Biological, prognostic and therapeutic impact of the epigenome in CLL

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Abstract

Chronic lymphocytic leukemia (CLL) is characterized by widespread alterations in the genetic and epigenetic landscapes which seem to underlie the variable clinical manifestations observed in patients. Over the last decade, epigenomic studies have described the whole-genome maps of DNA methylation and chromatin features of CLL and normal B cells, identifying distinct epigenetic mechanisms operating in tumoral cells. DNA methylation analyses have identified that the CLL methylome contains imprints of the cell of origin, as well as of the proliferative history of the tumor cells, with both being strong independent prognostic predictors. Moreover, single-cell analysis revealed a higher degree of DNA methylation noise in CLL cells, which associates with transcriptional plasticity and disease aggressivity. Integrative analysis of chromatin has uncovered chromatin signatures, as well as regulatory regions specifically active in each CLL subtype or in Richter transformed samples. Unique transcription factor (TF) binding motifs are overrepresented on those regions, suggesting that altered TF networks operate from disease initiation to progression as non-genetic factors mediating the oncogenic transcriptional profiles. Multi-omics analysis has identified that response to treatment is modulated by an epigenetic imprint, and that treatments affect chromatin through the activity of particular set of TFs. Additionally, the epigenome is an axis of therapeutic vulnerability in CLL, as it can be targeted by inhibitors of histone modifying enzymes, that have shown promising pre-clinical results. Altogether, this review aims at summarizing the major findings derived from published literature to distill how altered epigenomic mechanisms contribute to CLL origin, evolution, clinical behavior, and response to treatment.

Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia of the elderly in the Western world, and shows a highly heterogeneous spectrum of biological and clinical manifestations [1]. CLL is a paradigmatic model of cancer evolution and diversification with relevant clinical impact. Once CLL is diagnosed, some patients remain stable for decades whereas others progress rapidly requiring treatment with recurrent waves of responses and relapses after becoming resistant to conventional and novel therapies [2-4]. In some patients, the disease evolves into an aggressive lymphoma with dismal outcome known as Richter transformation [1]. At the other side of the clinical spectrum, some CLL patients remain stable for years or even undergo a spontaneous regression without any treatment [5]. This clinical heterogeneity is underlined by a variety of biological features. CLL patients can be globally classified into two main groups based on the level of somatic hypermutation in the variable region of the immunoglobulin heavy chain (IGHV) locus, which reflects if they are derived from a germinal center experienced B cell or not. Those patients whose leukemic cells have low or no somatic IGHV hypermutation (called unmutated- or U-CLL) show a more aggressive behavior than those with a mutated IGHV locus (named IGHV mutated- or M-CLL) which typically present a more benign clinical course [6,7]. The landscape of somatic genetic changes has been thoroughly characterized in CLL. Beyond the classical copy number changes involving del(13q), del(11q), del(17p) and trisomy 12, present in approximately 80% of the patients, CLL cells show a very heterogeneous pattern of mutated genes. Next-generation sequencing (NGS) approaches using large cohort of patients have identified few recurrent mutations that are present in more than 10% of the patients, being *SF3B1*, *NOTCH1*, *ATM* and *TP53* the most frequently mutated genes, while a large tail of genes is mutated only in a small fraction of the patients [8-10]. Interestingly, most mutations can be grouped into specific pathways, including NOTCH signaling (e.g., *NOTCH1*,*FBXW7*), B-cell receptor and Toll-like signaling (e.g., *MYD88*, *BCOR*, *ECR2*), MAPK-ERK

pathways (e.g., *MYD88*, *KRAS*), NF-kB signaling (e.g., *NFKBIE*, *EGR2*), or general processes such as DNA-damage and cell-cycle regulation (e.g., *ATM, TP53, POT1*), chromatin remodeling (e.g., *CHD2*, *SETD2*), and RNA/ribosomal processing (e.g., *SF3B1*, *XPO1, MED12*) [1]. While the prognostic value of the highly frequent mutations has been demonstrated, the precise role and clinical impact of many of these mutations remain to be elucidated.

Beyond the undisputed role of genetic alterations in the pathophysiology of CLL, decades of research have demonstrated that cancer cells in general, and CLL cells in particular, also undergo a widespread epigenetic reconfiguration. Epigenetics encodes a variety of molecular features that regulate gene expression and provides function to the information encoded in the genome [11]. The character of a cell is the result of specific pattern of gene expression, that is controlled by these epigenetic features, and are directed by transcription factors (TF) that selectively bind DNA regions for transcriptional activation or repression. The layers of epigenetic regulation include cytosine methylation at CpG dinucleotides, DNA accessibility, post-translational modifications of histones, and the three-dimensional network of connections between different regions of the genome. Over the last decade epigenomic studies have generated whole-genome maps of DNA methylation, chromatin features and 3D genome interactions in CLL and normal B cells at different differentiation stages. The goal of this review is to distil the information derived from these studies, and to summarize how altered epigenomic mechanisms contribute to CLL origin, clonal evolution, and clinical behavior. A specific focus on the TF networks driving epigenetic deregulation is reported. Finally, the impact epigenetic modifications have on the response to current treatments, as well as the therapeutic options that are derived from epigenetic regulators is discussed.

Epigenetic insights into the cellular origin of CLL.

The cellular origin of CLL has been a subject of intense research and debate, as different approaches can lead to apparently different interpretations [12,13]. Immunogenetic

studies suggest that M-CLL cells are derived from germinal center experienced B cells, that have undergone IGHV somatic hypermutation, while U-CLL have matured independently of a germinal center (GC) reaction. Indeed, transcriptional studies suggest that U-CLL derives from unmutated mature CD5+ B cells and M-CLL from a low abundant subpopulation of CD5+/CD27+ post-germinal center B cell [14]. However, it has been also described that all CLL cells show a common transcriptomic profile resembling a memory B cell [15]. DNA methylation analysis has much to add to this discussion, as it is a *bona fide* epigenetic marker related to cellular identity and gene regulation [16,17]. DNA methylation was initially described as a repressive mark, but it is nowadays established that its function is broader and depends on the genomic context where methylated CpGs are present [18,19]. During normal B cell differentiation, the DNA methylome is extensively modified, and each differentiation stage shows a specific epigenetic fingerprint [20,21]. In the context of CLL, genome-wide DNA methylation analyses revealed that most changes seem to be associated with epigenetic imprints of their putative cell of origin, and thus most differences were observed between M-CLL and U-CLL cases. However, DNA methylation profiling identified that it was more appropriate to categorize CLLs into three rather than two subtypes. These epigenetic CLL subtypes or “epitypes” are NBC-like (n-CLL) or low-programmed CLL (LP-CLL) large overlapping with U-CLL, MBC-like (m-CLL) or high-programmed CLL (HP-CLL) mostly composed by M-CLL cases, and an intermediate group (i-CLL) without a clear association to any specific normal B-cell subpopulation [22,23]. Recent findings have shown this intermediate group carries borderline immunoglobulin mutation load and show a bias toward light-chain use, with a high proportion of cases showing IGLV3-21 rearrangements [24]. Moreover, many i-CLL cases carry a mutation induced by SHM that produced a signal G>C substitution at the splice site between the immunoglobulin VDJ and constant genes, changing the glycine at position 110 to an arginine (the so-called R110 mutation). This R110 mutation is known to help trigger an aberrant BCR-BCR interaction inducing a constant BCR signaling in the cells [25]. The fact that the IGLV3-

