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

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AUTHOR CONTRIBUTIONS
Conception and design: NG-E, ESJ-E, FP and XA
Development of methodology: NG-E, ESJ-E, AC-L, LB, XA
Acquisition of data and assistance with experiments: NG-E, ESJ-E, AC-L, EU-C, EM, LG, BG-T, NG-C, LET-A, PR-O, PA-R, SA-M
Analysis and interpretation of data: 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: NG-E, ESJ-E, JIM-S, FP and XA
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COMPETING INTERESTS

The authors declare no competing interests.

DATA AVAILABILITY

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

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 BETs from the chromatin disrupting the transcriptional complex, including Transcription Factors (TFs) 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 lncRNAs[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-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 (1µM) of the BETi JQ1 (GI_{50}: 43.3µM for KMS-11, 56.2µM for RPMI 8226 and 19.6µM 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 the research of the University of Navarra). This analysis revealed a significant downregulation of more than ten percent 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 PCR (RT-qPCR) in the three MM cell lines used before (**Figure 1C**). The fact that pharmacological regulation of the lncRNAs 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 lncRNAs in MM.

To determine whether JQ1 was indeed regulating gene expression by modulation of *de novo* active chromatin regions, we performed ATAC-seq and ChIP-seq (for H3K27ac) in KMS-11, RPMI 8226 and KMS-12-BM cells after 72-hour of JQ1 treatment. We observed modest change in the percentage of chromatin activation marks or chromatin accessibility in *de novo* chromatin active regions (**Figure 1D**), not 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 expression of *SMILO* (**Figure 1E**). These results indicate that JQ1 may displace the transcriptional machinery, such as TFs, from *de novo* active regions and specifically from those related to *SMILO*.

To identify potential TFs bound to the active chromatin regions of *SMILO*, and thereby responsible for regulation of this lncRNA, we performed a Reverse-ChIP[10] in three accessible and *de novo* active chromatin regions of *SMILO* (**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) (**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 cell 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; Supplementary figure S2A**). Among these candidates, *FLI1* showed the highest positive correlation with *SMILO* expression (**Figure 2C; 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 out 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 (**Supplementary figure S3A**). However, *FLI1* presented *de novo* chromatin active marks in MM (**Supplementary figure S3B**), suggesting that overexpression of *FLI1* could be epigenetically regulated. High 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 (**Supplementary figures 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 ISS stage resulted in a significantly lower PFS (global $p<0.0001$). According to the number of events, patients were stratified into 5 risk groups (**Supplementary figure S3E-F**). Similarly, high expression of *FLI1* together with del13q, del17p, amp1q or ISS stage identified 6 groups with significant differences in their OS ($p<0.0001$) (**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.

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 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 RNAs 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 TFs 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 lncRNAs by affecting the TF that govern their regulation. These findings support the development of novel therapeutic strategies against RNA with a broad application to treatment of MM and in general to cancer.

