NEW INSIGHTS ON THE TRANSCRIPTIONAL REGULATION OF CD69 GENE THROUGH A POTENT ENHANCER LOCATED IN THE CONSERVED NON-CODING SEQUENCE 2

Teresa Laguna[1], Laura Notario[1] , Raffaella Pippa[2], Miguel G. Fontela[1], Berta N. Vázquez[1, 3], Miren Maicas[2, 4], Noemí Aguilera-Montilla[5], Ángel L. Corbí[5], María D. Odero[2], Pilar Lauzurica[1]

[1] Activación Immunológica Group, Carlos III National Health Institute (ISCIII), Majadahonda, Spain.

[2] Laboratory of Genetics, Division of Oncology, CIMA, University of Navarra, Pamplona, Spain.

[3] Current address: Department of Genetics, Human Genetics Institute of New Jersey, Rutgers University, Piscataway, New Jersey 08854, USA.

[4] Current address: Biomedicine Institute of Valencia (IBV), Valencia, Spain.

[5] Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

Address correspondence and reprint requests to Dr. Pilar Lauzurica, Activación Immunológica Group, Carlos III National Health Institute, Majadahonda, Spain. E-mail address: lauzurica@isciii.es

ABSTRACT

The CD69 type II C-type lectin is one of the earliest indicators of leukocyte activation acting in lymphocyte migration and cytokine secretion. CD69 expression in hematopoietic lineage undergoes rapid changes depending on the cell-lineage, the activation state or the localization of the cell where it is expressed, suggesting a complex and tightly controlled regulation. Here we provide new insights on the transcriptional regulation of CD69 gene in mammal species. Through *in silico* studies, we analyzed several regulatory features of the 4 upstream conserved non-coding sequences (CNS 1-4) previously described, confirming a major function of CNS2 in the transcriptional regulation of CD69. In addition, multiple transcription binding sites are identified in the CNS2 region by DNA cross-species conservation analysis. By functional approaches we defined a core region of 226 bp located within CNS2 as the main enhancer element of CD69 transcription in the hematopoietic cells analyzed. By chromatin immunoprecipitation, binding of RUNX1 to the core-CNS2 was shown in a T cell line. In addition, we found an activating but not essential role of RUNX1 in CD69 gene transcription by site-directed mutagenesis and RNA silencing, probably through the interaction with this potent enhancer specifically in the hematopoietic lineage. In summary, in this study we contribute with new evidences to the landscape of the transcriptional regulation of the CD69 gene.

1. INTRODUCTION

CD69 is an inducible receptor expressed in leukocytes. It is rapidly upregulated on the membrane of lymphocytes upon stimulation, as it is observed in T cells after 1 hour of treatment with PMA[1], while it reaches its maximum expression in myeloid populations in about 24 hours [2-5]. This time-specific regulation of CD69 expression is suggested to be in part due to distinct transcriptional regulation mechanisms, since several *cis*-acting elements have previously been found in CD69 locus with lineage-specific effects on transcription [6].

In the human and mouse CD69 promoters, regulatory elements binding NF-κB, AP-1, OCT, CREB and the Early Growth Response proteins (EGR) have been identified and proposed as responsible for inducible expression[7-10]. Apart from these, other *cis*-regulatory regions have been identified previously in the CD69 locus[6, 11]: four upstream conserved non-coding sequences (CNS 1-4) and a non-conserved hypersensitivity site (HS) located within the first intron of the CD69 gene. It has been previously shown that the four CNS are regulatory regions being in open conformation and possessing marks of active transcription on histones in mouse lymphocytes[6]. It was also observed a differential regulation between T and B cells in transgenic mice bearing the hCD2 reporter under the control of the CD69 promoter and different combinations of the CNSs [6]. Although transcriptional studies confirmed CNS2 as a potent transcriptional enhancer; in transgenic mouse lines, the construct formed by CNS2 plus CNS1 plus promoter showed an inhibition of the transgene expression[6].

Here we further analyzed the role of CNS2 in CD69 gene transcription, defining specific regulatory elements within this region and identifying transcription factors

which probably intervene in the enhancer mechanism. For that purposes, we employed both *in silico* and experimental procedures.

We performed data mining of predicted conserved Transcription Factor Binding Sites (TFBS) in CNS2, which permitted the finding of cis-acting elements on their basis of conservation during evolution [12]. This method has been successfully applied to find regulatory elements in other immune inducible genes, such as γ Interferon[14]. After comparing these results with data from ENCODE Consortium, we further analyzed the cis- and *trans*-acting elements of CNS2 by experimental means. These approaches allowed us to obtain new insights on the transcriptional regulation of CD69, such as the identification of a minimal enhancer sequence within CNS2 and the role of different transcription factors in this function. The attempt to delineate the function of RUNX1 in CD69 transcription regulation and the discussion of the results founded is presented.

2. MATERIALS & METHODS

2.1. Data from ENCODE consortium

Human open chromatin regions, histone H3K27Ac marks and transcription factor binding by Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) in different cell lines were obtained from the *ENCODE Consortium* [152] and displayed on the *University of California-Santa Cruz* (*UCSC) Genome Browser* (https://genome.ucsc.edu/ENCODE/). Input sequences employed from *UCSC* (https://genome-euro.ucsc.edu/cgi-bin/hgGateway/) were: Human 2009 chr12: 9,902,000-9,953,000 (Supplementary Figure 1); Human 2009 chr12: 9.912.000-9.920.000 (Figs. S2 and S4); Human 2009 chr12: 9,922,000-9,950,500 (Supplementary Figure 3 and Supplementary Figure 5).

2.2. Identification of predicted conserved transcription factor binding sites (TFBSs) within CNS2

Sequences of CNS2 for human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), rhesus (*Macaca mulatta*), dog (*Canis familiaris*) and horse (*Equus caballus*) species, were downloaded from the online platform *Vista-Point* from the portal *VISTA tools from comparative genomics* (http://genome.lbl.gov/vista/index.shtml) using as base genome the human genome version March 2006 from the *UCSC Genomic Browser* website. These sequences were introduced into the application *Genomatix DiAlign* on the *Genomatix* website (http://www.genomatix.de/), and the output data were depicted as arrows indicating the binding sites over a plot of sequence conservation in mammals obtained from the *UCSC Genome Browser* (human Mar 2006: chr12: 9,808,600-9,809,300).

2.3. Plasmids

Mouse CD69 promoter (-1 to -609, BAC clone RP24-188C4) was cloned into BglII and HindIII restriction enzyme (RE) cloning sites of the commercial luciferase vector pGL3 basic (Promega). After that, CNS2 region (mouse 2010 chr6: 129,234,359-129,235,318) was cloned into KpnI and XhoI RE sites, introducing an EcoRI site by KpnI for further cloning. Modified CNS2 constructs containing single and double deletions were generated by overlap PCR [16] employing custom primers (Supplementary Table 1) and cloned into EcoRI and XhoI RE sites in the plasmid containing the CD69 promoter.

2.4. Site-Directed Mutagenesis

The kit *QuikChange Lightning Site-Directed Mutagenesis kit* (Agilent) was employed following manufacturer instructions using primers to perform the mutations shown in Supplementary Table 2. Every PCR product and *DpnI* digestion was checked by agarose gel electrophoresis previous to transformation in bacteria.

2.5. Luciferase assays

Jurkat T cells (5-7 x 10^5), K562, U937 and C1R cells (2-3 x 10^5) were transfected with 1 µg of modified firefly luciferase plasmid (purified with *Plasmid Maxi Kit* from Qiagen) plus 20 ng of pRL-TK (Renilla luciferase plasmid from Promega, to standardize the luciferase activity independently of the efficiency of transfection between samples) using *Superfect* (Qiagen) following manufacturer's protocol. RAJI

cells (5-7 x 10^5) were transfected with 2 µg of firefly luciferase plasmid and 20 ng of renilla plasmid per condition employing 6 µl of *X-tremeGENE 9* reagent from Roche. After transfection, cells were cultured at 37 ºC with 5% CO_2 for 24 hours. Next, they were stimulated or not with 10 ng/ml of PMA and 500 ng/ml of Ionomycin, PMA alone or plate-bound anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (clone CD28.2; eBioscience) mouse antibodies (plated at 5 µg/ml) or were mock incubated, for other 24 hours. 48 h after transfection, cells were lysed using *Passive Lysis Buffer* (Promega) and luciferase activity (firefly/renilla) was measured with the *Dual Luciferase Kit* from Promega.

