

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

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21

22 **ABSTRACT**

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

41

## 42 1. INTRODUCTION

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

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

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

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

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

77 **2. MATERIALS & METHODS**

78 2.1. Data from ENCODE consortium

79 Human open chromatin regions, histone H3K27Ac marks and transcription factor  
80 binding by Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) in  
81 different cell lines were obtained from the *ENCODE Consortium*<sup>152</sup> and displayed on  
82 the *University of California-Santa Cruz (UCSC) Genome Browser*  
83 (<https://genome.ucsc.edu/ENCODE/>). Input sequences employed from *UCSC*  
84 (<https://genome-euro.ucsc.edu/cgi-bin/hgGateway/>) were: Human 2009 chr12:  
85 9,902,000-9,953,000 (Supplementary Figure 1); Human 2009 chr12: 9.912.000-  
86 9.920.000 (Figs. S2 and S4); Human 2009 chr12: 9,922,000-9,950,500 (Supplementary  
87 Figure 3 and Supplementary Figure 5).

88

89 2.2. Identification of predicted conserved transcription factor binding sites (TFBSs)  
90 within CNS2

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

101

### 102 2.3. Plasmids

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

110

### 111 2.4. Site-Directed Mutagenesis

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

116

### 117 2.5. Luciferase assays

118 Jurkat T cells ( $5-7 \times 10^5$ ), K562, U937 and C1R cells ( $2-3 \times 10^5$ ) were transfected with  
119 1  $\mu$ g of modified firefly luciferase plasmid (purified with *Plasmid Maxi Kit* from  
120 Qiagen) plus 20 ng of pRL-TK (Renilla luciferase plasmid from Promega, to  
121 standardize the luciferase activity independently of the efficiency of transfection  
122 between samples) using *Superfect* (Qiagen) following manufacturer's protocol. RAJI

123 cells ( $5-7 \times 10^5$ ) were transfected with 2  $\mu\text{g}$  of firefly luciferase plasmid and 20 ng of  
124 renilla plasmid per condition employing 6  $\mu\text{l}$  of *X-tremeGENE 9* reagent from Roche.  
125 After transfection, cells were cultured at 37 °C with 5% CO<sub>2</sub> for 24 hours. Next, they  
126 were stimulated or not with 10 ng/ml of PMA and 500 ng/ml of Ionomycin, PMA alone  
127 or plate-bound anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (clone CD28.2;  
128 eBioscience) mouse antibodies (plated at 5  $\mu\text{g}/\text{ml}$ ) or were mock incubated, for other 24  
129 hours. 48 h after transfection, cells were lysed using *Passive Lysis Buffer* (Promega) and  
130 luciferase activity (firefly/renilla) was measured with the *Dual Luciferase Kit* from  
131 Promega.

132

## 133 2.6. Nucleofection

134 RUNX1 RNA silencing experiments were performed using *Cell Line Nucleofector® Kit*  
135 *V* from Amaxa and siRNAs *siRUNX1-59* (ref: s2459) and *siNeg* were from Ambion.  $10^6$   
136 Jurkat cells were used per transfection. Cells were washed 3 times in 1x PBS and  
137 resuspended in 100  $\mu\text{l}$  of *Cell Line Nucleofector Solution V*. Then 600 ng of *siRUNX1* or  
138 *siNeg* were mixed with the cell suspension in an Amaxa certified cuvette and  
139 nucleofected applying the program X-05 in the Amaxa Nucleofector. After 10 min at  
140 room temperature, cells were harvested with 500  $\mu\text{l}$  of pre-warmed complete medium  
141 rinsing the cuvette, transferred to a 6-well culture dish and incubated at 37 °C and 5%  
142 CO<sub>2</sub> for 24 hours in a final volume of 1 ml of complete medium. Next, cells were  
143 harvested or stimulated with 10 ng/ml of PMA plus 500 ng/ml of Ionomycin for 24  
144 extra hours. Effective RUNX1 silencing at 24 hours was confirmed by western blot.

145

## 146 2.7. RNA extraction and Real-time PCR

147 Cells nucleofected for 24 hours (unstimulated) or nucleofected for 24 hours and then  
148 stimulated for 24 extra hours were washed in cold 1x PBS and resuspended in 350 µl of  
149 lysis buffer RP1 (Macherey-Nagel). RNA extraction was performed employing  
150 *NucleoSpin® RNA/Protein* kit from Macherey-Nagel following manufacturer directions.  
151 cDNA was synthesized using *AMV Reverse Transcriptase* from Promega according to  
152 manufacturer's instructions. Real-time PCR was performed using *LightCycler®*  
153 *FastStart DNA Master<sup>PLUS</sup> SYBR Green I* from Roche. Relative quantification was  
154 carried out amplifying hCD69 and 18s RNA (housekeeping control gene). Primers for  
155 hCD69 amplify a 50nt-amplicon located between exons 1 and 2. The primers used  
156 were: hCD69\_F: 5'-CAGTCCAACCCAGTGTTCCT-3';  
157 hCD69\_R: 5'-CGTGTTGAGAAATGGGGACT-3';  
158 RNA18S\_F: 5'-CTCAACACGGGAAACCTCAC-3';  
159 RNA18S\_R: 5'-CGCTCCACCAACTAAGAACG-3'. A touch-down protocol <sup>17</sup> was  
160 employed to avoid unspecific DNA amplification.

161

## 162 2.8. Chromatin Immunoprecipitation

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



171

172 2.9. Flow cytometry of human cell lines

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

178        3. **RESULTS**

179        3.1. CNS2 is a relevant regulatory element in hematopoietic cells

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

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

202 and displayed the data as arrows indicating the conserved TFBSs (in 6 species black, in  
203 4 species grey) over a plot of human-mice sequence conservation from *VISTA Browser*  
204 (Figure 3).<sup>13</sup> We found several conserved TFBSs, most of them common to mouse and  
205 human and as expected, generally located in the most conserved regions in CNS2  
206 (Figure 3). Among the binding sites for transcription factors related to the immune  
207 function are the RAR-related orphan receptor alpha (ROR $\alpha$ )<sup>19</sup>, RUNX<sup>20-22</sup> and the  
208 GA-binding protein alpha chain (GABPA)<sup>23,24</sup>, and NFAT<sup>25,26</sup>, the Interferon  
209 regulatory factors (IRF)<sup>27</sup> and c-Rel<sup>28-30</sup>. Other conserved binding sites are for  
210 transcription factors related to general processes occurring after activation, like  
211 cytoskeletal rearrangement for proliferation, such as SRF, or are targeted by several  
212 pathways affected by the immune response, such as the E-twenty six-like factor 1 (Elk-  
213 1), which is a target of the MAPK pathways<sup>31</sup>. This analysis suggests that these TFBSs  
214 undergo a strong trend to be conserved along all the mammal class, implying that they  
215 may have important roles in CD69 gene regulation. As expected, some predicted  
216 conserved TFBSs such as ELK1, GATA, SRF, RUNX and NFAT, were confirmed to  
217 bind to CNS2 obtained through CHIP assays from ENCODE data (Figure 2).

