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Disruption of DNA methylation and mitochondrial dysfunction in pre-leukemia

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DECLARATION

I, **Asrat E. Kahsay**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Hematopotiec stem cells (HSC) produces mature blood cells on demand and self-renew their own population in the bone marrow which is tightly regulated through metabolic and epigenetic remodeling such as DNA methylation. DNMT3A is family of methyltransferases that catalyzes de novo DNA methylation of cytosine and conversely oxidized by TET2 (TET Methylcytosine Dioxygenase 2) to hydroxymethylcytosine and modulate HSC differentiation. Mutations in DNMT3A and TET2 have been shown to promote self-renewal of HSC in animal models and considered as founding mutations in majority of acute myeloid leukemia (AML). As crucial epigenetic signaling hubs in HSCs mitochondria provide metabolites serving as cofactors and substrates for enzymes with epigenetic modification activity such as DNA methylation. Despite having antagonistic biochemical activities it is unclear how loss of function in DNMT3A and TET2 lead to HSC differentiation defects. Thus, we propose that mitochondrial dysfunction might be at the center of disease initiation in pre-leukemia. Therefore, the goal of this study was to assess mitochondrial dysfunction in DNMT3A and TET2 mutations through functional and molecular profiling of mitochondria phenotypes. Using shRNA mediated stable knock-down of DNMT3A and TET2 in HEK293T cells, we demonstrated that deficiency of DNMT3A increased mitochondrial biogenesis. Specifically, loss of DNMT3A significantly enhanced average and total mitochondrial volume in parallel with increased TFAM and mito-chondrial DNA. In addition, by utilizing aequorin and mito-GCaMP6m indicators our study showed previously unreported disruption of mitochondrial calcium uptake due to inactivation of TET2 and DNMT3A. Our mechanistic investigation reveals that shRNA loss of TET2 re-duced mitochondrial and cytosolic calcium as a result of defects in ER calcium release due to down regulation of IP3R3. In conclusion, our data not only demonstrated the critical function of TET2 but also identified novel targets that might contribute to calcium homeostasis in Hem-atopotiec and leukemic stem cell function.

RIASSUNTO

Le cellule staminali ematopotiche (HSC) producono cellule del sangue mature su richiesta e si auto-rinnovano la propria popolazione nel midollo osseo che è strettamente regolata attraverso il rimodellamento metabolico ed epigenetico come la metilazione del DNA. DNMT3A è una famiglia di metiltransferasi che catalizza la metilazione de novo del DNA della citosina e viceversa ossidata da TET2 (TET metilcitosina diossigenasi 2) a idrossimetilcitosina e modula la differenziazione delle HSC. È stato dimostrato che le mutazioni in DNMT3A e TET2 promuovono l'auto-rinnovamento dell'HSC in modelli animali e sono considerate mutazioni fondanti nella maggior parte della leucemia mieloide acuta (LMA). In quanto hub di segnalazione epigenetica cruciale nei mitocondri delle HSC, i mitocondri forniscono metaboliti che fungono da cofattori e substrati per enzimi con attività di modificazione epigenetica come la metilazione del DNA. Nonostante abbia attività biochimiche antagoniste, non è chiaro come la perdita di funzione in DNMT3A e TET2 porti a difetti di differenziazione HSC. Pertanto, proponiamo che la disfunzione mitocondriale potrebbe essere al centro dell'inizio della malattia nella pre-leucemia. Pertanto, l'obiettivo di questo studio era valutare la disfunzione mitocondriale nelle mutazioni DNMT3A e TET2 attraverso la profilazione funzionale e molecolare dei fenotipi mitocondriali. Usando il knockdown stabile mediato da shRNA di DNMT3A e TET2 nelle cellule HEK293T, abbiamo dimostrato che la carenza di DNMT3A ha aumentato la biogenesi mitocondriale. In particolare, la perdita di DNMT3A ha migliorato significativamente il volume mitocondriale medio e totale in parallelo con l'aumento del TFAM e del DNA mitocondriale. Inoltre, utilizzando gli indicatori ae-quorin e mito-GCaMP6m, il nostro studio ha mostrato un'interruzione precedentemente non segnalata dell'assorbimento di calcio mitocondriale a causa dell'inattivazione di TET2 e DNMT3A. La nostra indagine meccanicistica rivela che la perdita di shRNA di TET2 ha ridotto il calcio mitocondriale e citosolico a causa di difetti nel rilascio di calcio ER a causa della sottoregolazione di IP3R3. In conclusione, i nostri dati non solo hanno dimostrato la funzione critica di TET2, ma hanno anche identificato nuovi bersagli che potrebbero contribuire all'omeostasi del calcio in Hematopotiec e alla funzione delle cellule staminali leucemiche.

PUBLICATIONS

1. Bonora M, Patergnani S, Ramaccini D, Morciano G, Pedriali G, **Kahsay AE**, Bouhamida E, Giorgi C, Wieckowski MR, Pinton P (2020) Physiopathology of the Permeability Transition Pore: Molecular Mechanisms in Human Pathology. Biomolecules 10:998
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3. Morciano G, Rimessi A, Patergnani S, Vitto V.A.M, Danese A, **Kahsay A.E**, Palumbo L, Bonora M, Wieckowski M.R., Giorgi C, Pinton P (2022) Calcium dysregulation in heart diseases: Targeting calcium channels to achieve a correct calcium homeostasis. Pharmacol Res 2022 Feb 4;106119
4. Bouhamida E, Morciano G, Perrone M, **Kahsay A.E**, Della Sala M, Wieckowski M R, Fiorica F, Pinton P, Giorgi C, Patergnani S (2022) The Interplay of Hypoxia Signaling on Mitochondrial Dysfunction and Inflammation in Cardiovascular Diseases and Cancer: From Molecular Mechanisms to Therapeutic Approaches. Biology 11(2):300

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LIST OF ABBREVIATIONS

ALL:	Acute lymphoblastic leukemia
AML:	Acute Myeloid Leukemia
AMPK:	AMP-activated protein kinase
AraC:	Cytarabine
CHIP:	Clonal hematopoiesis of indeterminate potential
CMML:	Chronic myelomonocytic leukemia
CMP:	Common myeloid progenitors
CPT1A:	Carnitine palmitoyltransferase 1A
DAC:	5-aza-2′-deoxycytidine
DNMTs:	DNA Methyltransferase
DNR:	Daunorubicin
Drp1:	Dynamin-related protein 1
ETC:	Electron transport chain
FAO:	Fatty acid oxidation
FLT3-ITD:	FMS-like tyrosine kinase 3-internal tandem duplication
HSCs:	Hematopoietic stem cells
IDH2:	Isocitrate dehydrogenase-2
iPSs :	Induced pluripotent stem cells
LICs:	Leukemia-initiating cells
LT-HSCs:	Long-Term HSCs
MCU:	Mitochondrial Calcium Uniporter
MDS:	Myelodysplastic syndrome
MEP:	Megakaryocyte/ erythroid progenitors
MPPs:	Multipotent progenitors
MTCH2:	mitochondrial carrier homolog 2
mtDNA :	Mitochondrial DNA
mTOR:	Mammalian target of rapamycin

OXPHOS:	Oxidative phosphorylation
PDK:	Pyruvate kinase
PI:	Propidium Iodide
PMCA:	plasma membrane Ca^{2+} - ATPase
POLG:	DNA polymerase gamma
PTCL:	Peripheral T cell lymphoma
ROS:	Reactive oxygen species
SAM:	S-adenyl methionine
SDF-1:	Stromal cell-derived factor 1
SG:	SYTOX green
SOCE:	Store-operated calcium entry
ST-HSCs:	Short-Term HSCs
STIM1:	Stromal Interaction Molecule 1
TCA:	Tricarboxylic acid
TET2:	TET Methylcytosine Dioxygenase 2
ΔΨm:	Mitochondrial membrane potential
[Ca2+] c:	Cytosolic Ca^{2+} concentration
$[Ca^{2+}]$er:	Endoplasmic reticulum Ca^{2+} concentration
$[Ca^{2+}]$m:	Mitochondrial Ca^{2+} concentration
5hmC:	5-hydroxymethylcytosine

1. INTRODUCTION

1.1. Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are the source of all cell types found in peripheral blood as well as some of the cells found in all body tissues [1]. Hematopoiesis (hemo: blood; poiesis: creation), which predominately takes place in the bone marrow, is a dynamic process that requires close coordination of several cell-fate decisions in order to support the lifelong production of blood[2]. Hematopoietic stem cells, which are a rare population with multipotent (differentiate) and self-renewal qualities[3]. Asymmetrical and symmetrical divisions of the individual HSC are both possible during this process. The expansion of stem cells in the bone marrow is caused by the former, which produces two daughter HSCs. On the other hand, asymmetrical division produces two HSCs: one that is identical to the parental HSC and one that is a more developed progenitor that can develop into mature blood cells[4]. HSCs gradually lose their capacity for multi-lineage division and proliferation while gradually acquiring differentiated cell properties during differentiation. For such the HSC compartment can be divided into two groups: Short-Term HSCs (ST-HSCs), also known as progenitors, and Long-Term HSCs (LT-HSCs), which are determined by their ability to repopulate after twelve weeks of transplantation[5].

1.2. Dynamic regulation of HSC Hematopoiesis

Two fundamental characteristics are used to define HSCs, the capacity for self-renewal and the capacity to differentiate into multi lineage committed progenitor cells[6]. In order to maintain an adequate supply of primitive cells for lifelong hematopoiesis, intrinsic and extrinsic factors that integrate and respond to constantly changing physiological demands of the body precisely regulate the delicate balance between self-renewal and differentiation[7].

1.2.1. Extrinsic regulation of HSC Hematopoiesis

Extrinsic factors are soluble molecules such as cytokines and growth factors which are provided by the bone marrow microenvironment, serves as both a nourishing and restraining home[8]. In the bone marrow microenvironment in order to regulate HSCs the hypoxia-related transcriptional, metabolic, and sympathetic nervous system programs must be continuously activated [9]. Other cell types found in the bone marrow niche include mesenchymal stem cells (MSCs), osteoprogenitors, osteoblasts, osteocytes, and chondrocytes, work together with HSCs physically and contribute to their maintenance [8]. In sum, these extrinsic signals are essential for preserving a functional HSC pool and hematopoiesis.

1.2.2. Intrinsic regulation of HSCs

Intrinsic factors contain epigenetic and lineage-specific transcriptional regulators. This procedure is essential for controlling HSC maintenance, lineage commitment, and differentiation during hematopoiesis[10]. The molecular wiring within adult HSCs that mediates their continued maintenance in a quiescent, or G0, state while permitting their quick entry into the cell cycle in response to hematopoietic demand is an active area of research. Studies have shown , the retinoblastoma (Rb) family of transcriptional repressors, which consists of the pRb, p107, and p130 proteins, limits cell cycle entry by suppressing the transcription of the E2F gene of positive cell cycle regulators, such as E-type cyclins. Studies have also shown that conditional knockdown of all three Rb family members in adult mice caused a strong cell-intrinsic myeloproliferation phenotype resulted the animals to die 1-3 months after the gene was inactivated. This was associated with rise both HSC proliferation and cell numbers and severely impaired in HSC self-renewal reconstitution[7]. Similar to the Rb gene family, mice lacking just one D-cyclin, or just one of the two associated Cdks, exhibit only slight hematopoietic defects, demonstrating the substantial functional redundancy defending this complex. When it comes to regulating cell cycle initiation and progression, cyclins and Cdks work upstream of the Rb family members. Additionally, members of the Rb family cannot be phosphorylated, preventing their entry into the S phase, due to the antagonistic effects of the Ink4 family on the cyclin D-Cdk4/6 complex.

According to a number of data, the Ink4 family members are differentially regulated in adult HSCs to maintain the appropriate ratio between quiescence and proliferation [11].

Furthermore, it has also been shown that during lineage specification of HSCs certain chromatin modifications permit transcription of specific lineage-related genes while inactivating the unrelated genes[12]. Histone modifications are a crucial part of post-translational modification. The regulation of transcription, DNA replication, DNA repair, and DNA condensation depends on several histone modifications, the best known of which are histone acetylation and methylation[13].

Histone acetylation primarily refers to active transcription whereas histone methylation is a type of complex that depends on the particular methylated sites to regulate transcriptional states[14]. Histone methyltransferases are enzymes that control the reversible process of histone methylation. While histone 3 lysine 9 (H3K9) and 27 (H3K27) take place in transcriptional repression, methylation of histone H3 lysines 4 (H3K4), 36 (H3K36), and 79 (H3K79) are involved in transcriptional activation [15]. Polycomb group (PcG) proteins are epigenetic modifiers that selectively repress gene expression by forming multi-subunit complexes known as polycomb repressive complexes (PRCs) and mediating H3K27 methylation. The PRCs essentially come in two main forms, PRC1 and PRC2, and both of them cause covalent post-translational modifications of histones[16]. One of the most thoroughly studied PRC1 proteins is the B lymphoma Mo-MLV insertion region 1 homolog (BMI1), whose expression is known to be crucial for HSC's capacity for long-term self-renewal and repopulation[17]. On the other hand, it has been demonstrated that Enhancer of Zeste Homolog 2 (EZH2), a member of the PRC2 complex's catalytic component and involved in the di-/trimethylation of H3K27, stabilizes the chromatin structure and preserves the long-term self-renewal potential of HSCs by turning off pro-differentiation genes and enhancing a proliferation by up regulating the expression of genes that promote the cell cycle [18,19,20]. Additionally, the histone methyl transferases Mixed Lineage Leukemia 1 (MLL1), which adds mono-, di-, and trimethylation to H3K4, and disruptor of telomeric silencing 1-like (DOT1L), which catalyzes the mono-, di-, and trimethylation of H3K79 have been linked to transcriptional activation, which is crucial for the preservation of hematopoietic stem and progenitor cells [21,22,23].

Histone acetylation, which involves the transfer of one acetyl group by histone acetyl transferases (HATs) to lysine residues of histone proteins, is also a widely studied histone modification linked to transcriptional activation [24].

HATs that are crucial for hematopoiesis as well as the maintenance and differentiation of different stem cell types. The maintenance of hematopoietic stem cells is largely dependent on the monocytic leukemia zinc finger protein (MOZ). The number of HSPCs and B cell development in mice are both hampered by MOZ deficiency[25]. Along with histone alterations, DNA methylation is the other epigenetic modification that affects how genes are expressed. DNA methylation is a dynamic process that generates cell-type-specific, temporal, spatial, and heritable epigenetic marks [26]. By inducing transcriptional activators or repressors, which produce active or silent chromatin, respectively, DNA methylation modifies gene transcription[27]. Highly methylated genes are those that are necessary for keeping HSCs or progenitors in their undifferentiated state[28]. These genes shift toward lower levels of methylation pattern as progenitors prepare for lineage differentiation[29]. Thus, when HSCs differentiate into myeloid or lymphoid cells, DNA methylation levels are strictly controlled, and during hematopoietic differentiation, a specific cell type's DNA methylation pattern is present only in that cell type[30].

Taken together, intrinsic factors control HSC maintenance by regulating cell cycle entry and epigenetic modifications to permit transcription of specific lineage-related genes while inactivating the unrelated genes.

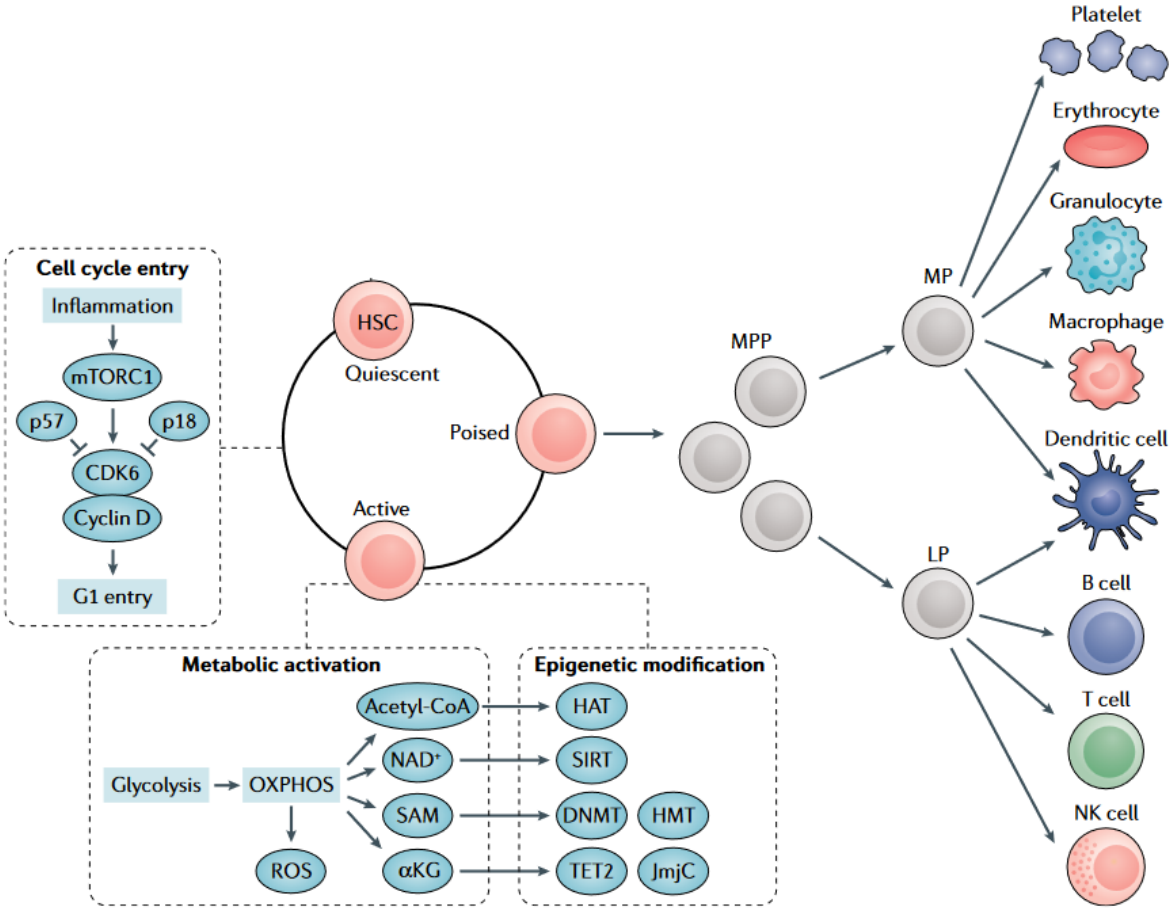

Fig 1. Dynamic regulation of HSC activity. In the steady state, the majority of hematopoietic stem cells (HSCs) are maintained in a quiescent, G0 state. Quiescent HSCs is reversibly regulated by cyclin D–CDK6 complex at cell cycle which is affected by CDK6 inhibitors such as p57 and p18 and activated through mTORC1 activity and enter the cell cycle. Proliferation of HSC requires activate mitochondrial oxidative phosphorylation (OXPHOS) while dormant HSCs rely on glycolysis for metabolic fuel. This metabolic change leads to enhanced the amounts of tricarboxylic acid (TCA) cycle products, including acetyl-CoA, nicotinamide adenine dinucleotide (NAD+), S-adenosylmethionine (SAM) and α-ketoglutarate (αKG), which are important key epigenetic modifiers, like histone acetyl transferase (HAT), sirtuins (SIRT), DNA methyltransferases (DNMT), histone methyltransferases (HMT), ten–eleven translocation 2 (TET2) and jumonji C domain-containing histone lysine demethylases (JmjC). Epigenetic alterations regulate HSC fate decisions, such as self-renewal versus differentiation, via modulation of main transcription factor activity. HSCs are strongly protected from programmed cell death mechanisms, such as apoptosis and necroptosis, through the increased stimulation of pro-survival BCL-2 genes and the TNF–NF-κB–p65–cIAP2 axis. The deactivation of cell cycle and switching their energy demands to glycolysis to some extent through autophagy mediated mitochondrial clearance HSCs return to the G0 state. LP, lymphoid progenitor; MP, myeloid progenitor; MPP, multipotent progenitor; NK cell, natural killer cell; ROS, reactive oxygen species. (Figure adopted from Nature Reviews Cancer 2020[31])

1.3. Dysregulation of HSC and leukemia Development

Pre-leukemia and leukemia states are both a part of the multi-step leukemogenesis process. Understanding the cellular and molecular mechanism of leukemogenesis begins with identifying and characterizing a leukemia initiating cell[32]. It has been suggested that leukemia might originate from long-lived HSCs because it would take time for multiple genetic and epigenetic alterations to accumulate in order for a fully transformed leukemia phenotype.

1.3.1. Pre-leukemia

HSCs live in a specialized niche that offers favorable conditions for preserving quiescence and stemness. However, dormant HSCs are inherently susceptible to mutagenesis caused by incorrect DNA repair and age-related single-base substitutions. In this situation, pre-leukemic HSCs acquire founder mutations that improve their competitive advantage relative to normal HSCs under certain selective pressure due to the BM niche microenvironment.

According to the cancer stem cell (CSC) hypothesis, a tumor develops from a small population of tumor cells that have stem cell properties and the ability to proliferate in a manner similar to clones. Evidence for CSC in AML showed that, in a serial transplantation assay, only a small population of leukemic cells known as leukemia-initiating cells (LICs) could start the disease while the majority of leukemic cells could not [33]. LICs have a high propensity for tumorigenesis and are similar to normal stem cells in terms of their capacity for self-renewal and differentiation. Some have speculated that LICs may be derived from regular stem cells [34]. LICs and typical stem cells share the same regulatory pathways for self-renewal and differentiation (e. g. signaling pathways for Notch, Hedgehog, and Wnt /β catenin). These extrinsic signals, which come from the bone marrow microenvironment, influence the self-renewal and differentiation functions of HSC, ultimately encouraging the formation of LIC[35]. Because LICs exhibit phenotypic heterogeneity among AML patients, they may also develop from committed progenitors that have developed the ability to self-renew[36]. For committed progenitors to be the source of LICs, two models have been put forth. According to the first model, an initiating mutation can happen in HSCs, and subsequent mutations can affect the progenitors downstream, giving rise to the LIC [37].

This idea is supported by a study using a C/EBPα knockdown AML mouse model that showed pre-leukemic HSCs expanded clonally and that leukemia originated from a downstream progenitor[38]. According to the second model, committed progenitors can serve as targets for the initial mutation and can produce LICs on their own. Hence, secondary recipients could contract AML from committed progenitors that had MLL-AF9 overexpressed by retrovirus method[39]. It has long been believed that the majority of malignancies develop before initiating mutations that cause clonal expansion and, consequently, a pre-malignant state. Clonal hematopoiesis of indeterminate potential (CHIP) is the name given to this phenomenon[40]. The genes that frequently underwent recurrent mutations included DNA methyltransferase 3A (DNMT3A), TET2, TP53 (encodes p53), SF3B1, additional sex combs-like 1 (ASXL1) and Janus kinase 2 (JAK2) [41]. Studies show that patients with CHIP had a low risk of developing overt malignancy during the time duration of the study, likely due to the requirement for additional mutations in the relatively small number of cooperating genes. Nevertheless there is currently no evidence to support the clinical necessity of screening asymptomatic patients for CHIP, especially the lack of treatment that could retrain polyclonal hemopoiesis [42]. HSCs obtain primary and secondary mutations that give them a competitive growth advantage during the pre-leukemia phase. Pre-leukemic HSCs can still self-renew and produce differentiated progeny cells[43]. Consequently, blast cells then out-compete regular cells and take control of the entire hematopoietic system, which results in bone marrow failure. Following the acquisition of additional mutations during the leukemic phase, pre-leukemic HSCs change into leukemia initiating cells[44].

1.3.2. Genetic aberrations in leukemia progression

A higher incidence of leukemia is linked to specific germ line mutations, both acquired and genetic, and clonal chromosomal abnormalities. As essential indicators for the diagnosis and prognosis of AML recurrent cytogenetic aberrations have long been recognized which is observed in 50-60% of AML patients and includes aneuploidies, translocations, deletions, insertions, and inversions [45]. Some inversions or translocations produce oncogenic fusion proteins that mess with the control of cell growth or differentiation [46]. A normal karyotype with somatic changes and/or altered gene expression can be found in about 45 percent of AML patients.

RUNX1, FLT3, NPM1, DNMT3A, and other genes with mutation frequencies greater than 5% in AML were identified by a genome-wide analysis. These mutations were grouped according to their biological activities and potential roles in the pathogenesis of AML[47]. Genomic instability and subsequent clonal evolution may be caused by the majority of mutations found in cancer genomes. The AML genome does not frequently exhibit genetic instability, though, as the mutational status does not change as the disease progresses[48,49,50].

1.3.3. Epigenetic aberrations during leukaemic progression

The epigenetic landscape of several cancer cells has been characterized as a result of recent developments in high-throughput sequencing. It has been demonstrated that mutations in epigenetic regulators are responsible for the emergence of a number of cancers, including AML. It is known that early events during the development of AML events that are crucial for the onset and/or progression of the disease involve mutations in epigenetic modifiers [51]. Studies have shown that epigenetic regulator mutations impair the self-renewal and/or differentiation functions of HSCs [52].

DNA methylation, among other epigenetic changes brought on by these mutations (e. g. mutations in DNMT3A, TET2, IDH, etc.) and histone alterations (MLL fusions, etc.) that cause malignant transformation (and thus mutation) in the AML genome[53,54]. It is well known that DNMT3A mutations are common in AML patients with a normal karyotype and that they may be the first events leading to AML development[55]. Accordingly, DNMT3A function loss led to an increase in self-renewal capacity and a decrease in multi-lineage differentiation, acting as a pre-leukemic HSC[56]. Additionally, it was demonstrated that DNMT3A mutation patients had global hypomethylation compared to DNMT3A normal. Hypomethylated genomic regions studies demonstrate enriched for HOX genes, which play promotes a role in hematopoiesis and leukemogenesis[57]. Furthermore, the loss of TET2 function resulted DNA hypermethlylation phenotype and diminishes histone demethylation by histone demethylase ablation. Because TET2 inactivation was found in pre-leukemic stem cells, TET2 mutations have been proposed as pre-leukemic events in AML[58]. Mutations in this enzyme leads to disruption of protein activity and reduced 5-hydroxymethylcytosine levels[59]. Likewise, TET2 mutation showed aberrant differentiation and enhanced HSC pool resulting the leukemic progression[60,61.]

1.4. Acute myeloid leukemia

Acute myeloid leukemia (AML) is an aggressive hematological disease with clinically and genetically heterogeneous group that can cause clonal immature blast cells proliferation at the expense of the normal production of their terminally differentiated counterparts[62]. AML results from the gradual acquisition of genetic and epigenetic alterations of leukemic phase variables such as clone heterogeneity, phase duration and environmental exposure [63,64]. These genetic alterations comprise single nucleotide variations, translocations, deletions or insertions, copy number variations and defect of heterozygosis. Each of these genetic alterations affects the pathogenesis of AML in a unique way, and when they combine within various sub-clones, they create a complex leukemic profile [65 ,66]. As a result of the accumulated mutations, normal HSC functions are compromised, differentiation is blocked, and the capacity for self-renewal is increased[67]. AML has a slight male predominance and a median age at diagnosis of about 70 years in the western world. Asian countries report much lower age-standardized incidence rates for AML than do western nations. According to estimates, the incidence of AML in Japan and China is roughly half that in the US and Europe[68].

1.4.1. Spectrum of acute myeloid leukemia

The French-American-British (FAB) classification was used to initially categorize AML, and it defined each subtype of AML based on its cellular makeup morphology following standard staining. The FAB classification does not offer prognostic value for the majority of patients, despite being useful in a few specific situations. According to the stage of differentiation of their originating cell, the FAB subtypes M0 to M7 were categorized. Leukocyte progenitors gave rise to subtypes M0 to M5, with M0 being the most primitive subtype (undifferentiated acute myeloblastic leukemia) and M6 and M7 being the most recent subtypes (acute erythroid leukemia and acute megakaryoblastic leukemia), respectively[69]. Moreover, the WHO classification include four main clinically significant groups based on genetics and immune phenotyping: AML with recurrent genetic abnormalities, AML with myelodysplastic changes, therapy-related AML and AML not otherwise specified[70].

1.4.2. Relapsed acute myeloid leukemia

Leukemia stem cells (LSCs) are a major factor in the biology of AML relapse and the subject of improved risk stratification and new treatments. Relapse can happen in a variety of ways. When leukaemic blasts are treated with conventional chemotherapeutics, but not LSCs, the leukemic hierarchy can once again be established. Alternately, the therapy-resistant pre-leukemic HSC clones can pick up additional driver mutations and develop into new LSC clones after the successful eradication of LSCs [66,71]. Despite the fact that in AML, LSCs can arise from a variety of stem and progenitor populations, they share characteristics with abnormal HSCs. In fact, the clinical outcome in AML is predicted by the stemness transcriptional programs in LSCs and HSCs, which have significant overlap[72]. Enrichment of LSC signature genes is highly indicative AML therapy failure across mutational subtypes, strongly supporting the existence of a conserved stemness resistance. LSCs with pre-leukemic HSC characteristics or blasts with a progenitor immunephenotype, both of which maintain potent stemness transcriptional signatures and functional LSC properties upon relapse, can also be the source of AML relapse[73]. These findings, which were clarified at the single-cell level, corroborate earlier findings from large-scale gene signature data and further support the idea that stemness is the primary mechanism causing AML therapy resistance[74].

