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Evidence for an oncogenic modifier role for mutant histone acetyltransferases in diffuse large B-cell lymphoma

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Abstract

Mutations in histone acetyltransferases (HATs) are among the most common mutations in diffuse large B-cell lymphoma (DLBCL). We previously showed that two human DLBCL cell lines, RC-K8 and SUDHL2, express C-terminally truncated, HAT domain-deficient p300 proteins (p300 C) that are required for optimal cell proliferation. Microarray analysis of mRNA expression in RC-K8 cells following p300 C knockdown shows upregulation of NF- κ B and p53 gene expression programs and down-regulation of a MYC gene expression program. Experiments indicate that these gene expression changes are due to inhibitory effects of p300 C on NF- κ B activity and on p53 protein levels and stimulatory effects on MYC protein levels, suggesting that p300 C mutants enhance the proliferation of DLBCL cells by adjusting the transcriptional output of cell-specific oncoproteins. We propose that p300/CBP gene truncation represents a new class of oncogenic mutation that optimizes the activity of context-specific oncogenic transcription factors. We propose “oncogenic modifier” to describe such mutations.

Keywords

Histone acetyltransferase; mutation; diffuse large B-cell lymphoma; p300; MYC; NF- κ B

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkins lymphoma [1]. Much research has investigated mutations and altered signaling pathways that contribute to DLBCL cell proliferation and survival, and this has led to targeted therapeutics and diagnoses [2, 3]. Mutations in the related histone acetyltransferases (HATs) p300 and CBP are among the most common mutations in DLBCL [2, 4–9].

Most p300/CBP mutations in DLBCL are point mutations within or near the HAT domain [9], and several such point mutations compromise CBP's ability to acetylate transcription factors BCL6 and p53 [4]. In a minority of DLBCL tumors and cell lines, frame-shifts, nonsense mutations or genomic alterations result in removal of the p300/CBP HAT domain

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Potential conflict of interest

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[4, 10]. Expression of truncated, HAT domain-deficient p300/CBP proteins is maintained in several DLBCL cell lines [4, 11, 12].

Although p300 and CBP are frequently mutated in DLBCL [2, 4-9], the effects of p300/CBP mutations on global gene expression patterns have not been extensively characterized. We previously characterized two human DLBCL cell lines—RC-K8 and SUDHL2—that express C-terminally truncated, HAT-deficient p300 proteins (p300 C-1087 and p300 C-820, respectively), but no wild-type p300 [4, 11, 12]. These p300 mutants lack acetyltransferase activity, but nevertheless, localize to discrete nuclear subdomains, interact with transcription factor REL, and act as weak co-activators in reporter gene assays [12, 13]. Moreover, knockdown of p300 C proteins reduces the growth of RC-K8 and SUDHL2 cells in liquid culture and soft agar [12, 13], indicating that p300 C proteins have pro-proliferative and oncogenic activity. Therefore, we hypothesized that knockdown of p300 C expression would impair specific oncogenic pathways that contribute to the growth of these cells. In this report, we have characterized the effect of knockdown of p300 C protein on overall gene expression in RC-K8 and SUDHL2 cells. Our results suggest that p300 C proteins provide oncogenic modifier activities that fine-tune specific gene expression pathways in DLBCL for optimal tumor cell proliferation.

MATERIALS AND METHODS

Plasmids

DNA manipulations were performed by standard methods [14]. Details of subclones and primers are in Supplementary Information.

Cell Culture

293T and RC-K8 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Biologos, Montgomery, IL) [15]. SUDHL2 cells were cultured in RPMI supplemented with 10% FBS. Transfection of 293T cells was performed using polyethylenimine [13]. The design of control and *EP300* short hairpin RNAs (shRNA), generation of retroviral stocks carrying these shRNAs, and infections were performed as described [13]. Virally transduced RC-K8 and SUDHL2 cells were selected with 1 µg/ml puromycin [12].

Western Blotting and Indirect Immunofluorescence

Whole-cell lysates were prepared in AT buffer and analyzed by Western blotting as described [11, 16], using primary antisera described in Supplementary Information. A modified transfer buffer was used for transfer of high molecular weight proteins (full-length p300) [11]. Bands on Western blots were quantified using ImageJ [17].

Indirect immunofluorescence was performed as described [12, 13, 15] using antisera listed in Supplementary Information. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were visualized using a FLUOVIEW Laser Scanner Microscope (Olympus, Center Valley, PA).

Electrophoretic Mobility Shift Assay (EMSA)

Whole-cell lysates were prepared and analyzed by EMSA as described [18]. Equalized lysates were then incubated with radiolabeled κ B-site [18] or LSF-site (provided by Ulla Hansen) [19] probes.

RNA Isolation, cDNA Generation, and Real-time qPCR

To stably knockdown p300 C, approximately 10^6 RC-K8 or SUHDL2 cells were virally transduced with control or p300 shRNA. After approximately four weeks of selection with puromycin, total cellular RNA was isolated from approximately 10^8 cells using TRIzol Reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocol, and cDNA was generated as described [12, 18]. PCRs were performed and Ct values were analyzed as described [12]. Fold change in mRNA was normalized to *GAPDH* mRNA expression (1.0) in the given cell type. Primers for qPCR are described in Supplementary Information.

