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Systematic profiling of *DNMT3A* variants reveals protein instability mediated by the DCAF8 E3 ubiquitin ligase adaptor

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Author Contributions

YHH and MAG conceived the project, discussed, and designed experiments. YHH, CWC, VS, AT, MS, OD, BRC, CG, ML and LB conducted experiments. DH, CJW and JMR performed sequencing and statistical analysis. KTB, MAS, LVM, and PJV assisted with clinical sample collection, storage, and management. YZ, YH, XC, AM, WL, JRB and BLE provided methodology assistance. YHH and MAG wrote the manuscript. All authors interpreted the results and edited the manuscript.

Declaration of Interests

The authors declare no competing financial interests.

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Abstract

Clonal hematopoiesis is a prevalent age-related condition associated with greatly increased risk of hematologic disease; mutations in DNA methyltransferase 3A (*DNMT3A*) are the most common driver of this state. *DNMT3A* variants occur across the gene with some particularly associated with malignancy, but the functional relevance and mechanisms of pathogenesis of the majority of mutations is unknown. Here, we systematically investigated the methyltransferase activity and protein stability of 253 disease-associated *DNMT3A* mutations, finding that 74% were loss-of-function mutations. Half of these variants exhibited reduced protein stability and, as a class, correlated with greater clonal expansion and AML development. We investigated the mechanisms underlying the instability using a CRISPR screen and uncovered regulated destruction of DNMT3A mediated by the DCAF8 E3 ubiquitin ligase adaptor. We establish a new paradigm to classify novel variants that has prognostic and potential therapeutic significance for patients with hematologic disease.

Keywords

Clonal hematopoiesis; hematological malignancies; *DNMT3A* variants; E3 ubiquitin ligases; protein instability

Introduction

One trillion new blood cells are produced each day through a complex orchestrated hierarchy rooted in long-lived hematopoietic stem cells (HSCs) in the bone marrow. Over time, all HSCs acquire somatic mutations, some of which confer a Darwinian fitness advantage enabling them to produce a disproportionate number of progeny, a condition known as *clonal hematopoiesis* (CH). CH is increasingly prevalent with age and is associated with increased risk of myelodysplastic syndrome, hematologic malignancies and all-cause mortality (1,2).

Virtually all individuals from middle-age on will have expanded HSC clones (3), but by the age of 70, about one fifth will have larger clones considered as CH comprising 4% of the peripheral blood (PB) (2% variant allele frequency, or VAF) (4). Broadly, larger clones are associated with a higher risk of hematologic malignancies (1). While mutations are randomly acquired throughout the genome, variants in a limited number of genes are recurrently observed (5). Understanding the mechanisms through which HSC clones expand and associate with malignancy development is therefore of significant interest (6).

DNA methyltransferase 3A (*DNMT3A*) is a critical tumor suppressor in the hematopoietic system, and is the most frequently mutated gene in CH. Mutations in *DNMT3A* are spread across all three functional domains, namely, Pro-Trp-Trp-Pro (PWWP), ATRX-DNMT3A-DNMT3L (ADD), and the methyltransferase (MTase) (Figure 1A). The R882 hotspot mutation in the dimerization region of the MTase domain that accounts for around 60% of mutations in acute myeloid leukemia (AML) has been shown to act as a dominant-negative leading to reduced DNA methylation activity (7-10), despite R882-specific hypermethylation through alteration of flanking sequence preferences has been reported (11). Importantly, in CH, as well as in lymphoid malignancies, R882 is found in only around 20% of cases, and the impact of the other 80% of mutations, many of which are predicted to be pathogenic, is largely unknown. Mouse models have shown that homozygous ablation of *Dnmt3a* leads to focal hypomethylation, stem cell expansion and predisposition to hematological malignancies (12-14). Therefore, we hypothesized that at least some of the other missense mutations would also exhibit loss of DNA methylation activity.

In addition to somatic mutations in CH and hematologic malignancies (15,16), germline mutations in *DNMT3A* are clinically relevant: they can lead to Tatton-Brown-Rahman overgrowth syndrome (TBRS) in which patients exhibit obesity, excessive height, and intellectual disability (17,18). Peripheral blood of one TBRS patient with an R882 mutation was shown to exhibit DNA hypomethylation patterns relative to its healthy sibling (19), but other mutations, many of which overlap with those found in hematologic disorders, have not been characterized. Importantly, a patient with microcephaly is reported to have a mutation leading to hyper-active DNMT3A (20). Together, these findings suggest that the precise dosage of DNMT3A is physiologically critical, with heterozygous mutations leading to either an excess, or a diminution, of DNMT3A activity with potentially dire consequences.

Here, we sought to systematically profile *DNMT3A* variants found in patients to gain insights into the mechanism of pathogenesis with a view toward risk stratification and identification of potential therapeutic strategies for hematological malignancies.

Results

Methyltransferase function of 253 Disease-associated *DNMT3A* Variants

In order to profile methyltransferase activity of *DNMT3A* variants in a high-throughput manner, we first sought to establish a fluorescent reporter capable of faithfully representing functionality of *DNMT3A* variants. Our previous work had shown that DNA methylation at the *HOXA5* promoter was a sensitive indicator of DNMT3A activity (21). Thus, we knocked in a Snrpn-blue fluorescence protein (BFP) reporter (22) to this locus in human

embryonic kidney 293T (HEK293T) cells at a reverse orientation such that increased DNA methylation at *HOXA5-Snrpn*-BFP would lead to decreased BFP. This strategy can provide read-out of methyltransferase functionality by flow cytometry (Figure 1B). To test the fidelity of the assay, we transduced the cells with lentiviral vectors carrying *DNMT3A*^{WT} and a known catalytically inactive variant *DNMT3A*^{E756A} (23,24). The proportion of BFP-negative cells in *DNMT3A*^{WT} transduced cells increased and plateaued at approximately 20% after 2 weeks (Figures 1C and 1D). Conversely, the proportion of BFP-negative cells in *DNMT3A*^{E756A} transduced cells remained virtually unchanged over this period (Figure 1D). Increased DNA methylation at the *HOXA5-Snrpn* reporter in cells transduced with WT-*DNMT3A* was verified (supplemental Figure 1A). These results confirmed that we could measure methyltransferase functionality of *DNMT3A* variants using the *HOXA5-Snrpn*-BFP reporter system.

We then selected 253 patient-associated *DNMT3A* variants, mostly missense mutations and some 3-bp in-frame deletions, distributed across three functional domains of the 912-residue protein (Figure 1A; supplemental table 1). The variants were compiled from reports of mutations in CH (1,2,25), cancer and TBRS (17,18) and cover a large portion of reported mutations. These variants were cloned into a lentiviral vector which was then transduced into the *HOXA5-Snrpn*-BFP reporter cells. The proportions of BFP-negative cells were monitored and normalized to *DNMT3A*^{WT} fifteen days later in order to measure the *DNMT3A* methyltransferase functionality. Across the protein, 74% (187 of 253) of the variants were classified as severe loss-of-function (Figures 1E; supplemental Figures 1B-D, 2A, 3A and 4A), with 85% (23 of 27) and 81% (114 of 140) of those in the PWWP and MTase domains respectively exhibiting little or no activity. In the ADD domain, a lower proportion (68%, 29 of 40) exhibited the same severity while other variants had modest loss of activity.

In order to verify the observed reduction of methyltransferase activity, we introduced a number of variants into methylation-deficient mouse embryonic stem cells (*Dnmt3a/b* double knock out (DKO) mESCs) with almost no background DNA methylation (~5% of average DNA methylation of genome-wide CpGs) (23) (supplemental Figure 1E). We focused on frequently-documented variants in CH, AML, and TBRS – a single amino acid in-frame Trp 297 deletion (W297Del) in the PWWP domain, a nonsynonymous Arg 749 missense variants (R749C), and the known catalytically inactive mutation Glu 756 (E756A) in the MTase domain as a control. After expression in DKO mESCs, the harvested cells were subjected to whole genome bisulfite sequencing (WGBS). *DNMT3A*^{WT} re-established DNA methylation of about 60% of genome-wide CpGs. In contrast, *DNMT3A*^{W297Del}, *DNMT3A*^{E756A} and the other *DNMT3A* variants failed to increase methylation (Figures 1F and supplemental Figure 1F). Notably, *DNMT3A*^{E756A} retained comparable protein expression to *DNMT3A*^{WT}. *DNMT3A*^{W297Del}, and the other *DNMT3A* variants, on the other hand, had lower protein expression (supplemental Figure 1G). Western blot confirmed that *DNMT3A*^{W297Del} expression was significantly reduced compared to *DNMT3A*^{WT} (Figure 2A), suggesting lack of methylation activity may be attributed to decreased protein levels.