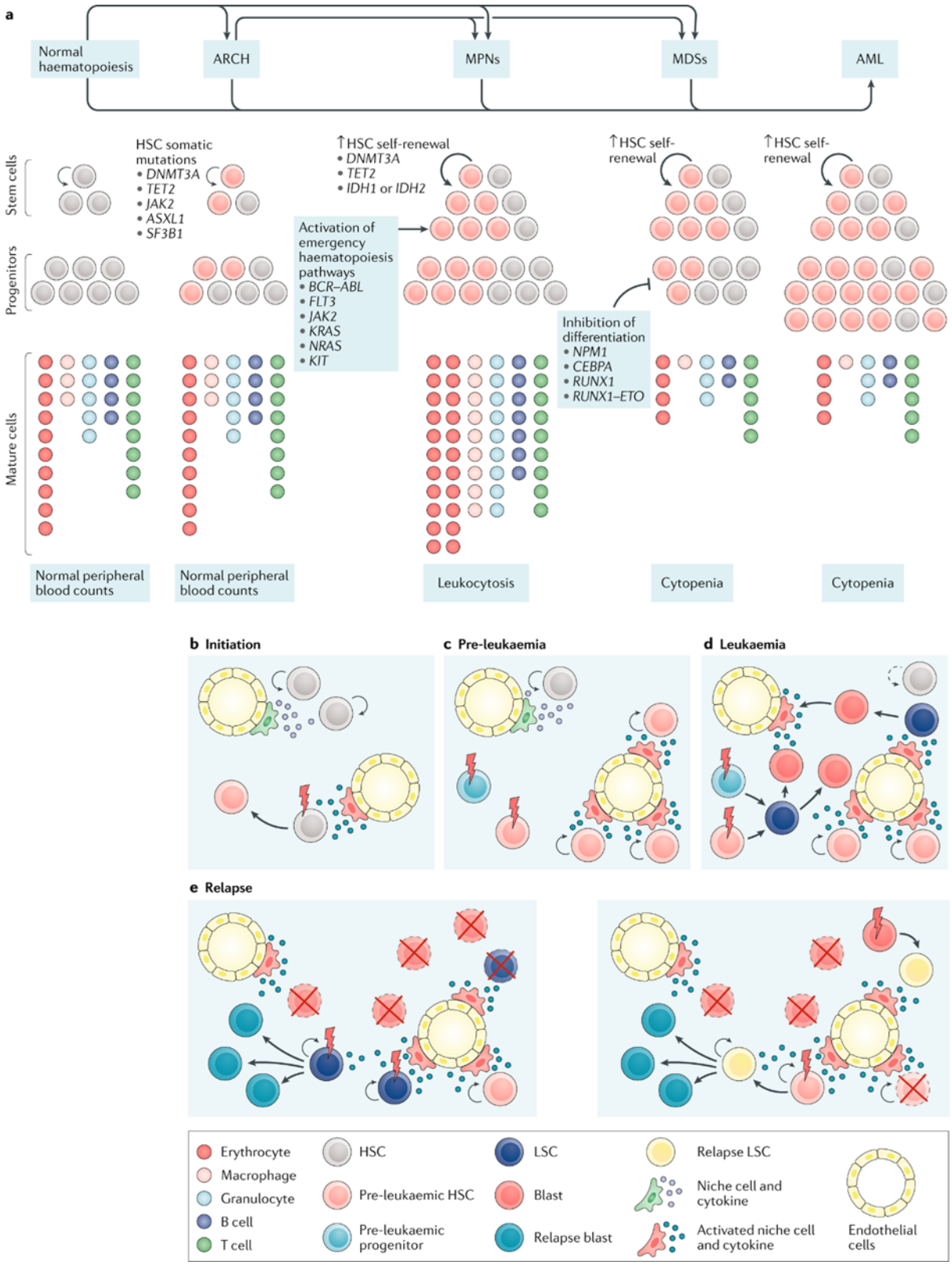

Fig 2. Dysregulation of HSC properties and leukemia development. (a) Leukemia develops through the accumulation of mutations that dysregulation hematopoietic stem cell (HSC) self-renewal, activate HSC proliferation and inhibit differentiation into progenitor cells. Somatic mutations in HSCs cause competitive fitness and the relative expansion of single clones in age-related clonal hematopoiesis (ARCH). In myeloproliferative neoplasms (MPNs), HSCs accumulate mutations that boost self-renewal which results in an excessive pro-

duction of mature cells. Distinctly, myelodysplastic syndromes (MDSs) develop due to enhanced HSC self-renewal diminished differentiation of progenitors resulting dysplasia and cytopenia. In acute myeloid leukemia (AML), drive aggressive proliferation and expansion of leukaemic blasts. **(b– e)**Leukemia also hijacks the bone marrow niche microenvironment to drive leukaemic stem cell (LSC) emergence and disease evolution. **(b**) Initiation phase: HSCs are intrinsically vulnerable to mutagenesis driven by erroneous DNA repair and age-associated single-base substitutions. (**c)** Pre-leukaemic phase: mutated pre-leukaemic HSCs gradually expand and also accumulate additional mutations resulting to the subsequent transformation of pre-leukaemic into LSCs. (**d)** Leukaemic phase: LSCs produce leukaemic blasts from pre-leukaemic HSCs or their progeny thus inhibiting normal HSC activity and hematopoiesis. (**e)** Relapse phase: relapse can develop upon treatment with chemotherapeutics which might permit pre-existing LSCs to reappear in leukaemic hierarchy. On the other hand, he treatment-resistant pre-leukemic HSC clones can pick up additional driver mutations and develop into new LSC clones after the LSCs have been successfully eradicated. (Figure Nat Rev Cancer. 2020)[31].

1.4.3. Therapeutic targets of acute myeloid leukemia

We now have a much better understanding of the molecular and phenotypic diversity of AML thanks to developments over the last 10 to 15 years. As a result of this new information, several intracellular signaling pathways that are essential for the development of various forms of AML have been identified, as well as numerous molecular and immunological therapeutic targets [75]. Studies on genetic lesions made it abundantly clear that AML had a complex genetic profile with clonal heterogeneity. Moreover, chemotherapy creates new mutations that may lead to relapse and therapy resistance, contributing to the diversity of AML clones[76]. Thus, the outcomes of patients following AML treatment is determined by the disease heterogeneity.

An intensive regimen of anthracyclines and cytarabine makes up induction therapy. The goal of induction therapy, which is administered for one week, is to achieve complete remission, which is defined as having less than 5% of blasts in the bone marrow [77]. In 60-85% of patients 60 years of age or younger, complete remission is achieved with the induction regimen. However, patients over the age of 60 are unable to tolerate the intense chemotherapy; as a result, less intense therapy is used in conjunction with a palliative regimen that includes hypomethylating agents[78].

Consolidation therapy entails two to four cycles of intermediate-dose Cytarabine and/or HSC transplantation in order to prevent relapse and eradicate minimal residual disease (MRD) in bone marrow. The most successful treatment for patients with AML is known to be consolidation therapy combined with allogeneic hematopoietic stem cell transplantation [79]. Patients who fail to achieve their first remission with induction therapy or who do so but later experience a relapse may benefit from stem cell transplantation[80].

Even though stem cell transplantation is gives best results AML treatment, it has serious side effects, such as graft versus host disease (GVHD), which increases the risk of morbidity and mortality associated with transplants[81]. To eliminate leukemic cells that might be chemo-resistant, radiotherapy is combined with chemotherapy. The graft-versus-leukemia effect, which is the immune responses mounted by donor hematopoietic cells eliminates any remaining leukemic cells [82]. For patients who are unable to receive intensive chemotherapy, DNA methyltransferase inhibitors (DNMTis) like azacitidine and decitabine have been widely used as first-line treatments. It has been demonstrated that an oral azacitidine derivative (CC486) can change the course of AML by increasing both OS and relapse free survival. When given as "maintenance therapy" in a phase III trial to patients older than 55 years who had undergone induction chemotherapy (with or without consolidation therapy) and had entered disease remission[83]. Therapies that target other epigenetic processes, as opposed to DNMTis, have largely fallen short of expectations. These include lysine demethylases and histone deacetylases. The inhibitors of transcriptional repressors like vorinostat, and pracinostat are also included[84 , 85 , 86]. Novel epigenetic therapies frequently fall short in their ability to show efficacy in patients with relapsed and/or refractory AML.

Table 1. Emerging and promising agents for the treatment of AML

Class of epigenetic regulator	Target	Compound	Phase of development
DNA methyltransferase	DNMTs	Azacitidine	Approved (see text)
		Decitabine	Approved (see text)
		Rationally designed novel inhibitors	Preclinical and clinical[94,95]
Regulator of methylation	IDH1, IDH2	Inhibitors of mutant IDH1/2	Clinical trials ongoing with compounds including IDH305 (ClinicalTrials.gov identifier: NCT02381886; targeted at IDH1 R132 mutation), AG-221 (NCT01915498; targeted at mutant IDH2), AG-120 (NCT02074839; targeted at mutant IDH1)
Histone lysine acetyltransferase	CREBBP (CBP)	CREBBP inhibitor	Preclinical[96]
	EP300 (p300)	EP300 inhibitor	Preclinical[97]
Histone deacetylase	HDACs	HDAC inhibitors	Several clinical trials ongoing, often in combination with other treatment modalities (eg, with DNMT inhibitors [examples ClinicalTrials.gov identifiers NCT01617226 and NCT00867672], conventional chemotherapy [example NCT01802333], or in conjunction with allogeneic stem cell transplantation [examples NCT01451268 and http://www.hovon.nl/studies/studies-per-ziektebeeld/aml.html?action=showstudie&studie_id=104&categorie_id=4])
Histone acetyl reader	Bromodomain containing proteins (BET proteins)	BET inhibitors	Several clinical trials ongoing with compounds including OTX-015 (ClinicalTrials.gov identifier: NCT01713582), CPI-0610 (NCT02158858), TEN-010 (NCT02308761), GSK525762 (NCT01943851)
Histone lysine methyltransferase	EZH2	EZH2 inhibitors	Preclinical[98,99]
	MLL-complexes	DOT1L inhibitors	Clinical trial with compounds including EPZ-5676 (ClinicalTrials.gov identifier: NCT01684150)
		Inhibitors of MLL-Menin interface	Preclinical[100]
		Inhibitors of MLL-LEDGF interface	Preclinical[101]
Histone lysine demethylase	LSD1	LSD1 inhibitors	Clinical trials with compounds including GSK2879552 (ClinicalTrials.gov identifier: NCT02177812) and tranylcypromine in combination with tretinoine (NCT02261779)
	Jumonji family of KDMs	Small molecular inhibitors competitive for 2-oxoglutarate	Preclinical[102,103]
Histone arginine methyltransferase	PRMTs	PRMT inhibitors	Preclinical[104,105]

Table from Stein EM, et al. Blood. 2016[87].

1.5. DNA methylation

DNA methylation is one of the most significant epigenetic modifications which crucial for the control of gene expression, genomic imprinting, X chromosome inactivation, and tumor development [88]. De novo DNA methylation, maintenance, and demethylation are the three stages of DNA methylation[89].

1.5.1. DNA methylation

A family of enzymes called DNA methyltransferases (Dnmts) are responsible for catalyzing the transfer of a methyl group from S-adenyl methionine (SAM) to the fifth carbon of cytosine residues to create 5mC, which is the result of DNA methylation. Three catalytically active DNA methyltransferases enzymes (DNMT1, DNMT3A, and DNMT3B) as well as a fourth, catalytically inactive enzyme, DNMT3L, have been identified in humans. A fifth protein, formerly known as DNMT2, is no longer regarded as a DNA methyltransferase[90].

Additionally, the DNMT3C novel DNA methyltransferase has recently been identified. These proteins have similar amino acid sequences and are highly conserved. The catalytic domain at the C-terminus is responsible for the enzymatic activity, and the regulatory domain at the N-terminus allows DNMTs to anchor in the nucleus and recognize nucleic acids or nucleoproteins [91]. The methylation process utilizes DNMT1, DNMT3A, and DNMT3B in various ways. All methylation in the genome must be maintained, which requires DNMT1. In order to restore the daughter strand's specific methylation pattern to that of the parental DNA during replication, DNMT1 is required. De novo methyl-transferases (DNMT3A and DNMT3B) are involved in the establishment of DNA methylation patterns during embryogenesis and the establishment of genomic imprints during the development of germ cells [92].

The expression of DNMT3A and DNMT3B decreases as cells differentiate, despite being highly expressed in early mammalian embryos. These two proteins display both spatial and temporal differences in their respective roles during embryonic development. When an embryo is developing late and especially after birth, DNMT3A methylate a specific set of genes and sequences, whereas DNMT3B modifies a larger region of genomic sequences in early embryos [92,93].

1.5.2. DNA demethylation

The TET methylcytosine dioxygenases actively demethylate DNA by oxidizing 5-methylcytosine (5mC) to hydroxymethylcytosine (5hmC), 5-Formylcytosine (5fC), and 5-carboxylcytosine (5caC). In the case of 5fC and 5caC, all the oxidized forms can encourage DNA demethylation during replication. Demethylation can also happen as a result of thymine DNA glycosylase (TDG) removing bases, which is followed by the activity of the base excision repair pathway[94]. The C-terminal catalytic domain of TET proteins is universal. TET1 and TET3 both have an additional CXXC domain that may help with DNA binding, but TET2 has a chromosomal inversion caused by evolution that splits it into two parts: the catalytic portion, or TET2, and the CXXC domain-containing portion, called IDAX/CXXC4 [95,96]. The expression patterns of various TET enzymes in vivo also differ. While TET2 and TET3 are expressed more widely, TET1 is primarily expressed in embryonic stem cells. Particularly in hematopoietic and neuronal lineages, TET2 expression is more prevalent in a variety of differentiated tissues[97]. TET2 expression is the most prevalent of the three proteins, and it is mutated in a variety of malignancies[98].

1.5.3. CpG sites

The majority of DNA methylation takes place at CpG sites, or cytosines that come before guanine nucleotides. CpG islands are known to control gene expression by transcriptional silencing of the corresponding gene [99]. There are differences in DNA methylation between introns and exons, according to a closer look at DNA methylation at gene bodies[100]. Age-related hypomethylation changes and tissue- and cancer-related methylation are linked to CpG shores[101]. Mammalian genomes are generally lacking in CpG sites, which may be related to the mutagenic potential of 5mC, which can deaminate to thymine [102,103]. DNA methylation is not only present at promoter CpG, and knowledge of the purposes of DNA methylation at other genomic elements is advancing. Traditionally, it has been assumed that methylation at gene bodies mutes the so-called repetitive elements (e.g. Alu elements, LINE-1, etc.) [104].

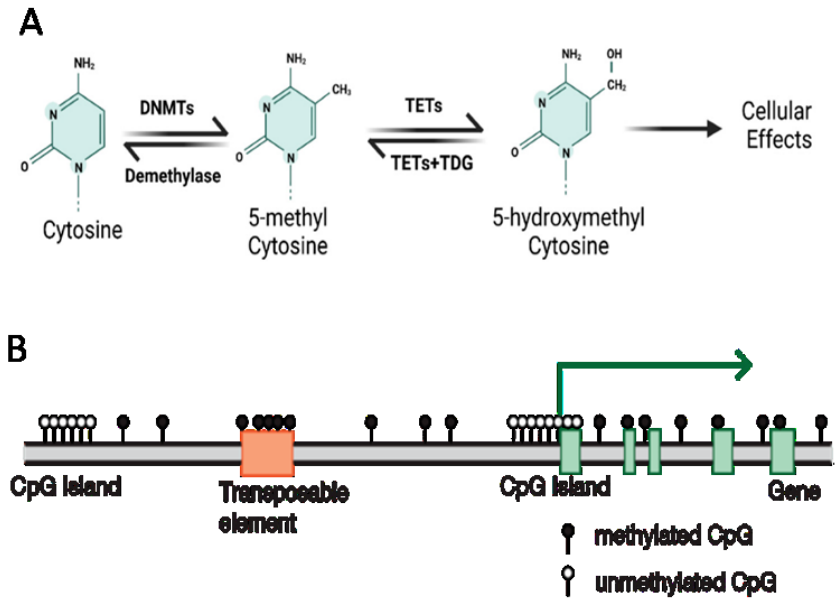

Fig 3. Schematic diagram of DNA methylation and CpG sites. **(A)** DNA methylation and demethylation mechanism. **(B)** Global CpG depletion resides in a specific category of GC- and CpG-rich sequences termed CpG islands. (figures A from Cancers 2022, 14, 5768)[105] and B from Mariuswalter 2016)

1.5.4. DNMT3A and TET2 in cancer

Intense research has been conducted on role of DNA methylation in cancer. The DNA methylation patterns of cancerous cells are drastically altered compared to those of normal cells [106]. In particular, elevated expression of DNMT3A has been observed in cancer cell lines and primary human tumor tissues. Deregulated or/and mutated DNMTs and TETs disrupt the balance between 5mC and 5hmC production, resulting in aggressive cancer growth[107]. Several models have been used to investigate DNMT3A's oncogenic role. The proliferation, migration, invasion, or colony-formation of melanoma cell lines are unaffected by the antisense-mediated knockdown of DNMT3A in vitro. However, DNMT3A depletion is linked to decreased melanoma growth and metastasis in the in vivo setting [108]. In line with this, high DNMT3A expression is found in gastric cancer tumors compared to normal tissue, and it is also associated with poor prognosis[109].

In gastric cancer cell lines, DNMT3A knockdown resulted a decrease in proliferation, whereas ectopic expression promotes growth. Depletion of p18INK4C leads to hypermethylation of its promoter, which in turn causes the growth acceleration associated with DNMT3A overexpression [110].

Additionally, both murine and human colon cancer have been shown to up regulate the expression of DNMT3A than normal. A 40% decrease in colon tumor development is seen in the Apcmin/+ model when Dnmt3a is conditionally knocked out[111].

Several different types of human solid tumors have also been found to contain TET gene mutations or express low levels TET proteins[112,113]. In some studies, up to 20% of patients, such as melanoma, and colorectal cancer were discovered to frequently carry missense mutations in one or more of the TET genes. The significance of these mutations is unknown, but they may have a negligible impact on TET activity [114]. The direct targeting of TET proteins by a number of microRNAs, some of which are overexpressed in cancer, has also been documented. All three TET family members were found to be negatively regulated by miR-22 in particular, and patients with breast cancer who have poor clinical outcomes have been associated with miR-22 expression [115]. In conclusion, these findings suggest that DNMT3A and TET2 have an oncogenic role and disruption of DNA methylation activity can lead to tumor development and proliferation.

1.5.5. DNMT3A and TET2 in myeloid malignancies

The first DNMT3A mutations linked to malignancy were only discovered in 2010, with three groups reporting mutations in AML with frequencies of up to 22%. This is despite the long-established link between aberrant DNA methylation and cancer[116]. Now, DNMT3A mutations have been identified in varying numbers in the majority of hematological malignancies. Deep sequencing of hematological malignancies revealed that DNMT3A mutations were frequently found at higher variant-allele frequencies (VAFs) than other accompanying mutations, indicating that they were among the first to appear[76]. Two separate studies discovered that human HSCs isolated from AML patients could contain DNMT3A mutations even in the absence of other typical leukemia-associated mutations[71,117]. Furthermore, at least in xenograft models, human DNMT3Amut HSCs appeared to be significantly more advantageous than wild-type HSCs, similar to what was seen in mice.

These studies show that human HSCs can carry DNMT3A mutations and still contribute to various blood lineages, existing in a pre-leukemic state before acquiring additional mutations that cause leukemia[71].

Adults with AML have the highest frequency of DNMT3A mutations in the myeloid lineage, with most studies reporting a 20–25 percent mutation frequency in DNMT3A in de novo disease[118]. Around 60% of DNMT3A mutations are found at the residue R882 in the methyltransferase domain, according to numerous studies that looked at all or nearly all of the coding region in AML[119]. Although less frequently than in AML, the R882 position is the most frequently mutated in other myeloid malignancies, such as myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPNs), and chronic myelomonocytic leukemia (CMML)[120]. DNMT3A mutations are typically heterozygous in all of these diseases, with non-R882 mutants being the only ones that exhibit biallelic involvement. DNMT3A mutations are also frequently found in tumors of the T lymphoid lineage, though they are distributed in a more varied way than in myeloid lineages. Less than 20% of DNMT3A mutations that affect the R882 position in peripheral T cell lymphoma (PTCL) cluster in the methyltransferase domain[121]. Similar percentages of mutations in T cell acute lymphoblastic leukemia (T-ALL) affect the R882 position, and biallelic involvement is very common, occurring in up to 62 percent of patients[122]. The mechanisms underlie how DNMT3A mutations cause leukemia unclear. Specifically at CpG islands, shores, and promoters, though some promoter hypermethylation also occurs, the R882 mutation in AML patients is correlated with global hypomethylation[123,124]. In sum, the biological function of DNMT3A is critical for the differentiation of self-renewing cells. The balance is shifted when something is lost or activity is decreased, which leads to a predisposition to cancer and other pathological effects.

TET2 mutations have also been identified in a variety of cancers, including myeloproliferative neoplasms (11.5%), myelodysplastic syndromes (17.2%), chronic myelomonocytic leukemia (45.8%), and acute myeloid leukemia (20.1%)[125]. When it comes to MDS, MPN, and AML, frame shift mutations predominate, whereas in CMML, missense mutations are more common. Due to the inactivating effects of these mutations, it can be concluded that the hematopoietic system, whose disruption causes leukemia, depends on the proper functioning of this TET2[125]. Additionally, studies have revealed that TET2 mutations typically manifest in the CD34 cells of patients, where CD34 serves as a marker for human hematopoietic stem cells. Patients with secondary AML also frequently carry TET2 mutations. Numerous studies have demonstrated the rapid progression to secondary AML in MPN patients with TET2 mutations[126].

There are two different kinds of TET2 mutations. The first category includes nonsense and frame shift mutations, which can happen anywhere along a gene. Specific missense mutations in TET2 catalytic domain, a crucial conserved domain, make up the second class of mutations[127]. All of the mutations cause the protein to become inactive, indicating that TET2 methyl-cytosine dioxygenase enzyme activity is crucial for hematological development. The fact that 5hmC may be crucial for the hematopoiesis process is highlighted by older patients with myeloid disorders who have TET2 mutations have a significant loss of 5hmC in the undifferentiated cells[98]. The conditional loss of TET2 activity brought on by the Vav: Cre-mediated removal of exon 3 in vivo resulted an expansion of the lineage negative Sca.1+ cKit+ (LSK) cells and promotes replating potential in a colony forming unit assay in vitro [60] suggesting that Tet2 knockdown could impact on stem/progenitor cell differentiation. These studies also demonstrated that TET2 KO bone marrow had increased stem cell function and self-renewal and was capable of competing with TET2WT bone marrow in transplant assays. Finally, CMML-like syndrome with expanded monocytes, increased spleen weight, and proliferative growth in the bone marrow, spleen, liver, and lung was observed in aged TET2KO mice[128]. Increased granulocyte macrophage counts were also caused by TET2 loss. Following TET2 loss, the knockout animal also displayed hepatosplenomegaly. Because the HSC differentiated more toward monocytes than erythrocytes, its differentiation was also impacted[129].

TET2 mutations are typically found in combination with other mutations in AML and myeloproliferative disorders. One of the most frequent AML mutations is in the FLT3 protein, which has point mutations in the tyrosine kinase domain and internal tandem duplications (ITDs) close to the juxtamembrane domain that cause the kinase to activate on its own [130]. TET2 regulates the hematopoietic system, as demonstrated in some studies using TET2 knockdown cells and TET2 depleted mice. However, the precise mechanism by which TET2 depletion alters the normal hematopoiesis is unknown[126].

In conclusion, TET2 mutations linked with poor prognosis in AML patients with intermediate-risk cytogenetic, particularly when it is shared with other adverse markers. The pathways and targets of TET2 are still unclear, despite the fact that numerous studies are still in progress trying to clarify the function of TET2 in healthy and cancerous hematopoiesis.

1.6. Mitochondria

Mitochondria are ubiquitous, semi-autonomous double membrane organelles with their own genome[131]. They are found in the cytosol of all nucleated eukaryotic cells responsible for generating the majority of the energy. Despite being initially associated with its function in energy production, it has now been proven that mitochondria can affect almost any cellular process. These organelles contain enzymes essential for the biosynthesis of several macromolecules, such as lipids, nucleotides, and iron-sulfur clusters, as well as enzymes that help the Krebs cycle and fatty acid β-oxidation facilitating the breakdown of other molecules[132]. Beyond metabolism, mitochondria integrate the pathways of various biological processes like cell death, calcium homeostasis, inflammation, and gene expression[133].

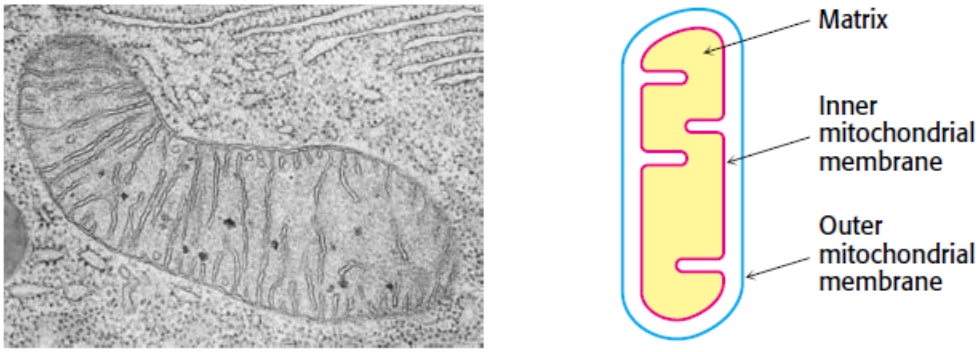

Fig 4. Mitochondrion. The double membrane of the mitochondrion is evident in this electron micrograph. The numerous invaginations of the inner mitochondrial membrane are called cristae. The oxidative decarboxylation of pyruvate and the sequence of reactions in the citric acid cycle take place within the matrix. [(Left) Omikron/Photo Researchers.] (Figure from stryer Berg et al., Biochemistry, 9e, © 2019 W. H. Freeman and Company)

1.6.1. Mitochondria in Hematopotiec stem cell

Mitochondria are remarkably heterogeneous organelles with cell-specific morphology and dynamics that enable them to perform specialized tasks to maintain cellular homeostasis and coordinate cellular adaptation to stressful situations. Mitochondrial dysfunction has become a significant factor in many pathological conditions, such as hematologic malignancies, in line with its multifunctional role.

In fact, it has been suggested that mitochondria are one of the factors that determine the fate of stem cells [134]. HSCs and other stem cell types experience significant changes in the quantity and quality of their mitochondria during proliferation and differentiation. The hematopoiesis process itself may be the cause of these changes in the mitochondrial landscape in HSCs, but there is mounting proof that mitochondria actively regulate hematopoiesis[135].

1.6.1.1. Mitochondria and ROS in the regulation of HSC

HSCs preferentially settle in bone marrow niches that are thought to be hypoxic and have low levels of reactive oxygen species (ROS) partly due to their sensitivity to the cellular redox state[136]. ROS levels must be balanced for proper HSC function. Accordingly mitochondrial metabolism is restricted in order to keep ROS levels at minimum and prevent HSC commitment and differentiation[137]. Low levels of ROS, regulated by intrinsic factors such as cellular respiration or NADPH oxidase activity, or extrinsic factors such as stem cell factor or prostaglandin E2 are necessary for maintenance self-renewal[138]. Moreover, a family of tightly controlled NADPH oxidases (NOXs) which are homologues of the phagocyte oxidase (Phox or NOX2) have been reported to produce ROS in cells. Human HSCs have been found to express NOX1, 2, and 4, as well as different NOX regulatory subunits[139]. Interestingly, recent studies showed that mononuclear cells from mouse BM express NOX1 and 2, but not NOX4 in Lin−Sca1+c-kit+ cells, while HPCs, Lin− cells. This suggests that NOX4 expression is down regulated upon HSC differentiation and that NOX4 may be crucial in the regulation of HSC function[140]. HSC differentiation and commitment has been associated with increased ROS levels, activation of the mTOR signaling pathway, and enhanced mitochondrial biogenesis[141]. However, an abnormal levels in ROS generation occur due to various pathological conditions, which can impair HSC self-renewal and promote HSC senescence, leading to early exhaustion of HSCs and hematopoietic dysfunction[142].

1.6.1.2. Mitochondrial respiratory complexes in regulation HSCs

To determine whether mitochondrial respiration is required for HSC maintenance studies have also shed light on ETC-dependent HSC regulation.

In fetal HSCs, loss of Uqcrfs1 a gene that encodes the Rieskeiron-sulfur protein (RISP), a crucial subunit of the mitochondrial CIII have been associated with dysregulation in HSC differentiation.

In activation of Uqcrfs1 lead to a reduction in stem cell genes and a loss of multi-lineage repopulation potential[143]. This is associated with hypo acetylation due to decreased citrate production in the mitochondria and elevated 2-HG levels, which may lower the NAD/NADH ratio and resulting in hyper methylation of DNA and histones[143]. Moreover, defective SDHD-ESR (encodes one of the mitochondrial CII subunits) in mouse, showed a significant decline in number of precursors of myeloid lineage known as the LK population. The decline in LK fraction is primarily due loss Megakaryocyte/erythroid progenitors (MEP), rather than common myeloid progenitors (CMP)[144]. This could be attributed to the fact that the SDHD gene encodes for a protein involved in both the Krebs cycle and the oxidative phosphorylation system. As a result, the mutant SDHD-ESR mouse allows for simultaneous impairment of mitochondrial bioenergetics and supply of metabolic intermediates, which may be a condition necessary to demonstrate the true mitochondrial dependence of HSC[144]. In addition, the hematopoietic NDUFS4-KO mouse, which modifies the assembly of mitochondrial CI, exhibited a decreased respiratory capacity, increased production of mitochondrial ROS, and higher levels of superoxide dismutase-2 mRNA expression in the BM[145]. Mitochondrial respiratory complexes can also affect HSCs repopulating capacity. For instance, SdhD deletion induced significant reduction in the long-term (LT) LT-HSC population capacity[144]. Similar decrease is also noted due to the loss of Uqcrfs1 in vivo in CD45.2 B cells, T cells and myeloid cells in the peripheral blood[143]. Additionally, in vitro colony-replacing capacity was decreased by pharmacological inhibition of CII but not CI, which is not inhibited[146].

Overall, these findings show that different respiratory complexes cause distinct cellular adaptations in HSCs.

1.6.1.3. mtDNA in HSC

Mammalian mtDNA is a 16.6 kb double-stranded DNA (dsDNA) molecule that is gene dense which encodes 11 messenger RNAs (mRNAs) (translated to 13 proteins), 2 ribosomal RNAs (rRNAs) (12S and 16S rRNA), and 22 tRNAs.

In higher metazoans, mitochondrial DNA (mtDNA), which is packaged into protein DNA complexes known as nucleoids, is tightly bound to the inner mitochondrial membrane (IMM) within the mitochondrial matrix[147]. The mitochondrial matrix is the site of the mtDNA transcription apparatus, which includes the mitochondrial RNA polymerase (POLRMT), the mitochondrial transcription actor B2 (TFB2M), and the mitochondrial transcription factor A (TFAM)[148].

Studies on mtDNA mutagenesis mouse models, demonstrated the critical role of mitochondria in HSCs. Loss of POLγ mutant mice revealed hematopoietic progenitor (HPC) dysfunction already from embryogenesis distinct differentiation blocks and/or disappearance of downstream progenitors as well as defects resembling premature aging of HSCs, including anemia, lymphopenia, and myeloid lineage skewing [149,150]. Furthermore, a decrease in mtDNA during the transition of HSCs to bipotent megakaryo-cytic/erythroid precursor cells (preMeg/Es), have been observed which gradually increased during differentiation through the erythroid committed progenitors[151]. Moreover, studies have shown that mito-mice with a pathogenic mtDNA mutation and BM cells carrying 77–83 percent mtDNA had significantly lower ATP content than WT mice[152]. Likewise, up regulation of nuclear genes that control mtDNA replication and transcription at various stages of hematopoietic differentiation have been demonstrated in early erythroid committed progenitors[151].
Taken together, these studies show that HSCs need intact and functional mtDNA at various stages of maintenance.

1.6.1.4. Mitochondrial mass in HSCs

Compared to multipotent progenitors (MPPs) and more restricted progenitors, HSCs have a sizable number of mitochondria that are relatively inactive. The self-renewing murine and human HSCs exhibit low mitochondrial activity and membrane potential[153,154]. Surprisingly, compared to the more committed progenitors, these long-term HSCs have a lower mitochondrial mass[155]. This is in line with studies that show inhibition mTOR pathway via TSC1 results in a decrease in mitochondrial mass and a blockade of HSC differentiation[156]. On the other hand, a decrease in MTCH2-dependent HSC self-renewal capacity is associated with an increase in OxPHOS activity and mitochondrial size/volume[157].

More importantly, HSCs exhibit significantly lower mitochondrial activity than lineage-committed progenitors, even under normoxic conditions[158]. In this sense, autophagy, which is necessary for HSC self-renewal potential, primarily functions as a gatekeeper [159]. Enhanced autophagy lowers the number of mitochondria that are active subsequently decrease mitochondrial mass as well as HSCs mitochondrial activity[160].

1.6.1.5. Mitochondrial metabolism in the regulation of HSC maintenance

HSCs are primarily dormant, and they differ from committed progenitors and other BM cells which are primarily lineage-differentiated cells in their metabolic wiring and reliance on glycolysis[137]. HSCs produce high levels of 1, 6-bisphosphate and other byproducts of glycolysis step. High pyruvate kinase activity (PDK), which is dependent on hypoxia-inducible factor 1α (HIF1α), is associated with such an increase in glycolytic by- product levels[161]. In turn, HIF1 drives and controls a metabolic program that maintains glycolysis as primary energy source and restricts the use of the TCA cycle[162]. Moreover, the functional identity of HSCs and maintenance of their population are also negatively impacted by increased mitochondrial activity. Loss of mitochondrial carrier homolog 2 (MTCH2) lead to increased OxPHOS activity and intracellular ROS levels, which causes HSCs to enter the cell cycle and lose their capacity for self-renewal[157]. In contrast, HSCs ability to self-renew in ex vivo cultures that typically induce differentiation is maintained when mitochondrial activity is reduced by chemically uncoupling the mitochondrial ETC[155]. These studies suggest that the self-renewal of HSCs heavily, but not exclusively, rely on glycolysis, highlighting the significance of limited mitochondrial activity and metabolism in hematopoiesis and HSC fate decisions.

