Alterations to *DNMT3A* in Hematologic Malignancies

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ABSTRACT

In the last decade, large-scale genomic studies in patients with hematologic malignancies identified recurrent somatic alterations in epigenetic modifier genes. Among these, the *de novo* DNA methyltransferase *DNMT3A* has emerged as one of the most frequently mutated genes in adult myeloid as well as lymphoid malignancies and in clonal hematopoiesis. In this review, we discuss recent advances in our understanding of the biochemical and structural consequences of *DNMT3A* mutations on DNA methylation catalysis and binding interactions and summarize their effects on epigenetic patterns and gene expression changes implicated in the pathogenesis of hematologic malignancies. We then review the role played by mutant *DNMT3A* in clonal hematopoiesis, accompanied by its effect on immune cell function and inflammatory responses. Finally, we discuss how this knowledge informs therapeutic approaches for hematologic malignancies with mutant *DNMT3A*.

Introduction

Since the discovery of recurrent DNA methyltransferase 3A (*DNMT3A*) mutations in acute myeloid leukemia (AML) a decade ago (1–3), the role of *DNMT3A* defects in hematologic malignancies has been a subject of intense investigation. In subsequent studies, *DNMT3A* alterations were identified at various frequencies in multiple myeloid and lymphoid neoplasms, often associated with poor prognosis, yet were virtually absent outside of the blood system (3–6). Mechanistic and functional studies established a role for *DNMT3A* in enforcing a tight balance between the hematopoietic stem cell (HSC) differentiation and self-renewal, through the maintenance of specific DNA methylation profiles that control gene expression programs (7–10). Patterns of co-occurrence with other leukemia-associated genetic lesions and evidence from pre–single-cell, bulk sequencing studies aiming to reconstruct clonal architecture implicated *DNMT3A* mutations as an early, preleukemic event (11–14). This was later confirmed by detection of mutant *DNMT3A* in non-malignant, preleukemic HSCs isolated from patients with AML (15), and culminated in the discovery of frequent somatic *DNMT3A* mutations in age-related clonal hematopoiesis (CH; refs. 16–19). At the same time, *de novo* mutations in *DNMT3A* were detected in individuals with a recently described Tatton-Brown-Rahman overgrowth and intellectual disability syndrome (20). Studies into the molecular mechanisms of these phenotypes effected by *DNMT3A* alterations, mostly believed to be loss of function, supplied a wealth of granular methylomic data including evidence of erosion of the DNA methylation canyons and explored cross-talk with other layers of epigenetic regulation (21–25). These early advances are already summarized in a number of excellent reviews (26–28). Since then, there were a plethora of studies in two key areas. First, structural determinants of the binding specificity of DNMT3A to DNA and chromatin, as well as protein–protein interactions. Second, the involvement of *DNMT3A* in hematopoietic lineage fate determination during differentiation, and its central role in CH, regulation of inflammatory states, and immune cell function. These recent advances, as well as emerging therapeutic approaches for hematologic conditions with mutant *DNMT3A*, are the main focus of this review.

DNMT3A Structure and Regulation of Catalysis

DNMT3A is a 130 kDa protein encoded by the *DNMT3A* gene spanning 23 exons on human chromosome 2 (or chromosome 12 in the mouse). It is expressed as two alternatively spliced isoforms: the ubiquitous DNMT3A1 (long), and DNMT3A2 (short), detected in the embryonic stem cells (ESC), early embryonic tissues, as well as testes, ovaries, spleen, and thymus. The long isoform contains extra amino acids that enhance anchoring to nucleosomes and binding to DNA *in vitro* (29–31).

Domain structure of mammalian DNMTs, also reviewed elsewhere (32–35), comprises the N-terminal regulatory part consisting of the PWWP and the ADD domains that promotes nuclear localization of the enzyme, targeting to chromatin and interactions with allosteric regulators, and the C-terminal domain that is mainly involved in DNA binding and methylation catalysis.

The Pro-Trp-Trp-Pro (PWWP) domain is required for targeting to tri- and especially dimethylated histone H3 lysine 36 (H3K36) marking gene bodies and intergenic regions respectively (36, 37). Binding to these marks allosterically increases the methyltransferase activity of DNMT3A and thus protects these genomic regions from spurious transcription initiation (38, 39). Conversely, phosphorylation by CK2 reduces DNMT3A activity while targeting it to heterochromatic regions (40). The ATRX-DNMT3L-DNMT3A (ADD) domain binds to unmethylated H3K4 that marks inactive chromatin and allosterically releases the autoinhibition of the enzymatic activity of DNMT3A (41). The ADD domain additionally interacts with epigenetic factors involved in transcriptional gene silencing such as polycomb-repressive complex (PRC) 2 catalytic subunit EZH2, H3K9-specific histone methyltransferase SUV39H1, and histone-lysine deacetylase HDAC1, and with transcription factors p53, PU.1, and MYC (42, 43). Conversely, a recent study in mouse neurons showed the interaction of the methylated DNA-binding protein MeCP2 with the ADD domain causes autoinhibition of the catalytic activity of DNMT3A (44).

