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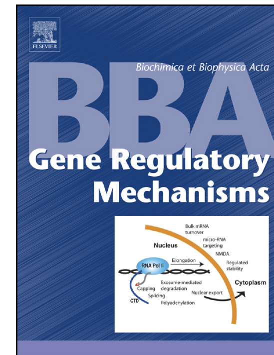

Glucocorticoids uncover a critical role for ASH2L on BCL-X expression regulation in leukemia cells

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Glucocorticoids uncover a critical role for ASH2L

on BCL-X expression regulation in leukemia cells

Luciana Rocha-Viegas[1*]; Micaela Silbermins[1]; María Florencia Ogara[1]; Joaquín Miguel Pellegrini[2]; Sol Yanel Nuñez[3]; Verónica Edith García[2,4]; Guillermo Pablo Vicent[5] and Adali Pecci[1,4]

[1]Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE-UBA-CONICET), Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, C1428EHA Buenos Aires, Argentina

[2]Instituto de Química Biológica Ciencias Exactas y Naturales (IQUIBICEN-UBA-CONICET), Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, C1428EHA Buenos Aires, Argentina

[3]Instituto de Biología y Medicina Experimental (IBYME-CONICET), Laboratorio de Fisiopatología de la Inmunidad Innata, Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina

[4]Departamento de Química Biológica, Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, C1428EHA Buenos Aires, Argentina

[5]Department of Molecular Genomics, Institute of Molecular Biology of Barcelona, IBMB-CSIC, Baldiri Reixac 4, 08028 Barcelona , Spain

*__Corresponding author__: Rocha-Viegas, Luciana, IFIBYNE-UBA-CONICET, Ciudad Universitaria, CABA, Buenos Aires, Argentina. Tel: 54-11-4576-3368, Fax: 54-11-4576-3321, email: lrochav@qb.fcen.uba.ar

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ABSTRACT

Targeting the apoptosis machinery is a promising therapeutic approach in myeloid malignancies. BCL2L1 is a well-known glucocorticoid-responsive gene and a key apoptosis regulator that, when over-expressed, can contribute to tumour development, progression and therapeutic resistance. Moreover, synthetic glucocorticoids, like dexamethasone, are frequently used in the treatment of hematopoietic diseases due to its pro-apoptotic properties. We report here that the trithorax protein ASH2L, considered one of the core subunits of H3K4-specific MLL/SET methyltransferase complexes, contributes to anti-apoptotic BCL-X_L over-expression and cell survival in patient-derived myeloid leukemia cells. We find that the unliganded glucocorticoid receptor (uGR) and ASH2L interact in a common protein complex through a chromatin looping determined by uGR and ASH2L binding to BCL2L1 specific +58 HRE and promoter region, respectively. Upon addition of dexamethasone, GR and ASH2L recruitment is reduced, BCL-X_L expression diminishes and apoptosis is induced consequently. Overall, our findings indicate that uGR and ASH2L may act as key regulatory players of BCL- X_L upregulation in AML cells.

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Keywords: Glucocorticoid receptor, BCL2L1 gene, ASH2L, myeloid leukemia

INTRODUCTION

The proper control of apoptosis is important in many aspects of life including development, homeostasis and disease biology [1]. Impaired cell death plays a central role in cancer development and limits the efficacy of conventional cytotoxic therapies [2, 3]. Since BCL-2 family proteins are pivotal regulators of apoptotic cell death and given their deregulation in myeloid malignancies, therapeutic agents for these targets are currently in preclinical or clinical development [4]. Accumulating evidence points to the existence of therapies targeting multiple anti-apoptotic BCL-2 family members, particularly MCL-1, BCL-2 and BCL-X_L. These therapies may sensitize tumor cells to apoptotic stimuli, such as chemotherapy or radiation without displaying single-agent cytotoxic activity [4-7]. Nevertheless, therapeutic progress in the treatment of acute and chronic leukemia has been slow and often neither adequate nor successful [4]. Therefore, understanding the contribution of individual anti-apoptotic members, such as BCL-X_L, to the supervision of hematopoietic homeostasis has been the topic of intense research for many years [1]. Deregulation of apoptosis is one of the important features of acute myeloid leukemia (AML) [8]. In this sense, to understand the molecular mechanism underlying cell death and its contribution to tumor progression, this study is focused on anti-apoptotic BCL-X_L expression regulation by GR and ASH2L.

BCL-X_L (BCL2L1) has been reported to be over-expressed in hematopoietic tumors and to play a crucial role in AML pathogenesis [7]. Its high expression also correlates with resistance to cytotoxic chemotherapy and to targeted agents [6, 8-13], supporting its usefulness as potential prognostic biomarker to predict response to anticancer therapeutics. Furthermore, other authors and our group have previously demonstrated that BCL2L1 is a well-known glucocorticoid-responsive gene [14-17]. Glucocorticoids

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have been identified as potent regulators controlling cell turnover with a striking tissue- and cell-context specificity, and their effects on apoptosis and cell survival are of substantial clinical relevance [18]. Synthetic glucocorticoids, like dexamethasone (Dex), are frequently used in the treatment of hematopoietic diseases due to its pro-apoptotic properties [19, 20]. This context-driven plasticity likely reflects that steroid hormones could represent an alternative and promising therapy in leukemia, although their mechanisms of action on these cells are still unclear. In that sense, BCL2L1 gene provides an interesting steroid-responsive model to follow the molecular events from actively transcribed to silent chromatin.

The trithorax protein ASH2L is considered to be one of the core subunits of all known H3K4-specific MLL/SET methyltransferase complexes and functions as an oncoprotein over-expressed in promyelocytic leukemia [21]. Importantly, during human myeloid leukemia U937 cell differentiation induced by retinoic acid (RA), ASH2L and the nuclear receptor RARα interact and are both recruited to promoter regions of RA-responsive genes [22].

Here, we report that the unliganded glucocorticoid receptor (uGR) and ASH2L are both recruited to BCL2L1 gene and consequently contribute to anti-apoptotic BCL-X_L over-expression in a patient-derived myeloid leukemia U937 cell line. ASH2L binding around gene promoter and uGR recruitment to a distal response element (+58 kbp) predicts a common protein complex due to chromatin looping occurring in these tumor cells. Moreover, the presence of glucocorticoids reduces both GR and ASH2L recruitment to chromatin, which results in BCL-X_L down-regulation and concomitant apoptosis induction. Interestingly, ASH2L interference facilitates myeloid leukemia cell death comparably and independently of glucocorticoid addition, suggesting that ASH2L may be playing a relevant role in GR-mediated BCL-X_L expression in this leukemic context.

MATERIALS AND METHODS

Steroids and Antibodies

Dex 50 nM and RU 38486 (RU486) 100 nM from Sigma were used for hormonal treatments. Hormones were dissolved (1000 x) in absolute ethanol.

Anti-BCL-$X_{L/S}$ (sc-L19), anti-GR (sc-1002), anti-ACTIN, anti-TUBULIN and anti-H3 for western blot assays were purchased from Santa Cruz Biotechnology. Anti-GR (ab3579) from Abcam, anti-ASH2L (A300-107A) from Bethyl Laboratories and anti-trimethyl H3K4 (07473) antibody from Millipore were used in ChIP and co-IP assays.