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FIGURE LEGENDS

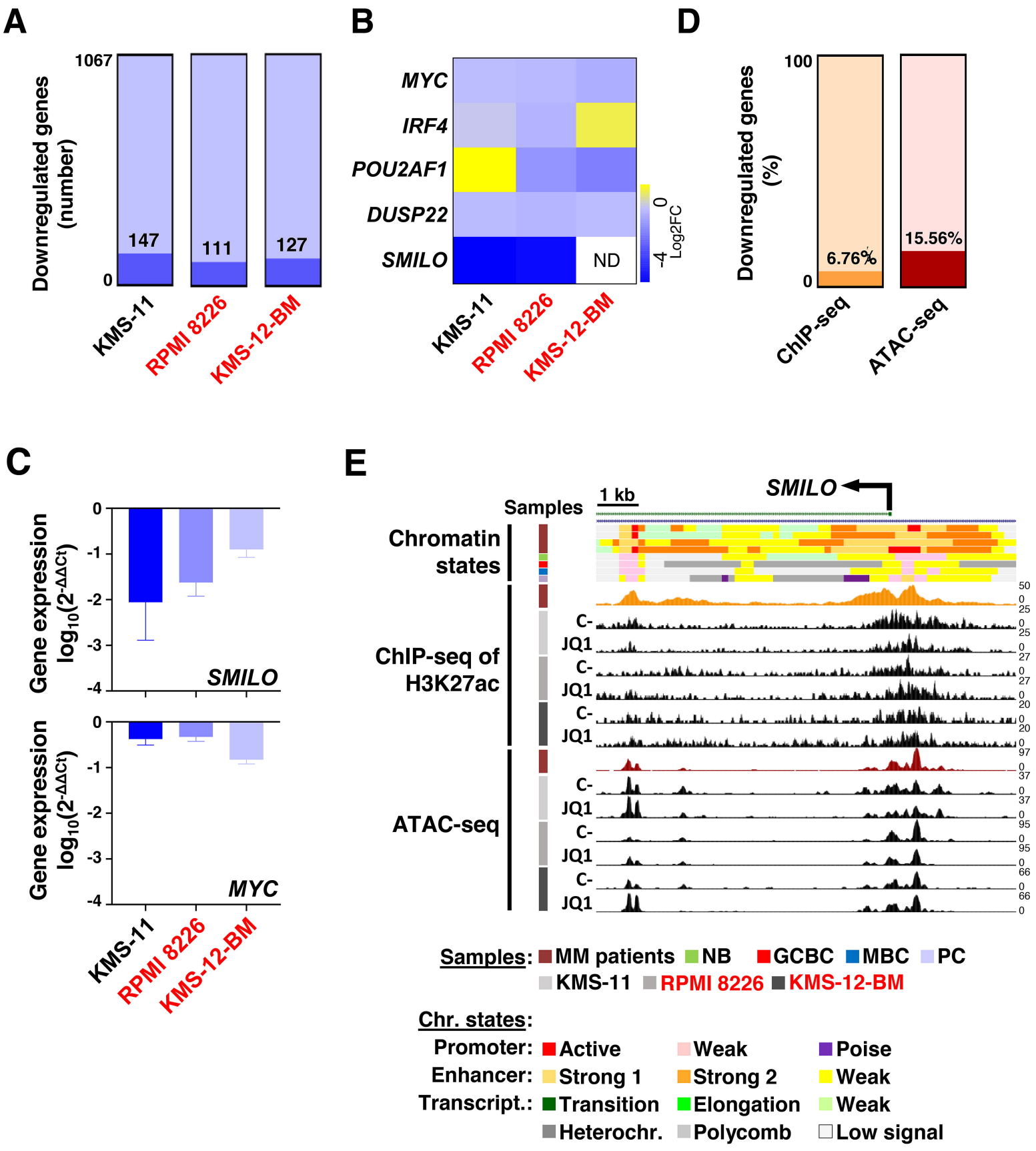
Figure 1. The expression of *de novo* active lncRNA *SMILO* decrease after JQ1 treatment. A) Graphical representation of the number of genes associated with *de novo* active chromatin regions that present a significant downregulation (p-value<0.05 and a log_2 Fold Change (LFC)<-1) of their expression after JQ1 treatment in each MM cell line. In this graph the expression of 1059 coding genes and 8 annotated lncRNAs from *de novo* active regions are shown. Two biological replicates were carried out in the three cell lines for the RNA-seq analysis; **B)** Gene expression of *SMILO* and the former targets of JQ1 *MYC, IRF4, POU2AF1, DUSP22* after JQ1 treatment in MM cell lines. (log2FC: Log_2 Fold Change; ND: not detected); **C)** Validation by RT-qPCR of *SMILO* and *MYC* expression after JQ1 in the three MM cell lines used before (experiment performed in three 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 ChIP-seq) and accessibility (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 LFC<-0.5 in two cell lines, having the same tendency in the third cell line (LFC<0); **E)** ChIP-seq of H3K27ac and ATAC-seq of *SMILO* chromatin region without (C-) and after JQ1 treatment in MM cell lines. NB: Naïve B cells; GCBC: germinal center B cells; MBC: memory B cells; PC: plasma cell; MM: multiple myeloma. RNA-seq, 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: * for p-value < 0.05, ** for p-value < 0.01, *** for p-value < 0.001, **** for p-value < 0.0001.

Figure 2. FLI1 bound to the active chromatin regions of *SMILO*. A) Reverse-ChIP results represented as a Venn diagram for each ATAC peak, resulting in 16 candidate proteins in common to KMS-11 and MM.1R cell lines; **B)** Gene expression of the four candidate genes, *FLI1, SUB1, PKP2* and *MAFG*, in different subpopulations of healthy B cells and 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 TF FLI1. A) Chromatin states of *FLI1* gene in MM samples and different subpopulations of healthy B cells together with the chromatin acetylation analyzed by ChIP-seq of the H3K27ac in two 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 RNAs 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 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). NB: Naïve B cells; GCBC: Germinal center B cells; MBC: Memory B cells; PC: Plasma cells; MM: Multiple myeloma. 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: * for p-value < 0.05, ** for p-value < 0.01, *** for p-value < 0.001, **** for p-value < 0.0001.