2.6. Nucleofection

RUNX1 RNA silencing experiments were performed using *Cell Line Nucleofector® Kit V* from Amaxa and siRNAs *siRUNX1-59* (ref: s2459) and *siNeg* were from Ambion. 10^6 Jurkat cells were used per transfection. Cells were washed 3 times in 1x PBS and resuspended in 100 µl of *Cell Line Nucleofector Solution V*. Then 600 ng of *siRUNX1* or *siNeg* were mixed with the cell suspension in an Amaxa certified cuvette and nucleofected applying the program X-05 in the Amaxa Nucleofector. After 10 min at room temperature, cells were harvested with 500 µl of pre-warmed complete medium rinsing the cuvette, transferred to a 6-well culture dish and incubated at 37 ºC and 5% CO_2 for 24 hours in a final volume of 1 ml of complete medium. Next, cells were harvested or stimulated with 10 ng/ml of PMA plus 500 ng/ml of Ionomycin for 24 extra hours. Effective RUNX1 silencing at 24 hours was confirmed by western blot.

2.7. RNA extraction and Real-time PCR

Cells nucleofected for 24 hours (unstimulated) or nucleofected for 24 hours and then stimulated for 24 extra hours were washed in cold 1x PBS and resuspended in 350 µl of lysis buffer RP1 (Macherey-Nagel). RNA extraction was performed employing *NucleoSpin® RNA/Protein* kit from Macherey-Nagel following manufacturer directions. cDNA was synthesized using *AMV Reverse Transcriptase* from Promega according to manufacturer's instructions. Real-time PCR was performed using *LightCycler®* *FastStart DNA Master^{PLUS} SYBR Green I* from Roche. Relative quantification was carried out amplifying hCD69 and 18s RNA (housekeeping control gene). Primers for hCD69 amplify a 50nt-amplicon located between exons 1 and 2. The primers used were: hCD69_F: 5'-CAGTCCAACCCAGTGTTCCT-3';
hCD69_R: 5'-CGTGTTGAGAAATGGGGACT-3';
RNA18S_F: 5'-CTCAACACGGGAAACCTCAC-3';
RNA18S_R: 5'-CGCTCCACCAACTAAGAACG-3'. A touch-down protocol [17] was employed to avoid unspecific DNA amplification.

2.8. Chromatin Immunoprecipitation

ChIP assay was performed as previously described [18]. Briefly, chromatin from cross-linked cells (20 x 10^6 HL-60 cells and 70 x 10^6 in Jurkat cells per condition) was sonicated, incubated overnight with goat anti-RUNX1 (C-19), rabbit anti-Elk-1 (I-20) (Santa Cruz Biotechnology, Inc.) and goat (RUNX1 IP) and rabbit (Elk-1 IP) anti-IgG antibodies (Sigma-Aldrich) in RIPA buffer, and precipitated with protein G/A-Sepharose. Cross-linkage of the co-precipitated DNA-protein complexes was reversed, and DNA was used as a template for quantitative PCR (qPCR). Primers employed are shown in Supplementary Table 3.

2.9. Flow cytometry of human cell lines

Staining was performed for 20 min at 4º C with PE-Cy7- or PE- conjugated anti-human CD69 antibody diluted in staining buffer (1x PBS supplemented with 2% of Fetal Bovine Serum and 2mM of EDTA). Samples were analyzed employing the flow cytometer *FACSCanto* (Becton Dickinson) and data was analyzed using *FACSDiva* software (Becton Dickinson).

3. RESULTS

3.1. CNS2 is a relevant regulatory element in hematopoietic cells

As a first approximation we performed data mining of several regulatory features of the different Conserved Non-Coding Sequences, CNS1-4, described in a previous work[6] (and figure 1), for distinct subpopulations of human cells (https://genome.ucsc.edu/ENCODE/). We observed that the chromatin in the four CNSs were accessible constitutively in the hematopoietic lineages, in agreement with the experimental results of our previous study[6]. Remarkably, the strength of the hypersensitivity signal is higher for CNS2 and CNS1-Promoter than for CNS3 and CNS4 (Figure 1). H3K27ac was also enriched at CNS2 in several hematopoietic cells lines consistent with its role as a potent enhacer. In addition, CNS2 also bound the highest number of transcription factors (Figure 2) when compared with CNS1, CNS3 and CNS4 These data provides additional evidence on the relevance of CNS2 to be a *cis*- regulatory element *in vivo.* Also, most of the factors described to bind to the promoter region were also find to bind to CNS2, which further supports a regulatory interaction between both regions.

Next we performed an *in silico* search with Genomatix program DiAlign plus TF to identify conserved TFBSs in the CNS2 region. This analysis identifies cis-acting elements on their basis of conservation during evolution [12], presumably due to the outcome of beneficial effects on species survival. It is based on the definition of a weight matrix pattern of probability for each family or subfamily of transcription factors to bind a specific sequence of DNA, representing the complete nucleotide statistical distribution for each single position of the binding sequence. For that purpose, we compared sequences of CNS2 from human, mouse, rat, rhesus, dog and horse species,

and displayed the data as arrows indicating the conserved TFBSs (in 6 species black, in 4 species grey) over a plot of human-mice sequence conservation from *VISTA Browser* (Figure 3). [13] We found several conserved TFBSs, most of them common to mouse and human and as expected, generally located in the most conserved regions in CNS2 (Figure 3). Among the binding sites for transcription factors related to the immune function are the RAR-related orphan receptor alpha (RORα) [19] , RUNX [20-22] and the GA-binding protein alpha chain (GABPA) [23, 24] , and NFAT [25, 26], the Interferon regulatory factors (IRF) [27] and c-Rel [28-30] . Other conserved binding sites are for transcription factors related to general processes occurring after activation, like cytoskeletal rearrangement for proliferation, such as SRF, or are targeted by several pathways affected by the immune response, such as the E-twenty six-like factor 1 (Elk-1), which is a target of the MAPK pathways [31]. This analysis suggests that these TFBSs undergo a strong trend to be conserved along all the mammal class, implying that they may have important roles in CD69 gene regulation. As expected, some predicted conserved TFBSs such as ELK1, GATA, SRF, RUNX and NFAT, were confirmed to bind to CNS2 obtained through CHIP assays from ENCODE data (Figure 2).

3.2. Regions of CNS2 responsible for its transcriptional enhancer function

CD69 receptor expression is upregulated in lymphocytes and other leukocytes[32] (Supplementary Figure 1) upon stimulation. To test the importance of the TFBSs in the transcriptional regulation capacity of CNS2, we analyzed the influence of deletions of the regions designated A, B, C and D, corresponding to regions that contain grouped TFBSs in CNS2 (Figure 3 and 4a).

The major effect in the enhancer activity was observed when the region B (which contains TFBSs for RUNX1, GABPA and Elk-1) was eliminated, in unstimulated and stimulated Jurkat cells, reaching a significant 55% reduction in luciferase activity under PMA stimulation (Figure 4b). We observed a similar reduction in the enhancer capacity of CNS2 in the absence of the region B in the monocytic U937 and myeloid K562 PMA-stimulated cell lines (Supplementary Figure 2). A smaller decrease of transcriptional activity with the construct lacking the region A was observed, with significant reductions in the unstimulated or antibody-stimulated Jurkat cell line (Figure 4b). Transcriptional activity of the constructs lacking regions C or D was not significantly different from the activity of the construct with the complete CNS2 (Figure 4b).