Cell Culture and Treatments

Wild type, shControl and shASH2L U937 monocytic leukemia cells obtained directly from Dr. Di Croce Lab. (Centro de Regulación Genómica, Barcelona, Spain) were cultured at 37ºC and 5% of CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin. Cells were then washed twice with PBS, plated in RPMI medium supplemented with 10% dextran-coated charcoal-stripped FBS (CS-FBS) and non-treated (vehicle) or treated with Dex (50 nM) to induce apoptosis and harvested at 24 h, 48 h and 72 h. Cell extracts were prepared as previously described [22]. pRS-based retrovirus was produced by transfecting GP2-293 packaging cells (Clontech). The collected retrovirus was subsequently used to transduce THPI or K562 cells by spin-infection (900 g, 90 min, 32ºC) in presence of protamine sulfate followed by an additional overnight incubation at 37ºC in 5% CO_2. The protocol was repeated for 2 consecutive days [22]. After 36 h of shRNA-ASH2L transduction, infected cells were first selected with puromycin for at least 72 h. Once further validation of knockdown was done by western blot to detect ASH2L

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protein levels, cells were treated with Dex and were harvested to perform chromatin immunoprecipitation and mRNA analyses. All cell lines used in this study were maintained in culture for 4 to 5 weeks until a new cryovial was defrost; routinely check-ups for mycoplasma contamination were performed and were always negative. Monocytes were isolated from healthy volunteers´ blood (provided by the Blood Bank of the Hospital Churruca-Visca, Buenos Aires, Argentina). The Institutional Review Committee has approved all the studies performed in this work. Moreover, informed consent of all participating subjects was obtained. Monocytes were isolated either by MACS (Miltenyi Biotec; $CD14^{+}$ purity > 90% as assessed by Flow Cytometry), or by isolation of peripheral blood mononuclear cells (PBMC) by centrifugation over Ficoll-Hypaque (GE Healthcare, 17-1440-03) and cultured of cells ($2x10^{6}$ cells/ml) in 250 ml cell-culture flasks with RPMI 1640 supplemented with L-glutamine, penicillin-streptomycin and 10% fetal bovine serum for 16 h without stimulus to allow monocyte adherence.

RNA purification and RT-qPCR analysis

Total RNA was extracted using TRI Reagent (Genbiotech) according to the manufacturer's protocol. Reverse transcription using M-MLV reverse transcriptase (Promega) and oligo-dT primer (Invitrogen) was performed according to the provider's instructions. StepOnePlus (Applied Biosystems) real-time PCR was used to carry out the RT-qPCR reactions in clear optical 96-well reaction plates with optical covers, according to the manufacturer's instructions. Gene expression levels were normalized using the endogenous control PUM-1 for each sample (as it has been shown to not vary with Dex treatment), and differences in target gene expression were determined using StepOne v2.3 software. All PCR conditions and primer sequences are available upon request.

Chromatin Immunoprecipitation (ChIP)

For ChIP assays, cells were cross-linked with 0.8% formaldehyde at room temperature for 6 min. ChIPs were performed and analysed as described previously [22]. Chromatin immunoprecipitates for proteins and methyl marks were amplified by real-time quantitative PCR (StepOnePlus, Applied Biosystems), normalized to input and calculated as % of input. The sequences of the PCR primers are available upon request.

Subcellular Fraction Separation and Coimmunoprecipitation (co-IPs)

For co-IP assays, cells non-treated (vehicle) or treated with Dex (50 nM for 72 h) were washed in PBS and lysed in lysis buffer for subcellular fraction separation [23]. Briefly, nuclear pellets were washed twice in ice-cold PBS buffer, resuspended in 100 μl of lysis buffer [20 mM Tris-HCl (pH 6.7); 70 mM NaCl; 10% glycerol; 1% Triton X-100; 0.5% Nonidet P-40; 300 U/ml DNase I (Sigma); 0.05 mM leupeptin; 1 mM PMSF; 1 μm pepstatin A], and incubated on ice-water bath for 30 min. After the addition of 0.5 M NaCl, the incubation was continued for additional 30 min. Samples were centrifuged at 12000 rpm for 5 min at 4 C, and supernatants were referred to as nuclear fraction. Nuclear cell extracts were incubated overnight at 4ºC with the indicated antibodies. Protein A/G plus agarose beads (sc-2003, Santa Cruz Biotechnology) were added to the lysates for 2 h and the immunoprecipitates were then washed and loaded on SDS/PAGE. Inputs and IPs were analysed by western blot using anti-ASH2L and anti-GR antibodies. Bands were detected by chemiluminescence and quantified using Image J.

Chromosomal Conformation Capture (3C)

3C experiments were carried out according to protocols previously described [24]. Briefly, U937 or monocyte cells (15×10^6 per experiment) were cross-linked with 0.8%

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formaldehyde, and cell pellets were homogenized in lysis buffer (10 mM Tris-Cl pH 8.0, 10 mM NaCl, 0.2% NP-40). Cells were overnight digested with 400 U of SacI (New England Biolabs) per 5×10^6 cells at 37°C and then overnight ligated with 150 U of T4 DNA ligase (New England Biolabs) at 16°C. Crosslinks were treated with RNase A, reversed by overnight digestion with proteinase K at 65°C and the 3C DNA template was purified by two rounds of phenol-chloroform extraction. Next, the DNA was ethanol precipitated and dissolved in Tris-EDTA buffer at pH 8.0. qPCR reactions were carried out with forward primers located beside SacI digestion sites and within the region between the BCL2L1 P1A/P2 promoter and the +58 kbp enhancer. Primers used to amplify the 3C reaction are available upon request.

Apoptosis Analysis

After 72 h of vehicle or Dex treatment in CS-FBS containing-medium, shControl and shASH2L U937, THPI and K562 cells were incubated with ANNEXIN V-FITC and Propidium Iodide (BD) following the manufacture's instructions. Apoptosis was analyzed on a FACS ARIA II flow cytometer (BD Biosciences). Data were processed using FlowJo software (v 7.6). Percentage of early plus late apoptotic cells was calculated as the ratio between ANNEXIN V FITC/PI stained cells and the total number of cells.

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Statistical Analysis

GraphPad Prism 6 was used for statistical analysis. Student's t-test and one-way or two-way ANOVA tests followed by Tukey's post-hoc test were used. Unless otherwise indicated in the figure legends, we analysed three biological replicates for each data point in all graphs represented as mean ± SEM, and the level of significance was as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

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RESULTS

Glucocorticoids promote anti-apoptotic BCL-X_L expression down-regulation

Accumulating evidence indicates that abnormal expression profile of the anti-apoptotic BCL-X_L isoform can contribute to tumour development, progression and therapeutic resistance in myeloid leukemia [4, 5, 10, 25]. When comparing endogenous BCL-X_L expression, normal human monocytes show significantly lower levels than U937 monocytic leukemia cells ([26] and Figure 1A). Moreover, RT-qPCR experiments reveal that enhanced BCL-X_L levels in non-treated (vehicle) U937 cells grown in 10% CS-FBS containing-medium are significantly reduced upon 48 h of glucocorticoid treatment (Figure 1A), without affecting the expression of the pro-apoptotic isoform BCL-X_S (which is much less abundant than BCL-X_L in this cell line, Figure S1A). Furthermore, BCL-X_L protein levels also diminish significantly when cells are incubated with Dex (Figure 1B). This decrease is abrogated by the co-incubation with the GR antagonist RU486 confirming the GR-mediated BCL-X_L down-regulation. In view of these results, we wondered about the mechanisms underlying BCL-X_L over-expression in leukemia cells and its down-regulation by glucocorticoids.