FIGURE 1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ND
0
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KMS-11
RPMI 8226
KMS-12-BM
D
Downregulated genes (%)
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0
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15.56%
ChIP-seq
ATAC-seq
C
Gene expression log10(2-ΔΔCt)
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-2
-3
-4
SMILO
MYC
KMS-11
RPMI 8226
KMS-12-BM
E
Samples
1 kb
SMILO
Chromatin states
ChIP-seq of H3K27ac
ATAC-seq
C-
JQ1
Samples: MM patients NB GCBC MBC PC
KMS-11 RPMI 8226 KMS-12-BM
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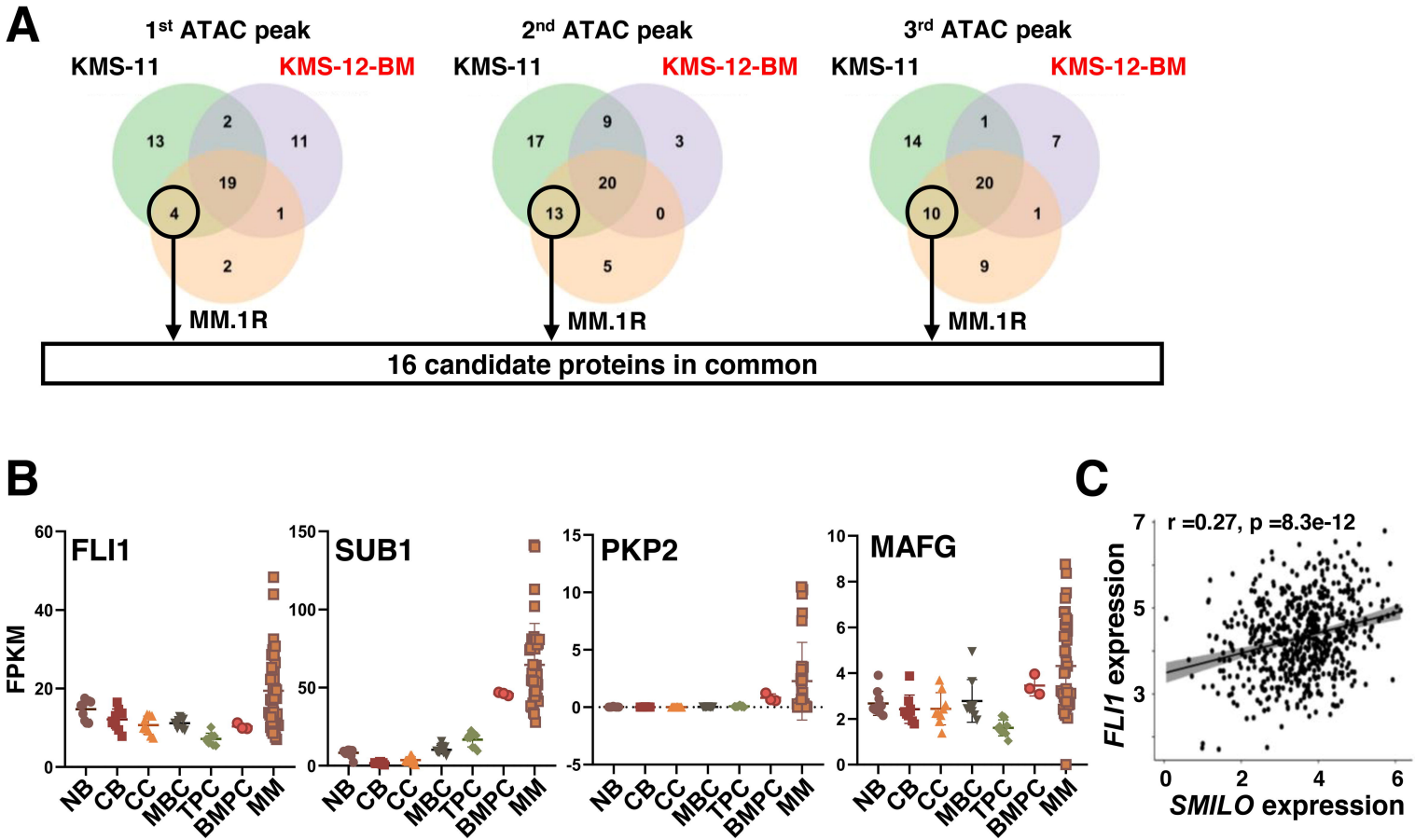
Enhancer: Strong 1 Strong 2 Weak
Transcript.: Transition Elongation Weak
Heterochr. Polycomb Low signal

