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

Master Thesis

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Summary

DNA methylation is essential for embryonic development and in diverse biological processes. The DNA methyltransferase Dnmt1 maintains parental cell methylation patterns on daughter DNA strands in mitotic cells. DNMT1 knock out/down (KO/KD) studies in hematopoietic lineages or in mice revealed that DNMT1 drives HSCs to myeloerythroid fate. In adult-stage erythroid cells, DNMT1 represses γ-globin gene expression by methylating its promoter and interacts with proteins (TR2/TR4, BCL11A) implicated in the silencing of γ-globin expression. Publications of our laboratory showed that DNMT1 co-purified with several hematopoietic transcription factors (GATA1, FOG-1, GFI-1b) involved in erythroid differentiation forming a core complex composed by DNMT1 and transcription factors ZBP89 and ZNF143, present also in non-hematopoietic cells, which interacts with distinct hematopoietic protein subcomplexes. Additionally, a short PCNA Binding domain (PBD) of DNMT1 proved necessary and sufficient for DNMT1 interaction with these transcription factors. Further evidence suggested that DNMT1 functions as a co-repressor of ZBP-89 and GATA1 acting through upstream regulatory elements of major hematopoietic transcription factors, the PU.1 and GATA1 gene loci. Moreover, DNMT1 KD in murine erythroleukemic (MEL) cells showed a clear lack in cell cycle arrest in erythroid differentiation due to the lack of repression of genes responsible for cell proliferation. The main aim of this study is to elucidate more clearly the role of DNMT1 in erythroid cell differentiation. Towards this aim, we attempted to generate and characterize DNMT1 KO MEL cells. Furthermore, we tried to further identify the protein partners of DNMT1 and exert its function, utilizing a biotin-tagging approach coupled to mass spectrometry. Another aim of this study is to identify gene targets of DNMT1 that are suppressed by its regulatory control through a methylation dependent or independent mechanism along global DNA demethylated erythroid genome, taking into account that DNMT1 is the main DNA methyltransferase expressed throughout murine fetal liver derived erythroid differentiation. Moreover, we tried to investigate whether the short PCNA binding domain (PBD) of DNMT1 renders DNMT1 a scaffolding protein for the recruitment of additional repressive partners inducing gene silencing performing phenotypic rescue assays of DNMT1 KO cells by stably transfecting DNMT1 deletion mutants.

Περίληψη

Η DNA μεθυλίωση είναι απαραίτητη κατά την εμβρυική ανάπτυξη και εμπλέκεται σε διάφορες βιολογικές διαδικασίες. Η DNA μεθυλοτρανσφεράση DNMT1 διατηρεί το πρότυπο μεθυλίωσης του προγονικού κυττάρου στους απογόνους του κατά την διαδικασία της αντιγραφής. Μελέτες DNMT1 Knock out/down (KO/KD) είτε σε αιμοποιητικές σειρές είτε σε ποντίκια έδειξαν ότι η DNMT1 καθοδηγεί τα HSCs να διαφοροποιηθούν προς κύτταρα της μυελικής σειράς. Η DNMT1 εμπλέκεται στη σίγηση της γ-σφαιρίνης των ερυθροκυττάρων στο ενήλικο στάδιο μεθυλιώνοντας τον υποκινητή της γ-σφαιρίνης και αλληλεπιδρώντας με πρωτεΐνες (TR2/TR4, BCL11A). Σε δημοσίευση του εργαστηρίου μας δείξαμε ότι η DNMT1 αλληλεπιδρά με διάφορους αιμοποιητικούς μεταγραφικούς παράγοντες (GATA1, FOG-1, GFI-1b) που εμπλέκονται στη διαφοροποίηση των ερυθροκυττάρων, δημιουργώντας ένα κύριο πρωτεϊνικό σύμπλοκο αποτελούμενο από την DNMT1 και τους μεταγραφικούς παράγοντες ZBP89 και ZNF143, το οποίο υφίστατο και σε μη αιμοποιητικά κύτταρα και αλληλεπιδρά με διακριτά υποσύμπλοκα αιμοποιητικών παραγόντων στα ερυθροκύτταρα. Επιπλέον, το μικρό PCNA Binding domain (PBD) της DNMT1 είναι αναγκαίο και ικανό για την αλληλεπίδραση της DNMT1 με μεταγραφικούς παράγοντες. Περαιτέρω στοιχεία υποδεικνύουν ότι η DNMT1 λειτουργεί ως συγκαταστολέας με ZBP89 και GATA1 δρώντας σε άνωθεν ρυθμίστηκα στοιχεία των γονιδίων των κύριων αιμοποιητικών παραγόντων PU.1 και GATA1. Ακόμη DNMT1 KD σε MEL κύτταρα έδειξε μια ξεκάθαρη αδυναμία στην παύση του κυτταρικού κύκλου κατά την διαφοροποίηση των ερυθροκυττάρων εξαιτίας της έλλειψης καταστολής γονιδίων υπεύθυνα για τον κυτταρικό πολλαπλασιασμό. Ο κύριος στόχος της συγκεκριμένης έρευνας είναι να διαλευκάνει περαιτέρω τον ρόλο της DNMT1 κατά την διαδικασία της διαφοροποίησης των ερυθροκυττάρων. Για τον σκοπό αυτό, προσπαθήσαμε να δημιουργήσουμε και να χαρακτηρίσουμε DNMT1 KO MEL κύτταρα. Επίσης, προσπαθήσαμε να εξακριβώσουμε περαιτέρω πρωτεϊνικούς παράγοντες με τους οποίους η DNMT1 αλληλεπιδρά ώστε να εντοπίσουμε τις λειτουργίες στις οποίες εμπλέκεται χρησιμοποιώντας μια μέθοδο, η οποία φέρει ως επίτοπο την βιοτίνη στο πρωτεϊνικό μόριο ενδιαφέροντος, συζευγμένη με φασματομετρία μάζας. Ένας ακόμα στόχος της συγκεκριμένης έρευνας είναι να εντοπίσουμε τους γονιδιακούς στόχους της DNMT1 οι οποίοι καταστέλλονται μέσω της δράσης ενός μηχανισμού εξαρτώμενου ή ανεξάρτητου της DNA μεθυλίωσης, δεδομένου ότι η DNMT1 είναι η κύρια μεθυλοτρανσφεράση που εκφράζεται στα ερυθροκύτταρα και τα χαμηλά επίπεδα μεθυλίωσης στο γονιδίωμα των ερυθροκυττάρων. Ακόμη, προσπαθήσαμε να διερευνήσουμε κατά πόσο το μικρό PCNA binding domain (PBD) της DNMT1 την καταστεί ικρίωμα για την προσέλκυση επιπρόσθετων κατασταλτικών παραγόντων που επάγουν την γονιδιακή σίγηση διεξάγοντας πειράματα επαναφοράς φαινοτύπου σε DNMT1 KO κύτταρα τα οποία διαμολύνονται μόνιμα με DNMT1 μεταλλάγματα απαλοιφής.

Introduction

Hematopoiesis is the process of generation of all the mature blood cells from a rare pool of hematopoietic stem cells (HSCs). Hematopoietic stem and multilineage progenitors (MLPs) produce more than 100 billion mature blood cells per day in a healthy individual which carry out functions such as oxygen transport, immunity, hemostasis, or tissue surveillance and remodeling. HSCs emerge from the hemangioblast which in murine embryos gives rise to endothelial, blood and vascular smooth muscle cells. Advances in live cell imaging allowed visualization of mammalian HSCs, emerging directly from endothelial cells(Klaus & Robin, 2017)(Eilken, Nishikawa, & Schroeder, 2009). Mature blood cells have a limited lifespan and need to be constantly replenished in order to maintain homeostasis of the organism. There are at least eight types of blood cells, varying in their appearance and function (Figure 1). The most abundant cells in the blood are the red blood cells or erythrocytes, whereas the rest of the cell types (white blood cells) are the platelets, the granulocytes (neutrophils, eosinophils and basophils), the monocytes and the lymphocytes (Orkin & Zon, 2008).