For qPCR analysis, validation of NF- κ B or MYC target gene expression was performed with six technical replicates of the same mRNA used for microarray studies. p-values were calculated with a two-sample, equal variance, two-tailed Students t-test, and significance was attributed to p-values < 0.05.

Microarray Analysis

As described above, p300 C-1087 was stably knocked down in RC-K8 cells by expression of retrovirally transduced shRNA, mRNA was then isolated using TRIzol, and purified using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Microarray analyses of RNA samples were then performed by the Boston University Microarray Core Facility. Gene expression changes were analyzed for fold change, by Ingenuity Pathway Analysis, and by Gene Set Enrichment Analysis as described in Supplementary Information.

RESULTS

Half-lives of p300 C Proteins in RC-K8 and SUDHL2 DLBCL Cells Are Similar to Wild-type p300

Consistent with previous reports [11, 12], the RC-K8 and SUDHL2 DLBCL cell lines each express a truncated p300 protein, but no full-length p300 [Figure 1(A)]. In contrast, the BJAB DLBCL cell line expresses only full-length p300.

To measure the turnover rate of p300 C proteins, RC-K8 and SUDHL2 cells were treated with cycloheximide for times up to 8 h, and then analyzed by anti-p300 Western blotting [Figure 1(B)]. Densitometric analysis of Western blots showed that the half-lives of p300 C-1087 (RC-K8) and p300 C-820 (SUDHL2) are approximately 4.5 h, which is similar to the half-life of wild-type p300 in BJAB cells [Figure 1(C)] and in cardiomyocytes [20]. Thus, C-terminal truncations of p300 in RC-K8 and SUDHL2 cells do not appreciably alter p300's stability, and the dynamics of activities common to p300 C and wild-type p300 would not be expected to be substantially different.

Knockdown of p300 C-1087 Changes Gene Expression Patterns in RC-K8 Cells

To determine the effects of p300 C-1087 knockdown on comprehensive gene expression, we performed cDNA microarray and qPCR analyses on mRNA from RC-K8 cells with p300 C-1087 knocked down versus control RC-K8 cells. Pools of RC-K8 cells stably transduced with a retroviral vector directing expression of either p300 shRNA or a control shRNA were generated. p300 C-1087 knockdown in two independent pairs of retroviral transductions was confirmed by Western blotting [Figure 2(A)]. In both experiments, expression of p300 C-1087 was reduced by approximately 90%, whereas there was no effect on levels of control protein β -tubulin [Figure 2(A)].

As a control for the effect of p300 C-1087 knockdown on gene expression, we used qPCR to confirm that mRNA levels of REL/NF- κ B target genes *A20*, *CCR7*, *IKBa*, and *TRAF1* increased in both samples isolated from RC-K8 cells in which p300 C-1087 was knocked down [Supplementary Figure 1]. This result is consistent with our previous study [12].

Global gene expression analysis using an Affymetrix Human Gene array (~24,000 probes) was performed with mRNA samples from RC-K8 cells. The change in mRNA expression for each gene was obtained by comparing expression values in p300 C-1087 knockdown cells ($n = 2$) to control shRNA cells ($n = 2$). Following p300 C-1087 knockdown, 560 genes showed statistically significant changes in expression of >1.5-fold at a q threshold of 0.05 and a 5% FDR [Supplementary Table I]. Of these 560 genes [Supplementary Table II], 210 showed increased expression and showed 350 decreased expression. As a control, we verified that p300 mRNA expression detected by probes against sequences encoding the N-terminal region of p300 (which are retained in p300 C-1087) was reduced in RC-K8 cells expressing p300 shRNA [Supplementary Figure 2]. The differential expression of the top 100 significantly altered genes upon p300 C-1087 knockdown are presented as a heat map [Supplementary Figure 3].

Knockdown of p300 C-1087 in RC-K8 Cells Differentially Affects NF- κ B- and MYC-dependent Gene Networks

Ingenuity Pathway Analysis (IPA) was used to identify upstream regulatory networks associated with the 560 genes whose expression changed following p300 C-1087 knockdown. Fifteen predicted upstream regulators passed our filtering criteria (bias-corrected z -score of ≥ 2.0 or ≤ -2.0 , and p -value of overlap < 0.01) [Table I]. These 15 regulators included ten upstream regulators with positive z -scores (i.e., regulators activated by p300 C-1087 knockdown) and five upstream regulators with negative z -scores (i.e., regulators inhibited by p300 C-1087 knockdown). Some of the 15 upstream regulators are either regulators of specific transcription factors (TNF, IL1 β) or transcription factors (NF- κ B, TP53, NUPR1, MYC) that are known to interact with p300 [9].

Because inhibition of NF- κ B reduces the proliferation of RC-K8 cells [21], we were interested to find that NF- κ B was an upstream regulator with a positive z -score following p300 C-1087 knockdown. Specifically, IPA upstream regulator analysis indicated that NF- κ B signaling increases upon knockdown of p300 C-1087 (NF- κ B, z -score +3.4 [Table II]),

which is consistent with our previous finding that p300 C-1087 knockdown increases expression of select NF- κ B target genes [Supplementary Figure 1, and [12]].

IPA also identified four significantly activated pathways (positive z-scores) where the apex molecule is either an NF- κ B target gene and/or an activator of NF- κ B. These activated NF- κ B-related factors are TNF [22], IL-1 β [23], TP53 [24, 25], and NUPR1 [26] (Table I). Thus, the activated output of p300 C-1087 knockdown in RC-K8 cells indicates significant activation and implementation of REL/NF- κ B signaling. At least two of the activated pathways—TP53 and TNF—can be inhibitors of cell growth [13].

