BET inhibitors downregulate the expression of the essential lncRNA *SMILO* in multiple myeloma through regulation of the transcription factor FLI1

Bromodomain and extraterminal domain (BET) proteins are epigenetic readers that bind to acetylated histones in active enhancer and promoter regions participating in their transcriptional regulation.[1-3] Several lines of evidence indicate the role of these BET proteins in oncogenic enhancers of human cancers providing the rationale to target BET proteins as a therapeutic strategy in different tumors. BET inhibitors (BETi) are small molecules that displace BET from the chromatin disrupting the transcriptional complex, including transcription factors (TF) and co-activators, leading to the inhibition of gene expression.[1,4]

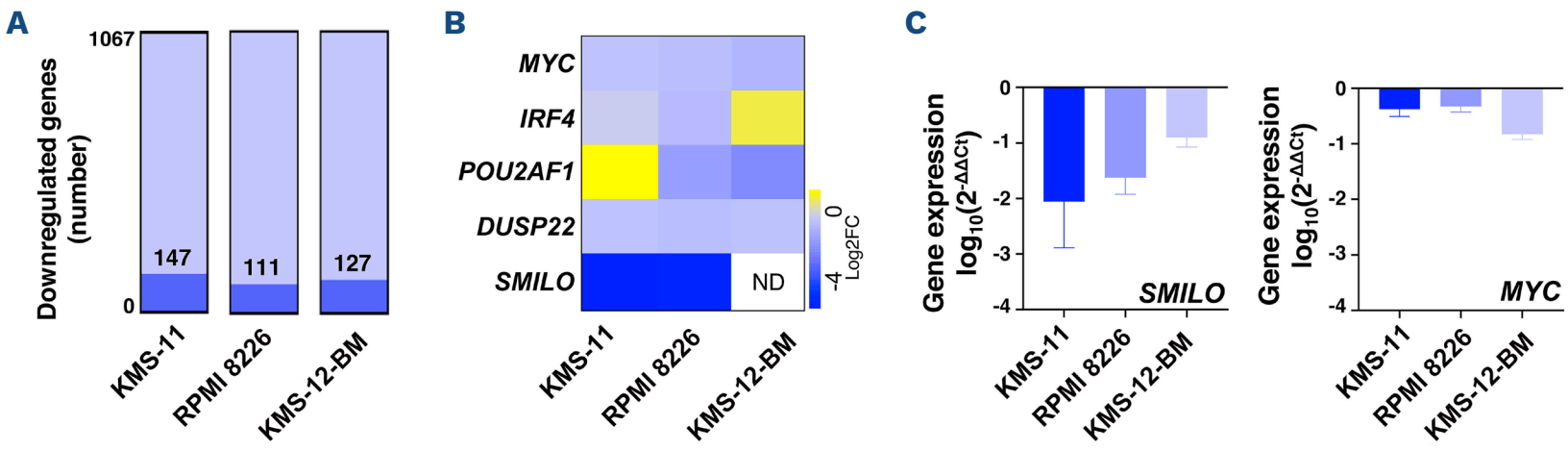
We have recently described that patients with multiple myeloma (MM) are characterized by the presence of an aberrant *de novo* activation of chromatin regions located mainly at promoters and enhancers that regulate genes involved in the pathogenesis of MM, including long non-coding RNA (lncRNA).[5,6] Based on these findings we hypothesized that the use of BETi in MM could modulate these *de novo* active regions contributing to the elimination of abnormal myeloma plasma cells.

