DNMT3A in Leukemia

Lorenzo Brunetti,^{1,2,3,6} Michael C. Gundry,^{1,2,4,6} and Margaret A. Goodell^{1,2,3,5}

¹Stem Cells and Regenerative Medicine Center, Baylor College of Medicine, Houston, Texas 77030

²Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas 77030

³CREO, University of Perugia, 06123 Perugia, Italy

⁴Department of Molecular & Human Genetics, Baylor College of Medicine, Houston, Texas 77030

⁵Texas Children's Hospital, and Houston Methodist Hospital, Houston, Texas 77030

Correspondence: goodell@bcm.edu

DNA methylation is an epigenetic process involved in development, aging, and cancer. Although the advent of new molecular techniques has enhanced our knowledge of how DNA methylation alters chromatin and subsequently affects gene expression, a direct link between epigenetic marks and tumorigenesis has not been established. DNMT3A is a de novo DNA methyltransferase that has recently gained relevance because of its frequent mutation in a large variety of immature and mature hematologic neoplasms. *DNMT3A* mutations are early events during cancer development and seem to confer poor prognosis to acute myeloid leukemia (AML) patients making this gene an attractive target for new therapies. Here, we discuss the biology of DNMT3A and its role in controlling hematopoietic stem cell fate decisions. In addition, we review how mutant DNMT3A may contribute to leukemogenesis and the clinical relevance of *DNMT3A* mutations in hematologic cancers.

DNA methylation is an epigenetic modification involved in key cellular processes such as transcriptional repression, genomic imprinting, and the suppression of repetitive elements. The first suggestion of a link between DNA methylation and cancer was the observation that human cancers tend to display global hypomethylation compared with normal controls (Feinberg and Vogelstein 1983). Subsequently, the field switched attention to focally hypermethylated regions with the hypothesis that epigenetic silencing of tumor suppressor genes through promoter hypermethylation would drive gene silencing, obviating the need for genetic inactivation of these pathways (Jones

and Laird 1999). Investigators then began looking for possible genes/pathways responsible for the observed methylation changes.

The deposition and maintenance of DNA methyl marks is orchestrated by DNA methyltransferases. In mammals, three genes encoding proteins with DNA methyltransferase activity have been identified: *DNMT1*, *DNMT3A*, and *DNMT3B* (Okano et al. 1998). DNMT3A and DNMT3B proteins are responsible for establishing the patterns of DNA methylation early in embryogenesis through de novo methylation of unmethylated CpG sites (Okano et al. 1999), and DNMT1 maintains such patterns throughout cell division by targeting hemimethylated

⁶These authors contributed equally to this work.

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DNA and copying the methyl mark onto the nascent DNA strand. All three proteins are essential for mammalian development (Okano et al. 1999).

Since the discovery of altered DNA methylation patterns in human cancers, an abundance of data has been collected on the expression levels of the DNMT genes in tumors, with overexpression of DNMT1 the most frequently observed change (Issa et al. 1993; Robertson et al. 1999). In addition, overexpression of the de novo methyltransferases (DNMT3A/DNMT3B) has been broadly reported in cancers (Mizuno et al. 2001; Girault et al. 2003; Oh et al. 2007; Rahman et al. 2010), supporting an oncogenic role for the DNMT family proteins. The success of DNA hypomethylating agents, including 5-azacytidine and decitabine, in the treatment of a multiple hematologic malignancies further supported this hypothesis (Santini 2012).

It, therefore, came as a surprise when genomic studies uncovered loss-of-function mutations in the DNMT family across multiple cancers (Gao et al. 2011; Yang et al. 2015). Most strikingly, *DNMT3A* mutations are frequently detected in a variety of adult hematologic malignancies, often occurring as early events during leukemogenesis (Roller et al. 2013; Shlush et al. 2014; Yang et al. 2015). Since this discovery, the development of mouse models and biochemical studies on DNMT3A have improved our understanding of the function of DNMT3A and its role in normal and malignant hematopoiesis. However, significant questions remain unanswered regarding both molecular mechanisms and clinical prognostic and therapeutic value. Herein, we review the known biology of DNMT3A and discuss recent clinical studies with therapeutic implications for *DNMT3A* mutations in cancer.

DNMT3A STRUCTURE

DNMT3A is a 130-kDa protein that is highly conserved in vertebrates, with 98% homology between humans and mice (Okano et al. 1998; Xie et al. 1999). The gene is encoded by 23 exons on human chromosome 2p23 and is expressed in two major forms: a long isoform,

DNMT3A1, and a short isoform, DNMT3A2 (Fig. 1), which has been the subject of most studies because of its predominant expression in mouse embryonic stem (mES) cells (Chen et al. 2002). DNMT3A2 has been shown to interact with DNMT3L, a catalytically inactive member of the methyltransferase family whose expression is restricted to germ cells and early embryogenesis (Hata et al. 2002). Disruption of this interaction through loss of either protein leads to defects in imprinting and spermatogenesis (Kaneda et al. 2004; Nimura et al. 2006).