FIGURE 2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KMS-12-BM
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2
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4
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MM.1R
2nd ATAC peak
KMS-11
KMS-12-BM
17
9
3
20
13
0
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MM.1R
3rd ATAC peak
KMS-11
KMS-12-BM
14
1
7
20
10
1
9
MM.1R
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FLI1
SUB1
PKP2
MAFG
FPKM
NB
CB
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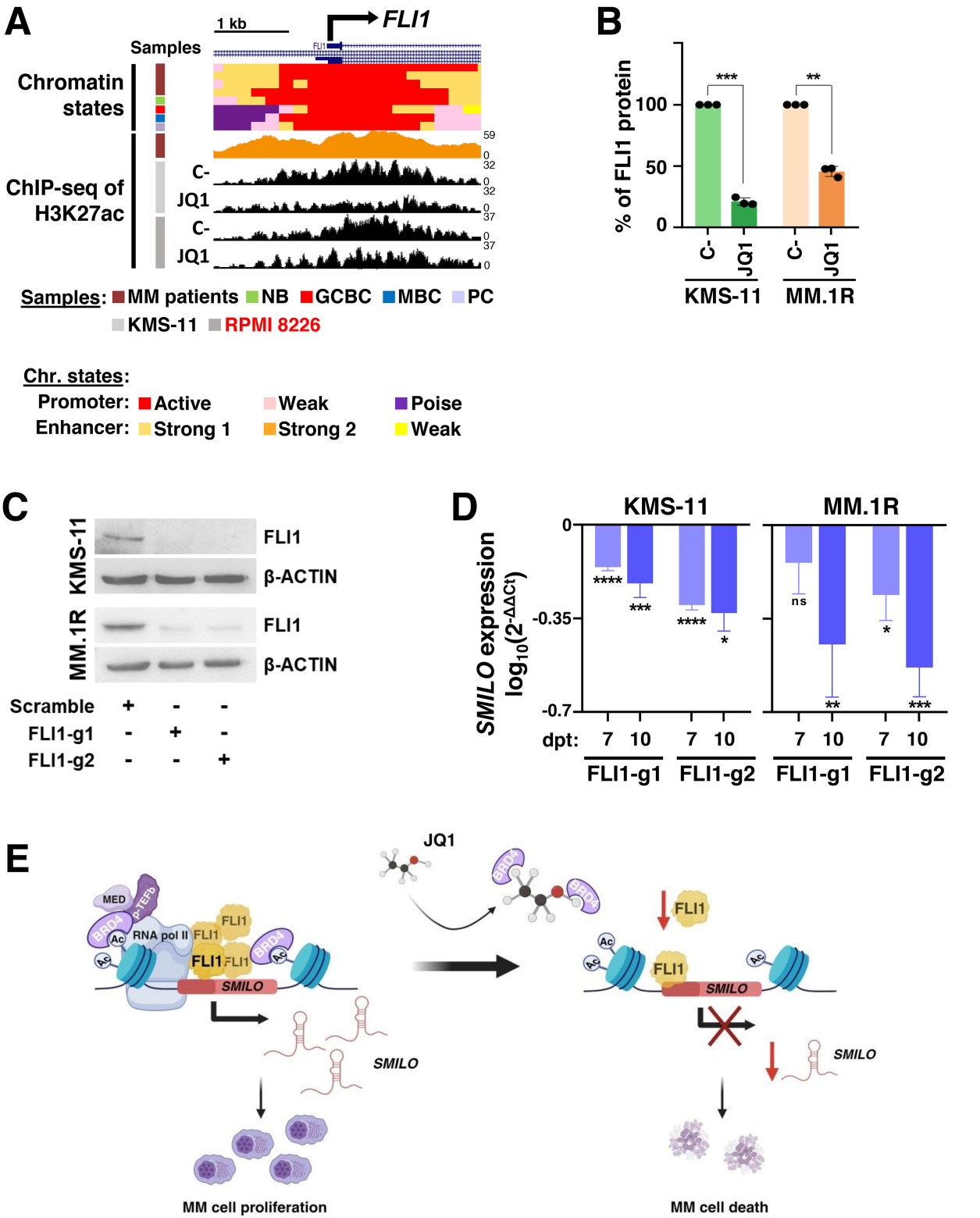
FLI1 expression
SMILO expression