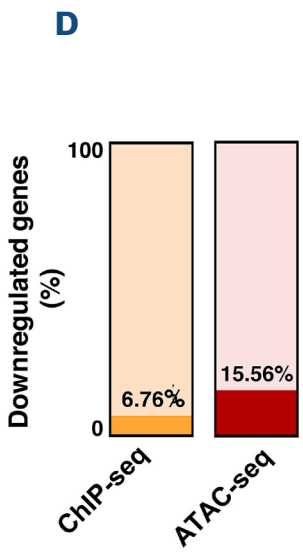
To assess this hypothesis, we conducted a RNA-sequencing (RNA-seq) analysis following a 72-hour treatment of the MM-derived cell lines KMS-11, RPMI 8226, and KMS-12-BM using a non-cytotoxic concentration (1 μM) of the BETi JQ1 (dose that inhibits the growth of cells by 50% [GI^{50}]: 43.3 μM for KMS-11, 56.2 μM for RPMI 8226 and 19.6 μM KMS-12-BM), a pan-BETi with a well-described mechanism of *MYC* inhibition in hematologic malignancies[1,2,4] (our research complies with all relevant ethical regulations and has been approved by the committee of ethics of research of the University of Navarra). This analysis revealed a significant downregulation of more than 10% of genes regulated by *de novo* active regions in MM cell lines (Figure 1A). Notably, previously identified BETi targets such as *MYC, IRF4, POU2AF1* and *DUSP22*[7] were also downregulated (Figure 1B). These results suggest that JQ1 is able to negatively modify the expression of genes regulated by *de novo* active regions in MM. Interestingly, we observed that one of the genes with greatest inhibition induced by JQ1 was the lncRNA *SMILO (LINC00582)* (Figure 1B). The inhibition of the expression of *SMILO* and *MYC* were validated by real-time quantitative polymerase chain reaction (RT-qPCR) in the three MM cell lines used before (Figure 1C). The fact that pharmacological regulation of the lncRNA has been proposed as a therapeutic approach[8,9] and that we have previously described that *SMILO* is overexpressed in 64% of MM patients and is an essential lncRNA for the proliferation of MM[6] suggested that JQ1 could be used not only to explore the mechanism of regulation of this lncRNA but also as a therapeutic approach to target lncRNA in MM.

In order to determine whether JQ1 was indeed regulating gene expression by modulation of *de novo* active chromatin regions, we performed the assay for transposase-accessible chromatin using sequencing (ATAC-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) for H3K27ac in KMS-11, RPMI 8226 and KMS-12-BM cells after 72 hours of JQ1 treatment. We observed a modest change in the percentage of chromatin activation marks or chromatin accessibility in *de novo* chromatin active regions (Figure 1D), even when we considered regulatory regions of genes downregulated by JQ1, with only 1.5% and 2.9% exhibiting a reduction in chromatin activation and accessibility, respectively. This is exemplified by the lack of changes in chromatin regions regulating the expression of *SMILO* (Figure 1E). These results indicate that JQ1 may displace the transcriptional machinery, such as TF, from *de novo* active regions and specifically from those related to *SMILO*.

In order to identify potential TF bound to the active chromatin regions of *SMILO*, thereby responsible for regulation of this lncRNA, we performed a Reverse-ChIP[10] in three accessible and *de novo* active chromatin regions of *SMILO* (*Online Supplementary Figure S1A*). The Reverse-ChIP was carried out in three MM cell lines, two with high *SMILO* expression (KMS-11 and MM.1R), and one exhibiting low levels of the lncRNA (KMS-12-BM) (*Online Supplementary Figure S1B*). We focused our analysis on proteins shared by KMS-11 and MM.1R, excluding those proteins that were present in KMS-12-BM. The analysis of the Reverse-ChIP identified 16 proteins associated with the three *SMILO* regions analyzed in KMS-11 and MM.1R cell lines (Figure 2A). When we analyzed the mRNA expression level of these 16 genes in B cells at different stages of differentiation (from naïve B cells to plasma cells), and MM patients we observed that four of them, *FLI1, SUB1, PKP2*, and *MAFG*, were overexpressed in MM (Figure 2B; *Online Supplementary Figure S2A*). Among these candidates, *FLI1* showed the highest positive correlation with *SMILO* expression (Figure 2C; *Online Supplementary Figure S2B*). Overexpression of *FLI1* has been linked to different genomic alterations and mutations not only in solid tumors but also in several hematological malignancies.[11,12] The analysis of a large cohort of MM patients indicated a very

low frequency of mutations of *FLI1* (3 of 604 patients from the CoMMpass MM patient's cohort). Furthermore, the expression of *FLI1* along with the expression of *SMILO* was not associated with classical genetic alterations in MM (*Online Supplementary Figure S3A*). However, *FLI1* presented *de novo* chromatin active marks in MM (*Online Supplementary Figure S3B*), suggesting that overexpression of *FLI1* could be epigenetically regulated. High