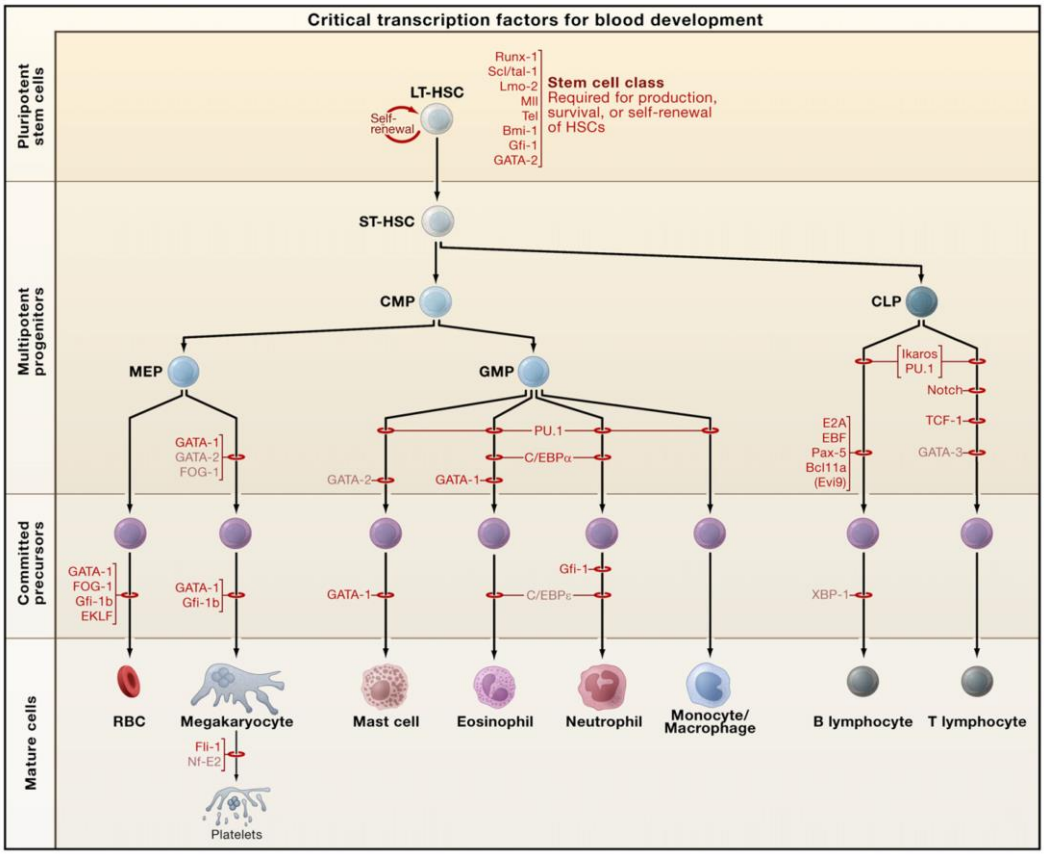


Figure 1. The hematopoietic tree. Schematic representation of the main lineage commitment steps in hematopoiesis. The long-term hematopoietic stem cell (LT-HSC) is the basis of the hematopoietic hierarchy and gives rise to multilineage progenitors (MLP)/ short-term hematopoietic stem cell (ST-HSC), which can differentiate into all the hematopoietic lineages. MLPs become lineage restricted to the lymphoid and myeloid lineages in the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP), respectively. CLPs can give rise exclusively to B and T cells, while CMPs can give rise to megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-monocyte progenitors (GMP). The stages at which hematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts, are indicated by red bars. The factors depicted in black have been associated with oncogenesis. Those factors in light font have not yet been found translocated or mutated in human/mouse hematologic malignancies (Orkin & Zon, 2008).

Erythropoiesis is a multistep tightly regulated process that involves differentiation of early erythroid progenitors to enucleated red blood cells. HSCs and MLP generate the common myeloid progenitors (CMP), which progress into megakaryocytic/erythroid progenitors (MEP). MEP cells, upon the action of growth factors and the combinatorial action of certain transcription factors, are differentiated into burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E). BFU-Es require stem cell factor (SCF), but not erythropoietin (EPO) for proliferation. In contrast, EPO is essential for the survival and proliferation of CFU-Es. Then proceeding to the last phase of erythropoiesis, terminal differentiation takes place. The CFU-Es differentiate into proerythroblast (ProE) cells, basophilic I and II (Baso1 and Baso2), polychromatophilic (Poly), and orthochromatic (Ortho) erythroblasts. During this process, the size of the cells gradually decreases, and they synthesize large amounts of hemoglobin (Hb) and reorganize their membrane with accompanying nuclear condensation. At the end of terminal erythroid differentiation, Ortho cells expel their nucleus, which is surrounded by plasma membrane with a small amount of cytoplasm, to generate a pyrenocyte, which is rapidly engulfed by macrophages of the erythroblastic niches, and a reticulocyte which completes its maturation in the bloodstream (Figures 1, 2, 3). Mature erythrocytes are biconcave disks without mitochondria or organelles, full of hemoglobin for maximal capacity to transport oxygen to the tissues (Tsiftsoglou, Vizirianakis, & Strouboulis, 2009)(Palis, 2014)(Baron, Isern, & Fraser, 2017)(Orkin & Zon, 2008).

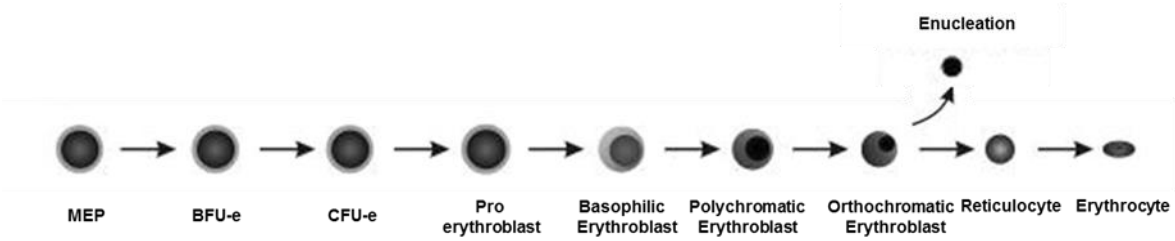


Figure 2. Schematic representation of the different stages in erythroid differentiation. Megakaryocytic/Erythroid progenitors (MEP) give rise to burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E), which are erythroid restricted progenitors. Terminal erythroid differentiation starts with the proerythroblast and terminates upon enucleation and the formation of erythrocytes.(Ferreira, Ohneda, Yamamoto, & Philipsen, 2005)

Erythropoiesis occurs in multiple waves and at distinct anatomical locations. The first wave occurs in blood islands in the yolk sac in both mouse and humans and produces primarily primitive erythroid nucleated cells (EryP). The second wave is also transient and arises in the yolk sac but is definitive, composed of enucleated erythroid cells. The third wave emerges from HSCs in the fetal liver, includes enucleated erythroid cells, and during ontogeny moves from the liver to the spleen and finally to the bone marrow (Figure 3)(Palis, 2014)(Baron et al., 2017).

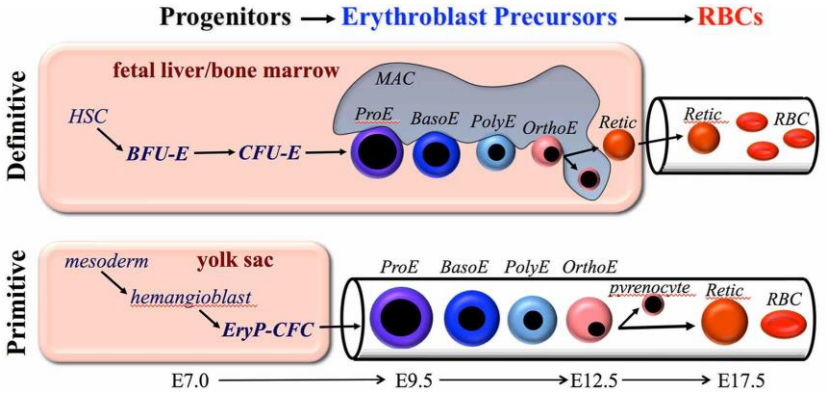


Figure 3. Overview of primitive and definitive erythropoiesis. Both forms of erythroid cell production are characterized by the progressive movement of cells through three compartments: progenitors, erythroblast precursors, and red blood cells (RBCs). Erythroid progenitors (BFU-E, CFU-E, and EryP-CFC) are defined by their capacity to form colonies of maturing erythroid cells in vitro. Erythroid precursors are defined morphologically as proerythroblasts (ProE), basophilic erythroblasts (Baso), polychromatophilic erythroblasts (PolyE), and orthochromatic erythroblasts (OrthoE). OrthoE enucleate to form a pyrenocyte, that contains the condensed nucleus, and a reticulocyte (Retic), that goes on to mature into a RBC. Definitive erythropoiesis in the adult organism is derived from hematopoietic stem cells (HSC), while primitive erythropoiesis occurs just once from mesoderm cells in the early embryo.(Palis, 2014)

Furthermore, the transition from primitive to definitive erythropoiesis coincides with switches in globin gene expression. Four globin chains and one heme group form the hemoglobin tetramers that bind oxygen or carbon dioxide. Hemoglobins are classified into embryonic, fetal and adult ones and differ in their affinity to bind the gas molecules. Primitive erythrocytes in human express the embryonic hemoglobins [Gower 1 (ζ2ε2), Gower 2 (α2ε2) and Portland (ζ2γ2)], definitive erythrocytes of fetal origin express the fetal hemoglobin (α2γ2) and definitive erythrocytes in the adult express hemoglobin which consists of two α and two β globins (α2β2). It should be noted though that globin switching is not lineage dependent. It has been reported that primitive erythroid cells, in addition to embryonic chains, produce fetal and adult globins and that definitive erythroid cells, in addition to fetal and adult globins, produce embryonic globins (Stamatoyannopoulos G, Constantoulakis P, Brice M, Kurachi S, 1987)(Tsiftsoglou et al., 2009).