More than a Third of *DNMT3A* Variants Lead to Protein Instability

Of the hundreds of identified *DNMT3A* mutations, none have been shown to result in high protein turnover. Furthermore, while algorithms can be used to predict whether amino acid changes are damaging protein function, they do not account for changes in protein stability. Therefore, we sought to systematically determine whether protein instability was a common molecular mechanism leading to loss of DNMT3A function. To investigate this, we used a bicistronic vector in which DNMT3A was fused to GFP as a sensor of DNMT3A protein level, linked to dsRed expression controlling for transfection efficiency (Figure 2B). Protein stability of 253 variants was measured by the ratio of green to red fluorescence after transfection into HEK293T cells (supplemental Figure 2B, 3B and 4B). Surprisingly, we found instability was a common feature, with 37% (94 of 253) of variants reducing protein stability (Figure 2C), with a nonrandom distribution across *DNMT3A*.

The observations of protein instability were also verified in patient samples and mESCs at a heterozygous state in the *Dnmt3a* endogenous loci. Lymphoblastoid cell lines (LCLs) were generated from the peripheral blood of patients with AML carrying mutant DNMT3A and DNMT3A protein levels were examined. All *DNMT3A* variants shown to be stable above had similar amounts of protein, while one sample bearing a heterozygous unstable mutant (DNMT3A^{P307S}) had lower DNMT3A (supplemental Figure 5A). Furthermore, *Dnmt3a* variants were introduced into the endogenous locus in mESCs using CRISPR-Cas9 genome editing. Single colonies bearing heterozygous knock-in of three unstable mutations (W293Del, G681R, and R732C) and two stable mutations (E752A and W856R), which correspond to W297, G685, R736, E756, and W860, in human DNMT3A, were validated by Sanger sequencing (supplemental Figure 5B). DNMT3A protein expression levels were significantly reduced in unstable mutants (DNMT3A^{W293Del}, DNMT3A^{G681R}, and DNMT3A^{R732C}) and increased in stable mutants (DNMT3A^{E752A} and DNMT3A^{W856R}) (Figure 2D) while the RNA expression levels of these *Dnmt3a* mutants remained comparable (Figure 2E). These data demonstrate that the reduced DNMT3A protein expression resulted from protein instability instead of defects in transcription. Together, data from the patient-derived LCLs and CRISPR-generated mESCs validate the finding of DNMT3A protein instability for multiple mutants in physiologically relevant heterozygous states.

To better understand whether particular features were associated with unstable and stable variants, we examined the structural distribution of the variants. Unstable PWWP domain (26) variants occurred across the entire domain (supplemental Figure 5C), in the hydrophobic core as well as in a region where intra-molecular polar/charged interactions occur that likely promote stability. Similarly, several unstable MTase domain variants (e.g. R729W and R736C) are located at the inter-molecular interface where DNMT3A interacts to form a highly active tetramer with DNMT3L (supplemental Figure 5D), a non-catalytic member of the DNMT3 family expressed during embryogenesis (27).

Overall, the majority of *DNMT3A* variants leading to protein instability were located in the PWWP and MTase domains, with 57% (16 of 27) and 44% (62 of 140) respectively classified as unstable (supplemental Figures 2B and 3B). In the MTase domain, the majority of unstable variants were located in the interior (supplemental Figure 5E). Others, (e.g.

P904L and E907V) were located at the intra-molecular interface between the MTase and the ADD domains (supplemental Figure 5F), indicating that inter-domain interactions may be critical for protein stability.

The MTase domain also contains mutations relatively resistant to instability, including the residues important for co-factor (S-adenosyl-L-methionine or SAM) binding (27), target recognition, catalysis, and DNMT3A-3A homodimer formation (28) (supplemental Figure 3B). These mutations likely lead to loss of function through effects on catalysis rather than loss of stability.

Finally, fewer variants in the ADD domain (17.5%, 7 of 40) led to protein instability (supplemental Figure 4B). All 7 variants impairing protein stability occurred at the cysteine residues and are involved in zinc coordination (supplemental Figure 5G), indicating the metal binding domains are important for overall protein structure and stability.

Together, these data establish that reduced DNMT3A stability is a common pathway to loss of function. Domains involved in protein-protein interactions had a higher proportion of variants leading to instability. Mutations appearing in TBRS and CH were equally likely to result in protein instability, underscoring a remarkable dose sensitivity of DNMT3A for normal human physiology.

Unstable Variants are Associated with Increased Clonal Expansion

Variants that lead to instability are expected to have around half the amount of WT DNMT3A protein, and, collectively, may be expected to behave similarly at a physiologic level. Conversely, LOF variants that retain stability may exert effects to varying degrees via multiple mechanisms such as diminished SAM or DNA binding. Thus, we sought to determine whether variants that led to protein instability would exhibit any phenotypic correlations.

Recent work has shown that variants with cell-intrinsic fitness advantages are major driving forces shaping clonal hematopoiesis (29). To determine whether *DNMT3A* variants associated with protein instability displayed fitness advantages over stable variants, we estimated the distribution of fitness effects for the two classes of variants. We plotted VAF density histograms for the stable and unstable variants detected in four studies that targeted over 99% of variants tested here (25,30-32), normalizing by the study size and study-specific mutation rate (29) (Figure 2F). Performing a maximum likelihood fit to a family of stretched exponential distributions (29), we found that the unstable group of variants, as a class, had a higher proportion of variants in the mid- to high fitness range compared to the stable group (supplemental Figure 6A). If destabilizing variants, on average, confer a larger fitness advantage compared to stable variants, we would expect to observe more unstable over stable variants after controlling for total mutation rate differences between the two classes. Indeed, we demonstrated a significant enrichment for unstable variants in data from four separate studies (supplemental Figure 6B), consistent with their having higher fitness effects, on average, than stable variants. Whereas destabilizing variants are predominantly in the moderate-high fitness class, expected of a “null” allele, stable variants have broader fitness effects, falling into both low and high fitness categories (supplemental Figure 6A). This is

consistent with our expectation that individual stable variants exert a more varied impact, with some representing a partial loss-of-function, and others, such as mutations like R882 that result in a dominant-negative effect (8,9,19), conferring an even higher fitness advantage (supplemental Figure 6C). These analyses again underscore the remarkable dose-sensitivity of DNMT3A function.

To determine whether unstable variants also are associated with leukemia development, we examined VAFs of *DNMT3A* variants in 60 patients with pre-AML (average 6.3 year before AML diagnosis) and 192 patients of age- and gender-matched controls identified from a publicly available dataset (33). While no significant differences in clonal expansion were observed in the control group (supplemental Figure 6D), in the pre-AML group, greater expansion was observed in patients with unstable vs stable variants (supplemental Figure 6E). This suggests that the increased fitness effects of unstable variants associated with CH also translate to a higher risk of AML development.

DNMT3A is degraded through proteasomal machinery

Given the striking prevalence of variants leading to protein instability, we sought to examine the molecular consequences of such mutations in a murine model. One mutation leading to severe protein instability was a 3 bp deletion resulting in an in-frame deletion of W297, equivalent to W293 in the mouse. We used CRISPR-Cas9 editing in murine zygotes to recreate this variant, generating a *Dnmt3a*^{W293Del} allele. To determine the severity of the W293Del variant compared to complete loss of DNMT3A, we intercrossed *Dnmt3a*^{+/-} and *Dnmt3a*^{W293Del/+} mice to generate compound heterozygous *Dnmt3a*^{W293Del/-} as well as homozygous mutant mice. Notably, both *Dnmt3a*^{W293Del/-} and *Dnmt3a*^{W293Del/W293Del} mice did not survive past postnatal day 24 (Figure 3A), resembling the phenotype of *Dnmt3a*^{-/-} mice (34), suggesting that the severity of the *Dnmt3a*^{W293Del} variant is comparable to the null variant. We then examined whole bone marrow cells from *Dnmt3a*^{W293Del} mice to determine whether they also exhibited a loss of DNMT3A. Indeed, DNMT3A was almost completely absent in homozygous mice, and about half the amount of WT protein was present in heterozygous mice (Figure 3B), despite normal mRNA levels (Figure 3C).

The *DNMT3A*^{W297Del} variant and its murine homolog showed similar severe protein instability; therefore, we sought to identify the responsible protein degradation pathway. Transfection of DNMT3A into HEK293T cells showed that after 12 hours, the amount of DNMT3A^{WT} was constant but DNMT3A^{W297Del} was approximately half of WT and subsequently decreased (Figure 3D). This confirmed that reduced DNMT3A^{W297Del} was not due to a translational defect but rather to impaired stability. We then used the stability assay described above to assess the relevance of major degradation pathways, treating *DNMT3A*^{W297Del} transfected cells with inhibitors of autophagy, the unfolded protein response, the proteasome machinery, and Cullin-RING E3 ubiquitin ligases (CRLs). Treatment with a proteasome inhibitor (MG132) rescued 2.5-fold expression of DNMT3A^{W297Del} (Figure 3E). The CRL inhibitor MLN4924 also led to a modest increase of mutant DNMT3A^{W297Del} expression in HEK293T cells (Figure 3E). These results

suggest that DNMT3A^{W297Del} is degraded through the proteasome, and that Cullin-RING E3 ubiquitin ligases may play a role in this process.

