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Epigenetics and B-cell Lymphoma

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STRUCTURED ABSTRACT

Purpose of review—It has only recently become apparent that mutations in epigenetic mechanisms and perturbation of epigenomic patterning are frequent events in B-cell lymphomas. The purpose of this review is to highlight these new findings and provide a conceptual framework for understanding how epigenetic modifications might contribute to lymphomagenesis.

Recent findings—Somatic mutations affecting histone methyltransferases such as EZH2 and MLL2, histone demethylases including UTX and JMJD2C and histone acetyltransferases including CBP and p300 are recurrent and common in lymphomas. These mutations result in disruption of chromatin structure and functions of other proteins, ultimately causing aberrant transcriptional programming affecting multiple gene networks. Widespread perturbation of cytosine methylation patterning now appears to be a hallmark of B-cell lymphomas and occurs in specific patterns that can distinguish disease subtypes. Therapeutic targeting strategies can overcome abnormal epigenetic mechanisms and potently kill lymphoma cells.

Summary—Newly discovered epigenetic lesions may provide critical insights into the genesis of B-cell lymphomas but further studies are required to understand how they affect biological mechanism. Epigenetic lesions offer tremendous opportunities for the development of improved biomarkers and treatments.

Keywords

DNA methylation; Chromatin modifications; Epigenetic programming; EZH2; Histone acetyltransferase

INTRODUCTION

Deregulated gene expression is a hallmark of cancer and is well documented in B-cell lymphomas. Expression patterning is reflective of the status of the various gene pathways that define cellular phenotype. For example, in activated B-cell (ABC) type diffuse large B-cell lymphoma (DLBCL) the presence of NFkB signatures indicated constitutive activation and biological dependence on this pathway. Likewise, DLBCLs with BCL6 target gene signatures are dependent on BCL6 for their survival. Gene expression is controlled not only by transcription factors but also by the regulatory state of chromatin. Chromatin modifications including cytosine methylation and histone modifications encode crucial DNA

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sequence independent (i.e. “epigenetic) information. Because chromatin architecture explains in large part how gene expression and cellular phenotypes are controlled, the elucidation of epigenetic mechanism is of great interest. This review highlights and explores how newly discovered mutations in chromatin modifying genes and perturbations in cytosine methylation patterning might contribute to lymphomagenesis.

Disequilibrium of histone methylation marks: a key event in lymphomagenesis (Figure 1)?

EZH2 is a histone methyltransferase component of Polycomb Repression Complex 2 (PRC2) that methylates histone 3 lysine 27(H3K27). EZH2 plays an essential role in programming pluripotency and self-renewal of embryonic stem cells [1]. During early B-cell development EZH2 is required for VDJ recombination [2], is subsequently downregulated in mature B-cells, but is highly expressed again after T-cell dependent activation in germinal center (GC) B-cells [3]. In GC B-cells EZH2 binds to genes involved in differentiation and suppressing cell growth and proliferation such as CDKN1A, CDKN1B and CDKN2A [3]. EZH2 targets displayed H3K27 trimethylation and were repressed. Many B-cell EZH2 targets (i.e.TGFβ pathway genes) are different from those in embryonic cells [3]. By epigenetically silencing these growth suppressive pathways EZH2 might facilitate the emergence of genetic mutations at these stages, which could lead to development of B-cell neoplasms. Diffuse large B-cell lymphomas (DLBCLs) and follicular lymphomas (FL) are derived from cells that have transited the GC reaction. EZH2 is often expressed in DLBCL where it represses its B-cell target genes [3].

Next generation sequencing of an FL patient identified a heterozygous somatic point mutation in *EZH2* exon 15, replacing tyrosine 641 with a histidine residue [4]. Resequencing of 221 additional FL patients yielded a 7.2% incidence of heterozygous $EZH2^{Y641}$ mutations with several amino acids substituting for tyrosine [4]. The incidence of $EZH2^{Y641}$ point mutations was 12% in another cohort of 221 patients [5]. 9.7% of DLBCLs (n=320) had *EZH2* mutations. However, these occurred only in GCB-DLBCLs (21.7%) and primary mediastinal B-cell lymphoma (PMBL, 4.2%) [4]. Presence of *EZH2* mutations did not appear to affect clinical outcome [4]. The Y641 residue is located within the EZH2 SET (catalytic) domain, and hence would be expected to affect enzymatic activity. Although initially thought to confer loss of function [4], more detailed studies indicated that $EZH2^{Y641}$ mutants display a shift in their histone methylation activity. While wild-type EZH2 readily catalyzes H3K27 mono and dimethylation, $EZH2^{Y641}$ is markedly more efficient at trimethylating H3K27 [6][7]. $EZH2^{Y641}$ DLBCL cells displayed greater levels of H3K27me3 and less H3K27me1 than wild-type *EZH2* cells [6][7]. $EZH2^{Y641}$ likely cooperates with and requires the wild-type allele to bring H3K27 to the fully trimethylated state. Although the biological impact of increased H3K27me3 is not known, it is likely significant since EZH2 siRNA induced profound growth arrest in an $EZH2^{Y641}$ DLBCL cell line [3]. EZH2 also has cytoplasmatic functions in cell signaling and actin polymerization [8], which raise the possibility that $EZH2^{Y641}$ might have effects beyond histone modification.

