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Targeting MYC Overexpressing Leukemia with Cardiac Glycoside Proscillaridin Through Downregulation of Histone Acetyltransferases

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ABSTRACT

Targeting MYC oncogene remains a major therapeutic goal in cancer chemotherapy. Here, we demonstrate that proscillaridin, a cardiac glycoside approved for heart failure treatment, causing Na⁺/K⁺ pump inhibition, targets efficiently MYC overexpressing cancer cells. At clinically relevant doses, proscillaridin induced rapid downregulation of MYC protein level, and produced growth inhibition preferentially against MYC overexpressing leukemic cell lines including lymphoid and myeloid stem cell populations. Transcriptomic profile of leukemic cells after treatment showed a downregulation of gene sets involved in MYC pathways, cell replication and an upregulation of genes involved in hematopoietic differentiation. Gene expression changes were associated with an epigenetic remodeling of chromatin active marks. Proscillaridin induced a significant loss of lysine acetylation in histone H3 (at lysine 9, 14, 18 and 27). In addition, loss of lysine acetylation was observed also in non-histone proteins such as MYC itself, MYC target proteins, and a series of histone acetylation regulators. Global loss of acetylation correlated with the rapid downregulation of histone acetyltransferase proteins (such as CBP and P300) involved in histone and MYC acetylation. Overall, these results strongly support the repurposing of proscillaridin in MYC overexpressing leukemia and suggest a novel strategy to target MYC by inducing the downregulation of histone acetyltransferases involved in its stability.

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INTRODUCTION

MYC (c-MYC) transcription factor is a major driver of oncogenic transcriptional programs. It contributes to gene dysregulation in cancer by promoting expression of genes involved in cell proliferation (1). High MYC expression drives tumor initiation, progression, and maintenance and is associated with aggressive cancers and poor prognoses (2,3). MYC is a potent driver in leukemia inducing cell proliferation and blocking cell differentiation (4). Moreover, MYC contributes to long-term self-renewal of leukemic stem cells (5). Conversely, genetic suppression of MYC in transgenic mouse models induces differentiation and cell growth arrest of leukemic cells (6-8). Therefore, targeting MYC addiction in leukemia is a major therapeutic goal. Since MYC lacks a catalytic site, its direct inhibition has been extremely challenging. Indirect MYC inhibition demonstrated therapeutic efficacy with bromodomain inhibitors (such as JQ1 or THZ1), by blocking MYC transcriptional effects (9-13). Unfortunately, cancer cells, such as leukemia, breast and ovarian cancers, develop resistance to these inhibitors by compensatory mechanisms using other bromodomain containing proteins or kinome reprogramming (14-16). Together, these studies highlight the need to develop new strategies to abrogate MYC addiction in cancer.

MYC stability is regulated by post-translational modifications and MYC acetylation increases its stability (17,18). The deposition of acetyl groups on lysine residues is catalyzed by lysine acetyltransferases (KATs), which acetylate also histone proteins causing chromatin opening and gene activation (19). Lysine acetyltransferase pharmacological inhibition represent an interesting strategy to target indirectly MYC by blocking upstream mechanisms involved in its stability. However, KATs have overlapping targets and commercially available KAT inhibitors require further optimization (20).

While screening more than 1,000 FDA-approved drugs for repurposing in oncology, we reported that cardiac glycosides, which are approved for heart failure treatment, exhibit significant epigenetic and

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anticancer effects (21,22). Cardiac glycosides, including digitoxin, digoxin, lanatoside, ouabain and proscillaridin, triggered reactivation of epigenetically silenced tumor suppressor genes (22). Moreover, all cardiac glycosides produced synergistic responses when used in combination with the epigenetic drug decitabine (demethylating agent), further supporting their epigenetic activity (21). Several epidemiological studies argue in favor of repurposing cardiac glycosides in oncology. Indeed, several reports showed that patients treated with cardiac glycosides for heart failure have a lower rate of cancer diagnosis as compared to the general population (23). Upon cancer diagnosis, these patients exhibit generally a less aggressive disease and responds better to therapy (23,24).

Repurposing cardiac glycosides in oncology is limited by their narrow therapeutic window, for which maximal plasmatic level is around 10 nanomolar, due to cardiac toxicities (23,25-27). Several *in vitro* and *in vivo* studies tested their anticancer activity at supra-pharmacological doses, which are not reachable in humans; in particular, in rodents who can tolerate high doses of these drugs due to structural differences in Na⁺/K⁺ pump as compared to human (26,28,29). Since the repurposing of cardiac glycosides is restricted to the low nanomolar range, we sought to identify cancer types highly sensitive to these drugs. To do so, we screened a panel of human cancer cell lines with proscillaridin, which was identified as the most potent cardiac glycoside in our previous screens (21,22). Proscillaridin produced antiproliferative effects with a preferential selectivity towards MYC overexpressing leukemia cells. We demonstrated that proscillaridin produced a global loss of acetylation in chromatin and MYC itself, producing epigenetic effects and MYC downregulation. These results provide compelling evidence for the repurposing of cardiac glycoside proscillaridin against leukemia driven by MYC oncogenic signature.

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MATERIALS AND METHODS

Cell Culture and Drug Treatments

A panel of 14 human cancer cell lines and *hTERT/SV40ER*-immortalized human primary fibroblasts transformed with *MYC*, *RAS*^{V12} or *MYC* and *RAS*^{V12} were used in the study. Cell types and culture conditions are described in Supplementary Materials and Methods. Proscillaridin was purchased from Santa Cruz Biotechnologies. IC₅₀ values were calculated with GraphPad Prism software.

Protein and Histone Extractions

Whole cell proteins were extracted using cold whole-cell lysis buffer (50 mM Tris-Cl pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton, 0.1 mM Na₃VO₄, and 1 mM PMSF), supplemented with Complete™ Protease Inhibitor Cocktail (Roche). Histones were harvested using acid-extraction method with cold Triton Extraction Buffer (TEB; 0.5% Triton, 2 mM PMSF, 0.02% NaN₃, 10 mM sodium butyrate), supplemented with protease inhibitor cocktail. Protein extracts were separated by SDS-PAGE and transferred onto a polyvinyl difluoride membrane. All experiments were performed in triplicate. Antibodies are listed in the Supplemental Materials and Methods section.

RNA Extraction, Sequencing and Analysis

QIAshredder was used to homogenize cell lysates and eliminate debris prior to RNA extraction using RNeasy Mini Kit. Briefly, 10 µg of purified RNA was treated with DNase and quantified by Agilent RNA 6,000 Nano kit bioanalyser chips. 1 µg of mRNA was used for library preparation with TruSeq Stranded mRNA LT. RNA sequencing was performed using HiSeq 2500. Experiments were performed in triplicate. Reads were aligned to human genome (hg19) using STAR v2.4.2 and differential gene expression analysis between untreated and treated cells was done using DESeq2 v1.10.1 (30,31). For

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bioinformatics analyses, data were processed using gene set enrichment analyses (GSEA, broadinstitute.org/gsea), metascape (metascape.org) and gene mania (genemania.org). MOLT-4 cells H3K27ac ChIP-seq data from publicly available dataset (GEO: GSM2037790) were used in associated with transcriptomic data. GSEA analysis of 8227 AML fractions and the LSC signatures was performed using the control sample data from GSE55814. GEO2R was used to generate a ranked list of LSC-related genes (6 LSC CD34⁺CD38⁻ samples vs 12 non-LSC CD34⁻ samples) used in GSEA analysis.