DNMT3A contains three well-studied domains. The Pro-Trp-Trp-Pro (PWWP) domain synergistically binds DNA and specific histone marks in gene bodies (see more below) (Wang et al. 2014). The ATRX-DNMT3-DNMT3L (ADD) domain binds H3 tail peptides (Otani et al. 2009). The S-adenosyl methionine (SAM)-dependent methyltransferase C5-type domain encodes the highly conserved methyltransferase (MTase) domain (Fig. 1), which recognizes and binds DNA, and after binding the cofactor SAM, attaches a methyl group to the C5 position of cytosine. The long isoform also contains 219 extra amino acids at the amino terminus, which have been shown to enhance the DNA-binding affinity and methylation activity of the protein in vitro (Suetake et al. 2011). De novo germline mutations in each of the three major domains occur in the recently described Tatton-Brown-Rahman syndrome (or *DNMT3A* overgrowth syndrome), which is characterized by tall stature, a distinctive facial appearance, and intellectual disability (Tatton-Brown et al. 2014).

In the nucleus, DNMT3A can exist in oligomeric form as dimers, tetramers, and larger structures through two distinct binding interfaces in the MTase domain. The oligomers are composed of homodimeric DNMT3A molecules or heterodimeric DNMT3A–DNMT3L molecules; however, the presence of a single binding interface in DNMT3L prevents further oligomerization past a 3L-3A-3A-3L tetramer state (Jurkowska et al. 2011). The structure of the tetramer allows for coordinated methylation of two independent CpG sites at an average distance of ~9 bp (Zhang et al. 2009). Importantly, both the DNA and SAM binding affinity of

Figure 1. DNMT3A genomic locus. The *DNMT3A* gene is composed of 23 exons distributed across ~110,000 bp on chromosome 2. The two major isoforms, *DNMT3A1* and *DNMT3A2*, are depicted along with the promoters and exons from which they are transcribed. The vertical dotted lines drawn on the different isoforms represent exon – exon junctions.

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DNMT3A, as well as the processivity of the enzyme, are tied to the oligomeric state of DNMT3A (Holz-Schietinger et al. 2011). In addition to programmed changes in oligomerization, such as those driven by developmental changes in DNMT3L expression and differential DNMT3A/3B isoform usage, a number of pathologic alterations including mutations at DNMT3A binding interfaces (Holz-Schietinger et al. 2012) have been shown to affect oligomerization and alter cell behavior. So far, oligomerization in adult somatic tissues, which lack DNMT3L, has not been assessed. It is possible that complex oligomers composed of different isoforms of DNMT3A and DNMT3B exist and that the dosages of these complexes affect regional de novo methylation. Further work is needed to better understand the regulation and importance of oligomerization on cellular phenotypes including cancer.

DNMT3A AND DNA METHYLATION

The distribution of mCpGs in the mammalian genome is nonrandom and the mechanism responsible for targeting DNMT3A and DNMT3B to specific genomic regions remains largely unknown. However, functional characterization of DNMT3A domains and studies on DNMT3A binding patterns and de novo methylation activity in embryonic stem cells (ESCs) have shed some light on the intrinsic and extrinsic factors controlling regional specificity.

Each of the three major domains in DNMT3A has been shown to associate with specific epigenetic marks. Crystal structure analysis recently showed an elegant autoinhibitory mechanism for DNMT3A, wherein the ADD domain physically blocks the MTase domain (Guo et al. 2015). When histone H3 with an unmodified lysine 4 tail is encountered (H3K4me0), the ADD–MTase interaction is disrupted, resulting in movement of the ADD domain enabling enzymatic activity. However, in the presence of amino-terminal H3 tail modifications, especially H3 lysine 4 trimethylation (H3K4me3), release from autoinhibition is significantly less efficient, providing a mechanism to ensure that actively expressed genes (marked

by H3K4me3) are not subject to DNA methylation (Ooi et al. 2007; Otani et al. 2009; Zhang et al. 2010). Mutations in the ADD domain are found in some hematologic cancers. Although most cancer-associated *DNMT3A* mutations are presumed to represent a loss of function, it is conceivable that ADD domain mutations could result in loss of autoinhibition, leading to a gain of function and aberrant hypermethylation of actively expressed genes marked by histone H3K4me3, ultimately leading to their suppression; this scenario remains to be tested.

Similarly, the PWWP domain binds DNA and trimethylated histone H3 lysine 36 (H3K36me3) (Dhayalan et al. 2010). H3K36me3 marks active chromatin and is highly enriched in gene bodies and repetitive elements, including pericentromeric DNA (Chantalat et al. 2011). Indeed, DNMT3A co-localizes with pericentromeric heterochromatic foci, and disruption of the PWWP domain in ES cells leads to a diffuse nuclear patterning of DNMT3A and a loss of methylation activity at major satellite repeats in pericentric heterochromatin (Chen et al. 2004). During gametogenesis and early embryogenesis, protection of the germline through suppression of repetitive elements is critical; this suppression may represent a major function of the PWWP domain during embryonic development. Furthermore, both the H3K36 methyltransferase SETD2 and the PWWP domain of DNMT3B have been shown to be required for targeting de novo methylation to transcribed genes (Baubec et al. 2015); thus, it is possible that the PWWP domain of DNMT3A acts in a similar manner.