A combination of signals and transcription factors define the transition from the primitive to the definitive erythropoiesis. In general, transcription factors play significant roles driving lineage-specific cellular maturation. They are responsible for the activation of the erythroid specific genes, the suppression of the alternative lineage transcriptional programs of the HSCs and MLPs as well as the suppression of cell division(Tsiftsoglou et al., 2009)(Palis, 2014)(Baron et al., 2017)(Orkin & Zon, 2008). Therefore, the epigenetic regulation of transcription factors, specifically, and the genome regulation, in general are critical for cell fate determination. Epigenetic signals include DNA methylation, covalent modifications of the histone proteins (such as acetylation, phosphorylation and methylation), incorporation of histone variants and non-coding RNAs. All these signals function synergistically to modulate chromatin structure and thereby determine the transcriptional activity of the genome.

DNA methylation, as already mentioned, is a major epigenetic modification catalyzed by DNA methyltransferases DNMTs, which transfer the methyl moiety from the methyl donor S-adenosylmethionine to 5th position on the cytosine ring. DNA methylation is most prevalent at CpG sites, although not all CGs are methylated and the pattern of distribution of

methylated and unmethylated CGs is cell type specific (Lister et al., 2009)(Arand et al., 2012). DNA methylation in regulatory regions of genes plays a role in silencing genes either by directly inhibiting the interaction of transcription factors with their regulatory sequences (Comb & Goodman, 1990) or by attracting methylated DNA-binding proteins, which in turn recruit histone deacetylases and histone methyltransferases, resulting in an inactive chromatin structure (Nan et al., 1998)(Jones et al., 1998). Genome wide DNA methylation patterns change during development and differentiation and have been implicated in various biological processes such as gene regulation, chromatin and nuclear structure, DNA replication and in developmental processes such as parental imprinting, X-chromosome inactivation in females and in carcinogenesis (Dai, 2015)(Jesús Espada & Esteller, 2010)(Jackson-Grusby et al., 2001)(Jurkowska, Jurkowski, & Jeltsch, 2011). In mammals, DNA methylation is carried out by the *de novo* DNA methyltransferases DNMT3a, DNMT3b, (Okano, Bell, Haber, & Li, 1999) and by the DNMT1 maintenance methyltransferase which adds methyl groups on newly synthesized hemimethylated strands of DNA following replication (Jurkowska et al., 2011). DNMT1 prefers hemimethylated substrates (A. Jeltsch et al., 2001)(Bacolla, Pradhan, Roberts, & Wells, 1999) although studies have shown it can catalyze methylation of unmethylated substrates as well (*de novo* methylation) (Jair et al., 2006)(Lorincz, Schu, Hutchinson, Dickerson, & Groudine, 2002)(Egger et al., 2006).

The functional relevance of DNMT1 during mammalian embryo development and in the homeostasis of adult tissues is highlighted by genetic experiments in mouse models and human somatic cells. Deletion of both Dnmt1 alleles in the mouse results in embryonic lethality at day 8.5 after midgestation. Notably, this rate of DNA demethylation has no apparent effect on cell viability from day 0 to day 8.5, during which time two sequential waves of complete demethylation (in primordial germ cells and in the pre-implantation embryo) and de novo methylation take place in the genome of the developing embryo (Li, Bestor, & Jaenisch, 1992). In line with this, mouse ES cells maintained in an undifferentiated state can survive to an almost complete CpG demethylation of the DNA molecule, but induction of a differentiation program in these demethylated ES cells rapidly results in cell death (Li et al., 1992) (Tsumura, Hayakawa, Kumaki, & Takebayashi, 2006). There are many publications that have linked DNMT1 function to cell growth regulation. HSCs from dnmt1 null homozygous mice resulted in no myeloid or lymphoid colony formation in in vitro differentiation assays, due to increased apoptosis (Bröske et al., 2009). In a similar way, depletion of DNMT1 in mouse fibroblasts causes p53-depentednt apoptosis within 5–6 days of DNMT1 loss (Jackson-Grusby et al., 2001) and genetic disruption of DNMT1 in HCT116 colon cancer cells causes mitotic catastrophe, G2/M cell-cycle arrest and eventually, apoptosis within 48 h (Tsujimoto, He, Zhang, Chen, & Hevi, 2007). Additionally, DNMT1 RNAi experiments in HCT116 decelerated proliferation rate and in primary human fibroblasts (IMR90) arrested in G1 phase (Barra, Schillaci, Lentini, Costa, & Leonardo, 2012). In human ESCs, null allele of Dnmt1 results in increased DNA damage and G1 arrest (Liao et al., 2015), whereas mice carrying a hypomorphic Dnmt1 allele are prone to tumor development due to chromosomal instability (Eden, Waghmare, & Jaenisch, 2003). Equally important, a causal link between DNMT1 mutations and human hereditary disease, namely a sensory neuropathy, has been reported (Klein et al., 2011).

DNMT1 is ubiquitously expressed and comprises 1620 amino acids in mouse and 1616 amino acids in human. The molecular weight of the protein is 183 kDa and is highly conserved between mouse and human with 77% protein identity. The C-terminal part of the protein contains the catalytic methyltransferase domain, whereas the N-terminal part contains a

number of functional 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) (Figure 4). The DMAP1 (DNA methyltransferase associated protein 1) interaction domain is involved in the interaction of Dnmt1 with Dmap1, a transcriptional repressor (Rountree, Bachman, & Baylin, 2000). Also, this domain has been shown to bind DNA at CG sites (A. Jeltsch et al., 2001)(Araujo et al., 2001). The PBD domain mediates interaction with PCNA thus targeting DNMT1 to replication foci (L.S. Chuang, H.I. Ian, T.W. Koh, H.H. Ng, G. Xu, 1997). The TS (targeting sequence) domain is involved in the targeting of DNMT1 to centromeric heterochromatin (Easwaran, Schermelleh, Leonhardt, & Cardoso, 2004), to replication foci (H. Bestor et al,. 1992) and also facilitates DNMT1 dimerization (Leonhardt, Fellinger, Rothbauer, Felle, & La, 2009). The PBHD consists of the BAH1 and BAH2 (Bromoadjacent homology 1 and 2) subdomains implicated in protein interactions (Yang & Xu, 2013). Furthermore, DNMT1 has been shown to interact with additional epigenetic factors, besides the aforementioned, such as HDACs (HDAC1, HDAC2) (Fuks, Burgers, Brehm, Hughes-davies, & Kouzarides, 2000) (Rountree et al., 2000)(Robertson et al., 2000), the Rb and E2F1 transcription factors (Robertson et al., 2000), the histone lysine methyltransferases Suv39H1(Hurd, Deplus, & Kouzarides, 2017), SET7/9 (Gyeong, Benner, Feehery, Samaranayake, & Este, 2009), G9a (Estève et al., 2006), EZH2(Brenner et al., 2006), the silencing effectors HP1 (heterochromatin protein 1) (Hurd et al., 2017), MeCP2(Sciences, 2003), the histone demethylase Lsd1 (Bajko et al., 2009) and the NuRD complex (Cai et al., 2013). Thus, the multi-domain interactive N-terminal region of DNMT1 underlies the multiple functional roles of DNMT1 into the nucleus besides its methyltransferase catalytic activity. Indeed, it has been shown that for a set of genes DNMT1 acts as a repressor independently of its methyltransferase activity, thus providing evidence that DNMT1 may also act as a scaffolding protein for the recruitment of additional repressive partners (Milutinovic, Brown, Zhuang, & Szyf, 2004), (Clements et al., 2012).