Methylation of H3K4 is associated with gene activation and acts as an opposing force to H3K27 methylation [9]. Although usually mutually exclusive, H3K4me3 and H3K27me3 can coexist in embryonic stem cells as “bivalent” marks primed to be either activated or repressed during subsequent lineage specification [9][10]. H3K4 trimethylation is mediated in part by the MLL/trithorax family of histone methyltransferases [9]. *MLL1* is frequently translocated in acute leukemias, and the resulting fusion proteins aberrantly activate oncogenic target genes such as *HOXA9* and *EVI1* [9][11]. Strikingly, it was recently reported that 89% of FLs (n=35) and 32% of DLBCLs (n=37) display truncation and frameshift mutations of *MLL2* [12]. All of these mutations disrupt the SET domain and so would be expected to result in deficient H3K4 methylation. Mutations in *MLL, MLL2* and

MLL3 were also recently identified in multiple myeloma [13]. Similar *MLL2* mutations occur in Kabuki syndrome, a congenital disorder associated with multiple organ and skeletal malformations and intellectual disability [14]. One study suggested a link between Kabuki syndrome and neuroblastoma [15]. MLL is also involved with the S-phase DNA replication checkpoint controlled by ATR, and loss of MLL resulted in DNA damage independent DNA synthesis [16]. It is interesting to speculate whether MLL2 might have a similar function in B-cells and thus whether *MLL2* mutations would lead lymphoma cells to tolerate genomic instability.

The frequent occurrence of MLL2 loss of function and EZH2 gain of function mutations underline the significance of H3K4me3 and H3K27me3 in aberrant epigenetic programming. Along these lines MLL2 forms a complex with UTX, an H3K27 demethylase that can oppose the actions of EZH2 [17]. In addition to potential reduction in H3K4me3, loss of MLL2 or other MLL proteins might also fail demethylate H3K27. In fact, homozygous or hemizygous inactivating mutations of UTX (which is located on the X chromosome) have been identified in multiple myeloma, and may represent an alternative way to alter H3K27 methylation in lymphoid malignancies [18]. UTX mutations were mutually exclusive with t(4;14) translocations, which overexpress another histone methyltransferase called MMSET. In contrast to *EZH2* and *UTX* mutation, MMSET overexpression is associated with reduction in cellular H3K27 methylation [19], as well as with increased H3K36 and H4K20 methylation [19][20]. These considerations underline the fact that histone modifying enzymes truly function in an integrated manner, with disruption of one enzyme affecting the actions of many others [17]. Notably, ~12% of patients with myelodysplastic syndrome or myeloproliferative neoplasms feature mono or bi-allelic *EZH2* loss of function mutations accompanied by a reduction in cellular H3K27me3 [21]. Therefore EZH2 and MLL family proteins may have opposing roles as tumor suppressors and oncogenes in myeloid development and mature B-cells.

Another example of epigenetic cooperativity involves the H3K9 demethylase JMJD2C, which is localized near the *JAK2* locus on a region of chromosome 9p24 amplified in PMBL and Hodgkin lymphoma (HL). JMJD2C demethylation of H3K9 and JAK2 phosphorylation of H3Y41 both inhibit binding of heterochromatin silencing protein HP1α [22]. Inhibition of JAK2 plus JMJD2C cooperatively increased H3K9 methylation and HP1α heterochromatin foci in PMBL cells [22]. One of the loci silenced in this manner was *MYC*, which is an important downstream mediator of JAK2 and JMJD2C [22]. Blockade or silencing of JAK2 and JMJD2C also resulted in enhanced death of PMBL and HL cell lines [22]. Collectively these instances offer some of the first proof that disruption of the proposed combinatorial “histone code” [17] is deleterious for the homeostasis of transcriptional regulation in B-cells and facilitates lymphomagenesis.

