Trends in Genetics

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Feature Review

Epigenetic Regulators as the Gatekeepers of Hematopoiesis

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Hematopoiesis is the process by which both fetal and adult organisms derive the full repertoire of blood cells from a single multipotent progenitor cell type, the hematopoietic stem cells (HSCs). Correct enactment of this process relies on a synergistic interplay between genetically encoded differentiation programs and a host of cell-intrinsic and cell-extrinsic factors. These include the influence of the HSC niche microenvironment, action of specific transcription factors, and alterations in intracellular metabolic state. The consolidation of these inputs with the genetically encoded program into a coherent differentiation program for each lineage is thought to rely on epigenetic modifiers. Recent work has delineated the precise contributions of different classes of epigenetic modifiers to HSC self-renewal as well as lineage specification and differentiation into various cell types. Here, we bring together what is currently known about chromatin status and the development of cells in the hematopoietic system under normal and abnormal conditions.

Highlights

The epigenetic landscape regulates HSC heterogeneity and hematopoietic cell fate decisions.

Chromatin analyses of hematopoietic cells have led to novel insights into HSC priming and biology.

Crosstalk between metabolism and chromatin is emerging as a crucial facet in regulating hematopoiesis.

Epigenetic regulators are promising targets for treatment of blood disorders including leukemia.

Epigenetic Factors Integrate Intrinsic and Extrinsic Factors to Enact Hematopoiesis

The mammalian blood system serves as an excellent paradigm to study adult stem cell renewal and cell fate commitment, since despite their tremendous diversity in terms of cell morphology, molecular features and functions – ranging from oxygen carriage to wound healing and pathogen defense – all the mature blood cells have a common progenitor: the **hematopoietic stem cells** (HSCs; see Glossary). These cells are a relatively rare population that sits at the apex of blood cell differentiation. Functionally, HSCs are defined by their capacity to reconstitute the entire blood system of a lethally irradiated recipient [1,2].

Hematopoietic differentiation or hematopoiesis is a dynamic process fine-tuned by both extrinsic and intrinsic factors [3–6]. The niche is a complex multicellular network composed of stem, progenitor and mature hematopoietic cells and nonhematopoietic cell types, and represents the most significant extrinsic cue. This microenvironment provides a variety of inputs such as exposure to cytokine or growth factor signaling, variable oxygen and pH conditions, as well as cell–cell contacts and extracellular matrix stiffness. Thereby, an intact niche provides the appropriate signals and conditions for maintaining HSC quiescence and promoting progenitor cell differentiation [7–9]. Moreover, different stimulations, for example immune response against viruses, will trigger the production of type one interferons such as IFNα. This cytokine can in turn act as a signal to break HSC quiescence, triggering a specific transcription network which will lean the differentiation output towards a particular cell-type program, preferentially myeloid output in this example [10,11]. For intrinsic cues, it is widely accepted that lineage regulators expressed in HSCs and progenitor cells instruct fate determination, as illustrated by the balance between the PU.1 and GATA1 transcription factors (TFs), where the first one elicits the myeloid program, and the second one governs the erythroid branch. Unbalanced expression of either leads to hematopoietic lineage skewing [12]. Therefore, the competing influences of various extrinsic and intrinsic inputs need to be coordinated to ensure balanced hematopoiesis.

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The classical view of hematopoiesis has been illustrated via a branching tree consisting of a stepwise series of bifurcations signposting progressive commitment to increasingly differentiated progenitor cell populations and ultimately to terminally differentiated cell types. Single-cell RNA sequencing (scRNA-seq) and *in vivo* lineage tracing experiments have recently overturned several aspects of this dogma (Box 1 and Figure 1, Key Figure). While HSCs remain at the top of all hematopoietic trajectories, the rigidity and hierarchical structure of the intermediate steps in the differentiation scheme have been reconsidered. While HSCs were previously thought of as homogeneous and having a blank slate with respect to differentiation, we now know that HSCs, in fact, represent a heterogeneous population consisting of cells with existing lineage biases which make them functionally uni-, bi-, or multipotent under steady-state conditions [13–18]. It has been hypothesized that these lineage biases are established through epigenetic priming during hematopoiesis [19,20]. Remarkably, single HSCs show a stereotypical behavior upon transplantation, in which the future lineage trajectory or fate of an individual HSC clone appears to be established prior to young adulthood and persistently manifests under various conditions. Furthermore, while the transcriptome is not consistently correlated with future trajectory, epigenetic features such as DNA methylation and chromatin accessibility reveal a confident association with the subsequent function of specific clones [20]. In agreement with this finding, short-term exposure to an immune threat such as bacteria can induce C/EBPβ-dependent changes in chromatin accessibility, which in turn prime the HSCs towards the myeloid compartment upon secondary infections [21]. Also, changes in the niche compartment have been shown to imprint a lasting influence on HSC chromatin accessibility that persists even following transplantation [8]. These data suggest that epigenetic memory represents an important factor in guiding HSC fate decisions.

Hematopoiesis as a Model to Understand Chromatin Regulation of Cell Fate

Factors capable of modulating the structure or function of chromatin are known as chromatin regulators. Chromatin or epigenetic factors can alter a cell's transcriptional output without eliciting changes in the underlying genetic sequence. Mechanisms of chromatin regulation involving direct modification of chromatin include DNA methylation, covalent histone modifications, and

Box 1. scRNA-seq and *In Vivo* Lineage Tracing Have Transformed Our View of Hematopoiesis

Even though the first observation of the HSCs' remarkable ability to restore the entire hematopoietic system was >50 years [159], the understanding of how – in terms of trajectory – they do so remains a fascinating question to this day. The classical dogma states that hematopoiesis consists of a stepwise differentiation, where asymmetric division of HSCs gives rise to one daughter cell which retains HSC characteristics and a second daughter cell which loses the self-renewal capacity but retains multipotent differentiation ability, known as a multipotent progenitor cell (MPP; see Figure 1 in main text). According to the classical model, MPPs will participate in sequential binary decisions [160,161] until they reach a fully differentiated mature blood cell type. The classical model has been predominantly derived from studies employing transplantation assays. Although transplants are valuable for their ability to determine self-renewal and lineage potential of HSCs, their reliance on surface markers to identify HSCs may mean that they miss the full scope of HSC heterogeneity.

The combination of next-generation techniques such as scRNA-seq with unperturbed *in vivo* tracking experiments and single-cell transplantations have challenged the classical hierarchical view of hematopoiesis. Recent work has uncovered new hematopoietic cell populations and unprecedented branching points that give rise to the notion that hematopoiesis is better represented as a continuum [13,162–171]. Nonetheless, despite the considerable advances promoted by those single-cell techniques they also have limitations and technical challenges that need to be considered. Barcode-based techniques are not able to fully assess rare HSC clones due to their exclusion by thresholding. Also, several lineage-tracing techniques rely on Cre lines, with all the associated caveats of specificity and selectivity. Researchers labelling HSCs with *Pdzk1ip1*-driven Cre showed that at steady state, blood lineage replenishment is highly dependent on HSCs [172]. In sharp contrast, researchers using the *Tie2*-Cre for the same purpose found that mature hematopoietic replenishment is not dependent on HSCs [13]. This discrepancy could only be resolved by engagement of independent techniques. Sleeping Beauty transposon-based tagging experiments and polylox barcoding supported the idea that, with the exception of megakaryocyte fate, MPPs likely represent the actual active differentiation compartment and are responsible for the replenishment of most of the mature blood cells at status quo [14,17,162]. This controversy emphasizes the need for the development and application of new approaches, models, and software to accurately deconvolute HSC fate *in vivo*.

