)

Erythropoiesis is the process of red blood cells forming by erythroid progenitors. As shown in Figure 1, HSCs give rise to CMPs (Common Myeloid Progenitors) and thereafter MEPs (Megakaryocyte/Erythroid Progenitors). Upon stimulation by growth and transcription factors,

MEPs commit into burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) cells. Terminal differentiation occurs as CFU-Es proceed to proerythroblast (ProE) cells, basophilic I and II (Baso1 and Baso2), polychromatophilic (Poly), and orthochromatic (Ortho) erythroblasts, as shown in Figure 2 [3]. During the differentiation pathway, cell size is gradually decreased, hemoglobin content is increased for maximal capacity to transport oxygen to the tissues, and eventually, enucleation takes place [4].

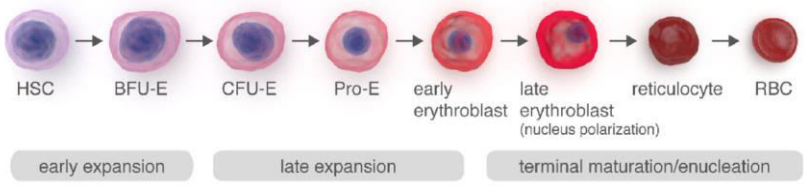


Figure 2 -Schematic representation of erythroid differentiation (Tolosano et al.)
As theprocess leads to terminal differentiation, the size of nucleus is reduced while hemoglobin content is increased**.**

The process of erythropoiesis is highly conserved in vertebrates and has been shown to occur in two "waves" during embryonic development. The first "wave" is called "primitive erythropoiesis" and takes place in the blood islands in the yolk sac in mouse as well as human. Its primary function concerns oxygenation as the embryo rapidly grows. This stage of erythropoiesis is characterized by fetal globin expression. As embryonic development proceeds, primitive erythropoiesis is replaced by "definitive" erythropoiesis. In this stage, the major hematopoietic organ is the fetal liver until the bone marrow is formed and takes its place [5].

Globin gene regulation

In humans, two gene clusters direct the synthesis of hemoglobins: the α locus, which contains the embryonic ζ gene and the two adult α genes, and the β locus, which consists of five genes arranged in chromosome 11 in the order in which they are expressed during development: ε, Gγ, Aγ, δ, and β genes (Figure 3). During human development, two switches in globin gene expression occur: first the embryonic to fetal globin switch, which coincides with the transition from embryonic (yolk sac) to definitive (fetal liver) hematopoiesis and, second, the fetal to adult switch, which occurs at the perinatal period [6]. The switches from ε to γ and from γ to β globin gene expression are controlled primarily at the transcriptional level. The ζ to α switch is controlled both at the transcriptional and post-transcriptional level REF. Unlike humans, most species –including mice- have only one switch, from embryonic to definitive globin expression occurring early in development [6]. Globin genes are expressed exclusively in erythroid cells in a strictly coordinated manner. Their expression is regulated from DNA regulatory elements, either in a short or a long distance from the genes. As far as the β-globin locus is concerned, DNA sequences in the 5' region of the cluster, up to 50 kb away from the gene, include four erythroid specific DNase I hypersensitive sites (HS) and a further upstream site (HS-5) that may function as

a boundary for the gene cluster [7]. HS are a hallmark of DNA-protein interactions and of chromatin modifications (forming an open structure) that facilitate the access of regulators and lower the threshold for activation of the linked genes [8]. Each HS is generally constituted of combinations of several DNA motifs for interacting transcription factors, among which the most important are GATA-1, EKLF and NF-E2 [8]. The five HS together are referred to as the Locus Control Region (LCR) of the β-globin gene cluster.

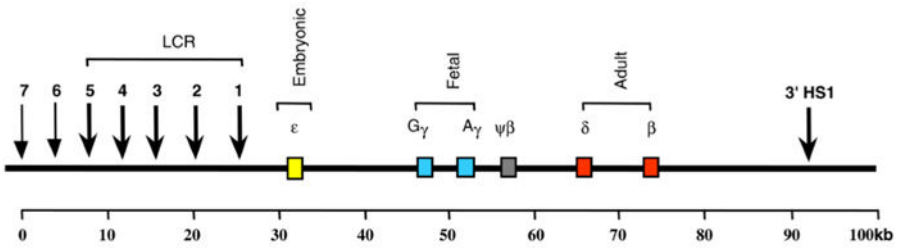


Figure 3 – Scheme of the human β globin locus (Stamatoyannopoulos, 2005)

Various models have been proposed for the LCR function: a looping, a tracking, a facilitated tracking, and a linking model [6]. The predominant model is the looping one, which suggests that the HSs of the LCR act as a complex, with the HS core elements forming an active site that interacts with only one globin gene at a time during erythroid development and binds transcription factors. This structure physically "loops" so that the LCR comes in close proximity to the appropriate gene. In addition, experimental evidence indicates that the LCR can flip-flop back and forth to activate adjacent genes such as the γ- and β-globin genes and that it is the total time spent by the LCR in each gene interaction that determines the prevailing chain in the cytoplasm. Thus, specific gene expression of is a result of a dynamic competition for interaction among the genes and the LCR [7]. Moreover, other variables that affect the strength and time of interaction between LCR and globin genes, and thus the overall expression, are the gene order, the distance from the LCR and the transcription factors regulating each stage of development.

One of the most interesting and best studied processes in erythropoiesis is the hemoglobin switching, which refers to the silencing of the γ-globin gene and the simultaneous activation of the β-globin gene, which occurs in the fetal to adult erythroid transition. Understanding this process is of great interest, as it is known that the γ-globin gene can function as a substitute for the defective β-globin gene in β-hemoglobinopathies such as β-thalassemia and sickle cell disease. Revealing the exact mechanism of γ-globin silencing may provide opportunities for its reactivation, which would have obvious therapeutic implications for these diseases. The first demonstration that fetal hemoglobin synthesis could be increased with drugs in adult erythroid cells was made in 1982, when it was observed that 5-azacytidine, a cytosine analog that cannot be methylated and hence can act as an inhibitor of DNA methyltransferases such as DNMT1, infused intravenously in anemic baboons stimulated sustained HbF production [9]. The drug is thought to act through demethylation of regulatory sequences.

Erythroid transcription factor BCL11A (B-cell lymphoma/leukemia A)

Much of the focus of research in erythroid cell differentiation and globin switching has been on the lineage-restricted transcriptional factors that mediate both procedures. Several erythroid transcription factors, such as GATA 1, FOG 1, EKLF, Gfi-1b and BCL11A were initially isolated through their binding to specific DNA binding motifs in the promoters of β-globin genes. The discovery of the B-cell lymphoma-leukemia A (BCL11A) identified this factor as an important regulator of HbF expression. The BCL11A transcription factor is a C2H2 type zinc-finger protein, expressed in several tissues and in various isoforms. The canonical isoform is also called BCL11A-XL and is approximately 91 kD. BCL11A has been previously shown to be critical in lymphopoiesis for the development of B cell precursors, however, subsequent studies have shown that it also binds to an intergenic region in the β-globin locus and has a dominant silencing effect on murine embryonic β-type βh1 and εγ-globin, as well as human ε- and γ-globin gene expression in β-globin locus YAC transgenic mice [10]. It has been shown, that only BCL11A-XL is capable of repressing the globin genes, as it contains 3 extra zinc finger domains compared to the BCL11A-L isoform. Recent studies have shown that BCL11A does so, by directly binding to the γ-globin promoters in adult erythroid cells via a TGACCA binding motif present in all embryonic and fetal globin genes in both human and mice [11].

In humans, BCL11A is expressed mostly in the bone marrow and spleen, as well as in lymph nodes, brain, heart and pancreas at lower levels; while in mice it is mainly expressed in the fetal liver, adult spleen, as well as in the brain and the central neuronal system of embryos. Moreover, only short isoforms of BCL11A are expressed in primitive erythropoiesis, followed by low expression of long isoforms in fetal liver and then robust expression in the adult bone marrow erythroblasts in humans, indicating a critical role *after birth*. On the contrary, in primitive murine erythropoiesis taking place in yolk sac there is no expression of BCL11A, followed by equal levels of expression of the long isoforms in fetal liver and later in bone marrow, suggesting a role *during terminal differentiation* [12], [13]. In the same study, it is shown that Bcl11a-/- mice fail to silence the expression of mouse embryonic β-like globins, as wells as of human ε-globin. Additional studies, showed that BCL11A also cooperates with GATA 1, FOG 1, SOX 6 and the NuRD complex, implying a possible role in chromatin remodeling during erythropoiesis [13],[14]. Moreover, erythroid transcriptional regulator KLF1 is known to be responsible for BCLL1A activation as it directly binds its promoter, and recent studies suggest a critical role of this interaction in erythroid maturation and hemoglobin switching [15].

A recent study showed that knockdown of BCLL1A in human primary erythroid cells leads to high expression of γ-globin and in a simultaneous reduction of β-globin in vitro. In addition, the same study described an innovative gene therapy approach for the treatment of sickle cell disease, using genetically modified sickle cell disease HSCs in transplantations. Modified cells carried an LCR-shRNA vector, which knocked down BCL11A and resulted in an improvement of disease-associated hematological parameters [16].