Finally, the MTase domain of DNMT3A has been shown to preferentially bind unmethylated DNA (Yokochi and Robertson 2002). In vitro experiments have shown that some CpG flanking bases, specifically AT-rich sequences, can enhance methylation activity by several orders of magnitude (Lin et al. 2002), consistent with genome-wide data on enriched sequence motifs at methylated CpGs (Handa and Jeltsch 2005). As discussed previously, the MTase domain also cooperatively oligomerizes on DNA, forming nucleoprotein filaments that enhance the activity of the enzyme and confer a processive-

like property to the protein (Emperle et al. 2014). The majority of somatic mutations in DNMT3A are heterozygous and act to disrupt this oligomerization, suggesting a key role for oligomerization in the normal function or stability of the protein.

Other chromatin-modifying enzymes have also been shown to associate with DNMT3A and may impact DNA methylation patterns. The majority of these binding partners associate either directly or indirectly with the ADD domain, including heterochromatin protein 1 (HP1), histone deacetylase 1 (HDAC1), UHRF1, and the histone lysine *N*-methyltransferases (EZH2, SUV39H1/2, SETDB1, and G9a) (Ayyanathan et al. 2003). The interplay between histone modifications and DNA methylation is complex, and the order of acquisition of histone marks and DNA methylation differs according to the specific histone mark and chromatin enzyme involved (Cedar and Bergman 2009). More data is needed on the complex relationships between histone marks and DNA methylation to trace the origin of epigenetic patterning during embryogenesis. Additionally, the relationship between these epigenetic marks in adult somatic tissues has not been studied and may be key to understanding the role of DNMT3A in cancer.

DNMT3A IN HEMATOPOIESIS

Although the majority of studies on DNMT3A have been performed in ESCs and germ cells, recent work indicates that DNMT3A may also regulate somatic stem cell differentiation (Challen et al. 2012; Hu et al. 2012; Wu et al. 2012; Dhawan et al. 2015). In the mouse hematopoietic system, deletion of *Dnmt3a* leads to a preference for self-renewal over differentiation and gradual expansion of the long-term hematopoietic stem cell (LT-HSC) compartment on serial transplantation (Fig. 2). A similar alteration in stem-cell fate is observed on loss of DNMT3A in neural stem cells (Hu et al. 2012) and on loss of DNMT3A/3B in mouse and human ES cells (Chen et al. 2003; Challen et al. 2012; Liao et al. 2015). Deletion of *Dnmt3b* does not appear to have a major effect on the LT-HSC

compartment, which is not surprising as the predominant DNMT3B isoform expressed in the hematopoietic system is enzymatically inactive (Challen et al. 2014). However, codeletion of *Dnmt3a/3b* enhanced LT-HSC expansion and virtually blocked differentiation, signifying residual DNMT3B activity.

Dnmt3a-null HSCs are phenotypically indistinguishable from wild-type cells, with similar levels of proliferation and apoptosis and an identical cell surface marker profile. *Dnmt3a*-null HSCs retain the ability to differentiate into all hematopoietic lineages, albeit with a lower differentiation quotient or reduced level of output per LT-HSC (Fig. 2). From a molecular standpoint, *Dnmt3a*-null HSCs display significant genome-wide hypomethylation with focal areas of hypermethylation that can be attributed to residual DNMT3B activity. Differentially methylated regions (DMRs) identified in *Dnmt3a*-null HSCs are distributed across all genomic features, but are significantly enriched at the edges of large hypomethylated regions known as methylation canyons (Jeong et al. 2014). These features were recently identified in multiple cell types (Xie et al. 2013), and often occur near developmental regulatory genes such as homeobox-containing genes. Analysis of gene expression changes after loss of DNMT3A revealed that many canyon-associated genes involved in HSC self-renewal are overexpressed in *Dnmt3a*-null HSCs and remain inappropriately turned on in differentiated progeny cells (Challen et al. 2012; Jeong et al. 2014). However, no clear relationship between canyon hypomethylation, altered gene expression, and phenotype has been established.

The expansion of *Dnmt3a*-null cells observed on competitive transplantation represents a potential mechanism for early leukemic transformation. In this model, cells that spontaneously acquire a mutation in *DNMT3A* would be more likely to self-renew than differentiate, and would therefore be protected from stem-cell exhaustion. Under conditions of stress, in which a larger fraction of the HSC pool is forced to cycle, selection and subsequent expansion of the *DNMT3A* mutant HSC pool could be even more pronounced. Indeed, trans-

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Figure 2. DNMT3A controls hematopoietic stem cell fate. In mice, serial competitive transplantation of wild-type (WT) hematopoietic stem cells (HSCs) (CD45.2) eventually leads to less efficient engraftment. The contribution of engrafted HSCs to hematopoiesis is proportional to their level of engraftment in the bone marrow (*top*). In contrast, *Dnmt3a*-null HSCs do not exhaust and instead begin to outcompete CD45.1 WT cells, resulting in an expanded contribution to the HSC compartment. The contribution of these *Dnmt3a*-null HSCs to blood production is minimal, reflecting an imbalance between self-renewal and differentiation driven by loss of DNMT3A (*bottom*).

planted *Dnmt3a*-null HSCs led to development of an array of myeloid and lymphoid neoplasms that recapitulate the full spectrum of human malignancies harboring *DNMT3A* mutations (Celik et al. 2015; Mayle et al. 2015).