As the single deletion of the region B in CNS2 showed an important reduction in its enhancer function, we tested afterwards double deletion of regions, combining the absence of the region B with the deletion of regions A, C or D (Figure 4c). The construct ΔAΔB reduced significantly the enhancer function of CNS2, decreasing transcription levels down to the levels of the promoter alone either in the T (Figure 4c) or in the B cell lines assayed (Supplementary Figure 2a). These data suggests that the region core of 226 bp embracing the regions A and B constitutes the most potent functional enhancer of the CD69 promoter in lymphocytes. To confirm these results, the region of 226bp of CNS2 covering the regions A and B were cloned independently upstream the promoter and assayed for their enhancer capacity. Remarkably, the enhancement of transcriptional activity by the construct with the region A-B of 226 bp was similar to the activity of the complete CNS2 sequence (Figure 4d). Therefore, these results defined the region of 226 bp containing multiple conserved transcription factors

binding elements as a core region that facilitate a cooperative effect of transcription factors occurring to produce the enhancement of CD69 transcription.

3.3. RUNX1 and other transcription factors may cooperate in the enhancer activity of CNS2

As the role of the RUNX transcription factors in thymocyte differentiation and in homeostasis of naive T cells has been described[33], its possible role in transcriptional regulation of CD69 trough CNS2 was further studied. First, the binding of RUNX1 to its conserved site in the region B of CNS2, was assayed by performing chromatin immunoprecipitation in hematopoietic cell lines. Indeed, we observed this binding (CNS2_RUNXBS) in Jurkat cell line after stimulation (Figure 5a). In addition, when RUNX1 is immunoprecipitated, the sequences of Elk TFBS was found enriched according with the proximity of RUNX and Elk transcription factors in the CNS2 region. Elk-1 binding to its own conserved TFBS in CNS2 (CNS2_ELK1BS) was observed in an inducible manner but not enrichment of RUNX1 TFBS was detected (Figure 5b).

To further investigate the role of RUNX1 and other different transcription factors possibly interacting with RUNX1 in CNS2 regulatory function, we tested the enhancer activity of different constructs mutated in several TFBSs within the core region of CNS2: RUNX, GABPA, SRF, RUNX plus SRF and RUNX plus SRF plus GABPA (Figure 6). No significant reduction of transcriptional activity was observed employing these constructs; suggesting that these transcription factor may be acting in a cooperative way. The only single mutation which produces in all experiments a

reduction of the transcriptional activity is the RUNX binding site mutation (Figure 5), although not reached a statistically significance.

We then analyzed if RUNX1 silencing affected CD69 transcription and expression in Jurkat cells. Indeed, CD69 mRNA levels were reduced when a silencer of RUNX1 (siRUNX1) was employed compared to the use of a control silencer (siNeg). This reduction was observed in all the experiments performed (a total of 4) and resulted to be significant (Figure 7) when the cell were unstimulated, however no reduction was observed in stimulated cells (data not shown). Since RUNX1 binding was not observed in the CNS2 region in unstimulated cells, this data suggests that RUNX1 transcription factor may regulate steady state CD69 transcriptional levels independently of CNS2. Moreover, these data indicates that the different transcription factors are collaborating in the enhancement of CD69 transcription carried out by CNS2 and other regions.

4. DISCUSSION

In this work we provide new data on CD69 gene transcriptional regulation: the description of a potent core enhancer in hematopoietic lineages which is located within the conserved non-coding sequence CNS2, and data pointing to a cooperative role of the different transcription factors, such as RUNX1, in the enhancer function through this region.

Data of chromatin accessibility and histone marks of active regulatory elements analyzed from ENCODE showed that the accessible regions match perfectly with the conserved non-coding sequences. Importantly, these open regions were mainly found in hematopoietic cell lines. The ones found in the promoter CNS1 and CNS2 had the highest signal in T and B lymphoid cells, an intermediate signal in erythroblastoid and progenitor cells and a moderate signal in myeloid cell lines. However, in non-hematopoietic cell lines this accessibility was markedly reduced. Therefore patterns of CD69 expression correlate with levels of open chromatin, suggesting that the regulation of the chromatin accessibility is a first control point in the transcriptional regulation of CD69 gene. The high number of transcription factors which bind to CNS2 observed in the ChIP-seq data from ENCODE and our previous results[6] point to this region as a different and relevant regulatory element in the regulation of CD69 transcription. In this work, we defined a region of 226 bp to be responsible of the enhancer role of CNS2 in different hematopoietic cells and analyzed the role of different transcription factors which bind to conserved sites within this core region. However, mutation of the different transcription binding sites did not result in any marked difference in the luciferase expression. This absence of effect may be due to redundancy of transcription factor complexes or due to limitations in the luciferase assay. Indeed, even though the luciferase assay have been widely used in cell lines to determine and characterize the

activity of promoters and enhances effects in regulating genes, it may not reflect the enhancer activity and the chromatin loop activity that occurs *in vivo*.

Although all known hematopoietic subpopulations show inducible expression of CD69 under stimulation by different molecules, the magnitude and the timing of the expression differs considerably [28]. This fact cannot be attributed to differences in the chromatin state of the different cell types, as their chromatin accessibility profiles, observed in both, the ENCODE data presented in this paper and in our previous data[6], were very similar among them. Similarly, according to the results of our transcriptional studies, all the hematopoietic cell lines analyzed show the same pattern of enhancement of the transcriptional activity of CD69 promoter by CNS2. Therefore, the differences in CD69 expression must be caused by different types of regulation, such as the action of different transcription factors on the regulatory regions of CD69. This hypothesis correlates with the observation of RUNX1 binding at basal state and under stimulation to different types of cells (Figures 2 and 5). As CNS2 regulatory region must show an open chromatin conformation without stimuli, the presence of RUNX1 binding seems to be related to the CD69 transcriptional activity (see mRNA expression of these cells at BioGPS). Similarly, the analysis of ELK1 binding to CNS2 in Jurkat cell line suggest it may playing a similar activating role as RUNX1 in transcription.

It has not been previously reported a relation between the transcription factors analyzed here and the lymphocyte activation under stimuli (which promotes the rapid expression of CD69, but not exclusively). However, it was observed that RUNX1 is required for the positive selection of thymocytes [33], the time point when CD69 is starting to be expressed during the thymocyte development [34]. Accordingly, conditional knockout mice of RUNX1 in CD4+ T cells show reduced expression of CD69 in thymocytes [33]. Although these evidences do not reveal a direct regulation of RUNX1 over CD69 gene,

335 it is likely that RUNX1, and the transcription factors which are upregulated after
336 activation, act over multiple gene targets which may include CD69.

337 Currently, there are proposed several mechanisms of activating transcription by
338 enhancers [35]. Our results from the mutagenesis and the ChIP experiments point to the
339 *billboard* mechanism as the most probable way of acting by the transcription factors
340 which bind to the core region of CNS2. Acting through this mechanism, the
341 transcription factors would be acting in a cooperative way, resulting in that any of them
342 would be required, and their action would be additive. Accordingly, it was previously
343 reported that RUNX1 forms highly stable protein-DNA complexes in cooperation with
344 E-twenty six (Ets) family of transcription factors (which include Elk-1), with
345 remarkably frequent binding to T-cell specific enhancers[36-39]. Specifically, RUNX1 and
346 Elk-1 have been proved to upregulate the EVI1 gene [40]. Besides the physical interaction
347 of the transcription factors, the chromatin conformation may be conforming a chromatin
348 loop [41], which has been frequently described for enhancers of several immune genes [42-
349 44]. This is supported by the fact that the vast majority of transcription factors which bind
350 to the promoter also bind to CNS2 in the hematopoietic cells studied in the ChIP
351 experiment from ENCODE (Figure 2), although further evidences are required.

352 Encompassing all these studies, we suggest a model of transcriptional regulation of the
353 CD69 gene (Figure 8), where transcription is controlled at a first level by chromatin
354 accessibility. In this model, in hematopoietic cells, CNS2, and more specifically its core
355 region, plays a major role in the enhancement of the transcription, being RUNX1 a
356 transcription factor which intervenes in that process in a positive manner, at least in T
357 lymphocytes. Depending on the subpopulation of the hematopoietic cells, different
358 transcription factors may be cooperating in the transcriptional regulation, giving
359 specificity and making possible a finely tuned regulation of CD69 protein levels. This

model does not exclude post-transcriptional regulation and needs further experimental analyses assessing the relevance of the complex regulation of CD69 expression in immune cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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5. REFERENCES

1. Hara T, Jung LK, Bjorndahl JM, Fu SM. Human T cell activation. III. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-o-tetradecanoyl phorbol-13-acetate, mitogens, and antigens. *The Journal of experimental medicine* 1986; **164**(6)**:** 1988-2005.