Acetylation Analysis by Immunoprecipitation and Mass Spectrometry

Whole cell protein extracts were incubated overnight with 5 µg/ml of MYC antibody (Abcam, AB32072). After immunoprecipitation and transfer, proteins were probed with lysine pan-acetyl antibody (1:2500 Cell Signaling 9681). For acetylome analysis by mass spectrometry, samples were prepared as previously described (32). Briefly, 4 biological replicates of untreated and proscillaridin-treated MOLT-4 cells (5 nM, 48h) were digested with trypsin. Peptides were analyzed by mass-spectrometry and data were extracted with the MaxQuant software package (version 1.5.5.1) and subsequently analyzed using an in-house computational pipeline for statistical analysis of relative quantification with fixed and/or mixed effect models, implemented in the MSstats Bioconductor package (version 3.3.10) (33,34). Peptides were searched with SwissProt human protein database.

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RESULTS

Cardiac Glycoside Proscillaridin Targets MYC-Driven Leukemic Cells

To identify cancer types with high sensitivity to proscillaridin in order to obtain concentrations within their therapeutic window, we screened a panel of 14 human cancer cell lines and measured cancer cell proliferation after a 24h treatment. Upon calculating the half-maximal inhibitory concentration (IC_{50}), we noticed a 2,800-fold difference in IC_{50} , with leukemic cells being more sensitive to proscillaridin (Supplementary Figure 1a). To explore the cause of this striking difference, we hypothesized that the oncogenic context might influence drug efficacy. By comparing protein expression of MYC oncogene (in untreated cancer cells) with proscillaridin IC_{50} values, we found that there was a significant inverse correlation ($p = 0.0172$; Supplementary Figure 1b). Proscillaridin produced a more potent growth inhibition in cells expressing high levels of MYC protein, such as acute lymphoblastic T-cell (MOLT-4) and B-cell (NALM-6) leukemia while being less effective in colorectal (SW48) and lung (A549) cancer cells expressing low levels of MYC (Figure 1a).

To evaluate MYC contribution in cancer cell sensitivity to proscillaridin, we investigated drug response using an isogenic cell system consisting of *hTERT/SV40ER*-immortalized human primary fibroblasts, transformed with different oncogenes including *MYC*, *RAS^{V12}* or the combination of both oncogenes. This system allowed exploring the effect of oncogenic transformation within the same genetic background. We choose this approach as opposed to MYC overexpression or knockout genetic manipulations in leukemic cell lines since MYC overexpression was shown to amplify more robustly already MYC-dependent genes, and MYC knockout in leukemia leads to proliferation arrest, apoptosis and/or senescence (6,35,36). After transfection, *MYC*-transformed fibroblasts had a small and round phenotype whereas *RAS^{V12}*-transformed cells displayed increased vacuole formation and large cytoplasm. *MYC* and *RAS^{V12}*-transformed cells exhibited a round phenotype with vacuole formation in

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the cytoplasm (Supplementary Figure 1c). High levels of MYC and RAS protein levels were detected after transfections when compared to non-transfected cells (Figure 1b). Using a wide range of proscillaridin concentrations (from 0.01 nM to 100 μ M) for 48h, we measured cell viability and calculated IC₅₀ values (Figure 1c). Untransformed fibroblasts were fully resistant to proscillaridin. Likewise, RAS^{V12} transformed cells were mildly affected by the treatment where proscillaridin at high doses failed to impact cell viability by more than 50%. Conversely, MYC transformed fibroblasts were highly sensitive to proscillaridin with an IC₅₀ value of 70 nM. Moreover, MYC and RAS^{V12} transformed fibroblasts (referred as to RAS^{V12}+MYC) had a low IC₅₀ value (132 nM) despite the presence of RAS^{V12}. Consequently, MYC overexpression was driving proscillaridin sensitivity in transformed fibroblasts. In comparison, after 48h treatment, MOLT-4 and NALM-6 cells (MYC overexpressing leukemic cells) showed IC₅₀ values of 2.3 nM and 3 nM, respectively, which correspond to clinically achievable concentrations (Supplementary Figure 1d).

To explore the mechanism by which MYC overexpression in cancer cells correlates with proscillaridin sensitivity, we compared its effects between MYC driven leukemic cells (MOLT-4 and NALM-6) and low expressing MYC cancers driven by KRAS mutations (SW48 colon and A549 lung cancer cells). We found that proscillaridin (5 nM; 48h) significantly reduced MYC protein level by more than 50% in MOLT-4 and NALM-6 cells but not in SW48 and A549 cells (Figure 1d). Time-course experiments with both leukemic cell lines (8h to 96h) showed that proscillaridin induced a significant (up to 80%) and rapid MYC downregulation (Figure 1d; Supplementary Figure 1e). These results demonstrate that low dose proscillaridin inhibits efficiently leukemia growth causing rapid MYC downregulation.

Proscillaridin Efficiently Targets MYC-Driven Leukemic Stem Cell Populations

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We sought to determine whether proscillaridin could target leukemic stem cells (LSCs) (37-39). To explore this possibility, we used two LSC models, a mouse model of T-ALL and a LSC model of human acute myeloid leukemia (AML) (37,40,41). First, pre-LSCs T-ALL cells were isolated from a transgenic mouse model that closely reproduces human T-ALL (42). We previously showed that these pre-LSCs are driven by the *SCL/TALI* and *LMO1* oncogenes, which depend on *NOTCH1-MYC* pathways, and are resistant to chemotherapeutic drugs used against leukemia (doxorubicin, camptothecin and dexamethasone) (5,37). Low concentrations (3-10 nM) of proscillaridin significantly decreased pre-LSC T-ALL viability by 70% after 4 days of treatment (Figure 2a). Despite being resistant to chemotherapeutic drugs, these pre-LSCs (T-ALL) were sensitive to proscillaridin at clinically relevant doses (37).

Then, we used primary human AML 8227 cells, which contain functional LSCs within the CD34⁺ sub-population, and non-LSC cells characterized by CD34⁻ with or without CD15⁺ expression (Figure 2b) (40,41). Gene set enrichment analysis from transcriptomic data published by Lechman et al. revealed that the LSCs-enriched fraction (CD34⁺/CD38⁻) in AML 8227 are enriched for MYC target genes expression as compared to non-LSCs (CD34⁻) (Figure 2c) (40). After 6 days of proscillaridin treatment, bulk AML 8227 cells had an IC₅₀ of 29 nM (Figure 2d). Likewise, CD34⁻ with or without CD15⁺ non-LSC cells had IC₅₀ of 38 nM and 29 nM, respectively. In contrast, all CD34⁺ AML cells (CD34⁺, CD34⁺/CD38⁺ and CD34⁺/CD38⁻) were more sensitive to proscillaridin with IC₅₀ values of 15 nM. Altogether, proscillaridin efficiently targets LSC-enriched populations, in both T-ALL and AML models marked by high MYC expression, further supporting its repurposing against MYC-dependent leukemia.

Proscillaridin Downregulates Cell Proliferation Programs and Induces T-Cell Differentiation

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To gain insight into proscillaridin effects against MYC-driven leukemic cells, we investigated drug-induced gene expression changes in T-ALL cells (MOLT-4). By quantitative RT-PCR (qPCR), we found that proscillaridin significantly downregulated MYC mRNA after 16h treatment and up to 90% after 48h (Figure 3a). Then, we used RNA-sequencing to explore transcriptomic effects of proscillaridin (5 nM; 48h) in MOLT-4 cells. After drug treatment, transcriptome analysis showed a downregulation of 2,759 genes ($\log_2FC < 0.5$; P-value adjusted < 0.05) and concomitant upregulation of 3,271 genes ($\log_2FC > 1$; P-value adjusted < 0.05 ; Supplementary Figures 2a and b). Using Metascape, gene ontology analysis revealed that downregulated genes were involved in DNA replication, biosynthesis and metabolic processes (Figure 3b; Supplementary Figure 2c). Consistent with qPCR results, *MYC* transcript was significantly downregulated in our RNA-sequencing data set (Figure 3c). Gene Set Enrichment Analysis showed that MYC PATHWAY (which includes 30 MYC target genes) was significantly downregulated (Figure 3c). Notably, these transcriptomic effects correlated with a 25% decrease of S-phase cells as measured by BrdU staining (Figure 3d; Supplemental Figure 2d). Proscillaridin also significantly downregulated 11 T-cell leukemia master transcription factors (Figure 3e) (43-45). These data support that proscillaridin efficiently inhibits proliferation programs in MYC-driven leukemia.