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FIGURE 1

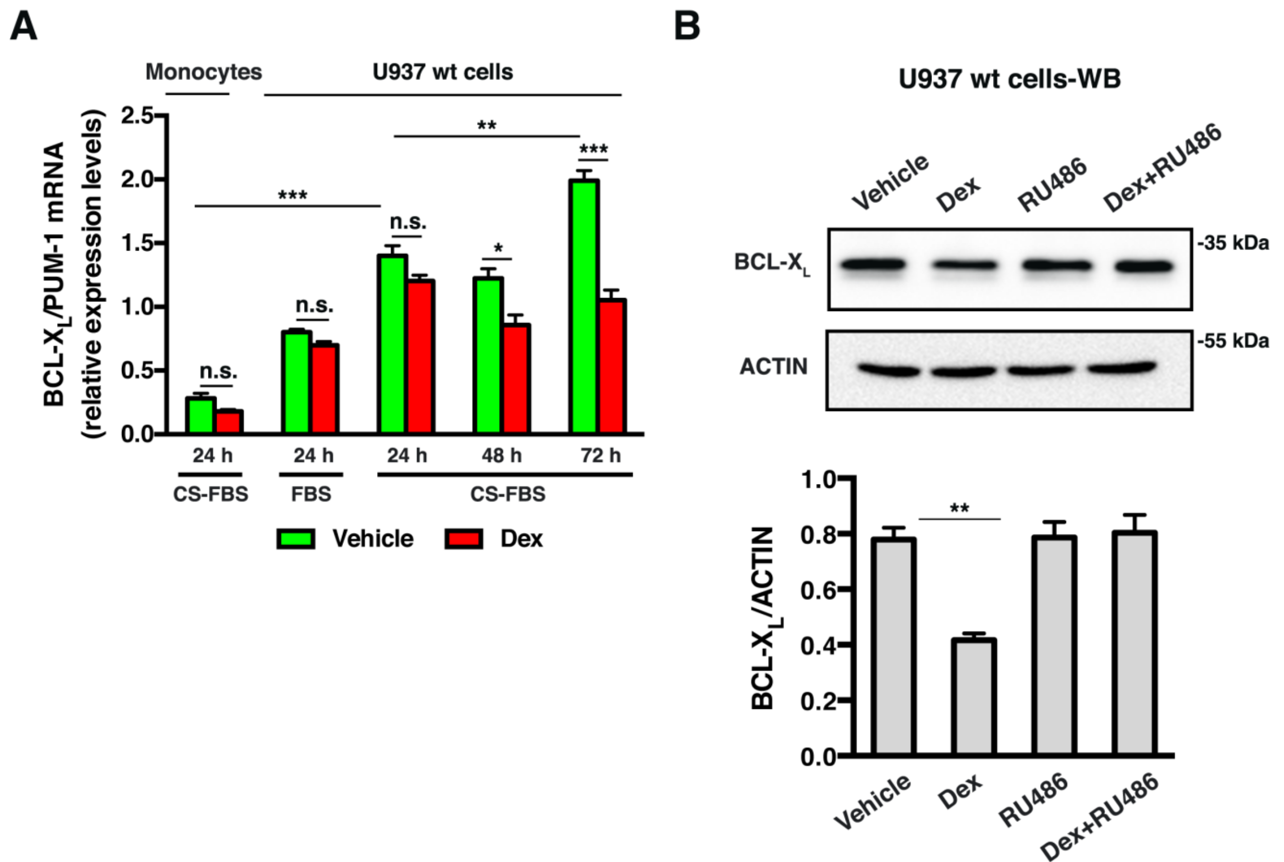

Figure 1. Glucocorticoids promote anti-apoptotic BCL-X$_L$ down-regulation

(A) Total RNA from monocytes grown on medium containing steroid-stripped serum (CS-FBS) or wild type U937 cells (U937 wt) grown on medium containing complete serum or CS-FBS, untreated (Vehicle) or treated with 50 nM Dexamethasone (Dex) for 24, 48 or 72 h was prepared. BCL-X$_L$ mRNA levels were quantified relative to PUM-1 control by RT-qPCR. Results expressed as a ratio are presented. **(B)** BCL-X$_L$ protein levels from total extracts of U937 wt cells grown on CS-FBS containing-medium untreated (Vehicle) or treated with 50 nM Dex (Dex), with the glucocorticoid antagonist 100 nM RU486 (RU486) or with Dex+RU 486 (Dex+RU) for 72 h, were analyzed by Western blot with the indicated antibody. Probing for β-ACTIN was used as a loading control. One representative gel is shown at the top, and the corresponding quantification expressed as a ratio for experiments performed in triplicate is shown at the bottom. Data are represented as mean ± SEM from at least three independent experiments. Statistical analysis corresponds to one-way ANOVA (A) and two-way ANOVA (B), *$p<0.05$; **$p<0.01$; ***$p<0.001$; n.s.: not significant.

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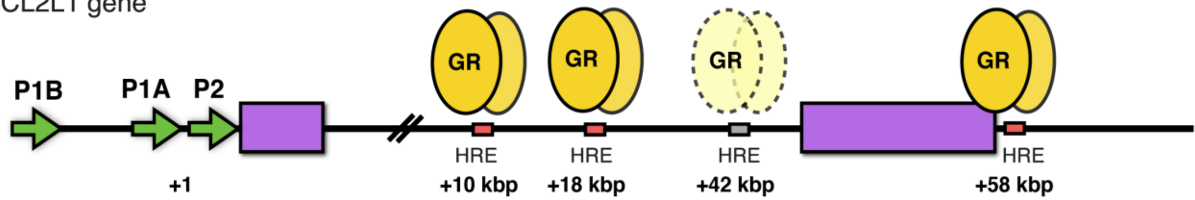
Unliganded GR is recruited to BCL2L1 gene in leukemia cells

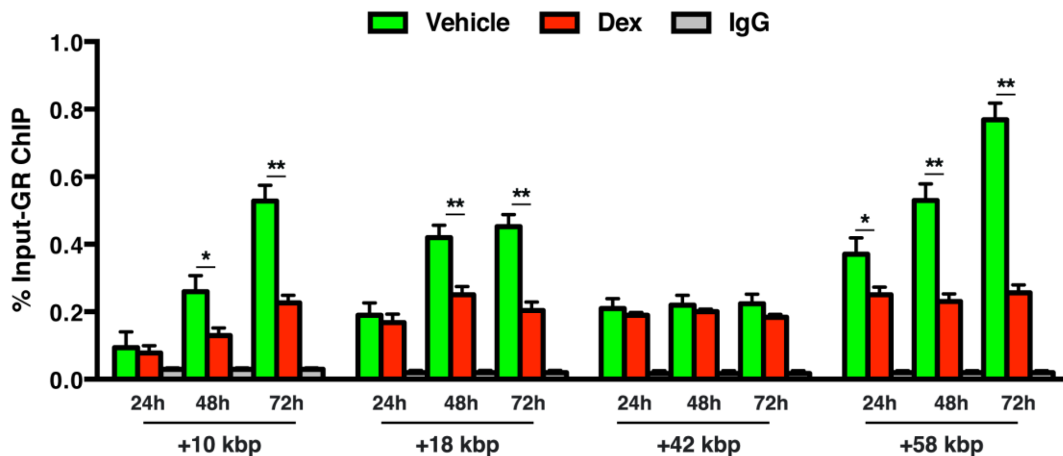
We first examined GR recruitment to hormone response elements (HREs) present in the human BCL2L1 gene and located in the intron at +10 and +18 kbp relative to the transcription start site (TSS), and at +58 kbp, at the end of the last gene exon (Figure 2A) [27, 28]. We also tested another HRE located at +42 kbp relative to TSS, previously described as a progesterone receptor response element [28]. Surprisingly, uGR is already present at all endogenous BCL2L1 HREs in non-treated (vehicle) U937 cells grown in 10% CS-FBS containing-medium for 24 h; and its recruitment to +10, +18 and +58 HREs increases in a time-dependent manner in this steroid-deprived condition (Figure 2A). No uGR recruitment is observed in normal monocytes (Figure S1B). In the presence of Dex, GR recruitment to HREs is significantly reduced after 48 h, maintaining basal binding levels. This profile is similar to that observed in Figure 1 where transcription of the BCL2L1 gene is significantly reduced upon 48 h of Dex treatment and more evident after 72 h (Figure 2A). As a control for ChIP assays, equal amounts of unrelated antibody (IgG) were included. Importantly, GR expression levels remain unchanged upon Dex treatment (Figure S1C), suggesting that differences in GR recruitment are not due to changes in nuclear receptor levels. Noteworthy, ChIP experiments along P1A BCL2L1 promoter also show significant uGR recruitment to this promoter region only after 72 h despite the absence of HREs (Figure 2B) that is remarkably reduced upon Dex addition, suggesting a GR trans-regulation throughout the intragenic HREs.