The highly conserved catalytic domain of DNMT3A catalyzes 5-cytosine methylation within CpG dinucleotides using S-adenosylmethionine as a methyl donor. Unlike the highly related enzyme

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DNMT3B that methylates multiple adjacent CpG sites processively through a noncooperative mechanism (45), DNMT3A forms large multimeric protein/DNA complexes with itself or other DNMT3s necessary for cooperative binding and efficient distributive catalysis (46). Most characterized is a heterotetrameric complex composed of a DNMT3A homodimer bound by two noncatalytic stimulatory DNMT3L subunits in a 3L-3A-3A-3L structure (47, 48). The DNMT3A–DNMT3A dimerization interface is stabilized by hydrophobic interactions between the phenylalanine residues, whereas the DNMT3A–DNMT3L interface is mediated by salt bridges and hydrogen bonding interactions. Two DNMT3A monomers comethylate two adjacent CpG sites separated by 14bp within the same DNA duplex (49). DNMT3B may also stimulate the activity of DNMT3A, especially in the absence of DNMT3L (47, 50, 51).

DNMT3A Mutations and Their Functional Consequences

Somatic mutations in *DNMT3A* found in hematologic malignancies are distributed throughout the open reading frame and generally fall into one of the following categories. First, nonsense, frameshift (splice and indel), and missense alterations in key residues, which are consistent with a loss of function. Second, a specific hotspot point mutation at arginine 882 (R882) at the dimerization interface, most often converted to histidine or cysteine. Finally, variants of unknown significance were represented by single amino acid substitutions with only sparse biochemical characterization. *De novo* germline or rare inherited mutations found in Tatton-Brown-Rahman overgrowth and intellectual disability syndrome (TBRS) have been shown to follow a similar distribution (20, 27, 52–54). The R882 mutations are believed to have a dominant-negative effect on the methyltransferase activity due to impaired oligomerization, although this notion is debated (55). Structural efforts found the R882 residue stabilizes the target recognition domain (TRD) through H-bonding within the DNA-binding domain. Consequently, R882 substitutions lead to defective DNA binding and impaired TRD-loop–mediated CpG recognition (49, 56, 57). This results in focal hypomethylation at specific loci that usually include developmental genes, resulting in increased HSC self-renewal and reduced differentiation, eventually driving leukemogenesis (28, 58).

Interestingly, the conformational change in the TRD loop of $DNMT3A^{R882H}$ resulted in an altered flanking sequence preference at positions +1, +2, and +3 that resembles the DNA substrates usually favored by DNMT3B (57, 59, 60). Consistently, $DNMT3A^{R882}$-specific hypermethylation of such DNMT3A/DNMT3B chimeric substrates (61) can be detected in primary AML samples along with hypomethylation of disfavored sequences, both of which are associated with a unique subset of genes (62), implying a gain-of-function effect (60). On the other hand, tetramerization interface mutations R736H and R771G or an internal W893S substitution exhibit a preference to methylating cytosines at non-CpG positions *in vitro* (63), which cannot be maintained at DNA replication and has also been observed for R882H (57).

In addition to multimerization and binding to DNA, other binding interactions can be affected by *DNMT3A* mutations. Examples include an increased interaction of $DNMT3A^{G543C}$ with histone H3 (1) and of $DNMT3A^{R882H}$ with the PRC1 components (64). Conversely, $DNMT3A^{R882}$ exhibited decreased binding to HDAC1 and 2, and haploinsufficient loss of *DNMT3A* was associated with a gain of H3K27ac histone acetylation and increased expression of PD-L1 in a TF-1 cell line model (65). Moreover, whereas a tumor suppressor p53 can compete with DNMT3L for binding at the tetramer interface and inhibit catalytic activity of wild-type DNMT3A, R882H allosterically relieves such negative regulation (63).

The PWWP domain preferentially targets DNMT3A to H3K36me2 and to a lesser extent to H3K36me3 (36). Loss of H3K36me2 marks resulting from *NSD1* haploinsufficiency leads to decreased DNA methylation observed in Sotos syndrome (66) and tracks closely with TBRS (67). Conversely, *de novo* missense mutations in the PWWP domain that do not impair protein stability, W330R or D333N, were identified in patients with microcephalic dwarfism (68). Mouse models (W326R or D329A) demonstrated a postnatal growth delay due to loss of interaction with H3K36me2/me3 and progressive hypermethylation of H3K27me3-marked bivalent chromatin and of DNA methylation canyons. This gain-of-function phenotype led to a transcriptional imbalance between key developmental genes, resulting in premature neuronal differentiation, impaired self-renewal, and growth retardation (68, 69).

The clinical and molecular overlap between overgrowth and intellectual disability syndromes caused by inactivating mutations in *DNMT3A* (Tatton-Brown-Rahman), *NSD1* (Sotos), PRC2 catalytic subunit *EZH2* (Weaver), as well as *SETD2* (Luscan–Lumish) and histone H1 (Rahman) highlights the molecular relationship between different layers of epigenetic regulation and chromatin. Disruption of these genes, characterized by shared yet unique DNA methylation landscapes (70), is inextricably related to hematologic malignancies. Further studies into the complexities of this cross-talk will be vital to our understanding of the *DNMT3A*-mutant–driven pathology.