Gene ontology analysis also revealed that upregulated genes were enriched for hematopoietic or lymphoid organ development, suggesting the onset of leukemia differentiation (Figure 3f and Supplemental Figure 2e). To probe the functional significance of this change, we measured T-cell differentiation markers in MOLT-4 cells before and after treatment. By qPCR, mRNA levels of T-cell differentiation markers *NOTCH3* and its target *HES1* were upregulated after 48h treatment and remained expressed for 2 days after drug removal (Figure 3g) (46,47). By flow cytometry, we measured a significant increase in TCR and CD3 expression, which lasted up to 4 days after drug removal,

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suggesting the onset of normal T-cell activation. Upregulation of these differentiation markers were in the same range than the levels measured after TPA treatment, a well-known inducer of leukemia differentiation (Figures 3h and i) (48,49). Altogether, proscillaridin treatment produced a transcriptomic shift from a proliferative program to the induction of T-cell leukemia differentiation.

Proscillaridin Induces Global Loss of Histone H3 Acetylation

Since proscillaridin induced gene expression and phenotypic changes, we hypothesized that it triggers epigenetic effects in high MYC-driven leukemia. We analyzed histone H3 and H4 post-translational modifications by western blotting after 16h to 96h of proscillaridin treatment (5 nM). We found that proscillaridin produced a significant time-dependent reduction (by 75%) of lysine acetylation at H3K9, H3K14, H3K18, H3K27 residues and global loss of H3 acetylation in MOLT-4 cells (Figure 4a; Supplemental Figure 3a). The dramatic reduction in H3K27ac level was confirmed by chromatin immunoprecipitation where H3K27ac antibody pulled-down similar levels of DNA than IgG after treatment (Supplemental Figure 3b). Similar results were obtained in NALM-6 cells after proscillaridin treatment (Supplemental Figure 3c). No change was detected on H4 acetylation or H3 methylation marks (Supplemental Figures 4a and b; and data not shown). Interestingly, loss of H3 acetylation induced global chromatin reorganization in MOLT-4 cells after treatment, as shown by DAPI staining (Supplemental Figure 4c).

We next asked if there was a correlation between loss of H3 acetylation and gene expression changes after proscillaridin treatment. To address this question, we combined our RNA-Seq data pre- and post-treatment with H3K27ac ChIP-seq data of untreated MOLT-4 (50) since this mark is associated with transcribed regions and is lost globally after treatment (Figure 4a) (23). Among 7,097 genes marked at their promoters with H3K27ac (-500 to +500 bp) in untreated MOLT-4 cells, 2,169 genes

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were differentially expressed after proscillaridin treatment. Seventy-four percent of those (1,608 genes), marked by H3K27ac in untreated cells, were significantly downregulated after treatment (Figure 4b), which is consistent with the loss of this active epigenetic mark (Figure 4c). Gene ontology analysis of these 1,608 downregulated genes showed a significant relationship with metabolism and proliferation processes (Figure 4d; Supplemental Figure 5a). Among these genes, all MYC PATHWAY genes (n=30) previously described (Figure 3c) were marked by H3K27ac in untreated MOLT-4 cells and were all downregulated by treatment (Figures 4 c and e). Network analysis showed that these MYC target genes are co-expressed simultaneously, and are known to exhibit protein-protein interactions with MYC, confirming the global effect of proscillaridin on MYC pathway (Supplemental Figures 6a and b). By contrast, upregulated genes marked by H3K27ac in untreated cells were associated with apoptosis, negative regulation of proliferation and cell differentiation (Supplemental Figure 5b), corroborating our transcriptomic and functional analyses. Collectively, these results demonstrate that proscillaridin produces global loss of H3 acetylation, which was associated with silencing of genes involved in proliferation and MYC pathway.

Proscillaridin Induces Loss of Lysine Acetylation in MYC Target Genes and Chromatin Regulators

We then asked whether depletion of lysine acetylation was extended to non-histone proteins after treatment. First, we measured MYC acetylation levels after 8, 16 and 24h of proscillaridin treatment (5 nM) in MOLT-4 cells, since this posttranslational modification plays a role in its stability (17,51). After MYC immunoprecipitation and probing with a pan-acetyl antibody, we measured a time dependent decrease (up to 75%) of MYC total acetylation (Figure 5a).

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To further characterize the extent of acetylation loss, we conducted an acetylome study by mass spectrometry on untreated and proscillaridin-treated (5 nM; 48h) MOLT-4 cells (Figure 5b). Two distinct MYC peptides showed a significant reduction in lysine acetylation after treatment, which confirmed our immunoprecipitation results (Supplemental Figure 7a). Mass spectrometry analysis showed that 28 peptides (including MYC) had a significant loss of lysine acetylation after treatment, associated with chromatin organization (Figure 5c). Among them, 8 are known MYC target proteins and 6 are involved in chromatin organization (Figures 5d and e). Networks analysis showed that these 28 proteins are generally co-expressed, suggesting a connection between their acetylation and expression levels (Supplemental Figure 7b). Interestingly, 8 out of 28 proteins were MYC target proteins, including MYC itself and 6 out of 28 are involved in histone acetylation regulation (Figures 5d and e) (52). Altogether, proscillaridin reduces lysine acetylation of MYC, its protein partners and several histone acetylation regulators.

Proscillaridin Efficiently Downregulates Histone Acetyltransferases Involved in MYC Acetylation

We next investigated whether acetylation loss was due to a dysregulation of histone acetyltransferases (KATs). We measured, by western blotting, KAT levels before and after proscillaridin treatment (5 nM; 8h-96h) in MOLT-4 cells. Proscillaridin produced a time-dependent reduction (up to 80%) of several KATs including KAT3A (CBP), KAT3B (P300), KAT5 (TIP60), KAT2A (GCN5) and KAT6A (MOZ) (Figure 6a, Supplemental Figure 8a). Expression of KAT2B (PCAF) and KAT7 (HBO1) were not altered by the treatment (Supplemental Figures 8a and b). No significant changes were observed in class I HDACs expression, suggesting that acetylation loss mainly involved KATs downregulation (data not shown). Interestingly, KAT downregulation was observed only at the protein level, since their mRNA levels were not altered after treatment (data not shown). Significant reduction in KAT protein expression

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including KAT2A, KAT3A, KAT3B and KAT6A, which target histone H3, occurred 8h prior to significant H3 acetylation loss. Despite KAT5 decrease, a KAT known to acetylate H2A, H3 and H4, no changes in H4 acetylation (total or on specific lysines) were measured after treatment (Supplemental Figure 4a) (53-57). This result can be explained by the fact that KAT7 (HBO1) expression, which is also involved in H4 acetylation, was not affected by the treatment (58,59). To confirm the effects of KAT downregulation in MOLT-4 cells, we used KAT3A/B pharmacological inhibitor C646. Similar to proscillaridin treatment, C646 (10 μ M; 48h) significantly reduced lysine acetylation (H3K14, H3K18, H3K27, and total H3-acetylation), depleted KATs (KAT3A, and KAT3B) and MYC protein levels (Figures 6b and c; Supplemental Figures 8c and d).

