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Research Article

Regulation of megakaryocytic differentiation of K562 cells by FosB, a member of the Fos family of AP-1 transcription factors

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Abstract. The regulation of megakaryocytic differentiation is poorly understood. Using K562 cells, which can partly recapitulate the process in response to phorbol 12-myristate 13-acetate (PMA), we performed microarray-based gene expression profiling to identify genes that play significant roles in megakaryopoiesis. Here, we describe the function of FosB, an AP-1 transcription factor. FosB is induced in PMA treated K562 cells in a sustained manner and forms an active AP-1 protein-DNA complex. Down-regulation of FosB with specific shRNAs inhibited the induction of CD41, a specific cell surface marker of megakaryocytes. We also show that activation of the PKC-MEK-ERK signaling pathway is required for induction of FosB and CD41. Finally, we cross-examined the microarray data in conjunction with gene function annotation data to identify additional target genes of FosB. We define 3 genes, INHBA, CD9, and ITGA2B as regulatory targets of FosB and show that CD9, in particular, is a direct target of FosB.

Keywords. FosB, AP-1, megakaryocyte, CD41, K562.

Introduction

Megakaryocytes represent one of the terminally differentiated cell types derived from hematopoietic stem cells. Like other cell lineages of the same origin, megakaryocytes differentiate through a series of lineage restricted precursor stages, each with characteristic cellular and molecular features. The observed phenotypic transitions include enlargement of size of up to 50 to 100 μm in diameter, endomitosis of up to 256N, and expression of specific cell surface markers such as CD41 and CD61 [1 – 4]. These cells eventually undergo apoptosis [5, 6] releasing platelets which subsequently mediate hemostasis and thrombosis [7, 8].

Although induction of megakaryocytic differentiation and production of platelets from primary hematopoietic stem cells and embryonic stem cells have been reported [9, 10], our understanding of signaling pathways and regulatory genes that mediate the dramatic and intricate cellular events is far from complete. The situation in part stems from the restricted access to cells during various stages of differentiation. Cell line models such as myelogenous leukemia K562 cells [11] and signaling molecules such as PMA (also called TPA) have thus been used extensively to model megakaryopoiesis [11, 12]. One of the established

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findings from such *in vitro* system is that PMA-induced ERK signaling activates AP-1 activity, which in turn mediates a subset of molecular events involved in megakaryocytic differentiation [13].

AP-1 describes transcription factors that function through TPA-responsive elements in response to various extracellular stimuli. Two groups of transcription factors form the core of AP-1, the Jun protein family (c-Jun, JunB, and JunD) and the Fos protein family (c-Fos, FosB, Fra-1, and Fra-2). The members are differentially expressed and regulated, and they dynamically homo- or hetero-dimerize to form a complex mixture of AP-1 factors in a given cell type [14, 15]. Given that various AP-1 dimers are known to physically and functionally associate with distinct transcription factors and thereby attain high binding sequence specificity and target selectivity, the changing of AP-1 composition within a given cell type is expected to be correlated with changes in the gene expression pattern [16]. We have carried out a time-dependent gene expression profiling of PMA-mediated megakaryocytic differentiation of K562 cells. In addition to the transient up-regulation of c-Fos as previously reported [13], we noticed a strong and sustained induction of FosB expression. We show that thus induced FosB forms an active AP-1 complex, is activated through MEK-ERK pathway, and is required for expression of CD41, a key marker for differentiating megakaryocytes. We also report that, in addition to CD41 encoded by an integrin gene ITGA2B (integrin α_{IIb}), INHBA (also called inhibin beta A or activin A) and CD9 are down-stream regulatory targets of FosB. Using comparative genomic analysis and chromatin immunoprecipitation assay, we show that CD9 in particular is a direct regulatory target of FosB. Our results reveal a novel function of FosB as a regulator of megakaryocytic differentiation and provide foundations for further dissection of the complex process.

Materials and methods

Cell culture. K562 cell line was obtained from the American Type Culture Collection. Routine cell culture was carried out in RPMI (GIBCO BRL Life Technologies Inc.) containing 10 % heat-inactivated fetal bovine serum (Hyclone), 100 unit/ml penicillin and 100 μg /ml streptomycin in a humidified chamber with 5 % CO_2 atmosphere at 37 °C. HEL cells were obtained from American Type Culture Collection and cultured under identical conditions. Megakaryocytic differentiation was induced by applying PMA (Calbiochem) either at the concentration of 20 nM or 1nM in dimethyl sulfoxide (DMSO) as indicated. Control cultures were treated with equal volumes of the solvent DMSO. Differentiation of K562 cells in the presence of PKC inhibitor GF109203X (3 μM), MEK inhibitor PD98059 (50 μM), p-38 inhibitor SB203580 (10 μM), and JNK inhibitor SP6000125 (10 μM) was initiated after 1 h of preincubation with one of the inhibitors. All of the inhibitors were purchased from Calbiochem and dissolved in DMSO.