Glossary

CRISPR: The CRISPR/Cas9 system is a harnessed system used to mediate genome editing in cells, including mammalian cells. It can be used to generate gene knockouts (via insertion/deletion) or knockins.

CRISPR Array Repair Lineage tracing (CARLIN): a technology developed by scientists at the Stem Cell Research program at Boston Children's Hospital and Dana–Farber Cancer Institute/Harvard Medical School to track every cell in the mouse body, from the embryonic stage until adulthood [157].

Granulopoiesis: granulocyte development.

Hematopoietic stem cells: adult stem cells that can give rise to all types of blood (hematopoietic) cells, including white blood cells, red blood cells, and platelets.

Histone variants: differ from the canonical histones by a few amino acids and are lowly expressed. Are typically inserted independently of replication.

Lineage and RNA recovery (LARRY): allows the generation of a library of clonally tagged cells with DNA barcodes. Reading these barcodes using single-cell sequencing enabled the reconstruction of genome-wide transcriptional trajectories from differentiating cell populations [15].

Nucleosome: the fundamental subunit of the chromatin. Each nucleosome is composed of an eight histone core, known as a histone octamer. Each histone octamer is composed of two copies each of the histone proteins H2A, H2B, H3, and H4.

SNPs: DNA sequence variation occurring when a single nucleotide in the genome differs between members of a species or paired chromosomes in an individual.

Key Figure

Models of HSC Lineage Commitment

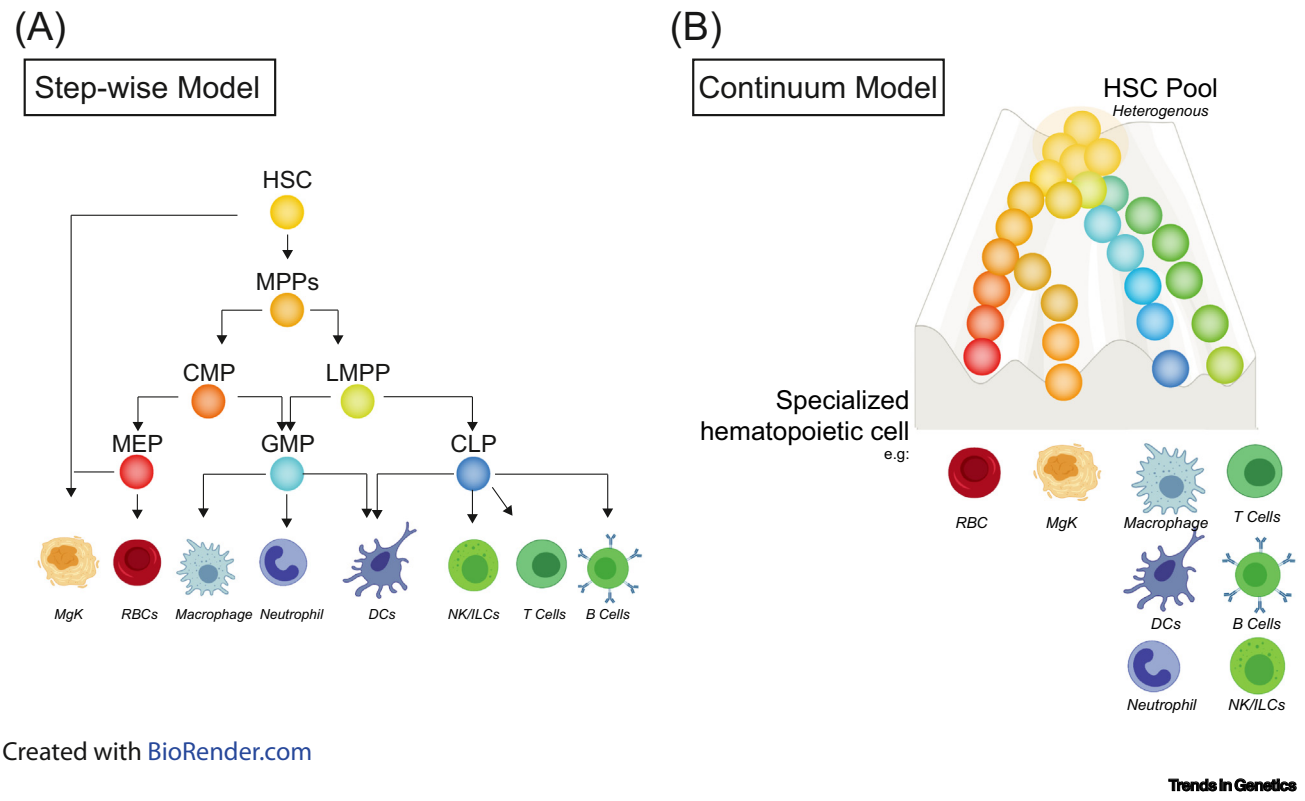

Figure 1. (A) Scheme depicting the canonical stepwise hematopoiesis pattern. Under this view, HSCs are thought to contribute to each lineage equally. (B) Revised model showing that hematopoiesis more closely represents a continuum. Recent findings indicate that only a small fraction of HSCs generate an equal outcome for all blood mature cells, while most HSCs exhibit a differentiation bias towards one lineage. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DCs, dendritic cells; GMP, granulocyte/macrophage progenitor; HSCs, hematopoietic stem cells; ILCs, innate-lymphocyte cells; LMPP, lymphoid/myeloid progenitor; MEP, megakaryocyte/erythroid progenitor; MgK, megakaryocyte; MPP, multipotent progenitor; NK, natural killer; RBCs, red blood cells..

nucleosome remodeling. These can be further influenced by miRNA, long noncoding RNA (lncRNA), specialized **histone variants**, and 3D chromatin conformation [22,23]. The concerted chromatin environment produced by the sum of these modifications and factors is known as the chromatin or epigenetic landscape. In general terms, chromatin modifiers elicit local chromatin alterations, which in turn either promote or repress transcription.

The chromatin landscape plays a decisive role from the start of the hematopoietic process. Multiple epigenetic factors collaborate to ensure the maintenance of the HSC pool by instructing these cells on which transcription program should be active or suppressed in order to maintain their stemness [24,25]. Dynamic chromatin reorganization is responsible for lineage priming by ensuring that the transcription network of a determinate cell type will be facilitated, while programs associated with an alternative fate will be prevented. In this regard, epigenetic modifiers exert a strong influence on lineage commitment decisions [26,27]. Unsurprisingly, chromatin deregulation also contributes to impaired hematopoiesis and leukemia [28,29]. Mouse knockouts

of enzymes responsible for DNA methylation or post-translational histone modifications frequently show hematopoietic defects ranging from bone marrow failure to leukemia [29,30].

Chromatin Dynamics Shaping Cell Fate Decisions

Nucleosomal position and density have a remarkable influence on chromatin accessibility and regulatory function by licensing the availability of binding sites to TFs and the transcription machinery [31,32] (Box 2). Accurate nucleosome phasing, density and assembly are therefore required to enable transcription at appropriate gene loci and time points. Local nucleosome occupancy and composition are finely tailored by a group of ATP-dependent enzymes termed nucleosome remodelers. They are subdivided into four major subfamilies named imitation switch (ISWI), chromodomain helicase DNA-binding (CHD/NuRD/Mi-2), switch/sucrose non-fermentable (SWI/SNF), and INO80. Mechanistically, ISWI and CHD have been shown to modulate nucleosome assembly and organization following DNA replication by regulating the maturation of nucleosomes and generating canonical nucleosomal spacing (Figure 2). The INO80 subfamily is mainly associated with the removal and replacement of a given histone by canonical or related variants. The SWI/SNF subfamily emerges as the primary remodeler responsible for regulating sliding of nucleosomes along the DNA or even releasing full nucleosomes from the chromatin. Hence, the SWI/SNF subfamily of nucleosome remodelers is essential to the regulation of nucleosome density or phasing: a process that is not only intricately linked with chromatin dynamics, but also with transcription, since nucleosomal packing hinders TF binding to motifs located in that region, while nucleosome-free regions permit rapid access to TFs [33]).