FIGURE 3


A
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FLI1
Samples
Chromatin states
ChIP-seq of H3K27ac
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JQ1
C-
JQ1
Samples: MM patients NB GCBC MBC PC
KMS-11 RPMI 8226
Chr. states:
Promoter: Active Weak Poise
Enhancer: Strong 1 Strong 2 Weak
B
% of FLI1 protein
100
50
0
***
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C-
JQ1
C-
JQ1
KMS-11
MM.1R
C
KMS-11
MM.1R
FLI1
β-ACTIN
FLI1
β-ACTIN
Scramble + - -
FLI1-g1 - + -
FLI1-g2 - - +
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MM.1R
SMILO expression
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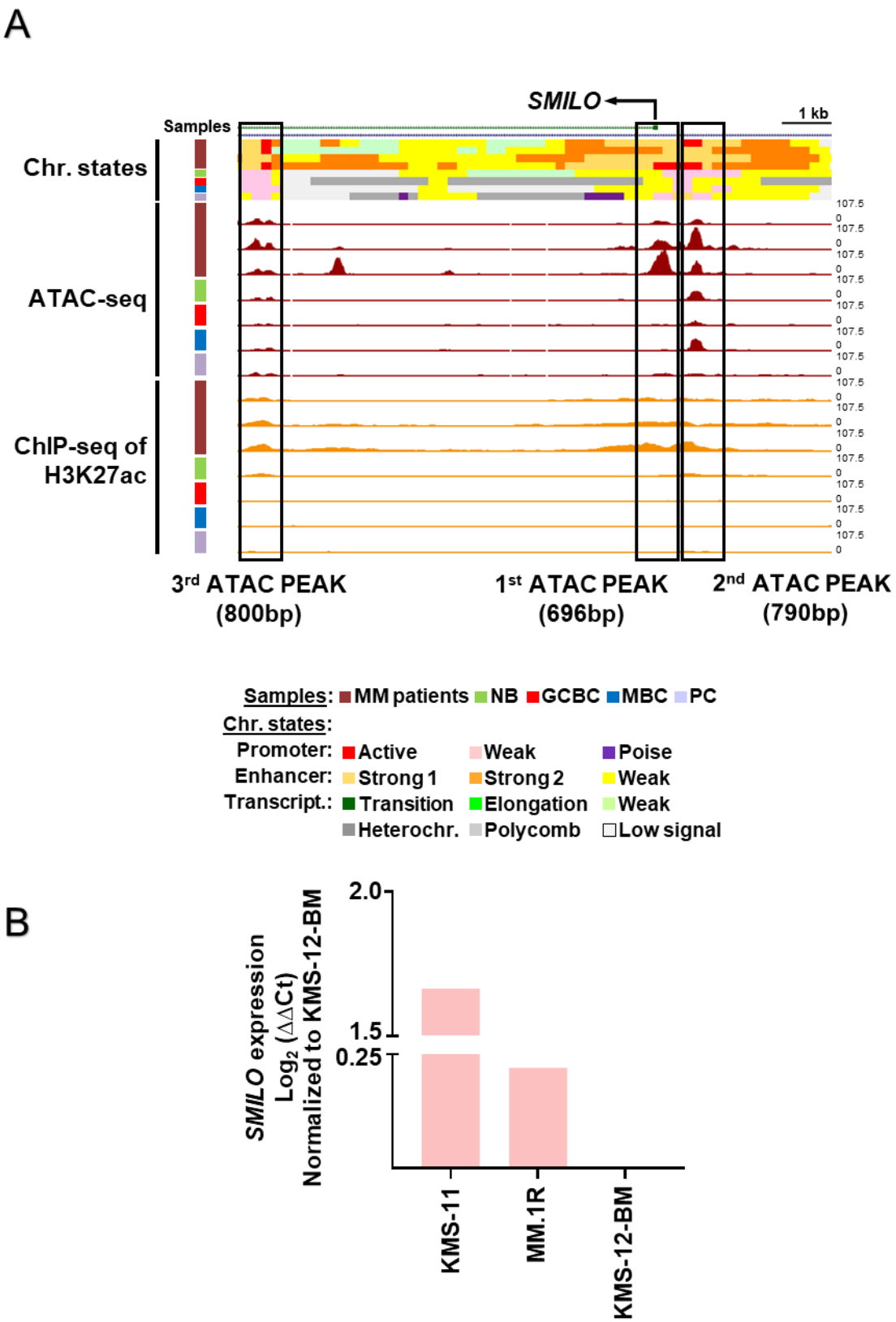
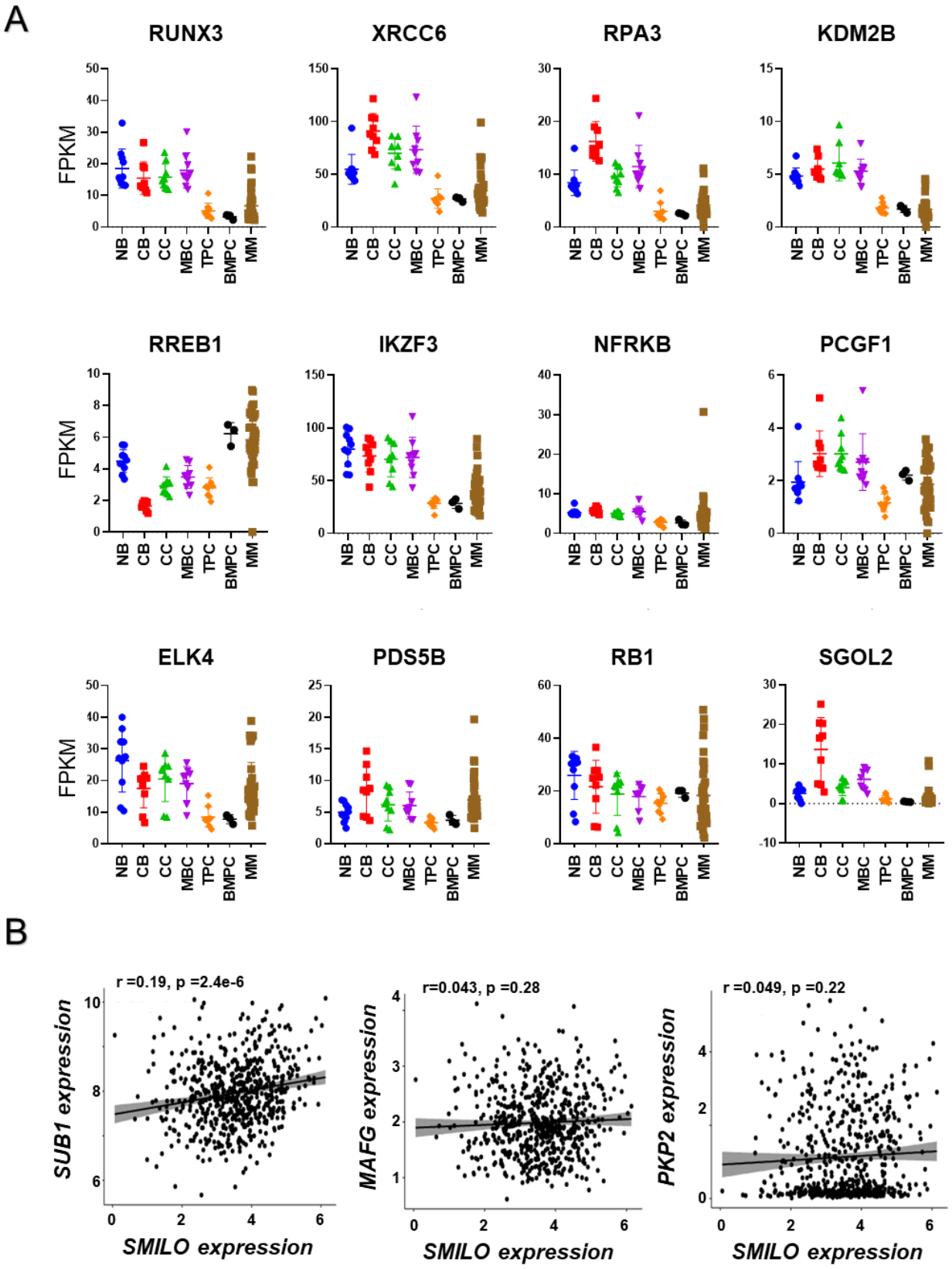