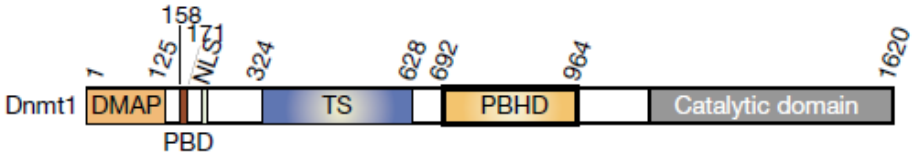


Figure 4: Schematic representation of DNMT1 domains. Dnmt1 is a large enzyme, comprising 1620 amino acids in mouse. The C-terminal end of the protein contains the catalytic methyltransferase domain, whereas multi-domain N-terminal region of DNMT1 contains the DMAP1 interacting domain (DMAP1), the PCNA binding domain (PBD), the nuclear localization signal (NLS), the targeting sequence (TS) and the polybromo homology domain (PBHD).

Implication of DNA methylation in hematopoiesis has been studied extensively, however the role of DNMT1 is still under investigation. Dnmt1 null ES cells are restricted in their differentiation potential, whereas conditional knockout of the Dnmt1 gene in the erythroid lineage using GATA1-Cre or EpoR-Cre mice results in embryonic lethality (Trowbridge, Snow, Kim, & Orkin, 2009)(Xu et al., 2013). Conditional DNMT1 knockout in hematopoietic stem cells (HSCs) restricts HSC differentiation to the myeloid progeny as they cannot differentiate into lymphoid cells (Bröske et al., 2009). Furthermore, dnmt1 knock-down studies in fetal liver cells showed that decreased levels of DNMT1 resulted in the acceleration of the erythroid specific gene induction suggesting that global demethylation may lead to the rapid removal of methylation marks at sites of massively induced erythroid genes (Shearstone et al., 2011). Observations in leukemic granulocyte-macrophage progenitors showed that DNA methylation-mediated silencing of bivalent chromatin domains is required for apoptosis,

tumor suppression and the hematopoietic lineage restriction (Trowbridge et al., 2012). In erythroid cells, it has been shown that DNMT1 represses γ-globin gene expression in adult-stage erythroid cells by methylating its promoter (Banzon et al., 2015)(Roosjen et al., 2014). Thus, DNMT1 inhibition results in reactivation of the human γ-globin gene expression in the adult stage, offering a potential therapeutic option for hemoglobinopathies treatment. Recent studies revealed that transcription factorsTR2/TR4 and BCL11A implicated in γ-globin silencing interact with DNMT1. Specifically, it was shown that DNMT1 co-purifies with the TR2/TR4 nuclear receptors, which bind to the embryonic and fetal β-type globin promoters acting probably as adult-stage specific repressors for these genes (Cui et al., 2011), whereas more recently DNMT1 was identified as a BCL11A-associated protein in the silencing of γ-globin expression in primary human adult erythroid cells (Xu et al., 2013).

Recent work from the Strouboulis laboratory provided evidence for the interaction of DNMT1 with several hematopoietic transcription factors (GATA1, FOG-1, GFI-1b) involved in erythroid differentiation. It was shown that DNMT1 formed distinct protein subcomplexes with specific transcription factors and it was proposed that a core complex composed by DNMT1 and transcription factors ZBP89 and ZNF143 existed, present also in non-hematopoietic cells (Papageorgiou et al., 2016). Additionally, a short PCNA Binding domain (PBD) of DNMT1 proved necessary and sufficient for DNMT1 interaction with these transcription factors. Furthermore, further evidence was provided suggesting that DNMT1 acted as a co-repressor of ZBP-89 and GATA1 acting through upstream regulatory elements of major hematopoietic transcription factors, the PU.1 and GATA1 gene loci (Papageorgiou et al., 2016). The fact that DNMT1 was found to interact with transcription factors implicated in erythropoiesis and globin gene regulation raised the prospect of DNMT1 being implicated in erythroid differentiation.

In order to investigate a potential role for DNMT1 in erythropoiesis, we knocked-down DNMT1 by lentiviral mediated shRNA in mouse erythroleukemic (MEL) cells (Karkoulia et al., unpublished work). Western blots and activity assays provided evidence for the high efficiency of the DNMT1 knock-down whereas FACS analysis confirmed no secondary effects. Three different assays were employed to quantitate cell differentiation in control and DNMT1 knock-down MEL cells. Preliminary expression profiling, cytospin preparations and FACS analysis in a set of at least three independent experiments showed a clear arrest in erythroid differentiation with defects in cell cycle regulation as a result of reduced levels of DNMT1. More precisely, cytospins showed that DMSO induced DNMT1 knock-down cells did not fully differentiate and rather resembled polychromatic/ orthochromatic red cells. FACS analysis showed increased rates of proliferation and microarray analysis showed impaired repression of the cell cycle promoting genes. Notably, all genes responsible for the erythroid transcription program were normally expressed. We therefore assume that the DNMT1 knock-down phenotype of impaired erythropoiesis results from cell cycle defects due to the lack of repression of genes responsible for cell proliferation. It is still unclear how DNMT1 is recruited to the cell cycle related genes, as DNMT1-ChIP experiments did not reveal binding of DNMT1 at their promoters. However, GATA-1 has been reported to regulate cell cycle related genes (Rylski et al., 2003) and thus it is possible that at least part of DNMT1's function in the cell cycle regulation may be mediated by its interaction with GATA-1.

Concluding, DNMT1 seems to have significant roles in many stages during the process of erythropoiesis. Initially, DNMT1 is responsible for driving HSCs to myeloerythroid fates (Bröske et al., 2009), and afterwards it is required for normal erythroid differentiation by

repressing genes responsible for cell proliferation. **Our main goal is to shed more light to the molecular basis of DNMT1 functions in regulating erythroid cell differentiation.** To this end, we tried to further identify the protein partners it interacts with to exert its function, utilizing a biotin-tagging approach coupled to mass spectrometry (Boer et al., 2003) and to characterize the consequences on erythroid differentiation of DNMT1 knockout. Another aim is to identify DNMT1 gene targets that are may be regulated through a methylation dependent or independent mechanism. Moreover, we tried to investigate whether the short PCNA binding domain (PBD) of DNMT1 renders DNMT1 a scaffolding protein for the recruitment of additional repressive partners inducing gene silencing, by performing rescue assays in MEL cell clones bearing deletion mutants of DNMT1.

Materials and Methods

Constructs

DNMT1 deletion mutants were kindly provided by Dr. C. Cardoso (TU, Darmstadt) (Easwaran et al., 2004). MTNG DNMT1 deletion mutant was recloned as a XbaI fragment containing GFP in pBUDneo vector, in order to acquire neomycin resistance and verified by sequencing. Mouse DNMT1 without the catalytic domain cDNA was PCR amplified using as template GMT1L (Figure 8A) and cloned as an Acc65I fragment into pEGFP-C2 vector (Addgene).

Cell cultures and Cell Transfections

C88 MEL cells were grown in DMEM-10% FBS-1% penicillin/streptomycin and induced to differentiate with 2% DMSO as previously described (Antoniou, 1991). C88 MEL cells expressing the BirA biotin ligase (Boer et al., 2003) and bio-tagged DNMT1 were cultured as above with the addition of G418 (for selecting Avi-tagged DNMT1 expression) and puromycin (for selecting BirA expression) as previously described (Papageorgiou et al., 2016).

HEK293T and HeLa were cultured in DMEM-10% FBS-1% penicillin/streptomycin (all from Life Technologies) and were transiently transfected using the calcium phosphate method and JetPEI (Polyplus transfection), respectively.

Nuclei from MEL cells, HEK 293T and HELA were prepared using the high salt extraction method. Cells were centrifuged at 1,100rpm for 5min at room temperature and washed twice with PBS and protease inhibitors at 4^0C. Cells were resuspended in 4 × cell volume of Buffer A (10 mM HEPES at pH 7.9, 1.5 mM MgCl2, 10 mM KCl, protease inhibitors). After incubation at 4^0C for 10 min, cell debris was pelleted at 13.000rpm, 1 min 4°C. The supernatant containing the cytoplasmic extract was removed after centrifugation and the pellet was resuspended in 1× cell volume of Buffer C (20 mM HEPES at pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 2 mM DTT, protease inhibitors). After incubation at 4^0C for 20 min, the soluble nuclear extracts were fractionated from the insoluble pellet by centrifugation at 13.000rpm, 10 min, 4°C.