Since KATs have overlapping enzymatic activities, we asked if the extent KAT protein downregulation was associated with proscillaridin sensitivity. We compared KAT protein levels before and after proscillaridin treatment in *MYC* overexpressing cancer cells (MOLT-4, NALM-6, *MYC* and *RAS*^{V12}+*MYC* transformed fibroblasts) versus low *MYC* expressing cancer cells (SW48, A549, and *RAS*^{V12} transformed fibroblasts; Figure 6d; Supplemental Figure 9a). Cancer cell lines were treated at 5 nM for 48h, which was clinically relevant and close the IC₅₀ values of leukemic cells. Transformed fibroblasts were treated at 70 nM for 48h, which was the IC₅₀ value of *MYC* transfected fibroblasts as described in Figure 1b. After treatment, we observed that KAT protein downregulation was more pronounced in drug-sensitive cells with high *MYC* expression as compared to drug-resistant cells with low *MYC* expression. Indeed, proscillaridin induced a significant downregulation of 4/7 KATs in MOLT-4 cells, 3/7 KATs in NALM-6 cells, 3/7 KATs in *RAS*^{V12}+*MYC* transformed fibroblasts, and 7/7 KATs in *MYC* transformed fibroblasts. Interestingly, downregulated KATs (KAT2A/GCN5, KAT3A/CBP, KAT3B/P300, KAT5/TIP60 and KAT6A/MOZ) in *MYC* overexpressing cells, were shown to acetylate MYC and increase its stability (17,51,60-64). In stark contrast, proscillaridin failed to

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downregulate more than one KATs in low *MYC* expressing cancer cells (SW48, A549 and *RAS*^{V12} transformed fibroblasts). Thus, proscillaridin-induced KAT proteins downregulation was more important in high *MYC* expressing cells, which correlated with IC₅₀ values within its therapeutic range.

Similar analysis was performed on histone H3 acetylation between high *MYC* expressing cancer cells versus low *MYC* expressing cancer cells (Supplemental Figures 9b and c). Proscillaridin induced a significant loss of H3 acetylation in drug-sensitive and *MYC* overexpressing cells (MOLT-4, NALM-6, *MYC* and *RAS*^{V12}+*MYC* transformed fibroblasts). In proscillaridin-resistant and low expressing *MYC* cancer cells, histone acetylation levels were unchanged in SW48 cells after treatment, which correlated with our previous report (Supplemental Figures 9b and c) (22). By contrast, A549 cells lost significantly H3 acetylation after treatment while *RAS*^{V12} transformed fibroblasts lost acetylation on some sites (K9, K27 and pan-acetyl) and other sites were not affected (K14 and K18) (Supplemental Figures 9b-c). These data suggest that proscillaridin sensitivity is not entirely dependent on histone acetylation loss, suggesting the importance of non-histone acetylation. In summary, proscillaridin antiproliferative effect was associated with its ability to downregulate several simultaneously KATs resulting loss of acetylation in histone and non-histone proteins (Figure 6e).

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Discussion

The repurposing potential of cardiac glycosides in oncology has been suggested several decades ago and is currently under intense clinical investigation either alone (in prostate cancers, NCT01162135; breast cancer, NCT01763931; and sarcoma, NCT00017446) or in combination with chemotherapy (digoxin with cisplatin in head and neck cancers, NCT02906800; or with epigenetic drug decitabine, NCT03113071) (65,66). Here, our data provide a strong rationale to repurpose proscillaridin specifically against leukemia with *MYC* oncogenic dependency.

Proscillaridin induced a rapid loss of MYC protein expression in *MYC*-driven leukemia cells. Importantly, proscillaridin efficiently targeted *MYC*-dependent leukemic stem cells, indicating the potential of controlling leukemia self-renewal capacity. We demonstrated that proscillaridin targeted *MYC* overexpressing leukemic cells by downregulating KATs involved specifically in *MYC* acetylation and transcriptional program (KAT2A, KAT3A, KAT3B, KAT5 and KAT6A) (17,51,60-64). These KATs are positive regulators of *MYC* stability and *MYC* pathway. Importantly, KATs have overlapping activity and targets, suggesting the relevance of targeting these enzymes simultaneously to efficiently reduce acetylation of *MYC* and its partners (17,61,62,64). Indeed, proscillaridin-induced downregulation of several KATs was observed in proscillaridin-sensitive and *MYC* overexpressing leukemic cells whereas this effect was sporadic in resistant and low *MYC* expressing cells. Proscillaridin treatment induced a significant loss of H3 acetylation levels in *MYC* overexpressing cells whereas loss of histone acetylation was not observed in SW48 cells but was significantly reduced in A549 and in two lysine residues in *RAS*^{v12}-transfected fibroblasts, suggesting that loss of H3 acetylation is not sufficient to modulate cell viability in these low *MYC* expressing cells. These data highlight the importance of acetylation levels in non-histone proteins as a potential therapeutic target in *MYC* overexpressing leukemia. Experiments are ongoing to address this specific question.

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Lysine acetylation is a dynamic process that can be modulated within minutes and it is maintained on histone and non-histone proteins by the redundant activity of KATs. Therefore, cancer cells may rapidly recover from incomplete pharmacological inhibition or from the specific inhibition of a particular KAT (53). Here, we showed that proscillaridin treatment in MYC overexpressing leukemia cells, led to the downregulation of several KATs, produced MYC inhibition, and induced persistent leukemia cell differentiation, which was maintained for several days after drug removal. Therefore, this study supports a strategy of simultaneously targeting several KATs to reduce efficiently acetylation in histone and in non-histone proteins, which overcome the redundant activity of KATs. The mechanism implicated in proscillaridin-induced KATs downregulation is under investigation. Overall, we conclude that proscillaridin downregulates MYC protein levels and MYC oncogenic pathway in leukemia through the downregulation of several KATs.

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Figure legends:

Fig. 1. Targeting High MYC Expressing Cancer Cells with Cardiac Glycoside Proscillaridin. **a** Cell viability and half-maximal inhibitory concentration (IC_{50}) calculations after a 24h proscillaridin treatment (ranging from 1 nM to 100 μ M) in a high MYC expressing human leukemia cell lines (MOLT-4 and NALM-6) and in low MYC expressing human cancer cell lines (SW48 and A549) (n=4). **b** MYC and RAS protein expression assessed by western blotting in immortalized fibroblasts and fibroblasts transfected with RAS^{V12} , MYC and RAS^{V12}/MYC (n=3). **c** Dose response curves after 48h proscillaridin treatment (0.01 nM to 50 μ M) in immortalized fibroblasts and fibroblasts transfected with RAS^{V12} , MYC and RAS^{V12}/MYC (n=4). **d** MYC protein expression after proscillaridin treatment (5 nM; 48h) in MOLT-4, NALM-6, SW48 (colon cancer) and A549 (lung cancer) cells assessed by western blotting. MYC expression is calculated as a ratio over ACTIN levels (*indicates $P < 0.05$; ANOVA; n = 3). **e** Time course experiment in MOLT-4 cells treated with proscillaridin at 5 nM (8h to 96h). MYC protein expression is calculated as a ratio over ACTIN levels (*indicates $P < 0.05$; ANOVA; n = 3).

Fig. 2. Proscillaridin Targets Leukemic Stem Cells (LSCs). **a** Cell viability assay of T-ALL pre-LSC co-cultured with MS5-DL4 cells. Proscillaridin (3 nM or 10 nM) was added 24h after co-culture, and cells were sorted for pre-LSC viability 4-days post treatment (*indicates $P < 0.05$; ANOVA; n \leq 3). **b** Cell viability assay of AML 8227 population composed of LSCs ($CD34^+$) and non-LSCs ($CD34^-/CD15^{+/-}$). AML 8227 were treated with proscillaridin (10 nM to 100 nM) for 6 days and cell viability was measured for each cell subgroup by flow cytometry. **c** Gene set enrichment analysis of MYC pathway between two AML 8227 subgroups: LSC-enriched population $CD34^+/CD38^-$ compared to non-LSC population $CD34^-$. Enrichment score (ES) and false discovery rate (FDR) rates are shown on the graph.