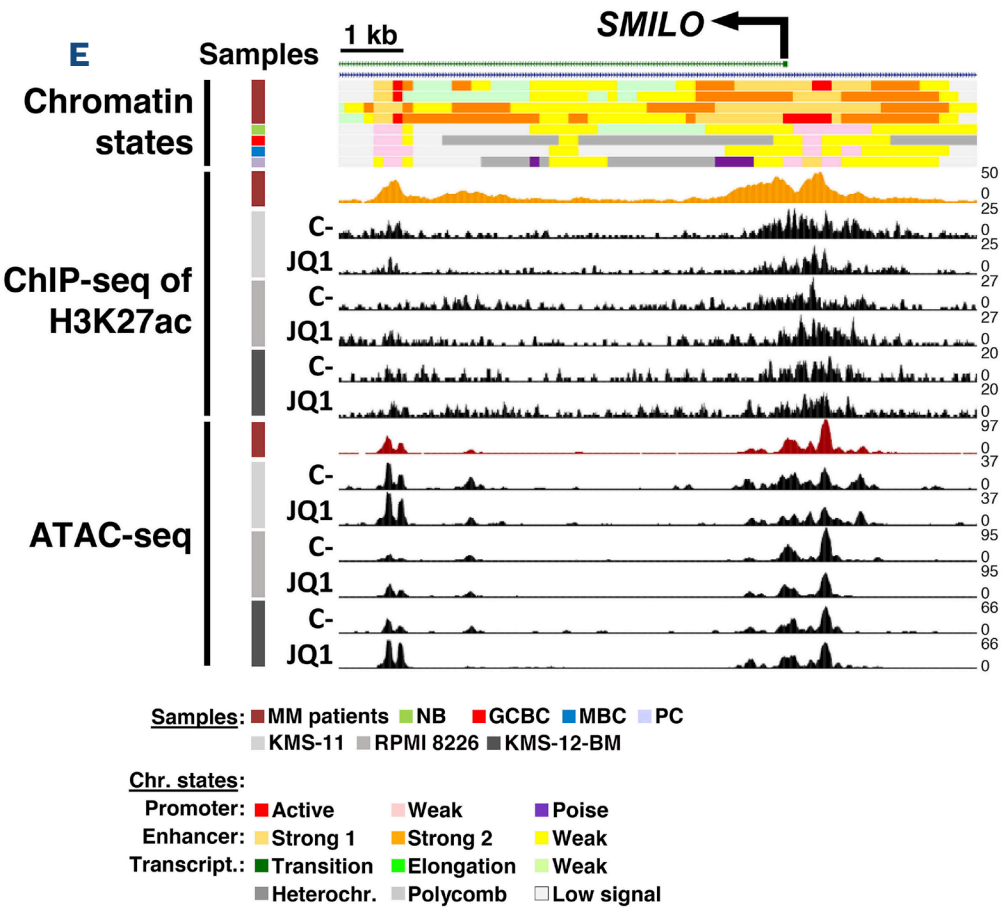


Figure 1. The expression of *de novo* active long non-coding RNA *SMILO* decrease after JQ1 treatment. (A) Graphical representation of the number of genes associated with *de novo* active chromatin regions that present a significant downregulation ($P<0.05$ and a log2 fold change [log2FC]<-1) of their expression after JQ1 treatment in each multiple myeloma (MM) cell line. In this graph the expression of 1,059 coding genes and 8 annotated long non-coding RNA (lncRNA) from *de novo* active regions are shown. Two biological replicates were carried out in the 3 cell lines for the RNA-sequencing analysis. (B) Gene expression of *SMILO* and the former targets of JQ1 *MYC, IRF4, POU2AF1*, *DUSP22* after JQ1 treatment in MM cell lines. ND: not detected. (C) Validation by real-time quantitative polymerase chain reaction (RT-qPCR) of *SMILO* and *MYC* expression after JQ1 in the 3 MM cell lines used before (experiment performed in 3 biological replicates); (*SMILO* forward [Fw] primer CACCTTGGGTCAGATGTGTG and reverse [Rv] primer TCGAGGTCAAGCAGAAAAGAA; *MYC* Fw primer TGCTCCATGAGGAGACACC and Rv primer CTCTGACCTTTTGCCAGGAG; and the house-keeping gene GUSβ Fw primer GAAAATATGTGGTTGGAGAGCTCATT and Rv primer CCGAGTGAAGATCCCCTTTTA). (D) Percentage of genes associated with *de novo* active regions presenting a decrease in chromatin acetylation (H3K27ac chromatin immunoprecipitation-sequencing [ChIP-seq] and accessibility (assay for transposase-accessible chromatin using sequencing [ATAC-seq]) after JQ1 treatment in MM cell lines. One replicate of the ChIP-seq for the H3K27ac and ATAC-seq upon JQ1 treatment experiments was carried out in each cell line. The chromatin regions downregulated for both H3K27ac and chromatin accessibility were determined as those presenting a log2FC<-0.5 in 2 cell lines, having the same tendency in the third cell line (log2FC<0). (E) ChIP-seq for H3K27ac and ATAC-seq of *SMILO* chromatin region without (C-) and after JQ1 treatment in MM cell lines. RNA sequencing, ChIP-seq for the H3K27ac and the ATAC-seq were conducted as previously described.[5] The normal distribution of the experimental data was assessed though Shapiro-Wilk test and the statistical differences were determined by one-tailed parametric *t* test or one-tailed non-parametric Mann-Whitney test (GraphPad Prism 8.0.1). The significance levels were denoted as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. NB: naïve B cells; GCBC: germinal center B cells; MBC: memory B cells; PC: plasma cell; MM: multiple myeloma.