Microarray expression profiling. 3×10^5 K562 cells/well were treated with 20 nM PMA for 0, 0.5, 1, 3, 6, 12, 24, and 48 h in a six-well plate, and total RNA was extracted using RNeasy Mini Extraction Kit (Qiagen). Biotinylated cRNA was prepared from 550 ng of total RNA per sample using Illumina TotalPrep™ RNA Amplification Kit (Ambion) and hybridized to the Illumina HumanRef-8 Expression BeadChip (Illumina, Inc.) following the manufacturer's instructions. The BeadChip was scanned using a high resolution Illumina BeadArray Reader Confocal scanner. Expression profiles were analyzed using Illumina BeadStudio (Gene Expression Module v3.1). Duplicate microarray data are deposited in the Gene Expression Omnibus (GEO) database [GEO:-GSE12736].

Western assay. Anti-c-Fos (sc-52X) and anti-FosB (sc-7203X) antibodies were purchased from Santa Cruz, Inc. For immunoblot assay, 25 μg of nuclear extract from each sample was resolved on a 12 % SDS-PAGE gel. Proteins were electrotransferred to nitrocellulose membranes which were exposed to primary antibodies and then to HRP-labeled secondary antibodies. The immunoblots were developed with an enhanced chemiluminescence (ECL) system (ECL Plus Western blotting detection kit, Amersham) subsequently.

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotide pair containing the AP-1 consensus sequence (5'-CGC TTG ATG AGT CAG CCG GAA-3'; 5'-TTC CGG CTG ACT CAT CAA GCG-3') was end-labelled with γ-^{32}P-ATP (Amersham) using T4 polynucleotide kinase and used as the probe. Unlabeled oligonucleotide probe and another oligonucleotide probe with the core sequence mutated (5'-CGC TTG ATA CCC TAG CCG GAA-3'; 5'-TTC CGG CTA GGG TAT CAA GCG-3') were used as the specific and non-specific competitors, respectively. Labeled AP-1 probe was incubated with 10 μg of nuclear extracts for 25 min at room temperature in 20 μl of reaction mixture [10 mM HEPES (pH 8.0), 5 % glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 1 % ficoll, and 2 μg of poly(dIdC)]. For competition experiments, nuclear extracts were pre-incubated with specified amount of unlabeled probes

for 15 min before labeled specific probe was added. For the supershift assay, nuclear extracts were incubated with 2 μg of anti-FosB-antibody or anti-c-Fos-antibody for 30 min at room temperature prior to addition of the ^{32}P-labeled specific probe. The protein-DNA complexes were resolved on 5% native polyacrylamide gel in 1× Tris acetate/EDTA buffer. The gel was dried and analyzed with BAS-2500 imaging analyzer (FUJIFILM).

RNA interference. Human FosB cDNA was examined to select 3 independent and non-overlapping sequences as targets of RNA interference. The target sequences for FosB were: 5'-AGGAACGTCTGGAGTTTGT-3', 5'-GCCAACCACAATTCAATGAA-3', and 5'-CTAGCAGCAGCTAAATGCA-3'. Negative control sequence (5'-CTTACGCTGAGTACTTCGA-3') represented a part of the firefly luciferase gene. The three specific sequences are unique to FosB, as determined by a publicly available siRNA evaluation Web tool (http://www.dharmacon.com/DesignCenter/). The target sequences were incorporated into pSUPER.retro.neo+GFP (OligoEngine) to generate shRNA expressing plasmid vectors following the manufacturer's protocol. The shRNA plasmids were electroporated into 1×10^6 K562 cells per sample using Cell Line Nucleofector Kit V (AMAXA) following the manufacturer's protocol. 48 h after electroporation, GFP positive cells were sorted using FACSAria cell sorter (BD Biosciences) and analyzed again on the same sorter to verify purities, which typically exceeded 98%. Two hours after sorting, 1 nM PMA was added to 3×10^5 GFP positive cells for 48 h followed by subsequent analyses.