Interestingly, recent studies provided evidence for a possible BCL11A-DNMT1 interaction during erythropoiesis. DNMT1 was identified as a BCL11A-associated protein in a proteomic screen, among various erythroid factors, suggesting that BCL11A-interacting proteins are differentially required for silencing of human embryonic or fetal globin expression, and that they may form distinct subcomplexes depending on the chromatin context [17]. In addition, the study shows that DNMT1 is involved in HbF silencing, as shRNA-mediated DNMT1 knockdown in primary human adult erythroid cells HbF expression was significantly induced, whereas the amount of total β-like globin mRNAs was modestly reduced. Collectively, these data imply that DNMT1 haploinsufficiency in combination with BCL11A deficiency can further enhance HbF expression [17]. In additional studies, double knockdowns of DNMT1 and BCL11A in stably transfected MEL cells with modified constructs, resulted in enhanced γ-globin expression and support a model in which Myb and BCL11A serve to define the developmental-stage specific repression of the embryonic and fetal β-like globin genes mediated by DNMT1 [18].

DNMT1-DNA methyltransferase 1

DNA methylation is the first well studied epigenetic mechanism that was associated with gene regulation and globin genes were among the first that a reverse correlation between cytosine methylation and transcription was observed [10]. It represents the major epigenetic modification during which a methyl group is added to the 5' C of a cytosine residue. DNA methylation is more frequent in CpG regions, although it can be observed to a lesser extent in non-CpG regions [19]. In mammals, the enzymes responsible for DNA methylation are the DNA methyltransferases DNMT1, DNMT3a και DNMT3b [20]. Studies in vertebrate models have indicated a close, cooperative association between DNA methylation and repressive histone modification in gene silencing [10].

DNMT1 is the methyltransferase responsible for the maintenance of the CpG nucleotide methylation after DNA replication. It shows a strong preference for hemi-methylated DNA as a substrate compared to non-methylated DNA [21]. During S phase, DNMT1 localizes in DNA replication foci, whereas during mesophase it appears diffuse [22]. Although DNMT1 functions mainly as a maintenance methyltransferase, it is also able to carry out *de novo* methylation [23].

DNMT1 is ubiquitously expressed and is critical for cell growth and differentiation in various tissues and cell types in mammals [24]. The Dnmt1 gene knockout in mice results in developmental defects and embryonic lethality after midgestation, while ESCs are viable after DNMT1 targeted disruption but die after differentiation is induced [22]. Several studies have correlated DNMT1 function with cellular growth regulation. For instance, DNMT1 complete inactivation in human cancer cells results in cell cycle arrest in G2 phase and therefore in mitotic catastrophe [25], while in human ESCs, DNMT1 knockout led to an increase of DNA damage and arrest in G1 phase[26].

DNMT1 is a protein of 1616 amino acids and 183 kDa, highly conserved between human and mice, with approximately 77% identity in protein level.

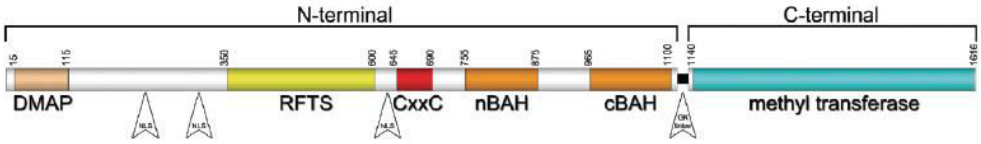


Figure 4 - DNMT1 structure (S.Dhe-Paganon, 2011)
N-terminal domain contains**:** DMAP1 binding site (15–115), PCNA binding site (160–240), RFTS (350–600), CxxC (645–690), BAH domains-1, 2 (BAH1 755-875) & BAH2 (965–1100), NLS (191–211, 259–378 and 630–757). Gly-Lys dipeptide linker separates the N-terminal and a catalytic C terminal domain
C-terminal domain (1140–1616) contains 10 conserved amino acid motifs, shared with many prokaryotic 5-methyl-cytosine methyltransferases. The catalytic center and coenzyme binding site reside within this domain.

As shown in Figure 4 [27], the N-terminal domain contains the regulatory domains: DMAP domain, responsible as its name suggests for the interaction with transcriptional co-repressor DMAP[28], the PBD domain (PCNA binding domain), the Replication Foci Targeting Sequence (RFTS) domain which guides the protein to the replication foci during the S phase of the cell cycle[29] and mediates DNMT1 dimerization[30], the CxxC zinc binding domain, essential for enzymatic activity [31] and two Bromo-Adjacent Homology (BAH) domains. The C-terminal part of the protein, starting from residue 1140, is responsible for the catalytic function of the methyltransferase. It has been shown, however, that the isolated catalytic domain is inactive, thus indicating a possible cooperative role for the N-terminal part in catalytic function. After DNMT1 binds to DNA, the catalytic domain interacts with an N-terminal region, allosterically activating the enzyme [27]. Moreover, other studies suggest that the catalytic domain participates in several DNMT1 interactions either in cooperation with the N-terminal domain or by itself, as shown in Figure 5 [32].

Despite various studies concerning the importance of DNA methylation in hemopoiesis, the precise role of DNMT1 remains to be elucidated. It has been shown that different DNA methylation patterns direct the alternative differentiation pathways of hematopoietic stem cells (HSCs) and that reduced DNMT1 activity can favor the myeloerythroid pathway at the expense of lymphoid lineage [33]. Additionally, DNMT1 has been found to be essential for HSCs self-

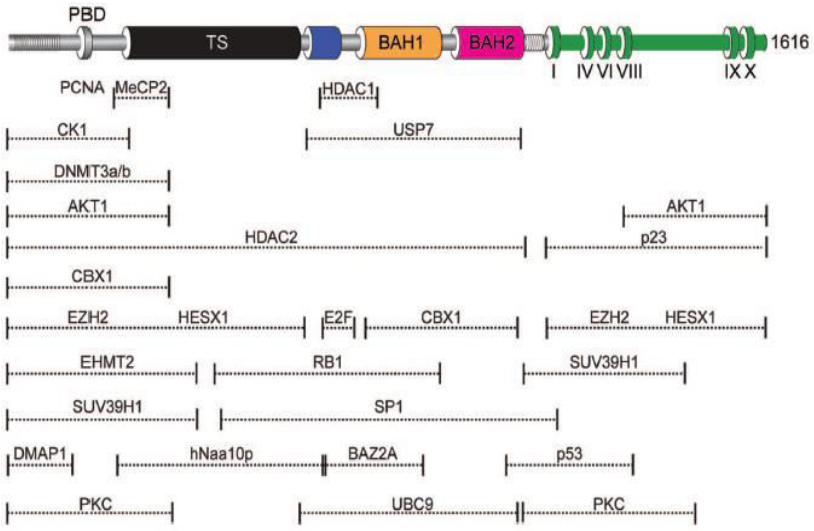


Figure 5 - Overview of DNMT1 interacting proteins (catalytic domain indicated in green colour) (W. Qin, H. Leonhardt & G. Pichler, 2011)

renewal and for establishing distinct gene expression patterns for each hematopoietic stem and progenitor cells [34].

In erythrocytes, DNA methylation has been extensively studied, as the chemical inhibition of DNMT1 activity results in reactivation of γ-globin gene expression in the adult stage, suggesting a possible therapeutic strategy for hemoglobinopathies [35]. Moreover, a pilot study using decitabine (5-aza-2'-deoxycytidine), a less toxic analog of 5-azacytidine which can deplete DNMT1, in β-thalassemia patients resulted in increased fetal globin levels and reduction of hemolysis [36].

Additionally, recent studies have shown that transcriptional factors implicated in γ-globin gene repression in adult erythrocytes, such as TR2/TR4 and BCL11A, interact with DNMT1 [37]. Furthermore, double knockdown of BCL11A and DNMT1 in MEL cells containing an intact 183-kb human β-globin locus, resulted in enhanced γ-globin expression[38].

Taking under consideration all the above, it is clear that DNMT1 plays a role in globin gene regulation and possibly also in erythropoiesis. Hence, understanding DNMT1 functions in erythropoiesis can reveal valuable information about the reactivation mechanism of fetal globin in adults, which could lead to possible novel therapy strategies for β-thalassemia and sickle cell disease.

Aim of study

The main goal of this study is to further elucidate the role of DNMT1 in erythropoiesis. As recent work in Strouboulis lab has shown, DNMT1 associates with transcriptional factors in separate complexes, thus indicating a crucial role for the protein in erythroid differentiation possibly as an epigenetic factor. Moreover, DNMT1 knockdown experiments, performed in mouse erythroleukemic (MEL) cells, resulted in impaired erythropoiesis, erythroid differentiation and gene expression. In order to further examine the potential role of DNMT1 in this process, CRISPR/Cas9 mediated DNMT1 knockout (KO) experiments were performed in mouse erythroleukemic (MEL) cells. Several experiments have been performed, showing a similar phenotype as the one previously observed in DNMT1 knockdown. This phenotype is characterized by impaired erythropoiesis as most cells do not reach terminal differentiation, while defects in cell cycle arrest were also noted, as a result of de-repression of cell cycle promoting genes.

Moreover, taking into consideration the recent findings showing a DNMT1-BCL11A association which may be important for γ-globin silencing, we tried to investigate further this interaction, by identifying the DNMT1 domain responsible for mediating it. Due to ineffective detection of endogenous BCL11A protein in MEL cells, we carried out tagged DNMT1 and BCL11A deletion mutant cotransfections in HEK cells, followed by immunoprecipitation and streptavidin pull down experiments, revealing revealed a possible mediating role for the DNMT1 catalytic domain in this interaction.

3. Materials and Methods

Plasmid constructs

DNMT1 deletion mutants GMT1L, GMTΔPBD, GMT, PBDQ162E were kindly provided by Dr. C. Cardoso (TU, Darmstadt) [39]. DMAP domain was PCR amplified from plasmid GMT1L and replaced in GMTΔPBD DNMT1 deletion mutant in order to acquire a construct missing only the PBD domain.