Ablation of nucleosome remodelers is associated with defective hematopoiesis. Deletion of *Arid1a*, the core component of the mammalian SWI/SNF complex, causes global reduction in accessible chromatin, affecting differentiation of both myeloid and lymphoid lineages [34]. Likewise, deletion of *Baf200,* another member of the SWI/SNF complex in mammals, leads to defective hematopoiesis, as illustrated by perinatal death from severe anemia due to defective erythropoiesis and impaired HSC expansion in the fetal liver [35]. In addition, MLL-AF9-driven leukemogenesis is accelerated in *Baf200*$^{-/-}$ tumor-bearing mice [35]. Moreover, the SNF2-like ATPase Mi2β of the NuRD complex was shown to be required for HSC self-renewal, as demonstrated by an increase in progenitor cell cycling and decrease in HSC quiescence observed shortly after its

Box 2. Interplay between TFs, Nucleosome Remodelers and Chromatin Dynamics

Nucleosome positioning, density and occupancy time (turnover) along the DNA can impose a physical barrier which attenuates chromatin accessibility and transcription. Nucleosome positioning is therefore intimately linked to gene expression. Strong promoters preferentially occur at regions displaying increased nucleosome turnover and low density, whereas weak promoters, despite having a high nucleosome turnover, show an increased nucleosome occupancy. Accordingly, facultative heterochromatin is characterized by low nucleosome turnover and occupancy. How do increased nucleosome turnover and reduced nucleosome occupancy translate into increased expression? TFs are proteins with specificity towards specific DNA sequences or motifs. Most TFs show affinity towards naked DNA, but are excluded from nucleosome-dense regions. One mechanistic model proposed for how TFs may gain access to histone-bound DNA is by exploiting nucleosome turnover; this model is known as passive competition for DNA binding. Alternatively, another model involves the active recruitment of nucleosome remodelers and the displacement of APs and/or linker histones (see Figure 2D in main text). In this multistep process, the TFs first bind to internucleosomal DNA and destabilize the proximal nucleosome, destabilization of the core histone particle will in turn recruit active remodelers which will ultimately lead to the establishment of DNA accessibility. Indeed both models are applicable to the DNA template in *cis*. Thus, a third method of nucleosome remodeling applies to TF binding in *trans*. In this model TFs binds to distal accessible regulatory elements, which will in turn recruit other cofactors to evict nucleosomes leading to maintenance of DNA accessibility in *trans* (see Figure 2D in main text). Finally, a special class of TF has the ability to directly bind to nucleosomal DNA which is sufficient to establish the open chromatin state. These TFs are termed pioneer factors and include key developmental TFs such as PU.1 and EBF1 (see Figure 2D in main text).

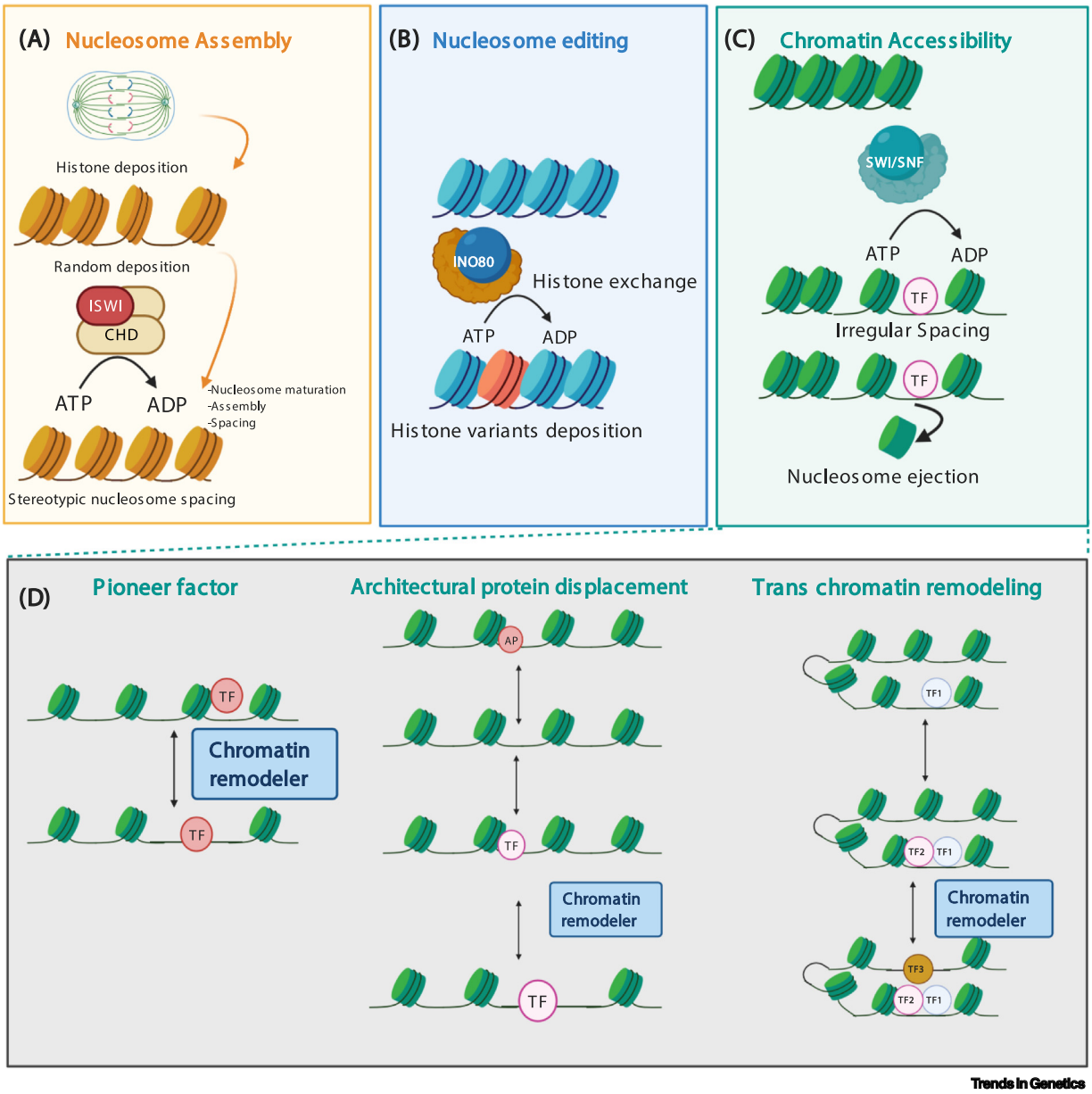

Figure 2. Nucleosome Remodelers Regulate Chromatin Dynamics. (A) ISWI and CHD coordinate nucleosome assembly and spacing following DNA replication. (B) INO80 performs nucleosome editing by changing the nucleosome composition. (C) The SWI/SNF subfamily modulates chromatin accessibility by repositioning nucleosomes, ejecting octamers or removing histones. (D) Left: Mechanism of pioneer TF binding. Middle: nucleosome remodeling in *cis* using an AP prior to pioneer TF binding. Right: nucleosome remodeling in *trans* via recruitment of active nucleosome remodelers and stabilizing secondary TFs (TF3 in this case). Abbreviations: AP, architecture protein; CHD, chromodomain helicase DNA-binding; ISWI, imitation switch; SWI/SNF, switch/sucrose non-fermentable; TF, transcription factor. Created with BioRender.com.