Considering the great significant differences in BCL-X_L regulation and GR recruitment observed, we decided to further analyse the effect of 10% CS-FBS containing-medium and glucocorticoid treatment at 72 h. Altogether, these observations support the idea that glucocorticoids could be reducing GR association to HREs and down-regulating BCL-X expression accordingly.

FIGURE 2

A





B

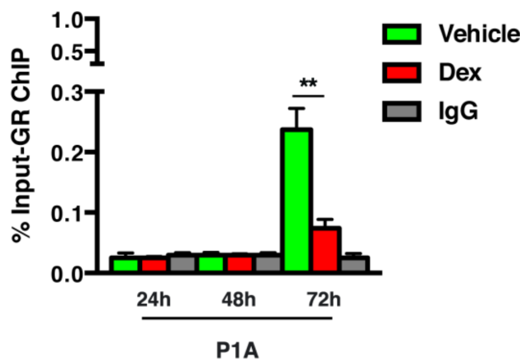


Figure 2. Unliganded GR is recruited to BCL2L1 gene in leukemia cells

(A and **B)** Schematic diagram of BCL2L1 gene showing reported GR regulatory regions (HREs) located at +10, +18 and +58 kbp from TSS (+1). Element located at +42 kbp was reported as a progesterone receptor HRE. GR occupancy in U937 wt cells untreated (Vehicle) or treated with 50 nM Dex (Dex) for 24, 48 and 72 h at BCL2L1 HREs **(A)** or at P1A promoter **(B)** analyzed by ChIP-qPCR using GR antibody. Element +42 and IgG were included as control. Data are presented as percent input and represented as mean ± SEM from at least three independent experiments. Statistical analysis corresponds to two-way ANOVA, *$p<0.05$; **$p<0.01$; n.s.: not significant.

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ASH2L is recruited to HREs and interacts with GR in vivo in leukemia cells

Since GR and ASH2L were already reported to interact in 293T cells [29] and considering that glucocorticoids induce apoptosis in U937 leukemia cells [18], we wondered whether ASH2L could participate in GR-mediated BCL-X_L regulation. ChIP assays showed that ASH2L occupancy is observed on the P1A BCL2L1 promoter and even on +10; +18 and +58 endogenous BCL2L1 HREs in non-treated (Vehicle) U937 cells grown in 10% CS-FBS containing-medium (Figure 3A). Concomitant to GR, lower levels of ASH2L occupancy are observed at +10 and +58 HREs, and at P1A BCL2L1 promoter region in Dex-treated cells (Figure 3A). Moreover, H3K4me3 epigenetic mark results significantly affected in this promoter region and in +58 HRE only after 72 h of Dex treatment (Figure S2A). These results suggest that Dex treatment reduces GR and ASH2L binding to BCL2L1 gene.

In view of the similar GR and ASH2L recruitment responses described above, we next explored the possibility of an endogenous GR-ASH2L association. Thus, we performed co-IP experiments using nuclear lysates from U937 cells non-treated (Vehicle) or treated with Dex. Immunoblot analysis of anti-GR (Figure 3B) or anti-ASH2L (Figure 3C) immunoprecipitates reveals the existence of an endogenous stable complex containing both GR and ASH2L even in the absence of stimulus. This interaction is enhanced by steroid treatment.

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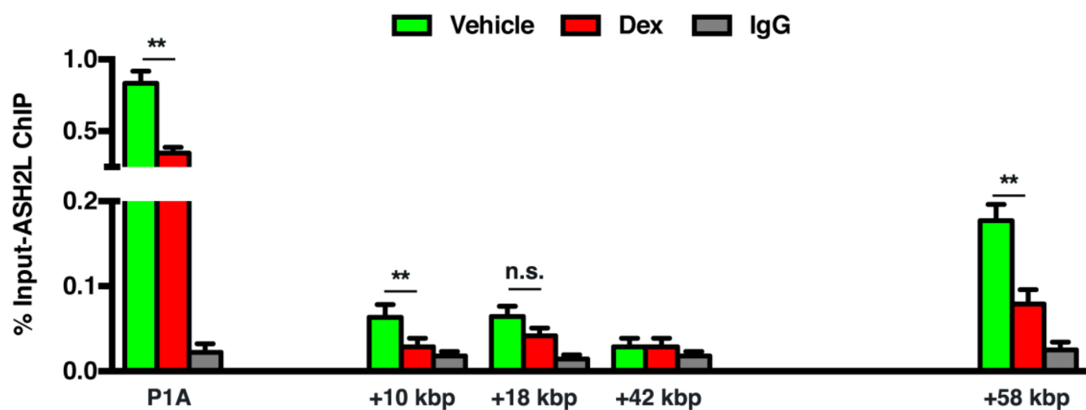
FIGURE 3

A

BCL2L1 gene



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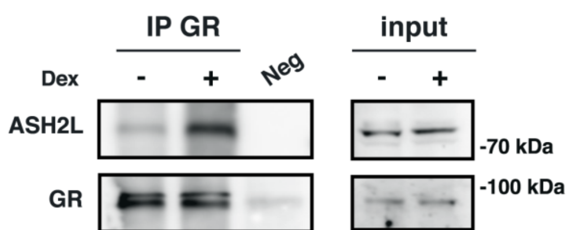

C

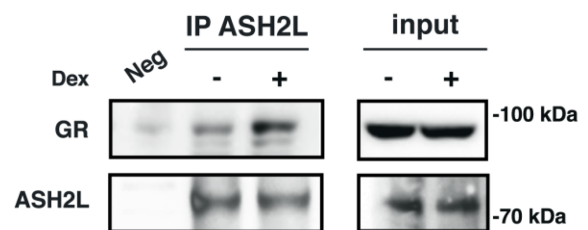

Figure 3. ASH2L is recruited to HREs and interacts with uGR in vivo in leukemia cells. **(A)** Schematic diagram of BCL2L1 gene showing GR HREs and ASH2L binding around promoter region. ASH2L occupancy in U937 wt cells untreated (Vehicle) or treated with 50 nM Dex (Dex) for 72 h at BCL2L1 P1A promoter and HREs analyzed by ChIP-qPCR using ASH2L antibody. Element +42 and IgG were included as control. Data are presented as percent input and represented as mean ± SEM from at least three independent experiments. Statistical analysis corresponds to two-way ANOVA, **p<0.01; n.s.: not significant. **(B** and **C)** Nuclear extracts from U937 wt cells untreated (–) or treated with 50 nM Dex (+) were immunoprecipitated with IgG antibody (Neg), with the anti-GR antibody (IP GR) **(B)** or with the anti-ASH2L antibody (IP ASH2L) **(C).** Western blots with input lysate or immunoprecipitates were analyzed using antisera against ASH2L or GR. One representative gel is shown.