Mouse DNMT1 without the catalytic domain (DNMT1 Δcatalytic) cDNA was PCR amplified using GMT1L as template and cloned as an XhoI/NotI fragment into C-terminal 3xFLAG-TEV-Avi pBUDneo vector [40], while mouse DNMT1 catalytic domain was PCR amplified using GMT1L and cloned as an XhoI/BglII fragment into N-terminal Avi-TEV-3xFLAG pBUDneo vector [40].

Cell cultures and Cell Transfections

C88 MEL cells were grown in DMEM-10% FBS-1% penicillin/streptomycin (all from Life Technologies) and induced to differentiate with 2% DMSO as previously described [41]. DNMT1 KO C88 MEL cells, resulted from CRISPR/Cas9 experiments by former master student Dimitra Vlachokosta, were cultured as above with the addition of G418 after transfection for selection as previously described [42].

HEK293T cells were cultured in DMEM-10% FBS-1% penicillin/streptomycin (all from Life Technologies) and were transiently transfected using JetPEI (Polyplus transfection).

Nuclei from MEL cells and HEK 293T were isolated using NP-40 Lysis Buffer and prepared using the RIPA extraction method. Cells were centrifuged at 1100rpm for 5min at room temperature and washed twice with PBS and protease inhibitors at 4°C. Cells were resuspended in NP-40 lysis Buffer (10 mM Tris-HCl, pH= 7.4, 10 mM NaCl 3mM $MgCl_2$ 0.5% NP-40, 1mM DTT with protease inhibitors or 1mM PMSF). After incubation at 4°C for 10 min, cell debris was pelleted at 1100rpm, 5 min 4°C. The supernatant containing the cytoplasmic extract was removed after centrifugation and the pellet was resuspended in RIPA(-SDS) Buffer (50 mM Tris-HCl pH 7.5, 1%NP-40, 0.25%Na-Deoxycholate, 150 mM NaCl, 1mM EDTA, 10% Glycerol, 1mM DTT with protease inhibitors or 1 mM PMSF). The nuclei are vortexed for 10 seconds and then transferred in a rotating platform in the cold room for 45min – 1h. After incubation, the soluble nuclear extracts were fractionated from the insoluble pellet by centrifugation at 13.000rpm, 35 min, 4°C. 10% glucose is then added to the extracts for better storage.

Benzidine and May Grünwald /Giemsa staining

$5x10^5$ live MEL induced and non-induced cells were resuspended in PBS and then treated with benzidine solution (60mg o-diansidine D9143 from Sigma Aldrich, 0,5ml glacial acetic acid diluted in 30 ml water) and 1/10 hydrogen peroxide in order to assess hemoglobin content. Cytospins (2000rpm for 2min) were performed for slide preparation.

For morphology observation, after the benzidine staining, the slides were stained with May Grünwald solution from Sigma Aldrich (undiluted) for 5 min. Then, washed with PBS and stained with Giemsa also from Sigma Aldrich (diluted 1/20 in PBS) for 15 min. They were then washed with PBS, air dried and visualize with phase contrast microscopy.

Annexin labelling

This was performed according to Annexin V Apoptosis Detection Kit: sc-4252 AK from Santa Cruz Biotechnology with DNMT1 KO MEL and MEL control induced and non-induced cells in order to determine apoptotic cells. Briefly, $5x10^5$ cells were pelleted (1500 rpm for 5 min), washed with PBS and resuspended in 100µl 1x Assay Buffer (10mM HEPES, 140mM NaCl, 2,5mM CaCl2, pH 7.4). After 0,5 µl of annexin V FITC was added, samples were incubated for 15 minutes at room temperature in the dark, washed with PBS and analyzed with Operetta High Content Imaging System. Propidium Iodide was added to determine the necrotic cells.

Propidium Iodide labelling

This labelling was performed with DNMT1 KO MEL and MEL control induced and non-induced cells in order to assess cell cycle stages. $5x10^5$ cells were pelleted (1500 rpm for 5 min), resuspended in PBS and fixed with 100% cold ethanol for 20 min in room temperature. Ethanol was removed and cell pellet was resuspended in PI-RNase solution (50 µg/ml PI from Sigma-Aldrich, 100µg/ml RNase Type I-A diluted in PBS), incubated at room temperature for 20 min. Analysis was operated with Operetta High Content Imaging System.

Western Blotting

SDS-PAGE and Western immunoblotting were carried out as previously described [43]. Membranes were subjected to enhanced chemiluminescence (ECL prime, GE Healthcare). Streptavidin-HRP (NEL 750, Perkin Elmer) was dissolved in 5% (w/v) gelatin from cold water fish skin (Sigma).

Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): HDAC-1 (10E2, sc-81598), GFP (B-2, sc-9996), as well as the secondary antibodies conjugated to horseradish peroxidase: sc-2357, sc-2314. Rabbit polyclonal DNMT1 antibody (1-248) was purchased from BioAcademia (Osaka, Japan), FLAG M2 antibody from Sigma Aldrich and BCL11A (ARP39322_P050) antibody was acquired from Aviva Systems Biotechnology.

Immunoprecipitations

Immunoprecipitations were carried out as previously described [44] using 50µl of Protein A or Protein G magnetic beads (Dynabeads, Invitrogen) per 500µg of nuclear extract and 100µl of magnetic beads for IgG control. Briefly, nuclear extract was immunoprecipitated with the beads and 10 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. Immunoprecipitates, IgG controls and supernatants were all analyzed by Western immunoblotting. Prior to immunoprecipitation, nuclear extracts were subjected to benzonase treatment for the removal of nucleic acids.

Streptavidin Pulldowns

Streptavidin pulldowns were done as previously described [43] using 50 µl of magnetic beads (Dynabeads® M-280 Streptavidin, Invitrogen) per 1mg of nuclear extract. Bound material was eluted by boiling for 10 min in 1x Laemmli sample loading buffer and analyzed by Western

immunoblotting. Prior to streptavidin pulldown, nuclear extracts were subjected to benzonase treatment for the removal of nucleic acids.

Real-Time quantitative PCR

SYBR Green-based quantitative qPCR (ABI SYBR Green PCR master mix, ABI) was performed on cDNA samples from DNMT1 KO MEL and MEL control induced and non-induced cells. Samples were analyzed in duplicates using an ABI StepOneTMReal-Time PCR System (ABI, Foster city, CA). Results were analyzed relative to a control sequence in the gapdh locus. Primer sequences are listed in Table 1.

Forward primer for hbb-b1	GCAACCTCAGAAACAGACATCATG
Reverse primer for hbb-b1	CCCCACAGGCCAGAGACA
Forward primer for hbb-βh1	AAAGACGGTGAACTCTGGGAAA
Reverse primer of hbb-βh1	GCATTGCACTAAGATAGTGTCCAGTAG
Forward primer for cdk1	CCAGTCCGACTCACGCCATA
Reverse primer of cdk1	AGTCCGGAACTGCGGTGT
Forward primer for cdca3	AAGCAAGTGGCTCGAGTAGC
Reverse primer of cdca3	GAGTAGGAGAGCGGGGATCT
Forward primer for cdca5	AGAAAGGCCATCGTCTTGAA
Reverse primer of cdca5	GGTCTCTGCTGTCCAGCTCT
Forward primer of cdca8	CTCCCAAGAAACGCAGCAG
Reverse primer of cdca8	TCAAGCCACTTCATCCCTTG
Forward primer of gapdh	CCAATGTGTCCCGTCGTGGATCT
Reverse primer of gapdh	GTTGAAGTCGCAGGAGACAAC

Table 1- Real Time PCR primers sequence

4. Results

4.1 DNMT1 KO MEL cells phenotype characterization

Previous studies showed that DNMT1 interacts in separate complexes with transcription factors involved in erythropoiesis and globin gene regulation, thus making DNMT1 a candidate epigenetic factor implicated in erythroid functions. To further investigate the role of DNMT1 in the process of erythropoiesis, DNMT1 knock-down experiments were performed in mouse erythroleukemic (MEL) cells (E. Karkoulia, unpublished data). These resulted in cell cycle defects deriving from a failure to inhibit genes that promote cell proliferation and resulting in impaired erythropoiesis (not shown). Specifically, DNMT1 knock-down MEL cells were arrested in the polychromatic or orthochromatic stage, not being able to proceed to terminal differentiation. Following these observations and in order for the role of DNMT1 in erythroid cell differentiation to be assessed more clearly, CRISPR/Cas9 mediated DNMT1 knockout (KO) experiments were performed in mouse erythroleukemic (MEL) cells (D. Vlachokosta, unpublished data).

The CRISPR/Cas9 system was employed in order to generate MEL DNMT1 KO clones by co-expressing three guide RNAs specific to the DNMT1 gene and the endonuclease Cas9. At least 80 clones were tested for DNMT1 protein expression and screening led to the selection of two clones (clones named C10 and 3D103G4) with clear absence of DNMT1 protein levels (Figure 6) for further studies.