Metabolic changes in mitochondria may also modify the availability of substrates for chromatin-modifying enzymes, resulting changes in epigenetic signatures that could alter chromatin accessibility and, consequently, gene transcription [163]. In fact, cells metabolize nutrients like glucose, fatty acids, and amino acids to produce a wide range of metabolites like acetyl-CoA, NAD, SAM, α-KG, ATP, and succinate, which serve as substrates or cofactors to modify chromatin and proteins. The acetylation modification process frequently involves acetyl-CoA derived from acetate. HATs catalyze the acetylation of histones, and lysine deacetylases (HDAC and SIRT) mediate this reversible reaction[164].

The metabolic process known as ketone body generates the metabolite β-hydroxybutyrate (βOHB), required for histone lysine β-hydroxybutyrylation (Kbhb) which mediated by the enzymes involved in acetylation modification[165].

Furthermore, iron, α-ketoglutarate (α-KG), and O_2 are required for the histone demethylation process carried out by iron-containing jumonji-domain (jmjC) demethylases [166] as well as DNA 5-methylcytosine demethylation catalyzed by the TET[167]. Therefore, metabolic changes in mitochondria provide substrates or cofactors that could modify chromatin and proteins alter gene expression.

1.6.1.6. Mitophagy/Autophagy in HSCs

Autophagy is evolutionarily conserved intracellular catabolic process that eliminates extracellular waste and/or damaged cytosolic components through the lysosomal pathway [168, 169]. HSCs regulate autophagy flux to modulate their maintenance, differentiation, and proliferation. Impairment of autophagy leads to premature aging, while maintenance of autophagy has been associated in the maintenance of different stem cells including HSCs [170, 171] .
For example, deletion of Atad3a, AAA+-ATPase regulator of Pink1-dependent mitophagy, resulted HSCs to expand in number with impaired lineage differentiation. Atad3a deficient HSPCs have the low mitochondrial content as a result of hyper activated mitophagy via Pink1 build-up[172]. In addition, Atg7-deficient mice show expansion of HSPCs with defective. Particularly, depletion of Atg7causes aberrant increase in mitochondrial mass, higher mitochondrial ROS level, and increased DNA damage [173]. Moreover, Atg5 Autophagy related deletion from hematopoietic lineage cells cause lymphopenia and anemia; impaired reconstitution potential. The deficiency causes impaired clearance of damaged mitochondria [174].

Likewise, conditional deletion of Rb1cc1 increased HSC proliferation and expansion of myeloid compartment. Furthermore, hematopoietic populations from Rb1cc1-null mice that are rich in HSCs and HPCs have higher levels of mitochondrial mass and ROS than those isolated from wild-type[175]. In addition, absence of ATG12 in HSCs resulted in a significant alteration in the DNA methylation profile, with an increase in hypo methylated regions that primarily encode phosphoproteins[158].

In sum, these studies demonstrates autophagy plays critical role that in preserving HSC quiescence and healthy hematopoietic differentiation.

1.6.1.7. Mitochondrial dynamics in the regulation of HSC maintenance

The mitochondrial fission/fusion machinery controls the dynamics and morphology of mitochondria and depends on the activity of proteins such as optic atrophy (OPA1), mitofusin-1 and mitofusin-2 (Mfn1 and Mfn2), and dynamin-related protein 1 (Drp1). Indeed , the antagonistic and balanced activities of fusion and fission proteins continuously define the shape of mitochondria [176]. Interestingly, the loss of MITCH2, which is associated to a decreased link of Drp1 with mitochondria, have been found to be responsible for the increase in mitochondrial size and the impairment of HSC function and number[157]. In addition, another study found that the fusion protein Mfn2, which raises intracellular Ca^{2+}buffering, is also necessary for the maintenance of the lymphoid potential of HSCs[177]. Studies conducted during the conversion of somatic cells into induced pluripotent stem cells (is) have also shown early wave of mitochondrial fragmentation caused by elevated fission and Drp1 activity[178]. Thus, findings collectively imply that mitochondrial dynamics affects the fate of HSCs via a variety of mechanisms.

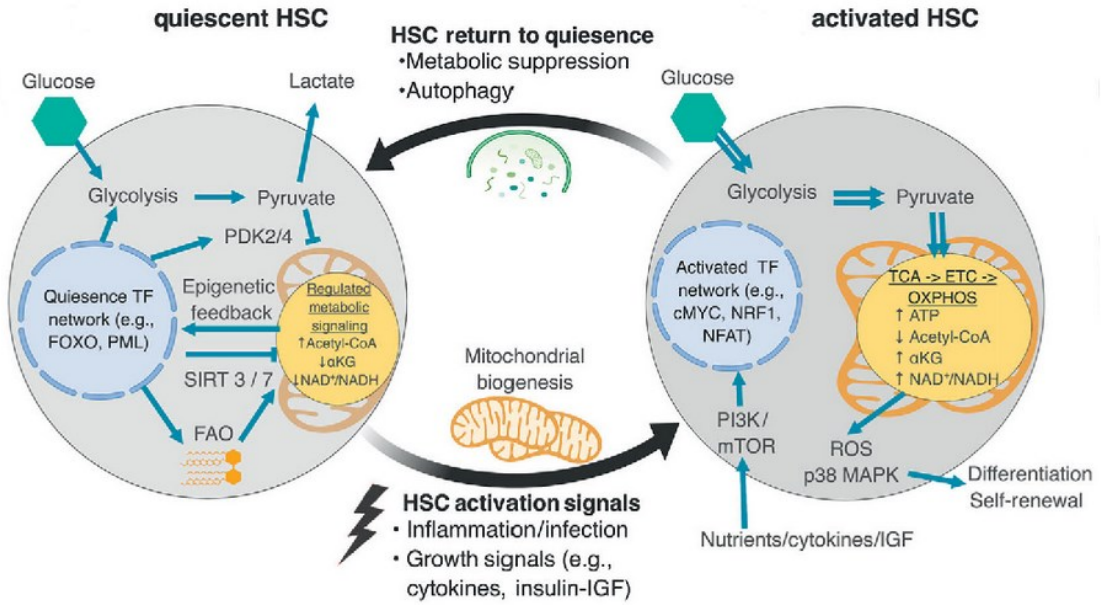

Fig 5. Changes in Metabolism and Epigenetics during Hematopoietic Stem Cell. HSCs, dormancy is sustained through a hardwired transcriptional regulatory network that enhances glycolysis and restrain pyruvate influx into the Krebs cycle. In addition, low aerobic flux and fatty acid oxidation (FAO) also offer key substrates that take part in metabolic and epigenetic regulation to maintain quiescence. When activated, HSCs use oxidative phosphorylation (OXPHOS), together with mitochondrial biogenesis, mTOR activation, and transcriptional reprogramming. The metabolic change induce reactive oxygen species (ROS) generation and MAPK activation and promotes HSC differentiation. When the hematopoietic system met their demand, metabolically activated mitochondria are removed via activate autophagy and return to quiescence by inhibiting oxidative metabolism (Figure taken from Molecular Medicine, 2019)[179].

1.6.1.8. Mitochondrial calcium in the regulation of HSC maintenance

Calcium signaling controls a number of mitochondrial processes, including the production of ATP. In fact, the important intracellular second messenger Ca^{2+}, can travel to the matrix and where it modulates the transporters, enzymes, and proteins involved in ATP synthesis [180]. The Calcium signaling toolkit is composed of various parts, including channels, pumps, anti-porters, and Ca^{2+} binding proteins, which work together to maintain cellular Ca^{2+} homeostasis [181]. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) is typically maintained at 100 nM; however, in response to various stimuli, $[Ca^{2+}]c$ can increase to 1–3 μM due to Ca^{2+} release from intracellular stores or /and Ca^{2+} enters through the plasma membrane (PM). The endoplasmic reticulum (ER), which is the principal intracellular Ca^{2+} store, is capable of releasing Ca^{2+} through ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs), which are found in ER membranes [182]. Both Ca^{2+} entry from the PM and ER Ca^{2+} release enhance cytosolic Ca^{2+} levels that can promote mitochondrial Ca^{2+} uptake. The fine-tuned process of mitochondrial calcium homeostasis is controlled through its ability of the mitochondria to handle calcium[183]. The outward transport of protons through the inner mitochondrial membrane (IMM), is established by the activity of the mitochondrial respiratory complexes which results negative membrane potential ($\Delta\Psi m$). Calcium influx into mitochondria depends on electrochemical potential and Mitochondrial Calcium Uniporter (MCU), a crucial protein channel of the IMM[184].

In the past ten years, there has been a lot of research done on the effect of mitochondria on HSC function[185,186,187]. Given that $\Delta\Psi m$ is the catalyst for mitochondrial Ca^{2+}, $[Ca^{2+}]_m$ it should be anticipated that HSC will likely have higher basal $[Ca^{2+}]_m$ than the differentiating progeny. Studies comparing $[Ca^{2+}]_m$ in HSC to progenitors or fully differentiated cells have not yet been reported.

Recently it has been demonstrated that isolated HSC cultured in low Ca^{2+} media significantly reduced the brightness of Rhod-2 the Ca^{2+} sensitive Rhoda mine derivative. In addition it has been noticed a relationship between respiration rates and the media Ca^{2+} availability[188]. This was accounted for Ca^{2+} dependence of Krebs cycle and ATP synthase.

Studies revealed that low cytosolic calcium (below 100nM), given that mitochondrial Ca^{2+} related dehydrogenases have a relatively low KD for calcium, it is conceivable at resting conditions HSC have a relatively high $[Ca^{2+}]_m$ [188].

In agreement with this, it has been reported that proper hematopoiesis requires mitofusin-2 (mfn2), well-known regulator of mitochondrial dynamics. HSC with Mfn2 deletion show a prototypical fragmentation phenotype and also resulted leads to significant decrease of mitochondrial Ca^{2+} uptake capacity. In HSC, mfn2 KO showed high $[Ca^{2+}]_c$ produced by stromal cell-derived factor 1 (SDF-1) and an increase of the NFAT-C1 ratio nuclear/cytoplasmic[177]. Finally, although biased for the lymphoid potential, mfn2 KO cells showed diminished reconstitution capacity in competitive BMT model experiments. Accordingly, S100a6 with reduced HSC function showed diminished $[Ca^{2+}]_m$ uptake as a result to SCF when compared to wild-type in addition to reduced $\Delta\Psi m$ and maximal respiratory capacity[189]. This suggests that the maintenance of HSC function depends on mitochondrial Ca^{2+} uptake, or at the very least, on its buffer capacity.

Investigation of HSC function in Drp1 KO cells, however, does not confirmed this model. The principal agent in the mitochondrial fission is Drp1. $[Ca^{2+}]_m$ has reportedly been impacted by its regulation of mitochondrial network dynamics[190]. In particular, overexpression of Drp1 leads to hyper-fragmentation, which isolates mitochondrial particles that do not directly contact the ER and lowers mitochondrial Ca^{2+} uptake. In addition, Drp1 inactivation results in a hyper fused mitochondrial network that favors $[Ca^{2+}]_m$ diffusion throughout the mitochondrial matrix. Despite the fact that $[Ca^{2+}]_m$ in HSC was never demonstrated during Drp1 modulation, it is anticipated that its genetic inactivation will result in a greater buffering capacity, which will favor HSC maintenance. A recent study, however, showed that $Drp1^{-/-}$ HSC have a decreased capacity for reconstitution, which was attributed to mitochondria's inability to properly segregate during HSC division[191].

A more recent study examined basal $[Ca^{2+}]_m$ in HSCs during stress hematopoiesis. Umemoto et al. demonstrated that basal $[Ca^{2+}]_c$, $\Delta\Psi m$ and basal $[Ca^{2+}]_m$ are increased following 5-FU intraperitoneal injection to stimulate HSC. The LTCC blocker Nifedipine also inhibited mitochondrial functions and prolonged the HSCs cell cycle phase, which slowed the recovery of stressed hematopoiesis[154]. Accordingly, a mouse HSC RNA sequencing study revealed that some MCU members (Micu1, Micu2, and Smdt1) have a tendency to increase their expression as cells transition from dormant to active MPP[192].

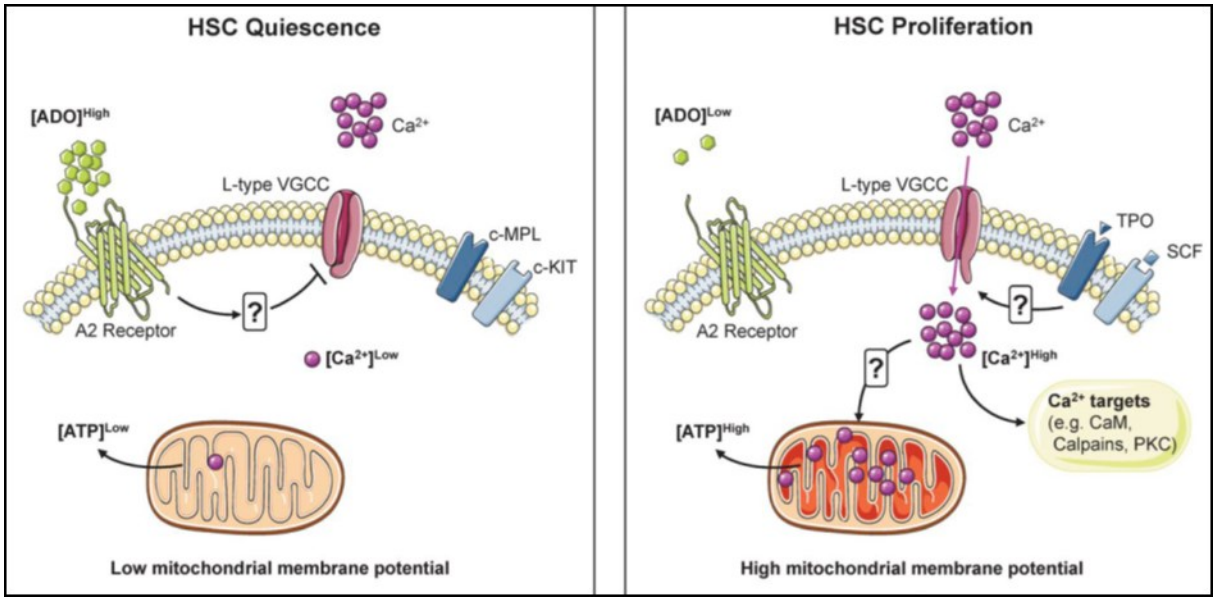

Fig 6. The Ca^{2+} mitochondria axis in the regulation of HSC division. Left: Adenosine (ADO) through adenosine A2 receptor suppresses Ca^{2+} entry to maintain low Ψm and maintain HSC dormancy or quiescence. Right: TPO and SCF induce Ca^{2+} influx in HSCs through L-type voltage-gated Ca^{2+} channel (VGCC) in turn p mitochondria, activate HSC division. Ca^{2+} influx also regulate different targets, such as calpains, CaM , and the PKC which modulate HSCs functions (Figure from J. Exp. Med. 2018) [193].

1.6.2. Mitochondrial dysfunction and cancer

Mitochondrial dysfunction is associated to a number of underlying mechanisms of deregulated cellular energetics. Such hallmark of tumor is that an increasing the production of metabolites and mitochondrial biogenesis. Important tumor suppressors and oncogenes control mitochondrial biogenesis. Indeed studies indicate, biogenesis is positively regulated by the c-My oncogene and suppressed by the p53 tumor suppressor.

For instance, the c-Myc oncogene induces PGC-1β expression, which in turn leads to increased expression of important mitochondrial proteins like Tfam, Polγ, and NRF1[194,195]. In addition, oncogene-induced ROS stimulates tumorigenesis in several ways, such as stabilization of hypoxia-inducible factor -α, activation of oxidative DNA bases, elevated calcium flux, down regulation of important phosphatases, like Pten and induction of both the NRF2 and NF-κB transcription factors [196, 197]. Moreover, a variety of mtDNA modifications, including insertions, point mutations, copy number changes and large-scale deletions have been found in human cancers[198]. The two most frequent changes to mtDNA in cancers are point mutations and copy number alterations[199].

An analysis of 859 patients with 20 different cancer types found that 66% of cancers had at least one somatic point mutation in the mtDNA, indicating that somatic point mutations in the mtDNA are a frequent occurrence in the development of human cancer. Of all 51 % of these mutations were found in the D-loop region of mtDNA, 40 % in the protein-coding region, 5 % in the rRNA genes, and 4 percent in the tRNA genes[200]. In addition, cancers cells appear to have specific tissue-specific changes in mtDNA copy number. In gastric cancers and breast cancers, a decreased mtDNA copy number is frequently observed [201, 202, 203]. Defects in nuclear-encoded mitochondrial enzymes, such as several TCA cycle enzymes and mitochondrial deacetylase SIRT3, may also contribute to deregulated cellular energetics in cancer cells in addition to somatic mtDNA alterations [204, 205, 206]. .

Together, these various lines of evidence point to mitochondrial dysfunction brought on by mtDNA mutations, mitochondrial enzyme deficiencies, or altered oncogenes/tumor suppressors as the underlying mechanisms of deregulated cellular energetics.

1.6.3. Mitochondria in Leukemogenesis

As cellular physiology changes, the structural and biochemical characteristics of mitochondria also change. Disruptions in mitochondrial processes can result in damaged or dysfunctional mitochondria and interfere with normal cellular processes, which can start or exacerbate pathophysiological conditions like hematological malignancy[207]. Leukemia are a group of extremely diverse blood cancers characterized by an excessive proliferation of lymphoid or myeloid-derived immature or mature cells in the peripheral blood and bone marrow. HSCs can give rise to leukemic cells through a series of progressive genetic mutations[208].

Mitochondrial ROS contributes to pathological progression in hematopoietic malignancies have been linked to chronic oxidative stress [209]. Some studies identified elevated extracellular superoxide generation in primary AML blasts compared with controls[210]. Furthermore, hematological malignancies have also been linked mitophagy. For instance mutations in a number of autophagy-related genes such as the BNIP3 and BNIP3 L genes exhibit aberrant expression in leukemic cell lines and AML has been reported[211]. In acute promyelocytic leukemia and AML, respectively, it has been shown significantly reduction in the expression of WIPI-1 (Atg18) and WIPI-2[212].

In addition, mitochondrial dynamic related proteins such as Drp1 were reported to have an effect in AML. FLT3 is constitutively activated as a result of mutations in FLT3-ITD, which promotes the proliferation and survival of leukemic cells. This promotes selective mitophagy and leukemic cell death. Mechanistic studies show activation of Drp1 becomes as result of deactivating protein kinase A (PKA), which is one of the downstream effectors of FLT3-ITD-dependent oncogenes[213]. Heydt et al., in contrast, reported FLT3-ITD cells were found to have higher basal autophagy[214].

Leukemic cells have also been found to undergo a metabolic switch from TCA to fatty acid oxidation (FAO), which has been associated to increased proliferation while improving FAO led to apoptosis [215]. This is consistent with the up regulation of the enzyme carnitine palmitoyltransferase 1A (CPT1A), which catalyzes the rate-limiting step of β-oxidation in the mitochondria of AML cells. In addition to enhancing CPT1 activity, leukemic cells also inhibit the negative regulator of FAO, malonyl CoA, which is produced by the enzyme acetyl-CoA carboxylase[216].

In addition to FAO, the up regulation of hexokinase-II, glucose transporter-1, lactate dehydrogenase (LDH), and hypoxia-induced factor 1α (HIF-1α) has been observed in AML patients with incomplete remission as compared to AML patients with complete remission[217]. The development of leukemia has also been linked to several additional TCA cycle enzymes. For example, in MEIS1/Hoxa9-driven leukemia, fumarate hydratase, which transforms fumarate into malate, was found crucial for the development but not maintenance of leukemia-initiating cells[218]. Moreover, in the AML K562 cell line enhanced Pyruvate carboxylase activity has been reported. This enzyme catalyzes carboxylation of pyruvate to oxaloacetate; the first reaction of gluconeogenesis [219]. Besides, accumulations of fumarate, 2-oxoglutarate, and succinate due to mutations in fumarate hydratase, IDH, and succinate dehydrogenase was also shown. This may prevent the conversion of 5-methylcytosine to 5-hydroxymethylcytosine by TET, which in turn inhibits the epigenetically regulated genes in AML. The significance of maintaining a balance between these cytosines demonstrate how TET-2 implicated in AML[220].

Finally, the dependence of leukemic cells on electron transport chain for energy needs and their poor prognosis have been described in several studies. For instance, low activity of ATP synthase in leukemia may be linked to a poor prognosis because β-F1-ATPase (part of complex V of the respiratory chain) is down regulated in AML patients with no remission compared to patients with complete remission[221].

Taken together, mitochondrial protein elements such mitophagy, intermediary metabolism enzymes, oncometabolites, and ROS generation may contribute to hematological malignancies, and they may be used as diagnostic and therapeutic targets in leukemia.

2. RATIONALE OF THE STUDY

Hematopoietic stem cells (HSC) can accumulate somatic mutation during lifetime. These can provide a positive selection leading to the expansion of selected clones. When clones expansion is not associated with cytopenia or other neoplastic signatures, creates a peculiar condition, named clonal hematopoiesis of indeterminate potential (CHIP). This condition is associated with increased risk of myeloid dysplastic syndrome (MDS) and acute myeloid leukemia, and it is now considered a pre-leukemic condition[222]. Interestingly, not all the subject displaying CHIP later develop neoplasia and CHIP can precedes non-malignant diseases.

The identification of the molecular mechanism leading to pre-leukemic establishment and, specially, its evolution to MDS or AML are of outstanding importance to improve diagnosis and treatment of these pathologies. Mitochondria gained considerable attention in both HSC maintenance [185] and MDS/leukemia development. These can indeed integrate metabolism, cell death and epigenetic landscape, making them a pivotal platform for signal transduction in normal and malignant conditions. In addition (1) Dnmt3a and TET2 (among the most mutated genes in pre-leukemia and MDS/AML) regulates epigenome through the mitochondria-related 5 methyl cytosine [223](2) both these enzymes can localize to mitochondria [224, 225]and (3) other mutations related to pre-leukemia have impact on mitochondrial functions. These consideration lead to the formulation of the rationale that mitochondria might participates in the establishment of pre-leukemia and/or its transition to secondary neoplasia.

3. OBJECTIVE

The aim of our work is to elucidate mitochondrial dysfunction in pre-leukemia stage of acute myeloid leukemia due to disruption of DNA methylation modifiers. Here the goal is to identify a common mitochondrial phenotype in DNMT3A and TET2 loss of function.

The specific objectives are:

1. To identify a mitochondrial phenotype in DNMT3A and TET2 mutations by assessing mitochondrial features such as bioenergetics, morphometric and interaction with intracellular structures, afferent and efferent signals, biogenesis or recycle (mitophagy) rates. Given that Ca^{2+} cycling and mitochondrial bioenergetics are interlinked subcellular analysis of Ca^{2+} homeostasis will be investigated (mitochondria, cytosol, ER, plasma membrane) will be achieved by: (I) the use of targeted aequorin chimeras for "in population" measurements (II) single cell imaging studies and in mechanism underlying in DNMT3A and TET2 mutations.

2. Characterization of mitochondrial route in pre leukemia /secondary neoplasia establishment will be investigated by generation of the mitochondrial phenotype. Major molecular determinants of mitochondrial physiology will be investigated to define the origin of the phenotype.

3. The final task will attempt to prove targetability of mitochondrial phenotype. We will exploit this repertoire to target the metastatic phenotype, giving priority to drugs already in clinical trials. Drugs will include: (I) inhibitors of mitochondrial fuel, signaling, respiration/biogenesis, (II) regulators of mitochondrial dynamics and stress response, and (III) inducers of cell death.

4. METHODS

4.1. Cell culture

HEK293T cells, were cultured in complete high glucose DMEM (containing 4 mM L-glutamine and 25 mM glucose) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin [226] and maintained at 37 °C in a humidified incubator containing 5% CO2.

4.2. ShRNA-Mediated Knockdown and Stable cell lines generation

Lentiviruses were produced by transfecting shRNA-targeting plasmids together with helper plasmids REV–VSVG and pMDL into HEK293T cells using Lipofectamin 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell supernatants were collected 48 h after transfection. After transfection, Lentiviruses particles in the medium were collected every 24 h, and after two collections the medium was filtered, then the target cell lines were infected or stored at -80°C. To obtain stable cell lines, cells were infected at confluence 60% for 24 h with lentiviral supernatants diluted 2:8 with normal culture media. Cells were subjected to Puromycin selection 24 h after infection. At 48 h after infection, 2 µl/ml Puromycin for shDNMT3A/PLKO and 500 µg/ml Hygromycin for shTET2 / TET2 CTRL (a plasmid of psi LVRH1H with 19 nucleotide length out shRNA mammalian target) were used selection for three weeks. Finally knockdown efficiency was confirmed using immunoblotting. In addition, HEK293T cells were seeded and the next day were treated with DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (DAC), at 5 µM or DMSO vehicle control for 48 h as has been described previously [227] and knockdown of DNMT3A was confirmed using immunoblotting.

4.3. Mitochondrial membrane potential

For mitochondrial membrane potential, cells were seeded at on 25-mm coverslips coated with Poly-L-lysine, allowed to grow for 24 h, and then incubated with 1 μM JC-1 (Invitrogen), in combination with Hoechst at 37 °C, or 30 min which is followed by wash. A decrease in mitochondrial depolarization patterns of cells by means of a shift from red fluorescence to green fluorescence was then examined using inverted fluorescent microscope.

4.4. TMRM assay for Complex I and II activity

To measure mitochondrial complex activity, cells were incubated for 10min 37 °C for TMRM containing solution MAS1 (Mannitol 220 mM, Sucrose 70 mM, KH2PO4 10 mM, $MgCl_2$ 5 mM, HEPES 2 mM, EGTA 1 mM, BSA 0.2% (w/v) and 10 nM TMRM) and equal amount of 50 μM digitonin contain MAS1 solution was added for Permeabilization for 10 min at 37 °C. Then all the buffer was removed and replaced with reducing equivalents containing solution MAS3 (MAS1, 4mM ADP, 10 mM pyruvic acid and 0.5 mM Malic Acid). Cells were installed into the Leyden chamber, incubate with 900 μl of MAS3 and images where taken NIKON with set acquisition of Speed: 1 frame per second, BIN: 2 Digitizer: 14MHz, in low laser, scanner: Slitta 30. Complexes activity were monitored by first acquiring 10 min of basal followed adding 20 μM of rotenone for complexes one inhibitor and 100 μM Succ, 200 μM TTFA, 10 μM FCCP in 5 min interval. TMRM fluorescence were calculated based on the maximum and minimum rates of complexes I and complexes II activities.

4.5. Mitochondrial Morphology Analysis

Cells were seeded at on 25-mm coverslips coated with Poly L lysine, allowed to grow for 24 h, and then infected with mtDsRed (Ex/Em: 556/586). After 36 h, the cells were imaged on a confocal Nikon Eclipse Ti system. Coverslips were placed in incubated chamber with controlled temperature and then z-stacks were acquired by 21 planes with 0.6 mm distance, to allow acquisition of the whole cell.

After acquisition, images were restored with the Autoquant 3D blind deconvolution module, installed on NISElements (Nikon Instruments Inc.), using a theoretical PSF. After restoration, images were loaded in Imaris 4.0 (Bitplane AG, Zurich, Switzerland), then subtracted of background and used to generate a threshold based isosurface object group. From each isosurface was calculated the number of objects and the average object volume expressed as voxel number.

4.6. Immunofluorescence

Cells were grown on 25-mm coverslips coated with poly L- lysine and washed with PBS and fixed in 4% formaldehyde for 10 min at 37°C. After washing three times with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 2 h at room temperature and then blocked with PBS-T containing 2% BSA at room temperature for 1h. Cells were then incubated with primary antibodies ATP5I (1: 100 anti-rabbit) and mTFAM (1:100 anti-mouse) overnight at 4°C, washed 3 times with PBS-T, and incubated with Alexa 594 goat anti-rabbit and Alexa 488 goat anti-mouse in BSA in room temperature secondary antibodies.

4.7. Fluo4 measurements

Coverslips with cell monolayers were incubated at 37°C for 30 min in DMEM supplemented with 5 μM Fluo 4-AM. After a brief wash with KRB / Ca^{2+} they were placed in an open Leyden chamber on the thermostated stage of a Zeiss Axiovert 100 inverted microscope, equipped with a sutter filter wheel and exposed to 340/380 wavelength light. Fluorescence data, collected with a Princeton instruments back-illuminated camera, were calculated with Metafluor software and expressed as emission ratios.

4.8. Aequorin measurements

For cytAEQ and $mtAEQ^{WT}$ at 36 h post-transfection, the coverslips were incubated with 5 μM coelenterazine for 1.5 h in Krebs-Ringer modified buffer (KRB) supplemented with 1 mM $CaCl_2$ (KRB: 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO4, 1 mM $MgSO_4$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4, at 37°C).

To reconstitute erAEQ with high efficiency, the luminal [Ca^{2+}] of the ER was first reduced by incubating the cells for 45 min at 4°C in KRB supplemented with 5 μM coelenterazine, the Ca^{2+} ionophore Ionomycin, and 600 μM EGTA. After incubation, the cells were extensively washed with KRB supplemented with 2% bovine serum albumin (BSA) and 2 mM EGTA before the luminescence measurement was initiated. Aequorin signals were measured in KRB supplemented with either 1 mM $CaCl_2$ or 100 mM EGTA using a purpose-built luminometer. The agonist (ATP at 100 μM) was added to the same medium. The experiments were terminated by lysing the cells with Triton X-100 in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The light signals were collected and calibrated with [Ca^{2+}] values. Further experimental details have been previously described[228].

4.9. Mitochondrial Ca^{2+} concentration measurements with 2mt-GCaMP6m

To test mitochondrial Ca^{2+} concentrations with high sensitivity, we used a new Ca^{2+} probe based on the last-generation GCaMP probe [229] targeted to the mitochondrial matrix. We chose the GCaMP6m version because it had the highest Ca^{2+} affinity. To measure the signal independent of variations in basal fluorescence intensity due to the variable expression levels of the probe, we took advantage of the isosbestic point in the GCaMP6m excitation spectrum; exciting GCaMP6m at 406 nm led to fluorescence emission that was not Ca^{2+} dependent. As a consequence, the ratio between the excitation wavelengths of 494 and 406 nm was proportional to the Ca^{2+} concentration and independent of probe expression levels. Cells were imaged with an IX-81 automated epifluorescence microscope (Olympus) equipped with a 40× oil immersion objective (numerical aperture 1.35; Olympus) and an ORCA-R2 charge-coupled device camera (Hamamatsu Photonics).

4.10. ER Ca^{2+} concentration measurements with ER-GCaMP6m-210

For the endoplasmic reticulum (ER) Ca^{2+} measurement we used ER-GCaMP6-210 to test the release of ER Ca^{2+} concentrations with high sensitivity, we used a new Ca^{2+} probe based on the last generation GCaMP targeted to the ER. We chose the GCaMP6m because it had the highest Ca^{2+} affinity. To evaluate its response to agonists, ATP was added to 1mM Ca^{2+}/Krebs-Ringer buffer, and the Ca^{2+} ratio was recorded.