218

### 219 3.2. Regions of CNS2 responsible for its transcriptional enhancer function

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

225 The major effect in the enhancer activity was observed when the region B (which  
226 contains TFBSs for RUNX1, GABPA and Elk-1) was eliminated, in unstimulated and  
227 stimulated Jurkat cells, reaching a significant 55% reduction in luciferase activity under  
228 PMA stimulation (Figure 4b). We observed a similar reduction in the enhancer capacity  
229 of CNS2 in the absence of the region B in the monocytic U937 and myeloid K562  
230 PMA-stimulated cell lines (Supplementary Figure 2). A smaller decrease of  
231 transcriptional activity with the construct lacking the region A was observed, with  
232 significant reductions in the unstimulated or antibody-stimulated Jurkat cell line (Figure  
233 4b). Transcriptional activity of the constructs lacking regions C or D was not  
234 significantly different from the activity of the construct with the complete CNS2 (Figure  
235 4b).

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

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

251

252 3.3. RUNX1 and other transcription factors may cooperate in the enhancer activity of  
253 CNS2

254 As the role of the RUNX transcription factors in thymocyte differentiation and in  
255 homeostasis of naive T cells has been described<sup>33</sup>, its possible role in transcriptional  
256 regulation of CD69 through CNS2 was further studied. First, the binding of RUNX1 to  
257 its conserved site in the region B of CNS2, was assayed by performing chromatin  
258 immunoprecipitation in hematopoietic cell lines. Indeed, we observed this binding  
259 (CNS2\_RUNXBS) in Jurkat cell line after stimulation (Figure 5a). In addition, when  
260 RUNX1 is immunoprecipitated, the sequences of Elk TFBS was found enriched  
261 according with the proximity of RUNX and Elk transcription factors in the CNS2  
262 region. Elk-1 binding to its own conserved TFBS in CNS2 (CNS2\_ELK1BS) was  
263 observed in an inducible manner but not enrichment of RUNX1 TFBS was detected  
264 (Figure 5b).

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

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

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

284

285 **4. DISCUSSION**

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

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

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

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

328 It has not been previously reported a relation between the transcription factors analyzed  
329 here and the lymphocyte activation under stimuli (which promotes the rapid expression  
330 of CD69, but not exclusively). However, it was observed that RUNX1 is required for  
331 the positive selection of thymocytes<sup>33</sup>, the time point when CD69 is starting to be  
332 expressed during the thymocyte development<sup>34</sup>. Accordingly, conditional knockout  
333 mice of RUNX1 in CD4+ T cells show reduced expression of CD69 in thymocytes<sup>33</sup>.  
334 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<sup>35</sup>. 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<sup>36-39</sup>. Specifically, RUNX1 and  
346 Elk-1 have been proved to upregulate the EVI1 gene<sup>40</sup>. Besides the physical interaction  
347 of the transcription factors, the chromatin conformation may be conforming a chromatin  
348 loop<sup>41</sup>, which has been frequently described for enhancers of several immune genes<sup>42-</sup>  
349<sup>44</sup>. 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

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

363

## 364 **CONFLICT OF INTEREST**

365 The authors declare no conflict of interest.

366

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373

## 374 **5. REFERENCES**

375

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

380

381 2. Yoshimura C, Yamaguchi M, Iikura M, Izumi S, Kudo K, Nagase H *et al.*  
382 Activation markers of human basophils: CD69 expression is strongly and  
383 preferentially induced by IL-3. *Journal of Allergy and Clinical Immunology*  
384 2002; **109**(5): 817-823.

385

- 386 3. Urasaki T, Takasaki J, Nagasawa T, Ninomiya H. Induction of the activation-  
387 related antigen CD69 on human eosinophils by type IIA phospholipase A2.  
388 *Inflammation Research* 2000; **49**(4): 177-183.
- 389  
390 4. Marzio R, Jirillo E, Ransijn A, Mauël J, Corradin SB. Expression and function  
391 of the early activation antigen CD69 in murine macrophages. *Journal of*  
392 *Leukocyte Biology* 1997; **62**(3): 349-55.
- 393  
394 5. Ochiai K, Kagami M, Nakazawa T, Sugiyama T, Sueishi M, Ito M *et al.*  
395 Regulation of CD69 Expression on Eosinophil Precursors by Interferon- $\hat{I}^3$ .  
396 *International Archives of Allergy and Immunology* 2000; **122**(Suppl. 1): 28-32.
- 397  
398 6. Vazquez BN, Laguna T, Carabana J, Krangel MS, Lauzurica P. CD69 gene is  
399 differentially regulated in T and B cells by evolutionarily conserved promoter-  
400 distal elements. *J Immunol* 2009; **183**(10): 6513-21.
- 401  
402 7. Ziegler SF, Levin SD, Johnson L, Copeland NG, Gilbert DJ, Jenkins NA *et al.*  
403 The mouse CD69 gene. Structure, expression, and mapping to the NK gene  
404 complex. *The Journal of Immunology* 1994; **152**(3): 1228-36.
- 405  
406 8. López-Cabrera M, Muñoz E, Blázquez MV, Ursa MA, Santis AG, Sánchez-  
407 Madrid F. Transcriptional Regulation of the Gene Encoding the Human C-type  
408 Lectin Leukocyte Receptor AIM/CD69 and Functional Characterization of Its  
409 Tumor Necrosis Factor- $\alpha$ -responsive Elements. *Journal of Biological Chemistry*  
410 1995; **270**(37): 21545-21551.
- 411  
412 9. Castellanos MC, Muñoz C, Montoya MC, Lara-Pezzi E, López-Cabrera M, de  
413 Landázuri MO. Expression of the leukocyte early activation antigen CD69 is  
414 regulated by the transcription factor AP-1. *The Journal of Immunology* 1997;  
415 **159**(11): 5463-73.
- 416  
417 10. del Carmen Castellanos M, López-Giral S, López-Cabrera M, O. de Landázuri  
418 M. Multiple cis-acting elements regulate the expression of the early T cell  
419 activation antigen CD69. *European Journal of Immunology* 2002; **32**(11): 3108-  
420 3117.
- 421  
422 11. Vazquez BN, Laguna T, Notario L, Lauzurica P. Evidence for an intronic cis-  
423 regulatory element within CD69 gene. *Genes and immunity* 2012; **13**(4): 356-  
424 62.
- 425  
426 12. Wang H, Zhang Y, Cheng Y, Zhou Y, King DC, Taylor J *et al.* Experimental  
427 validation of predicted mammalian erythroid cis-regulatory modules. *Genome*  
428 *Research* 2006; **16**(12): 1480-1492.