21^{R110} mutation is mediated by the SHM which associates with a GC reaction adds evidence to the idea that i-CLL cases are derived from a GC-experienced B cell. In summary, the integration of the DNA methylation profiles with the SHM status of the immunoglobulin genes has shed light on the origins of CLL. During their maturation process B cells circulate through the dark and light zones of the GC where they proliferate while enhancing their affinity to the antigen, until they are selected to leave. In this process B cells undergo several rounds of immunoglobulin genes mutagenesis but also a strong DNA methylation imprint [21, 26]. Thus, we can envision that n-CLL/LP-CLL are derived from GC-inexperienced cells, as they have no GC imprint at the mutagenic or DNA methylation levels; while m-CLL/HP-CLL show high SHM load and strong epigenetic programming and thus reflect they may derive from a B cell selected to leave a GC reaction after several maturation cycles. In this scenario, i-CLL could reflect a cell that has been positively selected early in the CG reaction, and thus show moderate SHM and intermediate DNA methylation pattern. Remarkably, from a clinical point of view the different cellular origins of the three epitypes are associated with distinct clinical features.

Epigenetic features help to predict CLL prognosis

Several studies have confirmed that the epigenetic classification into 3 CLL epitypes outcompetes the clinical prognosis using classical immunogenetic categorization [27-30]. These studies have confirmed n-CLL/LP-CLL have poorer prognosis and shorter time to first treatment, followed by i-CLL cases that show an intermediate prognosis, and being the m-CLL/HP-CLL the cases with better prognosis, and longer time to first treatment [27]. This DNA methylation-based classification could be implemented in the clinical setting, as this mark has been proven to be stable over time, and shows similar patterns in peripheral blood and lymph node samples [31,32]. Moreover, a prediction model using only five CpG sites was enough to successfully classify samples into the three epitypes [27]. However, recent studies have identified that the i-CLL cases can be

further divided into two prognostic groups based on the presence or absence of the IGLV3-21^{R110} mutation. Those i-CLL cases harboring an IGLV3-21^{R110} mutation are more aggressive and behave similarly to n-CLL/LP-CLL, while those lacking the mutation resemble the more indolent m-CLL/HP-CLLs [33]. Further studies are needed to determine if a DNA methylation classification that will also consider the R110 mutation has an added prognostic value to the standard *IGHV* gene profiling.

Beyond the classical role of DNA methylation as a gene regulator, an accumulative body of evidence supports the concept that methylation changes in late-replicating DNA regions do not have a direct impact on the expression of nearby genes but can be used to assess the accumulative number of cell divisions [34,35]. During mitosis cells accumulate hypomethylation in heterochromatic regions with low-CpG, and hypermethylation in high-CpG content regions marked with H3K27me3 chromatin states, known to be targets of the Polycomb repressive complex. This may be used as a mitotic clock to define the proliferative history of the cells [36]. In the context of normal and neoplastic B cells the epigenetically determined cumulative mitoses (epiCMIT) clock was developed, which is built considering both hyper- and hypo-methylation levels [32]. In normal B-cell maturation, the epiCMIT gradually augments as B cells proliferate, an increase that is particularly marked in highly proliferative GC B cells. Thus, the highest epiCMIT is observed in terminally differentiated plasma cell (PC), which do not proliferate but the mitoses accumulated throughout B cell maturation are imprinted in its DNA methylome. In neoplastic B cells, however, the interpretation is less trivial and must be divided into two components: the epiCMIT of the cell of origin, and the epiCMIT acquired during the neoplastic transformation and progression. Thus, considering the cell of origin for each of the CLL epitypes, a high epiCMIT was strongly associated with a worse prognosis, using time to first treatment as an endpoint variable. Additionally, using the epiCMIT as a continuous variable showed a highly significant independent prognostic impact in the context of other major prognostic factors including IGHV status and *TP53*

alterations [32]. The use of DNA methylation as a clinical surrogate of the proliferative history of a cell, and not its current proliferative status at the time of sampling, is particularly important. In the routine diagnostic setting, CLL cells are obtained from peripheral blood, where cells are quiescent, and not from lymph nodes where cells do proliferate under the adequate microenvironmental stimuli. Thus, the analysis of the epiCMIT in these samples will reflect the accumulated mitosis that had taken place in the proliferative compartment, despite being measured using quiescent cells, providing an easy-to-implement diagnostic tool to estimate clinical the clinical risk of each individual case.

Understanding clonal evolution through epigenetic profiling

Cancer epigenomes have been long appreciated to differ from their normal tissue counterparts. Furthermore, an intra-tumoral heterogeneity in the genetic and epigenetic layers has been described to cooperate in the evolutionary course of tumors. Although the DNA methylation is relatively stable over time, there is growing evidence of DNA methylation show an intra-tumoral heterogeneity, something that also plays a role in the evolution of the disease. It has been described that DNA methylation patterns in neoplastic B cells are more heterogeneous than in normal B cells, and that it also associates with a worst prognosis. CLL cells acquire DNA methylation in local sites in a stochastic manner, creating an “epigenetic noise”, which generate a plasticity that may facilitate tumor evolution, allowing cancer cells a greater degree of population diversity [37]. Furthermore, the integration of single cell DNA methylation analysis, single cell gene expression and genomic alterations allowed to trace the history of CLL cells and showed that intra-tumoral methylation correlates with genetic subclonal complexity [38]. Growing populations of CLL cells become diversified by stochastic changes in DNA methylation, in a process known as “epimutations”. The analysis of epimutations indicated that CLL cells undergo a rapid drift after initial steps of clonal expansions, giving rise to the early presence of subclones that expand the plasticity of the disease [38]. This plasticity allows

the competition between clones that will adapt to the changing microenvironment. In summary, the high degree of DNA methylation noise cooperates with transcriptional plasticity, and associates with unfavorable clinical outcomes.

Recently the evolution from CLL to Richter transformation (RT) has been studied at the single cell level. Surprisingly, evidence suggesting that RT subclonal diversification took place at least 19 years before the clinical manifestation of RT was identified [48]. These early seeds share genetic and transcriptional features of the expanded RT subclone, characterized by a BCR signaling pathway downregulation and an upregulation of the oxidative phosphorylation pathway [47]. The underlying mechanisms of this large time lapse before the subclone expansion and clinical manifestation of RT are currently not understood. In an attempt to understand this transcriptomic evolution from CLL to RT genome-wide profiles of DNA methylation, chromatin activation (i.e., histone H3 lysine 27 acetylation, H3K27ac) and chromatin accessibility (ATAC-seq) were also analyzed. The DNA methylome of RT mainly reflected the naïve and memory-like B cell derivation of their CLL counterpart. Chromatin activation and accessibility were remarkably different upon transformation, and further detailed studies are needed to identify if epigenetic plasticity is also playing a role in the process of subclonal selection to RT [47].

Epigenetic dysregulation contributes to CLL pathogenesis.