2. Yoshimura C, Yamaguchi M, Iikura M, Izumi S, Kudo K, Nagase H *et al.* Activation markers of human basophils: CD69 expression is strongly and preferentially induced by IL-3. *Journal of Allergy and Clinical Immunology* 2002; **109**(5)**:** 817-823.

3. Urasaki T, Takasaki J, Nagasawa T, Ninomiya H. Induction of the activation-related antigen CD69 on human eosinophils by type IIA phospholipase A2. *Inflammation Research* 2000; **49**(4)**:** 177-183.

4. Marzio R, Jirillo E, Ransijn A, Mauël J, Corradin SB. Expression and function of the early activation antigen CD69 in murine macrophages. *Journal of Leukocyte Biology* 1997; **62**(3)**:** 349-55.

5. Ochiai K, Kagami M, Nakazawa T, Sugiyama T, Sueishi M, Ito M *et al.* Regulation of CD69 Expression on Eosinophil Precursors by Interferon-Î³. *International Archives of Allergy and Immunology* 2000; **122**(Suppl. 1)**:** 28-32.

6. Vazquez BN, Laguna T, Carabana J, Krangel MS, Lauzurica P. CD69 gene is differentially regulated in T and B cells by evolutionarily conserved promoter-distal elements. *J Immunol* 2009; **183**(10)**:** 6513-21.

7. Ziegler SF, Levin SD, Johnson L, Copeland NG, Gilbert DJ, Jenkins NA *et al.* The mouse CD69 gene. Structure, expression, and mapping to the NK gene complex. *The Journal of Immunology* 1994; **152**(3)**:** 1228-36.

8. López-Cabrera M, Muñoz E, Blázquez MV, Ursa MA, Santis AG, Sánchez-Madrid F. Transcriptional Regulation of the Gene Encoding the Human C-type Lectin Leukocyte Receptor AIM/CD69 and Functional Characterization of Its Tumor Necrosis Factor-α-responsive Elements. *Journal of Biological Chemistry* 1995; **270**(37)**:** 21545-21551.

9. Castellanos MC, Muñoz C, Montoya MC, Lara-Pezzi E, López-Cabrera M, de Landázuri MO. Expression of the leukocyte early activation antigen CD69 is regulated by the transcription factor AP-1. *The Journal of Immunology* 1997; **159**(11)**:** 5463-73.

10. del Carmen Castellanos M, López-Giral S, López-Cabrera M, O. de Landázuri M. Multiple cis-acting elements regulate the expression of the early T cell activation antigen CD69. *European Journal of Immunology* 2002; **32**(11)**:** 3108-3117.

11. Vazquez BN, Laguna T, Notario L, Lauzurica P. Evidence for an intronic cis-regulatory element within CD69 gene. *Genes and immunity* 2012; **13**(4)**:** 356-62.

12. Wang H, Zhang Y, Cheng Y, Zhou Y, King DC, Taylor J *et al.* Experimental validation of predicted mammalian erythroid cis-regulatory modules. *Genome Research* 2006; **16**(12)**:** 1480-1492.

13. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A *et al.* MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 2005; **21**(13)**:** 2933-2942.

14. Hatton RD, Harrington LE, Luther RJ, Wakefield T, Janowski KM, Oliver JR *et al.* A distal conserved sequence element controls Ifng gene expression by T cells and NK cells. *Immunity* 2006; **25**(5)**:** 717-29.

15. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012; **489**(7414)**:** 57-74.

16. Higuchi R, Krummel B, Saiki R. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Research* 1988; **16**(15)**:** 7351-7367.

17. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* 1991; **19**(14)**:** 4008.

18. Pippa R, Espinosa L, Gundem G, Garcia-Escudero R, Dominguez A, Orlando S *et al.* p27Kip1 represses transcription by direct interaction with p130/E2F4 at the promoters of target genes. *Oncogene* 2012; **31**(38)**:** 4207-4220.

19. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y *et al.* T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ. *Immunity* 2008; **28**(1)**:** 29-39.

20. Collins A, Littman DR, Taniuchi I. RUNX proteins in transcription factor networks that regulate T-cell lineage choice. *Nat Rev Immunol* 2009; **9**(2)**:** 106-115.

21. Naito T, Tanaka H, Naoe Y, Taniuchi I. Transcriptional control of T-cell development. *International immunology* 2011; **23**(11)**:** 661-668.

22. Wong WF, Kohu K, Chiba T, Sato T, Satake M. Interplay of transcription factors in T-cell differentiation and function: the role of Runx. *Immunology* 2011; **132**(2)**:** 157-64.

23. Bannert N, Avots A, Baier M, Serfling E, Kurth R. GA-binding protein factors, in concert with the coactivator CREB binding protein/p300, control the induction of the interleukin 16 promoter in T lymphocytes. *Proceedings of the National Academy of Sciences* 1999; **96**(4)**:** 1541-1546.

24. Avots A, Hoffmeyer A, Flory E, Cimanis A, Rapp UR, Serfling E. GABP factors bind to a distal interleukin 2 (IL-2) enhancer and contribute to c-Raf-mediated increase in IL-2 induction. *Molecular and cellular biology* 1997; **17**(8)**:** 4381-9.

25. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT Family: Regulation and Function. *Annual Review of Immunology* 1997; **15**(1)**:** 707-747.

26. Macian F. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 2005; **5**(6)**:** 472-484.

27. Paun A, Pitha PM. The IRF family, revisited. *Biochimie* 2007; **89**(6–7)**:** 744-753.

28. Gilmore TD, Kalaitzidis D, Liang M-C, Starczynowski DT. The c-Rel transcription factor and B-cell proliferation: a deal with the devil. *Oncogene* 2004; **23**(13)**:** 2275-2286.

29. Fullard N, Wilson CL, Oakley F. Roles of c-Rel signalling in inflammation and disease. *The International Journal of Biochemistry & Cell Biology* 2012; **44**(6)**:** 851-860.

30. Visekruna A, Volkov A, Steinhoff U. A key role for NF-kappaB transcription factor c-Rel in T-lymphocyte-differentiation and effector functions. *Clinical & developmental immunology* 2012; **2012:** 239368.

31. Kasza A, Wyrzykowska P, Horwacik I, Tymoszuk P, Mizgalska D, Palmer K *et al.* Transcription factors Elk-1 and SRF are engaged in IL1-dependent regulation of ZC3H12A expression. *BMC molecular biology* 2010; **11:** 14.

32. Sancho D, Gómez M, Sánchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. *Trends in immunology* 2005; **26**(3)**:** 136-140.

33. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *The Journal of experimental medicine* 2008; **205**(8)**:** 1939.

34. Swat W, Dessing M, Boehmer HV, Kisielow P. CD 69 expression during selection and maturation of CD4+8+ thymocytes. *European Journal of Immunology* 1993; **23**(3)**:** 739-746.

35. Spitz F, Furlong EEM. Transcription factors: from enhancer binding to developmental control. *Nat Rev Genet* 2012; **13**(9)**:** 613-626.

36. Wotton D, Ghysdael J, Wang S, Speck NA, Owen MJ. Cooperative binding of Ets-1 and core binding factor to DNA. *Molecular and cellular biology* 1994; **14**(1)**:** 840-50.

37. Takeda J, Cheng A, Mauxion F, Nelson CA, Newberry RD, Sha WC *et al.* Functional analysis of the murine T-cell receptor beta enhancer and characteristics of its DNA-binding proteins. *Molecular and cellular biology* 1990; **10**(10)**:** 5027-35.

38. Gottschalk LR, Leiden JM. Identification and functional characterization of the human T-cell receptor beta gene transcriptional enhancer: common nuclear proteins interact with the transcriptional regulatory elements of the T-cell receptor alpha and beta genes. *Molecular and cellular biology* 1990; **10**(10)**:** 5486-95.