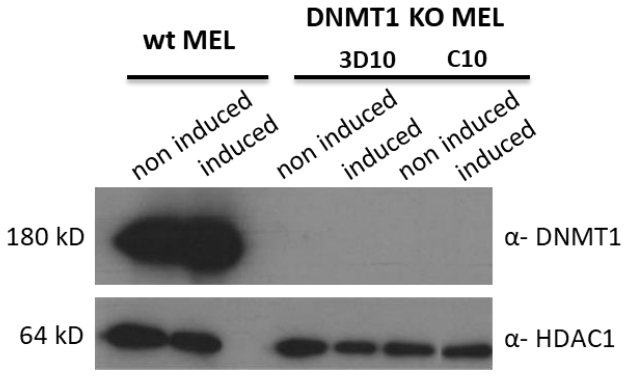
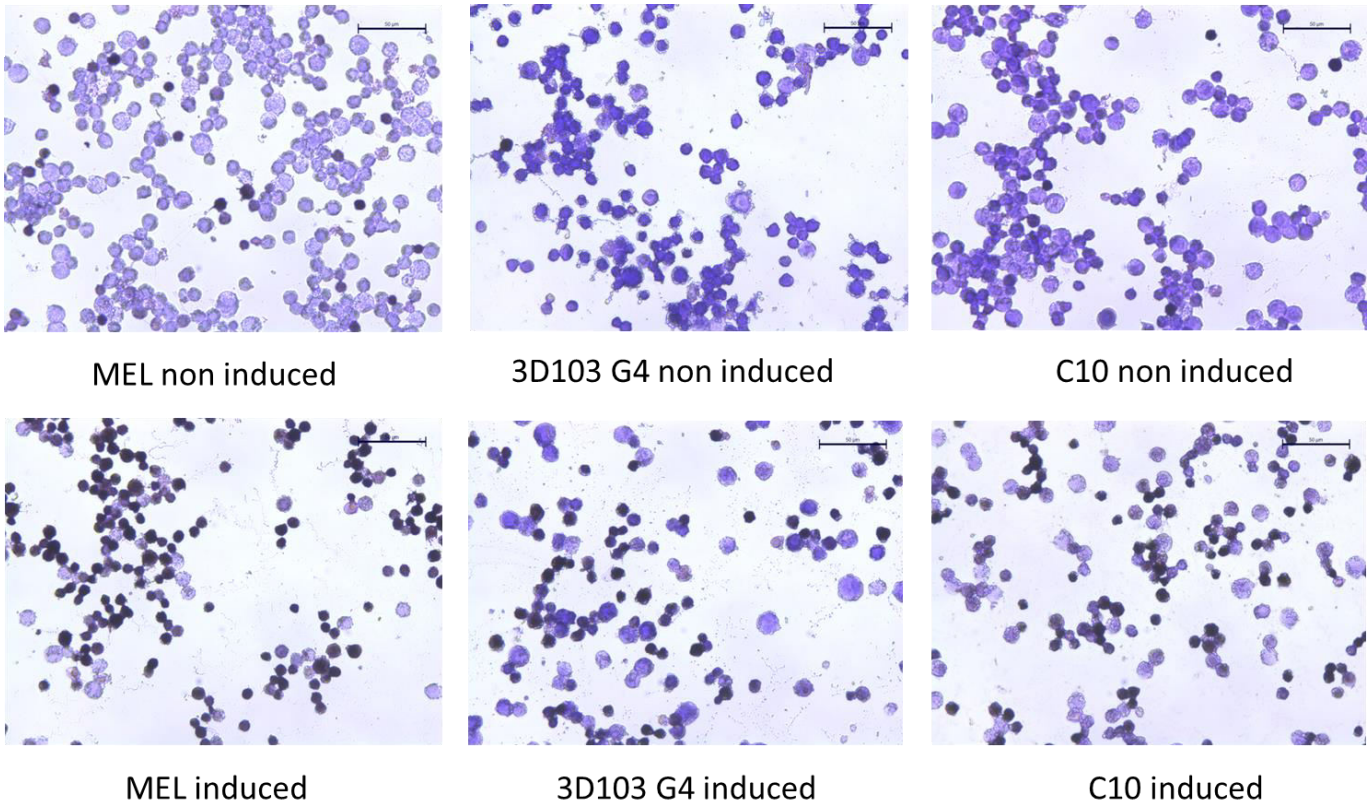


Figure 6- Detection of DNMT1 protein levels in MEL DNMT1 KO clones by Western blot. Nuclear extracts from transfected C88 MEL cells with DNMT1 CRISPR/Cas9 KO plasmid were immunoblotted with anti-DNMT1 antibody (top) and anti-HDAC1 (lower) antibody as protein loading control. Nuclear extracts from untransfected (wildtype=wt) MEL cells were used as control.

Subsequently, several assays were carried out in order to fully characterize the phenotype of the DNMT1 KO MEL cells. Firstly, effects on MEL differentiation were

A.



B.

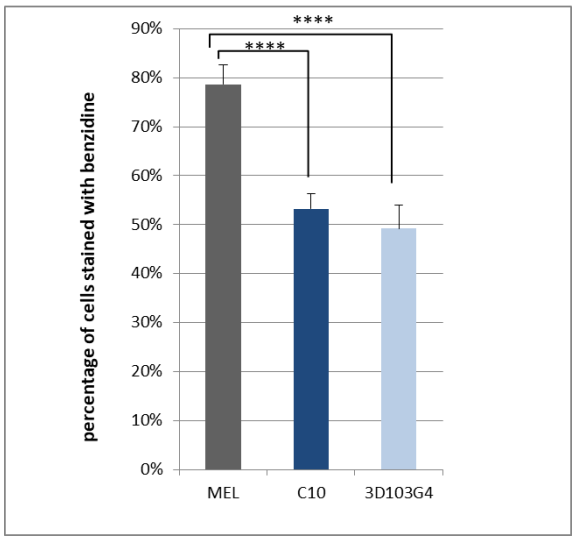


Figure 7- Induced and non-induced DNMT1 KO MEL cells (3D103G4 and C10) and control MEL cells stained with benzidine and May-Grünwald/ Giemsa.

A. Pictures from slides taken with phase-contrast microcopy. Darker color indicates benzidine staining and therefore, high hemoglobin content, while May-Grünwald/ Giemsa staining assess cell morphology. Fewer cells seem to be stained with benzidine comparing DNMT1 KO cells to control MEL cells, suggesting impaired differentiation. Moreover, DNMT1 knock-down MEL cells seem arrested in the polychromatic or orthochromatic stage.

B. Quantitation of benzidine positive cells in DMSO-induced control MEL cells and two DNMT1 KO clones. Four technical replicates were analyzed. P value= 0,00006 and 0,00008 for DNMT1 KO clone C10 and 3D103G4, respectively.

examined in terms of morphology and hemoglobinization. Induced and non-induced DNMT1 KO MEL cells and control MEL cells were stained with benzidine to assess hemoglobin content and afterwards, cytospins were performed and slides were stained

with May-Grünwald/ Giemsa dyes (see Materials and Methods) for morphology observation (Figure 7).

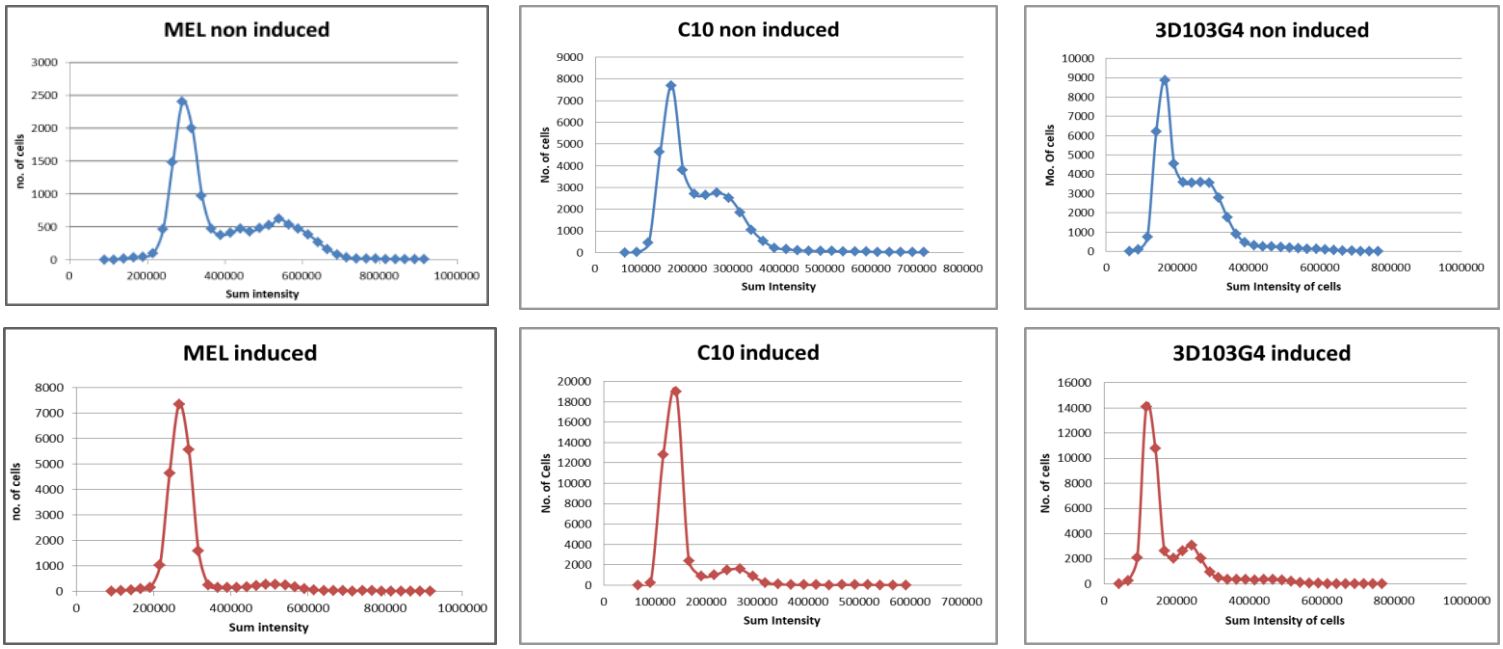
Darker color indicates benzidine staining and, therefore, high hemoglobin content, while May-Grünwald/ Giemsa staining assesses cell morphology. Fewer cells appear to be stained with benzidine when comparing DNMT1 KO cells to control MEL cells, suggesting impaired differentiation. Specifically, about 80% of control MEL cells observed in the microscope field of Figure 7 are benzidine positive, while this percentage for DNMT1 KO clones C10 and 3D103G4 is 53% and 49%, respectively. Moreover, DNMT1 KO MEL cells appear to be arrested in the polychromatic or orthochromatic stage, as previously observed. The polychromatic stage is the stage where hemoglobinization starts, as shown by the reduced size of erythroblasts and their nucleus. The orthochromatic stage is the last stage of hemoglobinization before enucleation and it is characterized by the smallest nucleated erythroid cells with very condensed chromatin and shrunk nuclei. The process is completed with the loss of nucleus (reticulocytes) and most cytoplasmic organelles (erythrocytes) [45]**.**

Secondly, cell viability and effects on cell cycle progression were assessed. Induced and non-induced DNMT1 KO cells and control MEL cells were analyzed with the Operetta High Content Imaging System, after annexin V-Propidium Iodide staining for apoptosis and necrosis detection. In addition, the cells were also stained with Propidium Iodide, following ethanol fixation, in order to study cell cycle progression (see Materials and Methods). The aforementioned experiments showed good cell viability in the absence of DNMT1, as shown in Table 2.