Unliganded GR and ASH2L are involved in BCL-X_L over-expression of myeloid leukemia cells

To further examine the role of ASH2L on BCL-X_L regulation in response to glucocorticoids, we studied the interplay between the MLL/SET methyltransferase complex and GR in a stable ASH2L knockdown U937 cell line (shASH2L) validated previously ([22] and Figure S3A). ChIP experiments show that whereas uGR binding to BCL2L1 HREs is insensitive to ASH2L knockdown in control cells grown in 10% CS-FBS containing-medium, no uGR recruitment is detected along P1A promoter in these conditions (Figure 4A), revealing the relevance of ASH2L for uGR binding to this promoter region. Surprisingly, upon treatment with Dex in ASH2L knockdown cells, receptor occupancy increases at +10 and +18 HREs, while no significant changes are observed around +58 HRE compared to control cells (Figure 4A). Control ChIP experiments in shControl U937 cells are also shown (Figure S3B). These results suggest that in a leukemia cell context, GR and ASH2L interaction on chromatin would be necessary for BCL2L1 active transcription in the absence of glucocorticoids.

As BCL-X_L over-expression in U937 wild type (wt) cells correlates with GR and ASH2L recruitment to chromatin, we then assessed the effect of ASH2L knockdown on BCL-X_L transcription. Interestingly, untreated (Vehicle) shASH2L cells grown in 10% CS-FBS containing-medium show reduced BCL-X_L expression levels similar to shControl cells under glucocorticoid treatment (Figure 4B and 4C). These results reinforce the idea that BCL-X_L active transcription in myeloid leukemia is not only controlled by uGR occupancy of BCL2L1 HREs but also by the critical ASH2L association with the BCL2L1 promoter region. Moreover, Dex treatment of ASH2L knockdown cells does not affect BCL-X_L mRNA or BCL-X_L protein basal amounts (Figure 4B and 4C), indicating that the effect of glucocorticoids on BCL2L1 regulation depends on ASH2L expression. ASH2L relevance

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is not only constraint to U937 cells, as similar results were obtained in THP1, another acute myeloid leukemia cell line (Figure S4). In fact, untreated (Vehicle) shASH2L THP1 cells show reduced BCL-X_L mRNA levels comparable to shControl cells under glucocorticoid treatment (Figure S4B). Furthermore, Dex treatment of ASH2L knockdown cells does not affect BCL-X_L mRNA or BCL-X_L protein basal amounts (Figure S4B and S4C). We also tested ASH2L role in chronic myeloid leukemia K562 cells. Despite BCL-X_L mRNA levels decrease in shASH2L K562 cells compare to shControl cells, neither Dex nor ASH2L knockdown affects BCL-X_L protein basal amounts in these cells.

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FIGURE 4

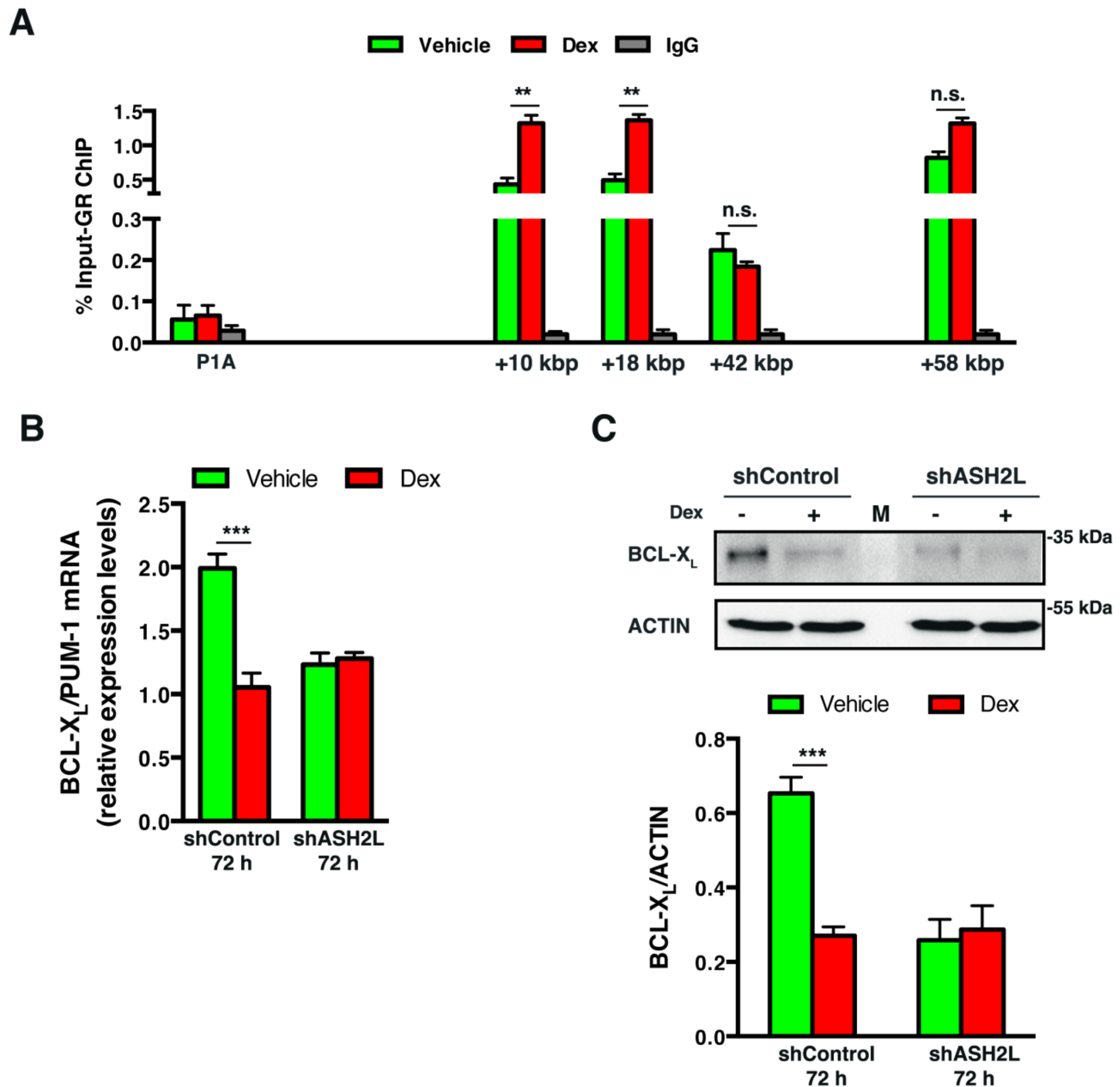

Figure 4. uGR and ASH2L are involved in BCL-X_L over-expression of myeloid leukemia cells. **(A)** shASH2L U937 cells untreated (Vehicle) or treated with 50 nM Dex (Dex) for 72 h were subjected to ChIP-qPCR analysis using GR antibody. Element +42 and IgG were included as a control. Data are presented as percent input. **(B** and **C)** Total RNA **(B)** or BCL-X_L protein **(C)** levels from total extracts of shControl and shASH2L U937 cells untreated (Control) or treated with 50 nM Dex (Dex) for 72 h were analyzed. **(B)** mRNA levels of BCL-X_L were quantified relative to PUM-1 control by RT-qPCR. Results expressed as a ratio are presented. **(C)** Western blot was performed with the indicated antibody. Probing for β-ACTIN was used as loading control. M, protein molecular weight marker. One representative gel is shown at the top, and the corresponding quantification expressed as a ratio is shown at the bottom. Data are represented as mean ± SEM from at least three independent experiments. Statistical analysis corresponds to two-way ANOVA, $^{**}p<0.01$; $^{***}p<0.001$; n.s.: not significant.