DNMT3A MUTATIONS AND CANCER

Since the first description of *DNMT3A* mutations in acute myeloid leukemia (AML) (Ley et al. 2010), multiple exome and targeted resequencing studies have identified *DNMT3A* mutations in AML (Shlush et al. 2014; Klco et al. 2015; Ivey et al. 2016) as well as in a variety of other adult myeloid and lymphoid neoplasms (Roller et al. 2013). Interestingly, *DNMT3A* mutations are very rare in pediatric blood cancers, with the few identified mutations found in adolescents (Ho et al. 2011; Thol et al. 2011b; Shiba et al. 2012).

The majority of *DNMT3A* mutations found in hematopoietic disorders occur within the

methytransferase domain (MTD), with a significant enrichment for mutations at codon R882 (Fig. 3) (Cancer Genome Atlas Research 2013; Roller et al. 2013). However, non-R882 missense and truncating mutations are found in each of the major domains (Cancer Genome Atlas Research 2013; Roller et al. 2013). In AML and other myeloid neoplasms, R882 mutations are usually heterozygous (Ley et al. 2010; Cancer Genome Atlas Research 2013; Gaidzik et al. 2013; Gale et al. 2015). However, in T-cell acute lymphoblastic leukemia (T-ALL), a high frequency of non-R882 biallelic mutations has been reported (Grossmann et al. 2013; Roller et al. 2013), suggesting different selective pressures on *DNMT3A* mutations in myeloid and lymphoid disorders. Data from *Dnmt3a*/*Flt3-ITD* double-mutant mice are consistent with an influence of DNMT3A dosage. *Dnmt3a*^{-/-} cells can generate both myeloid and lymphoid malignancies (Yang et al. 2016), whereas *Dnmt3a*^{+/-} cells are more likely to develop

Figure 3. *DNMT3A* mutations in acute myeloid leukemia (AML) and clonal hematopoiesis. The spectrum of *DNMT3A* mutations in AML (TCGA data) and clonal hematopoiesis (Jaiswal et al. 2014) are depicted on a figure of the long isoform. AML TCGA data include 57 *DNMT3A* mutations from 51 individuals (cohort of 200 patients), whereas clonal hematopoiesis data include 403 *DNMT3A* mutations from 398 individuals (cohort of 17,182 healthy patients). Each lollipop represents a nonsynonymous mutation, with the size of the lollipop correlating to the mutation count (within the respective groups) and the color indicating the type of mutation.

myeloid cancers (Meyer et al. 2016; Yang et al. 2016). Taken together, these data strongly indicate that *DNMT3A* is a haploinsufficient tumor suppressor gene in myeloid leukemias, when cooperating mutations are present.

In AML, ~65% of *DNMT3A* mutations are heterozygous missense mutations affecting codon R882 (Ley et al. 2010; Gaidzik et al. 2013; Gale et al. 2015). R882H mutation (or mouse R878H mutation) has been shown to exert a dominant-negative effect over the wild-type protein (Holz-Schietinger et al. 2012; Kim et al. 2013; Russler-Germain et al. 2014). The mutant protein can dimerize with WT *DNMT3A*, but tetramers, which comprise a substantially more active form of the protein, are not able to form (Holz-Schietinger et al. 2012; Russler-Germain et al. 2014). The resulting low levels of *DNMT3A* homotetramers result in a significant reduction of methyltransferase activity, providing an explanation for the genome-wide hypomethylation observed in patients with R882 mutations (Cancer Genome Atlas Research 2013; Qu et al. 2014; Meyer et al. 2016; Yang et al. 2016). In addition, the R882 mutant may acquire other deleterious interactions. Recent data suggest that it may interact with the Polycomb repressive complex 1

(PRC1), unlike WT *DNMT3A*, resulting in down-regulation of genes involved in promoting hematopoietic differentiation such as *PU.1* and *Cebpa* (Koya et al. 2016).

In contrast to R882 mutations, there is still a complete lack of data related to the biological impact of non-R882 missense mutations. It is possible that mutations in different *DNMT3A* domains lead to different unique neomorphic functions that result in specific underlying mechanisms of pathogenesis. Characterizing the epigenetic landscapes and phenotypic effects of non-R882 missense mutations in more detail will be vital to our understanding of *DNMT3A*-mutated cancer biology.

DNMT3A MUTATIONS IN AML

DNMT3A mutations are detectable in ~20% of patients with de novo AML, as well as in secondary AML (Table 1) evolving from previous myelodysplastic syndromes or myeloproliferative neoplasms (sAML) (Ley et al. 2010; Thol et al. 2011a; Fried et al. 2012; Gaidzik et al. 2013; Roller et al. 2013). Some clinical and pathologic features characterize *DNMT3A*^{mut} AML patients at diagnosis. Compared with wild-type *DNMT3A* (*DNMT3A*^{wt}) patients, they tend to

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Table 1. Prevalence of *DNMT3A* mutations in hematologic malignancies