- 429  
430 13. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A *et al.*  
431 MatInspector and beyond: promoter analysis based on transcription factor  
432 binding sites. *Bioinformatics* 2005; **21**(13): 2933-2942.
- 433  
434 14. Hatton RD, Harrington LE, Luther RJ, Wakefield T, Janowski KM, Oliver JR *et al.*  
435 A distal conserved sequence element controls Ifng gene expression by T cells  
436 and NK cells. *Immunity* 2006; **25**(5): 717-29.
- 437 15. An integrated encyclopedia of DNA elements in the human genome. *Nature*  
438 2012; **489**(7414): 57-74.
- 439  
440 16. Higuchi R, Krummel B, Saiki R. A general method of in vitro preparation and  
441 specific mutagenesis of DNA fragments: study of protein and DNA interactions.  
442 *Nucleic Acids Research* 1988; **16**(15): 7351-7367.
- 443  
444 17. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 'Touchdown' PCR to  
445 circumvent spurious priming during gene amplification. *Nucleic Acids Research*  
446 1991; **19**(14): 4008.
- 447  
448 18. Pippa R, Espinosa L, Gundem G, Garcia-Escudero R, Dominguez A, Orlando S  
449 *et al.* p27Kip1 represses transcription by direct interaction with p130/E2F4 at  
450 the promoters of target genes. *Oncogene* 2012; **31**(38): 4207-4220.
- 451  
452 19. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y *et al.* T  
453 Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors  
454 ROR $\alpha$  and ROR $\gamma$ . *Immunity* 2008; **28**(1): 29-39.
- 455  
456 20. Collins A, Littman DR, Taniuchi I. RUNX proteins in transcription factor  
457 networks that regulate T-cell lineage choice. *Nat Rev Immunol* 2009; **9**(2): 106-  
458 115.
- 459  
460 21. Naito T, Tanaka H, Naoe Y, Taniuchi I. Transcriptional control of T-cell  
461 development. *International immunology* 2011; **23**(11): 661-668.
- 462  
463 22. Wong WF, Kohu K, Chiba T, Sato T, Satake M. Interplay of transcription  
464 factors in T-cell differentiation and function: the role of Runx. *Immunology*  
465 2011; **132**(2): 157-64.
- 466  
467 23. Bannert N, Avots A, Baier M, Serfling E, Kurth R. GA-binding protein factors,  
468 in concert with the coactivator CREB binding protein/p300, control the  
469 induction of the interleukin 16 promoter in T lymphocytes. *Proceedings of the*  
470 *National Academy of Sciences* 1999; **96**(4): 1541-1546.

- 471  
472 24. Avots A, Hoffmeyer A, Flory E, Cimanis A, Rapp UR, Serfling E. GABP  
473 factors bind to a distal interleukin 2 (IL-2) enhancer and contribute to c-Raf-  
474 mediated increase in IL-2 induction. *Molecular and cellular biology* 1997;  
475 **17**(8): 4381-9.
- 476  
477 25. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT Family:  
478 Regulation and Function. *Annual Review of Immunology* 1997; **15**(1): 707-747.
- 479  
480 26. Macian F. NFAT proteins: key regulators of T-cell development and function.  
481 *Nat Rev Immunol* 2005; **5**(6): 472-484.
- 482  
483 27. Paun A, Pitha PM. The IRF family, revisited. *Biochimie* 2007; **89**(6-7): 744-  
484 753.
- 485  
486 28. Gilmore TD, Kalaitzidis D, Liang M-C, Starczynowski DT. The c-Rel  
487 transcription factor and B-cell proliferation: a deal with the devil. *Oncogene*  
488 2004; **23**(13): 2275-2286.
- 489  
490 29. Fullard N, Wilson CL, Oakley F. Roles of c-Rel signalling in inflammation and  
491 disease. *The International Journal of Biochemistry & Cell Biology* 2012; **44**(6):  
492 851-860.
- 493  
494 30. Visekruna A, Volkov A, Steinhoff U. A key role for NF-kappaB transcription  
495 factor c-Rel in T-lymphocyte-differentiation and effector functions. *Clinical &*  
496 *developmental immunology* 2012; **2012**: 239368.
- 497  
498 31. Kasza A, Wyrzykowska P, Horwacik I, Tymoszek P, Mizgalska D, Palmer K *et*  
499 *al.* Transcription factors Elk-1 and SRF are engaged in IL1-dependent regulation  
500 of ZC3H12A expression. *BMC molecular biology* 2010; **11**: 14.
- 501  
502 32. Sancho D, Gómez M, Sánchez-Madrid F. CD69 is an immunoregulatory  
503 molecule induced following activation. *Trends in immunology* 2005; **26**(3): 136-  
504 140.
- 505  
506 33. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR. The role of the Runx  
507 transcription factors in thymocyte differentiation and in homeostasis of naive T  
508 cells. *The Journal of experimental medicine* 2008; **205**(8): 1939.
- 509  
510 34. Swat W, Dessing M, Boehmer HV, Kisielow P. CD 69 expression during  
511 selection and maturation of CD4+8+ thymocytes. *European Journal of*  
512 *Immunology* 1993; **23**(3): 739-746.