Flow cytometric analysis. PMA-induced megakaryocytic differentiation of K562 cells was assayed by measuring the expression of specific cell surface antigens, CD41 and CD61 [17]. Cells were collected, washed and suspended in 190 μl of ice-cold PBS containing 1% bovine serum albumin (BSA). 10 μl of anti-CD41a-FITC-Ab (or anti-CD41a-PE-Cy5-Ab) or anti-CD61-PerCP-Ab (BD Pharmingen) was added for 30 min at room temperature, and the cells were washed three times with 1 ml of PBS containing 1% BSA. The cells were analyzed by flow cytometry in a FACSCalibur (BD Biosciences) using the BD Cell-Quest™ Pro version 4.0.1 software (BD Biosciences).

Microarray data analysis. Time course microarray data were obtained at eight independent time points as described above. Duplicate experiments were performed for each time point. Selecting genes with significant detection p-value produced ~14000 probes out of total 23920 probes. Quantile normalization was carried out for each dataset at eight time points using the average expression value. It was reasoned that significant genes should show over two-fold induction at least at one time point with respect to the control sample ($t = 0$; before PMA treatment), and 1779 probes satisfying this requirement have been determined. The K-means clustering method ($k = 12$) was used to classify these probes according to characteristic temporal expression patterns. For selection of FosB target gene candidates, the function/disease annotation data of the Ingenuity Pathway Analysis (IPA 6.0) was searched with "megakaryocyte" as the key word to identify 106 genes, 23 of which were also found in the list of 1779 PMA regulated probes. The final candidates were narrowed down to 10 genes that belonged to clusters 1, 3, 9, and 11.

Real-time PCR. The shRNA plasmid was electroporated into K562 cells as described above. Total RNA was prepared using a RNeasy Mini Extraction Kit (Qiagen, Hilden) from GFP positive cells that had been cultured for 48 h with or without PMA. cDNA synthesis was performed using a SuperScript II Reverse Transcriptase Kit (Invitrogen) with 2 μg of RNA per cellular sample. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and ABI Prism 7300 Sequence Detection System (Applied Biosystems). All data were analyzed after normalizing to the expression level of β-actin. Primers for amplification for CD9, INHBA, and ITGA2B sequences were designed using Primer3 program: CD9, 5'-TTGGTGATATTCGCCATTGA-3' (sense), 5'-ACGCATAGTGGATGGCTTTC-3' (antisense); INHBA, 5'-GTCGGCGGGGCTTGGAGTG-3' (sense), 5'-GGACCCGGACGTGCCTGCTAT-3' (antisense); ITGA2B, 5'-AGGTGAGAGGGAGCAGAACA-3' (sense), 5'-TCCACCTTGAGAGGGTTGAC-3' (antisense); β-actin, 5'-GGCTACAGCTTCACCACCAC-3' (sense), 5'-CAGGAAGGAAGGCTGGAAGAG-3' (antisense).

Chromatin immunoprecipitation (ChIP) assay. Chromatin immunoprecipitation assays were performed using EZ ChIP™ Chromatin immunoprecipitation kits (Upstate) following the manufacturer's protocols with minor modifications. Briefly, 4 x10^6 K562 cells/150-mm plate were treated with 0 or 20 nM PMA for 48 h, followed by chromatin cross-linking and preparation of cell lysates. Genomic DNA in the lysate (300 μl) was sheared by sonication subsequently. Detailed protocols are available upon request. Cell lysates (100 μl) were immunoprecipi-

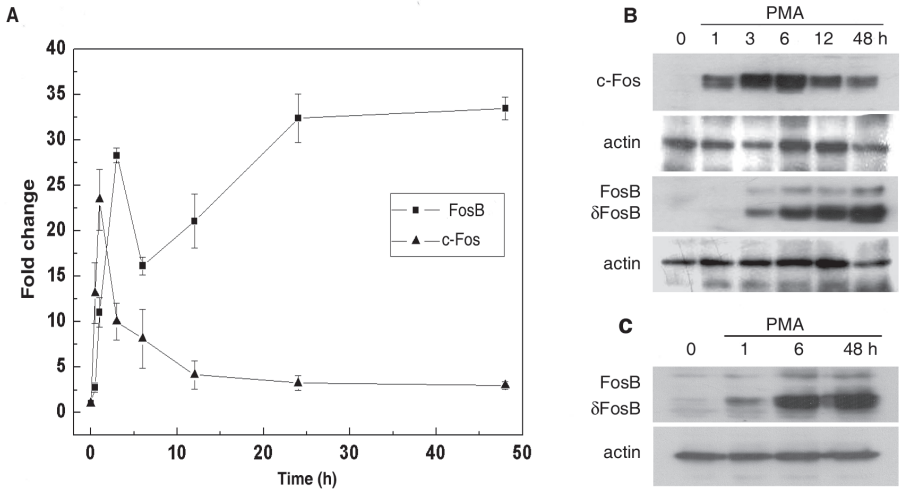

Figure 1. Induction of FosB by PMA in K562 cells. (*A*) Mean fold changes of FosB (■) and c-Fos (▲) genes during 48 h of PMA treatment are shown. Data are the average of two independent microarray experiments, and error bars represent standard deviations. (*B*) Immunoblot assays for c-Fos and FosB expression with K562 cells treated with 20 nM PMA for indicated times. (*C*) Immunoblot assay for FosB expression with HEL cells incubated for indicated times in the presence of PMA (10 nM).