Disease	Characteristics	Frequency (%)	References
Adult AML	De novo AML	62/281 (22.1)	Ley et al. 2010
	De novo CN-AML	36/123 (29.3)	Renneville et al. 2012
	Intermediate risk AML	272/914 (29.8)	Gale et al. 2015
	CN-AML	142/415 (34.2)	Marcucci et al. 2012
	89.5% de novo AML, 10.5% s-AML and t-AML	370/1770 (20.9)	Gaidzik et al. 2013
	t-AML and s-AML	t-AML 10/61 (16.4); s-AML 13/37 (35.1)	Fried et al. 2012
Pediatric AML	De novo AML	2/206 (1)	Liang et al. 2013
Adult T-ALL	T-ALL	Entire cohort 16/83 (19.3); early T-ALL 10/38 (26.3)	Grossmann et al. 2013
	ETP ALL	11/68 (16.2)	Neumann et al. 2013
MPAL	Adult T/myeloid MPAL	10/18 (55.6)	Wolach and Stone 2015
MDS	De novo MDS	127/944 (13.5)	Haferlach et al. 2014
MPN	All MPNs	10/155 (6.5)	Stegelmann et al. 2011
MDS/MPN	52 CMML, 20 s-AML evolved from CMML	CMML 2/52 (3.8); s-AML 6/20 (30)	Jankowska et al. 2011
	65 CMML-1, 38 CMML-2	CMML-1 2/65 (3.1); CMML-2 5/38 (13.2)	Roller et al. 2013
	JMML	3/100 (3)	Stieglitz et al. 2015
T-cell lymphomas	T-cell lymphomas	11/96 (11.4)	Couronné et al. 2012
	AITL and PTCL-NOS	21/79 (26.6)	Sakata-Yanagimoto et al. 2014
	CTCL	17/40 (42.5)	Choi et al. 2015

The large range of frequencies reported for AML depends on the subset of patients considered and whether the entire *DNMT3A* or only hotspots were sequenced.

AML, acute myeloid leukemia; CN-AML, caryotype normal-AML; t-AML, therapy-related AML; s-AML, secondary AML; T-ALL, T-cell acute lymphoblastic leukemia; ETP ALL, early T-cell precursor ALL; MPAL, mixed phenotype acute leukemia; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms; MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasms; CMML, chronic myelomonocytic leukemia; JMML, juvenile myelomonocytic leukemia; AITL, angioimmunoblastic lymphoma; PTCL-NOS, peripheral T-cell lymphomas not otherwise specified; CTCL, cutaneous T-cell lymphoma.

be older, have higher white blood cell counts, and be frequently diagnosed with cytogenetically normal AML (CN-AML) with myelomonocytic or monocytic (French–American–British M4 and M5) blast morphology (Thol et al. 2011a; Marcucci et al. 2012; Gaidzik et al. 2013; Gale et al. 2015).

DNMT3A^{mut} AMLs frequently harbor *NPM1* (*NPM1*^{c+}) and *FLT3* mutations, with 60%–80% of *DNMT3A*^{mut} cases having *NPM1*^{c+} and ~30% displaying both *NPM1* and *FLT3* mutations (Cancer Genome Atlas Research 2013; Gaidzik et al. 2013; Gale et al. 2015). Although there is evidence that *Npm1*^{c+}/*Flt3*-ITD mice (Mupo et al. 2013) and *Dnmt3a*^{mut}/*Flt3*-ITD mice (Meyer et al. 2016) develop leukemia with 100% penetrance,

no data have been published on *Dnmt3a*^{mut}/*Npm1*^{c+} mice. It therefore remains unclear whether any of these mutations plays a central role in determining such specific clinical phenotype.

Prognostic Impact of *DNMT3A* Mutations in AML

Although a large number of studies reported that *DNMT3A*^{mut} patients fare worse compared with *DNMT3A*^{wt} patients (Ley et al. 2010; Shen et al. 2011; Thol et al. 2011a; Hou et al. 2012; Marcucci et al. 2012; Renneville et al. 2012; Ribeiro et al. 2012; Tie et al. 2014; Gale et al. 2015), the impact of *DNMT3A* mutations in clinical decision-making is still a matter of debate (Patel

et al. 2012; Gaidzik et al. 2013). There is a lack of evidence that the increased risk of relapse is sufficient to indicate more aggressive strategies such as bone marrow transplantation during first complete remission for these patients. Interestingly, two groups reported that high-dose anthracyclines might overcome the negative impact of *DNMT3A* mutations (Patel et al. 2012; Sehgal et al. 2015), but larger prospective trials are needed to confirm this finding. Along with their classic topoisomerase II inhibitory properties, anthracyclines can cause nucleosome eviction from open chromatin, especially when delivered at high doses (Pang et al. 2013). Some data suggest that hypomethylated regions in *DNMT3A*^{mut} AMLs have a more open chromatin (Spencer et al. 2014), and it is possible that these regions are targets for nucleosome eviction induced by high-dose anthracycline.

Whether different types of mutations (e.g., missense vs. nonsense) in different amino acids (e.g., R882 vs. non-R882) have specific clinical consequences is unknown. The largest study reported worse outcomes for patients harboring R882 mutations, but not for patients with non-R882 mutations. Gale et al. (2015) investigated the impact of *DNMT3A* mutations in younger patients according to *NPM1* mutational status. R882 mutated patients had worse overall survival, in both *NPM1*^{c+} and *NPM1*^{wt} subgroups, whereas the impact of non-R882 missense mutations was evident only in the *NPM1*^{c+} patients, suggesting a less pronounced clinical impact for the non-R882 missense mutations. Although limited by small sample size, a sub-analysis revealed that patients with *DNMT3A* truncating mutations had the same prognosis as *DNMT3A*^{wt} patients. Ahn et al. reported different outcomes for patients with R882 and non-R882 mutations treated with allogeneic bone marrow transplantation. R882 mutations conferred significant adverse prognostic impact, especially when associated with *FLT3*-ITD, whereas patients harboring non-R882 mutations had a similar prognosis to *DNMT3A* wild-type patients (Gaidzik et al. 2013; Ahn et al. 2016).