- 513  
514 35. Spitz F, Furlong EEM. Transcription factors: from enhancer binding to  
515 developmental control. *Nat Rev Genet* 2012; **13**(9): 613-626.
- 516  
517 36. Wotton D, Ghysdael J, Wang S, Speck NA, Owen MJ. Cooperative binding of  
518 Ets-1 and core binding factor to DNA. *Molecular and cellular biology* 1994;  
519 **14**(1): 840-50.
- 520  
521 37. Takeda J, Cheng A, Mauxion F, Nelson CA, Newberry RD, Sha WC *et al.*  
522 Functional analysis of the murine T-cell receptor beta enhancer and  
523 characteristics of its DNA-binding proteins. *Molecular and cellular biology*  
524 1990; **10**(10): 5027-35.
- 525  
526 38. Gottschalk LR, Leiden JM. Identification and functional characterization of the  
527 human T-cell receptor beta gene transcriptional enhancer: common nuclear  
528 proteins interact with the transcriptional regulatory elements of the T-cell  
529 receptor alpha and beta genes. *Molecular and cellular biology* 1990; **10**(10):  
530 5486-95.
- 531  
532 39. Prosser HM, Wotton D, Gegonne A, Ghysdael J, Wang S, Speck NA *et al.* A  
533 phorbol ester response element within the human T-cell receptor beta-chain  
534 enhancer. *Proceedings of the National Academy of Sciences of the United States*  
535 *of America* 1992; **89**(20): 9934-8.
- 536  
537 40. Maicas M, Vazquez I, Vicente C, Garcia-Sanchez MA, Marcotegui N, Urquiza  
538 L *et al.* Functional characterization of the promoter region of the human EVI1  
539 gene in acute myeloid leukemia: RUNX1 and ELK1 directly regulate its  
540 transcription. *Oncogene* 2013; **32**(16): 2069-2078.
- 541  
542 41. Kulaeva OI, Nizovtseva EV, Polikanov YS, Ulianov SV, Studitsky VM. Distant  
543 Activation of Transcription: Mechanisms of Enhancer Action. *Molecular and*  
544 *cellular biology* 2012; **32**(24): 4892-4897.
- 545  
546 42. Tsytsykova AV, Rajsbaum R, Falvo JV, Ligeiro F, Neely SR, Goldfeld AE.  
547 Activation-dependent intrachromosomal interactions formed by the TNF gene  
548 promoter and two distal enhancers. *Proceedings of the National Academy of*  
549 *Sciences* 2007; **104**(43): 16850-16855.
- 550  
551 43. Schönheit J, Kuhl C, Gebhardt Marie L, Klett Francisco F, Riemke P, Scheller  
552 M *et al.* PU.1 Level-Directed Chromatin Structure Remodeling at the Irf8 Gene  
553 Drives Dendritic Cell Commitment. *Cell Reports* 2013; **3**(5): 1617-1628.
- 554

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

558

## 6. FIGURE LEGENDS

559  
560

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

572

573 Figure 2. ENCODE chromatin immunoprecipitation data for promoter, CNS1, CNS2,  
574 CNS3 and CNS4 of human CD69 gene. VISTA plot of conservation human (base) to  
575 mouse sequences, where the curve shows the percentage of conservation (left); grey  
576 zones, conserved non-coding sequences (CNSs). Base genome sequence: Human Feb.  
577 2009, chr12 9 905 000-9 950 000. ENCODE data is depicted through UCSC browser  
578 for TF binding obtained from ChIP. The darkness of the bars correlates with the  
579 intensity of the binding signal for each analysis.

580

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



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

593

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

604

605 Figure 5. RUNX1 binds to its TFBS in CNS2 in the hematopoietic lineage. Chromatin  
606 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  $2^{-\Delta\Delta C_t}$  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 transfection in every experiment was performed in  
623 duplicates or triplicates. *RLU*, Relative Luciferase Units.

624

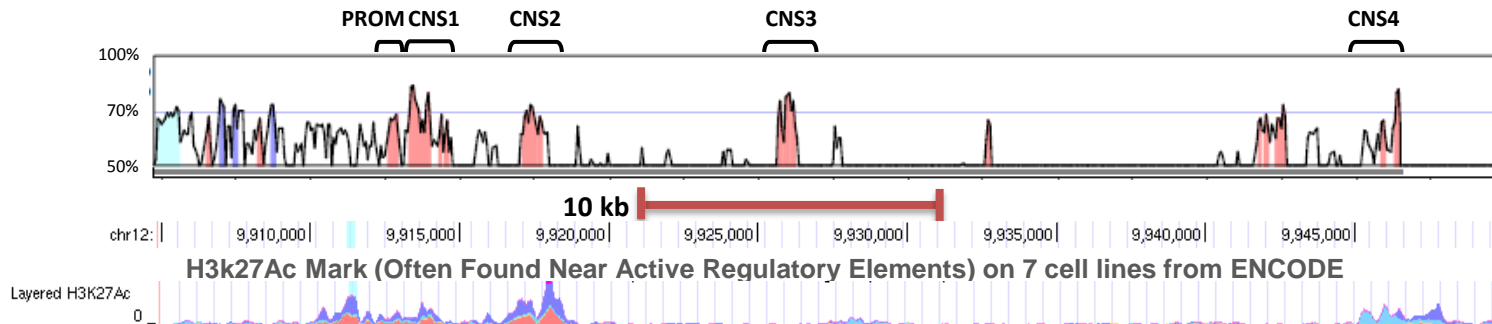
625 Figure 7. Down-regulation of hCD69 mRNA after RUNX1 silencing. Jurkat cells were  
626 nucleofected with RNA silencer of human RUNX1 (*siRUNX*) or a control silencer  
627 (*siNeg*) for 24 h and then RNA was extracted and analyzed by Real-Time PCR. Data are  
628 presented as Mean  $\pm$  SEM of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

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

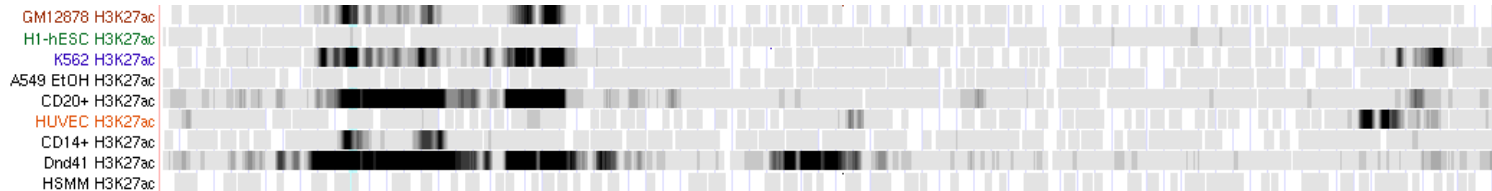
632

633 Figure 8. Proposed model of action of CNS2 in the regulation of the transcription of  
634 CD69 gene. CNS2 is only accessible in the hematopoietic lineage, being the regions A  
635 and B responsible for most all the enhancer activity of CNS2 on CD69 promoter.  
636 RUNX transcription factor binding site participates in this activity but needs the action  
637 of other TF in their respective binding sites in A and B. *Bottom*, one possible  
638 mechanism of action of CNS2 and TF in enhancement of promoter activity which  
639 consist in the formation of a loop between the two regions with the TF forming a  
640 complex, interacting at the same time with both regions and enhancing the transcription.