CLL has been the subject of several genome-wide studies characterizing chromatin accessibility, histone modifications and 3D interactions together with transcriptional profiling [39-43]. Interestingly, the integration of several of these layers of epigenetic regulation has revealed that CLL cells do have a unique chromatin activation signature, that is distinct from normal B cells at any differentiation stage. This unique repertoire of CLL-specific active regulatory regions (i.e., with increased H3K27ac) is also shows decreased DNA methylation, increased chromatin accessibility and local 3D interactions, and consequently increased expression of associated genes, including surface receptor signaling and lymphoid organ development related genes [41]. For example, they target

regulatory elements of genes such as *LEF1*, *CTLA4*, *FMOD* or *TCF4*, known to be involved in CLL pathogenesis [45]. Remarkably, the activation of these regulatory regions does not seem to be independent of each other, as their DNA sequence is enriched for binding sites of specific transcription factor families such as NFAT, FOX and TCF/LEF1. In keeping with this finding, other independent studies in CLL have also reported that regulatory regions with increased H3K27ac are enriched in binding sites of the same TF families [40,42,43]. This pattern is shared between M-CLL and U-CLLs, and therefore, seems to be a signature of CLLness, which contrasts with the extreme genetic heterogeneity observed in CLL patients. This indicates that leukemia evolution is linked to chromatin programming, from inactive regions in normal B cells to active enhancers in CLL and suggests that this onco-epigenetic process is an early essential event during leukemogenesis.

Moreover, some studies evaluated the coordination between different layers of epigenetic regulation. Unexpectedly the co-occurrence of typically mutually exclusive activating (H3K27ac) and repressing (H3K27me3) histone modifications was observed in CLL cells. The co-mapping of these marks has been observed in stem cells reflecting cellular heterogeneity due to admixture of different differentiation stages. This finding suggests epigenetic diversification is operating also in CLL, as a mix of cells with diverging epigenetic identities are found [43]. This may result in permissive chromatin states across cells, leading to greater cell-to-cell transcriptional variation. Similarly, while in normal B cells the combination of repressive H3K27me3 mark with the decrease of active marks such as H3K4me3 and H3K27ac generally associates with a uniform transcriptional repression output, in CLL these genes were associated with variable expression levels [43], indicating higher transcriptional plasticity and dysregulation. Thus, as it was proposed for DNA methylation [37], chromatin modifications could also contribute with intra-tumoral heterogeneity and subclonal evolution in CLL.

In addition to chromatin patterns shared globally by all CLL cases, studies have also identified clear distinct landscapes of chromatin accessibility and histone modifications between U-CLL and M-CLL subtypes [39, 41] When the differential chromatin regions were analyzed in the context of normal B cell subpopulations, different patterns were observed. First, patterns of *de novo* altered (i.e., not modulated in normal B cell maturation) chromatin accessibility in U-CLL or M-CLL were associated with transcription factor (TF) motifs markedly different from each other and from CLL as a whole [41,42]. Second, regions linking M-CLL to GC-experience cells (i.e., MBC), and U-CLL to GC-inexperienced cells (i.e., NBC) were observed. And third, M-CLL and U-CLL also show differences in regions showing different modulation patterns during normal B cell maturation. For instance, and unexpectedly, a fraction of the regulatory regions specifically active in U-CLL cases recapitulate a signature of active enhancers found in proliferative normal B cells (i.e., GCBC) [41]. This suggests U-CLLs may hijack some epigenetic mechanisms associated with the normal B cell proliferative mechanisms.

Distinct transcription factor networks drive the altered CLL epigenome

Taking advantage of the large epigenomic information derived from CLL primary cells, several studies aimed to identify the TFs involved that may mediate such epigenetic and transcriptional dysregulation. Interestingly, several independent studies pinpoint similar TFs candidates as potential drivers of the unique CLL activation of regulatory regions, including NFAT, TFC/LEF1 and FOX TF families [40-43]. Additionally, NFAT specific binding motif is found also enriched at the CLL specific hypomethylated regions, not shared by any other lymphoid malignancies or normal B cells [32]. Moreover, studies looking at genetic predisposition of CLL have identified SNPs that have a significant association with CLL development [46]. Some of this SNP are located within TF binding sites, and the presence of the risk allele may be related to an altered TF binding affinity. In particular, the SNPs that associates with higher risk of CLL development remarkably showed an increased binding affinity for the NFAT, TCF/LEF and FOX TF families [46].

By constructing the CLL-specific TF regulatory network using regulatory regions defined by H3K27ac and chromatin accessibility profiling, a study was able to identify the critical TFs nodes related to CLL active enhancers. Among the most highly connected CLL TFs identified there where TF known to be involved in B-cell differentiation such as IRF family members (i.e., IRF8, IRF2) and PAX5, together with MYC, FOXP1, RARA, and ETS1 [40]. CRISPR-Cas9 screening of the top TF candidates using CLL cell lines identify that transcription circuits mediated by PAX5, MYC, RARA, IKZF3 and IKZF1 were essential for CLL cell survival, being *PAX5* knock-out the one that conferred a most severe growth disadvantage [40]. The use of cell lines for this screening may have masked other TFs vulnerabilities that could be operating in primary cells, such as NFAT or LEF1, that are not essential in the Epstein-Barr transformed cellular models used.

Notably, de novo altered chromatin accessibility in U-CLL or M-CLL was associated with markedly different transcription factor (TF) motifs. Interestingly, regions gaining accessibility in U-CLL, the most aggressive subtype, were enriched in binding motifs of IRF, STAT1/2, AP-1, FOXP1, and GFI1 TFs, among others [41]. Many of these TFs are involved in cell cycle regulation and are, in part, operative in proliferative B cell subpopulations, suggesting U-CLL may exploit specific molecular mechanisms present in normal B cell subpopulations to achieve higher proliferation. Moreover, the chromatin activation and accessibility of Richter transformed samples has been described to be significantly different when compared to non-transformed CLL cells from the same patient [47]. The unique active regions found in Richter transformed cells were enriched in TF families different to those known to modulate the epigenome of CLL. Among them, the top was TEAD4, MAZ and E2F TF motifs, involved in oxidative phosphorylation and cell cycle [47]. The exact role of these TFs play in disease progression and transformation is mostly unexplored. Further research shall determine their role in the initiation and maintenance of CLL chromatin dysregulation, and whether their inhibition may represent a suitable therapeutic path.