Overall, 26 genes with statistically significant ($q < 0.05$) changes in gene expression (19 increased; 7 decreased) contribute to the identification of NF- κ B as an upstream regulator of a network of gene expression changes following p300 C-1087 knockdown in RC-K8 cells [Table II]. Of these 26 NF- κ B pathway genes, 16 were consistent with a program of NF- κ B activation following p300 C-1087 knockdown [Table II, genes 1-5, 7-17]. In Table II, the expression of a given gene is considered “activated” or “inhibited” as predicted by an increase or decrease, respectively, in associated NF- κ B activity.

To validate activation of NF- κ B target gene expression in RC-K8 cells upon p300 C-1087 knockdown, qPCR was performed on 11 NF- κ B pathway genes showing statistically significant ($q < 0.05$) increases of 1.8-fold following knockdown of p300 C-1087 (Table II). qPCR analysis confirmed that expression of all 11 genes was significantly upregulated ($p < 0.05$) by knockdown of p300 C-1087 [Figure 3(A)].

IPA also showed that MYC signaling was significantly inhibited (z-score = -3.929 , overlap p-value = $6.90E-05$) by p300 C-1087 knockdown [Table I], i.e., MYC target genes showed an expression pattern consistent with inhibition of MYC activity following p300 C-1087 knockdown. Furthermore, the MYC coactivator BRD4 [27] is among the upstream regulators inhibited (z-score = -2.018 , overlap p-value = $6.26E-04$) by knockdown of p300 C-1087. IPA identified 41 MYC targets with statistically significant ($q < 0.05$) expression changes following p300 C-1087 knockdown [Table III]. Approximately two-thirds (28) of the 41 differentially regulated MYC target genes change were consistent with a program of MYC inhibition following p300 C-1087 knockdown (i.e., two-thirds of the genes were either MYC-activated genes that showed downregulation, or MYC-inhibited genes that showed upregulation upon p300 C-1087 knockdown) [Table III]. Thus, IPA predicts that p300 C-1087 normally increases MYC transcriptional output in RC-K8 cells.

MYC is both a repressor and an activator of transcription, whereas NF- κ B is generally a transcriptional activator. Among 28 MYC-dependent genes whose expression change was consistent with an “inhibited” MYC profile following p300 C-1087 knockdown, there were 16 genes whose mRNA levels decreased [Table III, genes 1-16] and 12 genes whose mRNA levels increased [Table III, genes 17-28]. In contrast, all 16 of the NF- κ B-dependent genes with expression changes consistent with an “activated” NF- κ B program following p300 C-1087 knockdown showed increased expression [Table II]. Two genes (*ODC1* and *CD274*) are found on both the NF- κ B and MYC target gene lists [Tables II, III]. Given the direction of their changes (*ODC1* down-regulated by 3.2-fold; *CD274* up-regulated by 2.0-

fold), one might predict that in RC-K8 cells *ODC1* is normally activated by MYC, whereas *CD274* is primarily activated by NF- κ B.

To validate the prediction that p300 C-1087 knockdown is associated with decreased MYC target gene expression in RC-K8 cells, we used qPCR to measure expression ten MYC-dependent genes that, based on the microarray analysis, showed decreased expression of 1.8-fold following p300 C-1087 knockdown [Table III]. Results showed that mRNA levels of 9 of these 10 genes were significantly ($p < 0.05$) decreased in RC-K8 cells following p300 C-1087 knockdown [Figure 3(B)]. These results suggest that p300 C-1087 expression normally enhances MYC activity in RC-K8 cells.

To confirm that NF- κ B and MYC pathways were affected by knockdown of p300 C-1087 in RC-K8 cells, we used GSEA to determine whether there was a correlation between the RC-K8 gene expression profiles and specific gene sets from the Molecular Signatures Database (MSigDB) [28, 29]. GSEA showed that all ten MSigDB gene sets defined by either NF- κ B or MYC binding motifs were significantly enriched at the top or bottom, respectively, of the ranked gene expression list [Table IV, Supplementary Figure 5], supporting the hypothesis that the transcriptional activities of NF- κ B and MYC are reduced and enhanced, respectively, by expression of p300 C-1087 in RC-K8 cells.

Knockdown of p300 C-1087 Enhances NF- κ B Activity in RC-K8 and SUDHL2 Cells

To investigate the mechanism by which p300 C-1087 might dampen NF- κ B target gene expression, we first determined whether there was an effect of p300 C knockdown on expression of the NF- κ B transcription factors REL and RELA/p65 in RC-K8 or SUDHL2 cells. No change in the levels of REL or RELA/p65 was seen in either cell line following knockdown of p300 C [Figure 3(C)]. The levels of control protein β -tubulin were also not affected [Figure 3(C)]. Because NF- κ B DNA-binding activity is generally regulated by post-translational mechanisms (e.g., interaction with an I κ B inhibitor or nuclear translocation), we determined whether the DNA-binding activity of NF- κ B was affected by knockdown of p300 C. Knockdown of p300 C led to increased κ B site DNA-binding activity in both RC-K8 and SUDHL2 cells [Figure 3(D)]. In contrast, the DNA-binding activity of transcription factor LSF was not noticeably affected by p300 C knockdown [Figure 3(D), bottom panel]. These results suggest that NF- κ B DNA-binding activity and target gene expression are specifically reduced as a consequence of retained p300 C expression in RC-K8 and SUDHL2 cell lines.