DNA Methylation and Gene Expression Studies

DNMT3A mutations are now commonly considered preleukemic events, yet the consensus over their effects on DNA methylation landscapes and gene expression programs only recently emerged, due in part to the differences between model systems. Studies of complete hematopoietic-specific *Dnmt3a* loss in mice found hypomethylation of HSC-related genes that resulted in enhanced stem cell self-renewal at the expense of differentiation (7, 8, 10), even when other cooperating genetic lesions were present (22, 71–74). This leads to competitive advantage over normal HSCs and may predispose to the acquisition of cooperating proleukemogenic mutations in the expanded clone. Partial *Dnmt3a* loss or point mutations produced more subtle phenotypes such as focal hypomethylation of specific CpGs (24) with modest changes to global DNA methylation and transcriptional activity of genes nearest to differentially methylated regions. This was observed in the HSCs from both leukemic and nonleukemic primary samples with $DNMT3A^{R882H}$, suggesting that hypomethylation predates the onset of leukemia (23).

Studies focusing on the most common $DNMT3A^{R882}$ hotspot mutation found in AML or its mouse counterpart $Dnmt3a^{R878H}$ reported less consistent and highly context-specific phenotypes, which included focal hypomethylation at enhancer regions and undermethylated canyon edges, particularly at SMAD3- and NFκB-binding motifs (62). This was occasionally associated with increased expression of HSC-related, *Hoxa* cluster, *Meis1* (75), and *Mycn* genes (25), although negative enrichment of MYC and E2F target gene signatures was also reported in a variety of contexts (62, 71, 76). In addition, activation of mTOR and AML signaling pathways (77) and deregulation of cell-cycle–related gene signatures such as G_2–M checkpoint (71, 76) were identified. Downregulation of differentiation-associated genes (*Cepba, Cepbe*, and *Pu.1*) as a consequence of aberrant

DNMT3A^{R882} interaction with the PRC1 complex at target loci was also proposed (64). Overall, *DNMT3A*^{R882} resulted in deregulation of transcriptional programs related to cell identity and normal hematopoietic function, which may contribute to leukemogenesis (71).

Among these studies of hematopoiesis with altered *DNMT3A*, hypomethylation of active hematopoietic lineage-specific enhancers (10, 22, 62, 71–73, 78) as well as erosion at the DNA methylation canyon edges (21, 22) emerged as a unifying theme that could be extended to both lymphoid and myeloid malignancies with various comutational contexts, and even nonhematopoietic tissues (79). Consistently, in a T-cell acute lymphoblastic leukemia (T-ALL) model driven by *Dnmt3a*$^{-/-}$ combined with *Flt3*^{ITD}, hypomethylated enhancers were enriched for active histone marks H3K27ac and H3K4me1 (71). In *Dnmt3a* knockout with neomorphic *Idh2*^{R140Q}, this was accompanied by an increase in repressive H3K9me3 marks exacerbating the differentiation block (74). The DNA methylation and gene expression changes along with myeloid skewing could be partially restored upon re-expression of wild-type *Dnmt3a*, demonstrating that these phenotypes are reversible (72, 80).

In recent years, numerous RNA-sequencing studies supplied growing evidence for *Dnmt3a* involvement in megakaryocyte-erythroid differentiation and immune cell function, supporting previous more laborious phenotypic and functional observations (10, 81). Leukemia-initiating cells from *Dnmt3a*$^{-/-}$*:Idh2*^{R140Q} or *Dnmt3a*$^{-/-}$*:Tet2*$^{-/-}$ double knockout mice have a megakaryocyte-erythroid progenitor immunophenotype and repress corresponding gene expression programs (22, 74). Single-cell multiomics studies in *Dnmt3a*$^{-/-}$ HSCs showed skewed transcriptional priming toward erythroid over myelomonocytic lineage. This was due to hypomethylation and higher accessibility of the CpG-rich erythroid transcription factor motifs (82). In a T-ALL model driven by *Dnmt3a*$^{-/-}$ and constitutively active *Notch1*, enhancer regions showed profound hypomethylation, whereas gene sets associated with myeloid cell function, inflammation, and immune responses were upregulated (78). Cooperating *Dnmt3a*$^{-/-}$*:Jak2*^{V617F} in a model of myelofibrosis (MF) led to increased DNA accessibility at active enhancers driving activation of proinflammatory Tnfα/Nfκb signaling pathways for a fully penetrant myeloproliferative neoplasm (MPN; ref. 73). Gene networks related to mast cell degranulation and activation were enriched in the *Dnmt3a*$^{-/-}$ cells (83). In innate immunity, *Dnmt3a* regulates the production of type 1 IFNs by maintaining the expression of HDAC9 in macrophages (84), whereas DNMT3A-mediated hypermethylation redirects differentiation of primary monocytes from dendritic cells toward cancer tolerogenic myeloid-derived suppressor cells (85).