Treatment response and CLL epigenetic regulation

Multi-omics analysis is also a powerful strategy for dissecting the molecular response to targeted therapies and allows to define the temporal order of the induced changes and unravel underlying regulatory programs. The integrative sequential analysis of scRNA-seq and ATAC-seq in CLL samples during ibrutinib treatment identified that treatment very rapidly induces the decrease of NF-Kb TF binding. This was followed by the reduction of the regulatory activity of TFs involved in B cell development (such as EBF1, FOXM1, IRF4, PAX5 and PU.1). This decrease was accompanied by the downregulation of CLL-specific signatures, and a decrease of the CD5 and CD19 surface marker levels, indicating a broad erosion of CLL cell identity. The sustained ibrutinib therapy resulted in the acquisition of a quiescence-like gene signature including downregulation of *CXCR4* and *ZFP36L2*, with known functions in senescence and quiescence in hematopoietic cells. Most interestingly, single cell analyses allowed to identify patient-specific signatures present prior to treatment that could predict patient response to treatment [44]. Similarly, another study reported that prolonged ibrutinib treatment induces a global reduction of specific histone modifications such as H3K27ac and H3K27me3. Significant differences were found between samples from treatment-naïve patients or those that were resistant/refractory to other therapies before starting the ibrutinib regimen. Interestingly, resistant/refractory patients show a significant number of promoters that maintain a bivalency (i.e., H3K4me3 and H3K27me3) while, in treatment naïve samples, these promoters were fully silenced. This suggest that previous treatments left an epigenetic memory which could play a role in the decrease of their sensitivity to ibrutinib [48]. Furthermore, it was described that during ibrutinib treatment most of the regulatory regions that underwent significant changes in chromatin accessibly do so by becoming less accessible, suggesting an effect of ibrutinib on chromatin repression. Although this loss of chromatin accessibility was shared by all patients studied, some degree of patient-to-patient heterogeneity was observed. Regions with reduced accessibility were

CLL-specific regulatory regions marked by H3K4me1, and moreover, the binding motifs of PU.1/IRF8, TCF, EGR and NF-kb TFs were significantly enriched within them [49]. These genomic regions were linked to BCR and NF-kb signaling pathways, as well as DNA replication, which were previously shown to be affected by this treatment [50]. In contrast, those regions that increase chromatin accessibility upon ibrutinib treatment were associated with proteasome regulation and autophagy, among others. The combination of this chromatin data with chemosensitivity profiling identified an ibrutinib-induced gain of CLL cell selectivity for proteasome inhibitors, PLK1 inhibitors (which may target FoxO signaling) and mTOR inhibitors [49]. In these lines, chemo- and chemo-immunotherapies have been described to also affect the epigenome [51,52]. Through the sequential study of CLL samples pre-treatment and post-relapse to chemo-immunotherapy a global hypomethylation was observed, when compared with memory B cells [52]. Importantly recurrent DNA methylation changes were not observed at cohort levels, but through case-specific analysis, as was reported previously [26,31]. Those genomic regions that became hypomethylated pre-treatment and after relapse were enriched for the binding of GATA, STAT, HOX and FOX TFs. The EGR2 and E2F4 TF binding sites were associated with regions hypermethylated at relapse, suggesting a direct connection with relapse mechanisms [52]. A separate study identified differentially methylated regions followed chemotherapy, of which 31 were CLL specific. Among them methylation of the *HOXA4*, *SLCO3A1* and *MAFB* locus was associated with a reduced response to treatment [51].

A better understanding of the source of inter-patient heterogeneity is a prerequisite for improved cancer therapy. Through a multi-omics approach combining genomic, DNA methylation, transcriptomic and ex vivo drug response phenotype with unsupervised machine learning methods, a recent study has identified a risk signature, called “CLL progressive drive” (CLL-PD). In multivariable Cox regression such CLL-PD was a significant and independent predictor of overall survival and time to treatment. The CLL-

PD associates with lymphocyte doubling rate, global hypomethylation, accumulation of driver genomic aberrations and response to pro-proliferative stimuli. Interestingly, CLL-PD was linked with an increased activity of mTOR, MYC and OXPHOS pathways [53], suggesting targeting these pathways may be a potential therapeutic option.

Epigenetic regulators as a therapeutic target for CLL

Although DNA hypomethylating agents are currently approved for the treatment of some myeloid neoplasms, initial trials in CLL showed discouraging results [54.55]. However, an accumulating body of evidence indicates that inhibition of histone modifying enzymes may represent a more effective axis of epigenetic vulnerability to be exploited therapeutically in CLL. In keeping with the increased histone acetylation identified in CLL cases, the use of bromodomain and extra-terminal inhibitors (BET) has been explored [40,56]. BET family of proteins are transcriptional regulators that bind to acetylated histones and recruit the transcriptional machinery to control gene expression. Interestingly, CLL cell lines were most clearly affected by *BRD4* CRISPR-knock out than by the elimination of other BRD family members [40]. BRD4 associates with all active promoters and a significant fraction of active enhancers, and its inhibition has shown a selective anticancer activity in several preclinical models [57]. The use of a pan-BET inhibitor JQ1 inhibited cell proliferation of CLL primary cells and cell lines, and reduced tumor burden in xenografted mice [40]. Furthermore, the novel small molecule BET inhibitor PLX51107 showed to be highly selective against BRD4 and demonstrated potent antitumor activity in CLL preclinical models. This effect was driven by the disruption of BRD4-bound enhancers, including the regulatory regions of the *MIR21*, *IL4R*, *IL21R*, *IKZF3*, *IKZF1*, *mir155* and *TCL1A* genes [56].

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the Polycomb repressive complex (PRC2), which induces gene repression through H3K27me3. *EZH2* is upregulated in the proliferation centers of CLL lymph nodes [58] and its expression is regulated by the proliferative signals coming from the tumor microenvironment [59].

EZH2 overexpression is associated with clinical aggressiveness [60]. Particularly in U-CLL cases, EZH2 direct binding to PI3K pathway genes leading to an enhanced activity of the pathway [61]. Thus, the use of EZH2 inhibitors (i.e., GSK126 and GSK343) in preclinical settings in combination with ibrutinib, idelalisib and/or venetoclax induced a significant reduction of cell viability [59].

The histone deacetylases HDAC1 and HDAC2 form the catalytic core of numerous regulatory complexes. In CLL high HDAC activity is linked to inferior survival [62] and had been demonstrated to drive the silencing of several microRNAs [63]. A recent study has profiled the genome-wide occupancy of HDAC1 in CLL cells and demonstrated that in parallel to its activity as a transcriptional repressor, it is also recruited to a small number of super enhancers together with BRD4, operating as transcriptional activator of a set of genes involved in survival, BCR signaling and immune dysfunction (e.g., *CXCR4*, *PAX5*, *IKZF3* and *BLK*). HDAC pharmacological inhibition abolished both activation and repressive functions of HDAC1 [64]. Interestingly, using the aggressive *in vivo* CLL model Eu-TLC1 it has been shown that HDAC6 inhibition exerts immunomodulatory effects on CLL cells, via the reduced surface expression of programmed death-ligand 1 (PD-L1) and lowered interleukin-10 (IL-10) expression. Following this discovery, the combination of HDAC6 inhibitor ACY738 with PD-1/PD-L1 blockade augments antitumor efficiency in the Eu-TCL1 model [65].

A recent study has also used the Eu-TCL1A mice model to evaluate the role of the lysine-specific histone demethylase 1A (*KDM1A* or *LSD1*) in CLL aggressivity and as a potential therapeutic target. KDM1A forms part of different complexes and actively removes methyl groups from mono- or di-methylated lysine 4 or 9 on histone 3 (H3K4/K9me1/2) working as modulator of gene expression. Interestingly, the KDM1A^{high} gene expression signature associates with a more CLL aggressiveness. In keeping with this, the *Kdm1a* knock-down or pharmacological inhibition led to an increased histone methylation and CLL cell apoptosis, both in vitro and in vivo [66].

Conclusions and future perspectives.