CRISPR/Cas9 knockout (KO) plasmid transfection for in vitro DNMT1 gene knockout

The CRISPR/Cas9 system can be used to generate knockout cells by co-expressing a guide RNA (gRNA) specific to the target gene and the endonuclease Cas9 (Van der Oost J, Westra ER, Jackson RN, 2015). DNMT1 CRISPR/Cas9 KO plasmid containing Cas9 nuclease and a pool of three DNMT1-specific 20 nt gRNAs was purchased from Santa Cruz Biotechnology. Transfection of the plasmids was carried out in C88 MEL cells using UltraCruz® Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions with minor modifications. MEL cells were treated with two sequential transfections 24 hours apart and sorted for GFP expression 24 hours post-transfection. The acquired % of GFP cells was 1.72 and 0.41, respectively, two times that the CRISPR/Cas9 knockout assay was performed. Serial dilutions were carried out in order MEL DNMT1 KO clone be isolated.

Western Blotting

SDS-PAGE and Western immunoblotting were carried out as previously described (P Rodriguez et al., 2006). 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): N6 GATA-1 (sc-265), GFP (B-2) (sc-9996) and from Abcam HDAC1 (ab7028-50). Rabbit polyclonal DNMT1 antibody (1-248) was purchased from BioAcademia (Osaka, Japan) and nucleophosmin anti-B23 antibody was a kind gift from Pui K. Chan, Baylor College. Secondary antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (sc-2004, sc-2314, sc-2006).

Streptavidin Pulldowns

Streptavidin pulldowns were done as previously described (P Rodriguez et al., 2006) using 50 µl of resuspended beads (PureProteome™ Streptavidin Magnetic Beads) 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. For mass spectrometric (MS) analysis, 6 mg of nuclear extract were used for streptavidin pulldown. Prior to streptavidin pulldown, nuclear extracts were subjected to benzonase treatment for the removal of nucleic acids.

Mass Spectrometry

Mass spectrometry was carried out as previously described (Cui et al., 2011) with the elucidation that only on-bead trypsinization was carried out for the releasing of peptides.

DNMT1 ChIP assay

BirA MEL cells were harvested before (8 x10^7) or after (10^8) differentiation induction (with 2% DMSO for 5 days). Cells were cross-linked with 1% formaldehyde for 10min at room temperature. The reaction was quenched with a final concentration of 0.125M glycine; cell pellets were further processed as described (Schuh et al., 2005). Immunoprecipitations of sextuplicates per condition were performed using anti-DNMT1 antibody purchased from BioAcademia (70-201).

Day 1: 10^7 cells per sample were cross-linked with 1% formaldehyde for 10min with shaking at room temperature. The reaction was quenched with 0.125M glycine for 10min with shaking at room temperature. Cells were centrifuged at 3,000rpm for 5min at 4°C. Cell pellet was resuspended in 1ml ice cold PBS and 1% proteinase inhibitors, transferred in microfuge tubes and centrifuged at 3,000rpm for 5min at 4°C. Cells were lysed with 200µl ChIP Lysis buffer/ChIP reaction on ice for 10min. Chromatin was sonicated in a Diagenode bioruptor using high amplitude, 30sec on and 30sec off settings, for 19min to break up the DNA into fragments of maximum 500bp length. Tubes were centrifuged at maximum speed for 10min at 4°C. Supernatant was transferred in FACS tubes and 1/10 of the volume, i.e 20µl were transferred in a separate tube, mixed with 280µl of ChIP Dilution buffer and stored in -20°C to be used as the input sample. The 180µl of the lysate were mixed with 1.8ml of ChIP Dilution buffer and the samples were pre-cleared by adding 40µl of salmon sperm blocked protein A or G beads (Millipore)/ 2ml sample for 1hr with rotation at 4°C. This pre-clearing step was performed in a master mix tube for both the antibody and the no antibody samples. Tubes were centrifuged at 3,000rpm for 5min at 4°C. Supernatant was aliquoted into FACS tubes (2ml in each) and 5µg of the antibody or 5µl of the ChIP Dilution buffer (no

antibody control sample) were added for 4hrs with rotation at 4°C. 60µl of protein A or G beads were added to the samples for an overnight incubation with rotation at 4°C.

Day 2: FACS tubes were centrifuged at 3,000rpm for 5min at 4°C and supernatant was discarded. Beads were resuspended in 1ml of Low Salt ChIP buffer and transferred to fresh microfuge tubes followed by incubation for 10min with rotation at 4°C. Beads were concentrated by centrifuging at 1,000rpm for 1min at 4°C. Supernatant was discarded. Beads were washed with 1ml of High Salt buffer for 10min with rotation at 4°C and centrifuged at 1,000rpm for 1min at 4°C. Supernatant was discarded. Beads were washed with 1ml of $LiCl_2$ buffer for 10min with rotation at 4°C and centrifuged at 1,000rpm for 1min at 4°C. Supernatant was discarded. Beads were washed 2x with 1ml of TE buffer for 10min with rotation at 4°C and centrifuged at 1,000rpm for 1min at 4°C. Supernatant was discarded. Samples were briefly centrifuged at maximum speed and all of the supernatant was discarded. Complexes were eluted by adding 150µl of freshly made Elution buffer for 15min with rotation at room temperature. Beads were centrifuged at 3,000rpm for 5min at room temperature and supernatant was transferred in new tubes. The elution step was repeated by adding 150µl of freshly made Elution buffer for 15min with rotation at room temperature. Beads were centrifuged at 3,000rpm for 5min at room temperature and supernatant was added to the same tube. For the reverse cross-linking of the chromatin 12µl of 5M NaCl was added to each of the ChIP samples as well as in the input samples (stored at -20°C) for 4hrs at 65°C. DNA was cleaned up by the addition of 6µl 0.5M EDTA, 12µl 1M Tris-HCl pH 6.5, 1.2µl 10mg/ml proteinase K for 1hr at 45°C. 500µl of phenol/chloroform was added in each sample and tubes were centrifuged at 13,000rpm for 5min at room temperature. Supernatant was transferred in new eppendorfs and 1ml of 100% ethanol, 50µl 3M NaAc and 1µl glycogen carrier were added and tubes were stored overnight at -20°C.

Day 3: DNA was centrifuged at 13,000rpm for 10min at 4°C and pellet was washed with 70% ethanol. DNA pellet was air-dried after last step and resuspended in 200µl of MilliQ. Input samples were diluted 1:10 and 3µl of ChIP DNA or diluted input DNA per real time PCR were used.

ChIP Lysis Buffer: 50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS and 1% protease inhibitors

ChIP Dilution Buffer: 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl and 1% protease inhibitors

Low Salt Buffer: 20mM Tris-HCl pH 8.1, 2mM EDTA, 150mM NaCl, 1% Triton X-100, 0.1% SDS and 1% protease inhibitors

High Salt Buffer: 20mM Tris-HCl pH 8.1, 2mM EDTA, 500mM NaCl, 1% Triton X-100, 0.1% SDS and 1% protease inhibitors

LiCl Buffer: 10mM Tris-HCl pH 8.1, 0.5M LiCl, 1mM EDTA, 1% NP-40, 1% DOC and 1% protease inhibitors

TE Buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA and 1% protease inhibitors

Elution Buffer: 0.1M NaHCO3 and 1% SDS

Protease inhibitors were added fresh prior to using the buffers.

Real-Time PCR

Primers for dnmt-1 were designed using Primer Express. SYBR Green-based quantitative qPCR (ABI SYBR Green PCR master mix, ABI) was performed on input, DNMT-1 immunoprecipitated and no antibody control material. Samples were analyzed in duplicates using an ABI StepOneTMReal-Time PCR System (ABI, Foster city, CA). Enrichment was determined by dividing the amount of this in the immunoprecipitated fraction by the amount of target sequence in input DNA (Litt, Simpson, Recillas-targa, Prioleau, & Felsenfeld, 2001). Results were analyzed relative to a control sequence in the gapdh locus. No antibody ChIP enrichment values were subtracted from their corresponding DNMT-1 immunoprecipitated ChIP enrichment values. Primer sequences are listed in Table 1.