tated with 2 μg of FosB antibodies (sc-7203X, Santa Cruz, Inc.) or control IgG antibodies. For PCR amplification of the precipitated DNA, following primer pairs were used: CD9, 5'-GCTGCTACACCAGCCTTTCT-3' (sense), 5'-CTCATCGCTTACACCCCAAG-3' (antisense); CD9 cis control, 5'-CAGCTTTGCTGAAGGGTAGG-3' (sense), 5'-GTAGCGCAGAAATGGAGAGC-3' (antisense); HPRT, 5'-ACAGCTTGCTGGTGAAAAGG-3' (sense), 5'-ACTGGCAAATGTGCCTCTCT -3' (antisense).

Results

We carried out a microarray-based expression profiling of megakaryocytic differentiation of K562 cells in response to PMA. Cells treated with PMA were harvested at various time points between 0 and 48th h, and extracted RNA from each stage was subsequently applied to high-density microarray chips on which nearly 24000 human genes are represented (see Materials and methods). We noted that two of the well-established markers, integrin α_{IIb} and integrin β_3 whose expression is known to be induced during megakaryopoiesis, showed significant up-regulation in the microarray screen confirming the validity of the result. The complete microarray data from the duplicate trials have been deposited in the Gene Express Omnibus (GEO) database [GEO:GSE12736].

Also consistent with the previously published data was the expression pattern of an AP-1 transcription factor, c-Fos. In at least two independent studies, this member of the Fos gene family has been shown to be rapidly and transiently induced in K562 cells in response to PMA [13, 18]. In addition, one of the studies showed that c-Fos regulates $\alpha_2\beta_1$ integrin (CD49b) expression in an ERK-dependent manner during megakaryocytic differentiation of K562 cells. Our microarray data (Fig. 1A) and immunoblot analysis corroborate the published expression pattern of c-Fos (Fig. 1B). Interestingly, we noted that another member of the Fos family, FosB, was induced at a later time point and its expression was sustained to the last time point examined as shown by results from both the microarray and immunoblot assays (Fig. 1A, B). FosB induction was also seen in HEL cells in response to PMA with a similar temporal profile (Fig. 1C). As previously shown for a variety of human [19] and rodent cells [20], FosB is expressed in two different forms resulting from alternative splicing. In K562 cells and HEL cells, delta FosB, the smaller of the two, is clearly the more abundant variant. As the role of FosB in megakaryopoiesis has not been reported, we chose to focus on analyzing the function of this gene.

We first sought to determine if the induced FosB participates in forming AP-1 DNA binding activity. First, electrophoretic mobility shift assay was carried out. The consensus AP-1 binding sequence TGAGTCA, also known as the TPA-responsive element (TRE), was used as the specific AP-1 probe. Consistent with the previously published results [13], treatment with PMA led to an increase in AP-1 DNA binding activity (Fig. 2A). The protein DNA complex was readily competed away by the specific unlabeled probe but not by the control probe (Fig. 2A). The composition of AP-1 complex was examined by supershifting with an antibody specific to c-Fos or FosB. Consistent with the strong expression after 3 h of PMA treatment and down-regulation by 48th h, application of anti-c-Fos antibody resulted in a noticeable amount of supershifted protein–DNA complex in the 3 h sample which became virtually undetectable in the 48 h sample (Fig. 2B). In contrast,