deletion. These changes do not impair erythroid differentiation but affect myeloid and lymphoid lineages [36]. Furthermore, mice whose HSCs harbor a deletion of the gene encoding BPTF (a component of the NURF nucleosome remodeling complex) show impaired bone marrow reconstitution, culminating in bone marrow failure and anemia. Molecularly, *Bptf* knockout results in deregulated transcription of stemness programs, marked by decreased expression of genes

Box 3. Principles of Chromatin Accessibility

Eukaryotic chromatin is packaged into an array of nucleosomes, composed of a histone octamer core wrapped by 147 bp of DNA, and separated by linker DNA. The nucleosome core is formed by four histone proteins – H2A, H2B, H3, and H4 – that can be post-translationally modified, including methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation [173] or replaced by histone variants [174]. The nucleosomes themselves can also be moved, either through addition, removal or changes in internucleosomal spacing. Furthermore, the underlying DNA can also be modified through methylation. All of these epigenetic modifications come together to regulate chromatin dynamics and gene expression during developmental processes. Chromatin accessibility is measured by evaluating the vulnerability of its constituent DNA to enzymatic digestion or cleavage. The first evaluation of chromatin accessibility was conducted by Hewish and colleagues in 1973, who revealed regular nucleosome phasing using DNA endonucleases. Later, Southern blot hybridization revealed the precise 100–200-bp phasing pattern produced through DNase digestion of chromatin. Advances in sequencing technology have permitted the genome-wide mapping of chromatin accessible regions with increasing sensitivity [175,176]. We briefly describe the most widely used techniques in Box 4.

encoding MEIS1, PBX1, MN1, and LMO2, TFs essential for HSC maintenance [37]. Collectively, these findings shed light on the importance of appropriate nucleosome distribution in facilitating and instructing hematopoiesis.

Defects in chromatin accessibility are associated with altered transcription programs in hematopoietic progenitor cells and disbalanced mature cell output [38]. In this sense, methods which help ascertain the chromatin landscape (Boxes 3 and 4) have emerged as powerful tools in understanding cell fate decisions along a continuous trajectory. ATAC-seq experiments on distinct hematopoietic human cell types, including HSCs, have revealed that cell types can be identified much more precisely on the basis of their chromatin accessibility profiles than their transcriptomes [39]. This is mainly due to the fact that ATAC-seq data can reveal *cis*-regulatory elements that are specifically active at different stages of differentiation [39–41]. Moreover, the

Box 4. Selection of Modern Next-Generation-Sequencing-Based Technologies

MNase-seq: utilizes the MNase single-strand-specific endo-exonuclease to indirectly infer chromatin accessibility by unveiling the areas of the genome occupied by nucleosomes and regulatory factors. Thereby, this method resolves the genome-wide nucleosome distribution in a qualitative and quantitative manner.

DNase-seq: this technique exploits the capacity of double-stranded endonuclease DNase I to preferentially cleave within nucleosome-free regions, termed DNase I hypersensitive sites (DHSs). DNase-seq analysis uncovered that while a minority of DHSs is found within promoters and transcriptional start site-proximal regions, there is significant enrichment of DNase-accessible regions at distal enhancers. One major concern with this technology is that DNase has cleavage biases, thus directly affecting the TF footprint interpretation.

Assay for transposase-accessible chromatin using sequencing (ATAC-seq): represents one of the remarkable advances in the field given the protocol's ease and capacity to support low input material (500–50 000 cells). This protocol hijacks the ability of the Tn5 transposase to fragment DNA and randomly integrate into the genome. The Tn5 is further tagged/labeled with Illumina oligos or fluorescent probes which will allow the generation of sequencing libraries to map open chromatin and its related regulatory elements (ATAC-seq), or the single-cell visualization of global chromatin accessibility (ATAC-see). ATAC-seq not only maps chromatin accessibility, but is also suitable for TF footprinting. Recently, ATAC-seq has been adapted to a single-cell platform, allowing the inspection of chromatin dynamics during cell fate determination.

Bisulfite sequencing (WGBS/RRBS): bisulfite genomic sequencing is the gold-standard technology for detection of DNA methylation. This method relies on the observation that unmethylated cytosines are converted to uracils when exposed to sodium bisulfite whereas methylated forms of cytosine remain unchanged. The alignment can then be used to resolve methylation status at single nucleotide-level in a similar manner to detecting DNA variants from NGS data.

Nucleosome occupancy and methylome sequencing (NOMe-seq): this method provides simultaneous information on DNA accessibility and methylation. Instead of relying on DNA cleavage, NOMe-seq probes DNA status through chemical modification by treating the samples with a GpC methyltransferase (MTase from M.CviPI) that methylates open chromatin regions. Bisulfite conversion of nonmethylated cytosine to uracil nucleotides provides a single-nucleotide measure of accessibility. Given the abundance of GC regions in the genome, NOMe-seq generates high-resolution profiles, however, it requires a large number of sequencing reads which is associated with increased cost.

integration of RNA sequencing, ATAC-seq, and chromatin-immunoprecipitation sequencing (ChIP-seq) profiles of hematopoietic progenitor and mature cells has unveiled that hematopoiesis involves a gradual reorganization of the chromatin landscape, in which poised enhancers are established through gain of chromatin accessibility before onset of RNA expression from their target promoters. Although HSCs show extensive poised enhancers, the maximum poised enhancer peaks have been observed in MPPs, implying *de novo* gain and loss of enhancer accessibility during hematopoiesis [27].

3D genome conformation has also been proposed to play a role in regulating chromatin state dynamics in HSCs and derived lineages. Genome folding permits *cis*-regulatory regions to either increase or decrease their proximities and thereby interactions in 3D space. Genome conformation has been shown to facilitate the expression of appropriate transcriptional programs during cell fate specification of multiple tissues and organs [42–44], as illustrated by the fact that the enhancer–promoter interaction patterns are cell type specific and segregate with the hematopoietic tree [45]. Furthermore, 36% of the genome was found to undergo compartment switching during differentiation of human embryonic stem cells (ESCs) to one of the three germ layers [46]. However, little is known regarding how chromosome organization contributes to fate determination in HSCs. A recent study comparing the 3D genome organization of mouse fetal and adult HSCs uncovered that although topological associated domains (TADs), the main structural units of chromosome architecture, remain largely unchanged during fetal–adult transition, there is an increase in the separation between active and inactive compartments and sharper definition of TAD boundaries [47]. The authors additionally uncovered significant cell type-specific enhancer–promoter interactions, with more cell cycle and metabolism-related genes exhibiting enhancer–promoter contacts in fetal than adult HSCs. This strongly supports the idea that genome architecture contributes to adult HSC identity. Further evidence for a role of chromosome architecture in HSC identity comes from studies of *Stag2* null mice. HSCs harboring a deletion in chromosome architectural protein *Stag2* show changes in chromatin accessibility and transcription of lineage specification genes, including *Spi1/PU.1*, *Ebf1*, and *Pax5*, leading to reduced hematopoietic progenitor cell commitment towards the B cell lineage. Supporting the specific requirement for an appropriate chromatin landscape, this defect can be rescued by *Stag2* re-expression, but not by overexpression of *Spi1/PU.1* [48].