Epigenetic, gene expression, and functional changes observed in various models with *Dnmt3a* alterations are summarized in **Table 1**, along with cooperating genetic interactions in hematologic malignancies.

DNMT3A and Cooperating Mutations in Hematologic Malignancies

DNMT3A mutations tend to be an early event in hematologic malignancies that requires additional genetic lesions, summarized in **Table 1**. The spectrum of cooperating mutations is nonrandom and varies considerably between diseases. For example, *FLT3* internal tandem duplication (*FLT3*^{ITD}) and mutations in *NPM1* are most frequent in AML, whereas *TET2* mutations are found in both myeloid and lymphoid malignancies (11, 72, 76, 86–88). Furthermore, *DNMT3A* mutations are almost exclusive to adult leukemia; the rare *DNMT3A*-positive pediatric AML cases are likely associated with TBRS (52).

More detailed studies revealed distinct clinical and molecular implications associated with different *DNMT3A* mutation types and allelic dosage. *DNMT3A*^{R882} were more prevalent in the context of *NPM1* (89, 90) and *FLT3*^{ITD} (91, 92) mutations, more likely to be ancestral or "founder" event, and also associated with shorter overall survival (28, 93). By contrast, *IDH1* mutations tended to co-occur with truncating *DNMT3A* mutations (74, 93, 94), whereas non-R882 *DNMT3A* mutations were predominant in ALL (78, 95) where they were frequently biallelic (4, 5) and associated with older age, treatment resistance, and poor outcome (96). In comparison, in myeloid malignancies, mutations in *DNMT3A* are usually heterozygous (3). Genetic modeling in mice provided further evidence for the critical role of *Dnmt3a* dosage. In combination with *Flt3*^{ITD}, homozygous ablation of *Dnmt3a* was more likely to result in T-ALL, whereas loss of a single *Dnmt3a* allele led to AML (71, 72). *Dnmt3a* knockout in combination with *Idh1* mutation (74) or *Tet2* knockout (22) synergistically induces myeloid malignancies in animals. Similarly, cooperating mutations in *cKit* (97) and *Kras* (8) in *Dnmt3a*$^{-/-}$ HSCs drive malignant transformation. Although these studies provided invaluable insights into the mechanisms of mutational cooperativity in leukemia pathogenesis, the genetic makeup and disease phenotype observed in the clinic were only partially recapitulated. There is a growing interest in creating clinically accurate mouse models with the ultimate goal to empower therapeutic and drug development efforts. A *Dnmt3a*^{R878H}*:Flt3*^{ITD}*:Npm1*^{c} triple-mutant mouse that faithfully models an aggressive AML (11) enabled the discovery of a novel therapeutic resistance mechanism driven by altered chromatin regulation (76).

Furthermore, the temporal order of mutations influences clinical disease presentation. Studies in *DNMT3A*-mutated MPNs driven by *JAK2* or *MPL* alterations found that "*DNMT3A*^{mut}-first" patients had essential thrombocythemia, whereas "*JAK2*-first" patients were younger and more likely to present with polycythemia vera or MF (98). A recent study took these concepts one step further and modeled sequential acquisition of *Dnmt3a*^{R878H} and *Npm1*^{cA} mutations in mice, with varying latency between these genetic events. *Dnmt3a*^{R878H} produced an expansion of the HSC compartment (analogous to CH in humans; refs. 76, 77) that progressed to myeloproliferation/myelodysplasia after *Npm1*^{cA} and, with additional selective pressures of proliferative and/or proinflammatory stress, to AML (15, 86). Increasing the latency between *Dnmt3a* and the "second hit" mutation renders a more fulminant disease. Further reports unveiling the contributing cell-autonomous and cell-extrinsic mechanisms are eagerly awaited.

The strong requirement for cooperating oncogenic events highlights the role of mutant *DNMT3A* as an early event that facilitates leukemic transformation by other mechanisms rather than driving it *per se*. This premalignant role is well in alignment with its high prevalence in CH, discussed next.

DNMT3A Mutations in CH

CH is a clonal expansion of HSCs in the absence of hematologic disease; it is commonly detected by the presence of somatic mutations, often in presumed leukemia driver genes such as in *DNMT3A*. Incidence of CH sharply increases with age, spurring the term "age related clonal hematopoiesis". CH was first described in the 1990s based on increased X-inactivation skewing in women with age (99). More recently, modern sequencing technologies facilitated detection of sizeable hematopoietic clones (variant allele frequency >2%) in >30% of people aged 60+ (16–18, 100). Mutations in *DNMT3A* are by

Table 1. Molecular and phenotypic consequences of *DNMT3A* alterations and cooperating mutations in human disease and in animal models.