The wild-type REL transcription factor is primarily cytoplasmic when overexpressed in malignantly transformed chicken spleen cells [30]. Moreover, the majority of REL is present in cytosolic fractions of RC-K8 cells, despite the constitutively high nuclear REL DNA-binding activity in these cells [31]. To determine whether p300 C proteins can induce nuclear localization of REL and thereby increase REL DNA-binding activity, we infected 293T cells with retroviral vectors for expression of p300 C-1087 or p300 C-820 alone or in combination with REL [Supplementary Figure 4(A)]. Each vector directed expression of appropriately sized proteins in 293T cells [Supplementary Figure 4(B)]. By immunofluorescence, the p300 C proteins were localized to discrete nuclear “speckles” in 293T cells [Supplementary Figure 4(C)], whereas REL appeared entirely cytoplasmic in the

presence or absence of p300 C expression [Supplementary Figure 4(C)]. Thus, in 293T cells the p300 C proteins do not appear to enhance REL-dependent nuclear localization, suggesting that they do not increase REL/NF- κ B DNA-binding activity in RC-K8 cells by inducing nuclear translocation.

Knockdown of p300 C-1087 Reduces MYC Expression in RC-K8 and SUDHL2 Cells

Because MYC DNA-binding activity is primarily regulated at the level of protein expression [32], we compared the levels of MYC protein in control and p300 C knockdown cells for both RC-K8 cells and SUDHL2 cells. The levels of MYC were reduced by ~50-60% in both cell lines following p300 C knockdown [Figure 3(C)]. Of note, the levels of MYC were decreased to approximately the same extent in both cell types even though the basal levels of MYC are different in the two cell types.

Knockdown of p300 C-1087 Increases p53 Expression in RC-K8 Cells

IPA predicted that the p53 (TP53) tumor suppressor pathway is upregulated by p300 C-1087 knockdown (z-score = +3.147). To investigate how p300 C-1087 knockdown might enhance a p53-dependent gene expression network, we compared the levels of p53 protein in control and p300 C knockdown RC-K8 and SUDHL2 cells. Following knockdown of p300 C, the levels of p53 protein increased by ~2.5-fold in RC-K8 cells, but the levels of p53 did not substantially change in SUDHL2 cells [Figure 3(C)]. Of note, RC-K8 cells are p53 wild type (Leandro Cerchietti, personal communication), whereas the p53 mutation status in SUDHL2 cells is not known. In addition, basal expression of p53 in SUDHL2 cells is approximately 20-fold higher than in RC-K8 cells [see legend, Figure 3(C)].

DISCUSSION

In this report, we have investigated the role of mutant p300 proteins in regulating global gene expression in human DLBCL cells. Knockdown of p300 C-1087 activated an NF- κ B gene program and inhibited a MYC gene program in RC-K8 DLBCL cells. These effects on NF- κ B- and MYC-dependent gene expression programs appear to be due to effects on the transcription factors themselves, because knockdown of p300 C in both RC-K8 and SUDHL2 DLBCL cells was associated with increased NF- κ B DNA-binding activity and decreased MYC protein levels. These results suggest that p300 C proteins modify NF- κ B- and MYC-dependent gene expression programs that are required for the optimal proliferation of some DLBCL cells.

While mutations in p300/CBP occur in approximately 30% of DLBCL patients and cell lines, only a small subset of these mutations leads to expression of truncated p300 proteins. Three DLBCL cell lines (RC-K8, SUDHL2, OCI-LY10) have been reported to have p300-truncating mutations [11-13, 33]. These three cell lines are in the activated B-cell (ABC)-like molecular subtype of DLBCL, which is characterized by high NF- κ B activity that is required for cell proliferation [21, 31, 34-36]. Given that both reduced NF- κ B activity (effected by overexpression of I κ B α proteins) and exaggerated NF- κ B activity (by knockdown of p300 C-1087) slow the proliferation of RC-K8 and SUDHL2 cells [12, 13,

21], we think it likely that these cells were selected for increased but moderated levels of NF- κ B activity, which maintains an optimal oncogenic gene expression program. Consistent with this hypothesis, RC-K8 cells have high nuclear REL DNA-binding activity due to two mutations (I κ B α and A20) that increase REL nuclear localization, but also have two mutations (REL-NRG and p300 C-1087) that reduce REL-directed transactivation [11, 13, 21, 31, 35]. Moreover, in both RC-K8 and SUDHL2 cells, alteration of this balanced REL/NF- κ B activity by re-expression of wild-type A20 induces apoptosis [35] or by re-expression of I κ B α [21] or knockdown of p300 C-1087 [12, 13] slows cell growth. On a related note, high-level expression of REL/NF- κ B has been shown to be toxic in several cases [37, 38], and several reports have shown that mutations that reduce the high transactivation activity of REL can enhance its transforming activity [30, 39-42]. Thus, at least in part, the decreased growth of RC-K8 and SUDHL2 cells following knockdown of p300 C-1087 may be due to toxic effects of the consequently increased REL/NF- κ B activity.