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d Dose response curves and IC₅₀ values after a 6-day proscillaridin treatment (ranging from 10 nM to 100 nM) in each AML 8227 subgroup (n=3).

Fig. 3. Transcriptomic Profiles from Replicative To Differentiated Phenotype After Low Dose Proscillaridin Treatment in High MYC Expressing Leukemic Cells. **a** Quantitative PCR (qPCR) analysis of *MYC* mRNA expression after proscillaridin treatment (5 nM; 8h to 48h) in MOLT-4 cells, relative to untreated cells and normalized to β -2-microglobulin (t-test: P<0.05; n=3). **b** Transcriptomic analysis by RNA-Sequencing of untreated and proscillaridin-treated (5 nM; 48h) MOLT-4 cells (n = 3). Genes downregulated by proscillaridin treatment (Log₂ FC<-0.5) were analyzed by Metascape and the top 7 Gene Ontology (GO) pathways are displayed. **c** Left panel, *MYC* transcript (RPKM) expression after proscillaridin treatment (5 nM; 48h) in RNA-sequencing data set (*indicates P<0.001, t-test, n=3). Right panel, gene set enrichment analysis of *MYC* pathway before and after proscillaridin treatment (5 nM; 48h) in MOLT-4 cells. Enrichment score (ES) and false discovery rate (FDR) rates are shown on the graph. **d** Effect of proscillaridin treatment (5 nM; 48h) on the percentage of S phase cell population on MOLT-4 cells (* indicates P<0.017, t-test, n = 3). **e** Gene expression values (Log₂ fold change) obtained from RNA-sequencing in 11 genes downregulated after proscillaridin treatment (5 nM; 48h) in MOLT-4 cells. These genes were selected due to their role as master transcription factors associated in T-cell leukemia. **f** Heat map of gene expression levels (RPKM) involved in differentiation pathways (MOLT-4 cells) before and after proscillaridin treatment (5nM; 48h). **g** Quantitative PCR (qPCR) analysis of *NOTCH3* and *HES1* mRNA expression measured after proscillaridin treatment (5 nM; 48h) and 2 days post treatment, relative to untreated cells and normalized to GAPDH in MOLT-4 cells (n=2). **h** and **i** Left panel, T-cell differentiation markers TCR and CD3 are measured by flow cytometry in MOLT-4 cells after proscillaridin treatment (5 nM; 48h), as well as 2 and 4 days post treatment (n=3). Right panel, percentage of TCR and CD3 expression in MOLT-4 cells. TPA treatment (10 nM; 48h, followed by a 24h resting period) was used as positive control of T-cell differentiation (*indicates P<0.05; ANOVA; n=3).

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Fig. 4. Gene Reprogramming Induced by Proscillaridin Is Associated with Global Acetylation Loss in Histone H3. **a** Right panel, MOLT-4 cells were treated with proscillaridin (5 nM) and histones were acid-extracted after 8, 16, 24, 48, 72 and 96 hours. Histone 3 acetylation levels were assessed using antibodies against K9ac, K14ac, K18ac, K27ac, and total histone 3 acetylation. H3 was used as loading control. Left panel, H3 acetylation levels at 48h treatment were quantified and expressed as a percentage of untreated cells (* indicates $P < 0.05$; ANOVA; $n = 3$). **b** 2,169 genes are marked by H3K27ac in promoter regions (-500/+500 bp) in untreated MOLT-4 cells. RPKM values of differentially expressed genes ($FC > 1$; $FC < -0.5$) from RNA-sequencing data before and after proscillaridin treatment are displayed (* indicates $P < 0.05$; t-test; $n = 3$). **c** Pie chart shows percentage of upregulated (black) and downregulated (grey, including 30 MYC target) genes marked by H3K27ac in promoters of untreated MOLT-4 cells (5 nM, 48h). **d** Metascape analysis of genes marked by H3K27ac in promoters in untreated MOLT-4 cells and downregulated after proscillaridin treatment (5 nM; 48h). Top 9 GO pathways are displayed. **e** Metascape analysis of the 30 MYC target genes.

Fig. 5. Acetylation Decrease In MYC Targets And Chromatin Regulators Induced by Proscillaridin In High MYC Expressing Cells. **a** Right panel, immunoprecipitation (IP) of MYC total lysine acetylation (K-AC) after proscillaridin treatment (5 nM; 8h-16h-24h) in MOLT-4 cells. Left panel, total lysine acetylation level on MYC was quantified and expressed as a percentage of untreated cells (* indicates $P < 0.05$; ANOVA; $n = 3$). **b** Lysine acetylome profiling of MOLT-4 cells before and after proscillaridin treatment (5 nM; 48h). **c** Lysine acetylome metascape analysis in 28 peptides with significant loss of acetylation ($\text{Log}_2\text{FC} < -1$) after proscillaridin treatment (5 nM; 48h) in MOLT-4 cells. **d** Log_2FC of acetylation levels in MYC target proteins (untreated VS treated). **e** Log_2FC of acetylation levels of histone regulators (untreated VS treated).

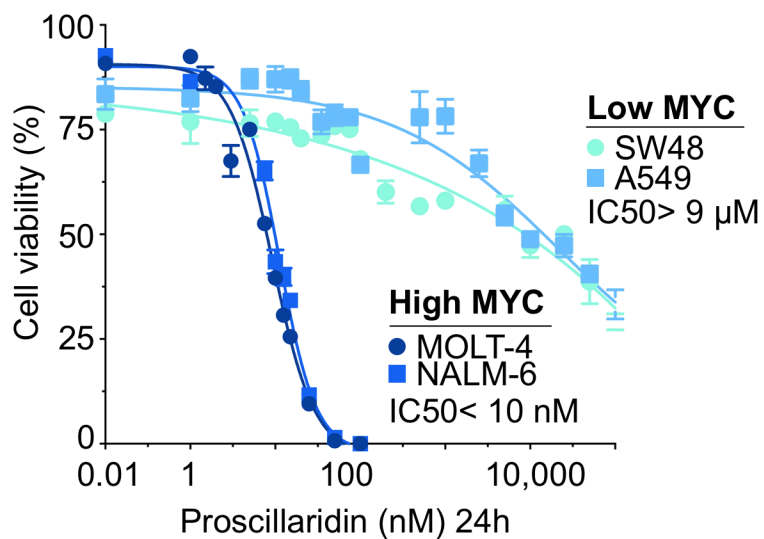
Fig. 6. Proscillaridin Treatment Induces Downregulation of KATs Involved In MYC Acetylation. **a** KAT2A (GCN5), KAT3A (CBP), KAT3B (P300), KAT5 (TIP60), and KAT6A (MOZ) expression