641



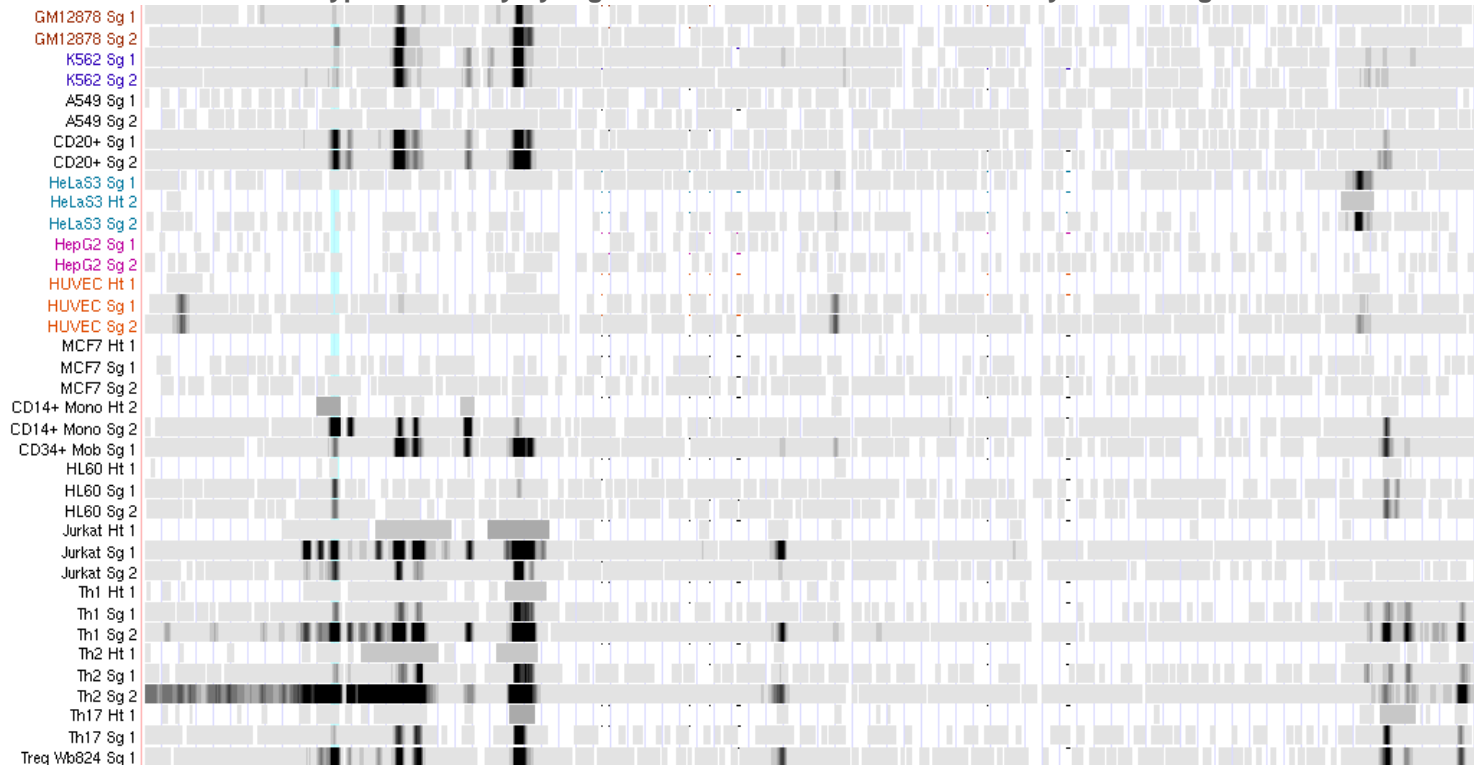
### Histone Modifications by ChiP-seq from ENCODE/Broad Institute