expression of *FLI1* was associated with a significant decrease in progression-free survival (PFS) and overall survival (OS) of patients with MM using the CoMMpass (IA18) database (*Online Supplementary Figure S3C, D*). On the multivariate analysis, when considering classic high risk genetic alterations in MM as described before,[13] high expression of *FLI1*, together with the del13q, amp1q and International Staging System (ISS) stage resulted in a significantly lower PFS (global $P<0.0001$). According to the number of events, patients were stratified into five risk groups (*Online Supplementary Figure S3E, F*). Similarly, high expression of *FLI1* together with del13q, del17p, amp1q or ISS stage identified six groups with significant differences in their OS ($P<0.0001$) (*Online Supplementary Figure S3G, H*). These results suggest that FLI1 is an important TF with a potential therapeutic as well as prognostic impact in patients with MM.

In order to determine the mechanism of FLI1-mediated downregulation of *SMILO*, we analyzed ChIP-seq data after treatment with JQ1. We observed a significant decrease in H3K27ac at the promoter region of *FLI1* (Figure 3A). Additionally, expression of protein levels of FLI1 were significantly decreased after JQ1 treatment in MM cell lines (Figure 3B), as previously described in acute myeloid leukemia (AML).[14] To support the hypothesis that FLI1 is causally involved in the expression of *SMILO*, FLI1 was silenced using CRISPR/Cas9 technology as previously explained.[5] The inhibition of FLI1 by two guide RNA led to a substantial reduction in protein levels after 7 days (Figure 3C) that was correlated with a significant reduction of the expression of *SMILO* further exacerbated by day 10 (Figure 3D). Given that JQ1 treatment induces a greater reduction in *SMILO* expression (Figure 1C) compared to the direct inhibition of FLI1 (Figure 3D), it is plausible that JQ1 may regulate *SMILO* through other mechanisms. All these results suggest a model where JQ1 leads to a substantial drop of *SMILO* expression, in part through the downregulation of the TF FLI1, displacing it from the chromatin of the lncRNA *SMILO* (Figure 3E). Altogether, our data posit FLI1 as one of the TF that directly regulate *SMILO* expression in MM cells and unlocks the opportunity of modulating an essential lncRNA through the inhibition of its associated TF. This is particularly promising provided the therapeutic potential of targeting FLI1.[11,15]

In conclusion, we have demonstrated the potential of BETi to modify gene expression through regulation of *de novo* active chromatin regions and highlight the possibility of modulating the expression of lncRNA by affecting the TF that govern their regulation. These findings support the development of novel therapeutic strategies against RNA