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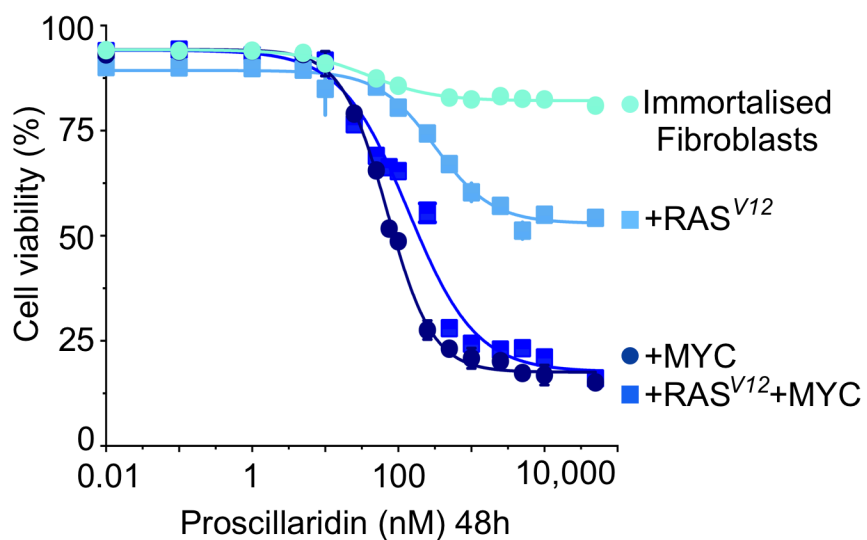
levels after proscillaridin treatment (5 nM, 48h) were assessed by western blotting in MOLT-4 cells (ACTIN was used as loading control). **b** Quantification of H3 acetylation levels after treatment with KAT3B/A inhibitor C646 (10 μ M, 48h) (* indicates $P < 0.05$; t-test; $n = 3$). **c** MYC protein expression after C646 treatment and proscillaridin treatment (5 nM; 48h). ACTIN was used as loading control. **d** MOLT-4, NALM-6, SW48 and A549 cell lines were treated with proscillaridin (5 nM, 48h) and fibroblasts transfected with RAS^{V12} , *MYC* and RAS^{V12}/MYC were treated with proscillaridin (70 nM, 48h). KAT3A (CBP), KAT3B (P300), KAT5 (TIP60), KAT2A (GCN5), KAT2B (PCAF), KAT6A (MOZ) and KAT7 (HBO1) expression levels were assessed by western blotting, quantified and expressed as percentage of untreated cells (* indicates $P < 0.05$; t-test; $n = 3$). **e** Scheme showing that proscillaridin targets high MYC expressing leukemic cells by inhibiting the histone acetyltransferases involved in MYC acetylation and stability.

Figure 1

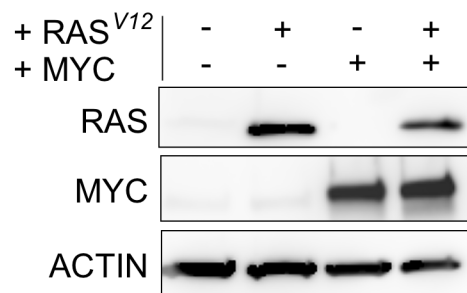
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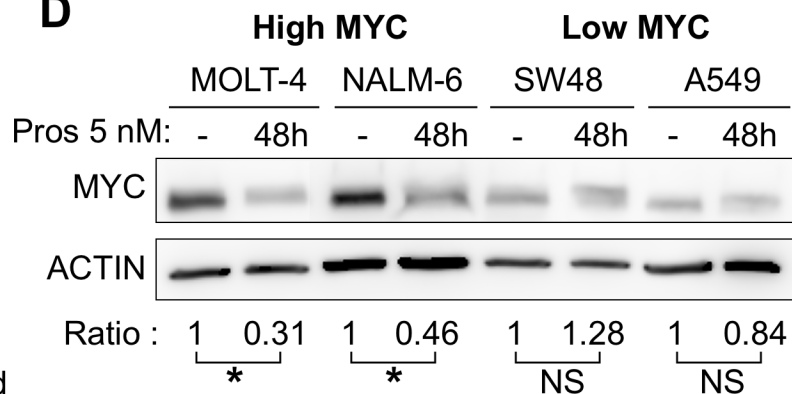
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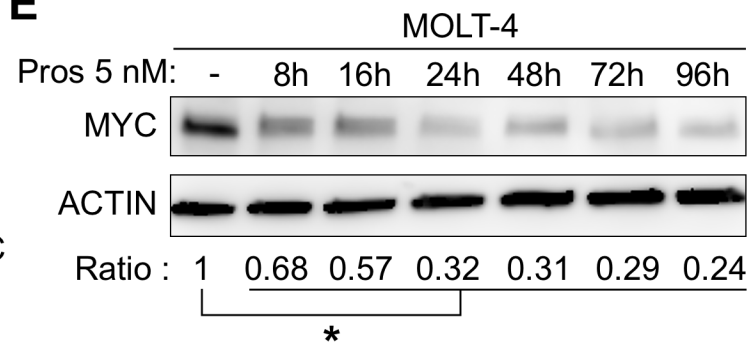
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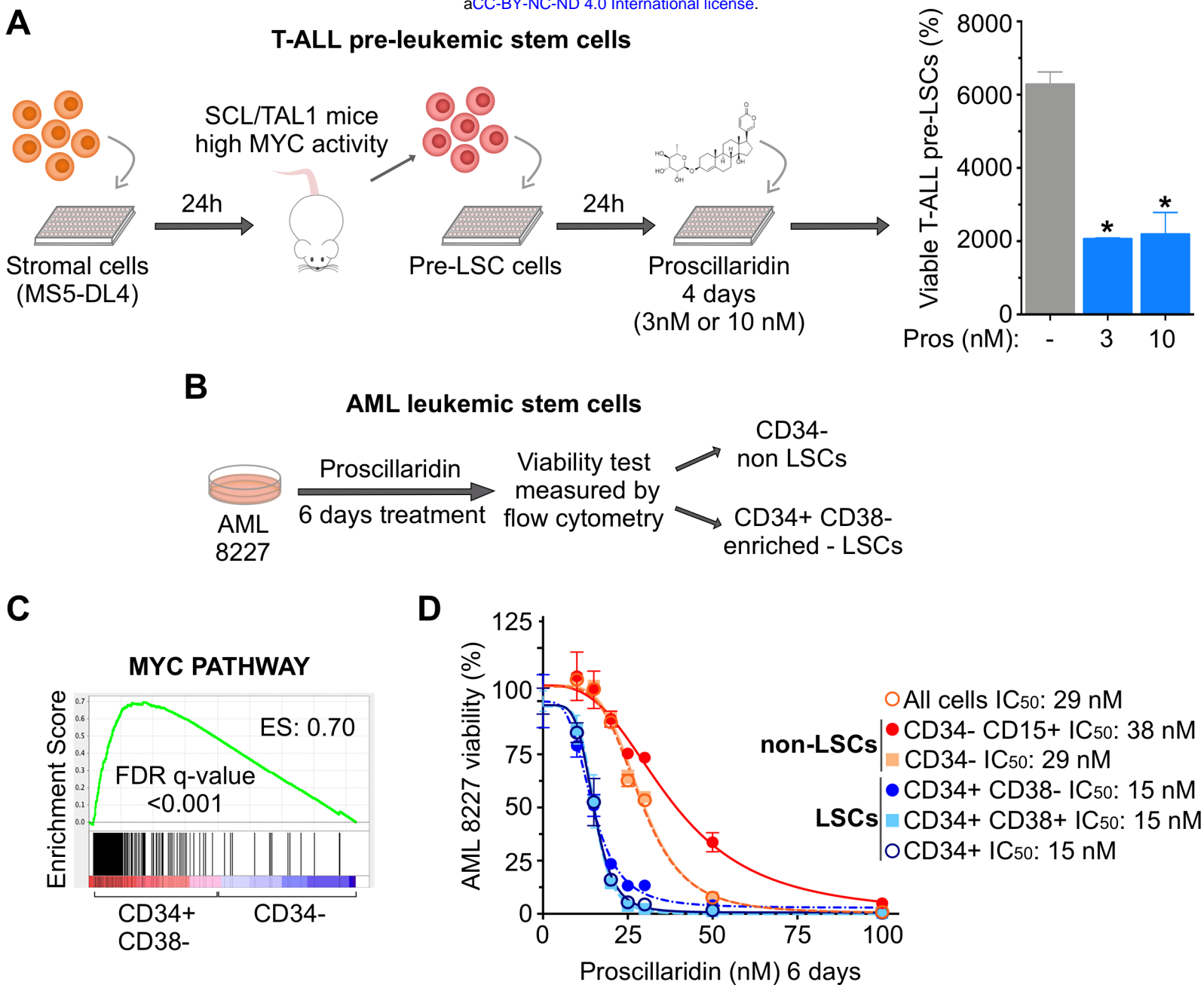