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BCL2L1 promoter and distal HRE adopt a looping conformation in leukemia cells

Since uGR and ASH2L are unexpectedly recruited to promoter and distal +58 HRE BCL2L1 sequences respectively, we sought to determine if this evidence reflects functional enhancer–promoter loops. For this, we analysed by chromosome conformation capture (3C) experiments DNA interactions all along BCL2L1 gene (Figure 5A) not only in wt, shControl and shASH2L U937 cells but also in normal monocytes under control conditions. Thus, cross-linked genomic DNA was digested with SacI and then religated. Fragments 2-4; 2-6 and 2-7 from BCL2L1 gene were amplified by qPCR (Figure 5). Interestingly, an association is localized between the promoter region encompassing P1A/P2 and +58 HRE (fragment 2-7) only in wt and shControl U937 leukemia cells (Figure 5B-D). This association was markedly down-regulated by glucocorticoids (Figure 5C). Instead, in shASH2L cells and normal monocytes this interaction was undetectable (Figure 5D). Based on these observations, we confirm a pivotal role of uGR-ASH2L complex for the promoter region encompassing P1A/P2 and the +58 BCL2L1 HRE physical interaction in leukemia cells. Upon hormone treatment, BCL2L1 looping is affected concomitantly with the GR/ASH2L impediment on chromatin, resulting in direct down-regulation of BCL-X_L expression.

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FIGURE 5

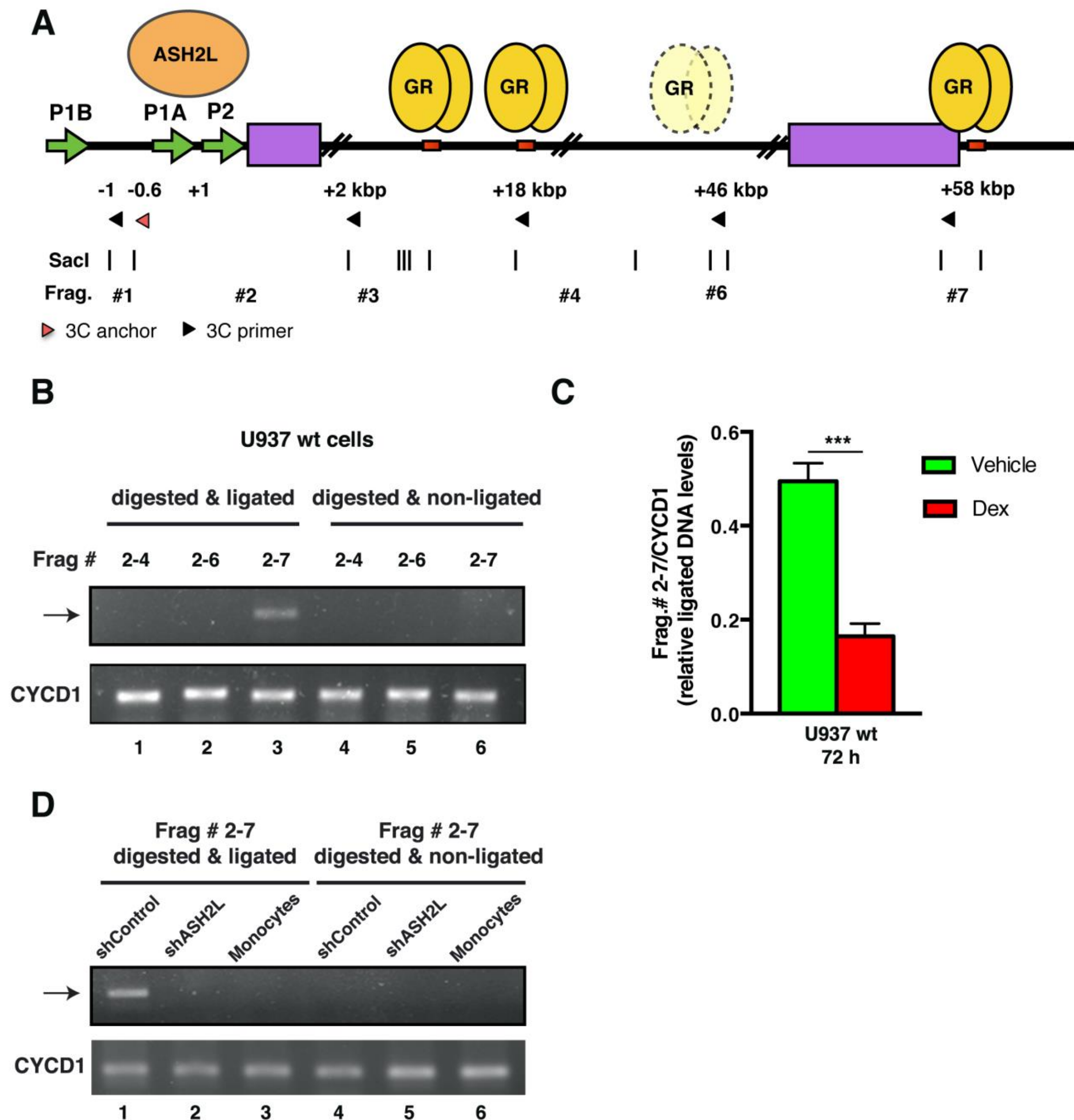

Figure 5. BCL2L1 promoter and distal HRE adopt a looping conformation in leukemia cells. **(A)** Design of 3C analysis along BCL2L1 gene. SacI restriction enzyme sites (black bars) and the resulting fragments (Frag.) are shown as numbers (#1-7) and distances (kbp) from the TSS. The 3C anchor primer shown as red arrow is pairing 3C primers indicated as black arrows. **(B)** 3C analysis with genomic DNA template from U937 wt cells is shown. The arrow indicates the expected qPCR product detecting each 3C fragment using either SacI digested and ligated (lanes 1–3) or SacI digested templates (lanes 4–6). PCRs are shown in the following order: anchor primer #2 pairing with the 3C primers located at Frag. #4, #6 and #7. No qPCR product was obtained using anchor primer #1 or 3C primer #3 (data not shown). An intronic region of the CYCD1 gene was used as a loading control (bottom). One representative result of three

independent 3C experiments is shown. **(C** and **D)** 3C analysis presented as Frag. # 2-7 qPCR products are shown. An intronic region of the CYCD1 gene was used as loading control. Data of genomic DNA template from U937 wt cells untreated (Vehicle) or treated with 50 nM Dex (Dex) for 72 h is relativized to CYCD1 DNA levels. Data are represented as mean ± SEM from at least three independent experiments. Statistical analysis corresponds to Student's t test, ***p< 0.001 **(C)**. Data of shControl U937, shASH2L U937 cells and monocytes is shown as one representative result of three independent 3C experiments **(D).**

ASH2L down-regulation facilitates myeloid leukemia apoptosis comparable to glucocorticoid treatment

Previous reports showed that glucocorticoids induce apoptosis in human U937 leukemia cells ([30] and Figure 6A and 6B). To further examine the contribution of ASH2L to cell death, both early (ANNEXIN V-positive/PI-negative) and late (ANNEXIN V/PI-double positive) apoptotic cells were assessed by Flow Cytometry after FITC-ANNEXIN V/PI staining. Interestingly, shASH2L cells show a pronounced onset of apoptosis upon 72 h of culture with CS-FBS containing-medium, and in the complete absence of steroid treatment (Figure 6A and 6C), strongly reinforcing that ASH2L expression has a crucial role in cell death inhibition of U937 leukemia cells. On the other hand, Dex treatment of shASH2L cells shows similar apoptosis levels compared to untreated (Vehicle) shASH2L cells (Figure 6A and 6C), revealing that ASH2L is required to induce leukemia cell death in response to the steroid treatment. ASH2L relevance is also evident in THPI cells (Figure S4D). Of note, in K562 cells, where BCL-X_L expression does not respond to the GR-ASH2L mechanism, apoptosis is neither affected by Dex nor by ASH2L knockdown (Figure S4D).