Histone acetyltransferases as tumor suppressors in B-cells (Figure 2)

Histone lysine residues are acetylated by histone acetyltransferases (HATs). Histone acetylation induces a more “open” chromatin structure and recruits bromodomain protein “readers” to facilitate transcriptional activation. At least 1750 proteins in addition to histones are modified by lysine acetylation in leukemia cells [17], indicating that HATs have broad cellular activities. Several recent studies implicate the HAT proteins CBP and p300 as tumor suppressors in B-cell neoplasms. CBP and p300 function as co-activators of transcription factors and acetylate proteins relevant to lymphomagenesis such as p53, NFkB, BCL6 and Hsp90 [24][25][26][27]In lymphoma, heterozygous p300 truncation mutants deleting the lysine acetyltransferase (KAT) domain were first revealed in the lymphoma cell line RCK8[28]. Truncated p300 disrupted the function of c-Rel, which normally uses p300 as a co-activator[29]. P300 is a direct target gene of the transcriptional repressor BCL6, a key lymphoma oncoprotein and BCL6 and p300 levels were inversely correlated in primary

DLBCLs [26]. BCL6 inhibitors induce p300 protein expression and acetyltransferase activity in DLBCL cells, with subsequent acetylation of p53 (which induces p53 transcriptional functions) and Hsp90 (which suppresses Hsp90 chaperone activity) [26]. Blockade of p300 activity in DLBCL cells rescued them from being killed by BCL6 inhibitors [26] and lymphoma cells with mutant p300 were resistant to BCL6 inhibitors. Induction of BAT3, a critical cofactor of p300 required to acetylate p53, was also required for BCL6 inhibitors to kill DLBCL cells [26]. Combining BCL6 and HDAC inhibitors led to even higher p300 activity and synergistic killing of lymphoma cells *in vitro* and *in vivo* [26]. Suppression of p300 either through BCL6 or inactivating mutations thus plays a key role in DLBCL.

Resequencing studies in patients with B-cell neoplasms provided definitive evidence that CBP and p300 are *bona fide* tumor suppressors [26][30][31]. Sequencing and copy number analysis of the *CREBBP* locus that encodes CBP in 134 DLBCLs identified truncation or HAT domain mutations, and monoallelic deletions in 29% of patients [30]. Like *EZH2* mutations, these more frequently occurred in GCB DLBCLs (41.5%) vs. ABC DLBCLs (12%) [30]. HAT domain inactivating mutations were also present in 15/46 FL cases (32.6 %) [30]. 18.3% of relapsed pediatric B-acute lymphoblastic leukemia cases harbored *CREBBP* mutations, and only half of these were present at diagnosis [31]. Only 1% of non-relapsing patients displayed *CREBBP* mutations at diagnosis [31], suggesting that these mutations contribute to chemotherapy resistance. Somatic mutations truncating or disrupting the HAT domain of p300 were identified in 9.7% of a cohort of 97 DLBCL patients[26**], in 10% of the above cohort of 134 DLBCL cases [30], and in 8.7% of the 46 FL cases [30]. *CREBBP* mutants affecting the HAT domain were deficient in binding acetylCoA, in acetylating p53, BCL6 and H3K18, and in reconstituting cyclic AMP signaling in *Crebbp/ Ep300* knockout MEFs [30][31].

Similar to the case of *EZH2* and *MLL2*, mutations of *CREBBP and EP300* are usually heterozygous [26][30][31], suggesting that mutant protein could function as dominant negative or that the reduction in active protein level is sufficient to disrupt biological functions. Along these lines transduction of two different HAT domain deleted p300 mutants in DLBCL cell lines rescued cells from BCL6 inhibitors, consistent with a dominant negative effect [26]. Conversely transfecting wild type p300 into RCK8 cells, which express truncated p300, partially sensitized them to the actions of BCL6 inhibitor. Therefore, the stoichiometry between wild type and mutant alleles may be important in determining the biological impact of mutant alleles. Importantly, CBP and p300 are not identical in their biological functions and so it is not clear whether their loss of function in DLBCL is completely bio-equivalent. While *Cebbp*+/− mice display hematopoietic defects and eventually develop hematologic tumors, *Ep300*+/− do not reproduce either phenotype [32]. CBP and p300 also mediate different effects on the biological output of WNT signaling and other pathways (reviewed in [33]). Careful study will thus be required to parse out the various mechanisms through which these mutant alleles contribute to lymphomagenesis.