IPA upstream regulator analysis and qPCR analysis of gene expression changes in RCK8 cells showed that a MYC target gene network is suppressed following knockdown of p300 C-1087, suggesting that expression of p300 C-1087 is normally required for full MYC activity in RC-K8 cells. Enhanced MYC signaling in RC-K8 cells is likely due to increased expression of MYC protein in the presence of p300 C-1087 given that p300 C-1087 knockdown results in reduced MYC protein. Therefore, MYC, like REL/NF- κ B, appears to be a transcription factor whose oncogenic activity can be optimized by expression of truncated p300 proteins. The inhibition of RC-K8 cell growth and MYC-dependent gene expression by p300 C-1087 knockdown suggest that p300 C-1087-dependent enhancement of MYC-directed gene expression contributes to optimal proliferation of these cells. Sustained MYC activity is also important for the survival of SUDHL2 cells [43].

In RC-K8 cells, p300 C-1087 knockdown is associated with increased p53 protein expression [Figure 3(C)], suggesting that reduced protein expression is one mechanism whereby p300 C-1087 expression can dampen a p53-dependent gene network [Table I]. In SUDHL2 cells, p53 protein levels were not affected by p300 C knockdown [Figure 3(C)]. Because SUDHL2 cells express high levels of p53 protein compared to RC-K8 cells and because increased p53 expression is associated with p53 mutation [44], the effect of p300 C on p53 expression may depend on the p53 mutation status and/or p53 expression level in a given cancer cell.

Even though they are defective for HAT activity, p300 C proteins may contribute to B-cell malignancy, at least in part, by acting as aberrant scaffolds that organize altered transcription complexes at a variety of gene promoters/enhancers to cause broad-range transcriptional deregulation. Our work demonstrates that dampening of REL/NF- κ B-dependent gene transcription and increasing of MYC-dependent gene expression are two oncogenic effects of p300 C proteins in DLBCL [12] and herein]. Based on current evidence, we propose that truncated HAT proteins represent a new class of “oncogenic modifier” protein. Furthermore, we propose that p300 C proteins represent gain-of-function mutations—in that these proteins possess new functions not normally exhibited by wild-type p300.

Emerging evidence also indicates that certain mutations in tumor suppressor p53 abolish its tumor suppressing activities while endowing it with new oncogenic activities [45, 46].

Oncogenic modifiers are unlikely to directly transform cells or increase proliferation or cell survival, but rather they serve to resolve the chaos and initially suboptimal growth-promoting effects caused by mutations in strong oncogenic drivers (such as oncogenes and tumor suppressor genes). Mutations in oncogenic modifiers would thus be selected for their ability to confer subtly increased growth and survival onto emerging tumors, which are driven by mutations in classic cell- or context-specific drivers. Thus, oncogenic modifier mutations differ from those of previously described cancer gene classes (namely, oncogenes, tumor suppressors and apoptosis regulators) in that epigenetic regulatory proteins may have thousands of protein and chromatin targets and effects, whereas classic driver mutations affect only single or small numbers of cellular pathways. Thus, mutations in oncogenic modifiers, such as p300, would be expected to provide a broad-based molecular milieu in which more classical oncogenic drivers can exert optimal effects. The broad effects of such mutant HATs may make cells expressing such mutant proteins especially difficult to target [47], and indeed, the RC-K8 cell line is generally resistant to histone deacetylase inhibitor-induced growth inhibition, which is restored by re-expression of wild-type p300 [2]. Thus, agents that target these mutant HATs combined with inhibitors of pathways misregulated by the mutant HATs in a given DLBCL (e.g., NF- κ B, MYC) may represent an appropriate therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Half-life of wild-type and mutant p300 C proteins in BJAB, RC-K8 and SUDHL2 DLBCL cell lines. (A) Anti-p300 Western blot of 25 μ g of whole-cell extracts from BJAB, SUDHL2 and RC-K8 cells. The relevant p300 proteins are indicated. (B) Anti-p300 Western blotting of whole-cell extracts (25 μ g) from SUDHL2, RC-K8, and BJAB cells treated with cycloheximide (CHX) for the indicated times. Western blotting was performed with anti- β -tubulin antiserum as a control. (C) Expression of the relevant p300 proteins was quantified by densitometric analysis and values are relative to the levels of the p300 protein in control cells that were not treated with CHX (0 h; relative value of 100). Shown is a representative result of at least two independent experiments.

Figure 2.

Expression of p300 C-1087 in RC-K8 cells expressing a control or p300-specific shRNA. Anti-p300 Western blotting was performed on 40 µg of whole-cell extract from four independent samples of RC-K8 cells stably expressing p300 or control shRNA (knockdowns (KD) 1, 2). Western blotting was performed with anti-β-tubulin antiserum as a control.

Figure 3.