Table 2-Cell viability assessed by Annexin V-Propidium Iodide staining and Operetta High Content Imaging System analysis. DNMT1 KO cell viability is in levels similar to that of control MEL cells.

	% apoptotic cells	**% necrotic cells**	**%cells apoptotic and necrotic**	**% live cells**
MEL non-induced	2,59	3,043	0,3026	**94,06**
MEL induced	1,526	9,179	0,9398	**88,36**
C10 non-induced	0,3138	6,196	0,4842	**93,01**
C10 induced	1,144	8,026	0,5885	**90,24**
3D103G4 non-induced	0,1114	2,795	0,134	**96,96**
3D10G4 induced	2,829	1,911	0,1391	**95,12**

A.



B.

%	MEL non induced	MEL induced	C10 non induced	C10 induced	3D103G4 non induced	3D103G4 induced
G1	47,3	86,3	51,7	75,1	49	68,6
S	15,3	4,2	12,1	9	17,7	11,3
G2	36,6	8,7	35,7	15,1	32,7	19,2

C.

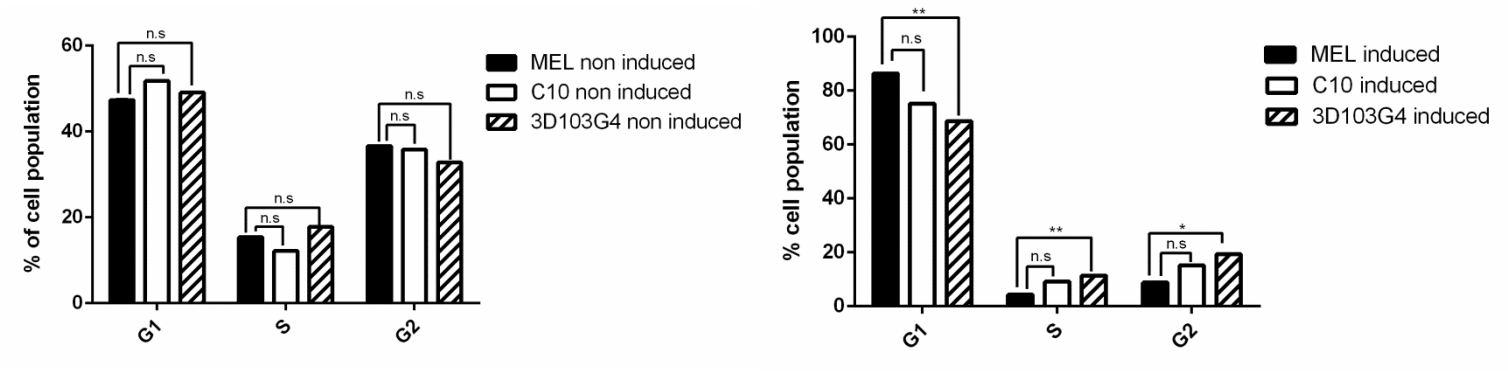


Figure 8 – Cell cycle analysis by DNA content

A. Graphs representing the number of cells corresponding to each cell cycle phase (G1, S, G2) acoording to Propidium Iodide intensity.

B. Percentage of cell population in each cell cycle phase. Average values from three independent experiments are shown.

C. Bar charts with quantitative analysis for the above results. No significant differences were observed between DNMT1 KO clones and control MEL cells before induction of differentiation (left). Upon induction, significant changes in DNMT1 KO clone 3D103G4 were detected compared to control MEL cells, while the results for DNMT1 KO clone C10 followed the same trend but with no statistical significance (right).

After confirmation that DNMT1 KO display similar cell survival levels to control MEL cells, we proceeded with analysis of cell cycle progression by treatment with Propidium Iodide followed by measurement of the DNA content of all cells (Figure 8). Graphs

represent the number of cells corresponding to each one of the cell cycle phases: G1, S or G2 phase (Figure 8A). The percentage of cell population corresponding to each phase was also measured from three independent experiments and is shown in Figure 8B. According to a previous study in the Strouboulis lab (E. Karkoulia, not shown), we expected to observe defects in cell cycle arrest post-differentiation. Indeed, an observable decrease in cell cycle arrest was noticed. As seen in Figure 8, about 86% of MEL cell population was found in G1 phase, i.e. these cells have stopped dividing and have entered the resting phase. Cells in G1 phase correspond to 75% of the cells for clone C10 and 68% for clone 3D103G4. In addition, 15,1% and 19,2% of DNMT1 clones C10 and ED10EG4 respectively, are in G2 phase, i.e synthesizing proteins and continue to grow to proceed in mitotic phase, as opposed to 8,7% of control MEL cells. As far as S phase is considered, i.e the cells synthesizing DNA, 9% of DNMT1 clone C10 and 11,3% of DNMT1 clone 3D103G4, while in control cells the percentage is only 4,2% (Figure 8B). Statistical analysis was performed for these results and showed no significant differences between the DNMT1 KO clones and control MEL cells as far as non-induced cultures are concerned. Upon induction of differentiation, however, DNMT1 KO clone 3D103G4 showed significant changes compared to control MEL cells, while DNMT1 KO clone C10 followed the same trend, albeit with no statistical significance (Figure 8C).

Moreover, in order to elucidate the implication of DNMT1 in erythroid cell differentiation, RNA expression profiling of select genes was assessed. In particular, Real Time-quantitative PCR was performed on Reverse Transcribed cDNA from RNA extracted from induced and non-induced DNMT1 KO MEL cells and control MEL cells (see Materials and Methods). We primarily evaluated the expression of the mouse β-globin embryonic hbb-βh1 and adult hbb-b1 genes, as well as of cell of cycle regulating genes cdk1, cdca8, cdca5, cdca3.

As shown in Figure 9B, hbb-b1 gene expression appears highly upregulated in induced control MEL cells, as expected in MEL terminal differentiation as the hbb-b1 gene encodes the adult beta major hemoglobin chain, which is robustly expressed in mature erythrocytes [13]. Hbb-βh1 gene encodes the embryonic β-like chain which is not expressed in MEL cells, as shown in Figure 9A. However, previous findings have shown that hbb-βh1 gene is de-repressed upon DNMT1 disruption, [17],[18]. We also find hbb-βh1 derepression in DNMT1 KO cells induced to undergo terminal differentiation (Figure 9A). Accordingly, hbb-b1 expression is reduced in DNMT1 knockout cells as a result of hbb- βh1 expression (Figure 9B).