Aberrant DNA methylation patterning in B-cell lymphomas

DNA methylation patterning contains much of the epigenetic information that determines the phenotype of normal and malignant cells [34]. Along these lines the methylome of multipotent and lineage-committed progenitors revealed numerous differentially methylated regions and a greater burden of methylation associated with lymphoid commitment [35]. Hypermethylation of gene regulatory regions is generally associated with silencing and hypomethylation with gene expression [34], although In reality DNA methylation patterning is more complex. For example, hypermethylation of a CpG-rich region within the first intron of *BCL6* was reported to maintain high levels of *BCL6* expression, at least in part by blocking binding of a negative regulator of this locus (CTCF) [36]. BCL6 levels decreased

in lymphoma cell lines exposed to the DNA methyltransferase inhibitor decitabine [36]. Aberrant hypomethylation of intergenic regions can lead to genomic instability and contribute to malignant transformation [34].

DNA methylation profiling studies indicate that cytosine methylation distribution is perturbed in lymphomas vs. normal B-cells, and that promoter methylation is generally inversely correlated with gene expression [3][37][38][39][40][41][42][43]. Several groups observed that certain aberrantly methylated genes in lymphomas are known targets of the PRC2 polycomb complex (which includes EZH2) in embryonic stem cells [3][39][40][41][42]. However, cytosine methylation and EZH2/H3K27me3 binding are almost entirely mutually exclusive in normal GC B-cells [3]. Another report found little overlap of SUZ12 (a PRC2 component) and H3K27me3 with differentially methylated regions [41]. In contrast, examination of methylation profiles of DLBCL patients revealed that EZH2 B-cell target genes frequently become hypermethylated [3] implying breakdown in lymphomas of the epigenetic barrier that separates PRC2 and cytosine methylation in normal B-cells. It is not known whether these events are linked to mutations in *EZH2* and *MLL2*, but is intriguing to consider that perturbations in chromatin structure could lead to mislocalization of cytosine methylation and consequent aberrant epigenetic silencing. It has also been speculated that methylation of PRC2 genes in lymphoma could represent oncogenic events occurring during early lymphoid development [39].

DNA methylation profiling can also used to classify lymphoma subtypes. Integrated DNA methylation and gene expression profiling of 69 DLBCL cases identified 239 genes differentially methylated in ABC vs. GCB DLBCLs indicating that these are epigenetically distinct entities [37]. Differentially methylated genes in ABC and GCB DLBCLs were centered on a TNFa/cytokine network implicating differential methylation of these genes as distinguishing ABC and GCB lymphoma biology [37]. The sixteen most differentially methylated and expressed genes between ABC and GCB predicted DLBCL subtype in an independent cohort of 203 DLBCL cases with 92% accuracy [37]. In a different study, comparison of DNA methylation profiles distinguished mediastinal gray zone lymphoma from the related PMBL and Hodgkin lymphomas and revealed small sets of differentially methylated genes associated with each subtype [43]. Although requiring further validation, diagnosis of morphologically difficult to distinguish lymphomas may thus be enhanced using DNA methylation biomarkers.

Cytosine methylation profiles of 22 primary mantle cell lymphomas (MCL) were compared to 10 sets of purified tonsilar naïve B-cells. Remarkably, MCLs displayed twice as many aberrantly hypomethylated as hypermethylated loci, suggesting that aberrant loss of methylation is a dominant feature of this disease [38]. The gene networks most heavily affecting by aberrant DNA methylation revolved around NFkB and HDAC1 [38]. Gene expression was inversely correlated with methylation at over 1400 loci [38]. *CD37*, an aberrantly hypomethylated and overexpressed gene in MCL, was expressed on the surface of MCL cells. CD37-SMIP therapeutic antibodies killed MCL cells suggesting targeting of a hypomethylated gene as a therapeutic approach for MCL [38]. Conversely, several tumor suppressors including *CDKN2B*, *HOXD8*, *MLF1* and *PCDH8* were hypermethylated and silenced in MCL. Treatment with decitabine and the HDAC inhibitor SAHA reactivated these genes and potently killed MCL cells [38].