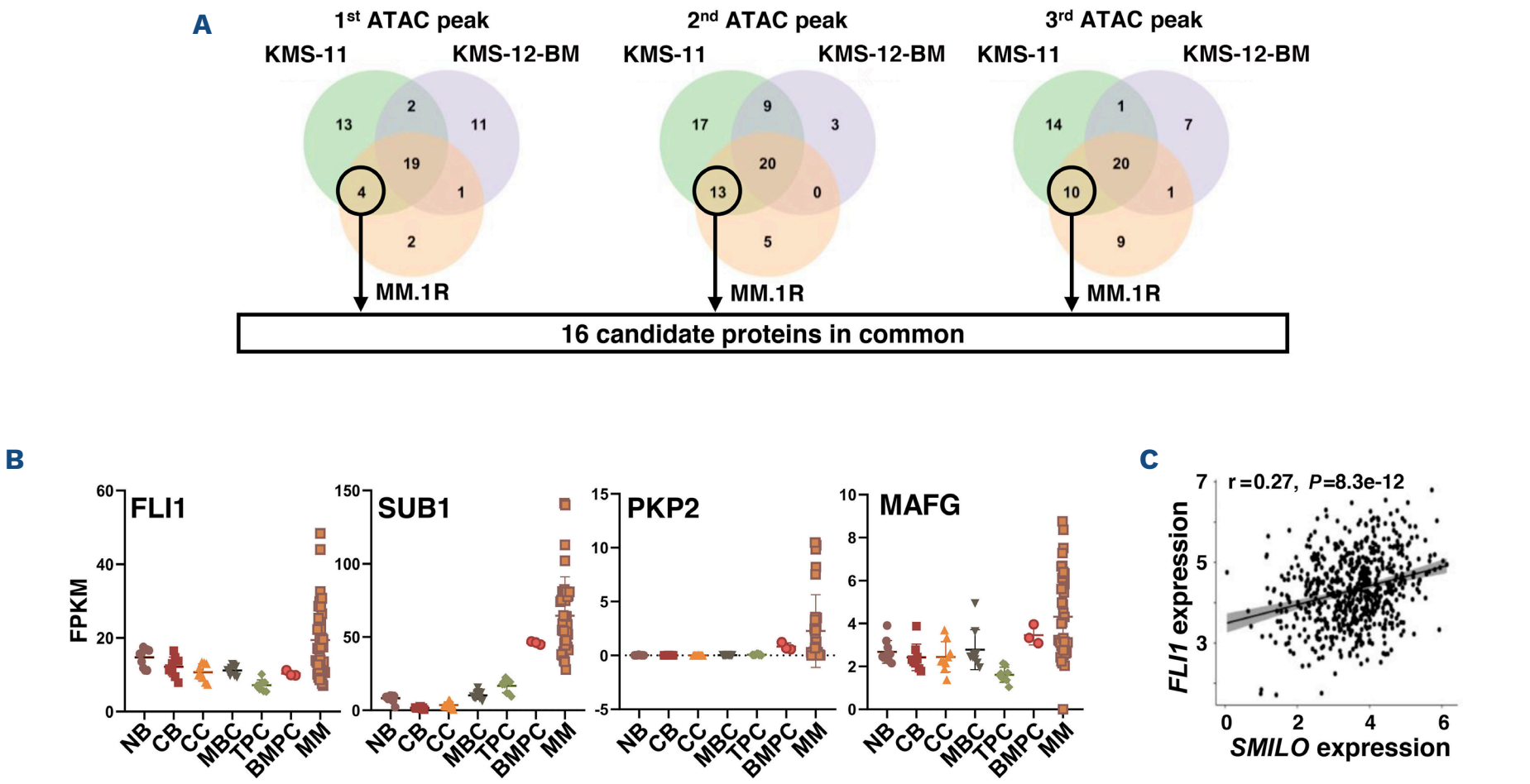


Figure 2. FLI1 bound to the active chromatin regions of *SMILO*. (A) Reverse chromatin immunoprecipitation (Reverse-ChIP) results represented as a Venn diagram for each assay for transposase-accessible chromatin using sequencing (ATAC) peak, resulting in 16 candidate proteins in common to KMS-11 and MM.1R cell lines. (B) Gene expression of the 4 candidate genes, *FLI1, SUB1, PKP2 and MAFG,* in different subpopulations of healthy B cells and multiple myeloma (MM) samples. (C) Expression correlation of *FLI1* with *SMILO*, using CoMMpass (IA18) cohort from MMRF (Multiple Myeloma Research Foundation). NB: naïve B cells; CB: centroblast B cells; CC: centrocyte B cells; MBC: memory B cells; TPC: tonsillar plasma cells; BMPC: bone marrow plasma cells; MM: multiple myeloma; FPKM: fragments per kilobase million.