39. Prosser HM, Wotton D, Gegonne A, Ghysdael J, Wang S, Speck NA *et al.* A phorbol ester response element within the human T-cell receptor beta-chain enhancer. *Proceedings of the National Academy of Sciences of the United States of America* 1992; **89**(20)**:** 9934-8.

40. Maicas M, Vazquez I, Vicente C, Garcia-Sanchez MA, Marcotegui N, Urquiza L *et al.* Functional characterization of the promoter region of the human EVI1 gene in acute myeloid leukemia: RUNX1 and ELK1 directly regulate its transcription. *Oncogene* 2013; **32**(16)**:** 2069-2078.

41. Kulaeva OI, Nizovtseva EV, Polikanov YS, Ulianov SV, Studitsky VM. Distant Activation of Transcription: Mechanisms of Enhancer Action. *Molecular and cellular biology* 2012; **32**(24)**:** 4892-4897.

42. Tsytsykova AV, Rajsbaum R, Falvo JV, Ligeiro F, Neely SR, Goldfeld AE. Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers. *Proceedings of the National Academy of Sciences* 2007; **104**(43)**:** 16850-16855.

43. Schönheit J, Kuhl C, Gebhardt Marie L, Klett Francisco F, Riemke P, Scheller M *et al.* PU.1 Level-Directed Chromatin Structure Remodeling at the Irf8 Gene Drives Dendritic Cell Commitment. *Cell Reports* 2013; **3**(5)**:** 1617-1628.

44. Li L, Zhang JA, Dose M, Kueh HY, Mosadeghi R, Gounari F *et al.* A far downstream enhancer for murine Bcl11b controls its T-cell specific expression. *Blood* 2013; **122**(6)**:** 902-911.

6. FIGURE LEGENDS

Figure 1. DNase hypersensitivity sites and active regulatory histone marks in intron I, promoter, CNS1,CNS2, CNS3 and CNS4 of CD69 gene for different cell lineages. VISTA plot of conservation human (base) to mouse sequences, where the curve shows the percentage of conservation (left); grey zones, conserved non-coding sequences (CNSs). Acetylation of Lysine 27 in histone 3 (H3K27Ac) marks from different human cell lines indicated on the left. Data extracted from ENCODE consortium and depicted in UCSC Browser, ENCODE DNase I hypersensitivity data, condensed and expanded, displayed for hematopoietic (GM12878, K562, CD20+, CD14+, CD34+, HL-60, Jurkat, Th1, Th2, Th17, Treg) and non hematopoietic cells (A549, HeLa S3, HepG2, HUVEC, MCF7, HSMM, H1hESC, NHEK, NHLF). Stronger signals are depicted in black and weaker in grey. Base genome sequence: Human Feb. 2009, chr12 9 905 000-9 950 000.

Figure 2. ENCODE chromatin immunoprecipitation data for promoter, CNS1, CNS2, CNS3 and CNS4 of human CD69 gene. VISTA plot of conservation human (base) to mouse sequences, where the curve shows the percentage of conservation (left); grey zones, conserved non-coding sequences (CNSs). Base genome sequence: Human Feb. 2009, chr12 9 905 000-9 950 000. ENCODE data is depicted through UCSC browser for TF binding obtained from ChIP. The darkness of the bars correlates with the intensity of the binding signal for each analysis.

Figure 3. Identification of conserved transcription factor binding sites related to the immune response in CNS2. VISTA conservation plot showing human and mouse CNS2

sequences comparison. Human sequence position is shown on the *x* axis and percentage similarity to mouse sequence on the *y* axis. Above, arrows mark the conserved transcription factor binding sites found using *Genomatix DiAlign* (see *Material & Methods*) (black arrows, TFBS conserved in the 6 species studied: human, mouse, rat, rhesus, dog, horse; grey arrows, TFBS conserved in 4 or 5 of those species). Every numbered arrow correspond to a TFBS indicated on the legend (right), where [1)] correspond to TFBS non conserved in mice and [2)] marks TFBS non-conserved in the human species (both in italic). Base sequence: human Mar 2006, chr12:9 808 600-9 809 300. Below, Conserved TFBS identified in mouse CNS2, grouped in 4 regions as for human CNS2.

Figure 4. <u>The regions A and B are mainly responsible for the enhancer activity of CNS2</u>. Jurkat cells were transfected with different modified pGL3 plasmids as indicated on the left. 24 hours later cells were stimulated or not with anti-mouse CD3 & anti-mCD28 (**a**) or PMA/Ionomycin (**a-c**), and after 24 extra hours luciferase activity was measured. Data represent the mean activity of each construct respect to the luciferase activity of the Promoter alone (*Prom*, RLU = 1) for each condition. Error bars represent SEM of 3 experiments. Each condition in every experiment was performed in triplicates. Statistics are calculated by one-way ANOVA with Bonferroni pair comparison method, where: *, p< 0,05; **, p< 0,01; ***, p<0,001. RLU, Relative Luciferase Units.

Figure 5. <u>RUNX1 binds to its TFBS in CNS2 in the hematopoietic lineage.</u> Chromatin immunoprecipitation with anti-RUNX1 (**a**) and anti-ELK1 (**b**) antibodies was

607 performed in untreated ("Unstim.") or 24 hours PMA-stimulated ("PMA") Jurkat cells.
608 Analysis of the co-immunoprecipitated sequences was performed by quantitative PCR
609 amplifying a region in the promoter (Prom), the conserved TFBS for RUNX in CNS2
610 (CNS2_RUNXBS), the conserved TFBS for RUNX in CNS3 (CNS3_RUNXBS) and
611 the conserved TFBS for ELK1 close to the RUNX binding site (CNS2_ELK1BS. qRT–
612 PCR results were calculated using the 2-ΔΔCt method, and they are presented as the
613 fold enrichment of chromatin DNA precipitated by the specific antibody versus
614 chromatin DNA precipitated by goat anti-IgG (for RUNX1) or rabbit anti- IgG (for
615 ELK1), as control. Data represent the mean of three different quantitative measures per
616 IP.