Integrating chromatin accessibility data with fine-mapped genome-wide association studies (GWASs) is valuable for uncovering **SNPs** in noncoding and intergenic regions linked to human diseases [39,49]. Recent GWAS studies on human hematopoietic cell types have discovered multiple SNPs associated with physiological traits such as RBC size, volume, and hemoglobin content [50,51]. Thus, we envision that integrating hematopoietic cell GWAS data with knowledge of cell type-specific regulatory element activity will enable us to build models for accurate prediction of genetic variants in blood-related diseases. Thereby, insights into transcription regulation and chromatin accessibility can be leveraged to understand human blood-related diseases.

Histone Variants Modulate Chromatin Accessibility during Hematopoiesis

Core histone variants such as H3.3 have been shown to play an instructive function during hematopoiesis [52]. Recently, the histone chaperone HIRA, responsible for depositing H3.3 onto chromatin, was shown to be pivotal for nucleosome remodeling in HSCs. Its depletion results in an overall decrease in chromatin accessibility and impaired HSC differentiation by hindrance of the transcription of genes coding for critical hematopoietic TFs [53].

Besides the canonical histone core, the linker histones, including variants of histone H1, are also capable of influencing chromatin accessibility. H1 modifies the angle at which DNA exits the

nucleosome, thus promoting the neutralization of the charge of linker DNA. As a consequence, chromatin folds into a more compact and less accessible state. Histone H1 was recently shown to promote **granulopoiesis** [54]. By contrast, overexpression of HMGN – a protein belonging to the family of nucleosome-binding proteins which competes with histone H1 – in hematopoietic progenitors results in impaired myeloid differentiation due to increased transcription of genes associated with stem identity and function [55]. The influence of H1 and linker DNA in regulating HSC fate are still to be defined.

Covalent Modifications at Histone Tails – Wagging between Histone Acetylation and Deacetylation

Chromatin accessibility is also influenced by the acetylation status of certain lysine residues in histone proteins. Addition of an acetyl group to lysine typically correlates with open chromatin structure and activation of gene expression. The levels of chromatin acetylation are tightly regulated by lysine acetyltransferases (KATs) and histone deacetylases (HDACs) [56,57].

Given the close association of histone acetylation and active transcription, KATs (p300, CBP, MOZ GCN5, HBO1, and MOF) have been extensively studied in the context of normal and malignant hematopoiesis [38,58–67]. For instance, the CBP-p300 family of KATs has been implicated in both HSC self-renewal and differentiation, as illustrated by specific binding of CBP/p300 to the c-Myb KIX domain which thereby regulates c-Myb-dependent gene expression [68].

Despite their homology and overlapping functions in other cell types, the role of CBP and p300 during hematopoiesis appear to be distinct and dependent on their expression levels [69]. Although *Cbp*$^{+/-}$ mice display a hematopoietic defect, *p300*$^{+/-}$ mice do not, suggesting either that CBP and p300 influence hematopoiesis through distinct networks, or that redundant pathways could rescue p300 but not CBP function [70–72]. Deletion of *Cbp* in the HSC compartment results in differentiation defects, which are reflected in impaired bone marrow reconstitution after transplantation [70]. In contrast, transplanted *p300*$^{-/-}$ HSCs only show mild differences with regards to differentiation [69], suggesting that changes in HSCs upon *p300* deletion might stem from the niche. Of note, despite its essential function in regulating adult HSC fate, CBP is not required to initiate HSC formation, but is essential for maintaining the HSC pool.

Ablation of the MYST family KAT MOZ (also known as KAT6A or MYST3) has been shown to regulate various aspects of hematopoiesis, ranging from HSC proliferation to self-renewal and hematopoietic lineage commitment [62]. Likewise, the KAT MOF (also known as KAT8 or MYST1) was found to specifically influence adult hematopoiesis [38,66], while being dispensable for the highly proliferative fetal counterpart [65]. MOF orchestrates erythropoiesis through its modulation of H4K16ac and chromatin accessibility in HSCs and erythroid progenitors (MPP2 and MEP). *Mof* expression during adult hematopoiesis is dynamic, showing a first peak at HSCs/progenitor cells and a second peak at the early erythroid progenitor state. The transcription factor GFI1B is responsible for a second peak in *Mof* expression, whose protein product binds chromatin and deposits H4K16ac in erythroid progenitor cells, generating a positive feedback loop which enforces the erythroid lineage. Removal of MOF or its enzymatic activity severely compromises erythroid differentiation in colony-formation assays. Consequently, both *Mof*$^{+/-}$ and *Vav1*-iCre *Mof*$^{flox/flox}$ animals suffer from anemia and display reduced numbers of erythroid progenitors and MEPs *in vivo* [38,66].

A fascinating feature of histone acetylation is its high dynamicity. Indeed, histone marks can be gained and lost repeatedly along a single hematopoietic trajectory. One example is the expression of *Mof,* which is dynamic during erythropoiesis, which starts out high in HSCs, drops in MPP1 and

pre-MEP cells, and then reaches another peak in MEPs [3]. This is also mirrored by fluctuations in global levels of the MOF target modification H4K16ac in these cell types. Fluctuations in the expression patterns of *Hdac4*, *Hdac2,* and *Sirt4* are also observed along differentiation through the erythroid lineage, raising the possibility that multiple histone acetylation sites besides H4K16ac are dynamically regulated [38].

Active histone deacetylation is the counterbalance of histone acetylation and promotes transcriptional repression. Modulating the levels of HDACs therefore also impacts HSC biology. For instance, HSCs lacking the histone deacetylase sirtuin 1 (SIRT1) exhibit defects in self-renewal and differentiation, resulting in reduced size of the lymphoid compartment and onset of anemia [73,74]. HDAC1 and HDAC2 seem to have redundant targets in HSCs, as only combined loss, but not single ablation, of these enzymes results in rapid hematopoietic failure from severe anemia and cytopenia. Of note, the phenotypes observed in HSCs upon *KAT* or *HDAC* knockout are strongly reminiscent of the phenotypes of aged HSCs, including self-renewal defects and myeloid bias. A deregulated histone acetyl profile may therefore be either a driver or consequence of physiological aging in HSCs. Consistent with this, the active mark H4K16ac appears to be downregulated in old murine HSCs [75]. Considering the significant number of stem cell maintenance pathways found to be regulated by MOF in young HSCs [38], one can speculate that decrease of H4K16ac may have detrimental effects on the ability of aging HSCs to maintain their stem identity. These findings indicate that pharmacological augmentation of cellular acetylation levels might be able to prevent functional decline in aged HSCs in humans.

DNA Methylation Represses Impromptu Programs in HSCs

Historically, DNA methylation has been described as a silencing mark, ensuring tissue-specific gene expression patterns in a heritable manner, including X-chromosome inactivation in mammals, silencing of transposon elements, and genome imprinting (reviewed by [76,77]). DNA methylation occurs predominantly at cytosine bases, producing 5-methylcytosine (5mC). In the mammalian genome, 70–80% of the cytosines adjacent to guanines (CpGs) are found methylated (5mC), except for gene regulatory regions close to promoters where CpGs remain unmethylated (CpG islands, CGIs) [78]. DNA methylation depends on the enzymatic activity of DNA methyltransferases (DNMTs). Three active DNMTs have been identified in mammals: DNMT1, DNMT3A, and DNMT3B [79,80]. While DNMT3A and DNMT3B recognize unmethylated regions and are responsible for establishing *de novo* CpG methylation [81], DNMT1 targets hemimethylated DNA and has a prominent role in maintaining DNA methylation patterns during DNA replication.