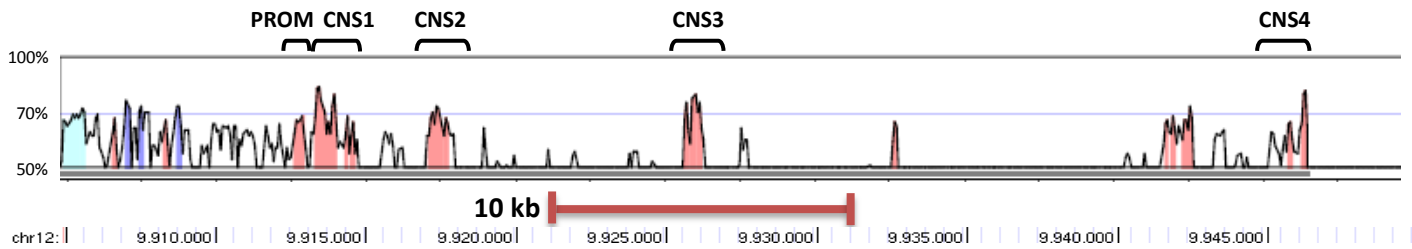


### DNaseI Hypersensitivity Clusters in 125 cell types from ENCODE (V3)

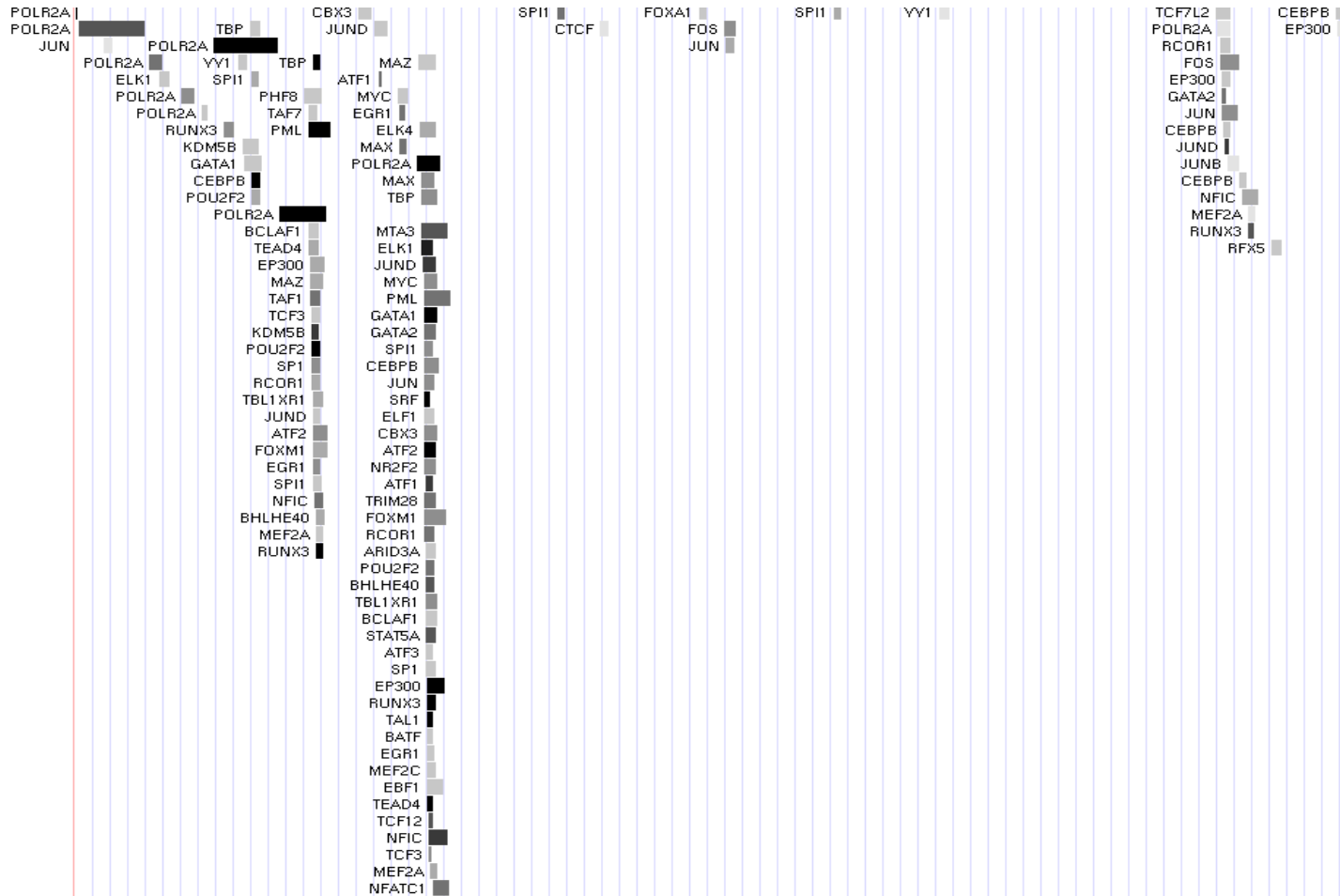
DNase Clusters

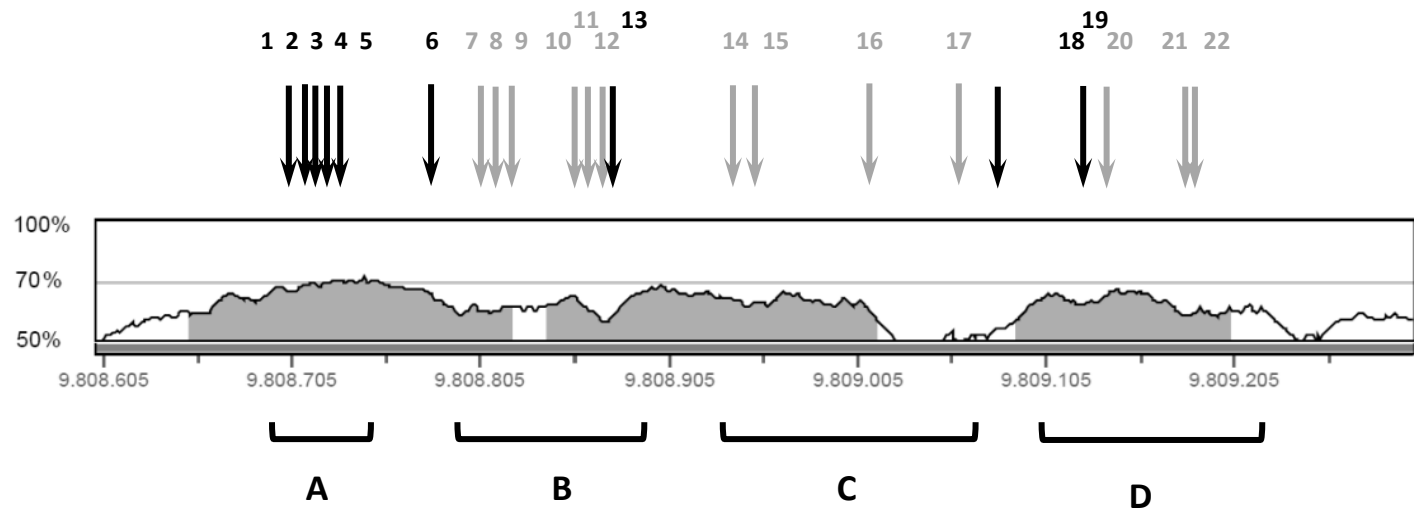
### DNaseI Hypersensitivity by Digital DNaseI from ENCODE/University of Washington