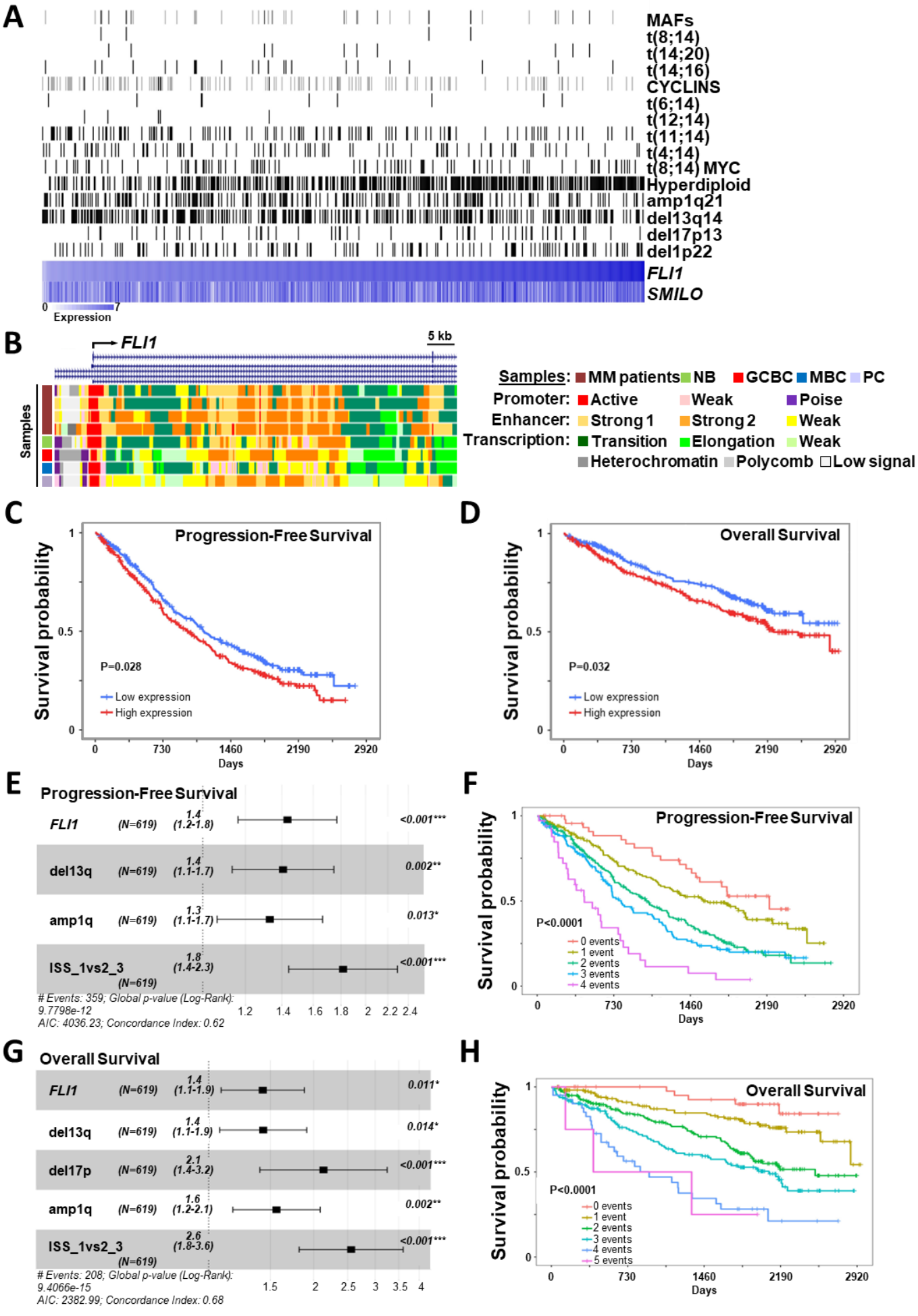
Supplementary figure S1. *SMILO* study in MM. A) Selected chromatin regions from *SMILO* to be analyzed by Reverse-ChIP technique: the figure shows the chromatin states, the chromatin accessibility (ATAC-seq) and the histone acetylation (H3K27ac ChIP-seq) in MM samples compared to healthy B cells. The three highlighted regions through black