Knockdown of p300 C expression is correlated with altered NF- κ B and MYC activity in RC-K8 and SUDHL2 cells. cDNA from reverse transcribed total RNA isolated from RC-K8 cells expressing p300 shRNA or a control shRNA was subjected to real-time qPCR using (A) NF- κ B or (B) MYC target gene specific primers. mRNA expression values from RC-K8 cells expressing p300 shRNA were compared to values from RC-K8 cells expressing control shRNA to determine the fold change in expression. Error bars represent standard error of the mean. Asterisks and gray shading represent significant ($p < 0.05$) fold changes as compared to control cells (1.0), using a one-tailed students t-test. Genes that did not show significant fold changes are shaded in white (*CAD*) (C) Anti-p65, anti-REL, anti-MYC, or anti-p53 Western blotting was performed using 25 μ g whole-cell extract from RC-K8 or SUDHL2 cells expressing p300 or control shRNA. The relative expression of MYC and p53 protein in p300 C knockdown cells was determined by densitometric analysis relative to control knockdown in the same cell line (1.0); values are shown below the corresponding Western blot. Because of the higher expression of MYC and p53 in SUDHL2 cells, the exposure times for the lanes containing these two proteins for SUDHL2 cells were less than those for RC-K8 cells (as indicated by the black lines between the panels). Western blotting was performed with anti- β -tubulin antiserum as a control. (D) EMSA using a κ B-site was performed using 25 μ g whole-cell extract from RC-K8 or SUDHL2 cells expressing p300 or control shRNA. In the bottom panel of the EMSA, 5 μ g of whole-cell extracts were incubated with an LSF binding site probe.

Table I

Upstream regulators of p300 C-dependent gene expression patterns in RC-K8 cells.

Upstream Regulator	Molecule Type	z-score	p-value of overlap
Upstream regulators activated by knockdown of p300 C-1087			
TNF	Cytokine	3.783	1.14E-04
TGM2	Enzyme	3.738	3.69E-03
IL1B	Cytokine	3.499	8.35E-03
NF- κ B	Transcription factor	3.400	4.36E-04
TP53	Transcription factor	3.147	1.95E-03
NUPR1	Transcription factor	3.083	4.37E-05
PTPRJ	Phosphatase	2.874	5.15E-03
SLC29A1	Transporter	2.282	6.39E-04
MAP4K4	Kinase	2.201	1.82E-04
PML	Transcription factor	2.109	4.08E-03
Upstream regulators inhibited by knockdown of p300 C-1087			
MYC	Transcription factor	-3.929	6.90E-05
PTPN1	Phosphatase	-2.286	3.02E-03
IGF1R	Transmembrane receptor	-2.175	1.12E-03
KLF15	Transcription factor	-2.042	3.98E-05
BRD4	Kinase and MYC co-activator	-2.018	6.26E-04

Fifteen predicted upstream regulators of the mRNA expression pattern in RC-K8 cells with p300 C-1087 knockdown compared to control RC-K8 cells identified by IPA and sorted by bias-corrected z-scores. The general function of each upstream regulator is listed under Molecule Type. Bias-corrected z-scores and p-values indicating the overlap between each target gene set and the microarray results are shown. The analysis was based on the fold changes of genes/molecules (n = 560) that were significantly differentially expressed ($q < 0.05$) in RC-K8 cells with p300 C-1087 knockdown compared to control RC-K8 cells. z-scores greater than 2.0 were considered significant for pathway activation, and z-scores less than 2.0 were considered significant for pathway inhibition following p300 C-1087 knockdown in RC-K8 cells.

Table II

NF- κ B target genes that are differentially expressed following p300 C knockdown in RC-K8 cells.

No.	Gene ID	Fold change	Ratio	Predicted NF- κ B status	q-value	p-value	Function
1	IFNG*	6.0	6.0	Activated	3.9E-02	1.2E-05	Interferon
2	TNFSF4*	2.5	2.5	Activated	4.0E-02	1.1E-04	Cytokine
3	HIP1*	2.4	2.4	Activated	3.9E-02	5.4E-05	Endocytosis/trafficking
4	TMOD2*	2.3	2.3	Activated	4.2E-02	3.5E-04	Actin-regulatory
5	GBP2*	2.3	2.3	Activated	4.0E-02	1.9E-04	GTPase
6	MYBL1*	2.2	2.2	Affected	4.0E-02	1.4E-04	Transcription factor
7	IRF1*	2.1	2.1	Activated	4.0E-02	1.1E-04	Transcription factor
8	CD274*	2.0	2.0	Activated	4.0E-02	1.8E-04	T-cell suppressing activity
9	CD80*	1.9	1.9	Activated	4.4E-02	9.1E-04	Membrane receptor
10	BIRC3*	1.9	1.9	Activated	4.4E-02	8.4E-04	Inhibitor of apoptosis
11	IL1A*	1.8	1.8	Activated	4.5E-02	1.0E-03	Cytokine
12	ZC3H12A	1.7	1.7	Activated	4.5E-02	9.5E-04	Cell death activator
13	SOCS1	1.7	1.7	Activated	4.9E-02	1.8E-03	Suppressor cytokine signaling
14	HLA-F	1.7	1.7	Activated	4.5E-02	1.1E-03	MHC heavy chain
15	GADD45B	1.7	1.7	Activated	4.4E-02	8.4E-04	Inhibitor cell growth
16	NFKBIZ	1.6	1.6	Activated	4.9E-02	1.8E-03	IL6 activator
17	E2F7	1.6	1.6	Activated	4.5E-02	1.1E-03	Transcription factor
18	GADD45A	1.6	1.6	Inhibited	4.5E-02	9.9E-04	Inhibitor cell growth
19	PEA15	1.6	1.6	Affected	4.8E-02	1.5E-03	Negative apoptotic regulator
20	LTB	-1.7	0.6	Inhibited	4.8E-02	1.5E-03	Inflammatory inducer
21	NOP14	-1.7	0.6	Affected	4.9E-02	1.7E-03	Ribosome biogenesis
22	PPIF	-1.8	0.6	Inhibited	4.9E-02	1.7E-03	Protein biosynthesis
23	IKBKE	-2.0	0.5	Inhibited	4.0E-02	1.8E-04	NF- κ B activation
24	ODC1*	-3.2	0.3	Inhibited	4.0E-02	8.8E-05	Protein biosynthesis
25	CCL17	-4.2	0.2	Inhibited	3.9E-02	4.2E-05	Cytokine
26	SFTPA1	-4.3	0.2	Affected	4.2E-02	4.1E-04	Pathogen defense