Although no definitive conclusion can be drawn, merging clinical and biological data, it seems likely that R882 mutations confer a neg-

ative prognosis to *DNMT3A*^{mut} patients. On the other hand, non-R882 mutations are extremely heterogeneous, encompassing a variety of mutations, and therefore understanding the impact of such mutations may be difficult. Until results from larger prospective trials become available, published data suggest that patients with *DNMT3A*^{mut} AML should be stratified according to more validated risk parameters such as age, karyotype, the persistence of residual disease after treatment (i.e., minimal residual disease; MRD), and other molecular abnormalities (e.g., *NPM1*^{c+}, *FLT3*-ITD).

***DNMT3A* Mutations, Minimal Residual Disease, and Relapse**

DNMT3A^{mut} cells are frequently detectable in patients with AML with long-lasting complete remission, raising the question of whether *DNMT3A* should be used to monitor MRD. Pløen et al. initially reported residual R882 *DNMT3A* mutations in a significant fraction of cells (mutant allele frequencies between <1% and 50%) in samples from 14 AML patients in complete remission. Five out of 14 patients did not relapse after a median follow-up of 53 months (Pløen et al. 2014). Recently, more studies have reported no clear association between the persistence of *DNMT3A* mutations in complete remission and worse clinical outcomes (Jeziskova et al. 2015; Ivey et al. 2016). The presence of *DNMT3A*^{mut} cells in patients with no evidence of residual leukemia is consistent with *DNMT3A* mutations being preleukemic events (Corces-Zimmerman et al. 2014; Shlush et al. 2014). The detection of *DNMT3A* mutations after successful treatment likely reflects the persistence of preleukemic clones instead of true leukemic cells, arguing against using *DNMT3A* as a marker for monitoring MRD.

The prevalence of residual *DNMT3A* mutations after treatment makes it reasonable to consider the hypothesis of AML relapse being, at least in some cases, the result of newly acquired mutations arising in a preleukemic *DNMT3A*-mutated clone. New prospective trials with large cohorts combined with genome-wide sequencing studies of samples acquired over time (e.g.,

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diagnosis, remission, and relapse) will help to answer this question over the next few years.

DNMT3A MUTATIONS IN MYELOYDYSPLASTIC SYNDROMES (MDSs)

As in AML, *DNMT3A* mutations are early events in MDSs (Walter et al. 2011; Papaemmanuil et al. 2013); however, their prevalence is significantly lower than in AML (Table 1) and their prognostic value unclear. Although some studies suggest reduced overall survival and higher probability of evolution to AML for *DNMT3A*^{mut} MDS patients (Thol et al. 2011c; Walter et al. 2011), others have shown no prognostic significance (Bejar et al. 2012; Haferlach et al. 2014).

MDS patients are often treated with hypomethylating agents such as 5-azacytidine or decitabine (Santini 2012), which are thought to inhibit DNMT1 and restore normal methylation levels at promoter regions of tumor suppressor genes (Paul et al. 2010). One study, although limited by small numbers, suggested that *DNMT3A* mutations are predictive of better response to hypomethylating agents (Traina et al. 2014), but the biological explanation for this behavior is unknown. Data from *Dnmt3a*-null HSCs and *DNMT3A*^{mut} AML patients (Yan et al. 2011; Challen et al. 2012; Qu et al. 2014) revealed that, along with global DNA hypomethylation, some regions of the genome instead gain methylation. It may be that hypomethylating agents are acting at these sites to restore normal methylation levels. Additionally, in the context of lower DNA methylation levels, 5-azacytidine and decitabine may further reduce global DNA methylation leading to a synthetically lethal phenotype and targeted cell death. More data are needed to assess the real clinical impact of *DNMT3A* mutations in MDS and the implications for therapeutic approaches.

DNMT3A MUTATIONS IN MYELOPROLIFERATIVE NEOPLASMS AND MYELOYDYSPLASTIC/ MYELOPROLIFERATIVE NEOPLASMS

Myeloproliferative neoplasms (MPNs) and myelodysplastic/myeloproliferative neoplasms

(MDS/MPNs) are chronic myeloid neoplasms classically characterized by the expansion of mature myeloid cells in the bone marrow and extramedullary sites (e.g., spleen), with or without dysplastic features. *DNMT3A* mutations are less frequent genetic events in MPNs and MDS/MPNs than in AML and MDSs (Table 1). *JAK2* is the most frequently mutated gene in MPNs and *JAK2*-V617F (*JAK2*^{V617F}) accounts for >95% of mutations of this gene (Kiladjian 2012). When co-occurring with *DNMT3A* mutations, the order of acquisition of *DNMT3A* and *JAK2* mutations may be relevant in defining different MPN disease phenotypes (Nangalia et al. 2015).