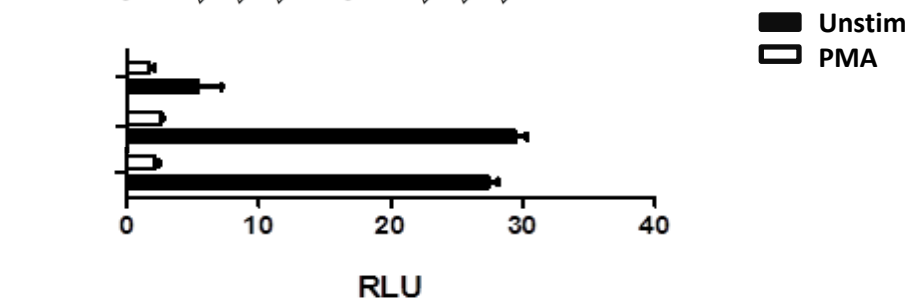
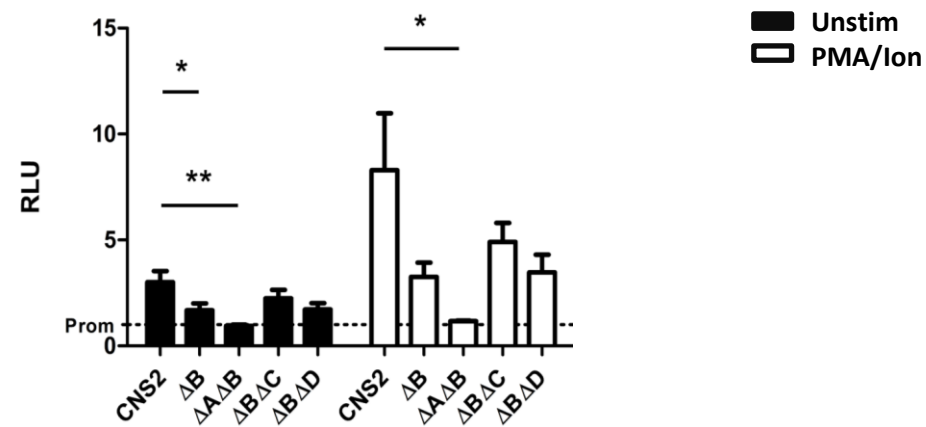
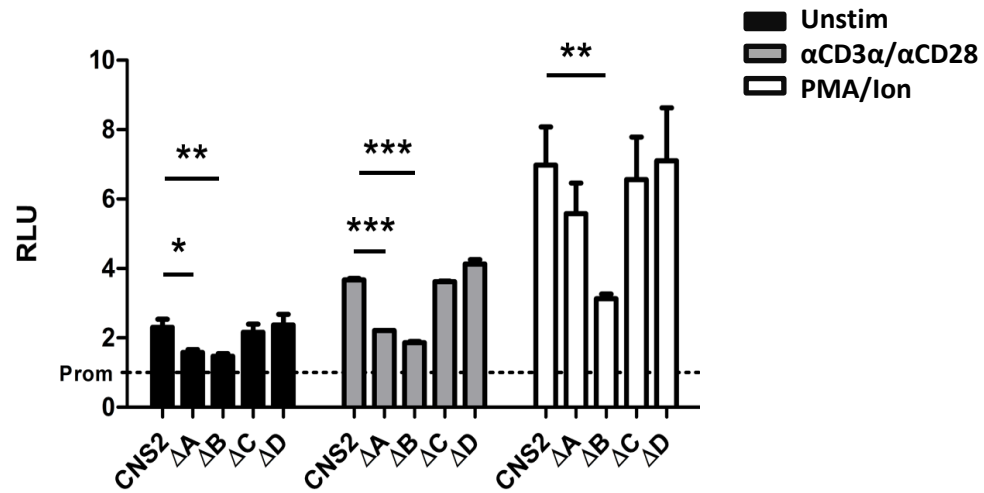
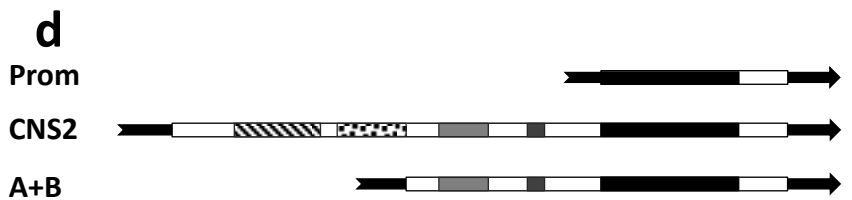
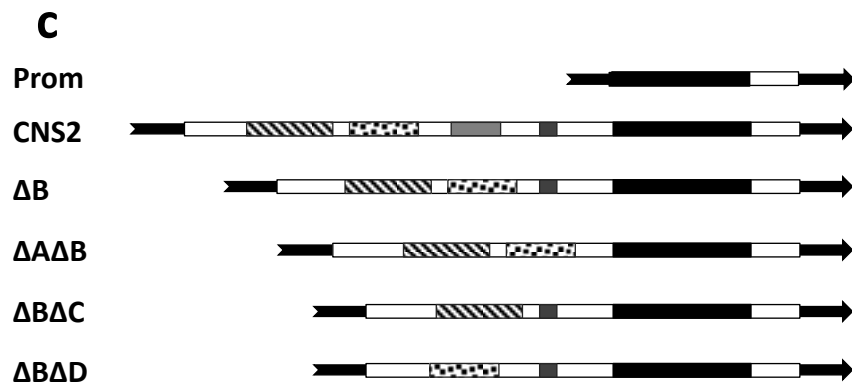
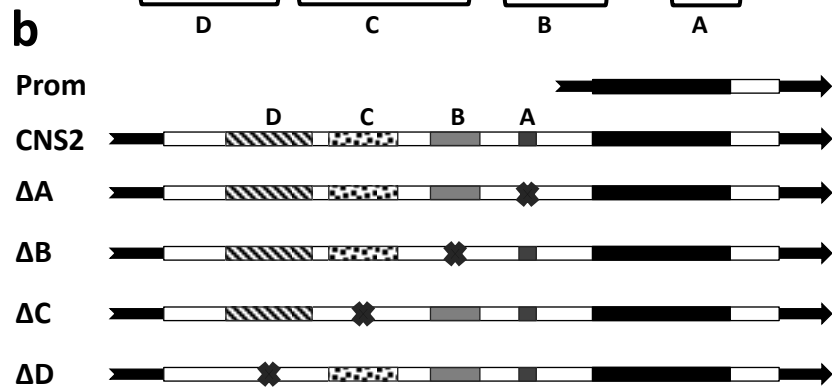
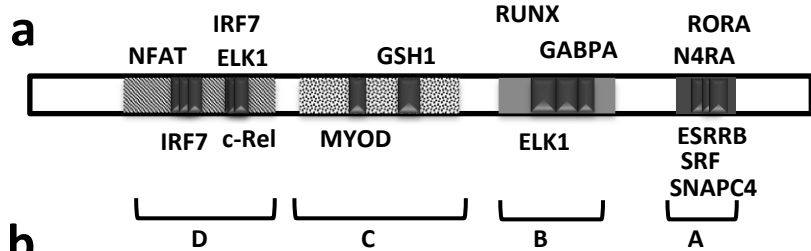


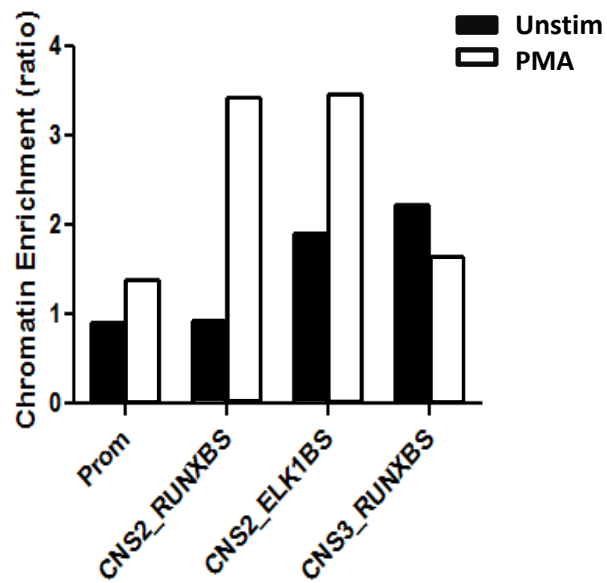
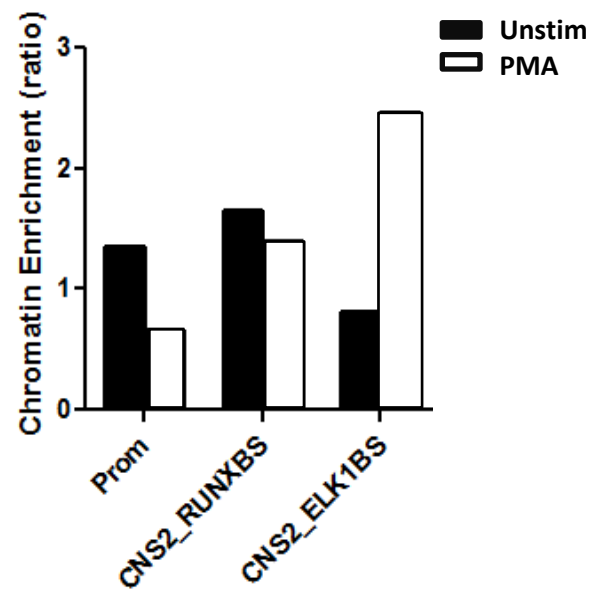
### Transcription Factor ChIP-seq (161 factors) from ENCODE with Factorbook Motifs