Given their distinct targets in DNA, DNMT1 and DNMT3A/B unsurprisingly influence cell fate in different ways. *Dnmt1*$^{-/-}$ HSCs show predisposition for myeloid-erythroid lineage commitment, accompanied by a significant decrease in the related lymphoid populations [82]. In contrast, scRNA-seq on conditional *Dnmt3a*$^{-/-}$ HSCs reveals a predisposition for the erythroid differentiation program, accompanied by increased chromatin accessibility at binding sites for critical erythroid TFs KLF1, GATA1, and TAL1 [83]. Transplantation experiments have revealed that the *Dnmt3a*$^{-/-}$ HSCs also exhibit enhanced self-renewal capacity and are able to regenerate over at least 12 transplant generations in mice, at the expense of cell differentiation [84–86]. This is in stark contrast to *Dnmt1*$^{-/-}$ HSCs, which suffer from drastically reduced self-renewal capacity [82]. The enhanced self-renewal capacity of *Dnmt3a*$^{-/-}$ HSCs might be connected with the fact that ~30% of hypomethylated regions in these cells are located at the edges of vast unmethylated regulatory regions, approximately 3.5 kb in length, termed canyons [87]. Overall, canyon-associated genes are enriched for stereotypical stemness genes, including the Hox family cluster [87], and show a gradual methylation loss upon serial transplantations, which

correlates with the striking extended lifespan of *Dnmt3a*$^{-/-}$ HSCs [86]. Thus, although deletion of either DNMT1 or DNMT3A/B results in hypomethylation, their downstream effects on HSC fate appear to be distinct. In mammals, DNMT1 promotes the self-renewal program of HSCs, while DNMT3A elicits differentiation programs, in particular by suppressing erythroid programs via *de novo* methylation of key TF motifs. Altered levels and mutations of DNMTs are associated with developmental disorders, including clonal hematopoiesis of indeterminate potential (CHIP) and acute myeloid leukemias in humans (AMLs) [88,89].

The discovery of the Ten-eleven translocation (TET) enzymes added another layer of complexity to the regulation of DNA methylation. The TET enzymes oxidize 5mC to 5-hydroxy-methylcytosine, which results in DNA demethylation [79]. Therefore, the interplay between DNMT and TET enzymes represents a powerful strategy to regulate gene activity through dynamic addition and removal of DNA methylation.

Among the TET enzymes, TET2 appears to be prominent in regulating HSC fate. Single-cell DNA methylation analyses (scRRBS; Box 4) on *Tet2*$^{-/-}$ HSCs revealed dramatic methylation changes at CpG sites contained within lineage-specific TF binding motifs, resulting in disruption of transcriptional priming [83]. While *Dnmt3a*-deficient HSCs appear to favor the erythroid differentiation program at the expense of monocyte lineage commitment [83,90], *Tet2*$^{-/-}$ HSCs show the opposite biases, with a skew towards the myelomonocytic progenitors. Furthermore, chromatin accessibility is decreased at motifs for TAL1 and KLF1 in *Tet2*$^{-/-}$ cells. Therefore, it is likely that TET2 and DNMT3A compete to maintain the methylation status quo at the same lineage-related loci but cooperate to repress the stem cell transcriptional network in HSCs. Mice receiving transplanted double knockout *Dnmt3a*$^{-/-}$ *Tet2*$^{-/-}$ HSCs exhibit a complex phenotype, with multiple signs of abnormal hematopoiesis, suggesting that TET2 and DNMT3A have a synergistic impact on hematopoiesis [91,92]. In fact, the interplay between TET and DNMT3 appears to be more complex than previously anticipated. Double knockout of *Dnmt3a* and *Tet2* in HSCs does not produce an epistatic phenotype, but rather elicits upregulation of erythroid signature genes in comparison to single *Tet2*$^{-/-}$ mutants. These findings are particularly important since single-mutant *Dnmt3a*$^{-/-}$ HSCs maintain their stem cell program, while the combination with *Tet2* deletion leads to derepression of lineage-specific regulators and augmentation of differentiation programs [91]. Taken together, these findings suggest that double null mutations may serve to elicit and accelerate hematopoietic disorders. Consistent with this hypothesis, *DNMT3A* and *TET2* mutations frequently co-occur in T cell lymphoma and AML in humans [91,93,94]. Moreover, *TET* loss of function in humans correlates with a unique pattern of global hypomethylation coupled to regional hypermethylation in diverse cancer genomes, including leukemias [95].

Polycomb Repressive Complexes: Repression beyond 5mC

As discussed earlier, DNA methylation has a valuable function in transcription suppression, but the fact that many unmethylated CpG islands show low or no expression hints towards the action of additional epigenetic repression machinery in HSCs. The Polycomb repressive complexes (PRCs) are another critical player in the regulation of chromatin permissibility (reviewed by [96,97]).

The PRCs can be further divided into PRC1 and PRC2. Both are multi-subunit complexes containing enzymatic members capable of catalyzing the monoubiquitylation of H2A (H2Aub1) and the addition of one or more methyl groups on lysine 27 at histone H3 (H3K27me). Both these histone modifications trigger transcriptional repression [98,99]. Considerable attention has been paid to studying both PRC1 and PRC2 dynamics during different developmental stages, ranging from ESCs, where they are responsible for repressing pluripotency, to adult stem cells,

in particular HSCs, since mutations in PRC components are strongly associated with blood-related disorders [97]. In this regard, pioneer ChIP-sequencing studies profiling histone methylation in young and old HSCs revealed the existence of bivalent genes, positive for both H3K27me3 and H3K4me3, whose functions are related to development and RNA metabolism [100].

PRC1 complexes exist in multiple forms, including canonical PRC1.2 and PRC1.4 as well as non-canonical variants [101]. Several members of the canonical PRC1.4 variant of the PRC1 complex (also known as PRC1–BMI1) are highly expressed in HSCs, where they regulate HSC self-renewal by repressing $p16^{INK4A}$ and $p19^{ARF}$ [102–106]. Furthermore, *Bmi1* null animals show a block in B cell differentiation via the silencing of *Ebf1* and *Pax5* [107]. Accordingly, *Bmi1* deletion in T cells leads to B cell reprogramming while BMI1 overexpression causes expansion of the HSC pool due to augmentation of their self-renewal capacity and increased symmetric division. In contrast, HSCs deficient for the PRC1.2 variant of the canonical PRC1 complex (*Mel-18* KO) are more quiescent and have less proliferation ability [108]. The expression of some PRC1 complex members appears to be dynamic during hematopoiesis. The chromobox (CBX) protein CBX7 is highly expressed in HSCs, whereas multipotent cells express CBX9 and repress CBX7 [109].

The noncanonical PRC1 complexes also contribute to determining HSC trajectory, underscored by the high rates of somatic mutations found in member *BCOR* and its homolog *BCORL1* in various blood-related diseases in humans [110,111]. In mice, *Pcgf1*-deficient hematopoietic progenitor cells produced augmented numbers of myeloid progenitor cells by downregulation of *HoxA* family genes *Hoxa7, Hoxa9*, and *Hoxa10* [112,113].

The PRC2 complex contains three core subunits: SUZ12, EED, and one of two methyltransferases: EZH1 or EZH2. The PRC2 complex subunits influence hematopoiesis at different stages in a dosage-dependent manner. For example, the homozygous deletion of *Suz12* impairs HSC function and promotes lymphopoiesis, whereas in heterozygosity, it enhances HSC self-renewal [114,115]. Also, embryonic deletion of *Ezh1* triggers the premature emergence of *bona fide* HSCs *in vivo* due to enhanced accessibility of binding sites for key HSC TFs, including HLF, FOXO1, and ARID5B [116]. Although *Ezh2* is indispensable for fetal hematopoiesis, its ablation in adult bone marrow results in only minor defects during lymphoid differentiation [117–119], suggesting that *Ezh2* regulation in HSCs can differ depending on the temporal expression and/or HSC state.