The profound analysis of bulk multi-layered epigenome in large cohorts of patients has provided valuable insights into the cell of origin and pathogenic mechanisms of CLL. Recent single-cell epigenetic and transcriptional profiling is adding clues on intraclonal heterogeneity as well as the clonal evolution taking place during disease development and in response to treatment. Altogether, this knowledge has increased our understanding of the molecular complexity underlying the initiation and progression of CLL. Epigenomic profiling has been able to identify TF families as potential regulators of these oncogenic mechanisms, but the exact role these TFs play in disease progression and transformation is mostly unexplored. Further research efforts shall develop *in vitro* models that preserve patient-specific characteristics while allowing to perform functional characterization of these TFs candidates. This research has the potential to identify novel disease vulnerabilities and therapeutic targets, that shall lead to the development of more effective treatments. In those lines, the inhibition of the chromatin modifying machinery in CLL has shown promising results in pre-clinical studies. Upcoming studies shall integrate new and previous knowledge on the mechanisms behind CLL epigenetic dysregulation, to move forward towards an individualized understanding of the disease, to develop personalized strategies for risk-assessment, treatment and clinical management.

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AUTHOR CONTRIBUTIONS

Both authors have written the manuscript and designed the figures.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

References

1. Delgado J, Nadeu F, Colomer D, Campo E. Chronic lymphocytic leukemia: from molecular pathogenesis to novel therapeutic strategies. Haematologica. 2020;105(9):2205-2217.
2. Ahn IE, Underbayev C, Albitar A, et al. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. Blood 2017; 129(11):1469-1479.
3. Herling CD, Abedpour N, Weiss J, et al. Clonal dynamics towards the development of venetoclax resistance in chronic lymphocytic leukemia. NatCommun 2018. 9(1):727.
4. Woyach JA, Johnson AJ. Targeted therapies in CLL: mechanisms of resistance and strategies for management. Blood 2015; 126(4):471-477.
5. Kwok M, Oldreive C, Rawstron AC, Goel A, Papatzikas G, Jones RE, et al. Integrative analysis of spontaneous CLL regression highlights genetic and microenvironmental interdependency in CLL. Blood. 2020;135(6):411–28
6. Hamblin TJ, Davis Z, Gardiner A, et al. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999; 94(6):1848–54.
7. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999; 94(6):1840–7.

8. Puente XS, Beà S, Valdés-Mas R, et al. Non-coding recurrent mutations in chronic lymphocytic leukaemia. Nature 2015; 526(7574):519–24.
9. Knisbacher BA, Lin Z, Hahn CK, et al. Molecular map of chronic lymphocytic leukemia and its impact on outcome. Nat Genet.2022; 54(11):1664-1674.
10. Landau DA, Tausch E, Taylor-Weiner AN, et al. Mutations driving CLL and their evolution in progression and relapse. Nature 2015; 526(7574):525–30.
11. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. Cell 2007; 128(4):669–81.
12. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. Blood 2011; 117(6):1781–91.
13. Ng A, Chiorazzi N. Potential Relevance of B-cell Maturation Pathways in Defining the Cell(s) of Origin for Chronic Lymphocytic Leukemia. Hematol Oncol Clin North Am. 2021;35(4):665–85.
14. Seifert M, Sellmann L, Bloehdorn J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. J Exp Med. 2012;209(12):2183–98.
15. Klein U, Tu Y, Stolovitzky GA, et al. Gene Expression Profiling of B Cell Chronic Lymphocytic Leukemia Reveals a Homogeneous Phenotype Related to Memory B Cells. J Exp Med.2001; 194(11):1625–38.
16. Roy, N. & Hebrok, M. Regulation of cellular identity in cancer. Dev. Cell 2015; 35, 674–684.
17. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002; 16(1):6–21.
18. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet. 2012;13(7):484–92.
19. Kulis M, Queirós AC, Beekman R, et al. Intragenic DNA methylation in transcriptional regulation, normal differentiation and cancer. Biochim Biophys Acta. 2013;1829(11):1161–74.
20. Martin-Subero JI, Oakes CC. Charting the dynamic epigenome during B-cell development. Semin Cancer Biol. 2018; 51:139–48.
21. Kulis M, Merkel A, Heath S, et al. Whole-genome fingerprint of the DNA methylome during human B cell differentiation. Nat Genet. 2015;47(7):746–56.
22. Kulis M, Heath S, Bibikova M, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nat Genet. 2012;44(11):1236–42.
23. Oakes CC, Seifert M, Assenov Y, et al. DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia. Nat Genet. 2016;48(3):253–64.
24. Nadeu F, Royo R, Clot G, et al. IGLV3-21R110 identifies an aggressive biological subtype of chronic lymphocytic leukemia with intermediate epigenetics. Blood. 2021;137(21):2935–46.
25. Maity PC, Bilal M, Koning MT, et al. IGLV3-21*01 is an inherited risk factor for CLL through the acquisition of a single-point mutation enabling autonomous BCR signaling. Proc Natl Acad Sci U S A. 2020;117(8):4320–7.
26. Shaknovich R, Cerchietti L, Tsikitas L, et al. DNA methyltransferase 1 and DNA methylation patterning contribute to germinal center B-cell differentiation. Blood. 2011;118(13):3559–69.
27. Queirós AC, Villamor N, Clot G, et al. A B-cell epigenetic signature defines three biologic subgroups of chronic lymphocytic leukemia with clinical impact. Leukemia 2015; 29(3):598–605.

28. Giacopelli B, Zhao Q, Ruppert AS, et al. Developmental subtypes assessed by DNA methylation-iPLEX forecast the natural history of chronic lymphocytic leukemia. Blood 2019; 134(8):688–98.
29. Bhoi S, Ljungström V, Baliakas P, et al. Prognostic impact of epigenetic classification in chronic lymphocytic leukemia: The case of subset #2. Epigenetics 2016; 11(6):449–55.
30. Wojdacz TK, Amarasinghe HE, Kadalayil L, et al. Clinical significance of DNA methylation in chronic lymphocytic leukemia patients: results from 3 UK clinical trials. Blood Adv. 2019; 3(16):2474–81.
31. Cahill N, Bergh AC, Kanduri M, et al. 450K-array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments. Leukemia 2013; 27(1):150–8.
32. Duran-Ferrer M, Clot G, Nadeu F, et al. The proliferative history shapes the DNA methylome of B-cell tumors and predicts clinical outcome. Nat Cancer 2020;1(11):1066–81.
33. Nadeu F, Syrykh C, Pons-Brun B, et al; IGLV3-21^{R110} mutation has prognostic value in patients with treatment-naive chronic lymphocytic leukemia. Blood Adv *2023*; bloodadvances.2023010132
34. Zhou W, Dinh HQ, Ramjan Z, et al. DNA methylation loss in late-replicating domains is linked to mitotic cell division. Nat Genet. 2018;50(4):591–602.
35. Yang Z, Wong A, Kuh D, et al. Correlation of an epigenetic mitotic clock with cancer risk. Genome Biol. 2016;17(1):205.
36. Horvath, S. & Raj, K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. Nat. Rev. Genet. 19, 371–384 (2018).
37. Landau DA, Clement K, Ziller MJ, et al. Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia. Cancer Cell 2014;26(6):813–25.
38. Gaiti F, Chaligne R, Gu H, et al. Epigenetic evolution and lineage histories of chronic lymphocytic leukaemia. Nature 2019; 569(7757):576–80.
39. Rendeiro AF, Schmidl C, Strefford JC, et al. Chromatin accessibility maps of chronic lymphocytic leukaemia identify subtype-specific epigenome signatures and transcription regulatory networks. Nat Commun 2016;7:11938.
40. Ott CJ, Federation AJ, Schwartz LS, et al. Enhancer Architecture and Essential Core Regulatory Circuitry of Chronic Lymphocytic Leukemia. Cancer Cell 2018;34(6):982-995.e7.
41. Beekman R, Chapaprieta V, Russiñol N, et al. The reference epigenome and regulatory chromatin landscape of chronic lymphocytic leukemia. Nat Med 2018;24(6):868–80.
42. Mallm JP, Iskar M, Ishaque N, et al. Linking aberrant chromatin features in chronic lymphocytic leukemia to transcription factor networks. Mol Syst Biol. 2019;15(5):e8339.
43. Pastore A, Gaiti F, Lu SX, et al. Corrupted coordination of epigenetic modifications leads to diverging chromatin states and transcriptional heterogeneity in CLL. Nat Commun. 2019; 10(1):1874.
44. Rendeiro AF, Krausgruber T, Fortelny N, et al. Chromatin mapping and single-cell immune profiling define the temporal dynamics of ibrutinib response in CLL. Nat Commun. 2020;11(1):577.
45. Navarro, A., Clot G, Martínez-Trillos A, et al. Improved classification of leukemic B-cell lymphoproliferative disorders using a transcriptional and genetic classifier. Haematologica 2017; 102(9):e360-e363.