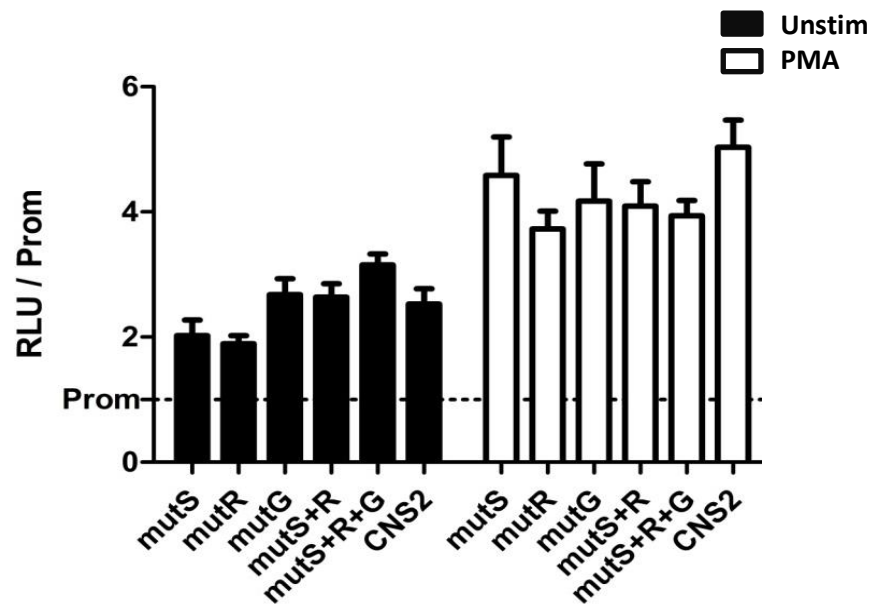
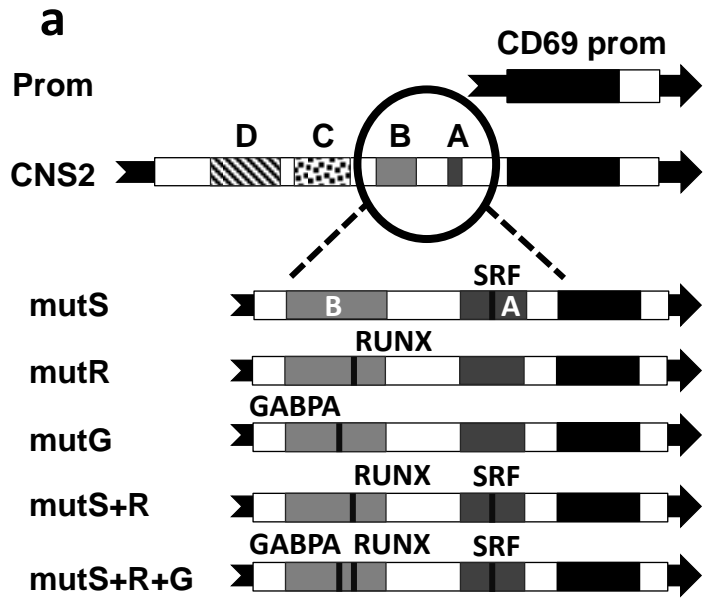


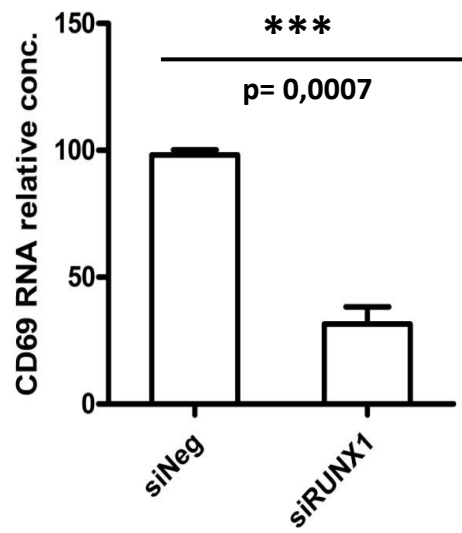
- 1 → RORA
- 2 → NR4A
- 3 → ESRBB
- 4 → SRF
- 5 → SNAP4
- 6 → OCT
- 7 → GATA
- 8 → MYOD
- 9 → *ETS*<sup>1)</sup>
- 10 → RUNX
- 11 → GABPA/NRF2 (ETS)
- 12 → *STAT1*<sup>1)</sup>
- 13 → ELK1 (ETS)
- 14 → *IRF4*<sup>1)</sup>
- 15 → *HOXF*<sup>1)</sup>
- 16 → GSH
- 17 → MYOD
- 18 → C-REL
- 19 → ETS
- 20 → *IRF7*<sup>2)</sup>
- 21 → NFAT
- 22 → IRF7



**a****RUNX1 IP - Jurkat****b****ELK1 IP - Jurkat**



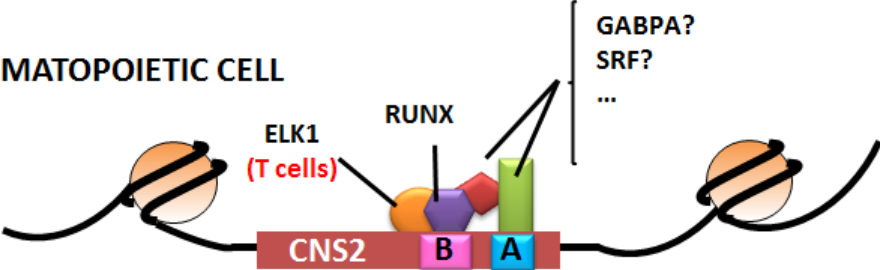




NON- HEMATOPOIETIC CELL



HEMATOPOIETIC CELL



↓ STIMULI