CpG dinucleotides are methylated by DNMT1, DNMT3A and DNMT3B. DNMT1 is predominantly involved in maintaining, while DNMT3A and 3B mediate *de novo* cytosine methylation[44]. DNMT1 also plays a critical role in replication, repair of double strand break and stem cell self renewal [45][46]. Analysis of DNMTs by immunohistochemistry in 81 DLBCL cases identified expression of DNMT1, 3A and 3B in 48%, 13% and 45% of

DLBCLs respectively [47]. Of these DNMT3B expression was independently associated with worse overall and progression free survival [47]. Interestingly, tumor cells often produce an aberrantly spliced isoform (*DNMT3B7*) lacking the C-terminal catalytic domain [48]. Transgenic mice expressing Dnmt3b7 in an Eμ-Myc background develop mediastinal B-cell lymphomas with markedly increased proliferative rates, increased genomic instability and altered methylation patterning [48]. The data suggest possible dominant negative and gain of function features of DNMT3B7 warranting exploration of this isoform in lymphoma patients.

CONCLUSION

Mutations affecting epigenetic and transcriptional modifiers now appear to be an almost universal feature of B-cell lymphomas. Large-scale disruptions of DNA methylation and histone modification patterning are emerging as a hallmark of these diseases. The challenge for the next years will be to understand the nature of the lymphoma epigenome and the biochemical and biological effects of mutant epigenetic factors. It will also be necessary to ascertain the utility of DNA methylation classifiers as biomarkers for diagnostic accuracy and therapeutic stratification. Because epigenetic marks are potentially reversible, the development of genuine epigenetic-targeted therapy drugs holds great promise. Already agents are emerging that target transcription factors, chromatin modifying enzymes and epigenetic reader proteins (e.g. [49][50]). In the long-term such agents might significantly advance the treatment of B-cell lymphomas with less toxicity to the normal immune system and other tissues.

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ABBREVIATIONS

DLBCL	Diffuse Large B-cell Lymphoma
PMBL	Primary Mediastinal B-cell Lymphoma
FL	Follicular Lymphoma
MCL	Mantle Cell Lymphoma
BCL6	B Cell Lymphoma 8
EZH2	Enhancer of Zeste 2
MLL	Mixed Lineage Leukemia
CREBBP	CREB Binding Protein
BAT3	HLA-B Associated Transcript 3 / BCL2-associated athanogene 6
MMSET	Multiple Myeloma SET domain protein / Wolf-Hirschhorn syndrome candidate 1
UTX	Ubiquitously-transcribed X chromosome tetratricopeptide repeat
WNT	Wingless-type MMTV integration site
TGFβ	Transforming Growth Factor Beta

SET domain	Suppressor of variegation 3-9, Enhancer of zeste, Trithorax- chromatin regulator
KAT domain	Lysine Acetyl Transferase
HAT	Histone Acetyl Transferase
JMJD2C	Jumonji Domain Containing 2C
JAK2	Janus Kinase 2
CTCF	CCCTC-binding factor

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KEY POINTS

- Gain of function mutations of EZH2 and loss of function mutations of MLL family members suggest that abundance and localization of histone methylation plays a fundamental role in lymphomagenesis.
- Frequent genetic lesions disrupting the histone acetyltransferases CBP and p300 implicate protein acetylation as a critical barrier preventing malignant transformation of B-cells.
- Aberrant DNA methylation patterning is emerging as a hallmark of B-cell lymphomas, and may cooperate with altered polycomb complexes to deregulate gene expression.
- Epigenetic lesions are promising biomarkers to improve diagnosis and predict outcomes in B-cell lymphomas.

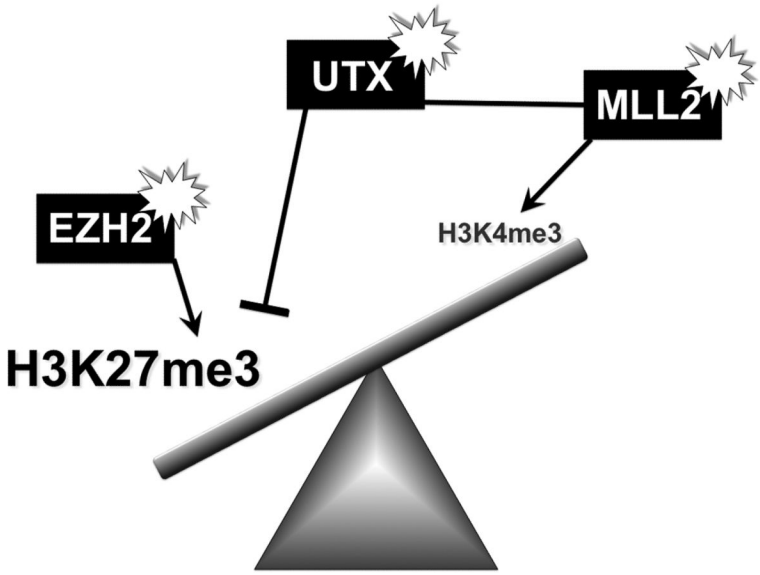

Figure 1. Possible disequilibrium of H3K27 and H3K4 trimethylation in B-cell lymphomas