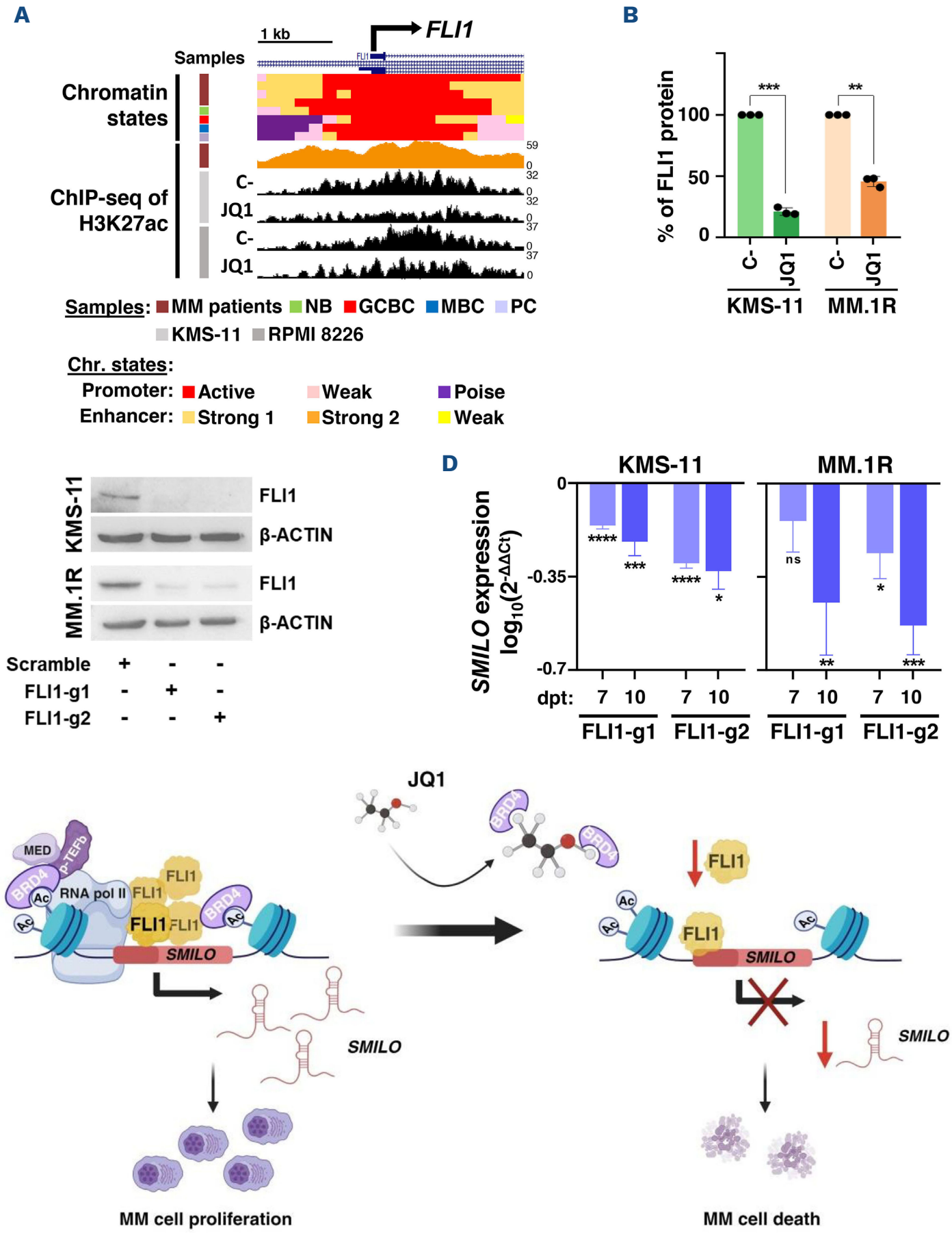


Figure 3. *SMILO* expression is regulated in part by the transcription factor FLI1. (A) Chromatin states of *FLI1* gene in multiple myeloma (MM) samples and different subpopulations of healthy B cells together with the chromatin acetylation analyzed by chromatin immunoprecipitation sequencing (ChIP-seq) for the H3K27ac in 2 MM cell lines (KMS-11 and RPMI 8226) without (C-) and with JQ1 treatment. (B) Quantification of the western blot of FLI1 after JQ1 treatment in the MM cell lines used for the Reverse-ChIP. (C) Western blot of FLI1 in KMS-11 and MM.1R after FLI1 knockdown by CRISPR/CAS9 (used primers to clone the guide RNA in the CRISPseq-BFP-backbone [AddGene #85707] vector: FLI1-g1 Fw primer CACCGCACAGGTCCTCCCCTTGGAG and Rv primer AAACCTCCAAGGGGAGGACCTGTGC; FLI1-g2 Fw primer CACCGGACGATGACTCTCCTCTCGT and Rv primer AAACACGAGAGGAGAGTCATCGTCC). (D) Expression of *SMILO* in KMS-11 and MM.1R cell lines after FLI1 knockdown by CRISPR/CAS9. (E) Graphical representation of the regulation mechanism of *SMILO* by JQ1 treatment: JQ1 degrades the FLI1 protein impairing its union to the chromatin of *SMILO*, giving rise to the downregulation of this essential long non-coding RNA (lncRNA) for MM cells. The used antibodies and conditions for western blots are: FLI1 (35980 Cell Signaling, 1:1000) and as loading control β-Actin (A5441 Sigma-Aldrich, 1:4000). The normal distribution of the experimental data was assessed though Shapiro-Wilk test and the statistical differences were determined by one-tailed parametric *t* test or one-tailed non-parametric Mann-Whitney test (GraphPad Prism 8.0.1). The significance levels were denoted as follows: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. NB: naïve B cells; GCBC: germinal center B cells; MBC: memory B cells; PC: plasma cells; Chr.: chromatin.