Table 1 (Real-Time PCR primer sequences):

Forward primer for PU1 URE	GGGAGGCAGAGCACACATG
Reverse primer for PU1 URE	GTTTCCACATCGGCAGCAG
Forward primer of gapdh	CCAATGTGTCCCGTCGTGGATCT
Reverse primer of gapdh	GTTGAAGTCGCAGGAGACAAC
Forward primer of Bach1 negative site	TCCCCAAACGCACACAAAG
Reverse primer of Bach1 negative site	CCAGCTCCAGCAGCTGTGTA

RESULTS

DNMT1 protein interacting partners in MEL cells

In order to characterize DNMT1 nuclear protein complexes in the proerythroblastic mouse erythroleukemia (MEL) cell model, which can be chemically induced to undergo terminal erythroid differentiation, we applied the biotin tagging system, which involves the fusion of a short (14-23aa) peptide tag to the protein of interest. The tag is recognized by the *E. coli* BirA protein biotin ligase which biotinylates it (Boer et al., 2003)(P Rodriguez et al., 2006)(Patrick Rodriguez et al., 2005). We made use of a stable MEL cell clone expressing the *E.coli* BirA biotin ligase and highly efficient biotinylated bio-tagged DNMT1 under the control of the human β-globin promoter and Locus Control Region (Needham et al., 1992)(Papageorgiou et al., 2016). The aforementioned bio-tagged DNMT1 MEL cell clone was cultured and induced to undergo terminal erythroid differentiation by treatment with DMSO for 72 hours. Large scale streptavidin pull-down experiments were executed using 6mg of bioDNMT1/BirA nuclear extracts. Streptavidin bound material was processed for mass spectrometry, following on-bead trypsinization.

A preliminary validation of the data from the mass spectrometry analysis was carried out by streptavidin pull-downs and Western blots for the detection of GATA1, a known interactor of DNMT1 (Figure 5)(Papageorgiou et al., 2016). The MS analysis revealed multiple nuclear factors co-eluting with DNMT1. Amongst them were known DNMT1 protein interactors such as PCNA (L.S. Chuang, H.I. Ian, T.W. Koh, H.H. Ng, G. Xu, 1997), DMAP1 (Rountree et al., 2000), MeCP2 (Sciences, 2003), HDAC1(Robertson et al., 2000) and members of the NuRD complex (Cai et al., 2013) validating the detection efficiency of MS (data not shown). MS analysis from two additional DNMT1 streptavidin pull-down experiments is under way. Also, further validation through streptavidin pull-downs and IPs is necessary in order to elucidate different protein interaction networks that DNMT1 participates in.

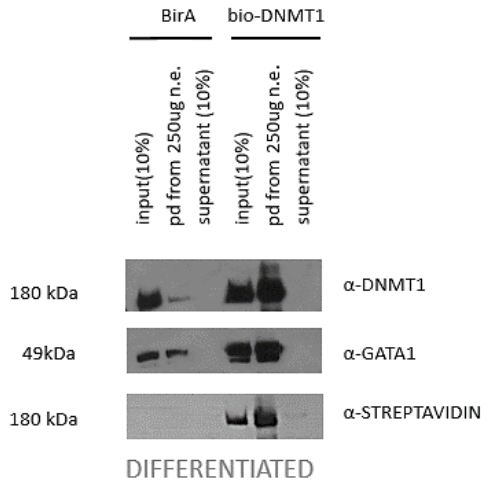


Figure 5. Detecting DNMT1 in streptavidin pull-downs from differentiated MEL cells by Western blots. Streptavidin pulldowns from nuclear extracts of DMSO induced MEL cells expressing biotintagged DNMT1 or control cells expressing only BirA. Samples were loaded in the following order: input, 10% of the material used for the pull-downs, pull-down samples (pd) and 10% of supernatant after pull-downs.

DNMT1-ChIP Sequencing between proliferating and differentiated BirA MEL cells.

In order to elucidate the genome-wide chromatin occupancy of DNMT1 and consequently reveal genes targets directly regulated by DNMT1 in erythropoiesis, we proceeded to carry out DNMT1 chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq). Formaldehyde cross-linked chromatin was prepared from untreated and chemically induced terminally differentiated MEL cells. Crosslinked chromatin was sonicated to minimize proportional presence of fragments larger than 500bp (Figure 6A). Six technical replicates using 10^7 cells were prepared per condition and immunoprecipitations were performed using an anti-DNMT1 antibody. In order to assess the efficiency of the ChIP assay, quantitative PCR (qPCR) was carried out using primers for the upstream regulatory element (URE) of the PU.1 gene (SPI1). As it has been shown in previous studies, DNMT1 and ZBP-89 bind to the murine PU.1 Upstream Regulatory Element (URE) in MEL cells (Papageorgiou et al., 2016)(X. Li, R.D. Romain, D. Park, D.T. Scadden, J.L. Merchant, 2015) operating as a repressive protein complex, presumably with GATA1 (Burda et al., 2016) by inhibiting expression of the PU.1 gene (Papageorgiou et al., 2016). Figure 6B shows that greater enrichment is evident in technical replicates 2, 6 from proliferating and 5, 6 from differentiated BirA MEL chromatin. These samples will be sent for massive parallel sequencing (ChIP-seq). We speculate that occupancy of DNMT1 in proliferating and differentiated BirA MEL cells will present differences and through this way we will characterise molecular mechanisms through which DNMT1 may regulate the process of terminal erythroid differentiation.

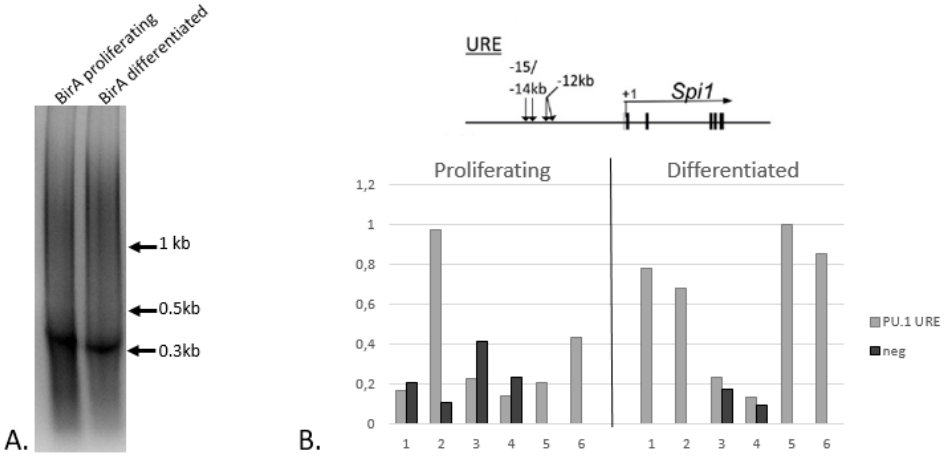


Figure 6. (A) Sonicated chromatin from proliferating and differentiated BirA MEL cells. Sonicated DNA is shown to be of a size below 500 bp. (B) **ChIP assay showing binding of DNMT1 to the murine URE of the PU.1 (Spi1) gene locus in crosslinked chromatin from mouse erythroleukemic MEL cells**. An unrelated sequence from Bach1 gene locus was used as negative control. The enrichment is the average of two replicate experiments normalized to the no antibody control material enrichment, compared to negative sites. Samples were analyzed relative to a sequence in the gapdh locus, using an ABI Prism 7000 sequence detection system.

DNMT1 knockout screening in MEL cells

Recent studies showing that DNMT1 interacts in separate complexes with transcription factors involved in erythropoiesis and globin gene regulation, raised the prospect of DNMT1 being implicated in erythroid differentiation (Papageorgiou et al., 2016)(Cui et al., 2011). To further investigate the implication of DNMT1 in the process of erythropoiesis, DNMT1 knock-down experiments were performed in mouse erythroleukemic (MEL) cells (E. Karkoulia, unpublished observations).

The efficiency of known-down was excellent (with no obvious viral secondary effects) verified by the undetectable protein levels of DMNT1 by western immunoblot analysis and reduced DNA methyltransferase activity (data not shown). DNMT1 knock-down resulted in impaired erythropoiesis due to cell cycle defects deriving from failure to inhibit genes that promote cell proliferation (not shown). DNMT1 knock-down MEL cells were arrested in the polychromatic or orthochromatic stage, not being able to proceed to terminal differentiation. In order to assess more clearly the role of DNMT1 in erythroid cell differentiation, we followed up these initial observations by performing CRISPR/Cas9 mediated DNMT1 knockout (KO) experiments in mouse erythroleukemic (MEL) cells.