List of differentially expressed NF- κ B target genes, $q < 0.05$, following p300 C knockdown in RC-K8 cells. Genes are numbered (No.) arbitrarily for referencing in text. Asterisks indicate genes whose change in expression was validated by qPCR [Figure 3(A)]. Fold change designates the fold change in expression in p300 C knockdown vs. control RC-K8 cells obtained by microarray analysis. Ratio equals the fold change for genes showing fold changes >1 ; ratio equals $-1/(\text{fold change})$ for genes showing fold changes <1 . 'Predicted NF- κ B status' indicates what the expression of the given gene observed in the microarray (i.e., fold change and ratio columns) indicates about overall NF- κ B signaling in RC-K8 cells. This is reported by IPA upstream regulator analysis and based on literature reports: 'activated' indicates the observed fold change in the given gene is consistent with an increase in NF- κ B signaling; 'inhibited' indicates the observed fold change in the given gene is consistent with a decrease in NF- κ B signaling; and 'affected' indicates the gene is regulated by NF- κ B, but there is no consensus on the directionality of this effect. q-values associated with the fold change for each gene are shown. p-value reports the p-value associated with the fold change for each gene (based on a modified t-test). 'Function' is a brief description of the known protein function; MHC, major histocompatibility complex.

Table III

MYC target genes that are differentially expressed following p300 C knockdown in RC-K8 cells.

No.	Gene ID	Fold change	Ratio	Predicted MYC status	q-value	p-value	Function
1	ODC1*	-3.2	0.31	Inhibited	4.0E-02	8.8E-05	Protein biosynthesis
2	FASN*	-2.9	0.34	Inhibited	4.2E-02	3.3E-04	Fatty acid synthesis
3	SRM*	-2.5	0.40	Inhibited	4.4E-02	7.3E-04	Spermidine biosynthesis
4	NME1*	-2.3	0.43	Inhibited	4.4E-02	7.2E-04	Kinase
5	CAD*	-2.2	0.45	Inhibited	4.2E-02	2.7E-04	Pyrimidine biosynthesis
6	PFAS*	-2.2	0.45	Inhibited	4.0E-02	2.1E-04	Purine biosynthesis
7	AIMP2*	-2.1	0.48	Inhibited	4.2E-02	2.6E-04	Unknown
8	LDHA*	-2.0	0.50	Inhibited	4.3E-02	5.0E-04	Anaerobic glycolysis
9	PGK1*	-2.0	0.50	Inhibited	4.5E-02	1.1E-03	Kinase
10	CDK4*	-1.8	0.56	Inhibited	4.5E-02	9.7E-04	Cyclin-dependent kinase
11	MINA	-1.8	0.56	Inhibited	4.4E-02	5.9E-04	Cell growth
12	PNO1	-1.8	0.56	Inhibited	4.4E-02	8.3E-04	Immunity
13	DNPH1	-1.7	0.59	Inhibited	4.4E-02	8.3E-04	Proliferation
14	NOLC1	-1.7	0.59	Inhibited	4.5E-02	9.7E-04	Unknown
15	PHB	-1.7	0.59	Inhibited	5.0E-02	2.1E-03	Tumor suppression
16	DBI	-1.5	0.67	Inhibited	5.0E-02	2.2E-03	Lipid metabolism
17	GADD45A	1.6	1.60	Inhibited	4.5E-02	9.9E-04	Inhibitor cell growth
18	CD48	1.7	1.70	Inhibited	4.9E-02	1.8E-03	Immune cell activation
19	HLA-B	1.7	1.70	Inhibited	4.4E-02	7.2E-04	MHC heavy chain
20	LAMB3	1.8	1.80	Inhibited	4.4E-02	7.6E-04	Basement membrane
21	CD274	2.0	2.00	Inhibited	4.0E-02	1.8E-04	T-cell suppressing activity
22	DDB2	2.0	2.00	Inhibited	4.2E-02	4.4E-04	DNA damage response
23	HLA-E	2.0	2.00	Inhibited	4.2E-02	3.9E-04	MHC heavy chain
24	SERPINE2	2.1	2.10	Inhibited	4.0E-02	1.8E-04	Protease inhibitor
25	FADS2	2.3	2.30	Inhibited	4.2E-02	2.4E-04	Fatty acid metabolism
26	GBP2	2.3	2.30	Inhibited	4.0E-02	1.9E-04	GTPase
27	CTSB	2.5	2.50	Inhibited	4.4E-02	6.1E-04	APP proteolytic processing
28	RRM2B	2.5	2.50	Inhibited	4.0E-02	1.3E-04	DNA synthesis
29	ACAT1	-2.1	0.48	Affected	4.0E-02	1.7E-04	Biosynthesis/metabolism
30	POLR2G	-2.1	0.48	Affected	4.2E-02	3.4E-04	Transcription initiation
31	RUVBL1	-2.1	0.48	Affected	4.2E-02	3.1E-04	ATPase
32	DHFR	-2.0	0.50	Affected	4.6E-02	1.2E-03	Purine synthesis
33	POLR1B	-1.9	0.53	Affected	4.5E-02	1.1E-03	rRNA transcription
34	MAT2A	-1.8	0.56	Affected	4.4E-02	6.7E-04	Biosynthesis
35	SGK1	1.6	1.60	Affected	4.9E-02	2.0E-03	Kinase
36	GADD45B	1.7	1.70	Affected	4.4E-02	8.4E-04	Inhibitor cell growth
37	PLA1A	2.0	2.00	Affected	4.4E-02	8.4E-04	Liposome activity