squares correspond to three regions studied by Reverse-ChIP named: first, second, and third ATAC peaks. The three DNA regions were selected as follows: the first ATAC peak was localized at the beginning of *SMILO*, presented *de novo* ATAC peak and *de novo* acetylation marks in MM patient samples; the second ATAC peak, was localized before the *SMILO* start site, contained an ATAC peak common to healthy B cells but presented *de novo* chromatin activation marks in MM patient samples; and the third ATAC peak, localized in the middle of *SMILO*, presented two small ATAC peaks common to healthy B cells with *de novo* acetylation marks in MM patient samples. The oligonucleotides used for the Reverse-ChIP were: CTTAAGCCCCAGGACATAAGAA and AGACTGGCTCACACGTAACA as forward (Fw) and reverse (Rv) primers, respectively, for the first peak; ATTGCACCTGGGATCCTGAA and GCCATCAACCAACCTATGCA as Fw and Rv, respectively, for the second peak; and ACGACCTTTTCCCCTGTTGT and CCATCTTTAAGCATCTCTGGGT as Fw and Rv, respectively, for the third peak. Chr: Chromatin; NBC: Naïve B cells; GCBC: Germinal center B cells; MBC: Memory B cells; PC: Plasma cells; MM: Multiple myeloma; **B)** qPCR of *SMILO* in different MM cell lines: the expression was analyzed in three MM cell lines (KMS-11, MM.1R, RPMI 8226) relative to the expression of *SMILO* in KMS-12-BM cell line.