46. Speedy HE, Beekman R, Chapaprieta V, et al. Insight into genetic predisposition to chronic lymphocytic leukemia from integrative epigenomics. Nat Commun. 2019;10(1):3615.
47. Nadeu F, Royo R, Massoni-Badosa R, et al. Detection of early seeding of Richter transformation in chronic lymphocytic leukemia. Nat Med. 2022; 28(8):1662–71.
48. Holmes KB, Sadreev II, Rawstron AC, et al. Ibrutinib induces chromatin reorganisation of chronic lymphocytic leukaemia cells. Oncogenesis 2019; 8(5):32.
49. Schmidl C, Vladimer GI, Rendeiro AF, et al. Combined chemosensitivity and chromatin profiling prioritizes drug combinations in CLL. Nat Chem Biol. 2019; 15(3):232–40.
50. Herman SE, Mustafa RZ, Gyamfi JA, et al. Ibrutinib inhibits BCR and NF-κ B signaling and reduces tumor proliferation in tissue-resident cells of patients with CLL. Blood 2014; 123(21):3286-95.
51. Barrow TM, Nakjang S, Lafta F, et al. Epigenome-wide analysis reveals functional modulators of drug sensitivity and post-treatment survival in chronic lymphocytic leukaemia. Br J Cancer. 2021; 124(2):474–83.
52. Tsagiopoulou M, Papakonstantinou N, Moysiadis T, et al. DNA methylation profiles in chronic lymphocytic leukemia patients treated with chemoimmunotherapy. Clin Epigenetics. 2019; 11(1):177.
53. Lu J, Cannizzaro E, Meier-Abt F, et al. Multi-omics reveals clinically relevant proliferative drive associated with mTOR-MYC-OXPHOS activity in chronic lymphocytic leukemia. Nat Cancer 2021;2(8):853-864.
54. Malik A, Shoukier M, Garcia-Manero G, Wierda W, Cortes J, Bickel S, Keating MJ, Estrov Z. Azacitidine in fludarabine-refractory chronic lymphocytic leukemia: a phase II study. Clin Lymphoma Myeloma Leuk. 2013;13(3):292-5.
55. Blum KA, Liu Z, Lucas DM, et al. Phase I trial of low dose decitabine targeting DNA hypermethylation in patients with chronic lymphocytic leukaemia and non-Hodgkin lymphoma: dose-limiting myelosuppression without evidence of DNA hypomethylation. Br J Haematol. 2010;150(2):189-95.
56. Ozer HG, El-Gamal D, Powell B, et al. BRD4 Profiling Identifies Critical Chronic Lymphocytic Leukemia Oncogenic Circuits and Reveals Sensitivity to PLX51107, a Novel Structurally Distinct BET Inhibitor. Cancer Discov. 2018;8(4):458–77.
57. Loven J, Hoke HA, Lin CY, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell 2013;153:320–34.
58. Szurián K, Csala I, Marosvári D, et al. EZH2 is upregulated in the proliferation centers of CLL/SLL lymph nodes. Exp Mol Pathol. 2018;105(2):161-165.
59. Chartomatsidou E, Ntoufa S, Kotta K, et al. Inhibition of EZH2 and immune signaling exerts synergistic antitumor effects in chronic lymphocytic leukemia. Blood Adv. 2019;3(12):1891-1896.
60. Rabello Ddo A, Lucena-Araujo AR, Alves-Silva JC, et al. Overexpression of EZH2 associates with a poor prognosis in chronic lymphocytic leukemia. Blood Cells Mol Dis. 2015;54(1):97-102.
61. Kosalai ST, Morsy MHA, Papakonstantinou N, et al. EZH2 upregulates the PI3K/AKT pathway through IGF1R and MYC in clinically aggressive chronic lymphocytic leukaemia. Epigenetics. 2019;14(11):1125-1140.
62. Van Damme M, Crompot E, Meuleman, et al. Global histone deacetylase enzymatic activity is an independent prognostic marker associated with a shorter overall survival in chronic lymphocytic leukemia patients. Epigenetics 2014;9(10):1374-81.

63. Sampath D, Liu C, Vasan K, et al. Histone deacetylases mediate the silencing of miR-15a, miR-16, and miR-29b in chronic lymphocytic leukemia. Blood 2012;119(5):1162-72.
64. Lai TH, Ozer HG, Gasparini P, et al. HDAC1 regulates the chromatin landscape to control transcriptional dependencies in chronic lymphocytic leukemia. Blood Adv. 2023;7(12):2897-2911.
65. Maharaj K, Powers JJ, Mediavilla-Varela M, et al. HDAC6 Inhibition Alleviates CLL-Induced T-Cell Dysfunction and Enhances Immune Checkpoint Blockade Efficacy in the Eμ-TCL1 Model. Front Immunol. 2020; 11:590072.
66. Jiang Q, Stachelscheid J, Bloehdorn J, et al. Oncogenic role and target properties of the lysine-specific demethylase KDM1A in chronic lymphocytic leukemia. Blood. 2023;142(1):44-61.

Figure legends

Figure 1. DNA methylation as a molecular mark to trace cell origin and clonal evolution. (A) Figure shows the dynamics of DNA methylation during normal B cell differentiation and leukemogenesis. DNA methylation patterns reflects both cell of origin and proliferative history of the tumor cells. The normal B cell differentiation entails widespread DNA methylation with a high degree of methylation programming occurring during germinal center reaction. Upon malignant transformation cells retain the methylation specific of their cell of origin, and develop a new oncogenic profile. (B) Boxplot represent the concept of the epiCMIT score, including DNA methylation changes coming from the cell of origin or the neoplastic development. (C) Kaplan-Meier curve exemplify the association between CLL subtypes and time to treatment, taking into account the DNA-methylation profiles and the IGLV3-21R110 mutation. NBC: Naive B cells. GCBC: Germinal center B cells. MBC: Memory B cells. PC: Plasma cells. n-CLL: naive-like CLL. i-CLL: intermediate CLL. m-CLL: memory-like CLL. LP-CLL: low programmed CLL. HP-CLL: high programmed CLL.