We next aimed to determine whether other unstable DNMT3A variants were degraded through the same mechanism. We treated cells expressing 20 of these variants distributed in the PWWP and MTase domains with inhibitors targeting multiple degradation pathways. Again, treatment with a proteasome inhibitor partially restored DNMT3A mutant protein expression in cells with all 20 mutations (Figure 3F). Notably, treatment with inhibitors of E1 ubiquitin-activating enzyme (MLN7243) conferred the highest rescue of DNMT3A variant expression (supplemental Figure 7A), indicating that DNMT3A is mainly degraded through ubiquitin-proteasome system. Treating cells expressing WT or DNMT3A variants with inhibitors of Cullin-RING E3 ubiquitin ligases (MLN4924), autophagy inhibitors (i.e., chloroquine) or the unfolded protein response (i.e., IREi) did not rescue protein expression (supplemental Figures 7B-E). In addition, an HSP90 inhibitor further decreased protein stability (supplemental Figure 7F), suggesting a role for HSP90 in stabilizing DNMT3A during protein synthesis.

A Proteasome Inhibitor Partially Restores Patient Methylome and Transcriptome Patterns

The finding that many *DNMT3A* variants lead to protein instability offers the possibility of therapeutic intervention. Focusing on the W297Del mutation, we first aimed to confirm that DNMT3A^{W297Del} is decreased in patient cells. We generated LCLs from the peripheral blood of a TBRS patient and confirmed that the DNMT3A protein level was indeed reduced (Figure 4A). Moreover, exposure to a proteasome inhibitor increased the level of DNMT3A in both WT and DNMT3A^{W297Del/+} LCLs (Figure 4A). To further examine whether proteasome inhibitor treatment would restore DNA methylation and correct gene expression levels, we cultured the DNMT3A^{W297Del/+} LCL cells in the presence of a low dose of proteasome inhibitor (1 μ M MG132) for 30 days and then conducted WGBS and RNAseq. Indeed, proteasome inhibitor treatment increased the average methylation in DNMT3A^{W297Del/+} LCLs from 59.4% to 63.5%, which is statistically identical to the 63.1% methylation ratio of genome-wide CpGs observed in WT LCLs (Figure 4B). Proteasome inhibitor treatment also increased the average methylation in WT LCLs by 2%. We then examined 25 chromatin marks across 127 reference genomes from the Epigenome Roadmap Project (35), to identify genomic regions that were most responsive to re-methylation in the presence of the proteasome inhibitors. We found that active enhancers, weak enhancers, heterochromatin, poised promoters and regions with repressive polycomb complexes showed the most dynamic changes (Figures 4C, 4D; supplemental Figures 8A-M), whereas regions close to the transcription start site (TSS) and transcribed regions (supplemental Figure 8N-W) remained largely inert to proteasome inhibitor administration.

We also examined the correlation between DNA methylation changes and gene expression. We identified genes that were expressed in the LCL cells and also harbored at least 5 CpGs in their promoter region. Within this group, upregulated genes were separated into two clusters using unsupervised consensus clustering. Genes whose promoters were hypomethylated in DNMT3A^{W297Del/+} compared to WT LCLs showed higher expression in DNMT3A^{W297Del/+} LCLs, with one particular group of genes (Cluster 1) particularly

strongly correlated. Treatment with proteasome inhibitors rescued this phenotype with reduced expression of Cluster 1, but not the more modestly affected Cluster 2 genes (Figure 4E). Genes on Clusters 1 and 2 were generally found in diverse cellular pathways although components associated with the plasma membrane were statistically enriched in the Cluster 1 genes (supplemental Table 2). Together, these data demonstrate that administration of proteasome inhibitor to cells harboring a *DNMT3A* variant leading to protein instability can at least partially restore the global methylome and transcriptome, opening up the possibility of therapeutic restoration of DNMT3A function in some contexts.

CRISPR screening links CUL4B^{DCAF8} ubiquitination complex to DNMT3A degradation

We next sought to identify the specific ubiquitination complex involved in DNMT3A turnover utilizing the unstable DNMT3A^{W297Del} variant, using a CRISPR screen (36). We engineered HEK293T cells to constitutively overexpress Cas9 protein and a bicistronic DNMT3A^{W297Del} stability reporter, and then transduced the cells with sgRNA libraries targeting ubiquitin ligases. Nine days later, we sorted for the cells with the highest (above 95th percentile) and the lowest (below 5th percentile) DNMT3A-GFP expression (Figure 5A) and then examined which sgRNAs were enriched in the cells with the highest compared to lowest DNMT3A-GFP expression. Strikingly, we found that *DCAF8*, *RBX1* and *CUL4B* were among the most enriched targets in the screen (Figure 5B). To verify the results, we examined whether the substrate adaptor DCAF8 physically interacts with DNMT3A. We conducted immunoprecipitation for DCAF8 and then probed for interaction with DNMT3A after culture in the absence or presence of a E1 ubiquitin-activating enzyme inhibitor. We observed co-immunoprecipitation between DNMT3A^{W297Del} and DCAF8 after 6 hours of inhibitor treatment, while weak interaction between WT DNMT3A and DCAF8 was also observed (Figure 5C). This suggested that DCAF8 had higher binding affinity to the variant protein and confirmed the possibility that CUL4B^{DCAF8} could be the ubiquitin ligase for DNMT3A.

We then sought to examine whether DNMT3A^{W297Del} expression was rescued when DCAF8 was ablated. Indeed, knock-out of *DCAF8* with sgRNAs rescued DNMT3A^{W297Del} level to a degree comparable to that of rescue by treatment with the proteasome inhibitor and E1 ubiquitin-activating enzymes (Figure 5D). Additionally, when we inhibited protein synthesis with cycloheximide (CHX) treatment, we observed rapid disappearance of DNMT3A protein; *DCAF8* KO enabled sustained DNMT3A expression (Figure 5E). Furthermore, knock-out of *DCAF8* in *DNMT3A*^{WT} LCLs also increased DNMT3A^{WT} expression (Figure 5F). Together, these results indicate that DCAF8 plays a major role in degradation of DNMT3A, both WT and unstable variants.

We next sought to verify whether DCAF8 regulates DNMT3A through facilitating ubiquitination. We observed that DNMT3A^{W297Del} showed high levels of ubiquitination relative to DNMT3A^{WT}. Ubiquitination of both DNMT3A^{WT} and DNMT3A^{W297Del} was significantly reduced in *DCAF8* KO cells (Figure 5G). Moreover, in mES cells harboring heterozygous knock-in of the unstable DNMT3A^{W293Del} and DNMT3A^{G681R} variants, *Dcaf8* ablation significantly rescued DNMT3A protein expression, whereas expression of stable DNMT3A variants (DNMT3A^{E752A} and DNMT3A^{W856R}) did not

change (Figure 5H). These results indicate that DCAF8 serves as a substrate adaptor for DNMT3A ubiquitination, and the ablation of DCAF8 prevents DNMT3A ubiquitination and degradation in a regulated manner. To determine whether DCAF8 also influenced stability of other unstable DNMT3A variants, we examined the turnover of the 20 selected unstable DNMT3A variants in *DCAF8*-KO HEK293T cells. *DCAF8* KO modestly rescued protein stability of most DNMT3A variants (Figure 5I). Importantly, the scaffold protein CUL4B was also enriched in the screen, as was RBX1, an E2 conjugating enzyme. These two components are both known to participate in a complex with the substrate adaptor DCAF8 (Figure 5J). Together, these results suggest a new complex for regulation of DNMT3A degradation and stability, some components of which could serve as potential therapeutic targets for rescuing DNMT3A expression in patients with unstable variants.

Discussion

The work presented here, characterizing patient-associated variants across approximately one-third of amino acids in DNMT3A, has revealed general principles of DNMT3A regulation and stability and has implications for the interpretation of novel variants across many disease types. While the concept of reduced stability is not unknown, that such a large portion of variants exhibit this phenotype and can be classified together was unexpected. Furthermore, our work illustrates that when unstable variants can be classified together, their biological impact is consistent. Finally, our discovery of DNMT3A destruction mediated by the CUL4B^{DCAF8} E3 ligase unveils an important new regulatory pathway for this protein whose biological role is remarkably dose-sensitive; this new pathway may offer new potential therapeutic avenues.