Furthermore, our data uncovers a fundamental role for ASH2L in promoting acute myeloid leukemia cell death inhibition through uGR association to BCL2L1 promoter region in U937 cells.

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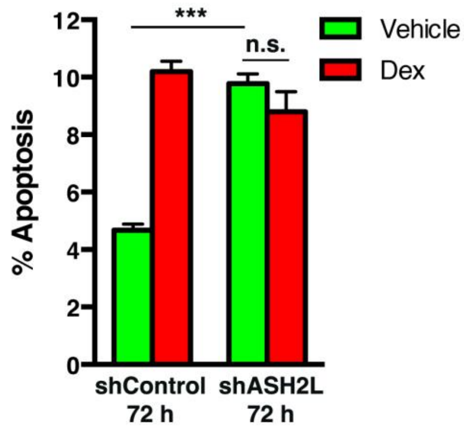
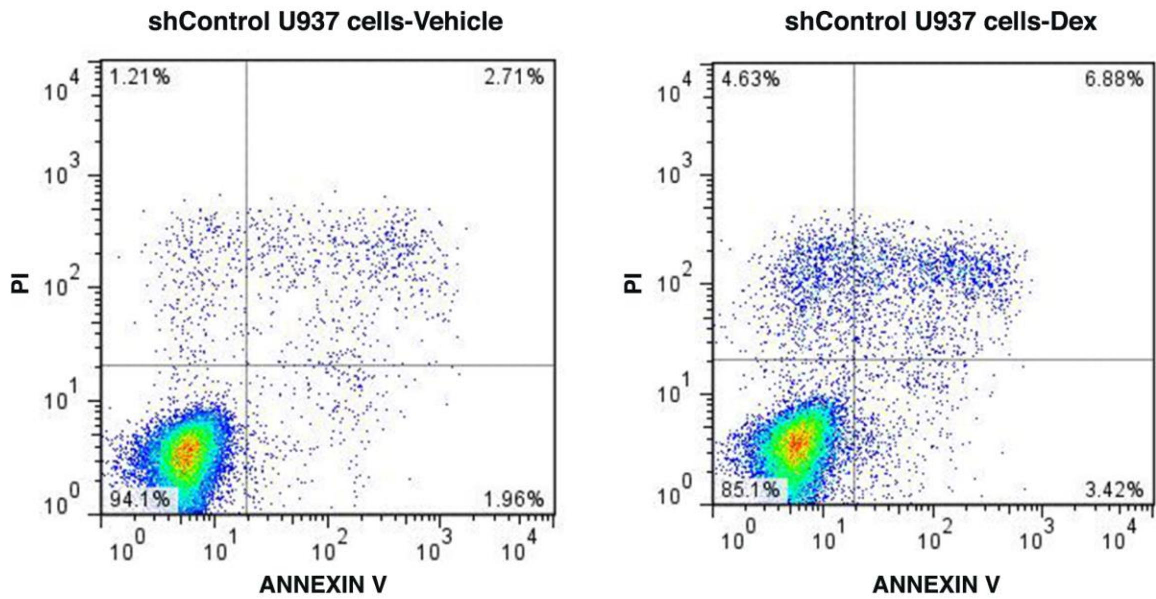
FIGURE 6





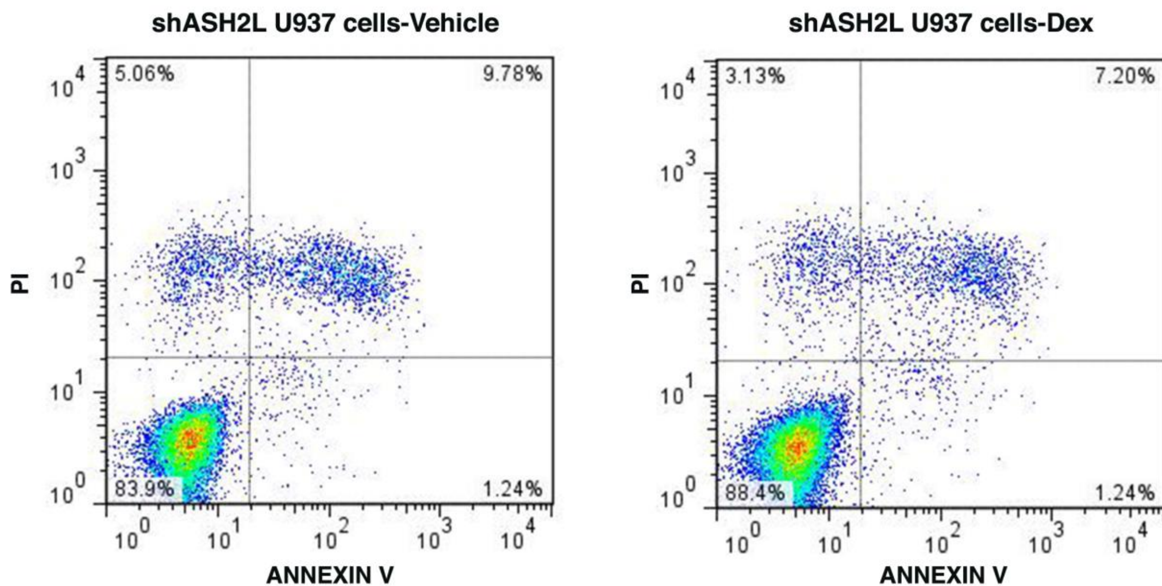


Figure 6. ASH2L down-regulation facilitates myeloid leukemia apoptosis comparable to glucocorticoid treatment

(A) The percentage of untreated (Vehicle) and treated (Dex) shControl and shASH2L U937 positive for ANNEXIN V staining (% apoptosis) is shown. Data are represented as mean ± SEM from at least three independent experiments. Statistical analysis corresponds to two-way ANOVA, ***p<0.001; n.s.: not significant. **(B** and **C)** Representative dotplots of apoptosis analysis of shControl **(B)** and shASH2L **(C)** U937 cells untreated (Vehicle) or treated with 50 nM Dex for 72 h, stained for ANNEXIN V/PI and analyzed by Flow Cytometry.

Taken together, our results suggest that uGR and ASH2L are mutually involved in a physical and transcriptional looping model dedicated to maintaining BCL-X_L over-expression. Moreover, the presence of glucocorticoids prevents the looping formation, represses BCL-X_L transcription and facilitates myeloid leukemia apoptosis (Figure 7).