Cooperating mutations in patients with *DNMT3A*-mutant disease

Cooperating mutation	*DNMT3A* mutation type(s)	Malignancy (AML/MDS/ MF/lymphoid)	Comments and references
FLT3-ITD	More likely R882	Adult AML	*DNMT3A, FLT3*-ITD, and *NPM1* mutations often co-occur (11, 93, 133–135)
NPM1	More likely R882	AML	*NPM1* is often acquired after *DNMT3A* mutation (11, 87, 89, 90, 93, 134)
FLT3-ITD and *NPM1*		AML	*DNMT3A, NPM1, and FLT3* mutations strongly co-occur, predict aggressive disease (11, 135)
IDH1/2	Truncating	AML, MDS, and other	Predicts poor survival (11, 74, 93, 94, 134)
TET2		T-cell lymphomas, MDS, AML	(88, 134, 136, 137)
JAK2		MPN, MF	(98)
NOTCH1	Non-R882	T-ALL, ETP-ALL	(78, 138)
RUNX1		AML, rarely MDS	Reduced survival, older age, poor treatment response (139–142)
KMT2A-PTD (*MLL*-PTD)	Enriched, mostly R882	AML	Poor survival (143, 144); mutually exclusive with MLL translocations in previous studies (98)
RAD21, STAG2, SMC3 (cohesin complex)			*DNMT3A* mutations may offset the survival disadvantage of *SMC3*-haploinsufficient cells (11, 134, 145, 146)
7q deletion		AML, MPN, MDS	*DNMT3A* mutations often ancestral (147); in MDS, often preceded by -7/del (7q) (148)
5q deletion		MDS, or MPN	(149)
9q deletion		AML	Del(9q) as sole cytogenetic abnormality; strong coassociation with *NPM1* mutation, *FLT3*-ITD rare (150)

***DNMT3A* and cooperating mutations in *in vitro* and animal *in vivo* models**

DNMT3A alteration	Cooperating mutation(s)	Malignancy or disease phenotype	Epigenetic changes	Gene expression and functional changes
Dnmt3a$^{-/-}$	*N/A*	Myeloid malignancies	Altered methylation patterns, focal loss of methylation at regulatory regions (8)	Upregulation of stemness genes and repression of differentiation factors (8), myeloid skewing (80)
Dnmt3a$^{-/-}$	*Tet2*$^{-/-}$	CMML and lymphoid malignancy	Hypomethylation of HSC-related gene enhancers	Activation of HSC genes, lineage-specific transcription factors, erythroid differentiation, JAK-STAT pathway (22)
	Idh2^{R140Q}	MDS, AML, and lymphoma	Gain of H3K9me3 and loss of H3K9ac (74)	Megakaryocyte-erythroid progenitor phenotype in leukemia-initiating cells
	Flt3^{ITD}	T-ALL	Profound hypomethylation at gene enhancers and canyon edges	Increased expression of inflammation, immune response, HSC- and myeloid-related genes, decreased expression of mature T cells genes (71)
	Activated *Notch1* signaling, through NICD expression	T-ALL	Enhancer and exon hypomethylation	Repression of proapoptotic genes, increased expression of myeloid, inflammation, and immune response genes (78)
	Jak2^{V617F}	MPN/MF	Enhancer hypomethylation	Proinflammatory signaling, HSC gene expression (73)
Dnmt3a$^{+/-}$	*Flt3*^{ITD}	AML, MPN	Modest changes in overall methylation. Hypomethylation at hematopoietic enhancers and canyon edges (71, 72). HSPC-like methylation in leukemic blasts	Increased expression of genes involved in cell fate specification (71). Enrichment for HSPC genes, genes downregulated during myeloid development, and c-Myc target genes (72)
DNMT3A$^{R882H/+}$ (human) or *Dnmt3a*$^{R878H/+}$ (mouse)	*Tet2*$^{-/-}$	T-ALL, T-cell lymphomas, MPN, and AML (88, 136)	Hypermethylation of tumor-suppressor genes and local hypomethylation of Notch pathway genes	Repression of tumor-suppressor genes and Wnt/β-catenin pathway. Activation of Notch pathway genes (151)
	Nras	AML	Focal hypomethylation at gene regulatory elements and gain of histone acetylation	Activation of stemness genes of the *Meis1-Mn1-Hoxa* node (25)

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Table 1. Molecular and phenotypic consequences of *DNMT3A* alterations and cooperating mutations in human disease and in animal models. (Cont'd)