Supplementary figure S2. Reverse-chip protein candidate selection criteria. **A)** Gene expression of the 12 protein candidates that were not overexpressed in MM in comparison to healthy subpopulations of normal B cells. **B)** Expression correlation of the three candidate proteins with *SMILO*. This analysis was performed using CoMMpass 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.


A
MAFs
t(8;14)
t(14;20)
t(14;16)
CYCLINS
t(6;14)
t(12;14)
t(11;14)
t(4;14)
t(8;14) MYC
Hyperdiploid
amp1q21
del13q14
del17p13
del1p22
FLI1
SMILO
0
7
Expression
B
FLI1
5 kb
Samples
Samples: MM patients NB GCBC MBC PC
Promoter: Active Weak Poise
Enhancer: Strong 1 Strong 2 Weak
Transcription: Transition Elongation Weak
Heterochromatin Polycomb Low signal
C
Progression-Free Survival
Survival probability
P=0.028
Low expression
High expression
Days
D
Overall Survival
Survival probability
P=0.032
Low expression
High expression
Days
E
Progression-Free Survival
FLI1 (N=619) 1.4 (1.2-1.8) <0.001***
del13q (N=619) 1.4 (1.1-1.7) 0.002**
amp1q (N=619) 1.3 (1.1-1.7) 0.013*
ISS_1vs2_3 (N=619) 1.8 (1.4-2.3) <0.001***
Events: 359; Global p-value (Log-Rank): 9.7798e-12
AIC: 4036.23; Concordance Index: 0.62
F
Progression-Free Survival
Survival probability
P<0.0001
0 events
1 event
2 events
3 events
4 events
Days
G
Overall Survival
FLI1 (N=619) 1.4 (1.1-1.9) 0.011*
del13q (N=619) 1.4 (1.1-1.9) 0.014*
del17p (N=619) 2.1 (1.4-3.2) <0.001***
amp1q (N=619) 1.6 (1.2-2.1) 0.002**
ISS_1vs2_3 (N=619) 2.6 (1.8-3.6) <0.001***
Events: 208; Global p-value (Log-Rank): 9.4066e-15
AIC: 2382.99; Concordance Index: 0.68
H
Overall Survival
Survival probability
P<0.0001
0 events
1 event
2 events
3 events
4 events
5 events
Days

Supplementary figure S3. FLI1 regulate the expression of *SMILO*. A) Correlation of *FLI1* and *SMILO* expression with classic genetic alterations of MM patients. This analysis was performed using CoMMpass cohort (IA18) from MMRF (Multiple Myeloma Research Foundation). **B)** Chromatin states of *FLI1* gene in B cell differentiation and MM patient samples. **C)** Univariate analyses of the Progression Free Survival (PFS) of the MM patients from CoMMpass cohort IA18 (619 MM patients) considering the expression of *FLI1*. **D)** Univariate analyses of the Overall Survival (OS) of the MM patients from CoMMpass cohort considering the expression of *FLI1*. **E)** Forest plot of the multivariate analysis representing the Progression Free Survival (PFS) in MM patients considering high expression of *FLI1* and classic high-risk genetic alterations in MM ((del(1p), amp(1q), del(17p), del(13q), hypodiploidy, mutations of TP53, t(4;14), t(11;14), t(12;14), t(6;14), t(8;14), t(14;16), and t(14;20)) and ISS stage). **F)** Kaplan-Meier curves showing the PFS of patients with MM considering expression of *FLI1* and different high-risk alterations in a multivariate analysis. **G)** Forest plot of the multivariate analysis representing the Overall Survival (OS) in MM patients considering *FLI1* high expression and classic high-risk genetic alterations in MM. **H)** Kaplan-Meier curves showing the OS of patients with MM considering expression of *FLI1* and different high-risk alterations in a multivariate analysis. NB: Naïve B cells; GCBC: Germinal center B cells; MBC: Memory B cells; PC: Plasma cells; MM: Multiple myeloma.