Figure 2. Distinct TF binding sites are found in leukemic associated chromatin activation signatures. Chromatin profiling studies have identified that a particular de novo active chromatin signature is found in CLL cases as a hole, as well as subtype- and Richter transformed-specific signatures. The most significantly TFBS associated with each group are summarized in the figure. Specific TFBS are also found in regions harboring SNPs associated with CLL development. The top TFs whose knock-out in cell lines confer growth disadvantages are shown. TFBS: transcription factor binding sites. NBC: Naive B cells. GCBC: Germinal center B cells. MBC: Memory B cells. M-CLL: IGHV mutated. U-CLL: IGHV unmutated. RT: Richter transformation.

Figure 3. Epigenetic drugs in CLL. Figure summarizing the data derived from pharmacological targeting epigenetic regulators in CLL, including in vitro and in vivo pre-clinical studies.

Figure 1

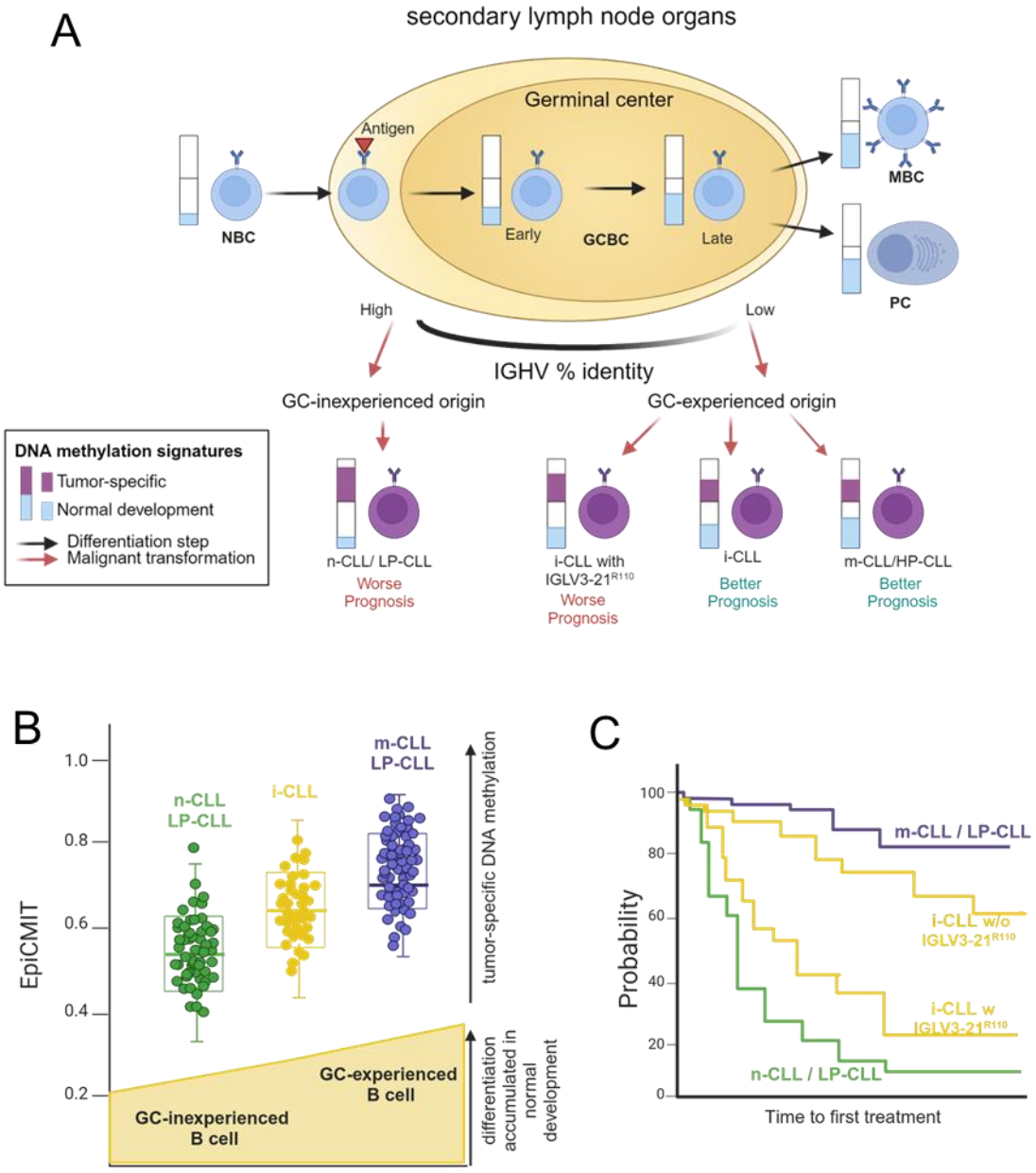

A
secondary lymph node organs
Germinal center
Antigen
NBC
Early
GCBC
Late
MBC
PC
High
Low
IGHV % identity
GC-inexperienced origin
GC-experienced origin
DNA methylation signatures
Tumor-specific
Normal development
Differentiation step
Malignant transformation
n-CLL/ LP-CLL
Worse
Prognosis
i-CLL with
IGLV3-21R110
Worse
Prognosis
i-CLL
Better
Prognosis
m-CLL/HP-CLL
Better
Prognosis
B
EpiCMIT
1.0
0.8
0.6
0.4
0.2
n-CLL
LP-CLL
i-CLL
m-CLL
LP-CLL
tumor-specific DNA methylation
GC-experienced
B cell
GC-inexperienced
B cell
differentiation
accumulated in
normal
development
C
Probability
100
80
60
40
20
0
m-CLL / LP-CLL
i-CLL w/o
IGLV3-21R110
i-CLL w
IGLV3-21R110
n-CLL / LP-CLL
Time to first treatment