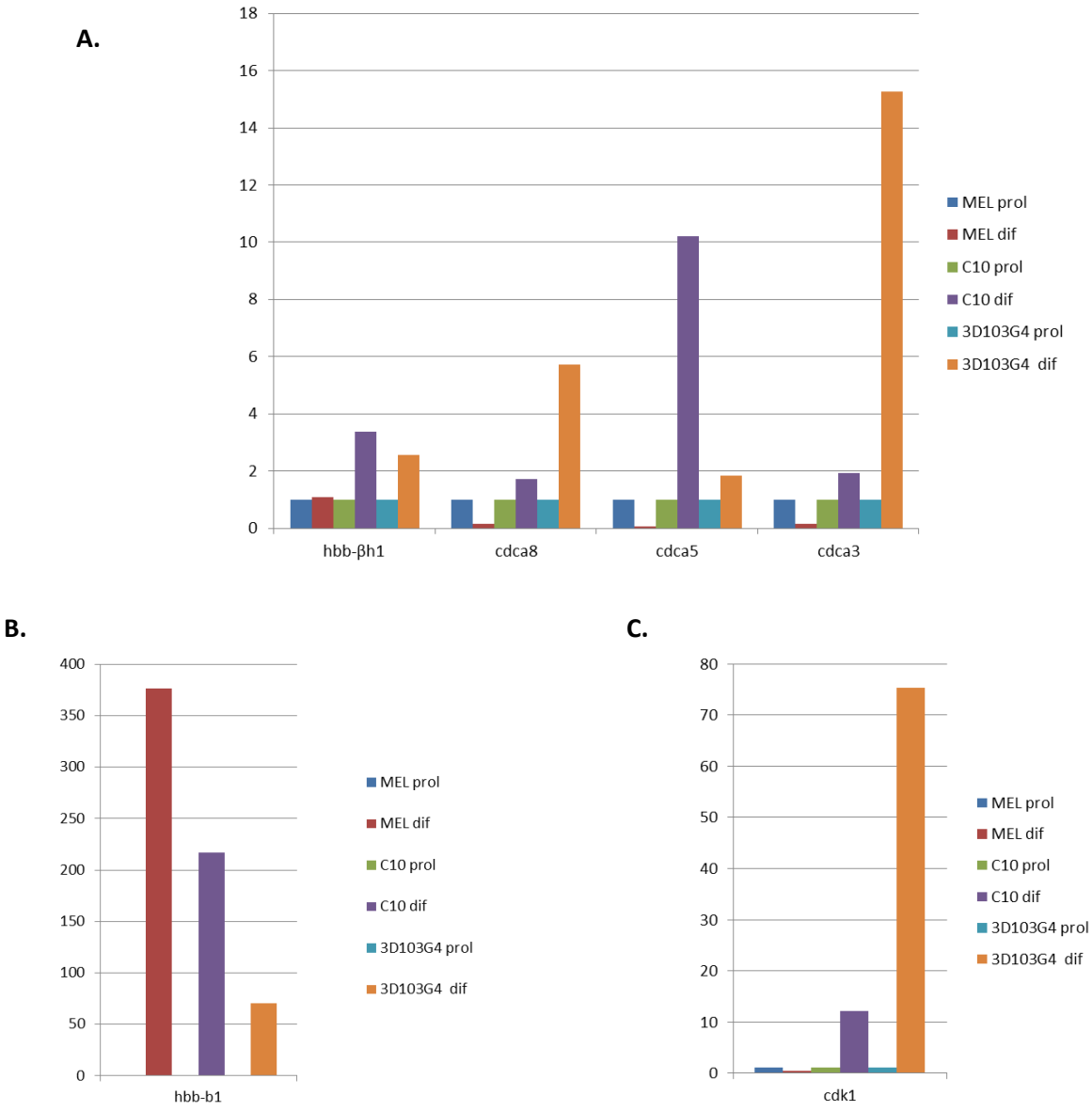


Figure 9 – Real Time-quantitative PCR. Gene expression analysis between induced and non-induced DNMT1 KO MEL cells and control MEL cells. Expression was normalized using gapdh gene expression levels.

A. Embryonic β-globin gene hbb-βh1 appears upregulated in induced DNMT1 KO MEL cells. Cell cycle progression genes cdca8, cdca5 and cdca3 also appear upregulated in induced DNMT1 KO MEL cells**.**

B. Adult β-globin gene hbb-b1 is increased upon differentiation, however at lower levels in DNMT1 KO MEL cells.

C. Cell cycle progression gene cdk1 is also upregulated in DNMT1 KO MEL cells upon differentiation, while no expression was detected in MEL cells.

CDK1 is a serine/threonine kinase known for its essential role in cell cycle progression [46], while CDCA3 is required for mitotic entry, participating in E3 ligase complex and therefore mediating the degradation of WEE1 kinase [47]. Also, overexpression of CDCA3 promotes oral cancer progression by preventing G1 phase arrest and enhancing cell proliferation [47]. CDCA5, also known as sororin, plays a critical role in mitosis as it is responsible for stabilization of chromatin and cohesion complex association [48],[49] and CDCA8, also known as borealin, is involved in the chromosomal passenger complex (CPC), which is the main regulatory complex in mitosis[50]. Its major function concerns the proper chromosome alignment and segregation in the mitotic spindle [51],[52]. All cell cycle genes appear upregulated to various levels in induced DNMT1 KO cells (Figure 9A and 9C), consistent with our previews observations (E. Karkoulia, unpublished data) and supporting the hypothesis that cell cycle defects are due to impaired inhibition of genes that promote cell proliferation, as all the aforementioned genes are required for cell cycle progress.

4.2 Investigation of DNMT1 and BCL11A interactions

In order to investigate the DNMT1-BCLL11A interaction previously described [17], immunoprecipitations were performed with MEL non induced nuclear extracts. DNMT1 was successfully precipitated; however, we were not able to detect any precipitated BCL11A protein, probably due to very low expression of the protein in this cell type (Figure 10).

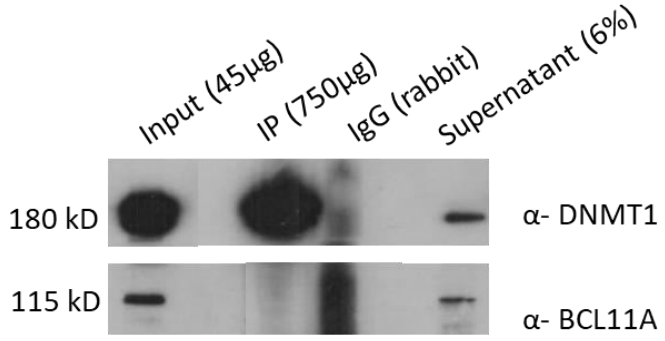


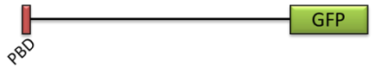
Figure 10- Detection of precipitated proteins after DNMT1 IP by Western blot. Nuclear extracts from non induced C88 MEL cells were immunoprecipitated with an anti-DNMT1 antibody. Immunoprecipitated material was immunoblotted with an anti-DNMT1 antibody (top) and an anti-BCL11A antibody (lower). DNMT1 was precipitated effieciently, however no interaction with BCL11A was detected.

During the immunoprecipitation procedure with anti-DNMT1 antibody and MEL nuclear extracts we encountered two major problems. Firstly, the smear we observed after Western blot,

probably because of the rabbit origin of the antibody. Secondly, the notably low expression of BCL11A in MEL cells as seen in the Input sample in Figure 1.

In order to address the problem of low Bcl11A levels in MEL cells, transient transfections were performed in HEK 293T cells in order to overexpress a BCL11A (XL isoform) plasmid carrying a FLAG tag. Moreover, we overexpressed multiple DNMT1 constructs, carrying variable tags, to assess the DNMT1 domains mediating the potential interaction between the two proteins. Among these constructs, we used a DNMT1 full length plasmid (GMT1L) as positive control. All the constructs are shown in Table 3.

Table 3- DNMT1 constructs

Plasmid (MW)	Structure	Description
GMT1L (205 kD)		Full length mouse DNMT1 carrying a GFP tag
GMTΔPBD (200 kD)		PBD domain missing - GFP tag
PBDQ162E (30 kD)		PBD domain with a mutation that abolishes the interaction with PCNA and replication foci - GFP tag
GMT (190 kD)		DMAP domain missing - GFP tag
DNMT1 Δcatalytic (160 kD)		Catalytic domain missing - FLAG,TEV and Avi tags- hBirA-GFP expressing vector
DNMT1 catalytic only (65 kD)		Catalytic domain only - FLAG,TEV and Avi tags- hBirA-GFP expressing vector

All plasmids were co-transfected with BCL11A-XL plasmid. GFP expression 24 hours after transfection was observed by fluorescent microscopy (Figure 11). Protein overexpression was also verified by Western blot as shown in Figure 11. For plasmids DNMT1 Δcatalytic and DNMT1 catalytic only, biotinylation was also checked as they carry an Avi tag and are cloned into hBirA expressing vectors.