No.	Gene ID	Fold change	Ratio	Predicted MYC status	q-value	p-value	Function
38	ITM2B	2.6	2.60	Affected	4.0E-02	1.2E-04	Inhibits amyloid deposition
39	FLNA	-1.7	0.59	Activated	4.4E-02	7.7E-04	Actin regulation
40	ASS1	1.8	1.80	Activated	4.9E-02	1.9E-03	Arginine biosynthesis
41	GH1	2.0	2.00	Activated	4.9E-02	1.8E-03	Growth hormone

List of differentially expressed MYC target genes, $q < 0.05$, in RC-K8 cells following p300 C-1087 knockdown. Genes are numbered (No.) arbitrarily for referencing in text. Asterisks indicate genes whose expression was validated by qPCR [Figure 3(B)]. Fold change designates the fold change in expression in p300 C knockdown vs. control RC-K8 cells obtained by microarray analysis. Ratio equals the fold change for genes showing fold changes >1 ; ratio equals $-1/(\text{fold change})$ for genes showing fold changes <1 . 'Predicted MYC status' indicates what the expression of the given gene observed in the microarray (i.e., fold change and ratio columns) indicates about overall MYC signaling in RC-K8 cells. This is reported by IPA upstream regulator analysis and based on literature reports: 'activated' indicates the observed fold change in the given gene is associated with activated MYC signaling; 'inhibited' indicates the observed fold change in the given gene is associated with inhibited MYC signaling; and 'affected' indicates the gene is regulated by MYC, but there is no consensus on the directionality of this effect. q-values associated with the fold change for each gene are shown. The p-value indicates the p-value associated with the fold change for each gene (based on a modified t-test). 'Function' is a brief description of the known protein function; MHC, major histocompatibility complex; APP, amyloid precursor protein.

Table IV

Gene Set Enrichment Analysis (GSEA) of NF- κ B and MYC defined gene sets in RC-K8 cells with p300 C-1087 knocked down

Gene Set Name	Size	NES	FDR q value	Nominal p value
V\$NFKAPPAB_01 www.broadinstitute.org/gsea/msigdb/cards/V\$NFKAPPAB_01	248	1.59	0.1326	< 0.001
V\$NFKB_Q6_01 broadinstitute.org/gsea/msigdb/cards/V\$NFKB_Q6_01	229	1.49	0.1634	2.25E-03
V\$NFKB_Q6 broadinstitute.org/gsea/msigdb/cards/V\$NFKB_Q6	249	1.44	0.1787	< 0.001
V\$NFKB_C broadinstitute.org/gsea/msigdb/cards/V\$NFKB_C	256	1.43	0.1880	8.99E-03
V\$NFKAPPAB65_01 broadinstitute.org/gsea/msigdb/cards/V\$NFKAPPAB65_01	235	1.36	0.2190	1.32E-02
V\$MYC_MAX_02 broadinstitute.org/gsea/msigdb/cards/V\$MYC_MAX_02	260	-1.29	0.1762	2.82E-02
V\$MYC_MAX_03 broadinstitute.org/gsea/msigdb/cards/V\$MYC_MAX_03	245	-1.46	0.0701	3.58E-03
V\$MYC_MAX_B broadinstitute.org/gsea/msigdb/cards/V\$MYC_MAX_B	259	-1.47	0.0649	1.83E-03
V\$NMYC_01 broadinstitute.org/gsea/msigdb/cards/V\$NMYC_01	266	-1.49	0.0596	1.80E-03
V\$MYC_MAX_01 broadinstitute.org/gsea/msigdb/cards/V\$MYC_MAX_01	247	-2.16	0.0001	< 0.001

Enrichment of NF- κ B or MYC target gene sets in RC-K8 cells with p300 C-1087 knocked down relative to control RC-K8 cells. Gene expression values from the microarray were ranked according to the moderated t-statistic and ranked lists were used to perform pre-ranked GSEA using NF- κ B or MYC DNA-binding site defined gene sets obtained from the Molecular Signatures Database (MSigDB, C3 Motif Gene Sets, TFT Transcription Factor Targets), version 4.0. Gene set name provides the name from the MSigDB and a link to the gene set; size lists the number of genes in the gene set that overlap with the genes in the ranked list; NES lists the normalized enrichment score (the enrichment score normalized to gene set size); FDR q-value lists the FDR-corrected p-value; nominal p-value lists the p-value computed from the NES and was used to compute significance of each gene set, which can be equal to 0 since it is computed by permutation. p-values of 0 are reported as $p < 0.001$ because 1000 permutations were run. Rows are sorted by descending NES. See Supplementary Information for more details.