The putative Polycomb group proteins additional sex combs like 1 and 2 (ASXL1 and 2) are not only commonly mutated in mammalian clonal hematopoiesis [120,121], but are also required for correct hematopoiesis acting in a gene dosage-dependent manner with non-overlapping biological functions between them [122–124].

Chromatin Dynamics Emerges as a Critical Facet of Blood-Related Disorders

Despite significant efforts in the clinic, AML accounts for ~4% of all cancer deaths[i] and patients who achieve remission have a relapse rate of ~50%, resulting in an overall survival rate of ~30% after 5 years. This dichotomy – remission and relapse – has been attributed to the persistence and maintenance of leukemic stem cells (LSCs) after treatment. Although LSCs cannot be precisely defined by surface markers, they can be defined by their capacity to regenerate and initiate AML upon transplantation into immunodeficient mice, analogous to how normal HSCs are capable of generating progenitors and mature blood cells [125].

Although the exact origin of LSCs remains a puzzle, it is accepted that the long lifespan of HSCs makes them susceptible to the accumulation of mutations over time, generating a potential reservoir of somatic mutations. One current hypothesis postulates that the acquisition of

additional driver mutations in already mutated HSCs can push them towards an LSC profile and enable them to gain the potential to promote leukemogenesis.

In support of this notion, analysis of hematopoietic cells from healthy elderly subjects revealed the presence of multilineage clones carrying somatic mutations without any apparent clinical phenotype [126–129]. Indeed, the frequency of mutations seems to gradually increase from HSCs to clonal hematopoiesis of indeterminate potential (CHIP) and even further in LSCs and AML in humans. HSCs isolated from patients with AML have been shown to harbor some but not all of the genetic alterations found in leukemic cells. Of note, the development of CHIP in humans has been recently associated with non-blood-related comorbidities, in particular noncommunicable diseases such as cardiovascular disorders [120,130–132].

Selection of mutations in genes encoding epigenetic regulators, specifically *DNMT3A*, *TET1*, *TET2*, *IDH2*, and *ASXL1*, are among the earliest events conferring competitive fitness to leukemic cells. LSCs harbor unique patterns of DNA methylation and histone modification compared to HSCs and blast cells. Furthermore, in spite of the intracellular heterogeneity, enhanced self-renewal capacity is a recurrent phenotype elicited by epigenetic regulator mutations [132]. One illustration of this is MLL deficiency, which leads to impaired *HOXA9* suppression, thereby promoting HSC self-renewal [67,133,134]. Similarly, hematopoietic cells from PRC2 mutant mice show aberrant expression of HSC-related genes due to impaired suppression of the 'stem program' [135].

Perturbations in histone acetylation levels have also been linked to emergence of LSCs and AML development. The histone acetyltransferase HBO-1, targeting lysine 14 on H3 (H3K14ac), facilitates expression of *Hoxa9* and *Hoxa10*, thereby sustaining the functional properties of LSCs [136]. Likewise, IKF2 appears to be dispensable for human and mouse HSCs, while its upregulation in LSCs promotes their self-renewal by inhibiting the C/EBP-driven differentiation program and thereby promoting AML onset [137].

Mutations in the RNA splicing machinery are another recurrent feature in CHIP, LSCs, and AML [138–140]. For instance, mutations of *IDH2* and the splicing factor *SRSF2* cooperatively drive the development of lethal myelodysplastic syndrome [141]. Evidence is also emerging of crosstalk between RNA-processing enzymes and epigenetic regulators in leukemic progenitors. The histone methyltransferase KDM4C enhances the chromatin accessibility and thereby expression of the *ALKBH5* gene encoding a N 6-methyladenosine (m6A) demethylase in LSCs. This upregulation leads to global changes in mRNA stability [142]. Moreover, the *METTL3* gene – encoding a m6A mRNA methyltransferase – was revealed as being essential for the ability of myeloid leukemia cells to establish the disease in immunodeficient mice. *METTL3*-deficient leukemia cells suffer from impaired cell proliferation and pronounced cell differentiation [143,144]. Thus, collectively these findings provide functional evidence that dysregulation of the HSC post-transcriptional machinery contributes to leukemogenesis, and that mutations in epigenetic regulators and spliceosome machinery components cooperate in the progression from clonal hematopoiesis to a pre-leukemic state.

Old Foes, New Promises – Epigenetic Regulators as Emerging Clinical Targets against Leukemia

The heterogeneity observed in AML due to the propensity of LSCs to clonally evolve poses a formidable challenge to the development of an effective and unique therapy for this disease. The majority of current strategies aim to disrupt LSC quiescence by restoring and promoting their differentiation capacity [132]. Epigenetic regulators are another class of attractive AML drug targets

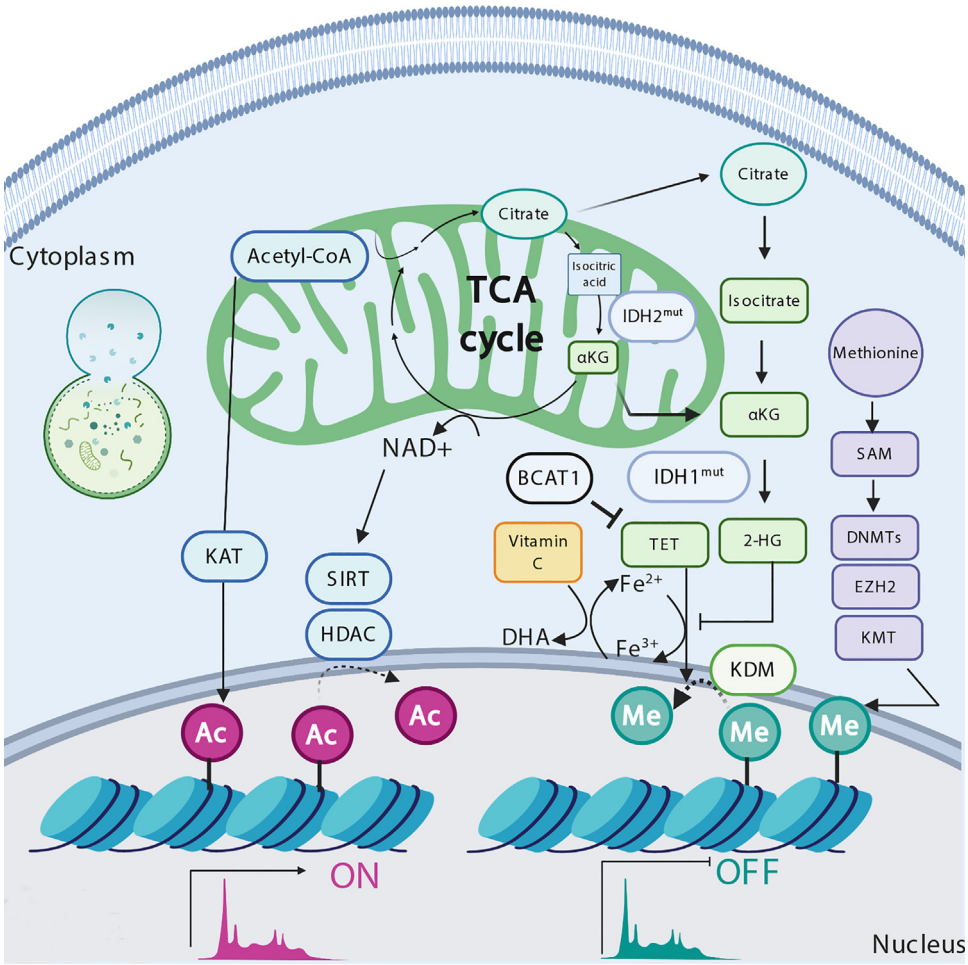

Figure 3. Metabolites Serve as Key Substrates for Histone-Modifying Enzymes. Scheme showing the relationship between intracellular metabolites and epigenetic regulation. The depicted molecules have been implicated in the regulation of both HSC and LSC fate. Genome-wide tracks represent active transcription (magenta) or repression (teal). Abbreviations: 2-HG, 2-hydroxyglutarate; Ac, acetyl-CoA; αKG, α-ketoglutarate; HDAC, histone deacetyltransferase; HSC, hematopoietic stem cell; KAT, lysine acetyltransferase; KDM, histone demethylation; KMT, histone methyltransferases; LSC, leukemic stem cell; Me, methyl group; SAM, S-adenosylmethionine; TET, ten-eleven translocation. Created with BioRender.com.

given the prevalence of LSC clones bearing mutations in them. However, this is hampered by the fact that most leukemia-associated mutations in epigenetic regulators result in loss of function.