FIGURE 7

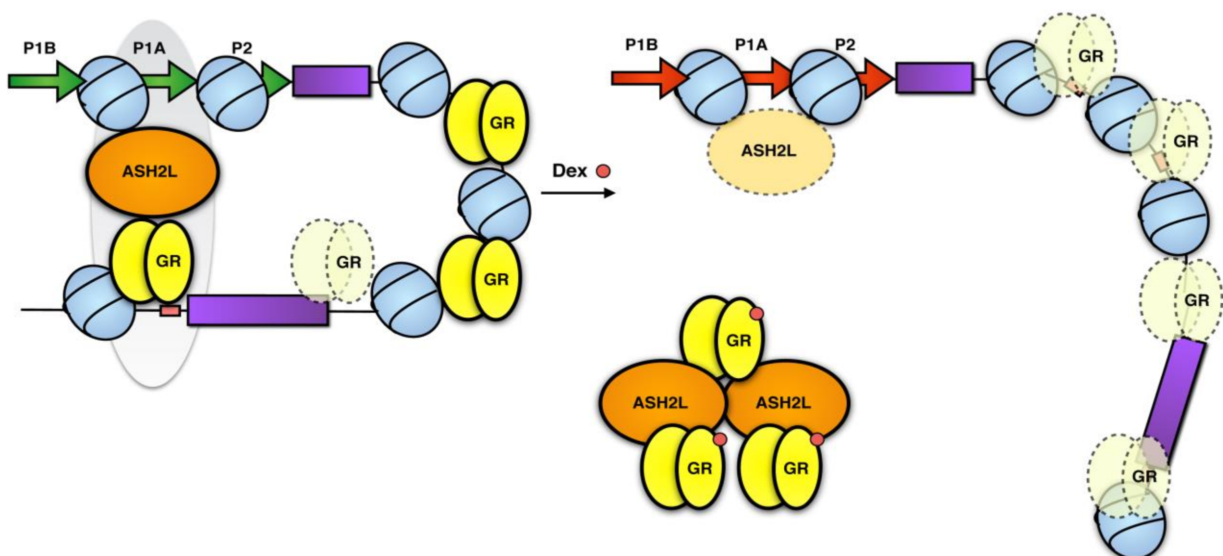


Figure 7. Model for the involvement of uGR and ASH2L in the transcriptional regulation of BCL2L1 gene in U937 leukemia cells.

Anti-apoptotic BCL-X_L isoform is over-expressed in U937 wt cells, whereby uGR and ASH2L contribute to a looping formation between P1A/P2 promoter and distal +58 HRE enhancer regions. Administration of Dex promotes BCL-X_L expression down-regulation by avoiding looping formation and a significant decrease in GR and ASH2L recruitment to chromatin. Solid-line oval icons indicate high recruitment and dash-line oval icons indicate low recruitment.

DISCUSSION

There is growing evidence supporting the idea that altered expression of BCL-2 family proteins might influence chemotherapy or radiation-induced apoptosis in malignant cells and therefore could confer multidrug resistance [2, 4]. Targeting the apoptosis machinery is a promising therapeutic approach in AML. In most cases, the main goal is to eliminate leukemia cells by activating cell death pathways, either throughout direct targeting of anti-apoptotic proteins, such as those of the BCL-2 family, or by reactivating the p53 response [31]. However, a large portion of patients harbours non-druggable genomic alterations, and how molecular alterations will correlate with clinical activity of modulators of apoptosis is currently largely unknown. The existence of such mechanisms demands the study of the regulation of genes in charge of controlling apoptosis in cancer. We have chosen BCL2L1 as our gene model, because in addition to its fundamental contribution to the regulation of cell death programs triggered by glucocorticoids [15, 17], BCL-X_L (BCL2L1) overexpression has been shown to play a crucial role in AML pathogenesis [6, 13]. Indeed, when comparing endogenous anti-apoptotic BCL-X_L expression, normal human monocytes show significantly lower levels of BCL-X_L as compared to malignant U937 cells ([26] and Figure 1). Moreover, we report here that these patient-derived myeloid leukemia cells grown in depleted serum-containing medium showed even higher BCL-X_L levels, suggesting that steroid deprivation stimulates the anti-apoptotic isoform expression. We therefore hypothesized that GR, in the absence of ligand, might modulate BCL2L1 gene expression in U937 cells. Interestingly, some reports argue that the uGR not only mainly localizes in the nucleus of different cell types, including human monocytes [32-34], but it is also transcriptionally active, modulating basal transcription of several endogenous genes [32, 35-37]. This idea is reinforced by our evidence that uGR is widely recruited to BCL2L1 gene in U937 leukemia cells upon 72 h of steroid deprived-

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containing medium (Figure 2). Oppositely, in the presence of glucocorticoids GR recruitment to BCL2L1 HREs diminishes, resulting in BCL-X_L down-regulation.

Butler and colleagues have recently reported that ASH2L over-expression in AML patients contributes to leukemogenesis by cooperating with other proteins that aberrantly up-regulate proliferation and survival pathways [38]. Strikingly, we provide evidence that ASH2L interacts with GR in myeloid leukemia cells and is recruited to BCL2L1 promoter region (Figure 3). Concomitant to glucocorticoid-dependent GR binding reduction to BCL2L1 HREs, ASH2L recruitment and H3K4me3 epigenetic mark also decrease, indicating that ASH2L is directly participating in this GR-mediated gene regulation. Moreover, uGR and ASH2L are both involved in BCL-X_L over-expression of myeloid leukemia cells (Figure 4), and the observation that BCL2L1 promoter region and distal +58 HRE adopt a looping conformation in the absence of hormone (Figure 5) could represent a potential elegant mechanism whereby uGR and ASH2L might contribute to AML pathology. We believe that +10 and +18 HREs that are not involved in this DNA structure may modulate another stage of the transcription process, *ie* transcription elongation. In this sense, our group has reported a novel mechanism complementary to the classical model of nuclear receptor dependent gene expression regulation, where progesterone receptor regulates BCL2L1 transcription in human mammary epithelial cells by modulating the elongation process through intragenic HREs [28].

In summary, we propose that in the absence of hormone it is the presence of ASH2L at P1A promoter what determines the looping formation with the uGR bound to +58 HRE. Upon Dex addition, GR and ASH2L show a stronger interaction between each other that leads to a decreased recruitment of both proteins to their corresponding binding regions. Consequently, chromatin looping formation is reduced. After ASHL2 silencing, looping formation does not occur, thus GR bound to +58 HRE is unable to drive transcription,

despite its recruitment to HREs is maintained or even increased in the presence of Dex. Further characterization and genome extent of this complex will be instrumental in defining a general epigenetic mechanism for myeloid leukemia cell progression.

A major impairment to successfully treat leukemia patients is glucocorticoid resistance, with consequent relapse of the disease [39, 40]. Therefore, identification of protein complexes that can effectively synergize with or provide an alternative to dexamethasone is of paramount relevance. Low expression of ASH2L protein was shown to correlate with a favourable outcome in AML [38] and, interestingly, our findings that ASH2L down-regulation facilitates U937 and THPI acute myeloid leukemia cell death in the absence of glucocorticoids (Figure 6 and Figure S4) strongly suggests that ASH2L activity may be playing a relevant role in GR-mediated BCL-X_L expression in this leukemic context.

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CONFLICT OF INTEREST

There is no conflict of interest to be declared by any author.

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CRediT author statement

Luciana Rocha-Viegas: Conceptualization, Investigation, Validation, Formal Analysis, Supervision, Visualization, Writing–Original Draft, Writing–Review & Editing, Project Administration, Funding Acquisition. Micaela Silbermins: Validation. María Florencia Ogara: Methodology. Joaquín Miguel Pellegrini: Resources. Sol Yanel Nuñez: Resources. Verónica Edith García: Resources. Writing–Review & Editing. Guillermo Pablo Vicent: Methodology, Writing–Review & Editing. Adali Pecci: Conceptualization, Supervision, Writing–Review & Editing, Funding Acquisition.

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Highlights

- GR and ASH2L are recruited to BCL2L1 gene and contribute to BCL-X_L over-expression
- GR and ASH2L interact in a common protein complex through a gene-loop conformation.
- Glucocorticoids decrease GR and ASH2L occupancy and promote BCL-X_L down-regulation
- ASH2L knockdown facilitates acute myeloid leukemia cell death.

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