As large numbers of novel germline and somatic variants across the genome are identified by genome sequencing of patients, it is becoming increasingly important to infer their impact on protein function. Numerous algorithms have been designed to identify the likelihood of a particular mutation being deleterious, but none have taken into account loss of protein stability. Not only do we show, for the first time, that a large portion of missense variants lead to protein instability, but our analysis of their relevance with regard to activity and protein structure offers a framework to apply these principles to novel variants in other genes.

DNMT3A mutations leading to protein instability have clinical significance. We found a relationship between protein instability and CH as well as hematologic malignancies. Greater clonal expansion was observed in individuals with unstable compared to stable mutations in the clonal hematopoiesis and pre-AML cohorts, suggesting that *DNMT3A* mutations leading to protein instability may increase the risk of AML development. While the stable dominant-negative R882 mutation has the greatest prognostic significance for development of AML, a comprehensive map of unstable mutations across *DNMT3A* may also have prognostic value. Because essentially all individuals will develop HSC clones bearing *DNMT3A* mutations during their lifetime (3), the functional implications of different variants are of broad interest.

Identification of DCAF8 as the substrate adaptor for DNMT3A protein degradation demonstrated a regulated turnover process that affects both mutant and WT protein, rather than general destruction of mis-folded protein. We hypothesize that DCAF8 has some affinity for WT DNMT3A protein, but that unstable mutations confer some degree of structural conformation change, further exposing potential degrons to DCAF8. This suggests a potential therapeutic target for patients with destabilizing mutations. A recent report showed that DNMT3A is normally recruited to intergenic regions by H3K36me2, but two destabilizing mutants of DNMT3A (W297Del and I310N) failed to bind to chromatin (37). We expect that CUL4B^{DCAF8} ubiquitin ligases may serve as a protein quality surveillance system for DNMT3A protein, eliminating protein that is not bound to chromatin. This would be consistent with other studies showing there is very little free cellular DNMT3A detected (38). While our data clearly show that DCAF8 is a major regulator of DNMT3A, we cannot preclude the existence of others. Inhibition of E1 activating enzymes shows stronger rescue of protein stability across multiple DNMT3A mutants than DCAF8 depletion (supplemental Figure 7A; Figure 5E), and treatment of *DCAF8* knock-out cell lines with the E1 inhibitor further increases DNMT3A protein slightly (Figure 5E). Together, these data suggest DCAF8 may not be the sole ubiquitin ligase responsible for DNMT3A degradation. Further work is needed to understand all the components and the context of DNMT3A protein quality control.

Finally, we showed that a proteasome inhibitor could partially restore the aberrant methylome and transcriptome in a patient-derived *DNMT3A*-mutant cell line. Several proteasome inhibitors have been approved by FDA for use in treating multiple myeloma (39,40); therefore, the use of proteasome inhibitors, or conceivably DCAF8 inhibitors, could be a potential therapeutic avenue for a subset of patients with TBRS, CH, and hematologic disorders. As the proteasome inhibitor also increased WT DNMT3A protein, proteasome inhibition could be a potential therapeutic strategy even in patients with deletions or other mutations. More broadly, identification of effects on stability of variants in other genes could be similarly used to develop new therapeutic strategies. A caveat of proteasome inhibition is the broad effects; it is possible that the restoration of methylome and transcriptome patterns could be indirect. Given that DNMT3A mutations in patients are typically heterozygous (1,2,15), testing low-dose and long-term proteasome inhibition on patient-derived *DNMT3A*^{mutant/+} cells may represent possible outcomes.

Given the rapid pace with which mutations involved in human disease are now being identified, there is an increasing need to be able to anticipate the impact of new variants. We believe the approach presented here, of comprehensive characterization of a large fraction of novel variants to understand protein function, can serve as a paradigm to gain broad insights into other recurrently mutated genes.

Methods

Cell lines, cell culture and lentiviral particle production

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by a density gradient centrifugation method using Ficoll. PBMCs (2×10^6) were incubated with concentrated supernatant from the EBV producer cell line B95-8 in a total of 200 μ L of

RPMI 1640 medium (containing 10% fetal calf serum and 5% L -glutamine) for 30 minutes. The cells were then plated at 10^6 cells/well in a flat-bottomed 96-well plate, as well as 1 μ g/mL cyclosporin A (Sandoz Pharmaceuticals, Washington, DC). Cells were fed bi-weekly until lymphoblastoid cell lines (LCLs) were established. The use of human samples had IRB approval from Baylor College of Medicine. Written informed consent was obtained from sample donors. All relevant ethical regulations on compliance were followed in this study.

Dnmt3a variants in previously established *Dnmt3a*-flagged mESC was generated using CRISPR-Cas9 genome editing. 1 μ g of sgRNA (Synthego) was incubated with 1 μ g of Cas9 protein (PNA Bio) for 30 min at room temperature to obtain Cas9-sgRNA RNPs. Then, 1.2 μ g of single-stranded DNA donor template (Integrated DNA Technologies) containing desired variant sequence was added prior to electroporation using a Neon transfection system (Thermo Fisher Scientific). 2×10^5 cells were electroporated using the optimized electroporation condition of 1,200 V, 20 ms, and 2 pulses. After electroporation, we collected single cell colonies for each of variants and the sequence of single colonies were validated by Sanger sequencing.

HEK293T cells are commercially available and DKO mESCs were established from Meissner lab (23). All cells used in this study were routinely tested to be mycoplasma-free (Mycoplasma Detection Kit, Minerva Biolabs). HEK293T cells were incubated in DMEM media with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (P/S) at 5 % CO₂ in 37°C incubator. DKO mESCs and their DNMT3A mutant-expressing derivatives were cultured in 12.5% Foundation ES serum, 1X Glutamax, 100 μ M β -mercaptoethanol, 1X NEAA and 1000U/mL leukemia inhibitory factor (LIF) and Knockout DMEM.

HEK293T cells were transfected with Pinducer20-BSD DNMT3A mutant plasmids using Lipofectamine 2000 (Life Technologies) and co-transfected with pMD2.G and psPAX2. Lentiviral particles were collected 48 hours and 72 hours after transfection. 4X PEG (32 % PEG6000, 0.4 M NaCl, and 0.04 M HEPES) was added to precipitate viral particles at 4°C overnight. Viral particles were then centrifuged at 1500g for 45 minutes and resuspended in mESCs medium. DKO mESCs were infected with Pinducer20 DNMT3A mutant lentiviral particles and 4 μ g/ml polybrene in the medium. 4 μ g/ml Blasticidin (BSD) selection was conducted for three days in the DNMT3A mutant infected mESCs and then recovered for seven days and conducted 4 μ g/ml BSD for a further seven days. DNMT3A mutant mESCs were treated with 2 μ g/ml doxycycline for 30 days and then collected for DNA and RNA extraction.

DNA Constructs

Full-length *DNMT3A* cDNA fused with a GFP sequence was cloned into pDONR223 using a Gateway cloning BP clonase II enzyme mix (Thermo Fisher Scientific). Pathogenic *DNMT3A* mutations listed in the COSMIC database and those causing TBRS and CH were selected and then modeled in pDONR-DNMT3A-GFP vectors using Quickchange II site-directed mutagenesis kits (Agilent Technologies). The full-length cDNA of every *DNMT3A* mutant in the pDONR vector used in subsequent experiments was verified by Sanger sequencing. For the DNA methylation assay, mutant DNMT3A pDONR vectors were further cloned into Pinducer20-BSD vectors using Gateway cloning LP clonase II enzyme

Generation of the *Dnmt3a*^{W293Del} murine model

The sequence of sgRNA-293 has been listed in the supplemental table and was generated by in vitro transcription (MEGAscript T7 Transcription kit). Single-stranded DNA template (ssODN) carrying W293Del and a *SacI* restriction site was synthesized. (Integrated DNA Technologies). Cas9 protein was purchased from PNA Bio. 100 ng/μl Cas9 protein, 50 ng/μl sgRNA-293, and 100 ng/μl ssODN diluted in nuclease-free PBS and further injected into fertilized C57BL/6 eggs. After culture, about 20-30 blastocysts were transferred into the uterus of pseudo-pregnant ICR females. Genotype identification of *Dnmt3a*^{W293Del} mice was firstly PCR by genotyping primers and followed by *SacI* restriction enzyme digestion for more than 4 hours. Detailed blood phenotypes of this murine model were published (43).

Quantitative PCR (qPCR)

RNA was extracted from whole bone marrow cells of mice using a RNeasy micro kit (Qiagen). 1 μg RNA of each sample was mixed with 1 μl oligo dTs and 1 μl 10mM dNTPs and ddH₂O. RNA mixtures were heated to 65°C for 5 minutes and incubated on ice for one minute. 4 μl 5X First-strand buffer, 1 μl 0.1 M DTT, 1 μl RNase inhibitor and 1 μl SuperscriptIII RT were added to RNA mixture, incubated at 50°C for 60 minutes and inactivated the reaction by heating to 70°C for 15 minutes. 0.5 μl cDNAs, 0.5 μl of 10 μM forward and reverse primers, 3.5 μl ddH₂O and 5 μl 2X SsoAdvanced universal SYBR Green supermix were added to PCR tubes, and quantitative PCR was conducted as the following program. First samples were heat activated with 95°C for 3 minutes, then secondly kept 95°C for 10 seconds, then 55°C for 10 seconds, 72°C for 30 seconds and repeated from the second step for 40 cycles.