EZH2 is a histone methyltransferase component of Polycomb Repressive Complex 2. Somatic heterozygous point mutations altering the catalytic activity of the catalytic SET domain of EZH2 occur in ~7-12% of follicular lymphomas, ~21% of GCB-DLBCLs and ~4% of PMBLs. Mutant EZH2 induces accumulation of the repressive H3K27 trimethylation chromatin mark in DLBCL cells. The MLL-trithorax histone methyltransferases oppose the actions of PRC2 and EZH2, and induce H3K4 trimethylation, an activating chromatin mark. Somatic heterozygous inactivating mutations of MLL2 occur in up to 89% of FL and 32% of DLBCL. MLL2 forms a complex with the H3K27 demethylase UTX, which is inactivated through mutations in multiple myeloma. These mutations would be expected to result in an increase in H3K27 trimethylation and reduction of H3K4 trimethylation. Presumably the consequence of this disequilibrium is oncogenic disruption of epigenetic programming facilitating lymphomagenesis. However these are all multifunctional proteins and may have numerous other downstream effects.

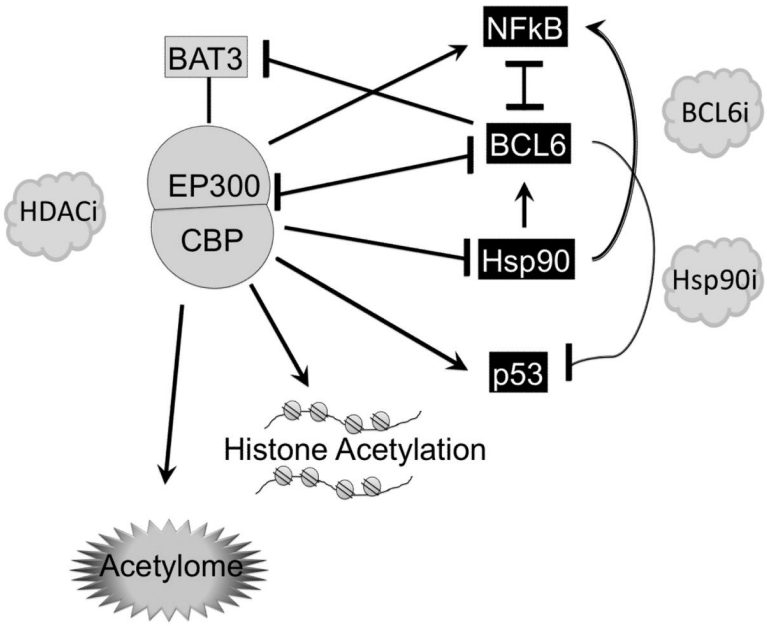

Figure 2. Disruption of CBP and p300 acetyltransferases in B-cell lymphomas

CBP and p300 have both overlapping and unique functions. p300 acts as a co-activator for NFkB, and activates p53 and on the other hand can attenuate Hsp90 chaperone functions and BCL6 transcriptional repressor functions. P300 and CBP also acetylate histones and potentially hundreds of other proteins in the acetylome. Deacetylated Hsp90 maintains BCL6 expression, which in turn suppresses p300 and its essential cofactor BAT3 and also represses p53. This vicious circle can be disrupted by treating lymphoma cells with BCL6 inhibitors, Hsp90 inhibitors or HDAC inhibitors, and these agents are synergistic when administered in combination. Blockade of p300 rescues DLBCL cells from being killed by BCL6 inhibitors and HDAC inhibitors. CBP also acetylates p53 and BCL6. Somatic heterozygous mutations or deletions of the *CREBBP* locus occur in 41.5% of GCB-DLBCLs, 12% of ABC-DLBCLs, and 32.6% of FL cases. EP300 mutations occur in 10% of DLBCLs. All cases seem to have in common disruption of the histone acetyltransferase catalytic domain, and the resulting truncated or mutant proteins may have dominant negative or gain of function properties, or may simply result in a reduced dosage of histone acetyltransferases. These effects would presumably lock DLBCL cells in a state with inactivated p53, and activated BCL6 and Hsp90, in addition to many other possible downstream effects.