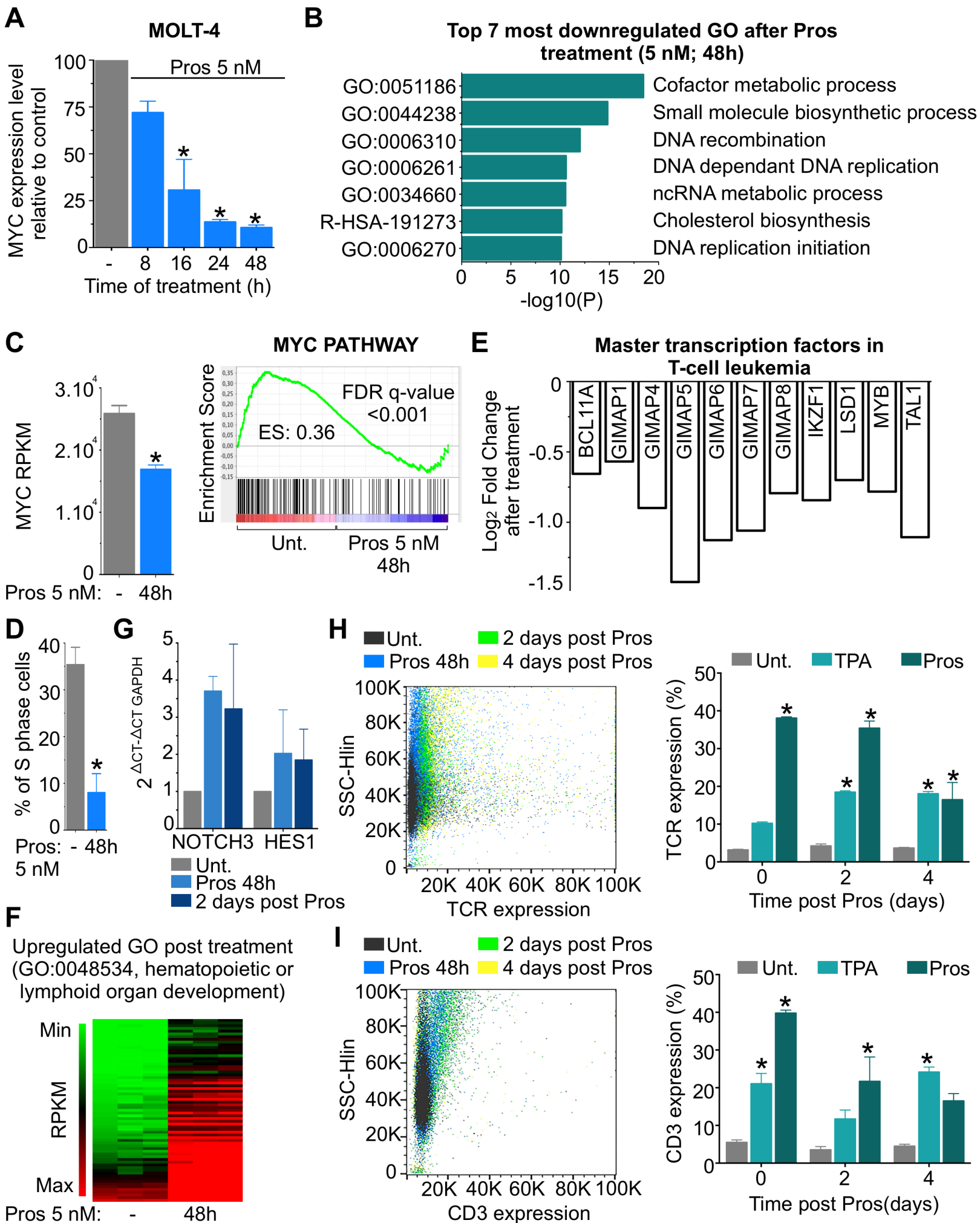
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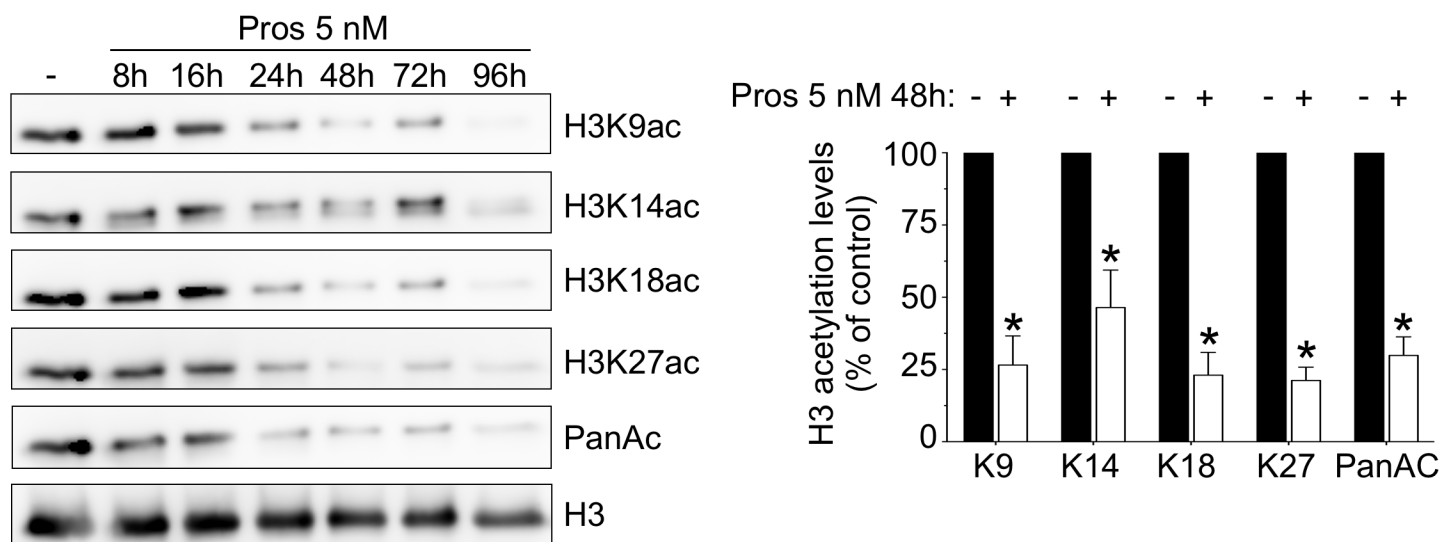
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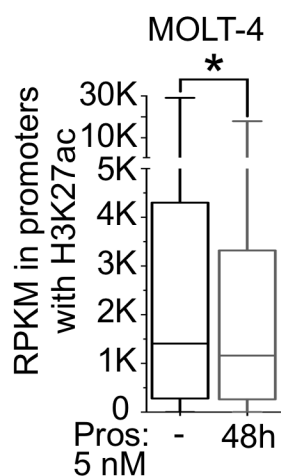




A



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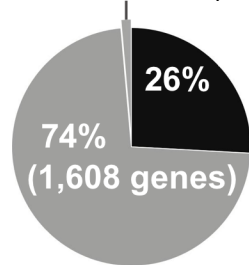