Modeling and analysis of clonal hematopoiesis mutations

We looked at studies that included 99% of the stabilizing and destabilizing variants (25,30-32). Deletions were excluded due to uncertainties estimating site-specific mutation rates for deletions. Data was trimmed to exclude variants at VAFs below each study's estimated limit of reliable variant detection (as described (29), Supplemental Methods 1).

To enable comparison between studies, the densities were normalized by dividing by [number of individuals in the study × bin widths] and the densities were then rescaled by dividing by 2μ , where μ is the study-specific haploid mutation rate summed across all the variants covered by the studies. Estimates for the distribution of fitness effects (s) were inferred by fixing $N\tau$ (total number of HSCs × time in years between successive symmetric cell differentiation divisions) to ~100,000 as inferred (29). The distribution of ages was assumed to be Gaussian with mean 60 years and standard deviation 15 years, which is the mean and standard deviation of participants in Coombs et al. (25), which contributed ~85% of the data from these four studies. We parameterized the distribution of fitness effects using a family of stretched exponential distributions with a maximum $s = s_{\max}$. We then performed a maximum likelihood procedure, optimizing the shape (β) and scale (d) of the distribution as well s_{\max} .

$$\rho(s) \sim \exp\left[-\left(\frac{s}{d}\right)^\beta\right]$$

For Coombs et al. (25), we only included the individuals that were both chemotherapy-naïve and radiotherapy-naïve. For Desai et al. (31) and Young et al. (32), we only included the ‘control’ participants. For the studies that reported replicate VAF measurements (Young et al. (30,32)), we required a variant to be detected in both replicate samples to be called and the average of the replicate values was taken as the VAF at that time-point. For studies that reported variants in participants from more than one timepoint (Young et al. (30,32) and Desai et al. (31)), we only included variants detected in the first blood sample for these studies.

RNA Sequencing and Analysis

RNA was extracted using a RNeasy micro kit (Qiagen) and quantified using Nanodrop. Truseq stranded mRNA library preparation was based on the manufacturer’s instruction (Illumina). Libraries were sequenced using Nextseq 500 sequencer. Paired end RNA-seq reads were mapped to the human genome (hg19) using TopHat 2.0.10. The Fragments Per Kilobase of exon per Million fragments mapped (FPKM) values were calculated by cufflinks 2.2.1. To examine whether proteasome inhibitors can restore transcriptome in *DNMT3A*^{W297Del/+} LCLs, we selected the genes which matched with the criteria as follows: at least 5 CpGs that were detected in the promotor of this particular gene and this gene has detectable expression in RNAseq. Within these gene (11,401 genes), upregulated genes (fold-change > 2, n=1208) and down-regulated genes (fold-change < -2, n=508) in *DNMT3A*^{W297Del/+} vs. *DNMT3A*^{+/+} sample were then identified. For visualization, we utilized unsupervised consensus clustering and separated genes into two clusters (Cluster 1 and 2) within upregulated genes. We used the DAVID Functional Annotation Tools (<https://david.ncifcrf.gov/tools.jsp>) to perform the gene function enrichment analysis in cluster 1 and 2 gene sets, and use all the detected genes as the background.

Whole Genome Bisulfite Sequencing (WGBS) and Analysis

100 ng DNA was used for WGBS libraries preparation using a TruSeq DNA methylation kit as the manufacturer’s instruction (Illumina). Libraries were sequencing using Nextseq 500 sequencer. For each WGBS profile, we used BSMAP to trim adaptor and low-quality sequence with default threshold, aligned bisulfite-treated reads to the human genome (hg19). Then the methylation ratio of each CpG covered with at least five reads was calculated by the module bsratio in BSMAP. Twenty-five chromatin states were defined using 12 epigenetic marks, including H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K27ac, H4K20me1, H3K79me2, H3K36me3, H3K9me3, H3K27me3, H2A.Z, and DNase, across all 127 reference epigenomes. All sequencing data are accessible at NCBI GEO database, accession GSE178798.

CRISPR – DNMT3A stability screen

The human BISON CRISPR-KO library, which contains 2852 guide RNAs, targets 713 E1, E2, E3, deubiquitinases, and control genes (36). It was cloned into the pXPR003 as

previously described (44) by the genome perturbation platform (GPP, Broad Institute). The lentivirus particles for the library were produced in a T-175 flask format. Briefly, 18×10^6 HEK293T cells were seeded in 25 mL DMEM medium supplemented with 10% FBS and Penicillin-Streptomycin-Glutamine. The next day a Packaging Mix was prepared: 40 μ g psPAX2, 4 μ g pVSV-G, and 32 μ g of the library in 1 ml OptiMem (Invitrogen) and incubated for 5 minutes at room temperature. This mix was combined with 244 μ L TransIT-LT1 (Mirus) in 5 mL of OptiMem, incubated for 30 minutes at room temperature, and then applied to cells. Two days post-transfection, cell debris was removed by centrifugation. The lentivirus particles containing medium were collected and stored at -80°C before use.

HEK293T cells were engineered with constitutively expressing Cas9 and bicistronic DNMT3A^{W297Del} reporter. 2×10^6 engineered HEK293T cells were added with 10% (v/v) of the human BISON CRISPR-KO in 2 mL of medium and spin-infected (2400 rpm, 2 hours, 37°C). 24h post-infection sgRNA infected cells were selected with 2 μ g/ml of Puromycin for two days. On the ninth day post-infection, populations were separated using fluorescence-activated cell sorting. Two populations were collected (top 5% and lowest 5%) based on the eGFP^{DNMT3A} to mCherry MFI ratio. Sorted cells were harvested by centrifugation and subjected to direct lysis buffer reactions (1 mM CaCl₂, 3 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, Tris pH 7.5 - with freshly supplemented 0.2 mg/mL Proteinase). The sgRNA sequence was amplified in a first PCR reaction with eight staggered forward primers. 20 μ L of direct lysed cells were mixed with 0.04U Titanium Taq (Takara Bio 639210), 0.5 x Titanium Taq buffer, 800 μ M dNTP mix, 200 nM P5-SBS3 forward primer, 200 nM SBS12-pXPR003 reverse primer in a 50 μ L reaction. The samples were heat activated with 94°C for 5 minutes, then secondly kept 94°C for 30 seconds, then 58°C for 10 seconds, 72°C for 30 seconds and repeated from the second step for 15 cycles, and lastly heated at 72°C for 2 minutes. 2 μ L of the primary PCR product was used as the template for 15 cycles of the secondary PCR, where Illumina adapters and barcodes were added (0.04U Titanium Taq (Takara Bio 639210), 1 x Titanium Taq buffer, 800 μ M dNTP mix, 200 nM SBS3-Stagger-pXPR003 forward primer, 200 nM P7-barcode-SBS12 reverse primer). An equal amount of all samples was pooled and subjected to preparative agarose electrophoresis followed by gel purification (Qiagen). Eluted DNA was further purified by NaOAc and Isopropanol precipitation. Amplified sgRNAs were quantified using the Illumina NextSeq platform. Read counts for all guides targeting the same gene were used to generate p-values. The data analysis pipeline comprised the following steps: (1) Each sample was normalized to the total read number. (2) For each guide, the ratio of reads in the stable vs. unstable sorted gate was calculated, and guide RNAs were ranked. (3) The ranks for each guide were summed for all replicates. (4) The gene rank was determined as the median rank of the four guides targeting it. (5) P-values were calculated, simulating a corresponding distribution over 100 iterations.

Immunoprecipitation

Cells from two 15 cm plates each condition were harvested in 8 ml IP lysis buffer with protease/phosphatase inhibitor. Cell lysate then was sonicated and centrifuged with the highest speed at 4°C . Pre-cleared cell lysate with empty protein A Dynabeads and determined protein concentration using a BCA kit (Thermo Scientific). Protein A Dynabeads

was washed twice in IP lysis buffer and bound with anti-DCAF8 antibody (A301-556A, Bethyl lab) at 4°C for one hour. Protein A Dynabeads with 4µg anti-DCAF8 antibody in 2mg cell lysate were then incubated at 4°C overnight. Wash the beads three times with 0.5 ml of the IP lysis buffer. Eluted with 40 µL of freshly prepared 1X sample buffer by heating at 90°C for 10 min and conducted western blotting.