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 (see Materials and Methods). At least three out of the 80 clones tested (clones: 2D10, 3D10, 4F7) resulted in clearly significant reduction of DNMT1 protein levels, whereas the rest of the clones showed variable levels of reduction in DNMT1 protein levels (Figure 7). The aforementioned three clones were subjected to further serial dilutions to derive single MEL DNMT1 KO clones. Also, clone 3D10 was obtained following an additional transfection of DNMT1 CRISPR/Cas9 KO constructs.

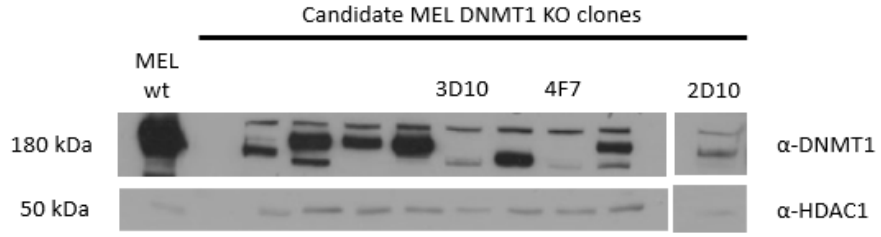


Figure 7. Detection of candidate MEL DNMT1 KO clones by Western blot. Nuclear extracts from transfected C88 MEL cells with DNMT1 CRISPR/Cas9 KO plasmid were immunoblotted with anti-DNMT1 antibody (top) and anti-HDAC1 (lower) antibody as protein loading control. Nuclear extracts from untransfected (wildtype=wt) MEL cells were used as control.

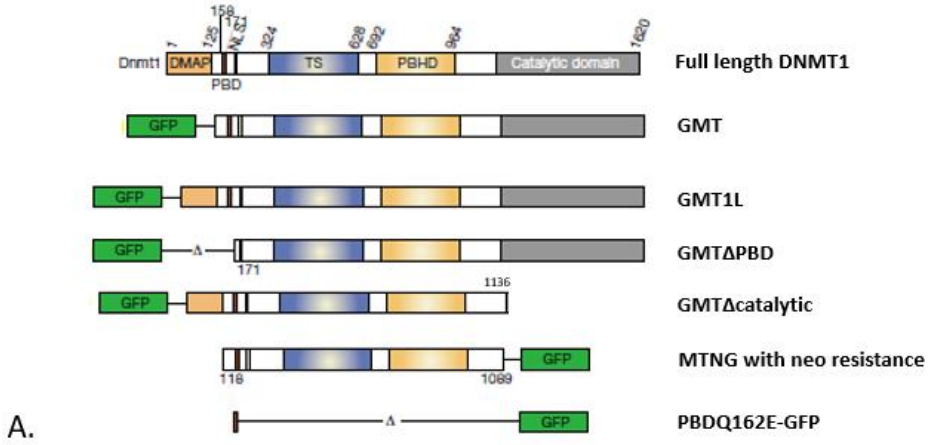
Further validation for the successful derivation of MEL DNMT1 KO clone will be through RNA extraction from candidate clones and interrogation for the presence or absence of dnmt1 transcripts. Furthermore, an additional experiment will be through the measurement of DNA methyltransferase activity in nuclear extracts. Cytospins, cell cycle analysis and expression profiling are ongoing experiments after the acquisition of MEL DNMT1 KO clone in order to elucidate the implication of DNMT1 in erythroid cell differentiation.

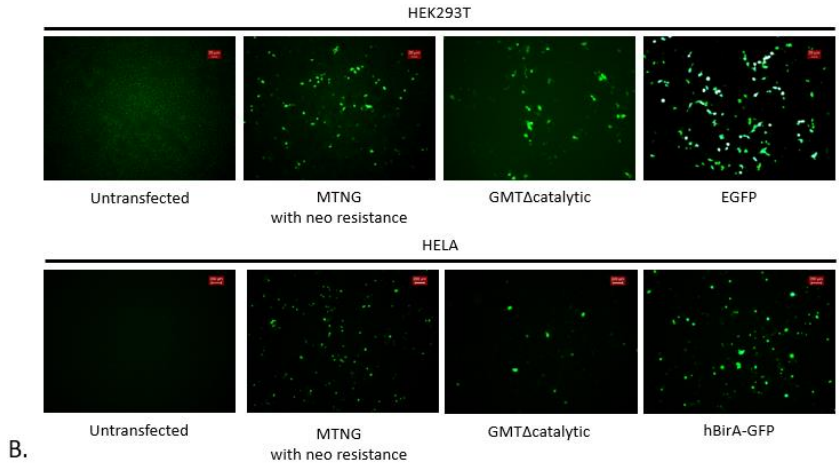
DNMT1 deletion mutants reveal a DNA methylation independent role of DNMT1 in erythropoiesis?

The role of the highly evolutionary conserved C-terminal catalytic DNA methyltransferase domain of DNMT1 is responsible for the maintenance of CpG methylation patterns in the genome and is associated with gene silencing (Comb & Goodman, 1990)(Nan et al., 1998)(Jones et al., 1998)(Robertson et al., 2000). The multi-domain N-terminal region of DNMT1 undergoes several protein-protein and protein-DNA interactions. Chromatin-modifying enzymes such as deacetylases (HDAC1, HDAC2) and histone methyltransferases (Suv39h1) are factors that DNMT1 recruits in order to maintain the methylation status and induce the appropriate histone H3 modification and subsequent silencing of genes (Fuks et al., 2000)(Jesus Espada et al., 2004). Recent data propose that DNMT1 is able to suppress gene transcription by a DNA methylation independent-mechanism, thus providing evidence that DNMT1 may also act as a scaffolding protein for the recruitment of additional repressive partners (Milutinovic et al., 2004)(Clements et al., 2012)(Jesús Espada, 2017). Recent work from the Strouboulis lab showed that the short PCNA binding domain (PBD) of DNMT1 is necessary and sufficient for interactions with transcription factors (Papageorgiou et al., 2016).

As outlined above, we have previously described the effects in erythroid differentiation of knocking down DNMT1. In order to identify the DNMT1 subdomain(s) that may be responsible for the phenotype of lack of cell cycle arrest at the polychromatic or orthochromatic stage and to assess if phenotype is related to a DNA methylation-independent mechanism, we decided to carry out rescue experiments of DNMT1 KD or KO MEL cells utilising DNMT1 deletion mutants tagged by fusion with GFP (Figure 8A)(Easwaran et al., 2004). The selection of a MEL cell clones expressing the DNMT1 deletion mutant will be done with neomycin (G418).

For the purpose of this experiment, two deletion mutants had to be created. A neomycin resistance gene was added to the MTNG DNMT1 deletion construct provided by Dr. C. Cardoso (MTNG with neo resistance, Figure 8A) and a new construct bearing a deletion of the catalytic domain was generated (GMTΔcatalytic). The two deletions mutants were transiently transfected in HEK293T and HeLa cells as preliminary validation of their correct expression. As depicted in Figure 8B, both DNMT1 deletion mutants expressed GFP. Western blot analysis using anti-GFP antibody and an anti-DNMT1 antibody to detect the DNMT1 deletion mutants, it was shown that the MTNG mutant fitted with neo was detected with anti-DNMT1 antibody in both cell lines, but GFP expression with an anti-GFP antibody was barely detectable in HeLa cells (Figure 8C). The GMTΔcatalytic deletion mutant was not detected either with an anti-GFP or with an anti-DNMT1 antibody (Figure 8C). Sequencing results of the GMTΔcatalytic construct revealed that the GFP fusion was not in-frame to the DNMT1 Δcatalytic domain part of the fusion protein.