***DNMT3A* and cooperating mutations in *in vitro* and animal *in vivo* models**				
***DNMT3A* alteration**	**Cooperating mutation(s)**	**Malignancy or disease phenotype**	**Epigenetic changes**	**Gene expression and functional changes**
	Idh2^{R140Q}	AML	Loss of differential methylation at enhancers, other regulatory regions	Activation of Ras signaling and apoptosis, repression of Myc targets, and heme metabolism (62)
	Flt3^{ITD}	AML	Hypomethylation of gene enhancers	Repression of *Myc, E2f*, and G2M checkpoint genes, upregulation of homeobox genes (71)
	N/A	AML	Focal hypomethylation at distal regulatory elements such as at canyon shores, enhancers and undermethylated canyons (25), attenuated CpG island hypermethylation (23)	Modest gene expression changes (23). Upregulation of stemness genes, *HoxA* cluster and *Meis1* (75), negative enrichment of G_2–M checkpoint genes (71, 76). Downregulation of differentiation genes, *Cepba, Cepbe, Pu.1* (64)
DNMT3A^{W330R}, *DNMT3A*^{D333N} (gain of function) and mouse models *Dnmt3a*^{W326R}, *Dnmt3a*^{D329A}		Microcephalic dwarfism, delayed growth	Hypermethylation at polycomb-marked DNA methylation valleys, loss of H3K27me3 and H3K4me3 bivalent chromatin at developmental genes (68)	Increased expression of neurogenic genes at the expense of pluripotency genes in mESCs differentiated into neurons *in vitro* (68, 69)
DNMT3A^{W297del} (mouse ^{W293del}), *DNMT3A*^{I310N} (mouse ^{I306N}), *DNMT3A*^{Y365C}		TBRS (overgrowth syndrome)	Hypomethylation at intergenic regions and decreased binding to H3K36me2	Aberrant chromatin localization and NSD1-DNMT3A cross-talk (36)

far the most common genetic event associated with CH (up to 40% of all CH cases). *DNMT3A*-driven CH was associated with prior environmental exposures including radiation, tobacco use, and iatrogenic interventions, although the causal relationship between these factors and initial acquisition of mutations or expansion of the mutant clone has not been established.

Because *Dnmt3a*$^{-/-}$ mice demonstrate enhanced HSC self-renewal (7, 8), it is possible that in CH *DNMT3A* mutations potentially compensate for aging-related HSC exhaustion (101). Conversely, it may provide the "first hit" toward leukemic transformation (102). Individuals with CH have a 0.5% to 1% chance per year to develop hematologic cancer, compared with <0.1% without CH. Yet, *DNMT3A* lesions predict only a moderately elevated risk of leukemic progression, in contrast to other common mutations such as in *TP53* (103, 104). In line with these observations, in a lymphoblastoid cell line from a mosaic individual with *DNMT3A*$^{R771Q/+}$-driven CH, stereotypical erosion of DNA methylation within regulatory regions of stem cell self-renewal and cancer-related genes, and not mutational frequency, favored clonal dominance and establishment of a cancer-poised epigenomic landscape (105). Although these studies provide a rationale for expanded screening for CH to identify individuals at an increased risk of leukemia, the clinically meaningful clone size and the cost–benefit ratio of monitoring are debated. A pivotal study modeling progression of *Dnmt3a*-driven CH to MPN and ultimately AML in mice suggested that a shift toward expansion of the myeloid-restricted progenitors of the mutant clone may serve as an early biomarker (86). Additional studies are critically needed to improve our understanding of the molecular and clinical implications of *DNMT3A* mutations in CH leading to better patient stratification algorithms.

Importantly, clinical observations from large cohorts unselected for hematologic disease revealed a strong relationship of CH with other comorbidities and increased all-cause mortality. Although clonally expanded HSCs appear functionally normal and give rise to mature, differentiated immune cell lineages that permeate nearly all tissues outside of the hematologic compartment, presence of CH mutations is likely to effect subtle changes in their function and, by extension, affect the physiology of surrounding tissues. Thus, CH is strongly associated with incidence and severity of cardiovascular disease (CVD; ref. 106), corroborated in a mouse model of CH driven by *Dnmt3a* loss (107). In a model of CH driven by CRISPR-mediated *Dnmt3a* loss, mature myeloid cells accentuated inflammation and exacerbated the extent of experimental atherosclerosis through increased secretion of a cluster of chemokines and cytokines (108). These results establish a causal role of *DNMT3A*-driven CH in CVD pathogenesis as well as other conditions with a prominent inflammatory component (109) including aplastic anemia (110) and solid tumors (111). In the latter study, presence of CH was associated with inferior overall survival due to progression of the primary malignancy. This suggests that CH can affect cancer pathophysiology through nontumor cell-autonomous mechanisms. Studies showed elevated inflammatory leukocytes and inflammation-related cytokines in the serum of colitis patients with *DNMT3A*-associated CH (112). Similar findings were reported in activated macrophages and mast cells after *DNMT3A* loss, which increased secretion of proinflammatory cytokines such as TNFα, IL6, and CXCL13 (83). On the other hand, inflammation signaling associated with aged bone marrow microenvironment contributed to CH through accentuated TNFα signaling and IFNγ response that primed the *Dnmt3a*-mutant HSCs and promoted their clonal expansion (113). Furthermore, cell-extrinsic environmental factors such as bacterial

infections bestow a fitness advantage to *Dnmt3a*-mutant hematopoietic clones (114). Additional studies exploring the link between *DNMT3A* mutations, CH, inflammation, and immune responses could yield many new exciting insights with biological and translational implications.