617

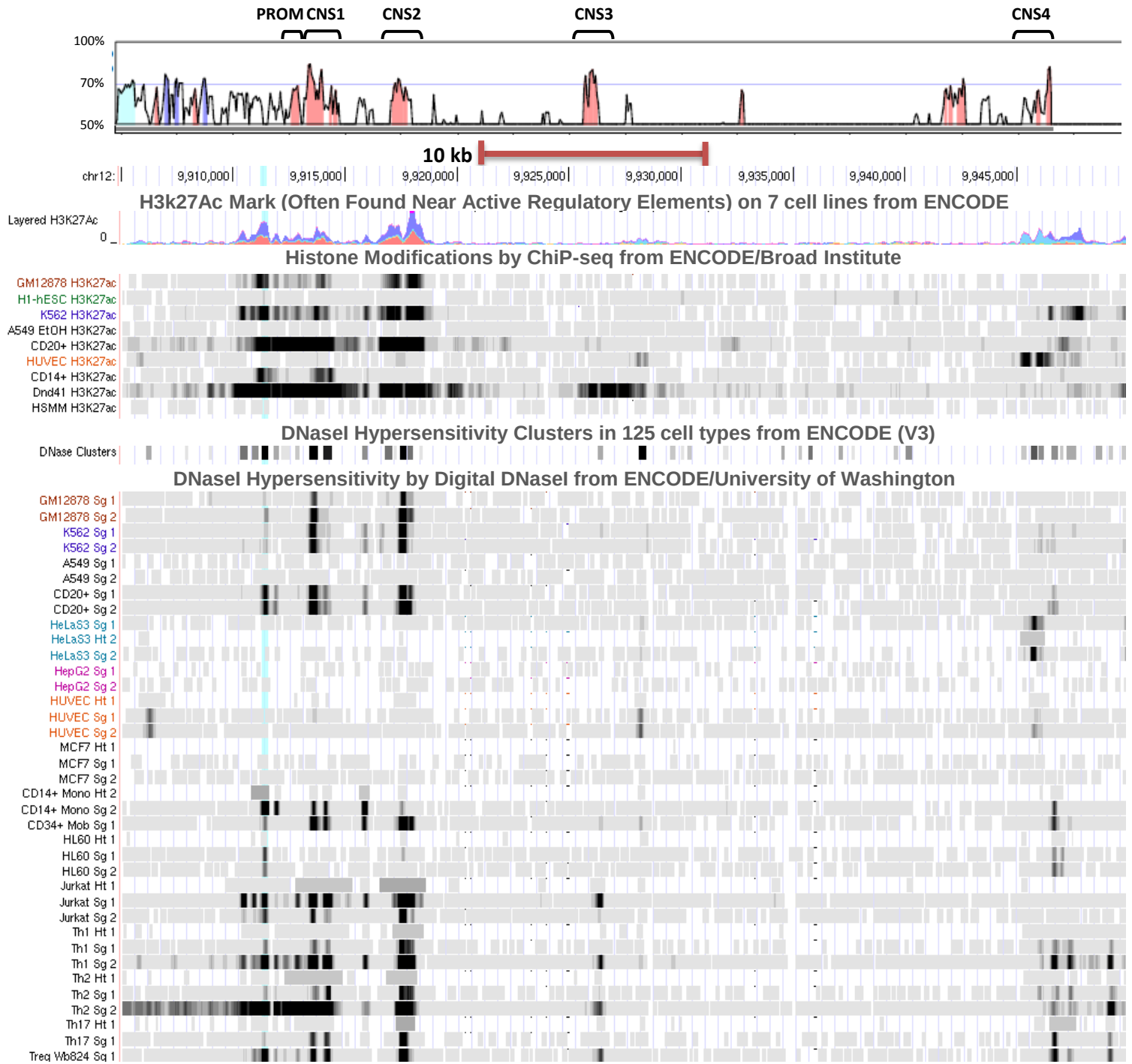
618 Figure 6. Contribution of RUNX, GABPA and SRF transcription factor in A-B
619 enhancer activity. Site-directed mutagenesis was designed for RUNX, GABPA, SRF
620 binding sites or combinations of them in CNS2 and transfection of the mutated plasmids
621 was performed into Jurkat cell line. Data are represented as Mean +/- SEM from 4
622 different experiments. Each trasnfection in every experiment was performed in
623 duplicates or triplicates. *RLU*, Relative Luciferase Units.

624

625 Figure 7. Down-regulation of hCD69 mRNA after RUNX1 silencing. Jurkat cells were
626 nucleofected with RNA silencer of human RUNX1 (*siRUNX*) or a control silencer
627 (*siNeg*) for 24 h and then RNA was extracted and analyzed by Real-Time PCR. Data are
628 presented as Mean ± SEM of 4 different experiments in which every transfection was
629 performed in triplicate. The mean value of cuadriplicates for siNeg transfection was

given an RNA relative concentration value of "100" and the siRUNX1 values were calculated accordingly.