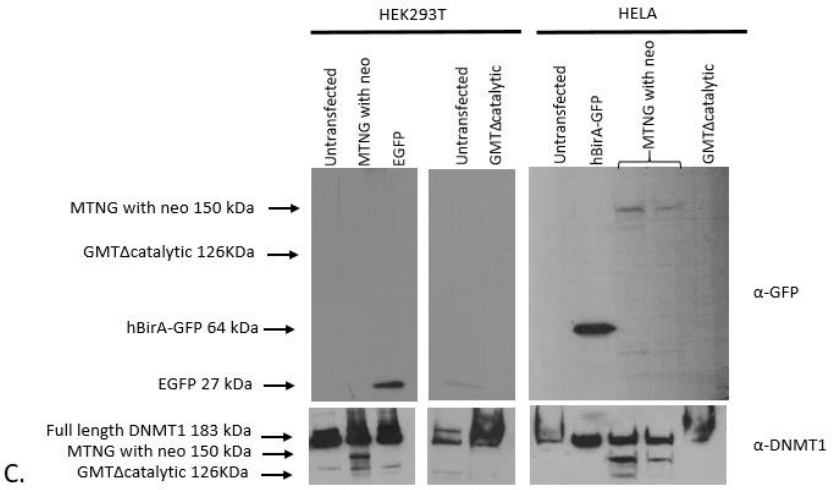


Figure 8. DNMT1 deletion mutants fused with GFP. (A) Schematic representation of the DNMT1 deletion mutants. DMAP: DMAP1 transcriptional repressor interacting domain; PBD: PCNA binding domain; NLS: Nuclear localization signal; TS: Targeting sequence; PBHD: Polybromo homology domain. (B) Expression of DNMT1 deletion mutants [MTNG with neo resistance (~150kDA), GMTΔcatalytic (~126 kDa)] in HEK293T and HeLa cell lines. Detection of GFP in microscope. EGFP and hBirA-GFP constitute control of transfection. (C) Detecting DNMT1 deletion mutants [MTNG with neo resistance (~150kDA), GMTΔcatalytic (~126 kDa)] by Western blot from HEK293T and HeLa nuclear extracts.

Discussion

Evidence from the Strouboulis laboratory indicating that DNMT1 interacts with transcription factors involved in erythropoiesis and globin gene regulation, raised the prospect of DNMT1 being implicated in erythroid differentiation (Papageorgiou et al., 2015) (Cui et al., 2011)(Dai, 2015). In agreement with this evidence, DNMT1 is the main DNA methyltransferase that is expressed throughout murine fetal liver derived erythroid differentiation (Shearstone et al., 2011) and DNMT1 conditional knockout in the erythroid lineage appears to be embryonic lethal (Trowbridge et al., 2009)(Xu et al., 2013). In addition, shRNA knockdown experiments have shown DNMT1 to be implicated in fetal γ-globin silencing in adult CD34+ derived human erythroid cells (Xu et al., 2013) and in mouse embryonic βh1 globin silencing in MEL cells (Roosjen et al., 2014). These observations identify DNMT1 as a possible candidate for treating hemoglobin disorders. Furthermore, dnmt1 knock-down studies in mouse fetal liver cells showed an acceleration of the erythroid specific gene activation suggesting that global demethylation may lead to the rapid removal of methylation marks at sites of induced erythroid genes (Shearstone et al., 2011). Unpublished data from the Strouboulis laboratory showed that shRNA-mediated knockdown of DNMT1 in MEL cells results in impaired erythropoiesis due to cell cycle defects deriving from failure to inhibit the genes that promote cell proliferation. All previous findings support the hypothesis that Dnmt1 is required for normal erythroid differentiation to be accomplished.

In this study, we tried to further elucidate DNMT1 functions in erythroid differentiation by identifying novel DNMT1 nuclear interactors and DNMT1 genome-wide occupancies in mouse erythroid cells utilizing a biotin-tagging approach coupled to mass spectrometry (Boer et al., 2003) and ChIP-sequencing, respectively. Mass spectrometry reveals many promising nuclear proteins involved in chromatin structure and modification, in chromosome structure, or in nuclear functions such as DNA replication and transcription (data not shown). Ongoing MS analysis will shed more light in characterizing the DNMT1 protein interactome. DNMT1 ChIP shows enrichment at the mouse *Pu.1* URE, a known binding site (Papageorgiou et al., 2016)(X. Li, R.D. Romain, D. Park, D.T. Scadden, J.L. Merchant, 2015), reassuring us to proceed to massive parallel sequencing (ChIP-seq). Furthermore, the molecular and functional characterization of the DNMT1 knock out MEL cell clones, by performing cytospins, cell cycle analysis, expression (RNA-seq) and methylation (MeDIP-seq) profiling will assist to fully elucidate the role of DNMT1 in terminal erythroid differentiation. Moreover, the comparison of methylation patterns between KO DNMT1 and wild type MEL cell lines could reveal genes that are differentially regulated by DNA methylation. We believe that these results will reveal new mechanisms on the epigenetic regulation of gene expression in erythropoiesis.

Another aim of this study is to identify gene targets of DNMT1 that are suppressed by its regulatory control. Taking into account that DNMT1 is the main DNA methyltransferase that is expressed throughout murine fetal liver derived erythroid differentiation and there is global demethylation during erythropoiesis (Shearstone et al., 2011), we assumed that DNMT1 functions at specific gene targets. We believe that DNMT1 ChIP-seq will reveal DNMT1 specific gene targets and we plan to compare these data with DNA methylation genome–wide patterns derived from MeDIP-seq in MEL cells (future experiments), in order to clarify that DNMT1 represses these genes through its methyltransferase activity. It is possible for a set of these genes that DNMT1 acts as repressor independently of its

methyltransferase activity (or not). There are data supporting the fact that DNMT1 may also act as a scaffolding protein for the recruitment of additional repressive partners (Milutinovic et al., 2004)(Clements et al., 2012)(Jesús Espada, 2017). This notion could be confirmed if the GMTΔcatalytic DNMT1 deletion mutant (Figure 8A) manages to rescue the phenotype of cell cycle arrest at the polychromatic/orthochromatic stage, as mentioned above. For further validation, impaired genes derived from expression profiling of GMTΔcatalytic DNMT1 deletion mutant would be compared with MeDIP-seq and DNMT1 ChIP-seq results from untreated MEL cells. This experimental procedure will contribute to identify DNMT1 gene targets and if DNMT1 uses a methylation dependent or independent mechanism for silencing its gene targets, or a set of them, in erythropoiesis.

In previous work in the Strouboulis lab it was shown that the short (~17 amino acid) PCNA binding domain (PBD) of DNMT1 is necessary and sufficient for interactions with transcription factors (Papageorgiou et al., 2016), independently of its function of mediating interactions with PCNA. Considering that the PBD-mediated DNMT1 interaction with PCNA was not critical for maintaining methylation patterns of newly replicated DNA (Schermelleh, Haemmer, Spada, Rosing, et al., 2007), nor is the PBD domain essential for DNMT1 methyltransferase activity, though it augments it (Spada et al., 2007), an intriguing question emerges: Is the PBD domain of DNMT1 the domain rendering DNMT1 as a scaffolding protein for the recruitment of additional repressive partners inducing gene silencing with a DNA methylation and DNA replication independent mechanism? Preliminary evidence for addressing this question will involve the comparison of rescue experiments of the DNMT1 deletion mutants [GMT, GMTΔPBD, MTNG (with neo resistance), GMTΔcatalytic and PBDQ162E-GFP, Figure 8A] and their expression profiling analysis. Specifically, the GMTΔcatalytic mutant will assess whether the catalytic activity of DNMT1 is required to rescue the cell cycle arrest at the polychromatic/orthochromatic stage and complete erythroid differentiation. Investigation of the GMT, GMTΔPBD, MTNG (with neo resistance) and GMTΔcatalytic mutants (Figure 8A) for their ability to rescue the phenotype of cell cycle arrest will correlate DNA methylation independent-mechanism of DNMT1 with the PBD domain. The GMT1L is the full length DNMT1 protein and represents the control for these rescue experiments. The PBDQ162E-GFP deletion mutant, which bears a single amino acid substitution at position Q162E abrogating DNMT1 interaction with PCNA (Schermelleh, Haemmer, Spada, Ro, et al., 2007) and therefore with replication foci (L.S. Chuang, H.I. Ian, T.W. Koh, H.H. Ng, G. Xu, 1997), will verify if the PDB domain is indeed sufficient to mediate DNMT1 interactions with repressive partners and could exclude the possibility that the DNMT1-repressive partners interactions mediated by PBD are related to the DNA replication machinery. Further characterization of the PBD could be achieved by creating a DNMT1-Q162E-C1229W MEL clone [bearing an inactivated catalytic domain, mutating the conserved Pro-Cys motif which is involved in the covalent complex formation (Chen, L. et al., 1991) by exchanging the cysteine for a tryptophan C1229W (Schermelleh et al., 2005) and a single amino acid substitution at Q162E abrogating interaction with replication foci (Schermelleh, Haemmer, Spada, Ro, et al., 2007)(L.S. Chuang, H.I. Ian, T.W. Koh, H.H. Ng, G. Xu, 1997)] for performing rescue experiments (as mentioned above), RNA-seq, ChIP-seq, and MS experiments. Comparing the experimental results of DNMT1-Q162E-C1229W with DNMT1Δcatalytic, we could address DNMT1 protein interactions and epigenetic regulation mediated by the PBD domain, independently of its methyltransferase activity and replication foci.

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