Western blotting

Whole bone marrow cells from different genotypes of mice, lymphoblastoid cell lines, and human cell lines were lysed using a cybuster protein extraction buffer (Millipore) with protease inhibitor cocktail (GenDEPOT). Standard immunoblotting analysis was performed using anti-GFP (1:1000, Novus, NB600-308), anti-DNMT3A antibody (1:1000, Santa Cruz, H-295; 1:1000, Abcam, ab16704), anti-DCAF8 antibody (1:500, Sigma-Aldrich, HPA027381), anti-DDB1 antibody (1:1000, Bethyl lab, A300-462A) and anti-GAPDH antibody (1:2000, Millipore, MAB374).

Ubiquitination assay

For detection of ubiquitination, 2×10^7 cells were harvest and lysed with 1 mL lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, with protease inhibitor) after 10 hr of MG132 treatment. Cell lysate was boiled for 10 min and sonicated. 9 mL renaturing buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton) was then added and incubated for 1 hr at 4°C. Clear cell lysate was obtained by centrifugation with the highest speed at 4°C. Protein A Dynabeads was washed twice in PBST and bound with 6µg anti-GFP antibody (NB600-308, Novus) at room temperature for 30 min. Protein A Dynabeads with anti-GFP antibody were then incubated with pre-cleared cell lysate at 4°C overnight. Beads were washed three times with 1 ml PBST, and protein eluted with 40 µL of freshly prepared 1X sample buffer by heating at 95°C for 10 min. Western blotting was performed using anti-ubiquitination antibody (3936S, Cell Signaling).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

DNMT3A has emerged as the most important epigenetic regulator and tumor suppressor in the hematopoietic system. Our study represents a systematic and high-throughput method to characterize the molecular impact of *DNMT3A* missense mutations, and the discovery of a regulated destruction mechanism of DNMT3A offering new prognostic and future therapeutic avenues.

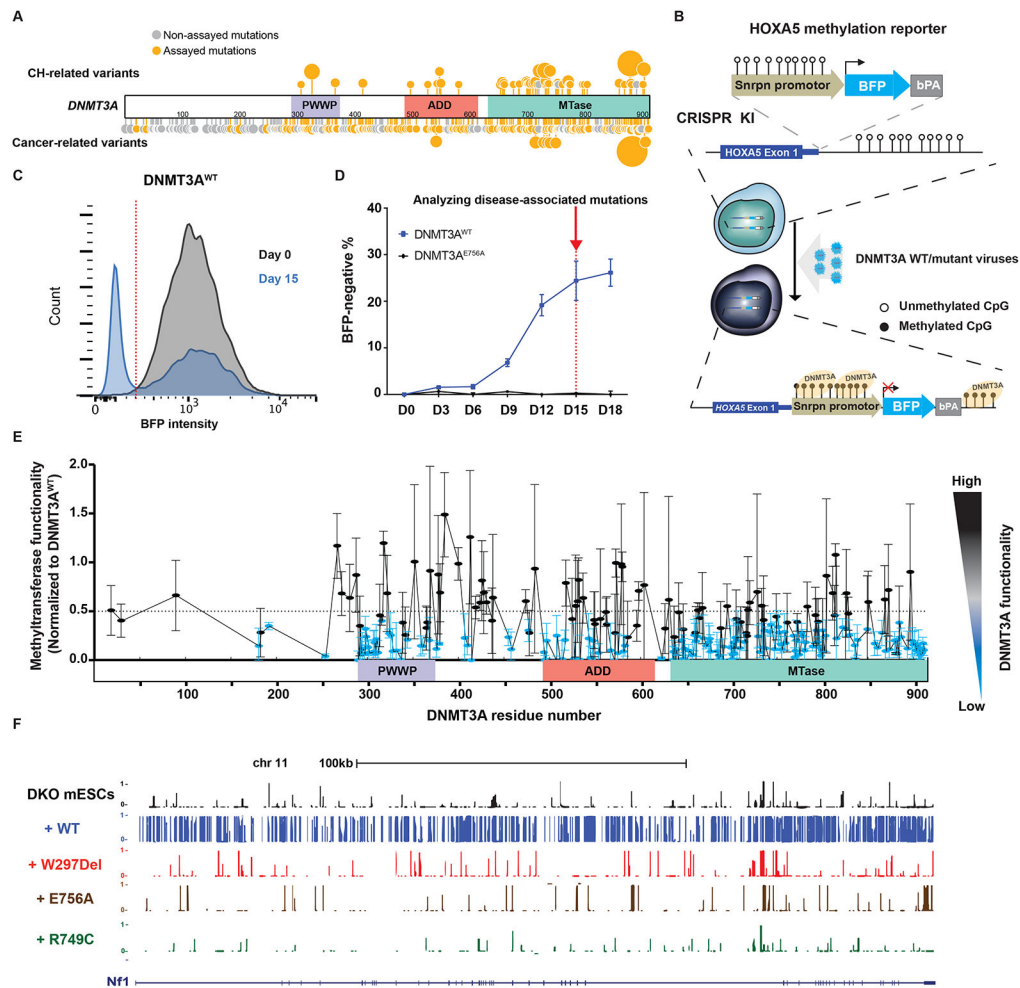


Figure 1: Methyltransferase activity analysis in 253 disease-associated *DNMT3A* missense mutations using the *HOXA5-Snrpn*-BFP methylation reporter. (A) Schematic of missense mutations across *DNMT3A* in cancer, CH, and TBRS. Sites marked in orange represent tested mutations (Supplemental Table 1); the larger circles indicate recurrent mutations. Sites in grey represent mutations seen in the databases but not assayed here. (B) Schematic of the methyltransferase activity assay using *HOXA5-Snrpn*-BFP methylation reporter. The *Snrpn* promoter region was linked to BFP and a bovine polyA signal and knocked in to the *HOXA5* locus in reverse orientation in HEK293T cells. These cells have high BFP fluorescence as shown in (C) in grey. When WT *DNMT3A* is introduced using lentiviral transduction, the methylation-sensitive *HOXA5* regions promotes DNA methylation, which leads to suppression of BFP expression (blue plot in (C)). (C) Methyltransferase activity assay. The graph depicts blue fluorescence intensity in the *DNMT3A*^{WT}-transduced cells as measured by flow cytometry on day 0 (grey) and day 15 (blue). (D) The panel represents the percent of BFP-negative cells after transduction with *DNMT3A*^{WT} or *DNMT3A*^{E756A} constructs measured by flow cytometry over 18 days. (E) Methyltransferase activity assay of 253 disease-associated *DNMT3A* missense mutations measured by flow cytometry. Lentiviral particles of each *DNMT3A* variant were infected in three biological replicates in *HOXA5-Snrpn*-BFP reporter cells. Activity

was normalized to the percent BFP-negative % cells in DNMT3A^{WT}-transduced cells 15 days later. To visualize results, we defined and colored mutations based on their levels of methyltransferase activity. Methyltransferase activity of those mutations labeled in were classified as having impaired methyltransferase activity (activity < 0.5), while those in black was considered to be in the range of WT DNMT3A (activity > 0.5). Data are presented as mean \pm s.e.m. (F) DNA methylation profile (WGBS) of cells transduced with the indicated DNMT3A mutants displayed on the UCSC genome browser. See also supplemental Figures 1-3.