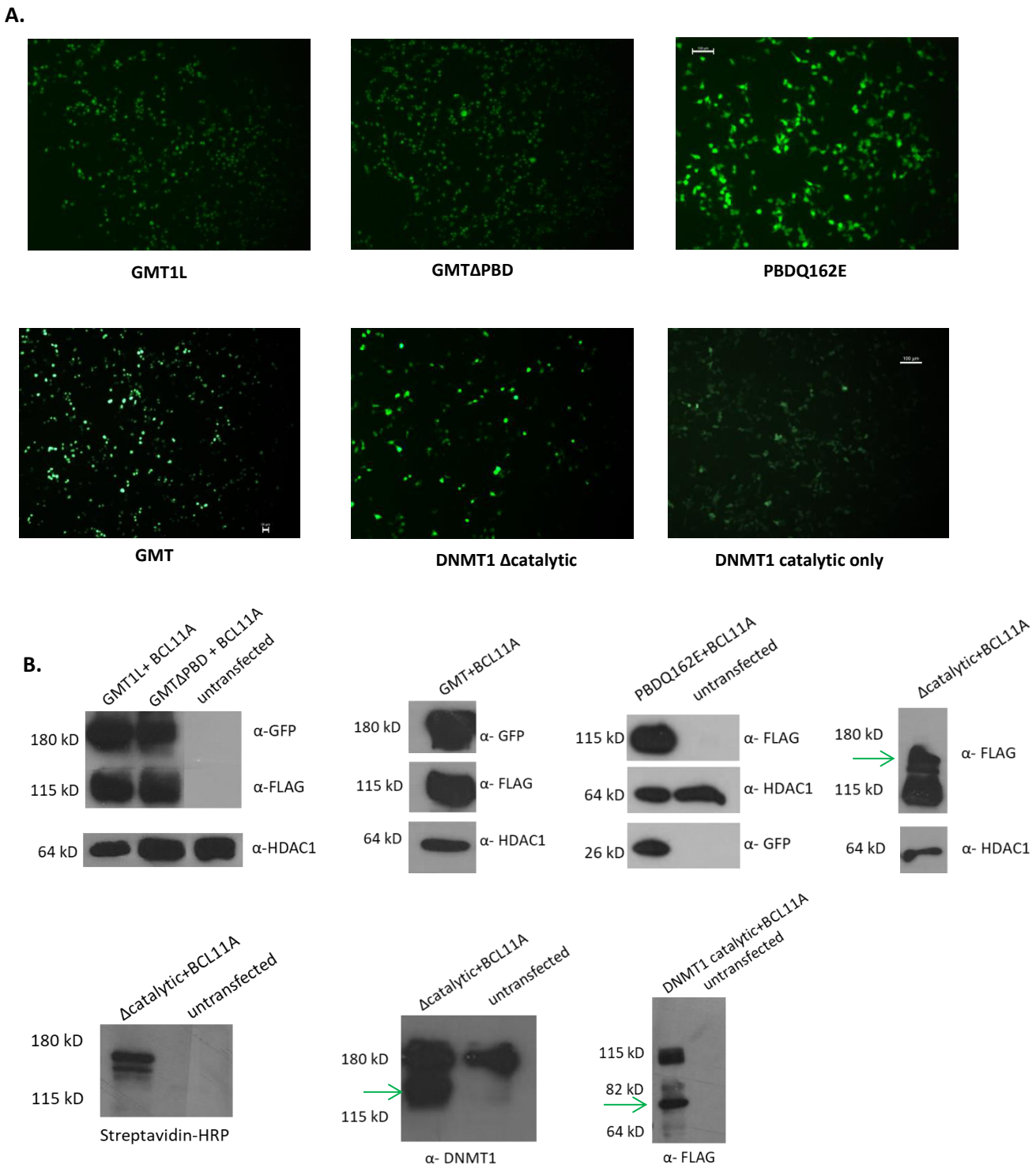


Figure 11 – HEK cells co-transfectioned with DNMT1 deletion mutants and BCL11A

A. GFP expression 24 hours after co-transfection of DNMT1 and BCL11A constructs in HEK cells by fluorescent microscopy. All DNMT1 constructs express GFP at adequate levels.

B. Plasmid expression analysis by Western blot. All nuclear extracts were blotted with an anti-FLAG antibody for BCL11A(115kD) plasmid detection. For DNMT1 plasmids GMT1L(205kD),GMTΔPBD (200kD), PBDQ162E (30kD) and GMT(190kD) detection was achieved with anti-GFP antibody. For plasmids DNMT1 Δcatalytic(160kD) and DNMT1 catalytic only (65kD) (indicated by green arrows), expression was verified with an anti-FLAG antibody, as well as streptavidin-HRP. For DNMT1 Δcatalytic an anti-DNMT1 antibody was also used. HDAC1 protein detection by an anti-HDAC1 antibody was used for loading control.

After plasmid expression was confirmed, we proceeded by carrying out immunoprecipitation (IP) experiments using nuclear extracts from the HEK co-transfected cells. Firstly, anti-GFP IP was performed with 500µg nuclear extracts from GMT1L and BCL11A co-transfected cells in order to confirm the interaction between the two plasmids. The IP efficiency was not optimal; however we detected the desirable interaction (Figure 12). We then proceeded with IP with nuclear extracts from GMTΔPBD and BCL11A co-transfected cells, to test whether the PBD domain, known to mediate DNMT1 interactions with other erythroid factors [42], would possibly also serve as the domain responsible for this interaction. However, the DNMT1/BCL11A interaction was detected in the absence of the PBD domain (Figure 12B).

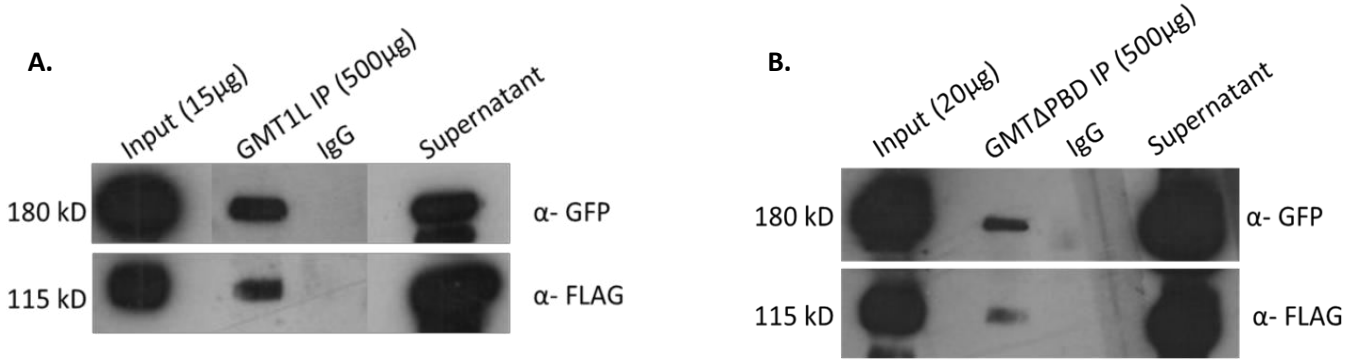


Figure 12 – Immunoprecipitation (IP) experiments
A. IP with 500µg nuclear exctracts from HEK cells co-transfected with GMT1L (205kD) and BCL11A (115kD) plasmids. Interaction was detected by Western blot, using anti-GFP antibody for GMT1L and anti-FLAG antibody for BCL11A.
B. IP with 500µg nuclear exctracts from HEK cells co-transfected with GMTΔPBD (190kD) and BCL11A (115kD) plasmids. Interaction was detected by Western blot, using anti-GFP antibody for GMTΔPBD and anti-FLAG antibody for BCL11A.

Considering the above result, we did not attempt immunoprecipitation with extracts from HEK cells co-transfected with PBDQ162E and BCL11A plasmids, but instead we went on with the GMT and BCL11A co-transfected cell extracts. The GMT construct lacks the DMAP domain, known for DNMT1 interactions with HDAC2 [27], yet we could still detect interaction between DNMT1 and BCL11A (Figure 13). Subsequently, we tried the plasmid lacking the C-terminal

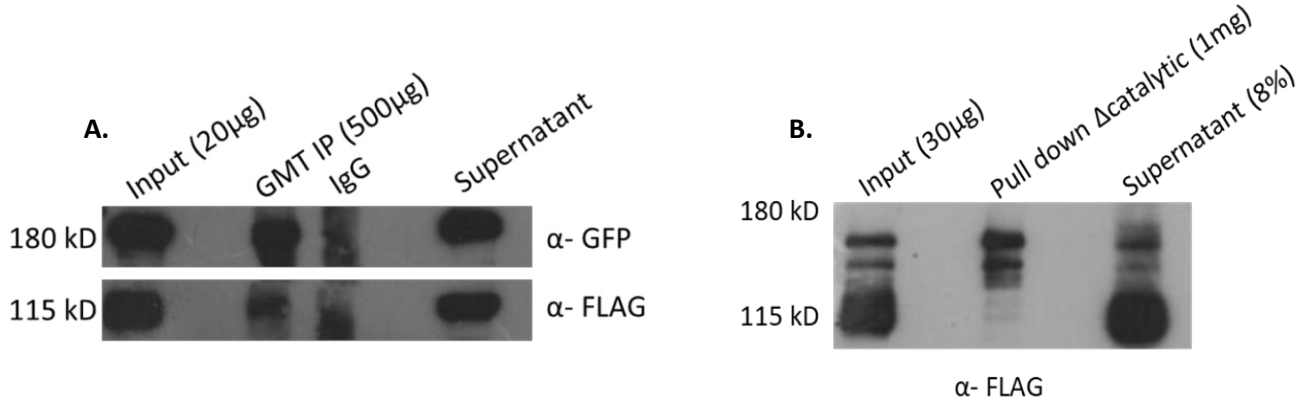


Figure 13 – Immunoprecipitation (IP) and Streptavidin Pull down experiments
A. IP with 500µg nuclear exctracts from HEK cells co-transfected with GMT (190kD) and BCL11A (115kD) plasmids. Interaction was detected by Western blot, using anti-GFP antibody for GMT and anti-FLAG antibody for BCL11A.
B. Streptavidin Pull down with 1mg nuclear extracts from HEK cells co-transfected with DNMT1 Δcatalytic (160kD) and BCL11A (115kD) plasmids. No interaction was detected by Western blot with an anti-FLAG antibody. Both proteins were detected with an anti-FLAG, as they are both fused to a FLAG tag.

catalytic domain of DNMT1. Since the DNMT1 Δcatalytic sequence is fused to an Avi tag cloned in an hBirA expressing vector, we performed streptavidin pull downs instead of immunoprecipitations, taking advantage of the considerably higher affinity between streptavidin and biotin. As shown in Figure 13, interaction was lost between DNMT1 and BCL11A, suggesting a crucial role of DNMT1 catalytic domain in mediating this interaction.

Following this result, we asked whether the catalytic domain was not only necessary, but also sufficient to mediate the interaction between DNMT1 and BCL11A. Thus, we constructed a plasmid with the DNMT1 catalytic domain only, fused to the FLAG and Avi tags in an hBirA expressing vector (see Table 3) and then performed streptavidin pulldowns. However, no interaction was detected as shown in Figure 14, suggesting a more complicated mediation of the cooperation between the two proteins.

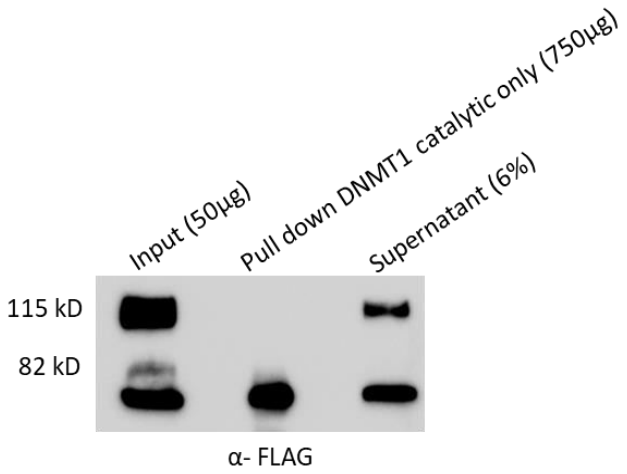
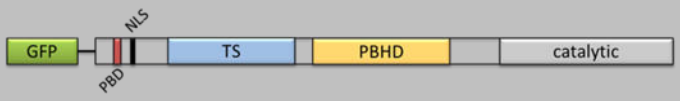


Figure 14- Streptavidin Pull down experiment
Streptavidin Pull down was performed with 750 µg nuclear extracts from HEK co-transfected with DNMT1 catalytic only (65kD) and BCL11A (115kD) plasmids. No interaction detected by Western blot with anti-FLAG antibody. Both proteins are fused with FLAG tag.