DNMT3A mutations are infrequent, but recurrent in MDS/MPNs. Indeed, they can be found in ~2% of chronic myelomonocytic leukemia (CMML) (Jankowska et al. 2011) and 3% of juvenile myelomonocytic leukemia (Table 1) (Stieglitz et al. 2015). Jankowska et al. reported that 25% of patients with sAML arising from previous CMML harbored *DNMT3A* mutations, possibly suggesting a higher probability of evolution to AML for CMML patients with *DNMT3A* mutations; however, this study investigated a small cohort (Jankowska et al. 2011) and larger studies are needed. As described for AML, *DNMT3A*^{mut} CMML is reported to display significantly reduced DNA methylation, mainly at intergenic and intronic regions (Meldi et al. 2015) and low-DNA methylation levels are also reported in a *Dnmt3a*-null MDS/MPN mouse model (Guryanova et al. 2015). However, whether this methylation pattern impacts the disease phenotype and eventually the prognosis of MDS/MPN patients is still unknown, and more studies are required to answer these important questions.

DNMT3A MUTATIONS IN LYMPHOID MALIGNANCIES

As reported for myeloid neoplasms, *DNMT3A* mutations in lymphoid malignancies are recurrent (Table 1) and thought to be early events (see below). In T-ALL, the prevalence of biallelic hits is noticeably higher than in myeloid cancers (Grossmann et al. 2013; Roller et al. 2013), sup-

porting the role of *DNMT3A* as a tumor suppressor in this context.

Adult early T-cell precursor ALL (ETP-ALL), a subset of T-ALL thought to arise from early thymocyte precursors that retain myeloid differentiating potential (Zhang et al. 2012), is particularly enriched for mutations in *DNMT3A* (Neumann et al. 2013). This finding creates a link between early immature T-ALL and myeloid malignancies, suggesting that some myeloid and lymphoid leukemias may arise from a shared *DNMT3A*^{mut} precursor that maintains both myeloid and lymphoid potential. More studies are needed to enhance our understanding of the mechanisms that drive neoplastic differentiation toward the myeloid or lymphoid lineages.

Although *DNMT3A*^{mut} T-ALL patients globally seem to fare worse compared with *DNMT3A*^{wt} leukemias, it should be noted that this difference might reflect the early-T phenotype, which has been linked to a more aggressive disease evolution, and not the *DNMT3A* mutational status itself (Grossmann et al. 2013; Roller et al. 2013; Van Vlierberghe et al. 2013). However, the limited data available so far suggest an independent impact of *DNMT3A* mutations and further studies will verify its value for risk stratification.

DNMT3A is also recurrently mutated in peripheral T-cell lymphomas (Table 1) (Couronné et al. 2012; Sakata-Yanagimoto et al. 2014; Choi et al. 2015). As in myeloid neoplasms, *DNMT3A*^{mut} tumor cells arise in an early progenitor that retains multilineage potential, with the mutation detectable in both the neoplastic T-cells and normal B-cells of these lymphoma patients (Couronné et al. 2012). Strikingly, ~70% of *DNMT3A*^{mut} cases also harbor *TET2* mutations (Couronné et al. 2012) and mouse models of *Dnmt3a* mutation and *Tet2* knockout develop both AML and T-cell malignancies (Scourzic et al. 2016; Zhang et al. 2016). The interaction between *DNMT3A*^{R882H} (thought to decrease global DNA methylation levels) and loss of *TET2* (thought to increase global DNA methylation levels) generated a complex methylation landscape with increased DNA methylation and reduced expression of

several tumor suppressors and hypomethylation and overexpression of T-cell key genes such as *Notch1* (Scourzic et al. 2016; Zhang et al. 2016). T-cell malignancies are still challenging to treat, thus a deeper understanding of these mechanisms is needed for the development of new therapeutic strategies.

***DNMT3A* MUTATIONS LINK AGE-RELATED CLONAL HEMATOPOIESIS, PRELEUKEMIC CLONES, AND AML**

High-throughput sequencing studies showed that some somatic mutations in known cancer driver genes could be present in the blood of elderly individuals before any hematopoietic neoplasm appears (Busque et al. 2012; Wong et al. 2015). These data imply the possibility that predisposing mutations can be acquired early on during cancer development and that only a fraction of subjects with such mutations would eventually develop cancer. Recently, four independent groups reported the presence of subclonal somatic mutations in the peripheral blood of individuals with no history of hematologic diseases (Genovese et al. 2014; Jaiswal et al. 2014; Xie et al. 2014; McKerrell et al. 2015). The prevalence of this phenomenon increased with age, with >10% of subjects over 70 having at least one detectable somatic mutation. *DNMT3A* was by far the most frequently mutated gene in clonal hematopoiesis (Genovese et al. 2014; Jaiswal et al. 2014; Xie et al. 2014).

Individuals with clonal hematopoiesis have approximately a 10-fold higher risk of developing hematologic cancers compared with individuals without clonal hematopoiesis (Genovese et al. 2014; Jaiswal et al. 2014). Although the association with an increased risk of blood cancers may seem intuitive, strikingly clonal hematopoiesis is also found to significantly increase the risk of all-cause and cardiovascular-related mortality and these risks are higher in subjects with higher levels of clonality (Genovese et al. 2014; Jaiswal et al. 2014). Because several cell types that play key roles in the pathogenesis of cardiovascular diseases (e.g., macrophages) arise from hematopoietic progenitors, mutant cells could display altered functions that

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lead to an increased risk of coronary heart disease and ischemic stroke. Yet, it is unclear whether different somatic mutations associated with clonal hematopoiesis have different impacts on the risk of developing different diseases.