One promising alternative is to modulate epigenetic regulators by altering the levels of their 'feedstock' through metabolic manipulation (Figure 3 and Box 5). This principle is illustrated by the mode of action of two clinically approved drugs: enasidenib and ivosidenib, inhibitors for mutant IDH2 and IDH1, respectively. Both compounds reduce levels of 2-HG, removing inappropriate inhibition of the TET enzymes, thereby restoring normal levels of DNA methylation and ultimately triggering LSC differentiation [145,146]. Vitamin C (ascorbic acid) can also act as a cofactor in the regulation of TET enzymes in mammals [147]. By hijacking this unique property, two groups independently showed that vitamin C can regulate murine and human HSC numbers and that supplementation of exogenous vitamin C attenuates leukemogenesis by restoring normal TET2 function [148,149].

Box 5. Crosstalk between Metabolites and Chromatin Environment during Hematopoiesis

Another crosstalk that is now receiving considerable attention is the relationship between cellular metabolism, epigenetic modifications and hematopoiesis (see Figure 3 in main text) [177–180]. A prime example of this is the observation that the demethylation activity of the TET enzymes can be attenuated by the activity of isocitrate dehydrogenase IDH1/DH2. Since these enzymes are involved in the oxidative decarboxylation of isocitrate, this reaction produces NADPH and 2-hydroxyglutarate (2-HG). The latter is a metabolite that inhibits TET2 activity. Indeed, mutations in *IDH1/2* are frequently found in leukemia and CHIP (see Chromatin Dynamics Emerges as a Critical Facet of Blood-Related Disorders in main text) and their deletion leads to impaired differentiation in cultured human HSCs [181]. Nevertheless, *Idh2*-mutant HSCs do not phenocopy the defects observed in *Tet2*-deficient animals [83], suggesting that modifications in DNA methylation likely precede metabolic changes in these cells. Indeed, AML patients show recurring gain-of-function mutations in *IDH1* and *IDH2* and increased 2-HG levels [182,183].

Moreover, changes in branched-chain amino acid (BCAA) metabolism have been associated with increased α-ketoglutaric acid levels in LSCs, resulting in indirect increases of 2-HG [184]. This metabolite, as discussed above, negatively regulates TET2, in turn causing DNA hypermethylation with significant effects on cell differentiation and proliferation [83,185,186].

Both HSCs and LSCs depend on glycolytic respiration, making them sensitive to the relatively low oxygen concentrations in the bone marrow microenvironment [9]. Perturbations in glycolysis are detrimental to both cell types, but LSCs exhibit a competitive advantage through their ability to increase mitochondrial mass and increase OXPHOS capacity and fitness [187]. In addition, by upregulating expression of ALOX5 and ALOX15, LSCs are able to colonize atypical niches rich in adipocytes, conferring them drug resistance [188,189]. Lysosomes also appear to have complex functions in HSC biology, as repression of lysosomal activity enhances both quiescence and differentiation capacity [190]. Even though the precise function of lysosomal activity in LSCs remains unclear, it has been shown that leukemic cells are more sensitive to lysosomal disruption when compared to normal progenitor cells [191]. Mitophagy reduction also preferentially affects abnormal progenitor cells, as it results in decreased numbers of LSCs and promotes AML differentiation [192]. Not surprisingly, targeting both mitochondria and lysosomes concomitantly represents a promising strategy against AML [193]. Altogether, the interplay between metabolism and epigenetic modifications is necessary in progression of cells from a pre-leukemic state to full-blown leukemia.

Outstanding Questions

Can changes in the niche metabolic microenvironment contribute to HSC fate determination by affecting epigenetic regulator activities?

How is temporal regulation of the chromatin landscape in different hematopoietic differentiation programs achieved?

Is the dosage of epigenetic regulators important for cell determination?

How do epigenetic regulators affect HSC interaction with the niche?

Advances in low-input ChIP-sequencing technologies should in future permit us to precisely assay the full extent of intercellular heterogeneity in histone post-translational modifications in HSCs.

Targeting epigenetic modifiers which repress differentiation programs also appears to be a promising strategy for avoiding AML relapse [150]. Preclinical studies using LSCs exposed to an inhibitor of the histone demethylase 1 (LSD1) showed increased differentiation output due to the return of the proper wiring of PU.1 and C/EBPα enhancers [151,152]. *Dnmt3a*-mutant HSCs showed increased H3K79me at the DNA-methylated canyons (see DNA Methylation Represses Impromptu Programs in HSCs), thereby suggesting that blocking its methylation could be a propitious strategy [153,154]. Consistently, reducing H3K79me either by downregulating disruptor of telomeric silencing 1-like (DOT1L) or administration of pinometostat (EPZ-5676) elicits efficient responses and decreased leukemogenic potential in a subset of *MLL*-rearranged advanced leukemias [132,155,156].

Concluding Remarks

In this review, we shed light on the multiple ways in which chromatin dynamics regulates hematopoiesis and HSC fate determination. HSC identity is ensured by their chromatin landscape, which provides and maintains their accurate transcription profile. In this regard, while TF binding and chromatin decompaction represent intrinsic cues, the niche can provide external cues to either reinforce cell differentiation or maintain an undifferentiated state. Importantly, perturbations in either can elicit leukemogenesis. Thereby, combining chromatin state, clonality, transcription and genomic profiles in an unperturbed/native system emerges as a powerful strategy to precisely decipher the onset of blood-related disorders. We envision that the engineered mouse encoding all the **CRISPR** editing components (**CRISPR array repair lineage tracing**, **CARLIN**) [157], and **lineage and RNA recovery (LARRY)** coupled with scRNA/ATAC-seq will be essential to disentangle HSC clone history and the importance of chromatin status. Another current issue rests on the fact that most barcode techniques are not suitable to study human hematopoiesis *in vivo*. Techniques taking advantage of the inherent intraindividual variation in mtDNA as an innate barcode to infer clonal relationships will prove critical in deciphering

progenitor fates of human hematopoiesis [158]. It is tempting to predict that the combination of clonal output with chromatin status profiling may provide insights into the mechanisms underpinning and conferring memory to lineage specification and HSC fate. In summary, despite exciting open questions to be addressed (See Outstanding Questions) the last decade has significantly advanced our understanding of how chromatin dynamics instruct cell transitions between developmental stages and cellular activation states. Further molecular insights into the action of chromatin factors in hematopoietic cell types will be valuable not only for epigenetic and stem cell researchers but also in providing sources of new druggable targets for the clinic in the coming years.

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Resources

[i]https://seer.cancer.gov/statfacts/html/leuks.html

[ii]https://biorender.com

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