We took advantage of the isosbestic point in the GCaMP6-210m excitation spectrum; exciting GCaMP6- 210 m at 406 nm led to fluorescence emission that was not Ca^{2+} dependent. As a consequence, the ratio between the excitation wavelengths of 494 and 402 nm was proportional to the Ca^{2+} concentration and independent of probe expression levels. Cells were imaged with an IX-81 automated epifluorescence microscope (Olympus) equipped with a 40x oil immersion objective (numerical aperture 1.35; Olympus) and an ORCAR2 charge-coupled device camera (Hamamatsu Photonics).

4.11. ROS measurement

To measure intracellular Reactive Oxygen Species (ROS) cells were seeded at on 25-mm coverslips coated with Poly-L-lysine, allowed to grow for 24 h, then incubated in 25 μM 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) containing PBS at 37°C for 30 minutes. Cells were then washed with PBS to remove the extra reagent that had not entered the cells, and the fluorescence intensity was immediately measured by fluoresces microscope.

4.12. Immunoblotting

Control and shDNMT3A and shTET2 HEK293T cells were harvested, washed, and pelleted (1100 rpm, 5 min, +4°C) in phosphate-buffered saline (PBS), suspended in RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (pH 7.4), and homogenized. After measurement of the concentrations, proteins were separated by SDS–PAGE on a 4–12% precast gel, and the levels of two protein markers for each were detected using the primary antibodies DNMT3A (Abcam ab2850, 1:1000), TET2 (Abcam, ab243323, 1:1000), TFAM (Cell signaling, 7495, 1:1000), PGC -1α (Boster, M00236., 1:1000), MCU (sigma , HPA016480, 1:1000), MICU1 (HPA037480. 1:1000), β -Actin (Merck, A1978, 1:5000), TOM20 (Santa Cruz, sc-136211, 1:1000), EMRE (Abcam ,157387, 1:1000), IP3R3 (Santa Cruz, sc-7277, 1:1000), IP3R1(Santa Cruz,, sc-6093, 1:1000), IP3R2 (Santa Cruz, sc-398434, 1:1000), Total oxphos (Abcam, ab110413, 1:1000), AMPK (cell signaling, 35277, 1:1000), P-AMPK (cell signaling, 2535, 1:1000), PMCA4 (Santa Cruz, sc-22079, 1:1000). STIM1 (Santa Cruz sc-68897, 1:1000), ORI1 (Santa Cruz, sc-68895, 1:1000), SERCA (Santa Cruz, sc-8095, 1:1000.

The membranes were then treated with specific HRP-labeled secondary antibodies, followed by chemiluminescence detection using a ChemiDoc Touch Gel Imaging System.

4.13. Mitochondrial DNA (mtDNA) quantification

Cells were grown on 25-mm coverslips coated with poly L- lysine and the amount of total mitochondrial DNA was measured using PicoGreen (Thermo Fisher Scientific) for nucleoid count and TMRM for mitochondria identification and analyzed with fluorescence microscopy.

4.14. Mitophagy assessment

Cells were grown on 25-mm coverslips coated with poly L- lysine and transfected with mtCFP as indicator of mitochondria and LAMP1-EGFP to identify lysosomes and confocal analysis of colcalization coefficient was used to characterize the degree of overlap between two channels and the degree of mitophagy were calculated between the ratio of mitochondria and lysosomes.

4.15. IP3R3 quantitative polymerase chain reaction

Total RNA was isolated using TRIzol and stored at -80 ºC until use. An Omniscript RT kit (205110, QIAGEN) was used for cDNA synthesis. Briefly, the template RNA (50 ng to 2 µg) was added to master mix containing buffer, dNTP mix, specific primers, RNAase inhibitor, and Omniscript reverse transcriptase and incubated for 60 min at 37 ºC after mixing thoroughly. Less than 5 µl of the finished reverse-transcription reaction containing cDNA was used for quantitative polymerase chain reaction (qPCR) by a QuantiNova SYBR green PCR kit (208052, QIAGEN) in a real-time cycler (Bio–Rad). The primer sequences (IP3R3 Forward 5'-CCAAGCAGACTAAGCAGGACA-3': IP3R3Reverse 5'-ACACTGCCATACTTCACGACA-3'. IP3R3 mRNA expression values were normalized to GAPDH, and the relative expression level was computed using standard methods ($2^{-\Delta\Delta Ct}$) with three independent technical repeats.

4.16. Determination of cell death: Propidium Iodide and SYTOX Green

Common chemotherapy drugs cytarabine (AraC) 10 μM for 72 h, and Daunorubicin (DNR) 5 μM for 48 h were used to assess resistance against shDNMT3A and shTET2 293T stable clones as previously described[230,231]. To quantify cell death after the experimental procedures, the cells were washed with normal Krebs and double stained with Hoechst and Propidium Iodide (PI) or SYTOX green (SG) for 25 min at 37°C in a phosphate buffer solution. Stained cells were examined immediately with a standard inverse fluorescence microscope at 480 nm and 546 nm. PI- and SG positive cells were counted in three representative high power fields of independent cultures and cell death was determined by the ratio of the number of PI positive cells/PI or SG stained positive cells.

4.17. Statistical analyses

All data were analyzed using Graph Prism Pad 8 (GraphPad software Inc, La Jolla, California). Quantitative analysis was expressed as means ± S.E.M, Anova test and paired t-test was used to generate P values for comparison between groups in each data set $P< 0.05$ is considered as significant.

5. RESULTS

5.1. Loss of DNMT3A but not TET2 promotes mitochondrial biogenesis in HEK293T cells

5.1.1. DNMT3A deletion through shRNA in HEK293T cells increase mitochondrial volume

Mitochondria are dynamic organelles that continually fuse and divide to form tubular networks [232]. This evolutionarily conserved activity requires several large GTPases and other mitochondrial proteins that affect membrane composition (IMM and OMM) [233]. To evaluate changes in mitochondrial morphology in our experimental model, we first established stable cell lines using Lentiviruses by transfecting shRNA-targeting plasmids of DNMT3A and TET2 into HEK293T cells and exposed for selection after infection. Immunoblotting analysis was conducted to confirm the resulting knockdown efficiency of shDNMT3A and TET2 in HEK293T as illustrated in figure 7A and B. To understand mitochondrial morphology features we used mitochondrial-targeted red fluorescence protein mtDsRed. Specifically, we transfected 293T cells with mtDsRed in shDNMT3A and shTET2 stable clones. Confocal images revealed an increase of mitochondria volume in shDNMT3A–transfected cells compared with the control PLKO (figure 7C and D). Moreover, shDNMT3A 293T cells also showed a higher average mitochondrial volume (figure 7E). On the other hand, analyses of the total mitochondrial network and volume revealed no significant differences in shRNA of TET2 in 293T cells (figure 7H, I and J). These observations indicate that DNMT3A ablation results in remodeling of the mitochondrial morphology with larger, relative distribution with no changes in number.

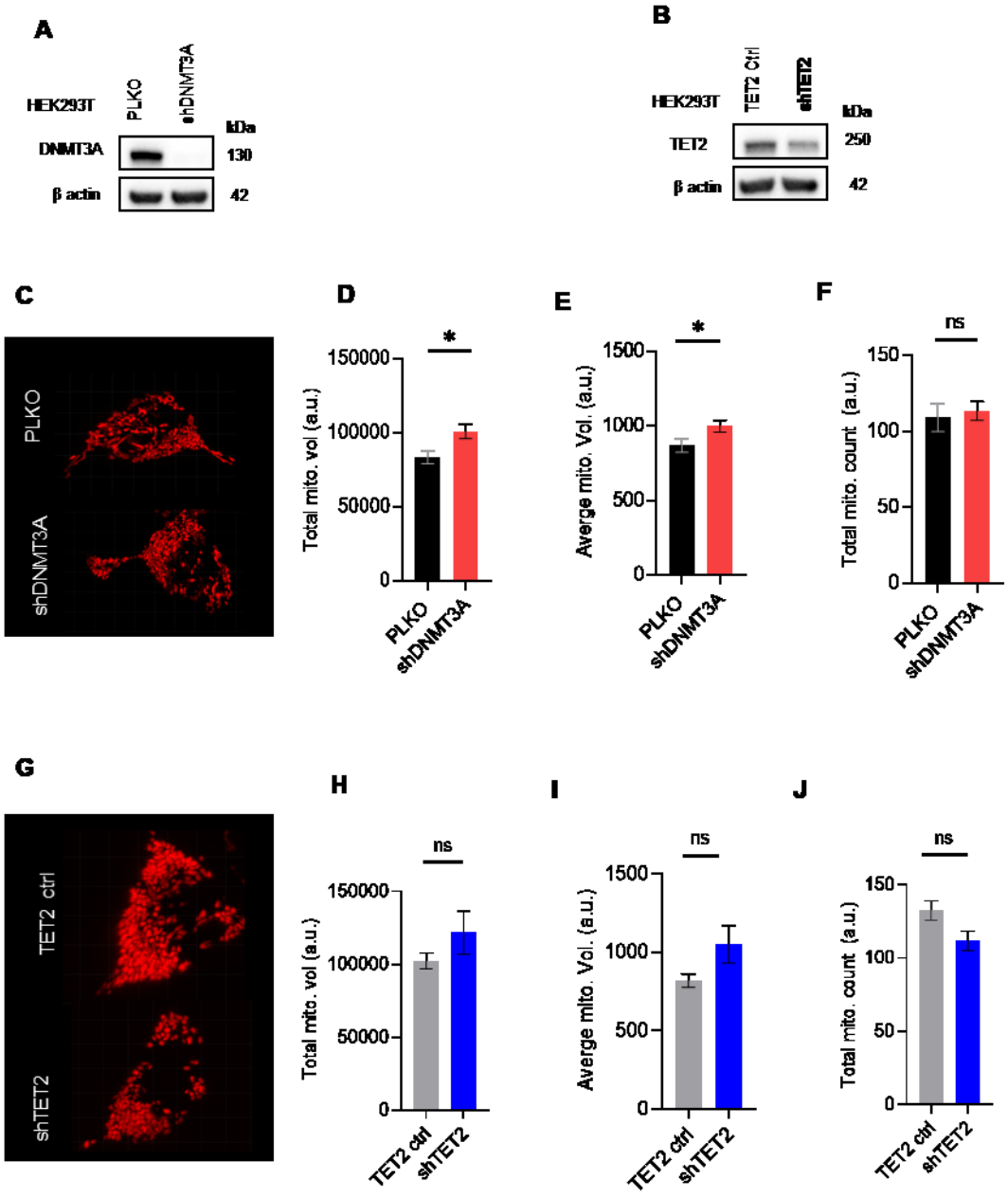

Fig. 7 Deletion of DNMT3A through shRNA in HEK293T cells increased mitochondrial volume. **(A)** Confirmation of slicing DNMT3A and **(B)** TET2 in HEK393T cells. Analysis of mitochondrial morphology was done by transfecting 293T cells with an mtDsRed for 36 h **(C)** representative image of mitochondrial morphology upper PLKO and lower shDNMT3A **(D)** increased total and **(E)** average mitochondrial volume with **(F)** no difference in mitochondrial count in shDNMT3A **(G)** representative image of mitochondrial morphology upper TET2 ctrl and lower shTET2 **(H)** indicating no alteration of mitochondrial morphology of total volume **(I)** average volume **(J)** and mitochondrial count in shTET2 under three experimental conditions via confocal microscope. Images acquired by confocal microscopy were deconvolved, 3D reconstructed and quantitatively analyzed. The data represent average ± SEM. Unpaired, two-tailed Student's t-test was used. Statistical significance: *$p \leq 0.05$.

5.1.2. ShDNMT3A increased mitochondrial TFAM and mitochondrial DNA

The size of the mitochondrial network at any given time is determined by combining effects of mitophagy and mitochondrial biogenesis [234]. Thus, we hypothesized that loss of DNMT3A might promote mitochondrial biogenesis. Mitochondrial biogenesis requires a complex interplay between nuclear and mitochondrial genomes [235]. Accumulating evidence indicate PGC-1α as a master regulator of mitochondrial biogenesis, strongly inducing the expression of downstream transcription factors such as mitochondrial transcription factor A (TFAM)[236]. The interactions between TFAM and mtDNA helps to regulate mitochondrial biogenesis and maintain the number of mtDNA[237, 238]. In fact, studies have shown that mtDNA copy number, mtDNA transcription, and translation of mtDNA-encoded polypeptides, as well as some mitochondrial functions, may closely reflect TFAM expression[237]. Thus, we ran immunofluorescence assays that specifically targeted the main mtDNA, TFAM and mitochondrial content, ATP5I. The confocal image analysis showed significant increase of TFAM to ATP5I ratio in shDNMT3A 293T cells (figure 8A). Furthermore, since mitochondria have their own genome, the amount of mitochondrial DNA is proportional to the number of mitochondria[239]. To further demonstrate this concept, we utilized a mitochondrial marker, TMRM to identify mitochondria and PicoGreen as a DNA dye to recognize mitochondrial DNA. Co-localization of the red TMRM and green PicoGreen in shDNMT3A HEK293T cells reveals an increase in mitochondrial DNA (figure 8B) which supports the up regulation of mitochondrial biogenesis. In addition, PGC-1α, is also regarded as master regulator of mitochondrial biogenesis and its interaction with mtTFA and NRF-1 has been described as a marker of mitochondrial biogenesis[240]. We thus, further confirmed the mitochondrial biogenesis-related protein expressions such us PGC-1α and TFAM by immunoblotting. Our results indicate up regulation of TFAM and PGC-1α in shDNMT3A (figure 8C) suggesting a positive regulation of mitochondrial biogenesis.

Mitochondrial homeostasis is maintained by delicate balance of two opposing processes, the production of new mitochondria through mitochondrial biogenesis and the degradation of damaged mitochondria through mitophagy[241]. To further confirm the increase mitochondrial biogenesis in shDNMT3A HEK293T cells, we transfected cells with mtCFP as indicator of mitochondria and LAMP1-EGFP as marker of lysosome. The amount of mitochondrial accumulation in lysosomes was then measured using confocal imaging. Colocalization coefficient analysis showed no significance difference in the rates of mitophagy (figure 8D). These results demonstrate that DNMT3A is a suppressor of mitochondrial biogenesis in HEK293T cells.

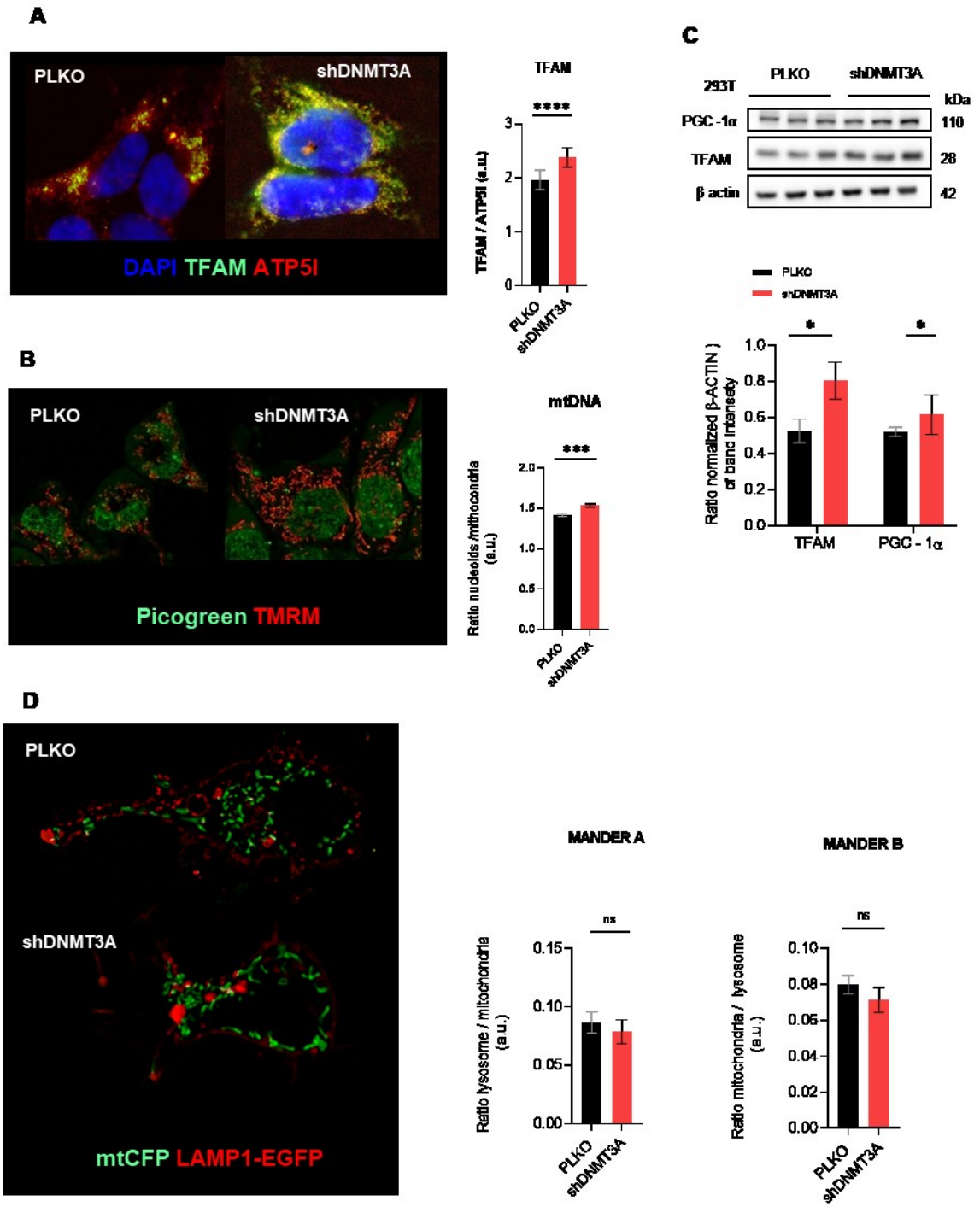

Fig.8 shDNMT3A increased mitochondrial biogenesis. Confocal microscopy assessment in control PLKO and shDNMT3A 293T cells show **(A)** increased TFAM to ATP5I ratio **(B)** increased mtDNA with PicoGreen (Green-DNA) and TMREM (red- mitochondria) **(C)** western blot analysis of PGC-1α , TFAM expression and normalized to β-actin **(D)** assessment of mitophagy were done by transfecting shDNM3A with mtGFP to visualize mitochondria (GREEN) and LAMP-GFP (RED) to indicate lysosomes. The degree of the signal of lysosomes with mitochondria (merge) was calculated via cell live imaging microscopy and Manders' overlap coefficient localization in the red channel overlaps localization in the green channel and vice versa. Graphs represent the amount of colcalization between lysosomes (red signal) and mitochondria (green signal). N = 10 visual fields per condition. The data represent average ± SEM. Unpaired, two-tailed Student's t-test was used. Statistical significance: *p ≤ 0.05; ***p ≤ 0.001. ; ****p ≤ 0.0001.

5.2. Energy status and mitochondrial complexes activity

5.2.1. Effects of disruption of DNMT3A and TET2 on intercellular reactive oxygen species

Evidence suggests that mitochondrial morphology and ROS production are inversely correlated. It has been suggested that mitochondrial shape and structure are closely related to the regulation of redox homeostasis by adjusting ROS as downstream signal [242] Moreover, mitochondria are major sources of superoxide and other downstream reactive oxygen species [243]. We thus, examined whether TET2 and DNMT3A – silenced 293T cells affect intracellular ROS accumulation. We tested the internal ROS production using 2′, 7′-dichlorofluorescein diacetate (DCF-DA) as reporter. The shRNA DNMT3A cells suppressed intracellular ROS accumulation (figure 9A) while TET2 increased ROS production (figure 9 B). A strong correlation between ROS generation and mitochondrial membrane potential (ΔΨm) has already also been reported[244,245]. The inner mitochondrial membrane potential can quickly depolarize in response to oxidative stresses brought on by ROS[244]. At present, it is widely accepted that mitochondria produce more ROS at high membrane potential. It has been shown that ROS production dramatically increases above 140 mV. Studies performed on mitochondria from Drosophila melanogaster showed that even a slight decrease in ΔΨm (10 mV) can lead to reduction in ROS production [246]. Interestingly, in certain pathological conditions, opposite correlations between ΔΨm and ROS production can also be observed[247, 248]. To assess the involvement of ΔΨm in ROS production of shTET2 and shDNMT3A, we measured ΔΨm using the Cell-permeant potentiometric dye JC-1 using fluorescence microscopy. When added into mitochondria the cationic carbocyanine dye JC-1 fluoresces in the green channel and exhibits large aggregates that fluoresce in the red channel. Fluorescent properties can be monitored as an indicator of ΔΨm. The shRNA silenced DNMT3A and TET2 stable clones in 293T cells showed no significant change ΔΨm (figure 9C and D). Therefore, alterations in ROS production observed cannot be related to changes in mitochondrial membrane potential in 293T cells.

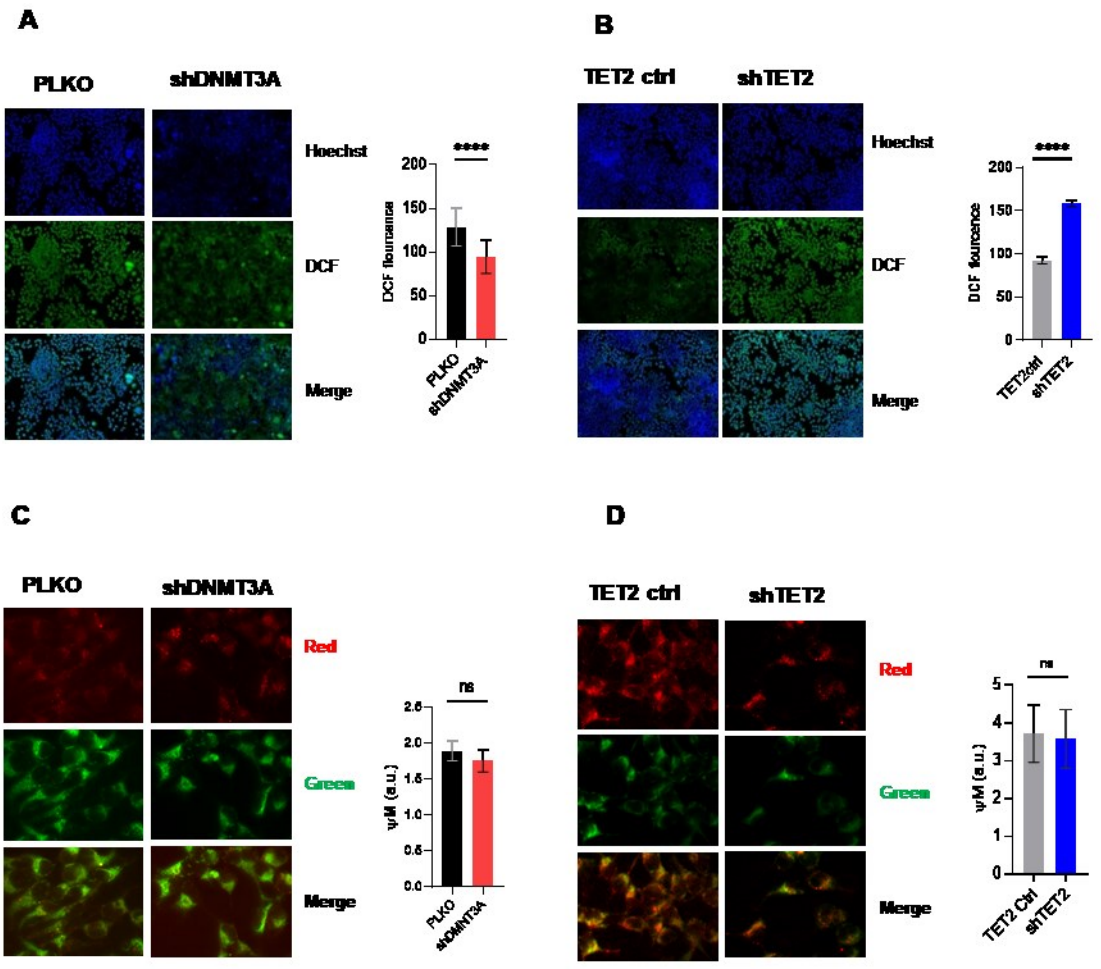

Fig 9. Mitochondrial membrane potential (ΨM) and intracellular ROS in shDNMT3A and shTET2. (A) Observation of the intracellular ROS production in shDNMT3A and (B) shTET2 using DCFH-DA as the detection probe (Blue for Hoechst, Green for DCF and scale bar=100 µm) (C) ΨM measurement using JC1 dye in shDNMT3A and (D) shTET2 using JC -1. Red (~590 nm)/green (~529 nm) fluorescence intensity ratio was used to quantify ΨM. Representative image scale bar=100 µm. The data represent average ± SEM of three independent experiment. Unpaired, two-tailed Student's t-test was used. Statistical significance: ****p ≤ 0.0001.

5.2.2. Loss of TET2 decreased mitochondrial complexes II activity

One of the major sources cellular ROS is mitochondrial respiratory chain typically during ATP synthesis. Consequently, ROS considered as by-products of energy perfusion to cell activities [249]. Moreover, metabolic alterations can affect epigenetic modifications through numerous metabolites act as substrates, inhibitors and cofactors for the enzymes that modify the epigenome; conversely, the epigenome can influence the transcription of metabolic genes[250].

In addition, it has been demonstrated that the pathogenesis of AML is influenced by the altered metabolic state of AML cells and the function of mitochondria[251]. For instance, oncogenic drivers of AML such as mutations in the mitochondrial enzyme isocitrate dehydrogenase-2 (IDH2) has been associated with reduced ATP synthase function and overall reduction in mitochondrial energy metabolism. IDH2 converts alpha-ketoglutarate into the R-enantiomer 2-hydroxyglutarate ((R)-2HG), which is key substrate in epigenetic modification such as DNA hypermethylation[252]. Based on these observations we aimed to assess whether metabolism is influenced by inactivating in epigenetic enzymes of DNMT3A and TET2. One such crucial metabolic energy sensor is AMP-activated protein kinase (AMPK) that become activated by increasing substrate adenosine monophosphate (AMP) levels under conditions of cellular stresses [253]. We first examined AMPK phosphorylation to assess cellular stress in shDNMT3A and shTET2. Our immunoblotting results show that the level of P-AMPK as measured in shDNMT3A (figure 10A) and shTET2 reveals no significant change (figure 10B).

Oxidative phosphorylation (OXPHOS) is a crucial intracellular process that produces ATP to fuel the cell. The OXPHOS system is composed of the mitochondrial complexes, formed by large four multimeric proteins and two groups of mobile electron carriers, plus ATP synthase complex that generates ATP [254]. Impairment or inefficient electron transfer through mitochondrial respiratory complexes has been linked to the deleterious causes human diseases due to reduced supply of energy metabolism and generation of ROS mainly through complexes I, II, and III[255]. In addition, hematological malignancies such as acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma have been associated with enhanced levels of mtDNA content and are highly dependent on OXPHOS for survival [256]. A growing number of studies have also suggest that the HSC and MPP fractions exhibit different levels of mitochondrial respiratory complexes expression[257,146]. Indeed, inactivation of Uqcrfs1 which encodes for the Rieskeiron-sulfur protein (RISP), an essential subunit of the mitochondrial CIII in fetal HSCs leads to decline in expression of genes required for self-renewal capacity and reduction in multi-lineage repopulation potential HSC [143]. This effect is attributed to decreased levels of citrate produced in mitochondria and increased 2-HG levels which could reduce NAD+/NADH ratio, causing DNA and histone hypermethylation[143]. Thus, in order to assess changes in protein expression of mitochondrial respiratory complexes due to loss of DNMT3A and TET2 we performed immunoblotting analysis using a "total OXPHOS (complexes I, II III, IV and V)" antibody cocktail.

The blot results showed an overall no significant differences in protein content in both conditions (figure 10C and D). However, there was a trend towards decrease in complex expression I, II and III.

Complex I, or NADH dehydrogenase pumps four H^+ into the inner membrane space by binding NADH and transfers two electrons to ubiquinone resulting NAD^+ and ubiquinol [258]. Complex I of the electron transport chain is particularly prone to dysfunction or damage, especially when ROS are present. In damaged mitochondria, succinate can potentially act as a fuel source thus bypass complex I to produce ATP and maintain membrane potential. Balancing ATP levels from dropping to critically low levels is essential in order to prevent the activation of cell death pathways[259]. A "shortcut" to ATP production through oxidative metabolism is made possible by succinate, an intermediate in the TCA cycle and is readily converted to fumarate, which interacts directly with the mitochondrial electron transport chain[260]. Succinate dehydrogenase (Complex II) oxidizes succinate and transfers two electrons via FAD to Quinone. However, this complex does not directly affect the proton gradient[261]. Hence, we sought to furfure understand mitochondrial complex activities in shDNMT3A and shTET2. To this purpose cells were incubated with TMRM and digitonin for mitochondrial staining and Permeabilization respectively. Before monitoring complexes' activity cells were supplemented with pyruvic acid and malic acid solutions to provide reduced equivalents. CI activity was monitored upon addition of strong inhibitor of complex I, rotenone which resulted no significance difference in both conditions (figure 10E). Immediately after being exposed to the uncoupler such as FCCP the maximal potential respiration achieved and proton gradient no longer regulates electron transfer. Complex II activity were obtained by subtracting the FCCP-stimulated rate from the maximum rate of complexes II following addition of succinate. The results showed a decreased complexes II activity in shTET2 with respect its control but not for shDNMT3A (figure 10F). This suggests loss of TET2 in shRNA 293T cells might contribute to metabolic and ROS related mitochondrial dysfunction.