Figure 2

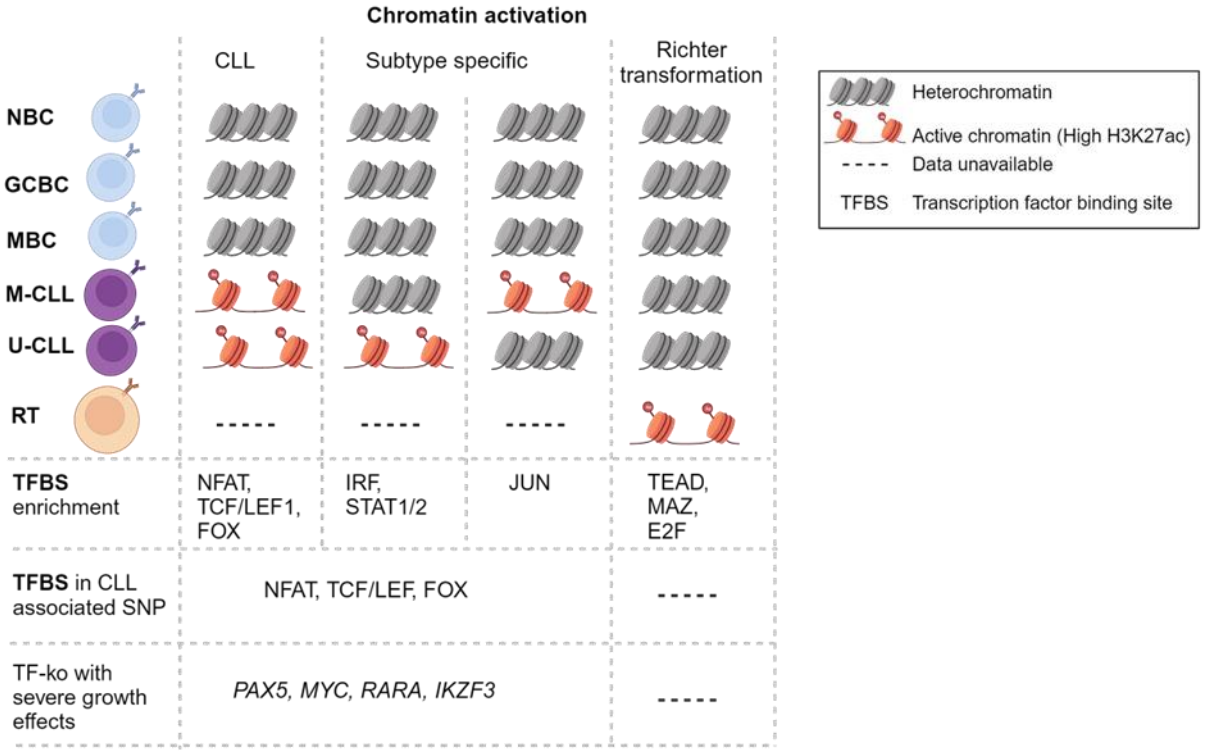
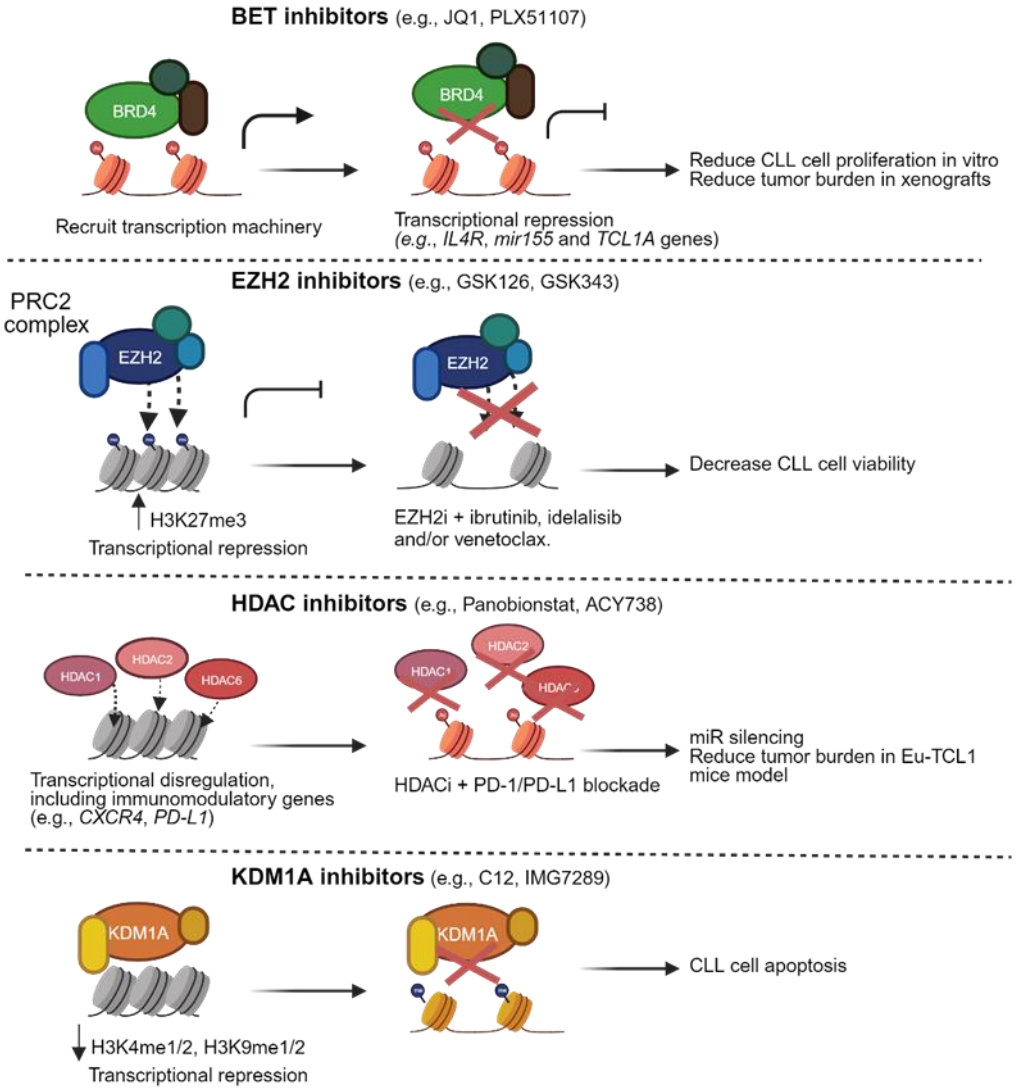

Chromatin activation
CLL
Subtype specific
Richter transformation
NBC
GCBC
MBC
M-CLL
U-CLL
RT
TFBS enrichment
NFAT, TCF/LEF1, FOX
IRF, STAT1/2
JUN
TEAD, MAZ, E2F
TFBS in CLL associated SNP
NFAT, TCF/LEF, FOX
TF-ko with severe growth effects
PAX5, MYC, RARA, IKZF3
Heterochromatin
Active chromatin (High H3K27ac)
Data unavailable
TFBS
Transcription factor binding site

Figure 3


BET inhibitors (e.g., JQ1, PLX51107)
BRD4
BRD4
Reduce CLL cell proliferation in vitro
Reduce tumor burden in xenografts
Recruit transcription machinery
Transcriptional repression
(e.g., IL4R, mir155 and TCL1A genes)
EZH2 inhibitors (e.g., GSK126, GSK343)
PRC2
complex
EZH2
EZH2
Decrease CLL cell viability
H3K27me3
Transcriptional repression
EZH2i + ibrutinib, idelalisib
and/or venetoclax.
HDAC inhibitors (e.g., Panobionstat, ACY738)
HDAC1
HDAC2
HDAC6
HDAC1
HDAC2
miR silencing
Reduce tumor burden in Eu-TCL1
mice model
Transcriptional disregulation,
including immunomodulatory genes
(e.g., CXCR4, PD-L1)
HDACi + PD-1/PD-L1 blockade
KDM1A inhibitors (e.g., C12, IMG7289)
KDM1A
KDM1A
CLL cell apoptosis
H3K4me1/2, H3K9me1/2
Transcriptional repression