with a broad application in the treatment of MM and in general of cancer.

Authors

Nahia Gómez-Echarte,[1,2*] Edurne San José-Enériz,[1,2,3*] Arantxa Carrasco-León,[1,2,3] Naroa Barrena,[4] Estibaliz Miranda,[1,2,3] Leire Garate,[2,3,5] Beatriz García-Torre,[6] Sandra Alonso-Moreno,[7,8] Naroa Gimenez-Camino,[1,2] Estibaliz Urizar-Compains,[1,2] Danel Olaverri-Mendizabal,[4] Paula Aguirre-Ruiz,[1,2] Beñat Ariceta,[1,2] Luis-Esteban Tamariz-Amador,[2,5] Paula Rodriguez-Otero,[2,5] Francisco J. Planes,[4] Laura Belver,[7,8] José Ignacio Martín-Subero,[6,9] Felipe Prosper[1,2,3,5#] and Xabier Agirre[1,2,3#]

[1]Centro de Investigación Médica Aplicada (CIMA), Instituto de Investigación Sanitaria de Navarra (IDISNA), Pamplona, Navarra; [2]Cancer Center Clínica Universidad de Navarra (CCUN), Pamplona, Navarra; [3]Centro de Investigación Biomédica en Red Cáncer (CIBERONC), Madrid; [4]Universidad de Navarra, Tecnun Escuela de Ingeniería, San Sebastián, País Vasco; [5]Servicio de Hematología y Terapia Celular, Clínica Universidad de Navarra (CUN), Pamplona, Navarra; [6]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona; [7]Josep Carreras Leukemia Research Institute (IJC), Badalona, Barcelona; [8]Catalan Institute of Oncology (ICO), Cancer Therapeutic Resistance Program (ProCURE), Badalona, Barcelona and [9]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

NG-E and ESJ-E contributed equally as first authors.

#FP and XA contributed equally as senior authors.

Correspondence:
F. PROSPER - fprosper@unav.es
X. AGIRRE - xaguirre@unav.es

https://doi.org/10.3324/haematol.2024.285966

Received: May 29, 2024.
Accepted: September 16, 2024.
Early view: September 26, 2024.