Figure 8. Proposed model of action of CNS2 in the regulation of the transcription of CD69 gene. CNS2 is only accessible in the hematopoietic lineage, being the regions A and B responsible for most all the enhancer activity of CNS2 on CD69 promoter. RUNX transcription factor binding site participates in this activity but needs the action of other TF in their respective binding sites in A and B. *Bottom*, one possible mechanism of action of CNS2 and TF in enhancement of promoter activity which consist in the formation of a loop between the two regions with the TF forming a complex, interacting at the same time with both regions and enhancing the transcription.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CNS2
CNS3
CNS4
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70%
50%
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H3k27Ac Mark (Often Found Near Active Regulatory Elements) on 7 cell lines from ENCODE
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GM12878 H3K27ac
H1-hESC H3K27ac
K562 H3K27ac
A549 EtOH H3K27ac
CD20+ H3K27ac
HUVEC H3K27ac
CD14+ H3K27ac
Dnd41 H3K27ac
HSMM H3K27ac
DNaseI Hypersensitivity Clusters in 125 cell types from ENCODE (V3)
DNase Clusters
DNaseI Hypersensitivity by Digital DNaseI from ENCODE/University of Washington
GM12878 Sg 1
GM12878 Sg 2
K562 Sg 1
K562 Sg 2
A549 Sg 1
A549 Sg 2
CD20+ Sg 1
CD20+ Sg 2
HeLaS3 Sg 1
HeLaS3 Ht 2
HeLaS3 Sg 2
HepG2 Sg 1
HepG2 Sg 2
HUVEC Ht 1
HUVEC Sg 1
HUVEC Sg 2
MCF7 Ht 1
MCF7 Sg 1
MCF7 Sg 2
CD14+ Mono Ht 2
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CD34+ Mob Sg 1
HL60 Ht 1
HL60 Sg 1
HL60 Sg 2
Jurkat Ht 1
Jurkat Sg 1
Jurkat Sg 2
Th1 Ht 1
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Th17 Sg 1
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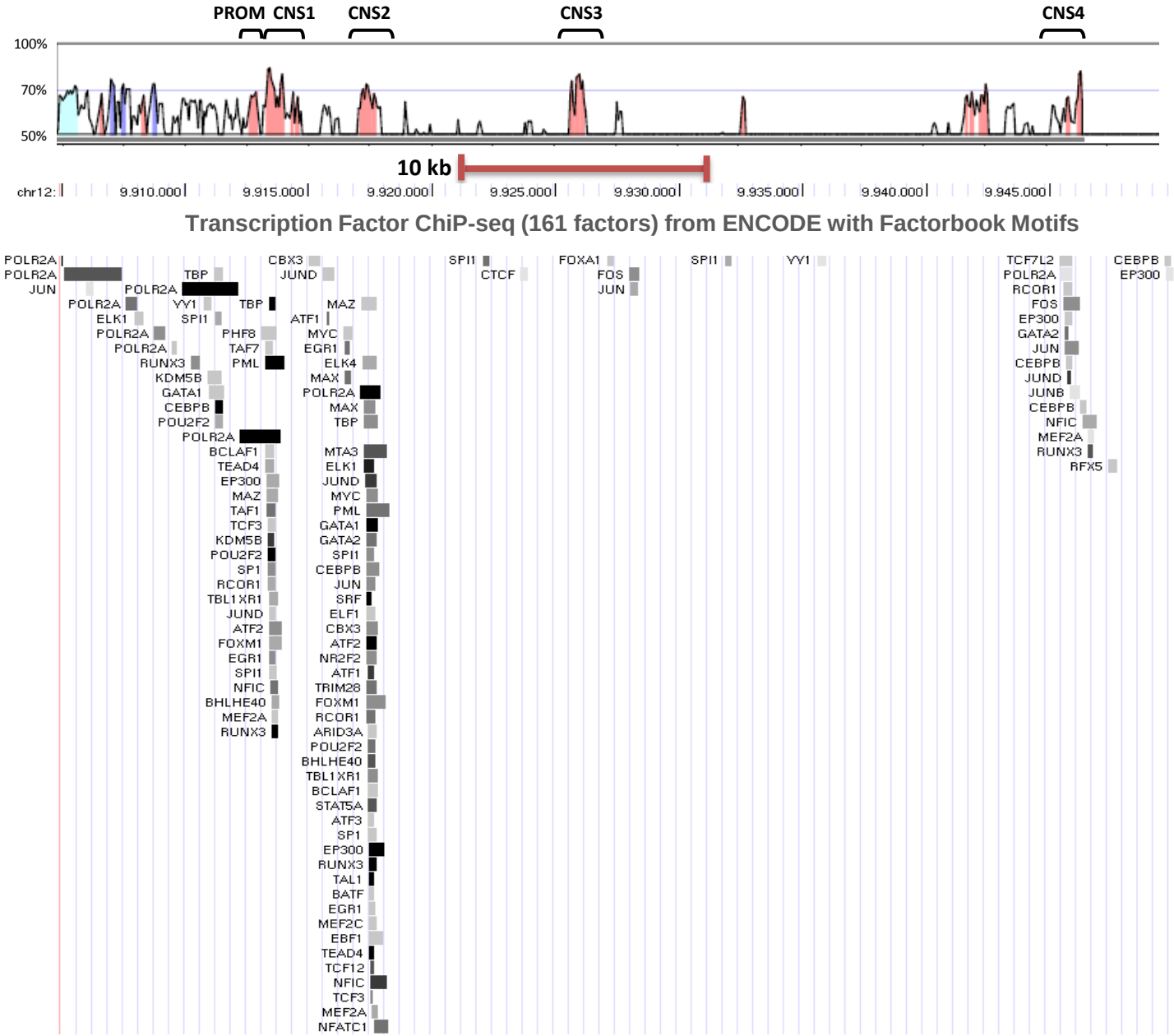
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CNS2
CNS3
CNS4
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70%
50%
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chr12:
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Transcription Factor ChiP-seq (161 factors) from ENCODE with Factorbook Motifs
POLR2A
POLR2A
JUN
TBP
POLR2A
POLR2A
YY1
TBP
ELK1
SPI1
POLR2A
PHF8
POLR2A
TAF7
RUNX3
PML
KDM5B
GATA1
CEBPB
POU2F2
POLR2A
BCLAF1
TEAD4
EP300
MAZ
TAF1
TCF3
KDM5B
POU2F2
SP1
RCOR1
TBL1XR1
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ATF2
FOXM1
EGR1
SPI1
NFIC
BHLHE40
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RUNX3
CBX3
JUND
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ATF1
MYC
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POLR2A
MAX
TBP
MTA3
ELK1
JUND
MYC
PML
GATA1
GATA2
SPI1
CEBPB
JUN
SRF
ELF1
CBX3
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STAT5A
ATF3
SP1
EP300
RUNX3
TAL1
BATF
EGR1
MEF2C
EBF1
TEAD4
TCF12
NFIC
TCF3
MEF2A
NFATC1
SPI1
CTCF
FOXA1
FOS
JUN
SPI1
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FOS
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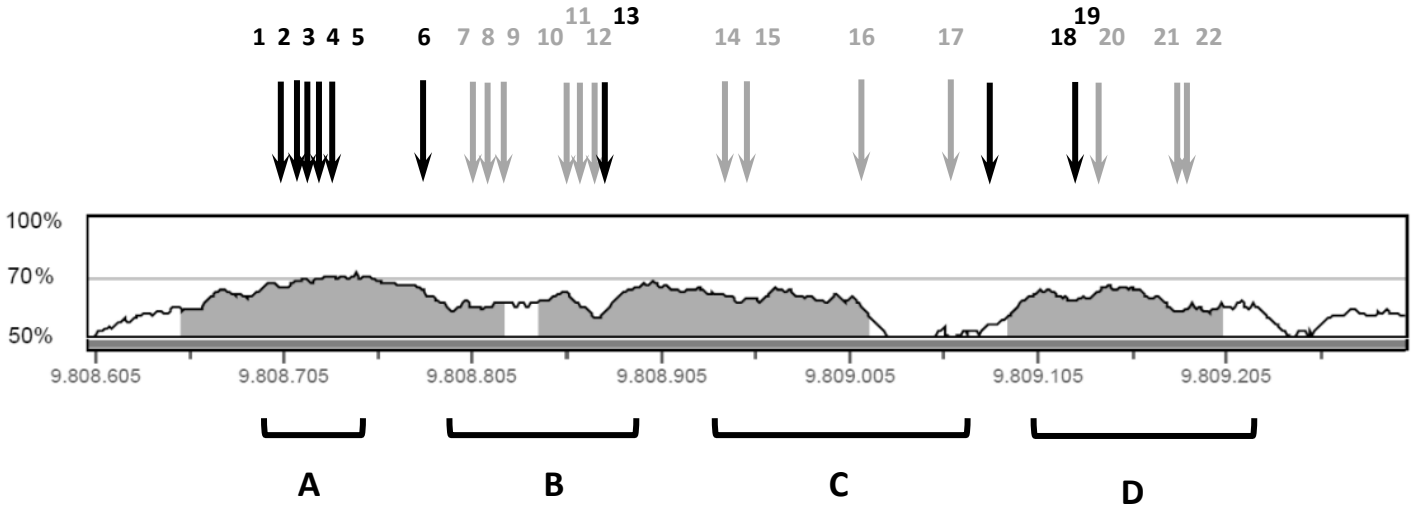
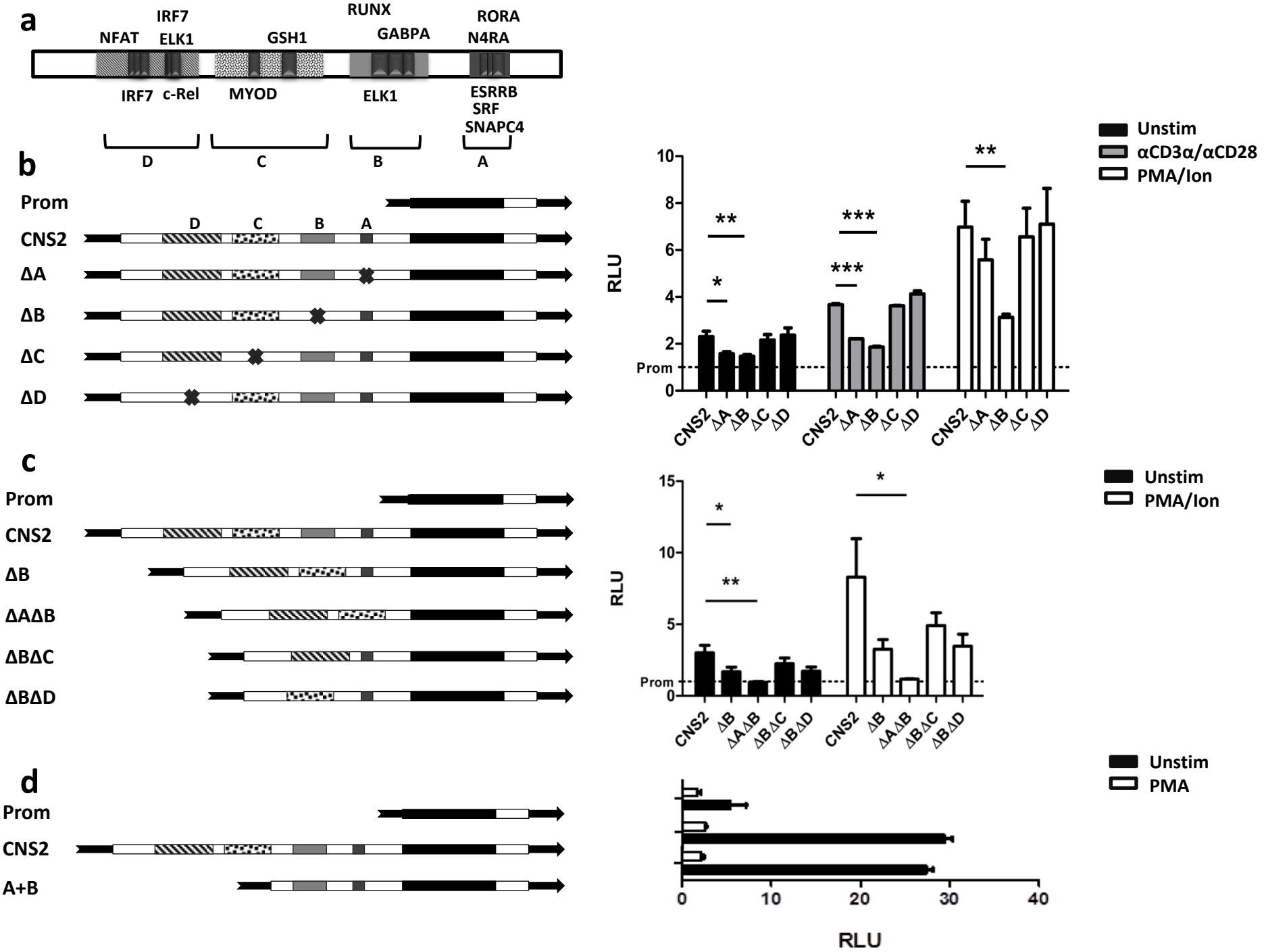