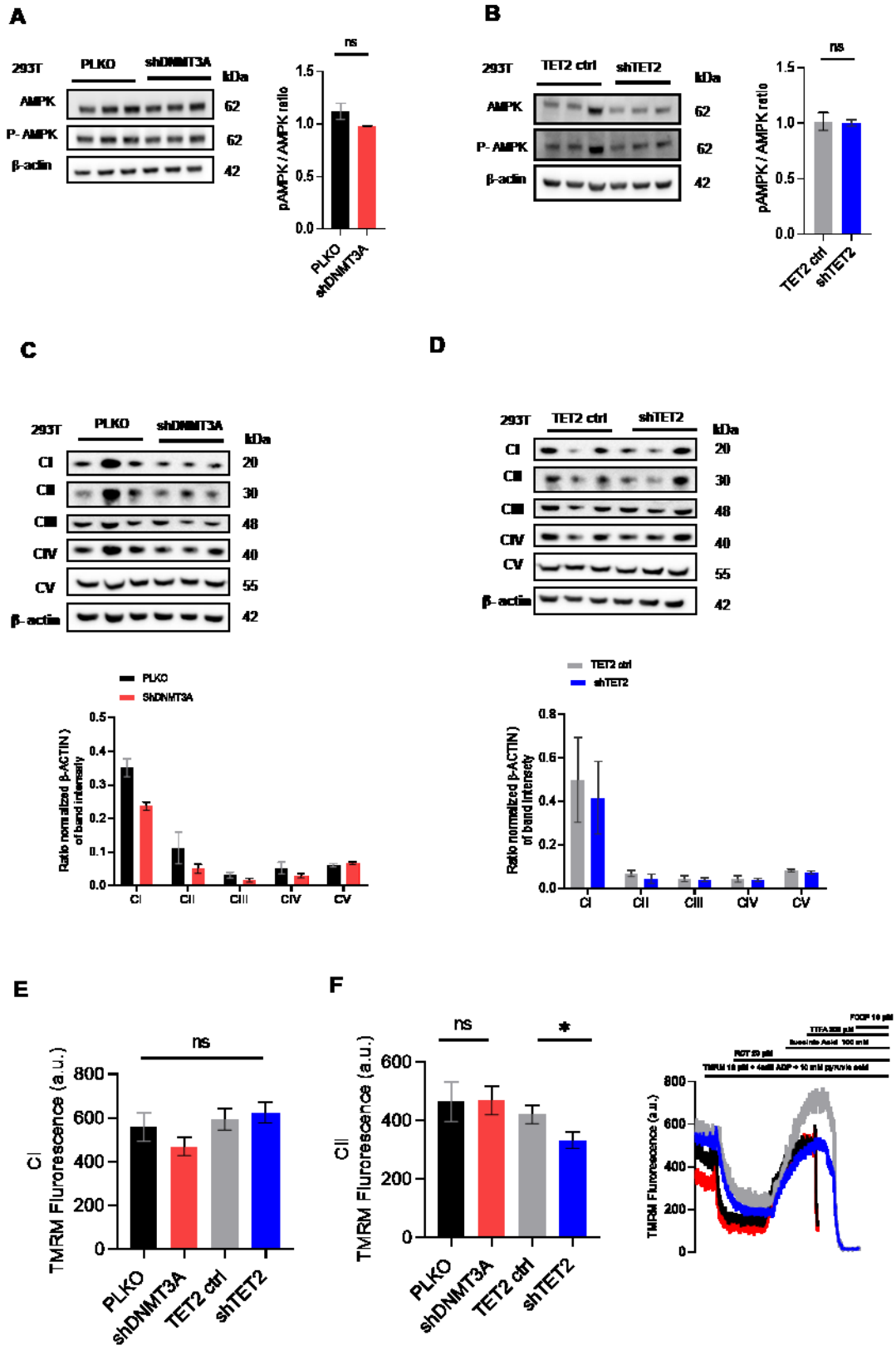
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Fig.10 mitochondrial functional assessment. (A) Western blot analysis of AMP-activated protein kinase α (AMPK), and the phosphorylated (activated) form of AMPK α (P-AMPK) for shDNMT3A 293T cells and **(B)** shTET2 normalized to β-actin **(C)** An antibody cocktail against proteins representing the five mitochondrial oxidative phosphorylation complexes (complexes I, II, III, IV and V) was used to examine the expression of mitochondrial oxphos proteins in shDNMT3A and **(D)** shTET2 293T cells normalized to β-actin **(E)** Complex I (CI) and **(F)** complex II (CII) activity measured by the increment in TMREM intensity followed by the addition of Rotenone, Succinate, and FCCP detected by Nikon- Fluorescence microscope. The data represent average ± SEM of three independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance: *$p \leq 0.05$.

5.3. Disruption of DNA methylation alters calcium hemostasis

5.3.1. Deletion of TET2 and DNAMT3A in shRNA 293T cells reduces mitochondrial calcium uptake

Early evidence on HSC activity and Ca^{2+} signaling demonstrated how calcium affects HSC function. Studies reveal that specific knockout of mitofusin 2 (Mfn2) diminished the function of HSCs with extensive lymphoid potential. Deletion of Mfn2 elevated intracellular calcium resulting the nuclear translocation of NFAT in HSC[177]. Mfn2 mediate outer mitochondrial membrane fusion crucial to the maintenance of the mitochondrial morphology and operation of the mitochondrial network[262]. This suggests that mitochondria biogenesis affects HSCs fate determination through alteration of calcium dynamics. Likewise studies have also highlighted that HSCs are endowed with lower intracellular calcium levels overall when compared to progenitors. This finding was supported, among other things, by the higher expression and activity of the plasma membrane Ca^{2+} pumps (PMCA) in HSCs compared to other hematopoietic cells [263]. Moreover, a Ca^{2+}- Calpain axis was also suggested to regulate HSC function. One of the predicted targets for Calpains is TET2 enzymes which are necessary for the demethylation of 5mC[188]. The handling of $[Ca^{2+}]_m$ in pre-leukemic HSC or malignant stem cells in comparison to their normal counterparts has not yet been directly assessed. Accordingly, we propose that inactivation of DNMT3A or TET2 might affect mitochondrial calcium handling. Thus we examined the effect of DNMT3A and TET2 knockdown on mitochondrial Ca^{2+} uptake using aequorin-based mitochondrial Ca^{2+} probe and 4mtGCaMP6m indicator which specifically localized in the mitochondrial matrix. Under all conditions, we monitored Ca^{2+} response to ATP, which activates Gq-coupled receptors to produce IP3, inositol 1, 4, 5 triphosphate.

Aequorin-based transfection of shTET2 and shDNMT3A with mitochondrial Ca^{2+} probe revealed that the genetic inactivation of both proteins significantly reduced $[Ca^{2+}]_m$ uptake (figure 11A and B). This is also evident using the mito-GCaMP6m indicator, which shows lower $[Ca^{2+}]_m$ levels in shDNMT3A (figure 11C) and TET2 (figure 11D).

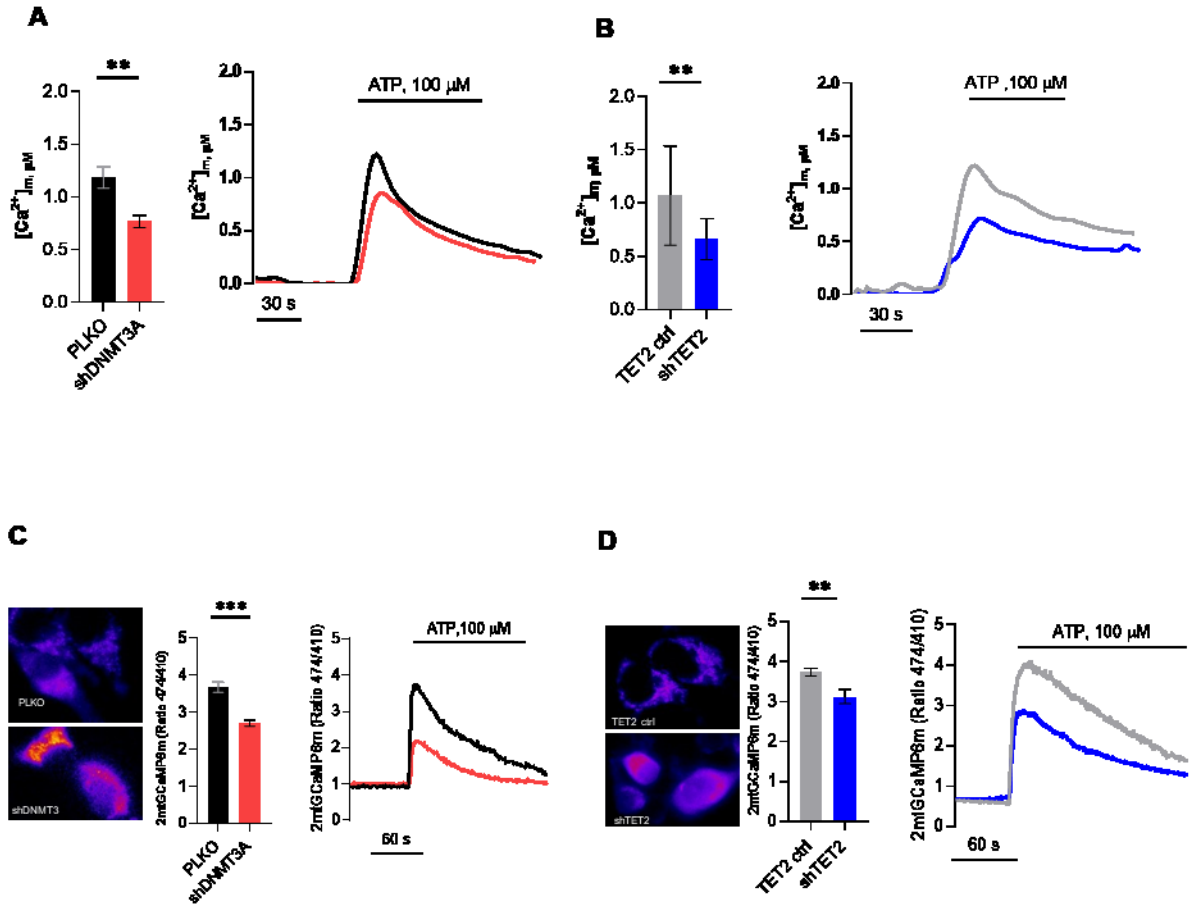

Fig.11 loss of TET2 and DNAMT3A shRNA lowers mitochondrial Ca^{2+} uptake. (A) Representative kinetics and analysis of aequorin-based $[Ca^{2+}]_m$ measurements in **(A)** ShRNA DNMT3A and **(B)** TET2 HEK293T cells and challenged with 100 µM ATP. **(C)** Mitochondrial calcium levels evaluated through ratiometric imaging of the mitochondrial-targeted GCaMP6m, in ShRNA DNMT3A and **(D)** in shTET2 stable clone HEK293T cells and stimulated with 100 µM ATP. The data represent average ± SEM four independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance: **p ≤ 0.01; ***p ≤ 0.001

5.3.2. Loss of TET2 in shRNA HEK293T cells reduces cytosolic calcium

One important aspect of the mitochondrial Ca^{2+} uptake is that sigmoidal or dose dependent response to cytoplasmic Ca^{2+} levels. Particularly, mitochondrial $[Ca^{2+}]$ uptake is minimal at low cytosolic $[Ca^{2+}]$ whereas it exponentially increases at higher threshold [264].

To investigate whether the shTET2 and shDNMT3A might specifically regulate mitochondrial Ca^{2+} homeostasis, we measured the Ca^{2+} concentrations in the cytosol using the aequorin technique and a cell-permeable Ca^{2+} indicator. As expected a profound reduction of cytosolic calcium was observed in shTET2 HEK293T cells compared to its controls (figure 12A). However, shDNMT3A 293T cells did not cause any change in cytosolic calcium (figure 12B). This phenomena is also observed using Fluo-4 AM, a cell-permeable Ca^{2+} indicator which shows a decrease $[Ca^{2+}]_c$ in shTET2 (figure 12C) but not in shDNMT3A (figure 12D).

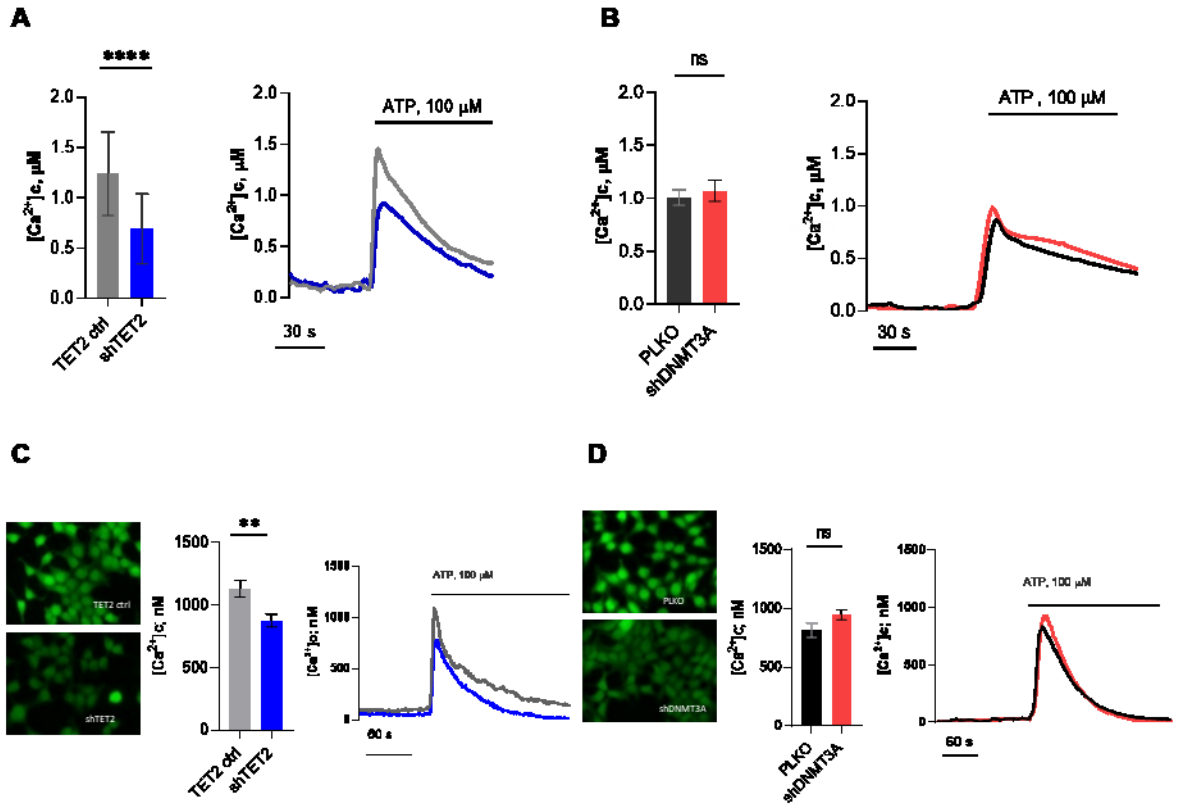

Fig. 12. Deletion of TET2 lowers cytosolic Ca^{2+} levels. Representative kinetics and analysis of aequorin-based $[Ca^{2+}]_c$ measurements in **(A)** shTET2 and **(B)** shDNMT3A HEK293T stable clones and challenged with 100 µM ATP . **(C)** Cytosolic calcium levels, evaluated using fluo-4 Am, in ShRNA TET2 and **(D)** DNMT3A HEK293T cells. The data represent average ± SEM four independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance: $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$

5.3.3. **Hypomethylating agent 5-aza-2′-deoxycytidine reduced $[Ca^{2+}]_m$ uptake**

According to our earlier observation, shDNMT3A only modifies mitochondria calcium levels but not the cytosol (figure 12B and D).

Therefore, to understand whether DNMT3A inactivation might specifically regulate mitochondrial Ca^{2+} homeostasis, we used the DNMT inhibitor 5-aza-2′-deoxycytidine (DAC), which inhibits all three DNMTs. Using mitochondrial-targeted aequorin in response to ATP stimulation, we measured mitochondrial Ca^{2+} uptake after DAC inhibition of DNMT was confirmed by immunoblotting (figure 13A). Specifically, 48 h of treatment in HEK293T cells with DAC significantly reduced $[Ca^{2+}]_m$ uptake in contrast to vehicle (DMSO) (figure 13B). Interestingly, DAC treatment had no effect on cytosolic levels despite the decrease in mitochondrial calcium (figure 13C). Consequently, these results support the effect of our genetic KD of DNMT3A in mitochondrial calcium uptake.

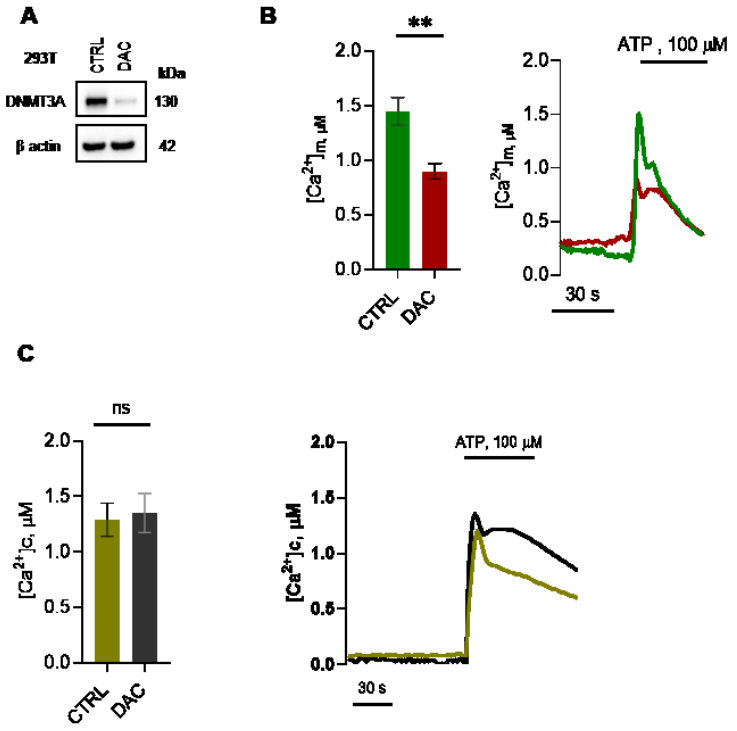

Fig.13. DAC lowers mitochondrial Ca^{2+} uptake. **(A)** Western blot representative of slicing of DNMT3A with DAC treatment and normalized to β-actin **(B)** Representative kinetics and analysis of aequorin-based $[Ca^{2+}]_m$ measurements and **(C)** $[Ca^{2+}]_c$ measurements in DAC treated HEK293T cells and control vehicle (DMSO). The data represent average ± SEM four independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance: $^{**}p \leq 0.01$.

5.3.4. MCU complex expression in shDNMT3A and DAC treated 293T cells

Mitochondrial Ca^{2+} uptake into the inner mitochondrial matrix is tightly controlled by the pore-forming molecule called mitochondrial calcium uniporter MCU [265].

MCU is regulated on variety of levels from protein-protein interaction with its regulatory elements such as MCUa and MCUb. Studies demonstrate relative quantities of these regulatory subunits influence Ca^{2+} transport capability of MCU itself [266]. Moreover, mitochondrial calcium uptake 1 and 2 (MICU1 and MICU2) [267] and the essential MICU regulator (EMRE), are also components of the uniporter complex and play a crucial role in regulating the integrity of the complex [268,269]. Abnormal variations in the expression levels or functional roles of one or more MCU complex members can cause changes in mitochondrial calcium uptake and have been implicated in various cancers[270]. As a result, we investigated the expression of MCU and regulatory subunits in shDNMT3A and DAC treated HEK293T cells. The immunoblotting analysis shows no significant difference in the expression of MCU and its regulatory proteins, MICU1, MCUb and EMRE in shDNMT3A (figure 14A) and DAC treated cells (figure 14B). These results demonstrate that the expression levels of MCU complex and its regulatory subunits are not responsible for the decrease mitochondrial uptake observed in shDNMT3A and DAC treated 293T cells. The decrease in $[Ca^{2+}]_m$ therefore might be due to mitochondrial biogenesis in KD of DNMT3A.

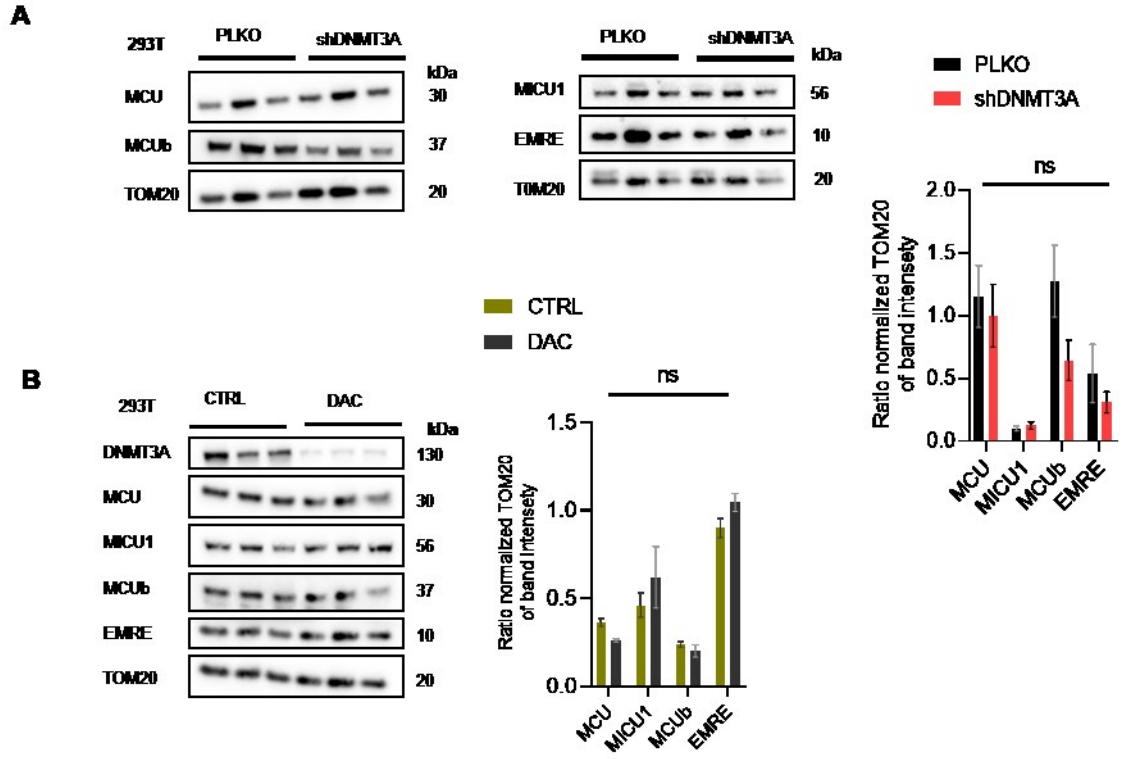

Fig.14 MCU complex expression in shDNMT3A and DAC treated 93T cells. **(A)** Immunoblotting analysis of MCU, MICU1, MCUb and EMRE protein levels in shDNMT3A and **(B)** DAC treated HEK293T cells of three independent experiments is shown. Statistical significance of specified densitometric ratios is indicated on the right. The data represent average ± SEM. Unpaired, two-tailed Student's t-test was used. Statistical significance: ns ≥ 0.05.

5.3.5. Analysis of $[Ca^{2+}]_c$ after manipulation of $[Ca^{2+}]_e$ in HEK293T upon KD of TET2

Two fundamental mechanisms can cause changes in intracellular $[Ca^{2+}]$. The first $[Ca^{2+}]$ mobilization from intracellular stores, primarily the endoplasmic reticulum (ER), and the second entry from the extracellular milieu[271]. To identify the source of the reduction in cytosolic calcium levels in shTET2 (figure 12A and C), we monitored $[Ca^{2+}]_c$ uptake after retention of external $[Ca^{2+}]$. Reconstitution of co-elenterazine was done in a calcium free buffer and then monitored the calcium entry from the plasma membrane upon reintroduction of Ca^{2+} 1mM in the extracellular mileu. The capacitative calcium influx in shTET2 show no difference in cytosolic calcium levels (figure 15A).

The capacitative calcium entry is an important pathway for calcium signaling at the plasma membrane is through store-operated calcium channels (SOCs). These channels produce the store-operated calcium entry (SOCE) each with a unique activation mechanism. SOCs are activated when ER Ca^{2+} stores are reduced which is sensed by the ER membrane-located Stromal Interaction Molecule 1 (STIM1) and induces STIM1 oligomerization and subsequent interaction with the PM-localized calcium channel ORAI1 enabling the calcium influx and refilling of ER Ca^{2+}- stores [272, 273]. Another crucial type of channel that transports calcium out of the cell in order to maintain the electrochemical gradient is the plasma membrane Ca^{2+}-ATPase (PMCA) which keeps cytosolic Ca^{2+} at or near its basal level [274]. Thus, we performed immunoblotting analysis of STIM1, PMCA, and ORAI1 proteins in shRNA TET2 HEK293T cells. Our data revealed no significant difference in expression of those store-operated calcium entry proteins (figure 15B). This suggests that the calcium influx from extracellular mileu is properly functioning. Therefore, we next monitored cytosolic calcium in the absence of extracellular influx. When the extracellular calcium is removed and Ca^{2+}- free buffer supplemented with 100 µM EGTA upon stimulation with ATP a significant decline in cytosolic calcium was observed in shTET2 compared to its control (figure 15C). Collectively, these results show that changes in cytosolic calcium levels observed in shTET2 might be due to intracellular Ca^{2+} store rather than influxes from extracellular milieu.

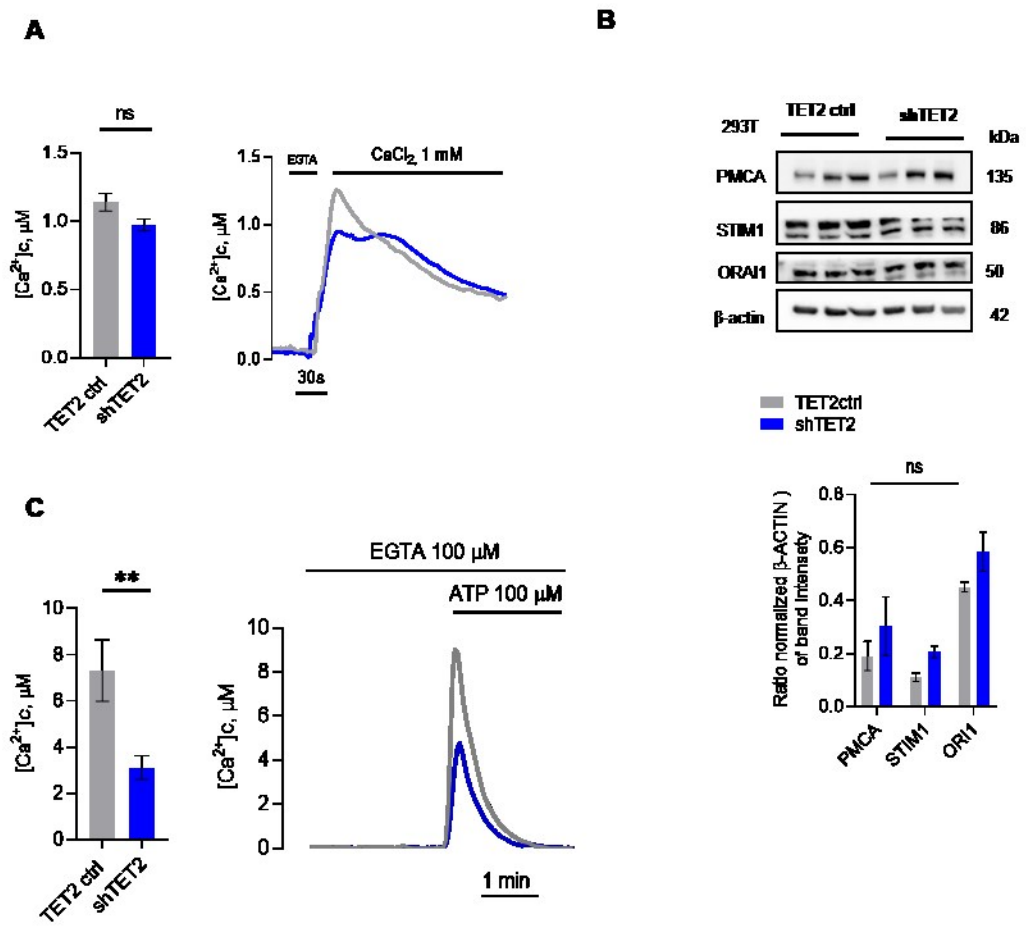

Fig. 15. Analysis of $[Ca^{2+}]_c$ after manipulation of $[Ca^{2+}]_e$ in HEK293T upon KD of TET2.
(A) Capacitive $[Ca^{2+}]_c$ influx measured in calcium free buffer (CFB) stimulated with $CaCl_2$ (1mM) **(B)** Western blot analysis of PMCA , STIM1 and ORA1levels in shRNA TET2 293T and normalized to β-actin in three independent experiments **(C)** intracellular cytosolic $[Ca^{2+}]_c$ was measured in CFB in the presence of EGTA (100 µM). The data represent average ± SEM four independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance: $^{**}p \leq 0.01$.

5.3.6. The KD of TET2 reduces Ip3 Receptor mediated release of Ca^{2+} from the ER

The ER is the main intracellular Ca^{2+}-storage organelle. Ca^{2+} release from the ER induce an increase in cytosol $[Ca^{2+}]$ and also other organelles, such as the mitochondria [275, 276]. Thus, we measured $[Ca^{2+}]_{er}$ in shTET2 HEK293T cells transfected with ER-targeted aequorin (erAEQ). In these experiments, the ER Ca^{2+} store was first depleted in the presence of Ionomycin of Ca^{2+}, during the phase of aequorin reconstitution in Ca^{2+}- free medium. When Ca^{2+} was reintroduced to the KRB perfusion medium, neither the uptake (figure 16A) nor the total content accumulation $[Ca^{2+}]_{er}$ (figure 16B) changes in shTET2.

After the $[Ca^{2+}]_{er}$ reached a plateau, cells were stimulated with ATP which activate a Gq coupled plasma membrane receptors and activates IP3, releasing Ca^{2+} from the ER via the IP3Rs. Here, we observed that, when compared to control cells, shTET2 cells exhibited a very different behavior during the release phase, with a particular decrease in the fast release (figure 16C) and slow release (figure 16D) which can be noted in representative kinetics. Likewise, we have also used the ER-targeted Ca^{2+} biosensor, GCaMP6m-210 to further confirm $[Ca^{2+}]_{er}$ handling in shTET2. When bound to Ca^{2+}, the GCaMP6m maintains high responsiveness and enables reliable measurements to monitor $[Ca^{2+}]_{er}$ [277]. We observed fluorescence changes in ER-GCaMP with decrease in the release of Ca^{2+} in shTET2 compared to control (figure 16E) which is also illustrated in representative kinetics figure 16F. Taken together, these results suggest the reduction in the release of Ca^{2+} in shTET2 might be due to the IP3-gated ER channels.