Results from Immunoprecipitation and Streptavidin Pull down experiments are summarized in Table 4.

Table 4 – Summary of Immunoprecipitation/Streptavidin pull down results

DNMT1 plasmids cotransfected with BCL11A	Structure	Detection of interaction
GMT1L (full length DNMT1)		YES
GMTΔPBD (PBD missing)		YES
GMT (DMAP missing)		YES
DNMT1 Δcatalytic (catalytic domain missing)		NO
DNMT1 catalytic (catalytic domain only)		NO

5. Discussion

Previous work in the Strouboulis lab, described interactions between DNMT1 and transcription factors with important erythroid functions such as GATA 1, ZNF143 and ZBP89, indicating a critical role for DNMT1 in erythroid differentiation. Moreover, the PBD domain was shown to be responsible for mediating these interactions [42]. Furthermore, DNMT1 has been previously implicated in fetal globin gene repression [37].

DNMT1 knockdown experiments were previously performed (Karkoulia E., unpublished data), showing defective erythropoiesis in MEL cells. In order to further investigate the potential role of DNMT1 in erythroid differentiation, CRISPR/Cas9 DNMT1 knockout experiments were carried out (D. Vlachokosta, unpublished) and led to similar results. DNMT1 KO MEL cells demonstrated impaired differentiation, as shown in Figure 7, in which fewer cells appear to be stained for hemoglobin accumulation (53% for clone C10 and 49% for clone 3D103G4 compared to 80% for control MEL cells). Cells that have not reached terminal differentiation appear to be arrested in earlier stages, mostly polychromatic or orthochromatic, as shown by May-Grünwald/Giemsa staining (Figure 7). The polychromatic stage is the stage where hemoglobinization starts, characterized by the reduced size of erythroblasts and their nucleus. The orthochromatic stage is the last stage of hemoglobinization before enucleation and it is characterized by the smallest nucleated erythroid cells with very condensed chromatin and shrunk nuclei. The process is completed with the loss of nucleus (reticulocytes) and most cytoplasmic organelles (erythrocytes) [45].

Moreover, defects in cell cycle arrest were noted in DNMT1 KO MEL cells. More specifically, about 86% of the MEL cell population was found in the G1 phase, with the same percentage reaching 75% for clone C10 and 68% for clone 3D103G4. In addition, 15,1% and 19,2% of DNMT1 clones C10 and 3D103G4 respectively, were in G2 phase, i.e. synthesizing proteins and continuing to grow to proceed to the mitotic phase, as opposed to 8,7% of control MEL cells. As far as the S phase is concerned, i.e the cells synthesizing DNA, 9% of DNMT1 clone C10 and 11,3% of DNMT1 clone 3D103G4, while in control cells the percentage is only 4,2% (Figure 8).

As expression profiling experiments confirmed, these defects appear to be due to inefficient repression of cell cycle progression genes such as cdk1, cdca3, cdca5 and cdca8 (Figure 9). These genes were selected for their known critical role in cell cycle. CDK1 is a cyclin dependent serine/threonine kinase known for its essential role in initiation of DNA replication and G1/S transition, as well as in maintaining genome stability throughout the cell cycle [46]. Another major process controlled by CDK1 is the unidirectional progress for the cell cycle. Irreversibility of cell cycle is essential for cell viability; therefore cells have developed mechanisms to ensure it. One of those mechanisms involves regulation of distinct transcriptional programs during the different phases of the cell cycle. Regulation of the cell cycle's transcriptional programs is highly complex, and Cdk1 appears to be playing a critical role in this process [46]. Cdk1 becomes active in late G1 phase and regulates a phosphorylation cascade mediating the initiation of DNA replication in S phase; afterwards, it prevents the re-replication of DNA in G2/M phases [46].

Moreover, cdk1 has important functions in chromosome segregation, as it regulates various proteins involved in chromosome cohesion and condensation, as well as regulation of spindle pole bodies. Last but not least, disruption of Cdk1 function results in DNA damage [46].

CDCA3 is an F-box protein, member of the cell division cyclin-associated protein family that is required for mitotic entry. It associates with Skp1-cullin-F-box (SCF) forming an ubiquitin ligase complex which mediates the degradation of WEE1 kinase [47]. Overexpression of CDCA3 promotes oral cancer progression by preventing G1 phase arrest and enhancing cell proliferation [47]. CDCA5, also known as sororin, plays a critical role in mitosis as a positive regulator of cohesion ensuring the maintenance of sister chromatin cohesion during metaphase [48],[49]. CDCA8, also known as borealin, is part of the chromosomal passenger complex (CPC), which is the main regulatory complex in mitosis [50]. Its dimerization has been proved to mediate optimal CPC checkpoint function by enhancing localization to centromeres and kinetochores [51]. Also, depletion of Borealin by RNA interference has been shown to cause spindle abnormalities and failure of cytokinesis, suggesting that it is required for kinetochore error correction and stability of the bipolar mitotic spindle [52]. Interestingly, combined overexpression of the CDCA3/5/8 genes in breast cancer cells has been shown to result in reduced survival of patients, due to rapid tumor proliferation [53].

We also examined the role of DNMT1 in β-globin gene regulation. We found that the adult hbb-b1 gene expression, which is greatly upregulated in induced MEL cells, was mildly reduced in DNMT1 KO MEL cells. By contrast, the embryonic hbb-βh1 gene, which is not normally expressed in MEL cells (Figure 9), was de-repressed to clearly detectable levels in DNMT1 KO MEL cells. This de-repression may contribute to hbb-b1 reduced expression levels observed in DNMT1 KO cells compared to MEL control cells, as the hbb-βh1 gene promoter competes with hbb-b1 gene promoter for interaction with the LCR interaction [6]. Alternatively, or in addition, the lower levels of hbb-b1 expression in DNMT1 KO cells may be due to impaired terminal differentiation. Our observations agree with several previous studies [17][18] strongly suggesting that upon knockdown of DNMT1 expression, fetal globin gene silencing is reversed, leading to an upregulation of the expression levels of these genes, sufficient to alleviate the phenotype of hemoglobinopathies [54].

In order to fully reveal the role of DNMT1 in terminal erythroid differentiation, further analysis is to be made concerning the DNMT1 KO MEL clones. Global gene expression profiling remains to be elaborated by RNA sequencing (ongoing). Moreover, assessment of methylation patterns by MeDIP-seq would be essential to understand the effect of the absence of DNMT1 in methylation profiles during murine erythropoiesis. Subsequently, rescue assays should be carried out by stably transfecting DNMT1 KO MEL cells with DNMT1 deletion mutants in order to investigate which domain of the protein is critical for this phenotype.

A further aim of this study was to investigate the interaction between DNMT1 and BCL11A, suggested by previous studies[17][18] as being involved in human fetal globin gene repression. We first attempted to detect the interaction between the endogenous proteins in MEL cells. We

did not succeed, presumably due to the low levels of expression of BCL11A protein in this cell type. As a result, we carried on with transient co-transfections of tagged DNMT1 and BCL11A proteins in HEK cells. After the interaction between the overexpressed proteins was confirmed, we investigated which domain of the DNMT1 protein was responsible for mediating it, our hypothesis being that the PBD domain would play this role as previously described for other DNMT1 interactions with erythroid factors[42]. To our surprise, the DNMT1 plasmid lacking the PBD domain was still capable of interacting with BCL11A. Following this result, we carried on testing other domains. Eventually, the catalytic domain was found to be critical as the interaction was abolished in its absence. Subsequently, we asked whether the catalytic domain alone would be sufficient to accomplish the interaction. However, this was not the case. These results imply a more complicated regulatory mechanism mediating the interaction between DNMT1 and BCL11A. It is known from several studies that the DNMT1 isolated catalytic domain is inactivated in the absence of the regulatory domains residing at the N-terminal of the protein [27]. In addition, dimerization of DNMT1 is regulated by the interaction between the N- and C-terminal domains and is required for its enzymatic activation [27], [55]. Moreover, DNMT1 catalytic domain has been shown to participate in DNMT1 interactions in cooperation with the N-terminal part of the protein [32]. On the other hand, several studies have shown that there are genes for which DNMT1 acts as a transcriptional repressor independent from its methyltransferase function, and therefore deletion of the catalytic domain from DNMT1 does not abolish its repressive activity. These findings suggest a role for a scaffolding function of the DNMT1 leading to recruitment of repressors at target genes [56]. Altogether, these studies indicate that the DNMT1 catalytic domain is indeed involved in protein interactions, however other parts of the protein, or perhaps even other interacting factors, may be required in order to regulate these interactions. In addition, it could be possible that it is not the catalytic domain *per se* that is required for the interaction, but the catalytic function of the methyltransferase, as DNMT1 is known to regulate gene expression by methylating gene promoters [35]. Unfortunately, our results are not sufficient to favor any one of those hypotheses.

Shedding light in the interplay between DNMT1 and BCL11A would be of great importance as it could reveal new approaches for hemoglobinopathies treatment.

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