Therapeutic Implications

The high frequency of *DNMT3A* mutations in myeloid neoplasms (about a quarter of AML and ∼10% of MPN and MDS cases), its truncal, or early, timing in tumor evolution, and the association with increased risk of relapse and poor overall prognosis position *DNMT3A* alterations and their molecular consequences as an attractive therapeutic target. Yet, despite significant advances in the understanding of the molecular pathophysiology of *DNMT3A*-mutant disease, the need for satisfactory treatment approaches that balance efficacy and toxicity remains unmet. To date, therapy development efforts have focused on four main areas (**Fig. 1**): (i) validate and fine-tune existing combinations already approved for AML, MPN, or MDS; (ii) inhibit aberrantly activated signaling pathways; (iii) target co-occurring actionable mutations and their downstream consequences; and (iv) exploit structural changes in the mutant DNMT3A protein.

In AML clinical trials, adverse outcomes bestowed by *DNMT3A* mutations could be improved by dose-intensified anthracyclines during induction, suggesting that cells with mutant *DNMT3A* are less sensitive to these agents (115, 116). A follow-up study in a model of *Dnmt3a*-mutant hematopoiesis revealed that the relative resistance to anthracyclines was due to abnormal chromatin remodeling and impaired DNA damage sensing (76). As a significant proportion of patients with *DNMT3A*-positive AML fall into the advanced age category with frequent comorbidities, the increased toxicity and treatment-related mortality of dose-dense anthracyclines may not be acceptable, necessitating less aggressive treatment strategies. A low-intensity regimen of nucleoside analogs cladribine combined with alternating cytarabine and decitabine can be an acceptable treatment option for older patients with AML that particularly benefits those with *DNMT3A* mutations (117). Mechanistically, cells expressing mutant DNMT3A treated with cytarabine had a defect in replication fork restart leading to persistent replication stress and accumulation of unrepaired DNA damage (118). Hypomethylating agents (HMA) such as azacitidine and decitabine are the backbone of the low-intensity regimens for AML and MDS. These cytidine analogs are incorporated into DNA and function as covalent suicide inhibitors of DNMTs and as DNA damage inducers by forming bulky adducts. Small clinical studies reported favorable responses in AML and MDS with *DNMT3A* mutations (119–121). This seemingly counterintuitive observation may be explained by the altered flanking sequence preference of the mutant DNMT3A enzyme that causes aberrant hypermethylation at noncanonical gene loci, or by defects in DNA damage response in the presence of mutant DNMT3A protein. Thus, bone marrow cells from mice expressing $Dnmt3a^{R878H}$ readily underwent differentiation after decitabine exposure, whereas $Dnmt3a^{-/-}$ bone marrow accumulated immature $cKit^{+}$ cells (122). Further research is needed to shed light on the mechanistic and therapeutic implications of different types of *DNMT3A* mutations. Furthermore, combinations of HMAs with other targeted agents have shown promise in patients with *DNMT3A* mutations (123).

Patterns of comutation may help guide targeted treatment strategies for *DNMT3A*-mutant disease. A landmark integrative precision oncology Beat AML trial found a strong correlation between *FLT3-ITD, NPM1*, and *DNMT3A* mutational triad and sensitivity to ibrutinib, a BTK and TEC inhibitor FDA approved for the treatment of B-cell chronic lymphocytic leukemia (124). The FLT3 inhibitor AC220/quizartinib was shown to preferentially elicit a differentiation response in the triple-mutant AML; in contrast, *DNMT3A* mutations were rare in patients with cytotoxic responses (125). In another *ex vivo* study, primary AML cells harboring *DNMT3A* mutations were slightly more sensitive to the JAK1/2 kinase inhibitor ruxolitinib plus venetoclax (an