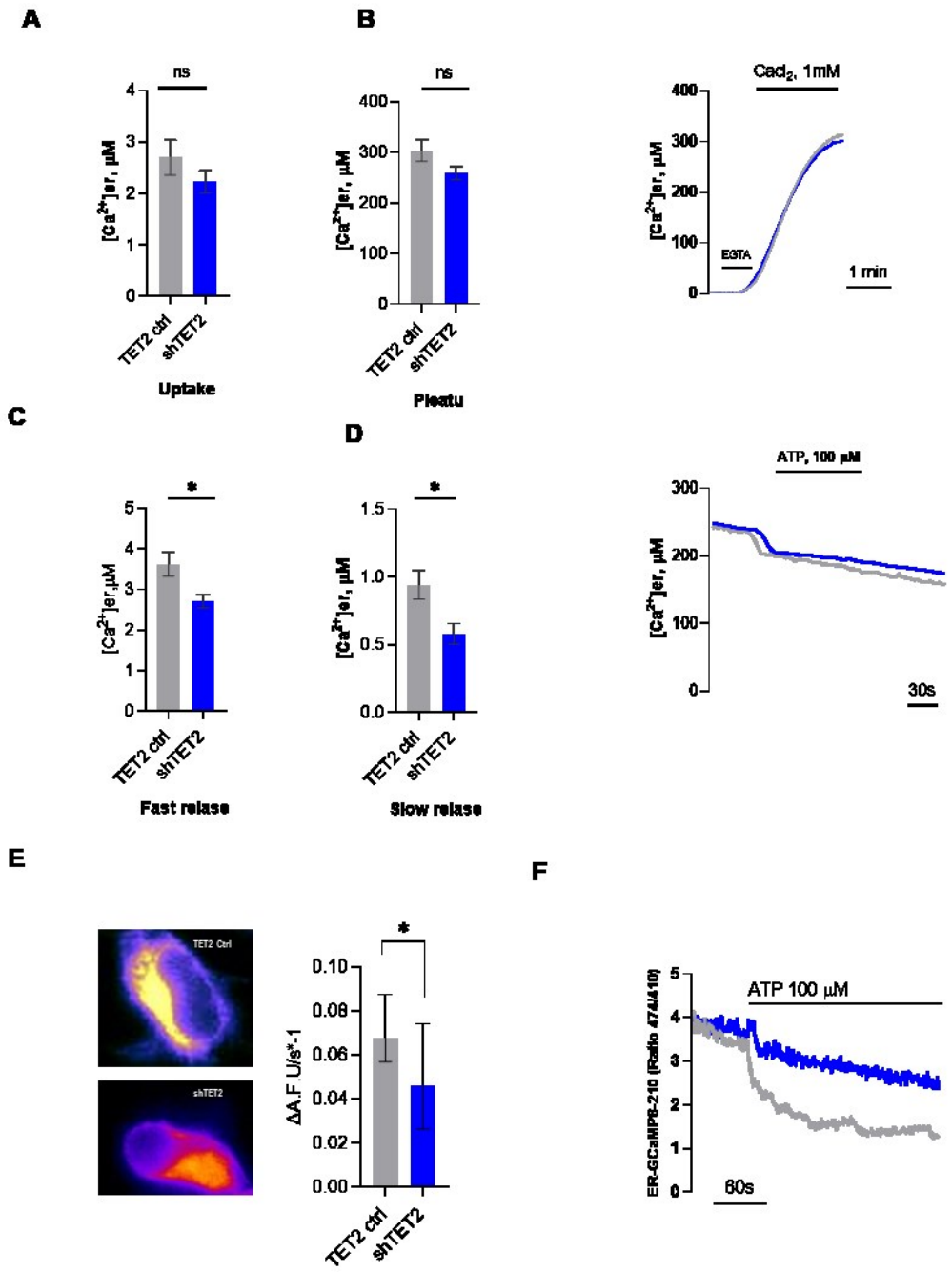

Fig.16. ER Ca^{2+} handling in shTET2 HEK293T cells. **(A)** Ability of ER Ca^{2+} concentration ([Ca2+]er) uptake in CFB **(B)** Capacity of calcium content when reached plateau in shTET2 HEK293T cells and represented kinetics **(C)** fast ER Ca^{2+} release and **(D)** slow release in shTET2 HEK293T cells when stimulated with 100 µM ATP with representative kinetics **(E)** ER calcium levels, evaluated through ratiometric imaging of the ER-targeted GCaMP6m and **(F)** representative kinetics of release in shRNA TET2 HEK293T cells challenged with 100 µM ATP. The data represent average ± SEM of three independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance: *p ≤ 0.05

5.3.7. The shRNA TET2 knockdown affects IP3R3 levels

ER-resident proteins such as the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and inositol 1,4,5- trisphosphate receptors (IP3R) are involved in Ca^{2+} transfer. IP3Rs are large cation channels that mediate Ca^{2+} release from the ER, giving rise to $[Ca^{2+}]_c$ levels which in turn delivered to mitochondrial matrix through MCU to regulate multiple aspects of cellular activities [278,279]. There are three isoforms of IP3Rs, each encoded by different genes and regulated by ATP, IP3, Ca^{2+} and post-translational modifications which all play important role in ER Ca^{2+} release[280]. Another crucial pump which pumps calcium from the cytosol to back to the sarcoplasmic reticulum (SR) is SERCA. These are membrane embedded large family of the P-type ATPases which regulate calcium concentrations [281]. We therefore performed immunoblotting to assess the expression levels of IP3Rs (IP3R1, IP3R2 and IP3R3), SERCA and MCU in shTET2. Interestingly, we found down-regulation of IP3R3 isoform in shRNA mediated loss of TET2 while IP3R1, IP3R2, SERCA and MCU remain unaffected (figure 17A). Likewise, the low levels of IP3R3 was also confirmed on mRNA level (figure 17B). Taken together, these results demonstrate that loss of TET2 in 293T cells are associated with reduced mitochondrial and cytosolic calcium levels as result of decreased ER-Ca^{2+} release due to down regulation of IP3R3.

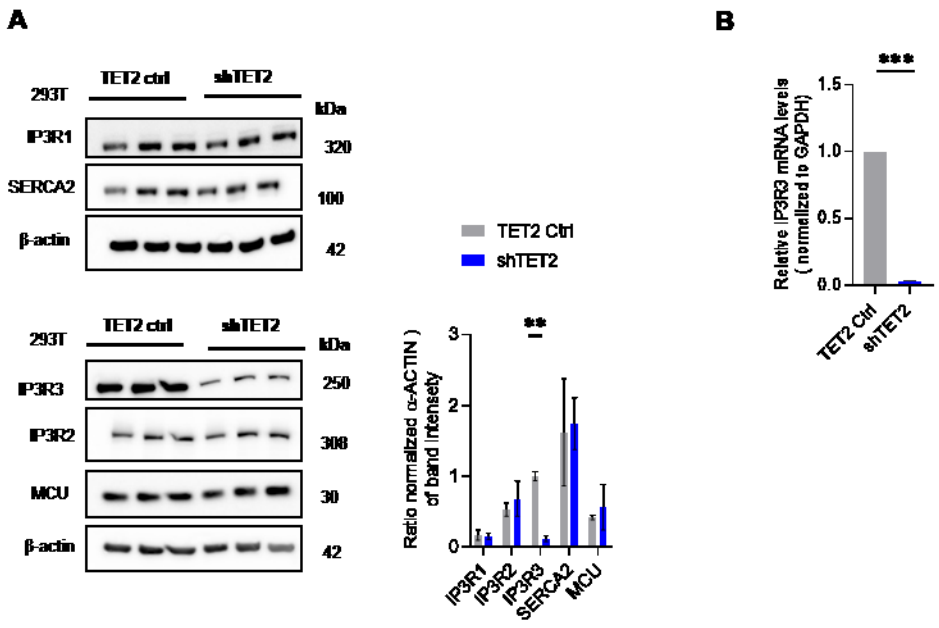

Fig 17. ShRNA TET2 loss affects IP3R3 levels. **(A)** Western blot analysis of IP3R1-3, SERCA2 and MCU of three independent experiments is shown. The data are normalized to β-actin. Statistical significance of specified densitometric ratios is indicated on the right. **(B)** Relative mRNA levels of IP3R3 studied by real-time RT-PCR. The data represent average ± SEM of three independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance; **p ≤ 0.01: ***p ≤ 0.001.

5.4. The shRNA mediated depletion of TET2 promotes resistance to cytarabine in HEK293T cells

Although several new therapeutic approaches have been introduced in recently, cytarabine (AraC) and daunorubicin (DNR) treatment for AML patients continues to be the gold standard of care. Numerous malignant tumors have been found to exhibit epigenetic dysregulation of DNA methylation or histone modifications and considered as contributing factors to the onset and progression cancer[282,283]. Dysregulation of epigenetic markers such as DNMT3A and TET2 have been implicated in the initiation and maintenance of AML in numerous studies, and also noted as significant disease reservoirs upon relapse[284 , 285]. Moreover, the high patient mortality rate in AML is frequently associated with therapy resistance while the mechanisms are still largely unknown. Therefore, identification of patients who are most likely to gain from personalized medicine as therapeutic strategy is necessary in the context of DNMT3A and TET2 mutations.

Thus, we aim to investigate whether the common chemotherapy drugs Cytarabine (AraC) and Daunorubicin (DNR) are resistance against the loss of function DNMT3A and TET2 293T cells. We used fluorescence microscope imaging techniques using propidium iodide (PI) and sytox green (SG), fluorescent DNA-binding dyes in conjunction with Hoechst. PI and SG can freely pass through the cell membranes of dead or dying cells, but they cannot enter healthy cells which excellent feature to assess cell death [286, 287]. After treating cells with 10 µM of AraC for 72 h, shDNMT3A 293T cells showed no sensitivity (figure 18A) while we identified more resistant against cytarabine in shRNA TET2 293T cells (figure 18B). Moreover, after being exposed to DNR at concentrations of 5 µM for 48 hours, neither shDNMT3A (figure 18C) nor shTET2 (figure 18D) showed any effect in DNR sensitivity.

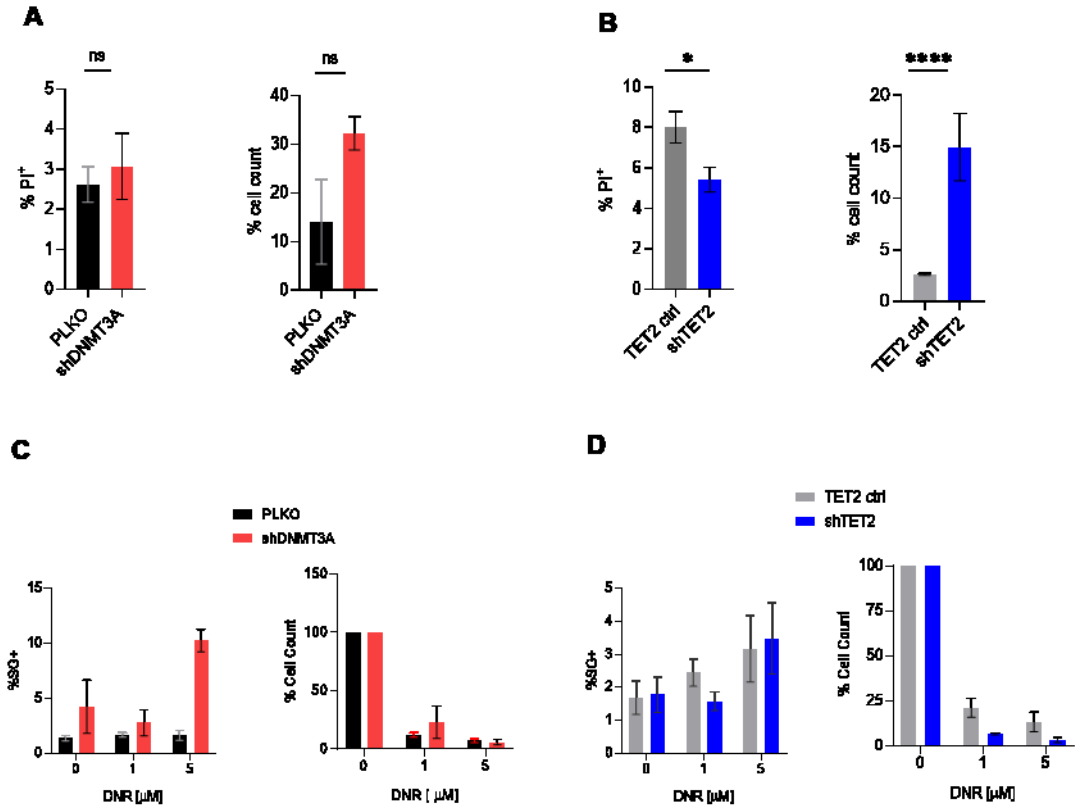

Fig. 18. ShRNA TET2 depletion promotes resistance against cytarabine in HEK293T cells. AraC treatment in **(A)** shDNMT3A **(B)** shTET2HEK293T cells with 10 µM for 72 h and **(C)** Cells were treated for 48 h with different concentrations of DNR in shDNMT3A and **(D)** shTET2 293T. Data represented percent of viable cells relative to untreated control. The data represent average ± SEM of three independent experiments. Unpaired, two-tailed Student's t-test was used. Statistical significance; *p ≤0.05; ****p ≤ 0.0001.

6. DISCUSSION

Hematopoiesis is process by which the different cellular constituents of the blood are made from Hematopoietic stem cells (HSCs) throughout the life of the organism in the bone marrow (BM)[186]. These unique cells are capable of lifelong self-renewal and commitment to multipotent progenitors (MPP) and involves a significant alteration in the metabolic program of the cells[185]. At steady state, quiescent and self-renewing HSCs heavily relay on glycolysis and undergo a metabolic switch to OXPHOS and produce elevated levels of ATP during activation and differentiation [288].

The profound metabolic switch that occur when HSCs transition from quiescence to proliferation are also accompanied by substantial epigenetic modifications. DNA demethylation is noted on metabolic activation of HSCs and during differentiation as demonstrated in direct down-stream MPP[158, 289]. In this regard, mitochondria may serve as crucial epigenetic signaling hubs in HSCs, with metabolic flux controlling the availability of metabolites acting as cofactors and substrates for enzymes with epigenetic modification activity such as DNA methylation [290, 291]. For example, OXPHOS regenerates NAD^+ pools to continue the TCA cycle, and αKG is in turn produced by the TCA cycle [292]. The αKG-dependent TET2 induces DNA demethylation by catalyzing the oxidation of 5-methylcytosine to 5- hydroxymethylcytosine, subsequently leading to DNA demethylation and modulating HSC differentiation. The αKG cofactor ascorbate accumulation in HSCs has been demonstrated in HSCs and promote TET2 function [293] and deletion of TET2 causes defective self-renewal activity of HSCs in mice[126, 294]. DNA Methyltransferase such as DNMT3A utilize the product of folate metabolism, S-adenosyl methionine (SAM), and are required for the establishment and maintenance of methylated nucleotides. Acute silencing of Dnmt3a decline differentiation and promotes self-renewal and immortalizes HSCs in mice[295, 296].

Furthermore, DNA methylation and demethylation enzymes have also been implicated in hematopoietic disorders such as acute myeloid leukemia and myelodysplastic syndrome[297,298]. Indeed, some of the most common 'early' mutational events in AML and several pre-leukaemic mutations involve aberration in DNA methylation. Clinical studies reported, 20–25% of AML patients with de novo DNMT3A mutations [56] and 24%-32% patients with TET2 mutation[299]. Several studies have also shown that DNMT3A and TET2 mutations cause chemotherapy resistance and trigger AML relapse[117,300].

It is unclear how loss of enzymes that methylate and demethylate DNA both lead to HSC differentiation defects, but these results point to an essential role for the dynamic regulation of DNA methylation and demethylation in controlling HSC function. As a result, the focus of a lot of current research has shifted to the metabolic cues involved in regulation of HSC function. Here, our study is particularly interested to assess how DNMT3A and TET2 mutations affect mitochondrial physiology. We therefore, applied shRNA mediated slicing of DNMT3A and TET2 to uncover mitochondrial profile in HEK293T cell line. Our results demonstrated that loss of DNMT3A leads to an increase in mitochondrial volume and is associated with higher levels of TFAM and mtDNA indicating up regulation mitochondrial biogenesis. However, shRNA deletion of TET2 in 293T cells showed no significant changes in mitochondrial morphology even though there is a trend towards increasing in total and average mitochondrial volume. This could be attributed to the lower knockdown efficiency of shRNA TET2 as compared to shDNMT3A. In general these observations highlight a potential association of mitochondrial biogenesis with DNA methylation in leukemic development.

Multiple molecular mechanisms have been implicated on regulation of methylation on mitochondrial biogenesis. According to a prior studies, increased TFAM expression resulted in deceased methylation at CpG sites of mtDNA promoter regions where TFAM binds with high affinity[301]. Moreover, DNA methylation of PGC-1α has been associated with an increase in mtDNA and mitochondrial biogenesis [302]. PGC-1α stimulates nuclear respiratory factors 1 and 2, which in turn promotes the transcription of TFAM and B2 (TFB2M), to catalyse mitochondrial biogenesis [303,241]. In contrast to our observation, DNMT1 knockout in adipocytes [304] and DNMT3B in hESCs[305] showed a reduction in the abundance of mtDNA. In addition, it has been demonstrated that TET2 shRNA-treated Human umbilical vein endothelial cells (HUVECs) with increased mitochondrial volume, shortened and altered mitochondrial cristae[306]. Moreover, adult HSC homeostasis is negatively impacted by both excessive and insufficient mitochondrial function. Altered mitochondrial biogenesis has been implicated to affect HSC physiology. Indeed, mTOR up regulation of mitochondria synthesis through PGC-1α has been reported to promote HSC expansion, exhaustion, and leukemogenesis due to enhanced oxidative stress[307].

In addition to changes in mitochondrial biogenesis, our study observed that loss of TET2 and DNMT3A alters intracellular ROS production. Global DNA hypo methylation has generally been linked to elevated ROS levels[308]. However, mounting evidence indicates that these findings cannot be taken as a whole and that ROS actually have different effects on local and global DNA methylation. Indeed, our findings revealed that TET2 knockdown elevated ROS levels while the loss of DNMT3A suppresses ROS production. Recent research findings that are consistent with our observations show that TET2 shRNA-treated HUVECs produced more ROS[306] while DNMT3A- R882C/H mutation reduced the production of ROS in myeloid cells[309]. In contrast to our observation, an increased ROS production has been associated with Dnmt3a-deficient muscle and this is attributed to ALDH1L1-dependent activation of NADPH oxidase as a factor in excess ROS production, mainly in oxidative muscles[310]. These studies demonstrate ROS levels are modulated by changes in DNA methylation in various tissues. Indeed, studies have suggested a potential explanation for the opposite effects ROS production due loss of DNMT3A and TET2. For instance, leukemic cells with altered metabolic process can increase ROS particular in mutations such as isocitrate dehydrogenase 1 IDH1 or IDH2 [311] which favors conversion of isocitrate to 2-hydroxyglutarate rather than α-ketoglutarate [312]. This shift in activity has several knock-on effects, the most relevant being an increase in intracellular ROS, mediated by 2-hydroxyglutarate[313]. This is important because TET proteins are α-ketoglutarate-dependent dioxygenases involved in the conversion of 5-methylcytosines (5-mC) to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine and 5-carboxycytosine. Moreover, Feng *et al.* demonstrated that inhibition of TET2 reduces mitochondrial 5hmC expression which in turn increased ROS generation[314].

Modification of DNA bases by ROS has also been studied. For example, hydroxyl radicals can lead to the formation of 5hmC from 5mC, initiated by abstraction of an H-atom from the methyl group[315]. On the other hand, ROS can also induce DNA hypermethylation by increasing expression of DNMTs. Increased levels of DNMT1, DNMT3A and DNMT3B prompted by the hypoxia-inducible transcription factor HIF1α were observed. This resulted either in global DNA hypermethylation and enhanced profibrotic gene expression or in specific hypermethylation of CpG islands in the SOD2 gene and subsequent loss of SOD2 expression[316,317,318].

To sum up, alterations in DNA methylation and ROS generation could be a prime molecular mechanism responsible for the disruption of HSC development which requires further research.

Mitochondria furthermore play crucial role in calcium homeostasis. It is not surprising that Ca^{2+} signaling research has started in recent years, shedding light on complex patterns of signals regulating HSC activity. Previous studies have shown that the mitochondrial fusion protein, mitofusin 2 (Mfn2), is essential for the maintenance of HSCs with extensive lymphoid potential, but not for myeloid-dominant. Mfn2 enhanced buffering of intracellular calcium, an effect mediated via ER-mitochondria tethering activity indicating a role of mitochondrial biogenesis and calcium in HSCs [319]. Furthermore, HSCs overall are endowed with reduced intracellular calcium compared to progenitors strongly enhances their maintenance in part through inactivation of calpain activity and resulting stabilization of TET enzymes[188]. In addition, some studies in AML indicate the association between differentiation and calcium signaling. It has been shown that the modulation of expression SERCA pumps, is an integral part of myeloid differentiation and indicates that lineage specific modification of the ER occurs during maturation. Moreover, it was demonstrated that SERCA isoforms may serve as important indicators for myeloid differentiation[320].

Interestingly, to date, no direct measurement of $[Ca^{2+}]_m$ handling in leukemic or hematopoietic stem cell compared to their normal counterparts has been reported. Here our data demonstrated that inactivation of DNMT3A and TET2 through shRNA mediated approach in HEK293T cell line significantly reduced mitochondrial calcium uptake. Loss of DNMT3A and TET2 affecting mitochondrial calcium uptake has not been previously described. Furthermore, our mechanistic analysis shows that defects in calcium release from ER stores are the cause of decrease cytosolic calcium levels shTET2. Our data clearly shows that TET2 deletion results in a down regulation of IP3R3 levels. The IP3R3-Ca^{2+} pathway is responsible for Ca^{2+} release from the ER [321]. Our findings established TET2 critical role in calcium handling and identified novel target that support calcium homeostasis.

Bioinformatics studies might explain the effect of inactivation of TET2 on IP3R3. Numerous CpG islands in the ITPR3 promoter region have the potential to be methylated that can suppress gene expression. The CpG islands in the ITPR3 promoter region might be highly methylated [322]. Though our experiment did not show methylation status of IP3R3 it could be hypothesized that deletion of TET2 might cause DNA hypermethylation which in turn reduced the expression of genes responsible for maintaining calcium homeostasis.

Likewise, consistent with our observations recent study revealed loss TET2-induced reduction in cytosolic calcium in muscle stem cells[323]. In addition, studies have shown that loss of IP3Rs impairs hematopoietic lineage differentiation, which is characterized by a decrease in type and number of hematopoietic progenitor cells[324]. According some reports, loss of TET2 have been also shown to impair HSC differentiation and increase self-renewal capacity [4] thus further studies could address TET2 –IP3R3 mediated regulation of HSCs.

Aside from the enhancer of zeste homolog 2 (EZH2), none of the genes known to date that are targets of pre-leukemic mutations were related to $[Ca^{2+}]_m$ homeostasis. It has been noted that pharmacological inhibition of EZH2 or shRNA deletion of EZH2 significantly reduces the expression of mitochondrial calcium uptake protein 1 (MICU1)[325]. MICU1 is the gatekeeper of the MCU that confers to the channel with low affinity for Ca^{2+}. MICU1 down-regulation, increases channel permeability which leads elevated basal $[Ca^{2+}]_m$[269]. Furthermore, the decrease in mitochondrial calcium that we observed in shDNMT3A is not related to cytosolic calcium levels or expression of the MCU and its regulatory proteins, or mitochondrial membrane potential. To gain a deeper understanding, we used pharmacological inhibitor of DNMTs, which decrease the expression DNMT3A as it has been also shown by others[326, 327, 328, 329]. Similar to our genetic inactivation of DNMT3A, DAC treated 293T cells also decreased mitochondrial calcium uptake without altering cytosolic calcium levels. For now our study does not provide the exact explanation to how and why the reduction of mitochondrial calcium in shDNMT3A and DAC treated HEK293T cells. However, based on our observations of increased mitochondrial volume in shDNMT3A, we speculate that a potential cause could be an increase in mitochondrial biogenesis. Studies show that the size, morphology, and proximity of mitochondria to the Ca^{2+} release sites have an impact on mitochondrial Ca^{2+} uptake[330]. In addition, alteration ER – mitochondria contact sites might also affect mitochondrial Ca^{2+} uptake[331,332]. The ER-mitochondria proximity is modulated by many structural proteins, which guarantee or disrupt when lost[333], therefore, it would be useful to evaluate proteins implicated in ER-to-mitochondrial Ca^{2+} signal propagation in shDNMT3A. Moreover, mitochondrial Ca^{2+} uptake is also dependent on $\Delta\Psi m$, which is an important parameter to consider. Though our data indicates shDNM3A and shTET2 had no effect on $\Delta\Psi m$, it would have been useful to measure $\Delta\Psi m$ simultaneously with Ca^{2+} signals rather than separately for absolute verification[334].

Finally, we looked into how two standard drugs affect shTET2 and shDNMT3A in HEK293T cell lines. Our data demonstrate cytarabine resistance to TET2 loss-of-function.

According earlier studies, TET2 mutation linked to a better myelodysplastic syndrome (MDS) prognosis after receiving hypomethylating chemotherapy[335]. Consistent with our observation loss-of-function mutations in the histone methyltransferase such EZH2 have been shown to confer resistance to cytarabine[336].

7. CONCLUSION

In line with the emerging role of mitochondrial regulation of HSCs and establishment of pre leukemia our data showed that DNMT3A deficiency caused increased mitochondrial biogenesis along with increased average and total mitochondrial volume, TFAM, and mitochondrial DNA. Interestingly, both loss of function in DNMT3A and TET2 reduced mitochondrial calcium uptake. Our mechanistic examination showed that loss of TET2 decreased mitochondrial and cytosolic calcium uptake due to defects in ER calcium release as result of IP3R3 down regulation. Alteration of Ca^{2+} hemostasis as result of inactivation of DNMT3A and TET2 in HEK293T cells might expose unexpected therapeutic strategies through manipulation of mitochondrial Ca^{2+} signalling to treat hematological malignancies.

8. REFERENCES

1 Seo, Y., Shin, K. H., Kim, H. H. & Kim, H. S. Current Advances in Red Blood Cell Generation Using Stem Cells from Diverse Sources. *Stem cells international* **2019**, 9281329, doi:10.1155/2019/9281329 (2019).

2 Yamamoto, R. *et al.* Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* **154**, 1112-1126, doi:10.1016/j.cell.2013.08.007 (2013).

3 Olson, O. C., Kang, Y. A. & Passegue, E. Normal Hematopoiesis Is a Balancing Act of Self-Renewal and Regeneration. *Cold Spring Harbor perspectives in medicine* **10**, doi:10.1101/cshperspect.a035519 (2020).

4 Nakamura-Ishizu, A., Takizawa, H. & Suda, T. The analysis, roles and regulation of quiescence in hematopoietic stem cells. *Development* **141**, 4656-4666, doi:10.1242/dev.106575 (2014).

5 Cheng, H., Zheng, Z. & Cheng, T. New paradigms on hematopoietic stem cell differentiation. *Protein & cell* **11**, 34-44, doi:10.1007/s13238-019-0633-0 (2020).

6 Seita, J. & Weissman, I. L. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley interdisciplinary reviews. Systems biology and medicine* **2**, 640-653, doi:10.1002/wsbm.86 (2010).

7 Swain, A., Inoue, T., Tan, K. S., Nakanishi, Y. & Sugiyama, D. Intrinsic and extrinsic regulation of mammalian hematopoiesis in the fetal liver. *Histology and histopathology* **29**, 1077-1082, doi:10.14670/HH-29.1077 (2014).

8 Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* **505**, 327-334, doi:10.1038/nature12984 (2014).

9 Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nature reviews. Immunology* **6**, 93-106, doi:10.1038/nri1779 (2006).

10 Cullen, S. M., Mayle, A., Rossi, L. & Goodell, M. A. Hematopoietic stem cell development: an epigenetic journey. *Current topics in developmental biology* **107**, 39-75, doi:10.1016/B978-0-12-416022-4.00002-0 (2014).

11 Pietras, E. M., Warr, M. R. & Passegue, E. Cell cycle regulation in hematopoietic stem cells. *The Journal of cell biology* **195**, 709-720, doi:10.1083/jcb.201102131 (2011).

12 Cui, K. *et al.* Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell stem cell* **4**, 80-93, doi:10.1016/j.stem.2008.11.011 (2009).

13 Surace, A. E. A. & Hedrich, C. M. The Role of Epigenetics in Autoimmune/Inflammatory Disease. *Frontiers in immunology* **10**, 1525, doi:10.3389/fimmu.2019.01525 (2019).

14 Heintzman, N. D. *et al.* Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* **459**, 108-112, doi:10.1038/nature07829 (2009).

15 Goyama, S. & Kitamura, T. Epigenetics in normal and malignant hematopoiesis: An overview and update 2017. *Cancer science* **108**, 553-562, doi:10.1111/cas.13168 (2017).

16 Cao, R. *et al.* Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**, 1039-1043, doi:10.1126/science.1076997 (2002).

17 Rizo, A. *et al.* Repression of BMI1 in normal and leukemic human CD34(+) cells impairs self-renewal and induces apoptosis. *Blood* **114**, 1498-1505, doi:10.1182/blood-2009-03-209734 (2009).

18 Li, J. *et al.* EZH2-mediated H3K27 trimethylation mediates neurodegeneration in ataxia-telangiectasia. *Nature neuroscience* **16**, 1745-1753, doi:10.1038/nn.3564 (2013).

19 Kamminga, L. M. *et al.* The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood* **107**, 2170-2179, doi:10.1182/blood-2005-09-3585 (2006).

20 Ezhkova, E. *et al.* Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell* **136**, 1122-1135, doi:10.1016/j.cell.2008.12.043 (2009).

21 Argiropoulos, B. & Humphries, R. K. Hox genes in hematopoiesis and leukemogenesis. *Oncogene* **26**, 6766-6776, doi:10.1038/sj.onc.1210760 (2007).
22 Jo, S. Y., Granowicz, E. M., Maillard, I., Thomas, D. & Hess, J. L. Requirement for Dot1l in murine postnatal hematopoiesis and leukemogenesis by MLL translocation. *Blood* **117**, 4759-4768, doi:10.1182/blood-2010-12-327668 (2011).
23 Feng, Y. *et al.* Early mammalian erythropoiesis requires the Dot1L methyltransferase. *Blood* **116**, 4483-4491, doi:10.1182/blood-2010-03-276501 (2010).
24 He, R., Dantas, A. & Riabowol, K. Histone Acetyltransferases and Stem Cell Identity. *Cancers* **13**, doi:10.3390/cancers13102407 (2021).
25 Thomas, T. *et al.* Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells. *Genes & development* **20**, 1175-1186, doi:10.1101/gad.1382606 (2006).
26 Liu, H. *et al.* DNA methylation dynamics: identification and functional annotation. *Briefings in functional genomics* **15**, 470-484, doi:10.1093/bfgp/elw029 (2016).
27 Ambrosi, C., Manzo, M. & Baubec, T. Dynamics and Context-Dependent Roles of DNA Methylation. *Journal of molecular biology* **429**, 1459-1475, doi:10.1016/j.jmb.2017.02.008 (2017).
28 Kosan, C. & Godmann, M. Genetic and Epigenetic Mechanisms That Maintain Hematopoietic Stem Cell Function. *Stem cells international* **2016**, 5178965, doi:10.1155/2016/5178965 (2016).
29 Hogart, A. *et al.* Genome-wide DNA methylation profiles in hematopoietic stem and progenitor cells reveal overrepresentation of ETS transcription factor binding sites. *Genome research* **22**, 1407-1418, doi:10.1101/gr.132878.111 (2012).
30 Farlik, M. *et al.* DNA Methylation Dynamics of Human Hematopoietic Stem Cell Differentiation. *Cell stem cell* **19**, 808-822, doi:10.1016/j.stem.2016.10.019 (2016).
31 Yamashita, M., Dellorusso, P. V., Olson, O. C. & Passegue, E. Dysregulated haematopoietic stem cell behaviour in myeloid leukaemogenesis. *Nature reviews. Cancer* **20**, 365-382, doi:10.1038/s41568-020-0260-3 (2020).
32 Moshaver, B. *et al.* Identification of a small subpopulation of candidate leukemia-initiating cells in the side population of patients with acute myeloid leukemia. *Stem cells* **26**, 3059-3067, doi:10.1634/stemcells.2007-0861 (2008).
33 Rahman, M. *et al.* The cancer stem cell hypothesis: failures and pitfalls. *Neurosurgery* **68**, 531-545; discussion 545, doi:10.1227/NEU.0b013e3181ff9eb5 (2011).
34 Zhang, Z., Yang, K. & Zhang, H. Targeting Leukemia-Initiating Cells and Leukemic Niches: The Next Therapy Station for T-Cell Acute Lymphoblastic Leukemia? *Cancers* **14**, doi:10.3390/cancers14225655 (2022).
35 Heidel, F. H., Mar, B. G. & Armstrong, S. A. Self-renewal related signaling in myeloid leukemia stem cells. *International journal of hematology* **94**, 109-117, doi:10.1007/s12185-011-0901-0 (2011).
36 Krivtsov, A. V. *et al.* Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* **442**, 818-822, doi:10.1038/nature04980 (2006).
37 Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111, doi:10.1038/35102167 (2001).
38 Kirstetter, P. *et al.* Modeling of C/EBPalpha mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. *Cancer cell* **13**, 299-310, doi:10.1016/j.ccr.2008.02.008 (2008).
39 Krivtsov, A. V. *et al.* Cell of origin determines clinically relevant subtypes of MLL-rearranged AML. *Leukemia* **27**, 852-860, doi:10.1038/leu.2012.363 (2013).
40 von Bonin, M. *et al.* Clonal hematopoiesis and its emerging effects on cellular therapies. *Leukemia* **35**, 2752-2758, doi:10.1038/s41375-021-01337-8 (2021).