C

Promoters marked by H3K27ac in untreated cells

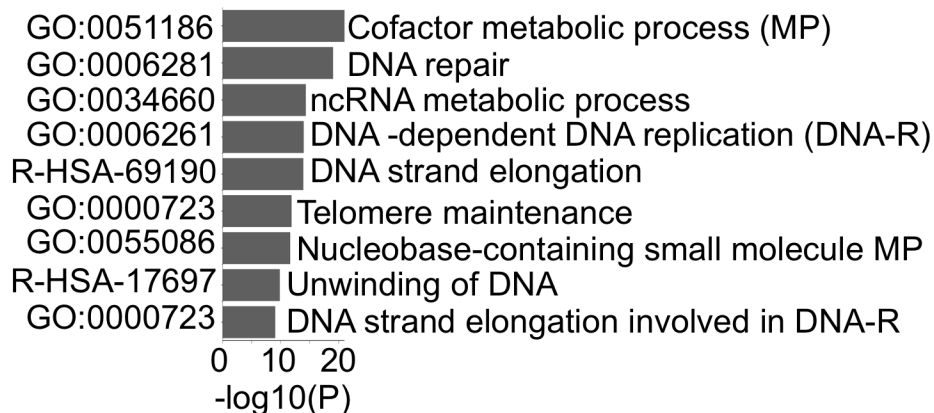
■ Upregulated
■ Downregulated

MYC PATHWAY (n=30)



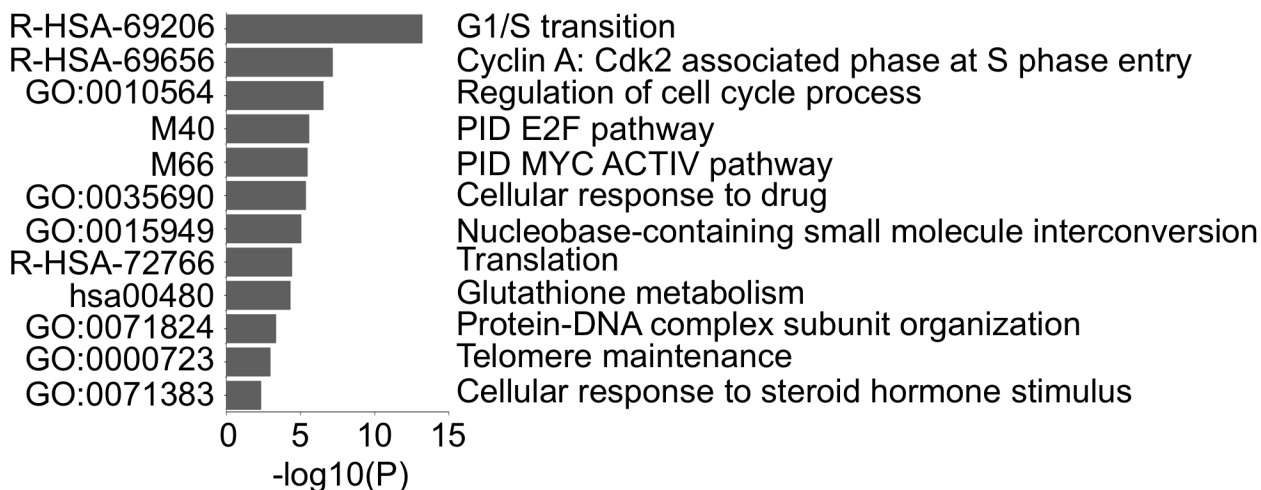
D

GO of Pros (5 nM; 48h) -induced downregulated genes with promoters marked by H3K27ac in untreated cells



E

GO of Pros (5 nM; 48h) -induced downregulated genes with promoters marked by MYC binding and H3K27ac in untreated cells



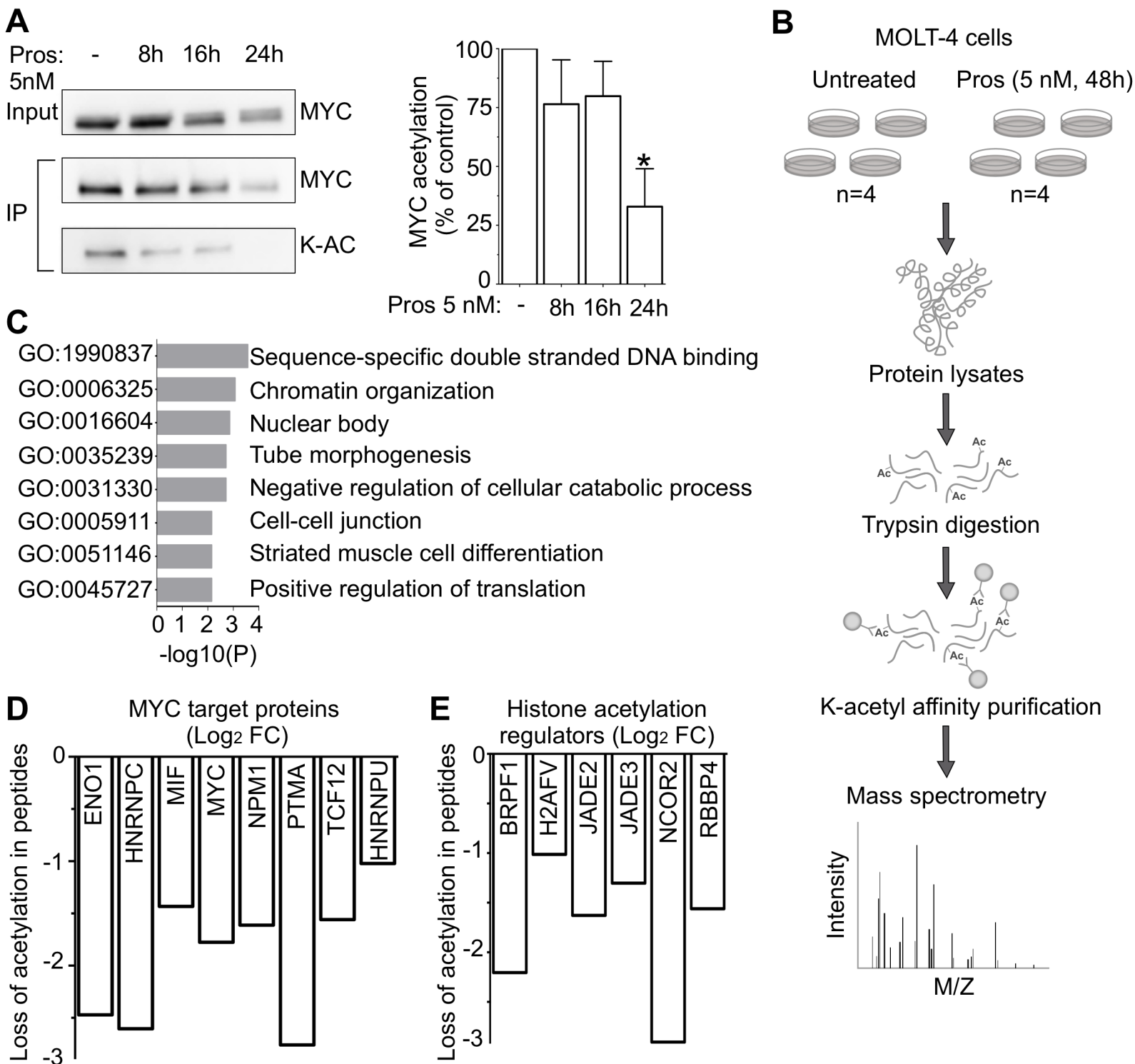


Figure 6