Organismal aging is known to be associated with decreased HSPCs fitness and alterations in the bone marrow microenvironment. Somatic mutations in some leukemia driver genes may allow for clonal expansion through increasing cellular fitness and promoting HSC self-renewal independent of age-related environmental changes. Alternatively, positive selection for cells with mutations in some genes may require the altered fitness landscape of aged bone marrow (McKerrell and Vassiliou 2015). *DNMT3A* mutations may confer a selective advantage to the cells in which they arise through both mechanisms. Although it has been shown that *Dnmt3a*-null HSCs have an enhanced serial engraftment capacity (Challen et al. 2012), less is known about the interaction of *DNMT3A* mutant cells and the surrounding environment. The aging microenvironment may select for the expansion of *DNMT3A* mutant cells at the expense of wild-type cells. Indeed, it has recently been reported that patients with autoimmune bone marrow failures such as aplastic anemia frequently harbor *DNMT3A* mutated clones (Yoshizato et al. 2015). These clones may expand as a result of stem cell exhaustion of wild-type HSCs forced to enter the cell cycle because of the profound cytopenia induced by marrow failure.

Whichever the mechanism leading to clonal expansion of hematopoietic progenitors, it seems likely that clonal hematopoiesis represents a primitive stage in the development of hematologic neoplasms. The vast majority of the genes reported as mutated in individuals with clonal hematopoiesis are well-known drivers of leukemia or MDS and many of them have been found in preleukemic clones in patients with AML (Corces-Zimmerman et al. 2014; Shlush et al. 2014). Preleukemic HSPCs may represent progenitors of patients with a history of clonal hematopoiesis that, after acquiring secondary genetic events, developed AML. It remains to be established why only a small frac-

tion of patients with clonal hematopoiesis develop hematologic cancers and what are the molecular and biological mechanisms that regulate the transition from clonal hematopoiesis to pre-leukemia and eventually to cancer. Addressing these issues would significantly enhance the development of new, targeted therapies to treat patients with MDS and leukemias.

THERAPEUTIC PERSPECTIVES AND FUTURE DIRECTIONS

DNMT3A mutations are one of the earliest genetic events during hematopoietic cancer development, making this gene an attractive target for new therapeutic approaches. Moreover, the negative overall worse prognosis for *DNMT3A*^{mut} patients necessitates more efficient therapies. However, we still need to better understand how DNA methylation is regulated and how it impacts gene expression and phenotype. Although loss of *DNMT3A* may promote expression of self-renewal genes (Challen et al. 2012), whether these are viable candidates for therapeutic targets is unknown. The development of genome-wide CRISPR-Cas9 screens may facilitate the rapid detection of synthetically lethal phenotypes in normal and malignant hematopoietic cells (Koike-Yusa et al. 2014; Shi et al. 2015). The application of these screens to mouse and cell-based models will allow us to unravel the possible downstream effects of *DNMT3A*^{mut} HSCs and discover potential targets for *DNMT3A* mutated cancers.

Beside high-dose anthracycline, there is a lack of data related to new strategies for *DNMT3A*^{mut} patients with AML or other hematologic cancers. *DNMT3A* mutants may play a role in up-regulating *HOX* genes in AML. Novel H3-methyltransferase inhibitors such as DOT1L inhibitors (McLean et al. 2014), MLL–menin interaction inhibitors (Borkin et al. 2015), and MLL inhibitors (Cao et al. 2014) showed efficient knockdown of *HOX* cluster genes and leukemic cell death, both in vitro and in vivo. Although these compounds were mainly tested in the context of *MLL*-rearranged leukemia, a wider spectrum of leukemias, including *DNMT3A*^{mut} AML, may be sensitive

to this treatment. The bone marrow niche plays a relevant role in both hematopoietic and leukemic stem cell survival and function and it has been already proposed as a possible target for novel therapies (Krause et al. 2013). Both mouse and human data support the idea of a *DNMT3A*^{mut} HSC having a competitive advantage over the wild-type counterpart (Fig. 2), but no study assessed the interplay between *DNMT3A*^{mut} cells and the microenvironment so far. Specific connections between *DNMT3A*^{mut} HSCs and the niche are likely to occur and they may promote expansion of *DNMT3A*^{mut} clones, supporting age-related clonal hematopoiesis and leukemogenesis. A better understanding of these interactions will provide new insight for the development of novel therapeutic approaches.

CONCLUDING REMARKS

DNMT3A has recently been identified as one of the most commonly mutated genes in adult hematologic malignancies. The development of *Dnmt3a*^{mut} mouse models has increased our understanding of how *DNMT3A* controls differentiation and self-renewal in the hematopoietic system and confirmed its role as a driver of leukemogenesis. The observation that *Dnmt3a*-null HSCs have a selective advantage over wild-type cells is consistent with the high prevalence of *DNMT3A* mutations in patients with clonal hematopoiesis, as well as with the detection of mutant *DNMT3A* in preleukemic clones, and with the persistence of *DNMT3A*-mutant cells in patients with AML in complete remission. However, whether these mutant cells are a long-term risk factor for leukemia relapse and need to be cleared with more aggressive therapies, such as allogeneic bone marrow transplantation, remains unclear. To develop new therapeutic strategies for the treatment of patients with *DNMT3A* mutations, it is crucial to better characterize the role of mutant *DNMT3A* in initiating and maintaining tumorigenesis.

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