41 Sperling, A. S., Gibson, C. J. & Ebert, B. L. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nature reviews. Cancer* **17**, 5-19, doi:10.1038/nrc.2016.112 (2017).
42 Kjaer, L. Clonal Hematopoiesis and Mutations of Myeloproliferative Neoplasms. *Cancers* **12**, doi:10.3390/cancers12082100 (2020).
43 Corces-Zimmerman, M. R. & Majeti, R. Pre-leukemic evolution of hematopoietic stem cells: the importance of early mutations in leukemogenesis. *Leukemia* **28**, 2276-2282, doi:10.1038/leu.2014.211 (2014).
44 Majeti, R. Clonal evolution of pre-leukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Best practice & research. Clinical haematology* **27**, 229-234, doi:10.1016/j.beha.2014.10.003 (2014).
45 Kumar, C. C. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. *Genes & cancer* **2**, 95-107, doi:10.1177/1947601911408076 (2011).
46 Tuna, M., Amos, C. I. & Mills, G. B. Molecular mechanisms and pathobiology of oncogenic fusion transcripts in epithelial tumors. *Oncotarget* **10**, 2095-2111, doi:10.18632/oncotarget.26777 (2019).
47 Shin, S. Y. *et al.* Mutation profiling of 19 candidate genes in acute myeloid leukemia suggests significance of DNMT3A mutations. *Oncotarget* **7**, 54825-54837, doi:10.18632/oncotarget.10240 (2016).
48 Dicker, F. *et al.* Mutation analysis for RUNX1, MLL-PTD, FLT3-ITD, NPM1 and NRAS in 269 patients with MDS or secondary AML. *Leukemia* **24**, 1528-1532, doi:10.1038/leu.2010.124 (2010).
49 Yu, J., Li, Y., Zhang, D., Wan, D. & Jiang, Z. Clinical implications of recurrent gene mutations in acute myeloid leukemia. *Experimental hematology & oncology* **9**, 4, doi:10.1186/s40164-020-00161-7 (2020).
50 Welch, J. S. *et al.* The origin and evolution of mutations in acute myeloid leukemia. *Cell* **150**, 264-278, doi:10.1016/j.cell.2012.06.023 (2012).
51 Abdel-Wahab, O. & Levine, R. L. Mutations in epigenetic modifiers in the pathogenesis and therapy of acute myeloid leukemia. *Blood* **121**, 3563-3572, doi:10.1182/blood-2013-01-451781 (2013).
52 Jiang, P. *et al.* Epigenetic regulation of hematopoietic stem cell homeostasis. *Blood science* **1**, 19-28, doi:10.1097/BS9.0000000000000018 (2019).
53 Mehdipour, P., Santoro, F. & Minucci, S. Epigenetic alterations in acute myeloid leukemias. *The FEBS journal* **282**, 1786-1800, doi:10.1111/febs.13142 (2015).
54 Feng, J. *et al.* Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nature neuroscience* **13**, 423-430, doi:10.1038/nn.2514 (2010).
55 Sun, Y. *et al.* Persistent DNMT3A mutation burden in DNMT3A mutated adult cytogenetically normal acute myeloid leukemia patients in long-term remission. *Leukemia research* **49**, 102-107, doi:10.1016/j.leukres.2016.09.001 (2016).
56 Ley, T. J. *et al.* DNMT3A mutations in acute myeloid leukemia. *The New England journal of medicine* **363**, 2424-2433, doi:10.1056/NEJMoa1005143 (2010).
57 Qu, Y. *et al.* Cancer-specific changes in DNA methylation reveal aberrant silencing and activation of enhancers in leukemia. *Blood* **129**, e13-e25, doi:10.1182/blood-2016-07-726877 (2017).
58 Metzeler, K. H. *et al.* TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29**, 1373-1381, doi:10.1200/JCO.2010.32.7742 (2011).
59 Liu, X. *et al.* Decreased 5-hydroxymethylcytosine levels are associated with TET2 mutation and unfavorable overall survival in myelodysplastic syndromes. *Leukemia & lymphoma* **54**, 2466-2473, doi:10.3109/10428194.2013.778408 (2013).

60 Moran-Crusio, K. *et al.* Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer cell* **20**, 11-24, doi:10.1016/j.ccr.2011.06.001 (2011).
61 Shide, K. *et al.* TET2 is essential for survival and hematopoietic stem cell homeostasis. *Leukemia* **26**, 2216-2223, doi:10.1038/leu.2012.94 (2012).
62 Khwaja, A. *et al.* Acute myeloid leukaemia. *Nature reviews. Disease primers* **2**, 16010, doi:10.1038/nrdp.2016.10 (2016).
63 Tuval, A. & Shlush, L. I. Evolutionary trajectory of leukemic clones and its clinical implications. *Haematologica* **104**, 872-880, doi:10.3324/haematol.2018.195289 (2019).
64 Dohner, H. *et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474, doi:10.1182/blood-2009-07-235358 (2010).
65 Vosberg, S. & Greif, P. A. Clonal evolution of acute myeloid leukemia from diagnosis to relapse. *Genes, chromosomes & cancer* **58**, 839-849, doi:10.1002/gcc.22806 (2019).
66 Jan, M. & Majeti, R. Clonal evolution of acute leukemia genomes. *Oncogene* **32**, 135-140, doi:10.1038/onc.2012.48 (2013).
67 Bereshchenko, O. *et al.* Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPalpha mutant AML. *Cancer cell* **16**, 390-400, doi:10.1016/j.ccr.2009.09.036 (2009).
68 Yi, M. *et al.* The global burden and attributable risk factor analysis of acute myeloid leukemia in 195 countries and territories from 1990 to 2017: estimates based on the global burden of disease study 2017. *Journal of hematology & oncology* **13**, 72, doi:10.1186/s13045-020-00908-z (2020).
69 Bennett, J. M. *et al.* Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *British journal of haematology* **33**, 451-458, doi:10.1111/j.1365-2141.1976.tb03563.x (1976).
70 Arber, D. A. *et al.* The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* **127**, 2391-2405, doi:10.1182/blood-2016-03-643544 (2016).
71 Corces-Zimmerman, M. R., Hong, W. J., Weissman, I. L., Medeiros, B. C. & Majeti, R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 2548-2553, doi:10.1073/pnas.1324297111 (2014).
72 Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature medicine* **17**, 1086-1093, doi:10.1038/nm.2415 (2011).
73 Shlush, L. I. *et al.* Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature* **547**, 104-108, doi:10.1038/nature22993 (2017).
74 van Galen, P. *et al.* Single-Cell RNA-Seq Reveals AML Hierarchies Relevant to Disease Progression and Immunity. *Cell* **176**, 1265-1281 e1224, doi:10.1016/j.cell.2019.01.031 (2019).
75 Wingelhofer, B. & Somervaille, T. C. P. Emerging Epigenetic Therapeutic Targets in Acute Myeloid Leukemia. *Frontiers in oncology* **9**, 850, doi:10.3389/fonc.2019.00850 (2019).
76 Ding, L. *et al.* Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* **481**, 506-510, doi:10.1038/nature10738 (2012).
77 Islam, A. Induction treatment of acute myeloid leukemia in an elderly patient with intramarrow injection/administration of cytarabine: first report of a case. *Clinical case reports* **3**, 1026-1029, doi:10.1002/ccr3.429 (2015).
78 Al-Ali, H. K., Jaekel, N. & Niederwieser, D. The role of hypomethylating agents in the treatment of elderly patients with AML. *Journal of geriatric oncology* **5**, 89-105, doi:10.1016/j.jgo.2013.08.004 (2014).
79 Baek, D. W. *et al.* Favorable long-term survival using consolidation chemotherapy without allogeneic hematopoietic cell transplantation for acute myeloid leukemia with wild-type NPM1 without FLT3-ITD. *Blood research* **54**, 189-197, doi:10.5045/br.2019.54.3.189 (2019).

80 Armand, P. *et al.* Validation and refinement of the Disease Risk Index for allogeneic stem cell transplantation. *Blood* **123**, 3664-3671, doi:10.1182/blood-2014-01-552984 (2014).
81 Ustun, C. *et al.* Allogeneic hematopoietic cell transplantation compared to chemotherapy consolidation in older acute myeloid leukemia (AML) patients 60-75 years in first complete remission (CR1): an alliance (A151509), SWOG, ECOG-ACRIN, and CIBMTR study. *Leukemia* **33**, 2599-2609, doi:10.1038/s41375-019-0477-x (2019).
82 Gupta, V., Tallman, M. S. & Weisdorf, D. J. Allogeneic hematopoietic cell transplantation for adults with acute myeloid leukemia: myths, controversies, and unknowns. *Blood* **117**, 2307-2318, doi:10.1182/blood-2010-10-265603 (2011).
83 Wei, A. H. *et al.* Oral Azacitidine Maintenance Therapy for Acute Myeloid Leukemia in First Remission. *The New England journal of medicine* **383**, 2526-2537, doi:10.1056/NEJMoa2004444 (2020).
84 Garcia-Manero, G. *et al.* A phase 1b/2b multicenter study of oral panobinostat plus azacitidine in adults with MDS, CMML or AML with ⩽30% blasts. *Leukemia* **31**, 2799-2806, doi:10.1038/leu.2017.159 (2017).
85 Cherry, E. M. *et al.* Venetoclax and azacitidine compared with induction chemotherapy for newly diagnosed patients with acute myeloid leukemia. *Blood advances* **5**, 5565-5573, doi:10.1182/bloodadvances.2021005538 (2021).
86 Garcia-Manero, G. *et al.* Pracinostat plus azacitidine in older patients with newly diagnosed acute myeloid leukemia: results of a phase 2 study. *Blood advances* **3**, 508-518, doi:10.1182/bloodadvances.2018027409 (2019).
87 Stein, E. M. & Tallman, M. S. Emerging therapeutic drugs for AML. *Blood* **127**, 71-78, doi:10.1182/blood-2015-07-604538 (2016).
88 Hashimoto, H., Vertino, P. M. & Cheng, X. Molecular coupling of DNA methylation and histone methylation. *Epigenomics* **2**, 657-669, doi:10.2217/epi.10.44 (2010).
89 Chen, Z. & Zhang, Y. Role of Mammalian DNA Methyltransferases in Development. *Annual review of biochemistry* **89**, 135-158, doi:10.1146/annurev-biochem-103019-102815 (2020).
90 Gujar, H., Weisenberger, D. J. & Liang, G. The Roles of Human DNA Methyltransferases and Their Isoforms in Shaping the Epigenome. *Genes* **10**, doi:10.3390/genes10020172 (2019).
91 Chen, T. & Li, E. Structure and function of eukaryotic DNA methyltransferases. *Current topics in developmental biology* **60**, 55-89, doi:10.1016/S0070-2153(04)60003-2 (2004).
92 Li, E. & Zhang, Y. DNA methylation in mammals. *Cold Spring Harbor perspectives in biology* **6**, a019133, doi:10.1101/cshperspect.a019133 (2014).
93 Smith, Z. D. & Meissner, A. DNA methylation: roles in mammalian development. *Nature reviews. Genetics* **14**, 204-220, doi:10.1038/nrg3354 (2013).
94 Greenberg, M. V. C. & Bourc'his, D. The diverse roles of DNA methylation in mammalian development and disease. *Nature reviews. Molecular cell biology* **20**, 590-607, doi:10.1038/s41580-019-0159-6 (2019).
95 Williams, K., Christensen, J. & Helin, K. DNA methylation: TET proteins-guardians of CpG islands? *EMBO reports* **13**, 28-35, doi:10.1038/embor.2011.233 (2011).
96 Ko, M. *et al.* Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX. *Nature* **497**, 122-126, doi:10.1038/nature12052 (2013).
97 Montagner, S. *et al.* TET2 Regulates Mast Cell Differentiation and Proliferation through Catalytic and Non-catalytic Activities. *Cell reports* **15**, 1566-1579, doi:10.1016/j.celrep.2016.04.044 (2016).
98 Bowman, R. L. & Levine, R. L. TET2 in Normal and Malignant Hematopoiesis. *Cold Spring Harbor perspectives in medicine* **7**, doi:10.1101/cshperspect.a026518 (2017).
99 Lim, W. J., Kim, K. H., Kim, J. Y., Jeong, S. & Kim, N. Identification of DNA-Methylated CpG Islands Associated With Gene Silencing in the Adult Body Tissues of the Ogye Chicken Using RNA-Seq and Reduced Representation Bisulfite Sequencing. *Frontiers in genetics* **10**, 346, doi:10.3389/fgene.2019.00346 (2019).

100 Shayevitch, R., Askayo, D., Keydar, I. & Ast, G. The importance of DNA methylation of exons on alternative splicing. *Rna* **24**, 1351-1362, doi:10.1261/rna.064865.117 (2018).
101 Shimoda, N. *et al.* Decrease in cytosine methylation at CpG island shores and increase in DNA fragmentation during zebrafish aging. *Age* **36**, 103-115, doi:10.1007/s11357-013-9548-5 (2014).
102 Li, D., Guo, B., Wu, H., Tan, L. & Lu, Q. TET Family of Dioxygenases: Crucial Roles and Underlying Mechanisms. *Cytogenetic and genome research* **146**, 171-180, doi:10.1159/000438853 (2015).
103 Pinney, S. E. Mammalian Non-CpG Methylation: Stem Cells and Beyond. *Biology* **3**, 739-751, doi:10.3390/biology3040739 (2014).
104 Pappalardo, X. G. & Barra, V. Losing DNA methylation at repetitive elements and breaking bad. *Epigenetics & chromatin* **14**, 25, doi:10.1186/s13072-021-00400-z (2021).
105 Zhao, L., Islam, R., Wang, Y., Zhang, X. & Liu, L. Z. Epigenetic Regulation in Chromium-, Nickel- and Cadmium-Induced Carcinogenesis. *Cancers* **14**, doi:10.3390/cancers14235768 (2022).
106 Baylin, S. B. & Herman, J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends in genetics : TIG* **16**, 168-174, doi:10.1016/s0168-9525(99)01971-x (2000).
107 Shen, N. *et al.* Inactivation of Receptor Tyrosine Kinases Reverts Aberrant DNA Methylation in Acute Myeloid Leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research* **23**, 6254-6266, doi:10.1158/1078-0432.CCR-17-0235 (2017).
108 Micevic, G., Theodosakis, N. & Bosenberg, M. Aberrant DNA methylation in melanoma: biomarker and therapeutic opportunities. *Clinical epigenetics* **9**, 34, doi:10.1186/s13148-017-0332-8 (2017).
109 Cao, X. Y. *et al.* DNA methyltransferase3a expression is an independent poor prognostic indicator in gastric cancer. *World journal of gastroenterology* **20**, 8201-8208, doi:10.3748/wjg.v20.i25.8201 (2014).
110 Mensah, I. K. *et al.* Misregulation of the expression and activity of DNA methyltransferases in cancer. *NAR cancer* **3**, zcab045, doi:10.1093/narcan/zcab045 (2021).
111 Linhart, H. G. *et al.* Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes & development* **21**, 3110-3122, doi:10.1101/gad.1594007 (2007).
112 Wang, K. C. *et al.* Ten-eleven translocation 1 dysfunction reduces 5-hydroxymethylcytosine expression levels in gastric cancer cells. *Oncology letters* **15**, 278-284, doi:10.3892/ol.2017.7264 (2018).
113 Kamdar, S. *et al.* Exploring targets of TET2-mediated methylation reprogramming as potential discriminators of prostate cancer progression. *Clinical epigenetics* **11**, 54, doi:10.1186/s13148-019-0651-z (2019).
114 Scourzic, L., Mouly, E. & Bernard, O. A. TET proteins and the control of cytosine demethylation in cancer. *Genome medicine* **7**, 9, doi:10.1186/s13073-015-0134-6 (2015).
115 Song, S. J. & Pandolfi, P. P. miR-22 in tumorigenesis. *Cell Cycle* **13**, 11-12, doi:10.4161/cc.27027 (2014).
116 Papaemmanuil, E. *et al.* Genomic Classification and Prognosis in Acute Myeloid Leukemia. *The New England journal of medicine* **374**, 2209-2221, doi:10.1056/NEJMoa1516192 (2016).
117 Shlush, L. I. *et al.* Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* **506**, 328-333, doi:10.1038/nature13038 (2014).
118 Genovese, G. *et al.* Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *The New England journal of medicine* **371**, 2477-2487, doi:10.1056/NEJMoa1409405 (2014).
119 Gaidzik, V. I. *et al.* Clinical impact of DNMT3A mutations in younger adult patients with acute myeloid leukemia: results of the AML Study Group (AMLSG). *Blood* **121**, 4769-4777, doi:10.1182/blood-2012-10-461624 (2013).

120 Jankowska, A. M. *et al.* Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. *Blood* **118**, 3932-3941, doi:10.1182/blood-2010-10-311019 (2011).
121 Sakata-Yanagimoto, M. *et al.* Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. *Nature genetics* **46**, 171-175, doi:10.1038/ng.2872 (2014).
122 Grossmann, V. *et al.* The molecular profile of adult T-cell acute lymphoblastic leukemia: mutations in RUNX1 and DNMT3A are associated with poor prognosis in T-ALL. *Genes, chromosomes & cancer* **52**, 410-422, doi:10.1002/gcc.22039 (2013).
123 Russler-Germain, D. A. *et al.* The R882H DNMT3A mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers. *Cancer cell* **25**, 442-454, doi:10.1016/j.ccr.2014.02.010 (2014).
124 Qu, Y. *et al.* Differential methylation in CN-AML preferentially targets non-CGI regions and is dictated by DNMT3A mutational status and associated with predominant hypomethylation of HOX genes. *Epigenetics* **9**, 1108-1119, doi:10.4161/epi.29315 (2014).
125 Euba, B. *et al.* A meta-analysis of TET2 mutations shows a distinct distribution pattern in de novo acute myeloid leukemia and chronic myelomonocytic leukemia. *Leukemia & lymphoma* **53**, 1230-1233, doi:10.3109/10428194.2011.639878 (2012).
126 Ko, M. *et al.* Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 14566-14571, doi:10.1073/pnas.1112317108 (2011).
127 Itzykson, R. *et al.* Clonal architecture of chronic myelomonocytic leukemias. *Blood* **121**, 2186-2198, doi:10.1182/blood-2012-06-440347 (2013).
128 Li, Z. *et al.* Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood* **118**, 4509-4518, doi:10.1182/blood-2010-12-325241 (2011).
129 Fuster, J. J. *et al.* Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science* **355**, 842-847, doi:10.1126/science.aag1381 (2017).
130 Levis, M. & Small, D. FLT3: ITDoes matter in leukemia. *Leukemia* **17**, 1738-1752, doi:10.1038/sj.leu.2403099 (2003).
131 Sercel, A. J. *et al.* Stable transplantation of human mitochondrial DNA by high-throughput, pressurized isolated mitochondrial delivery. *eLife* **10**, doi:10.7554/eLife.63102 (2021).
132 Spinelli, J. B. & Haigis, M. C. The multifaceted contributions of mitochondria to cellular metabolism. *Nature cell biology* **20**, 745-754, doi:10.1038/s41556-018-0124-1 (2018).
133 Missiroli, S., Perrone, M., Genovese, I., Pinton, P. & Giorgi, C. Cancer metabolism and mitochondria: Finding novel mechanisms to fight tumours. *EBioMedicine* **59**, 102943, doi:10.1016/j.ebiom.2020.102943 (2020).
134 Parker, G. C., Acsadi, G. & Brenner, C. A. Mitochondria: determinants of stem cell fate? *Stem cells and development* **18**, 803-806, doi:10.1089/scd.2009.1806.edi (2009).
135 Morganti, C. & Ito, K. Mitochondrial Contributions to Hematopoietic Stem Cell Aging. *International journal of molecular sciences* **22**, doi:10.3390/ijms222011117 (2021).
136 Naka, K., Muraguchi, T., Hoshii, T. & Hirao, A. Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxidants & redox signaling* **10**, 1883-1894, doi:10.1089/ars.2008.2114 (2008).
137 Takubo, K. *et al.* Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell stem cell* **12**, 49-61, doi:10.1016/j.stem.2012.10.011 (2013).
138 Ludin, A. *et al.* Reactive oxygen species regulate hematopoietic stem cell self-renewal, migration and development, as well as their bone marrow microenvironment. *Antioxidants & redox signaling* **21**, 1605-1619, doi:10.1089/ars.2014.5941 (2014).

139 Lambeth, J. D. Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. *Free radical biology & medicine* **43**, 332-347, doi:10.1016/j.freeradbiomed.2007.03.027 (2007).
140 Wang, Y. *et al.* Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells. *Free radical biology & medicine* **48**, 348-356, doi:10.1016/j.freeradbiomed.2009.11.005 (2010).
141 Liu, X. *et al.* Regulation of mitochondrial biogenesis in erythropoiesis by mTORC1-mediated protein translation. *Nature cell biology* **19**, 626-638, doi:10.1038/ncb3527 (2017).
142 Shao, L. *et al.* Reactive oxygen species and hematopoietic stem cell senescence. *International journal of hematology* **94**, 24-32, doi:10.1007/s12185-011-0872-1 (2011).
143 Anso, E. *et al.* The mitochondrial respiratory chain is essential for haematopoietic stem cell function. *Nature cell biology* **19**, 614-625, doi:10.1038/ncb3529 (2017).
144 Bejarano-Garcia, J. A. *et al.* Sensitivity of hematopoietic stem cells to mitochondrial dysfunction by SdhD gene deletion. *Cell death & disease* **7**, e2516, doi:10.1038/cddis.2016.411 (2016).
145 Bertaux, A. *et al.* Mitochondrial OXPHOS influences immune cell fate: lessons from hematopoietic AIF-deficient and NDUFS4-deficient mouse models. *Cell death & disease* **9**, 581, doi:10.1038/s41419-018-0583-0 (2018).
146 Morganti, C., Bonora, M., Ito, K. & Ito, K. Electron transport chain complex II sustains high mitochondrial membrane potential in hematopoietic stem and progenitor cells. *Stem cell research* **40**, 101573, doi:10.1016/j.scr.2019.101573 (2019).
147 Gustafsson, C. M., Falkenberg, M. & Larsson, N. G. Maintenance and Expression of Mammalian Mitochondrial DNA. *Annual review of biochemistry* **85**, 133-160, doi:10.1146/annurev-biochem-060815-014402 (2016).
148 Spitkovsky, D. *et al.* Activity of complex III of the mitochondrial electron transport chain is essential for early heart muscle cell differentiation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18**, 1300-1302, doi:10.1096/fj.03-0520fje (2004).
149 Ahlqvist, K. J. *et al.* Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in Polg mutator mice. *Cell metabolism* **15**, 100-109, doi:10.1016/j.cmet.2011.11.012 (2012).
150 Chen, M. L. *et al.* Erythroid dysplasia, megaloblastic anemia, and impaired lymphopoiesis arising from mitochondrial dysfunction. *Blood* **114**, 4045-4053, doi:10.1182/blood-2008-08-169474 (2009).
151 Norddahl, G. L. *et al.* Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. *Cell stem cell* **8**, 499-510, doi:10.1016/j.stem.2011.03.009 (2011).
152 Inoue, S., Yokota, M., Nakada, K., Miyoshi, H. & Hayashi, J. Pathogenic mitochondrial DNA-induced respiration defects in hematopoietic cells result in anemia by suppressing erythroid differentiation. *FEBS letters* **581**, 1910-1916, doi:10.1016/j.febslet.2007.03.092 (2007).
153 Notta, F. *et al.* Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* **333**, 218-221, doi:10.1126/science.1201219 (2011).
154 Umemoto, T., Hashimoto, M., Matsumura, T., Nakamura-Ishizu, A. & Suda, T. Ca(2+)-mitochondria axis drives cell division in hematopoietic stem cells. *The Journal of experimental medicine* **215**, 2097-2113, doi:10.1084/jem.20180421 (2018).
155 Vannini, N. *et al.* Specification of haematopoietic stem cell fate via modulation of mitochondrial activity. *Nature communications* **7**, 13125, doi:10.1038/ncomms13125 (2016).
156 Chen, C. *et al.* TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. *The Journal of experimental medicine* **205**, 2397-2408, doi:10.1084/jem.20081297 (2008).

157 Maryanovich, M. *et al.* An MTCH2 pathway repressing mitochondria metabolism regulates haematopoietic stem cell fate. *Nature communications* **6**, 7901, doi:10.1038/ncomms8901 (2015).
158 Ho, T. T. *et al.* Autophagy maintains the metabolism and function of young and old stem cells. *Nature* **543**, 205-210, doi:10.1038/nature21388 (2017).
159 Nguyen-McCarty, M. & Klein, P. S. Autophagy is a signature of a signaling network that maintains hematopoietic stem cells. *PloS one* **12**, e0177054, doi:10.1371/journal.pone.0177054 (2017).
160 Ito, K. *et al.* Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science* **354**, 1156-1160, doi:10.1126/science.aaf5530 (2016).
161 Tothova, Z. *et al.* FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* **128**, 325-339, doi:10.1016/j.cell.2007.01.003 (2007).
162 Takubo, K. *et al.* Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell stem cell* **7**, 391-402, doi:10.1016/j.stem.2010.06.020 (2010).
163 Cyr, A. R. & Domann, F. E. The redox basis of epigenetic modifications: from mechanisms to functional consequences. *Antioxidants & redox signaling* **15**, 551-589, doi:10.1089/ars.2010.3492 (2011).
164 Sabari, B. R., Zhang, D., Allis, C. D. & Zhao, Y. Metabolic regulation of gene expression through histone acylations. *Nature reviews. Molecular cell biology* **18**, 90-101, doi:10.1038/nrm.2016.140 (2017).
165 Shimazu, T. *et al.* Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* **339**, 211-214, doi:10.1126/science.1227166 (2013).
166 Thakur, C. & Chen, F. Connections between metabolism and epigenetics in cancers. *Seminars in cancer biology* **57**, 52-58, doi:10.1016/j.semcancer.2019.06.006 (2019).
167 Delatte, B., Deplus, R. & Fuks, F. Playing TETris with DNA modifications. *The EMBO journal* **33**, 1198-1211, doi:10.15252/embj.201488290 (2014).
168 Ashrafi, G. & Schwarz, T. L. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell death and differentiation* **20**, 31-42, doi:10.1038/cdd.2012.81 (2013).
169 Yang, Z. & Klionsky, D. J. Eaten alive: a history of macroautophagy. *Nature cell biology* **12**, 814-822, doi:10.1038/ncb0910-814 (2010).
170 Mihaylova, M. M. & Shaw, R. J. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nature cell biology* **13**, 1016-1023, doi:10.1038/ncb2329 (2011).
171 Revuelta, M. & Matheu, A. Autophagy in stem cell aging. *Aging cell* **16**, 912-915, doi:10.1111/acel.12655 (2017).
172 Jin, G. *et al.* Atad3a suppresses Pink1-dependent mitophagy to maintain homeostasis of hematopoietic progenitor cells. *Nature immunology* **19**, 29-40, doi:10.1038/s41590-017-0002-1 (2018).
173 Mizushima, N. & Levine, B. Autophagy in mammalian development and differentiation. *Nature cell biology* **12**, 823-830, doi:10.1038/ncb0910-823 (2010).
174 Jung, H. E., Shim, Y. R., Oh, J. E., Oh, D. S. & Lee, H. K. The autophagy Protein Atg5 Plays a Crucial Role in the Maintenance and Reconstitution Ability of Hematopoietic Stem Cells. *Immune network* **19**, e12, doi:10.4110/in.2019.19.e12 (2019).
175 Liu, F. *et al.* FIP200 is required for the cell-autonomous maintenance of fetal hematopoietic stem cells. *Blood* **116**, 4806-4814, doi:10.1182/blood-2010-06-288589 (2010).
176 Westermann, B. Mitochondrial fusion and fission in cell life and death. *Nature reviews. Molecular cell biology* **11**, 872-884, doi:10.1038/nrm3013 (2010).
177 Luchsinger, L. L., de Almeida, M. J., Corrigan, D. J., Mumau, M. & Snoeck, H. W. Mitofusin 2 maintains haematopoietic stem cells with extensive lymphoid potential. *Nature* **529**, 528-531, doi:10.1038/nature16500 (2016).

178 Prieto, J. *et al.* Early ERK1/2 activation promotes DRP1-dependent mitochondrial fission necessary for cell reprogramming. *Nature communications* **7**, 11124, doi:10.1038/ncomms11124 (2016).
179 Verovskaya, E. V., Dellorusso, P. V. & Passegue, E. Losing Sense of Self and Surroundings: Hematopoietic Stem Cell Aging and Leukemic Transformation. *Trends in molecular medicine* **25**, 494-515, doi:10.1016/j.molmed.2019.04.006 (2019).
180 Contreras, L., Drago, I., Zampese, E. & Pozzan, T. Mitochondria: the calcium connection. *Biochimica et biophysica acta* **1797**, 607-618, doi:10.1016/j.bbabio.2010.05.005 (2010).
181 Berridge, M. J., Bootman, M. D. & Roderick, H. L. Calcium signalling: dynamics, homeostasis and remodelling. *Nature reviews. Molecular cell biology* **4**, 517-529, doi:10.1038/nrm1155 (2003).
182 Zampese, E. & Pizzo, P. Intracellular organelles in the saga of Ca2+ homeostasis: different molecules for different purposes? *Cellular and molecular life sciences : CMLS* **69**, 1077-1104, doi:10.1007/s00018-011-0845-9 (2012).
183 Chen, L. *et al.* HINT2 triggers mitochondrial Ca(2+) influx by regulating the mitochondrial Ca(2+) uniporter (MCU) complex and enhances gemcitabine apoptotic effect in pancreatic cancer. *Cancer letters* **411**, 106-116, doi:10.1016/j.canlet.2017.09.020 (2017).
184 De Stefani, D., Raffaello, A., Teardo, E., Szabo, I. & Rizzuto, R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* **476**, 336-340, doi:10.1038/nature10230 (2011).
185 Ito, K., Bonora, M. & Ito, K. Metabolism as master of hematopoietic stem cell fate. *International journal of hematology* **109**, 18-27, doi:10.1007/s12185-018-2534-z (2019).
186 Nakamura-Ishizu, A., Ito, K. & Suda, T. Hematopoietic Stem Cell Metabolism during Development and Aging. *Developmental cell* **54**, 239-255, doi:10.1016/j.devcel.2020.06.029 (2020).
187 Rimmele, P. *et al.* Mitochondrial metabolism in hematopoietic stem cells requires functional FOXO3. *EMBO reports* **16**, 1164-1176, doi:10.15252/embr.201439704 (2015).
188 Luchsinger, L. L. *et al.* Harnessing Hematopoietic Stem Cell Low Intracellular Calcium Improves Their Maintenance In Vitro. *Cell stem cell* **25**, 225-240 e227, doi:10.1016/j.stem.2019.05.002 (2019).
189 Grahn, T. H. M. *et al.* S100A6 is a critical regulator of hematopoietic stem cells. *Leukemia* **34**, 3323-3337, doi:10.1038/s41375-020-0901-2 (2020).
190 Favaro, G. *et al.* DRP1-mediated mitochondrial shape controls calcium homeostasis and muscle mass. *Nature communications* **10**, 2576, doi:10.1038/s41467-019-10226-9 (2019).
191 Hinge, A. *et al.* Asymmetrically Segregated Mitochondria Provide Cellular Memory of Hematopoietic Stem Cell Replicative History and Drive HSC Attrition. *Cell stem cell* **26**, 420-430 e426, doi:10.1016/j.stem.2020.01.016 (2020).
192 Cabezas-Wallscheid, N. *et al.* Vitamin A-Retinoic Acid Signaling Regulates Hematopoietic Stem Cell Dormancy. *Cell* **169**, 807-823 e819, doi:10.1016/j.cell.2017.04.018 (2017).
193 Guitart, A. V., Finch, A. J. & Kranc, K. R. Ca(2+)tapulting HSCs into action. *The Journal of experimental medicine* **215**, 1971-1973, doi:10.1084/jem.20181097 (2018).
194 Zhang, H. *et al.* HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. *Cancer cell* **11**, 407-420, doi:10.1016/j.ccr.2007.04.001 (2007).
195 Sahin, E. *et al.* Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* **470**, 359-365, doi:10.1038/nature09787 (2011).
196 Sena, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species. *Molecular cell* **48**, 158-167, doi:10.1016/j.molcel.2012.09.025 (2012).
197 Hamanaka, R. B. & Chandel, N. S. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends in biochemical sciences* **35**, 505-513, doi:10.1016/j.tibs.2010.04.002 (2010).