Disclosures

No conflicts of interest to disclose.

Contributions

Conception and design by NG-E, ESJ-E, FP and XA. Development of methodology by NG-E, ESJ-E, AC-L, LB and XA. Acquisition of data and assistance with experiments by NG-E, ESJ-E, AC-L, EU-C, EM, LG, BG-T, NG-C, LET-A, PR-O, PA-R and SA-M. Analysis and interpretation of data by NG-E, ESJ-E, NB, DO-M, BA, PR-O, FJ-P, LB, JIM-S, FP and XA. Writing, review, and/or revision of the manuscript by NG-E, ESJ-E, JIM-S, FP and XA. Study supervision by ESJ-E, FP and XA.

Funding

This research was funded by grants from the Instituto de Salud Carlos III (ISCIII) and co-financed by FEDER: PI22/00947, PI23/00488, CIBERONC CB16/12/00489, Gobierno de Navarra (GNS_44-2021; GN2023/11, NanoRC 0011-1411-2022-000068) and Fundación BBVA. The study was also supported by Paula and Rodger Riney Foundation. NG-E was supported by Gobierno de Navarra (0011-0537-2021-000032) and FIMA AC 2020.

Data-sharing statement

The datasets generated in this work are available at GSE260566 accession code from GEO database.

References

1. Lovén J, Hoke HA, Lin CY, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell. 2013;153(2):320-334.
2. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell 2011;146(6):904-917.
3. Borck PC, Guo L-W, Plutzky J. BET Epigenetic reader proteins in cardiovascular transcriptional programs. Circ Res. 2020;126(9):1190-1208.
4. Schwalm MP, Knapp S. BET bromodomain inhibitors. Curr Opin Chem Biol. 2022;68:102148.
5. Ordoñez R, Kulis M, Russiñol N, et al. Chromatin activation as a unifying principle underlying pathogenic mechanisms in multiple myeloma. Genome Res. 2020;30(9):1217-1227.
6. Carrasco-Leon A, Ezponda T, Meydan C, et al. Characterization of complete lncRNAs transcriptome reveals the functional and clinical impact of lncRNAs in multiple myeloma. Leukemia. 2021;35(5):1438-1450.
7. Welsh SJ, Barwick BG, Meermeier EW, et al. Transcriptional heterogeneity overcomes disrupting drug combinations in multiple myeloma. Blood Cancer Discov. 2024;5(1):34-55.
8. Handa H, Honma K, Oda T, et al. Long noncoding RNA PTV1 Is regulated by bromodomain protein BRD4 in multiple myeloma and is associated with disease progression. Int J Mol Sci. 2020;21(19):7121.
9. Carrasco-León A, Amundarain A, Gómez-Echarte N, Prósper F, Agirre X. The Role of lncRNAs in the pathobiology and clinical behavior of multiple myeloma. Cancers (Basel). 2021;13(8):1976.
10. Belver L, Yang AY, Albero R, et al. Gata3-controlled nucleosome eviction drives Myc enhancer activity in T-cell development and leukemia. Cancer Discov. 2019;9(12):1774-1791.
11. Li Y, Luo H, Liu T, Zacksenhaus E, Ben-David Y. The ets transcription factor Fli-1 in development, cancer and disease. Oncogene. 2015;34(16):2022-2031.
12. Stockley J, Morgan N V, Bem D, et al. Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects. Blood. 2013;122(25):4090-4093.

13. Amundarain A, Valcárcel L V, Ordoñez R, et al. Landscape and clinical significance of long noncoding RNAs involved in multiple myeloma expressed fusion transcripts. Am J Hematol. 2022;97(3):E113-E117.
14. Roe J-S, Mercan F, Rivera K, Pappin DJ, Vakoc CR. BET bromodomain inhibition suppresses the functional output of hematopoietic transcription factors in acute myeloid leukemia. Mol Cell. 2015;58(6):1028-1039.
15. Song J, Yuan C, Yang J, et al. Novel flavagline-like compounds with potent Fli-1 inhibitory activity suppress diverse types of leukemia. FEBS J. 2018;285(24):4631-4645.