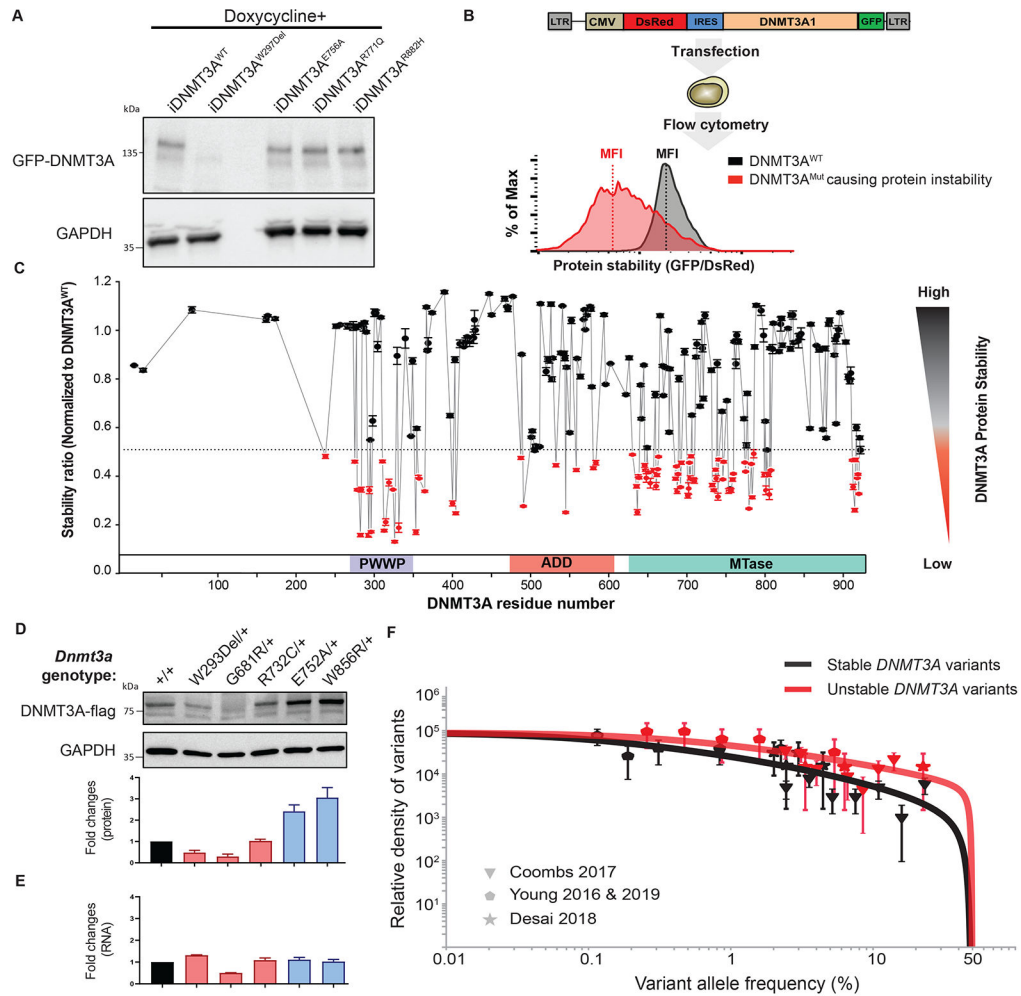


Figure 2: More than a third of missense mutations across *DNMT3A* decrease protein stability. (A) Western blot analysis of GFP-DNMT3A fusion protein and GAPDH control expression measured in DKO ESCs after doxycycline induction. (B) Schematic of protein stability assay using the DsRed-IRES-DNMT3A-GFP bicistronic vector. The vector produces a DNMT3A-GFP fusion protein, such that the level of GFP fluorescence allows inference of the amount of DNMT3A protein present. The fusion protein transcript is downstream of DsRed, following an internal ribosome entry site (IRES) so that DsRed serves as an internal control for differences in transcription efficiencies of the constructs. The construct is transfected into the HEK293T cells and 48 hours later, green and red fluorescence is measured by flow cytometry (right panel). The ratio of the mean fluorescence intensity (MFI) of the green and red fluorescence is used to indicate the level of GFP-tagged DNMTA protein in the cells. The fluorescence ratios in cells transfected with DNMT3A-mutant constructs is normalized to that of the DNMT3A-WT-expressing constructs. (C) The graph depicts protein stability of 253 *DNMT3A* missense mutations measured as described in (B). To visualize results, we defined and colored mutations based on their levels of severity of impaired protein stability. Mutations marked in red indicate those with severely impaired protein stability (Stability ratio < 0.5), whereas those in black was considered to be comparable to DNMT3A^{WT} (Stability ratio > 0.5). The mutation labeled in red is one

identified above as unstable. Data are presented as mean \pm s.e.m. (D) Western blotting analysis and quantification of heterozygous mESC unstable mutants (DNMT3A^{W293Del}, DNMT3A^{G681R}, DNMT3A^{R732C}) and stable mutants (DNMT3A^{E752A} DNMT3A^{W856R}) normalized to GAPDH. (E) Quantification of RNA expression levels in mESC mutants described in (D) normalized to GAPDH. (F) The distribution of variant allele frequencies of stable (grey data points) and unstable (red data points) variants plotted after controlling for study size, panel size and mutation rate compared to maximum-likelihood VAF distributions (red and grey lines) that assume a parameterized form for the distribution of fitness effects (29).

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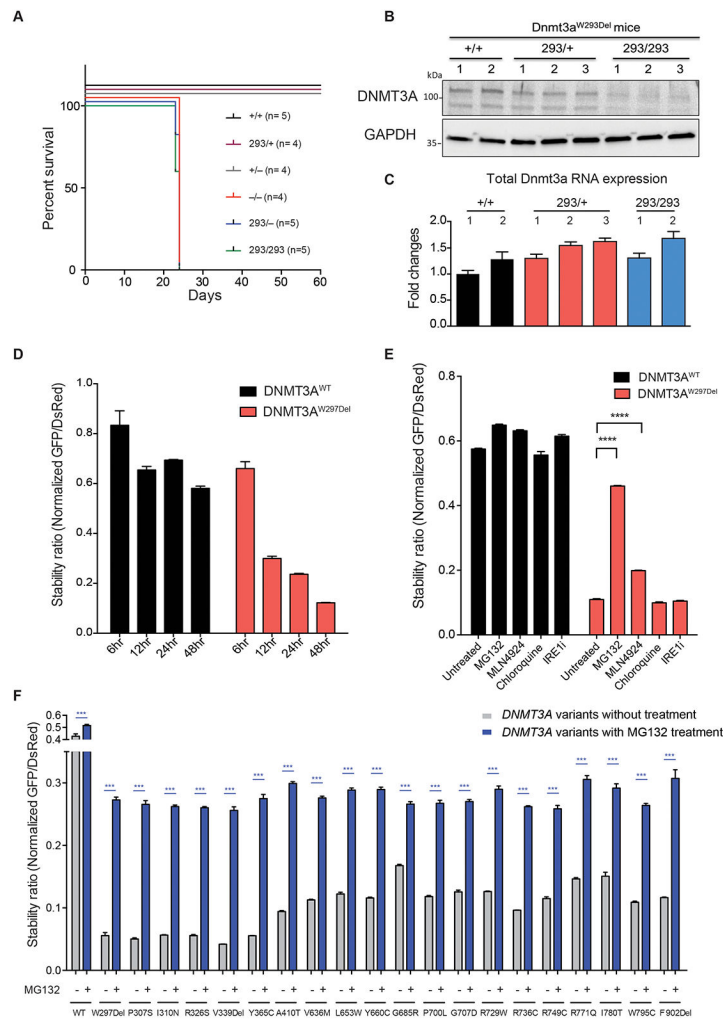


Figure 3: DNMT3A is degraded through the ubiquitin-proteasome system.

(A) Survival after the birth of mice with the indicated genotypes. No *Dnmt3a*^{-/-}, *Dnmt3a*^{W293Del/-}, or *Dnmt3a*^{W293Del/W293Del} mice survive beyond postnatal day 24. (B) Western blot of DNMT3A and GAPDH protein expression in whole bone marrow cells of mice. (C) Gene expression analysis of *Dnmt3a* in whole bone marrow measured by quantitative PCR and normalized for *Gapdh* mRNA expression. (D) The stability ratio of the WT and W297Del mutant over the indicated times, calculated as described in Figure 2C. (E) Alterations in DNMT3A protein stability after administration of proteasome inhibitor (MG132), a Cullin-RING E3 ubiquitin ligase inhibitor (MLN4924), an autophagy inhibitor (chloroquine) and an unfolded protein response inhibitor (IREi). (F) Stability ratio of mean fluorescence intensity (MFI) of DNMT3A-GFP versus MFI of DsRed before and after treatment with the proteasome inhibitor (MG132) as measured by flow cytometry 48 hours after transfection. Data are presented as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ using the unpaired t test. See also supplemental Figure 7.