198 Lee, H. C. *et al.* Mitochondrial genome instability and mtDNA depletion in human cancers. *Annals of the New York Academy of Sciences* **1042**, 109-122, doi:10.1196/annals.1338.011 (2005).
199 Lee, H. C., Chang, C. M. & Chi, C. W. Somatic mutations of mitochondrial DNA in aging and cancer progression. *Ageing research reviews* **9 Suppl 1**, S47-58, doi:10.1016/j.arr.2010.08.009 (2010).
200 Lee, H. C., Huang, K. H., Yeh, T. S. & Chi, C. W. Somatic alterations in mitochondrial DNA and mitochondrial dysfunction in gastric cancer progression. *World journal of gastroenterology* **20**, 3950-3959, doi:10.3748/wjg.v20.i14.3950 (2014).
201 Lee, H. C. *et al.* Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma. *Mutation research* **547**, 71-78, doi:10.1016/j.mrfmmm.2003.12.011 (2004).
202 Tseng, L. M. *et al.* Mitochondrial DNA mutations and mitochondrial DNA depletion in breast cancer. *Genes, chromosomes & cancer* **45**, 629-638, doi:10.1002/gcc.20326 (2006).
203 Wu, C. W. *et al.* Mitochondrial DNA mutations and mitochondrial DNA depletion in gastric cancer. *Genes, chromosomes & cancer* **44**, 19-28, doi:10.1002/gcc.20213 (2005).
204 Akram, M. Citric acid cycle and role of its intermediates in metabolism. *Cell biochemistry and biophysics* **68**, 475-478, doi:10.1007/s12013-013-9750-1 (2014).
205 Dang, L. *et al.* Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**, 739-744, doi:10.1038/nature08617 (2009).
206 Ricketts, C. *et al.* Germline SDHB mutations and familial renal cell carcinoma. *Journal of the National Cancer Institute* **100**, 1260-1262, doi:10.1093/jnci/djn254 (2008).
207 Wai, T. & Langer, T. Mitochondrial Dynamics and Metabolic Regulation. *Trends in endocrinology and metabolism: TEM* **27**, 105-117, doi:10.1016/j.tem.2015.12.001 (2016).
208 Katajisto, P. *et al.* Stem cells. Asymmetric apportioning of aged mitochondria between daughter cells is required for stemness. *Science* **348**, 340-343, doi:10.1126/science.1260384 (2015).
209 Hole, P. S., Darley, R. L. & Tonks, A. Do reactive oxygen species play a role in myeloid leukemias? *Blood* **117**, 5816-5826, doi:10.1182/blood-2011-01-326025 (2011).
210 Hole, P. S. *et al.* Overproduction of NOX-derived ROS in AML promotes proliferation and is associated with defective oxidative stress signaling. *Blood* **122**, 3322-3330, doi:10.1182/blood-2013-04-491944 (2013).
211 Lazarini, M. *et al.* BNIP3L in myelodysplastic syndromes and acute myeloid leukemia: impact on disease outcome and cellular response to decitabine. *Haematologica* **101**, e445-e448, doi:10.3324/haematol.2016.142521 (2016).
212 Brigger, D., Proikas-Cezanne, T. & Tschan, M. P. WIPI-dependent autophagy during neutrophil differentiation of NB4 acute promyelocytic leukemia cells. *Cell death & disease* **5**, e1315, doi:10.1038/cddis.2014.261 (2014).
213 Dany, M. *et al.* Targeting FLT3-ITD signaling mediates ceramide-dependent mitophagy and attenuates drug resistance in AML. *Blood* **128**, 1944-1958, doi:10.1182/blood-2016-04-708750 (2016).
214 Heydt, Q. *et al.* Oncogenic FLT3-ITD supports autophagy via ATF4 in acute myeloid leukemia. *Oncogene* **37**, 787-797, doi:10.1038/onc.2017.376 (2018).
215 Velez, J. *et al.* Mitochondrial uncoupling and the reprograming of intermediary metabolism in leukemia cells. *Frontiers in oncology* **3**, 67, doi:10.3389/fonc.2013.00067 (2013).
216 Shi, J. *et al.* High Expression of CPT1A Predicts Adverse Outcomes: A Potential Therapeutic Target for Acute Myeloid Leukemia. *EBioMedicine* **14**, 55-64, doi:10.1016/j.ebiom.2016.11.025 (2016).
217 Shen, N. *et al.* Expression of hypoxia-inducible factor 1alpha, glucose transporter 1, and hexokinase 2 in primary central nervous system lymphoma and the correlation with the biological behaviors. *Brain and behavior* **10**, e01718, doi:10.1002/brb3.1718 (2020).

218 Guitart, A. V. *et al.* Fumarate hydratase is a critical metabolic regulator of hematopoietic stem cell functions. *The Journal of experimental medicine* **214**, 719-735, doi:10.1084/jem.20161087 (2017).
219 Reed, M. A. C., Ludwig, C., Bunce, C. M., Khanim, F. L. & Gunther, U. L. Malonate as a ROS product is associated with pyruvate carboxylase activity in acute myeloid leukaemia cells. *Cancer & metabolism* **4**, 15, doi:10.1186/s40170-016-0155-7 (2016).
220 Laukka, T. *et al.* Fumarate and Succinate Regulate Expression of Hypoxia-inducible Genes via TET Enzymes. *The Journal of biological chemistry* **291**, 4256-4265, doi:10.1074/jbc.M115.688762 (2016).
221 Song, K. *et al.* Resistance to chemotherapy is associated with altered glucose metabolism in acute myeloid leukemia. *Oncology letters* **12**, 334-342, doi:10.3892/ol.2016.4600 (2016).
222 Bowman, R. L., Busque, L. & Levine, R. L. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. *Cell stem cell* **22**, 157-170, doi:10.1016/j.stem.2018.01.011 (2018).
223 Chan, S. M. & Majeti, R. Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. *International journal of hematology* **98**, 648-657, doi:10.1007/s12185-013-1407-8 (2013).
224 Bellizzi, D. *et al.* The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern. *DNA research : an international journal for rapid publication of reports on genes and genomes* **20**, 537-547, doi:10.1093/dnares/dst029 (2013).
225 Wong, M., Gertz, B., Chestnut, B. A. & Martin, L. J. Mitochondrial DNMT3A and DNA methylation in skeletal muscle and CNS of transgenic mouse models of ALS. *Frontiers in cellular neuroscience* **7**, 279, doi:10.3389/fncel.2013.00279 (2013).
226 Bonora, M. *et al.* ATP synthesis and storage. *Purinergic signalling* **8**, 343-357, doi:10.1007/s11302-012-9305-8 (2012).
227 Hagemann, S., Heil, O., Lyko, F. & Brueckner, B. Azacytidine and decitabine induce gene-specific and non-random DNA demethylation in human cancer cell lines. *PloS one* **6**, e17388, doi:10.1371/journal.pone.0017388 (2011).
228 Bonora, M. *et al.* Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. *Nature protocols* **8**, 2105-2118, doi:10.1038/nprot.2013.127 (2013).
229 Chen, T. W. *et al.* Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **499**, 295-300, doi:10.1038/nature12354 (2013).
230 Bai, H., Xu, R., Cao, Z., Wei, D. & Wang, C. Involvement of miR-21 in resistance to daunorubicin by regulating PTEN expression in the leukaemia K562 cell line. *FEBS letters* **585**, 402-408, doi:10.1016/j.febslet.2010.12.027 (2011).
231 Heo, S. K. *et al.* Radotinib enhances cytarabine (Ara-C)-induced acute myeloid leukemia cell death. *BMC cancer* **20**, 1193, doi:10.1186/s12885-020-07701-8 (2020).
232 Roy, M., Reddy, P. H., Iijima, M. & Sesaki, H. Mitochondrial division and fusion in metabolism. *Current opinion in cell biology* **33**, 111-118, doi:10.1016/j.ceb.2015.02.001 (2015).
233 Chan, D. C. Mitochondria: dynamic organelles in disease, aging, and development. *Cell* **125**, 1241-1252, doi:10.1016/j.cell.2006.06.010 (2006).
234 Rafelski, S. M. Mitochondrial network morphology: building an integrative, geometrical view. *BMC biology* **11**, 71, doi:10.1186/1741-7007-11-71 (2013).
235 Scarpulla, R. C. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiological reviews* **88**, 611-638, doi:10.1152/physrev.00025.2007 (2008).
236 Wu, Z. *et al.* Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115-124, doi:10.1016/S0092-8674(00)80611-X (1999).
237 Kanki, T. *et al.* Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA. *Molecular and cellular biology* **24**, 9823-9834, doi:10.1128/MCB.24.22.9823-9834.2004 (2004).

238 Larsson, N. G. *et al.* Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature genetics* **18**, 231-236, doi:10.1038/ng0398-231 (1998).
239 Medeiros, D. M. Assessing mitochondria biogenesis. *Methods* **46**, 288-294, doi:10.1016/j.ymeth.2008.09.026 (2008).
240 Puigserver, P. *et al.* A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829-839, doi:10.1016/s0092-8674(00)81410-5 (1998).
241 Ploumi, C., Daskalaki, I. & Tavernarakis, N. Mitochondrial biogenesis and clearance: a balancing act. *The FEBS journal* **284**, 183-195, doi:10.1111/febs.13820 (2017).
242 Kim, B. & Song, Y. S. Mitochondrial dynamics altered by oxidative stress in cancer. *Free radical research* **50**, 1065-1070, doi:10.1080/10715762.2016.1210141 (2016).
243 Bae, Y. S., Oh, H., Rhee, S. G. & Yoo, Y. D. Regulation of reactive oxygen species generation in cell signaling. *Molecules and cells* **32**, 491-509, doi:10.1007/s10059-011-0276-3 (2011).
244 Suski, J. *et al.* Relation Between Mitochondrial Membrane Potential and ROS Formation. *Methods in molecular biology* **1782**, 357-381, doi:10.1007/978-1-4939-7831-1_22 (2018).
245 Deshwal, S., Antonucci, S., Kaludercic, N. & Di Lisa, F. Measurement of Mitochondrial ROS Formation. *Methods in molecular biology* **1782**, 403-418, doi:10.1007/978-1-4939-7831-1_24 (2018).
246 Miwa, S. & Brand, M. D. Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochemical Society transactions* **31**, 1300-1301, doi:10.1042/bst0311300 (2003).
247 Geromel, V. *et al.* Superoxide-induced massive apoptosis in cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the ATPase-6 gene of the mitochondrial DNA. *Human molecular genetics* **10**, 1221-1228, doi:10.1093/hmg/10.11.1221 (2001).
248 Lebiedzinska, M. *et al.* Oxidative stress-dependent p66Shc phosphorylation in skin fibroblasts of children with mitochondrial disorders. *Biochimica et biophysica acta* **1797**, 952-960, doi:10.1016/j.bbabio.2010.03.005 (2010).
249 Bedard, K. & Krause, K. H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews* **87**, 245-313, doi:10.1152/physrev.00044.2005 (2007).
250 Fanucchi, S., Dominguez-Andres, J., Joosten, L. A. B., Netea, M. G. & Mhlanga, M. M. The Intersection of Epigenetics and Metabolism in Trained Immunity. *Immunity* **54**, 32-43, doi:10.1016/j.immuni.2020.10.011 (2021).
251 Sriskanthadevan, S. *et al.* AML cells have low spare reserve capacity in their respiratory chain that renders them susceptible to oxidative metabolic stress. *Blood* **125**, 2120-2130, doi:10.1182/blood-2014-08-594408 (2015).
252 Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer cell* **18**, 553-567, doi:10.1016/j.ccr.2010.11.015 (2010).
253 Hardie, D. G. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes & development* **25**, 1895-1908, doi:10.1101/gad.17420111 (2011).
254 Fernandez-Vizarra, E. & Zeviani, M. Mitochondrial disorders of the OXPHOS system. *FEBS letters* **595**, 1062-1106, doi:10.1002/1873-3468.13995 (2021).
255 Bhatti, J. S., Bhatti, G. K. & Reddy, P. H. Mitochondrial dysfunction and oxidative stress in metabolic disorders - A step towards mitochondria based therapeutic strategies. *Biochimica et biophysica acta. Molecular basis of disease* **1863**, 1066-1077, doi:10.1016/j.bbadis.2016.11.010 (2017).
256 Yu, M. Generation, function and diagnostic value of mitochondrial DNA copy number alterations in human cancers. *Life sciences* **89**, 65-71, doi:10.1016/j.lfs.2011.05.010 (2011).
257 de Almeida, M. J., Luchsinger, L. L., Corrigan, D. J., Williams, L. J. & Snoeck, H. W. Dye-Independent Methods Reveal Elevated Mitochondrial Mass in Hematopoietic Stem Cells. *Cell stem cell* **21**, 725-729 e724, doi:10.1016/j.stem.2017.11.002 (2017).

258 Protti, A. & Singer, M. Bench-to-bedside review: potential strategies to protect or reverse mitochondrial dysfunction in sepsis-induced organ failure. *Critical care* **10**, 228, doi:10.1186/cc5014 (2006).
259 Eleff, S. *et al.* 31P NMR study of improvement in oxidative phosphorylation by vitamins K3 and C in a patient with a defect in electron transport at complex III in skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 3529-3533, doi:10.1073/pnas.81.11.3529 (1984).
260 Jalloh, I. *et al.* Focally perfused succinate potentiates brain metabolism in head injury patients. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **37**, 2626-2638, doi:10.1177/0271678X16672665 (2017).
261 Chaban, Y., Boekema, E. J. & Dudkina, N. V. Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation. *Biochimica et biophysica acta* **1837**, 418-426, doi:10.1016/j.bbabio.2013.10.004 (2014).
262 Fang, D., Yan, S., Yu, Q., Chen, D. & Yan, S. S. Mfn2 is Required for Mitochondrial Development and Synapse Formation in Human Induced Pluripotent Stem Cells/hiPSC Derived Cortical Neurons. *Scientific reports* **6**, 31462, doi:10.1038/srep31462 (2016).
263 Tu, C. L., Chang, W. & Bikle, D. D. The extracellular calcium-sensing receptor is required for calcium-induced differentiation in human keratinocytes. *The Journal of biological chemistry* **276**, 41079-41085, doi:10.1074/jbc.M107122200 (2001).
264 Gherardi, G., Monticelli, H., Rizzuto, R. & Mammucari, C. The Mitochondrial Ca(2+) Uptake and the Fine-Tuning of Aerobic Metabolism. *Frontiers in physiology* **11**, 554904, doi:10.3389/fphys.2020.554904 (2020).
265 Granatiero, V., De Stefani, D. & Rizzuto, R. Mitochondrial Calcium Handling in Physiology and Disease. *Advances in experimental medicine and biology* **982**, 25-47, doi:10.1007/978-3-319-55330-6_2 (2017).
266 De Stefani, D., Patron, M. & Rizzuto, R. Structure and function of the mitochondrial calcium uniporter complex. *Biochimica et biophysica acta* **1853**, 2006-2011, doi:10.1016/j.bbamcr.2015.04.008 (2015).
267 Yamamoto, T. *et al.* Functional analysis of coiled-coil domains of MCU in mitochondrial calcium uptake. *Biochimica et biophysica acta. Bioenergetics* **1860**, 148061, doi:10.1016/j.bbabio.2019.148061 (2019).
268 Rai, K., Pilarski, R., Cebulla, C. M. & Abdel-Rahman, M. H. Comprehensive review of BAP1 tumor predisposition syndrome with report of two new cases. *Clinical genetics* **89**, 285-294, doi:10.1111/cge.12630 (2016).
269 Marchi, S., Giorgi, C., Galluzzi, L. & Pinton, P. Ca(2+) Fluxes and Cancer. *Molecular cell* **78**, 1055-1069, doi:10.1016/j.molcel.2020.04.017 (2020).
270 Vultur, A., Gibhardt, C. S., Stanisz, H. & Bogeski, I. The role of the mitochondrial calcium uniporter (MCU) complex in cancer. *Pflugers Archiv : European journal of physiology* **470**, 1149-1163, doi:10.1007/s00424-018-2162-8 (2018).
271 Rizzuto, R. *et al.* Ca(2+) transfer from the ER to mitochondria: when, how and why. *Biochimica et biophysica acta* **1787**, 1342-1351, doi:10.1016/j.bbabio.2009.03.015 (2009).
272 Alvarez, J. & Montero, M. Measuring [Ca2+] in the endoplasmic reticulum with aequorin. *Cell calcium* **32**, 251-260, doi:10.1016/s0143416002001860 (2002).
273 Villalobos, C., Gutierrez, L. G., Hernandez-Morales, M., Del Bosque, D. & Nunez, L. Mitochondrial control of store-operated Ca(2+) channels in cancer: Pharmacological implications. *Pharmacol Res* **135**, 136-143, doi:10.1016/j.phrs.2018.08.001 (2018).
274 Noble, D. & Herchuelz, A. Role of Na/Ca exchange and the plasma membrane Ca2+-ATPase in cell function. Conference on Na/Ca exchange. *EMBO reports* **8**, 228-232, doi:10.1038/sj.embor.7400914 (2007).

275 Raturi, A. & Simmen, T. Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *Biochimica et biophysica acta* **1833**, 213-224, doi:10.1016/j.bbamcr.2012.04.013 (2013).
276 Pinton, P., Rimessi, A., Romagnoli, A., Prandini, A. & Rizzuto, R. Biosensors for the detection of calcium and pH. *Methods in cell biology* **80**, 297-325, doi:10.1016/S0091-679X(06)80015-4 (2007).
277 de Juan-Sanz, J. *et al.* Axonal Endoplasmic Reticulum Ca(2+) Content Controls Release Probability in CNS Nerve Terminals. *Neuron* **93**, 867-881 e866, doi:10.1016/j.neuron.2017.01.010 (2017).
278 Rizzuto, R., De Stefani, D., Raffaello, A. & Mammucari, C. Mitochondria as sensors and regulators of calcium signalling. *Nature reviews. Molecular cell biology* **13**, 566-578, doi:10.1038/nrm3412 (2012).
279 Pathak, T. & Trebak, M. Mitochondrial Ca(2+) signaling. *Pharmacology & therapeutics* **192**, 112-123, doi:10.1016/j.pharmthera.2018.07.001 (2018).
280 Ivanova, H. *et al.* Inositol 1,4,5-trisphosphate receptor-isoform diversity in cell death and survival. *Biochimica et biophysica acta* **1843**, 2164-2183, doi:10.1016/j.bbamcr.2014.03.007 (2014).
281 Xu, H. & Van Remmen, H. The SarcoEndoplasmic Reticulum Calcium ATPase (SERCA) pump: a potential target for intervention in aging and skeletal muscle pathologies. *Skeletal muscle* **11**, 25, doi:10.1186/s13395-021-00280-7 (2021).
282 Marcucci, G. *et al.* Age-related prognostic impact of different types of DNMT3A mutations in adults with primary cytogenetically normal acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 742-750, doi:10.1200/JCO.2011.39.2092 (2012).
283 Cashen, A. F., Schiller, G. J., O'Donnell, M. R. & DiPersio, J. F. Multicenter, phase II study of decitabine for the first-line treatment of older patients with acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 556-561, doi:10.1200/JCO.2009.23.9178 (2010).
284 Sato, H., Wheat, J. C., Steidl, U. & Ito, K. DNMT3A and TET2 in the Pre-Leukemic Phase of Hematopoietic Disorders. *Frontiers in oncology* **6**, 187, doi:10.3389/fonc.2016.00187 (2016).
285 Pandolfi, A. & Steidl, U. HLX in AML: novel prognostic and therapeutic target. *Oncotarget* **3**, 1059-1060, doi:10.18632/oncotarget.705 (2012).
286 Demchenko, A. P. Beyond annexin V: fluorescence response of cellular membranes to apoptosis. *Cytotechnology* **65**, 157-172, doi:10.1007/s10616-012-9481-y (2013).
287 Lebaron, P., Catala, P. & Parthuisot, N. Effectiveness of SYTOX Green stain for bacterial viability assessment. *Applied and environmental microbiology* **64**, 2697-2700, doi:10.1128/AEM.64.7.2697-2700.1998 (1998).
288 Papa, L., Djedaini, M. & Hoffman, R. Mitochondrial Role in Stemness and Differentiation of Hematopoietic Stem Cells. *Stem cells international* **2019**, 4067162, doi:10.1155/2019/4067162 (2019).
289 Cabezas-Wallscheid, N. *et al.* Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell stem cell* **15**, 507-522, doi:10.1016/j.stem.2014.07.005 (2014).
290 Snoeck, H. W. Mitochondrial regulation of hematopoietic stem cells. *Current opinion in cell biology* **49**, 91-98, doi:10.1016/j.ceb.2017.12.010 (2017).
291 Martinez-Reyes, I. *et al.* TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions. *Molecular cell* **61**, 199-209, doi:10.1016/j.molcel.2015.12.002 (2016).
292 Shyh-Chang, N. & Ng, H. H. The metabolic programming of stem cells. *Genes & development* **31**, 336-346, doi:10.1101/gad.293167.116 (2017).

293 Agathocleous, M. *et al.* Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. *Nature* **549**, 476-481, doi:10.1038/nature23876 (2017).
294 Cimmino, L. *et al.* Restoration of TET2 Function Blocks Aberrant Self-Renewal and Leukemia Progression. *Cell* **170**, 1079-1095 e1020, doi:10.1016/j.cell.2017.07.032 (2017).
295 Challen, G. A. *et al.* Dnmt3a is essential for hematopoietic stem cell differentiation. *Nature genetics* **44**, 23-31, doi:10.1038/ng.1009 (2011).
296 Jeong, M. *et al.* Loss of Dnmt3a Immortalizes Hematopoietic Stem Cells In Vivo. *Cell reports* **23**, 1-10, doi:10.1016/j.celrep.2018.03.025 (2018).
297 Roller, A. *et al.* Landmark analysis of DNMT3A mutations in hematological malignancies. *Leukemia* **27**, 1573-1578, doi:10.1038/leu.2013.65 (2013).
298 Abdel-Wahab, O. *et al.* Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood* **114**, 144-147, doi:10.1182/blood-2009-03-210039 (2009).
299 Chou, W. C. *et al.* TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. *Blood* **118**, 3803-3810, doi:10.1182/blood-2011-02-339747 (2011).
300 Rothenberg-Thurley, M. *et al.* Persistence of pre-leukemic clones during first remission and risk of relapse in acute myeloid leukemia. *Leukemia* **32**, 1598-1608, doi:10.1038/s41375-018-0034-z (2018).
301 Rebelo, A. P., Williams, S. L. & Moraes, C. T. In vivo methylation of mtDNA reveals the dynamics of protein-mtDNA interactions. *Nucleic acids research* **37**, 6701-6715, doi:10.1093/nar/gkp727 (2009).
302 Bam, S., Buchanan, E., Mahony, C. & O'Ryan, C. DNA Methylation of PGC-1alpha Is Associated With Elevated mtDNA Copy Number and Altered Urinary Metabolites in Autism Spectrum Disorder. *Frontiers in cell and developmental biology* **9**, 696428, doi:10.3389/fcell.2021.696428 (2021).
303 Ventura-Clapier, R., Garnier, A. & Veksler, V. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. *Cardiovascular research* **79**, 208-217, doi:10.1093/cvr/cvn098 (2008).
304 Park, Y. J. *et al.* DNMT1 maintains metabolic fitness of adipocytes through acting as an epigenetic safeguard of mitochondrial dynamics. *Proceedings of the National Academy of Sciences of the United States of America* **118**, doi:10.1073/pnas.2021073118 (2021).
305 Cieslar-Pobuda, A. *et al.* DNMT3B deficiency alters mitochondrial biogenesis and alpha-ketoglutarate levels in human embryonic stem cells. *Stem cells* **38**, 1409-1422, doi:10.1002/stem.3256 (2020).
306 Chen, J. *et al.* Low shear stress induced vascular endothelial cell pyroptosis by TET2/SDHB/ROS pathway. *Free radical biology & medicine* **162**, 582-591, doi:10.1016/j.freeradbiomed.2020.11.017 (2021).
307 Kalaitzidis, D. *et al.* mTOR complex 1 plays critical roles in hematopoiesis and Pten-loss-evoked leukemogenesis. *Cell stem cell* **11**, 429-439, doi:10.1016/j.stem.2012.06.009 (2012).
308 Cyr, A. R., Hitchler, M. J. & Domann, F. E. Regulation of SOD2 in cancer by histone modifications and CpG methylation: closing the loop between redox biology and epigenetics. *Antioxidants & redox signaling* **18**, 1946-1955, doi:10.1089/ars.2012.4850 (2013).
309 Bera, R. *et al.* Genetic and Epigenetic Perturbations by DNMT3A-R882 Mutants Impaired Apoptosis through Augmentation of PRDX2 in Myeloid Leukemia Cells. *Neoplasia* **20**, 1106-1120, doi:10.1016/j.neo.2018.08.013 (2018).
310 Damal Villivalam, S. *et al.* A necessary role of DNMT3A in endurance exercise by suppressing ALDH1L1-mediated oxidative stress. *The EMBO journal* **40**, e106491, doi:10.15252/embj.2020106491 (2021).
311 Dang, L., Jin, S. & Su, S. M. IDH mutations in glioma and acute myeloid leukemia. *Trends in molecular medicine* **16**, 387-397, doi:10.1016/j.molmed.2010.07.002 (2010).

312 Ward, P. S. *et al.* The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer cell* **17**, 225-234, doi:10.1016/j.ccr.2010.01.020 (2010).
313 Latini, A. *et al.* Induction of oxidative stress by L-2-hydroxyglutaric acid in rat brain. *Journal of neuroscience research* **74**, 103-110, doi:10.1002/jnr.10735 (2003).
314 Feng, J. *et al.* Role of Tet1 and 5-hydroxymethylcytosine in cocaine action. *Nature neuroscience* **18**, 536-544, doi:10.1038/nn.3976 (2015).
315 Madugundu, G. S., Cadet, J. & Wagner, J. R. Hydroxyl-radical-induced oxidation of 5-methylcytosine in isolated and cellular DNA. *Nucleic acids research* **42**, 7450-7460, doi:10.1093/nar/gku334 (2014).
316 Kim, D. I. *et al.* PRMT1 and PRMT4 Regulate Oxidative Stress-Induced Retinal Pigment Epithelial Cell Damage in SIRT1-Dependent and SIRT1-Independent Manners. *Oxidative medicine and cellular longevity* **2015**, 617919, doi:10.1155/2015/617919 (2015).
317 Wu, X. *et al.* Epigenetic signature of chronic cerebral hypoperfusion and beneficial effects of S-adenosylmethionine in rats. *Molecular neurobiology* **50**, 839-851, doi:10.1007/s12035-014-8698-5 (2014).
318 Afanas'ev, I. New nucleophilic mechanisms of ros-dependent epigenetic modifications: comparison of aging and cancer. *Aging and disease* **5**, 52-62, doi:10.14336/AD.2014.050052 (2014).
319 de Brito, O. M. & Scorrano, L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* **456**, 605-610, doi:10.1038/nature07534 (2008).
320 Launay, S. *et al.* Lineage-specific modulation of calcium pump expression during myeloid differentiation. *Blood* **93**, 4395-4405 (1999).
321 Mikoshiba, K. The IP3 receptor/Ca2+ channel and its cellular function. *Biochemical Society symposium*, 9-22, doi:10.1042/BSS0740009 (2007).
322 Correction: Expression of the type 3 InsP3 receptor is a final common event in the development of hepatocellular carcinoma. *Gut* **69**, e1, doi:10.1136/gutjnl-2018-317811corr1 (2020).
323 Zhang, H. *et al.* Disturbance of calcium homeostasis and myogenesis caused by TET2 deletion in muscle stem cells. *Cell death discovery* **8**, 236, doi:10.1038/s41420-022-01041-1 (2022).
324 Wang, Y. J. *et al.* IP3R-mediated Ca2+ signals govern hematopoietic and cardiac divergence of Flk1+ cells via the calcineurin-NFATc3-Etv2 pathway. *Journal of molecular cell biology* **9**, 274-288, doi:10.1093/jmcb/mjx014 (2017).
325 Zhou, X. *et al.* Targeting EZH2 regulates tumor growth and apoptosis through modulating mitochondria dependent cell-death pathway in HNSCC. *Oncotarget* **6**, 33720-33732, doi:10.18632/oncotarget.5606 (2015).
326 Li, Q., Bartlett, D. L., Gorry, M. C., O'Malley, M. E. & Guo, Z. S. Three epigenetic drugs up-regulate homeobox gene Rhox5 in cancer cells through overlapping and distinct molecular mechanisms. *Molecular pharmacology* **76**, 1072-1081, doi:10.1124/mol.109.056291 (2009).
327 Li, H. *et al.* Elevated TWIST1 expression in myelodysplastic syndromes/acute myeloid leukemia reduces efficacy of hypomethylating therapy with decitabine. *Haematologica* **105**, e502, doi:10.3324/haematol.2019.235325 (2020).
328 Cao, L. *et al.* Inhibiting DNA Methylation Improves Survival in Severe Sepsis by Regulating NF-kappaB Pathway. *Frontiers in immunology* **11**, 1360, doi:10.3389/fimmu.2020.01360 (2020).
329 Kundakovic, M., Chen, Y., Guidotti, A. & Grayson, D. R. The reelin and GAD67 promoters are activated by epigenetic drugs that facilitate the disruption of local repressor complexes. *Molecular pharmacology* **75**, 342-354, doi:10.1124/mol.108.051763 (2009).
330 Bravo-Sagua, R. *et al.* Calcium Transport and Signaling in Mitochondria. *Comprehensive Physiology* **7**, 623-634, doi:10.1002/cphy.c160013 (2017).

331 Rizzuto, R. *et al.* Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Science* **280**, 1763-1766, doi:10.1126/science.280.5370.1763 (1998).
332 Csordas, G. *et al.* Structural and functional features and significance of the physical linkage between ER and mitochondria. *The Journal of cell biology* **174**, 915-921, doi:10.1083/jcb.200604016 (2006).
333 Csordas, G., Weaver, D. & Hajnoczky, G. Endoplasmic Reticulum-Mitochondrial Contactology: Structure and Signaling Functions. *Trends in cell biology* **28**, 523-540, doi:10.1016/j.tcb.2018.02.009 (2018).
334 McKenzie, M., Lim, S. C. & Duchen, M. R. Simultaneous Measurement of Mitochondrial Calcium and Mitochondrial Membrane Potential in Live Cells by Fluorescent Microscopy. *Journal of visualized experiments : JoVE*, doi:10.3791/55166 (2017).
335 Bejar, R. *et al.* TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood* **124**, 2705-2712, doi:10.1182/blood-2014-06-582809 (2014).
336 Kempf, J. M. *et al.* Loss-of-function mutations in the histone methyltransferase EZH2 promote chemotherapy resistance in AML. *Scientific reports* **11**, 5838, doi:10.1038/s41598-021-84708-6 (2021).