1 → RORA
2 → NR4A
3 → ESRBB
4 → SRF
5 → SNAP4
6 → OCT
7 → GATA
8 → MYOD
9 → ETS [1)]
10 → RUNX
11 → GABPA/NRF2 (ETS)
12 → STAT1 [1)]
13 → ELK1 (ETS)
14 → IRF4 [1)]
15 → HOXF [1)]
16 → GSH
17 → MYOD
18 → C-REL
19 → ETS
20 → IRF7 [2)]
21 → NFAT
22 → IRF7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IRF7
ELK1
GSH1
RUNX
GABPA
RORA
N4RA
IRF7
c-Rel
MYOD
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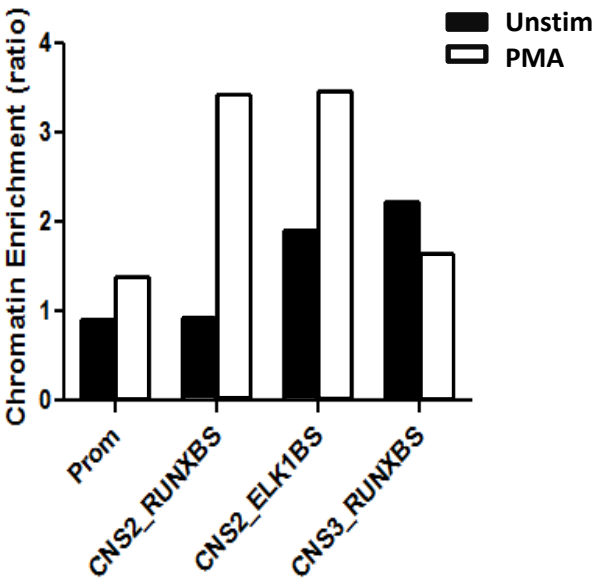
SRF
SNAPC4
D
C
B
A
b
Prom
CNS2
ΔA
ΔB
ΔC
ΔD
Unstim
αCD3α/αCD28
PMA/Ion
RLU
c
ΔAΔB
ΔBΔC
ΔBΔD
d
A+B
PMA

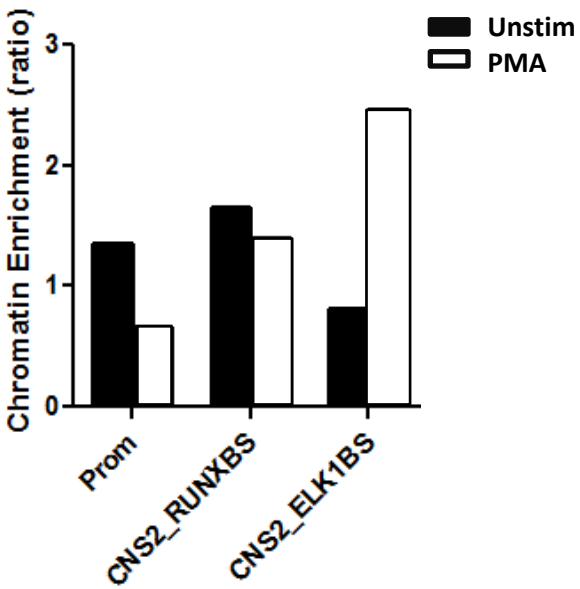
a

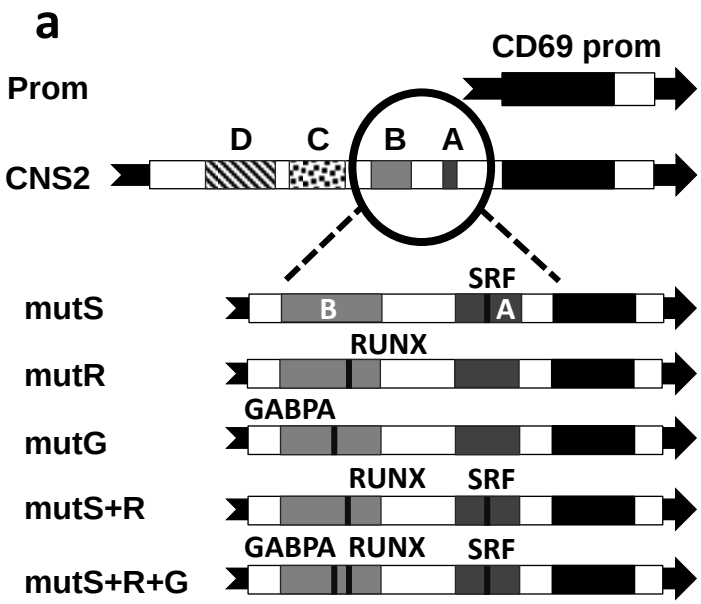
RUNX1 IP - Jurkat

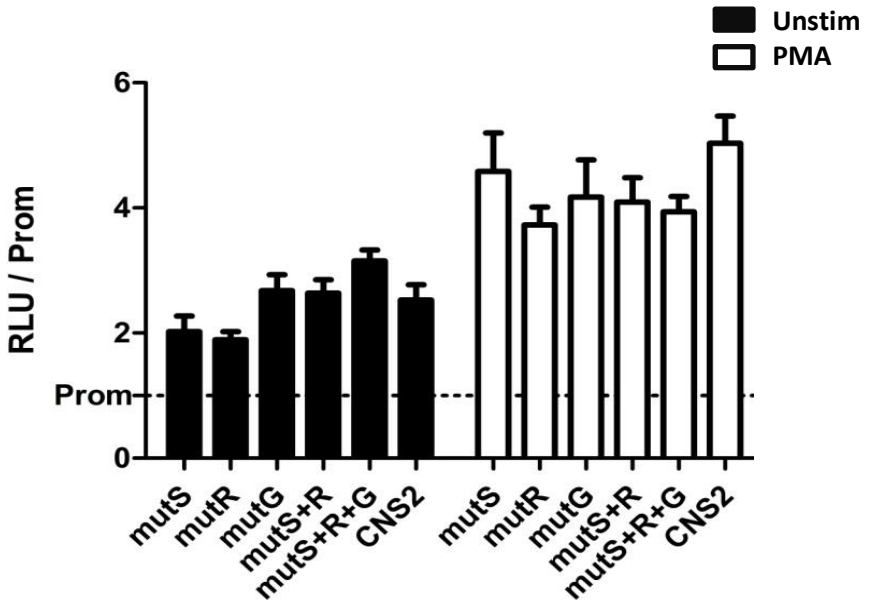


b

ELK1 IP - Jurkat




a
CD69 prom
Prom
D
C
B
A
CNS2
SRF
mutS
B
A
RUNX
mutR
GABPA
mutG
RUNX
SRF
mutS+R
GABPA
RUNX
SRF
mutS+R+G


Unstim
PMA
RLU / Prom
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4
2
Prom
0
mutS
mutR
mutG
mutS+R
mutS+R+G
CNS2
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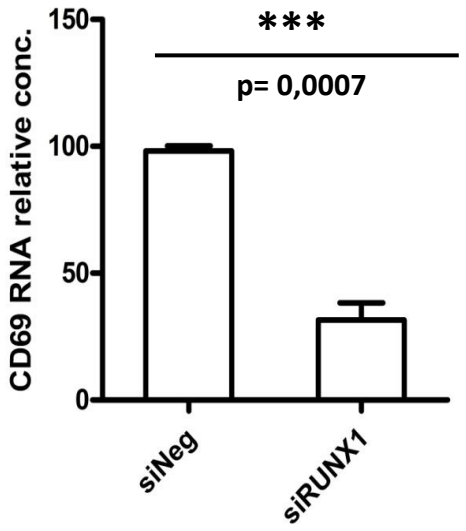
mutR
mutG
mutS+R
mutS+R+G
CNS2


***
p= 0,0007
CD69 RNA relative conc.
150
100
50
0
siNeg
siRUNX1

NON- HEMATOPOIETIC CELL

HEMATOPOIETIC CELL