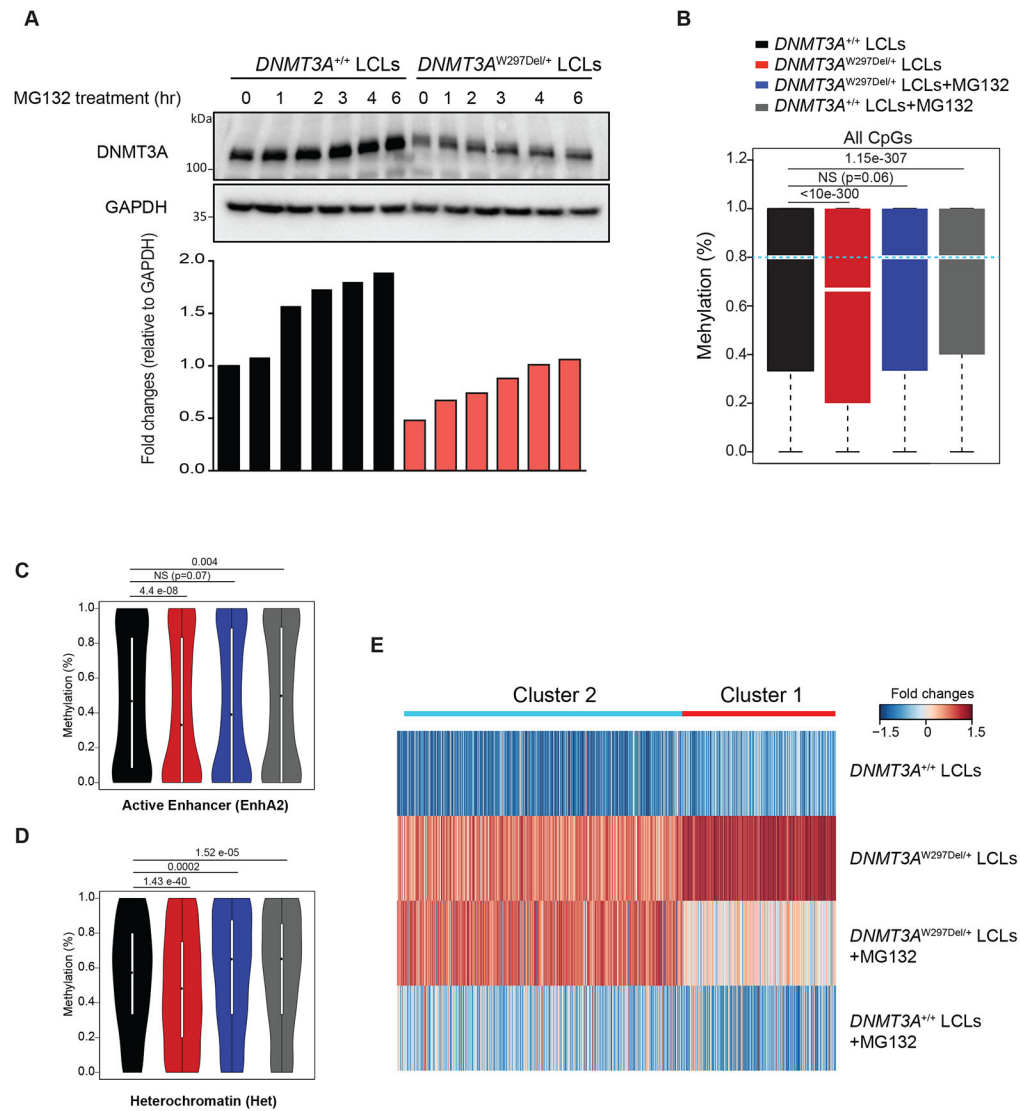


Figure 4: Proteasome inhibitors partially restore the methylome and transcriptome in patient-derived cells.

(A) Western blotting and fold-change quantification of DNMT3A and GAPDH protein expression in LCL cells from WT or a TBRS (*W297del*) patient. DNMT3A was normalized to GAPDH. (B) Boxplots showing DNA methylation distribution analyzed by whole-genome bisulfite sequencing in the indicated LCL cells with and without proteasome inhibition through MG132 treatment. The bars represent methylation ratios between the 1st and 3rd quartiles, with the median distribution, shown by a gap in the bars, of 66.7% (*DNMT3A*^{W297Del/+}) and 80% (all other samples). The whiskers represent methylation in the 1st and 4th quartiles. The statistical values represent the Wilcox rank-sum test. (C) Violin plots of DNA methylation distribution in 12,896 active enhancer regions as defined in the Roadmap Epigenomic Project. (D) Violin plot of DNA methylation ratios in 10,973 heterochromatic regions as defined in the Roadmap Epigenomic Project. (E) Gene expression heatmap of the indicated cells and treatments. Cluster 1 genes were hypomethylated in *DNMT3A*^{W297Del/+} compared to WT LCLs and responsive

to proteasome inhibitors. Cluster 2 genes were hypomethylated in *DNMT3A*^{W297Del/+} compared to WT LCLs but inert to proteasome inhibitors. See also supplemental Figure 8.

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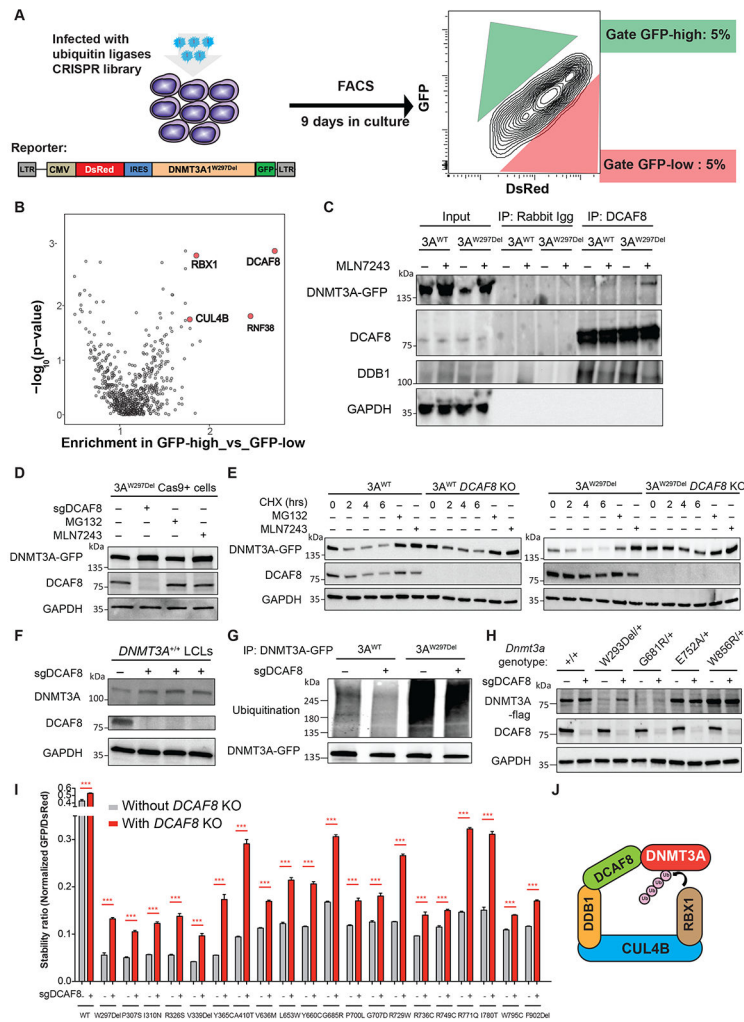


Figure 5: Targeted CRISPR screening identifies CUL4B^{DCAF8} ubiquitin ligase complexes essential for DNMT3A protein degradation.

(A) Schematic of targeted CRISPR screening to identify ubiquitin ligases essential for DNMT3A protein degradation. HEK293T cells were engineered to constitutively overexpress Cas9 and the indicated bicistronic DNMT3A^{W297Del} reporter and then infected with sgRNA libraries targeting ubiquitin ligases. Nine days after infection, we sorted both the top and bottom 5% of cells for DNMT3A-GFP expression. (B) The graph depicts the gene enrichment score and p-value in targeted CRISPR screening for ubiquitin ligases. DCAF8, RBX1, and CUL4B genes (red) were enriched and statistically significant. (C) The image depicts DCAF8 immunoprecipitation in DNMT3A-WT or W297del expressing cells after incubation for 6 hours with an inhibitor of E1 ubiquitin-activating enzymes (MLN7243), followed by blotting using GFP, DCAF8, DDB1, and GAPDH antibodies. (D) DNMT3A^{W297del} expressing HEK293T cells in which treating with sgRNA targeting DCAF8, proteasome inhibitor and E1 ubiquitin-activating enzyme, followed by western blot analysis for DNMT3A-GFP, DCAF8, and GAPDH. (E) Cycloheximide (CHX) treatment with 0, 2, 4, 6 hours of DNMT3A^{WT} and DNMT3A^{W297Del} HEK293T cells with or without DCAF8-KO followed by western blot for DNMT3A-GFP, DCAF8, and GAPDH.

Inhibitor treatments of proteasome and E1 ubiquitin-activating enzyme in DNMT3A^{WT} and DNMT3A^{W297Del} HEK293 cells serve as rescue control of experiment. (F) Western blot analysis of DNMT3A, DCAF8 and GAPDH in DCAF8-KO DNMT3A^{WT} LCLs. (G) The image depicts the levels of ubiquitination in DNMT3A^{WT} and DNMT3A^{W297del} HEK293T cells with or without DCAF8-KO. DNMT3A-GFP was pull-down by a GFP antibody and followed by western blot analysis using ubiquitin and GFP antibody. (H) Western blotting analysis of heterozygous mESC unstable mutants (DNMT3A^{W293Del} and DNMT3A^{G681R}) and stable mutants (DNMT3A^{E752A} DNMT3A^{W856R}) with or without *Dcaf8*-KO. (I) Stability of variants before and after DCAF8-KO measured as described in Figure 2C. (J) Schematic of the RBX1-CUL4B-DCAF8 complex serving as the ubiquitin ligase for DNMT3A protein. *** P< 0.001 using the unpaired t test.