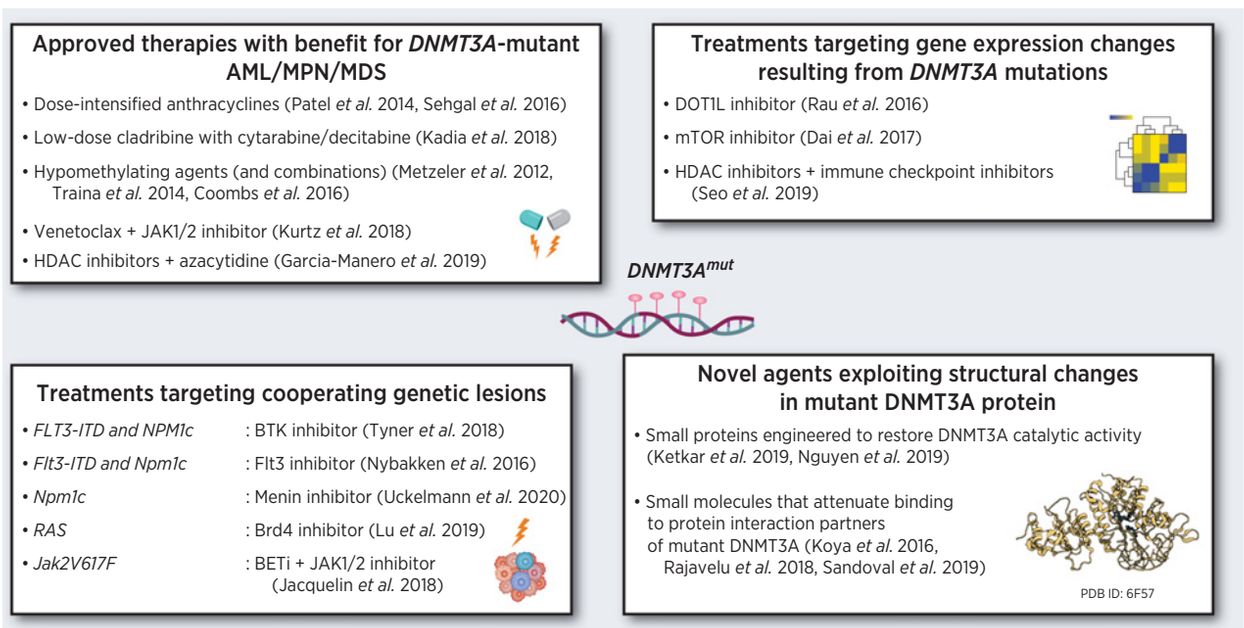

Figure 1.
Emerging therapeutic approaches for myeloid malignancies with *DNMT3A* mutations. Images created with BioRender.com.

inhibitor of antiapoptotic BCL-2 protein) combination, independently from *FLT3* and *NPM1* status (126).

Treatments targeting gene expression or methylation changes associated with *DNMT3A* mutations are also gaining traction. Several studies identified upregulation of the homeobox cluster A and B (*HOXA/B*) genes, which promote HSC self-renewal and are associated with poor prognosis in AML (1, 64, 75). Small-molecule inhibitors of the histone methyltransferase DOT1L restored repression of the *HOXA/B* genes both *in vitro* and *in vivo*, and proved effective for *DNMT3A*-mutant leukemia (127). The mTOR pathway, another regulator of the *HOX* gene expression, was found to be activated in the *DNMT3A*-mutant context. mTOR inhibitor rapamycin was effective against cells with *DNMT3A* mutations *in vitro* (77); it will be important to validate its therapeutic potential in preclinical models. *DNMT3A* mutations co-occur with *NPM1c* mutations in the preleukemic setting (60%–80%) and in AML. $Npm1^{c}$:$Dnmt3a^{R878H}$ double-mutant mice exhibited increased self-renewal in myeloid progenitor cells, associated with further activation of *HoxA/B genes* and *Meis1*. A menin inhibitor VTP-50469, previously shown to disrupt critical gene expression networks in *NPM1*-mutant AML cell line (128), was effective in eradicating preleukemic progenitors and preventing progression to AML in this model (129).

Bromodomain inhibitors, specifically an inhibitor of the histone acetylation reader BRD4, were effective in a study of AML with concurrent $DNMT3A^{R882}$ and *RAS* mutations, in both *in vitro* and *in vivo* models. Pharmacologic inhibition of BRD4 suppressed a subset of aberrantly activated gene targets that likely contribute to leukemogenesis, consistent with increased H3K27ac levels in TF-1 cells (130). In a model of MF, loss of *Dnmt3a* in hematopoietic cells expressing $Jak2^{V617F}$ resulted in high expression of TNFα via NFκB pathway accompanied by increased secretion of proinflammatory cytokines. Combining BET inhibitors with JAK1/2 kinase inhibitors could have therapeutic relevance (73).

Strategies related to engineering small proteins to restore the full catalytic activity of mutant DNMT3A or the ability of wild-type DNMT3A to heterotetramerize by disrupting the wild-type–mutant binding interface have also been proposed and could potentially offer therapeutic benefit (56, 80). With better understanding of the protein–protein binding repertoire of mutant DNMT3A such as p53, MeCP2, TDGs, and PRC1, pharmacologic interventions to attenuate these interactions may open additional therapeutic avenues to combat *DNMT3A*-mutant AML (44, 63, 64).

Concluding Remarks and Future Perspectives

Although mutations in *DNMT3A* are found in malignancies of virtually every hematopoietic lineage, the molecular understanding of its impact on malignant transformation is only beginning to emerge. Recent biochemical, structural, and -omics studies have shed light on the nature of aberrant methylation patterns, cross-talk with other layers of epigenetic regulation, and subsequent changes in gene expression profiles that contribute to clonal expansion and promote leukemogenesis. Further refinement and unification of our knowledge of these programs, including in the various comutational contexts that define disease subtypes and/or clonal architecture (28, 131, 132), are expected to translate into more effective therapies for patients with *DNMT3A*-mutant AML and other malignancies.

Recent years saw an explosion of research into the role of *DNMT3A* mutations in CH and its comorbidities. Abundant evidence supports accentuated self-renewal creating an expanded pool of cancer-poised HSCs, yet the definitive factors effecting malignant transformation await to be discovered. Once identified, these will be game-changing for CH prognostication and preventative interventions. In addition, cells with *DNMT3A* mutations propagate an inflammatory microenvironment leading to positive feedback to mutant clone self-renewal and proliferation and may exacerbate other nonhematologic disease conditions such as CVD. Characterizing the cell-extrinsic and -intrinsic factors and the mechanisms that promote the inception of CH in the *DNMT3A*-mutant context is crucial to the development of therapeutic strategies.

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