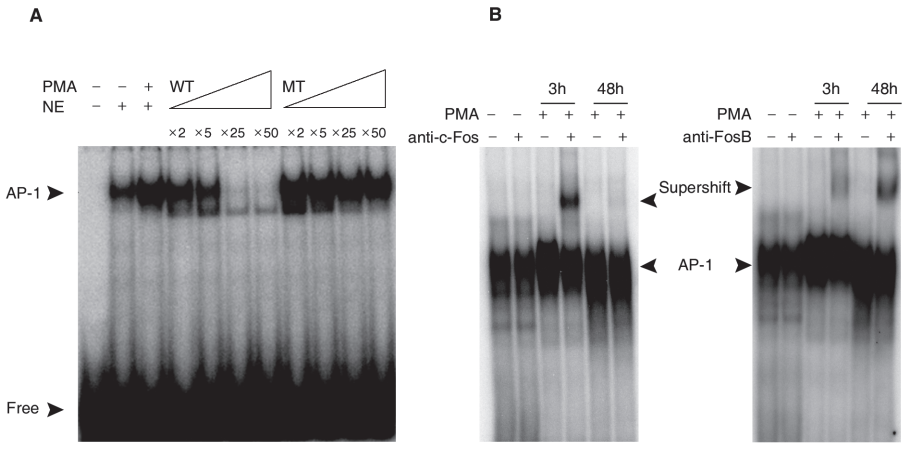

Figure 2. FosB forms active AP-1complex. (*A*) Analysis of AP-1 DNA binding activity from K562 cells. Nuclear extracts were prepared from untreated and PMA-treated K562 cells and analyzed by EMSA using an oligonucleotide probe containing AP-1 site. The upper arrowhead indicates the AP1-DNA probe complex, and the lower arrowhead indicates the free probe. For competition experiments, unlabeled AP-1 or mutated AP-1 probe was added to the indicated excess levels. (*B*) Analysis of the constitution of AP-1-DNA complex in PMA-treated K562 cells. Nuclear extracts isolated at 3rd and 48th h after treatment with PMA were incubated in the presence of antibodies against c-Fos and FosB proteins as indicated prior to the gel shift assay. Lower arrowheads indicate the AP-1-DNA complex, and the upper arrowheads indicate the complex consisting of AP-1, DNA and antibody.

supershifted FosB containing AP-1 complex was observed at a significant level in the 48 h sample but not in the 3 h sample (Fig. 2C). This is consistent with the expression pattern indicated by the microarray data and immunoblotting and confirms that FosB can function as an active component of AP-1 activity.

In order to test the function of FosB in megakaryocytic differentiation, we generated pSuperior plasmid constructs designed to express shRNAs specific to FosB and in tandem the green fluorescent protein (GFP). Three pSuperior plasmid constructs expressing distinct shRNAs targeting different portions of FosB were used in order to ascertain that the effects we obtain are not off-target effects. The control shRNA targets firefly luciferase and matches to no known mammalian sequence. K562 cells were transfected with one of the shRNA plasmid vectors targeting FosB or with the control plasmid by electroporation and selected based on expression of GFP by FACS prior to treatment with PMA. Immunoblotting assay showed that the FosB was highly induced by PMA in control shRNA expressing cells while the three FosB specific shRNAs (designated as shFosB1, 2, 3) visibly lowered the induced protein level (Fig. 3A). Specifically, it is shown that shFosB1 and shFosB3 dramatically down-regulated FosB while shFosB2 was somewhat less effective. We examined the expression of two of the megakaryocytic cell surface markers, CD41 and CD61 by flow cytometric analysis (Fig. 3B, C). Compared to control shRNA electroporated cells, FosB specific shRNA treated cells showed a significantly lowered level of CD41 induction. Of note, shFosB1 and shFosB3 were far more effective than shFosB2 in down-regulating CD41 induction. Although shFosB1 and shFosB3 were highly effective in down-regulating FosB induction, CD41 expression was down-regulated to a lesser extent suggesting that other factors than FosB also contribute to induction of CD41 expression. In contrast to CD41, CD61 induction was not affected detectably, if at all, by inhibition of FosB expression (Fig. 3C), suggesting that FosB regulates a portion of cellular response to PMA and is not a general regulator of megakaryocytic differentiation. A similar conclusion had been drawn for c-Fos with respect to the extent of its control over the whole megakaryopoiesis [21–23]. CD41 expression in K562 cells has been shown to be under the control of the MEK-ERK pathway and PKC up-stream to it. We used inhibitors to the various kinases to examine their effect on the induction of FosB and CD41. GF109203X, a general PKC inhibitor strongly inhibited induction of both FosB (Fig. 4A) and CD41 (Fig. 4B). Inhibition of MEK with PD98059 was somewhat less but still highly effective in inhibiting FosB induction (Fig. 4A) and had a more modest but clear inhibitory effect on CD41 induction as well (Fig. 4B). The inhibitor of p38 (SB203580) and the inhibitor of JNK (SP6000125) did not show inhibitory effect on the induction of FosB, although they subtly altered the level of CD41 expression. The extent of the effect of PD98059 is highly reminiscent of what was seen with shFosB1 and shFosB3 in that FosB was strongly down-regulated while CD41 induction was inhibited to a somewhat lesser extent. Taken together, these data suggest that PMA induces the expression of FosB via PKC-MEK-

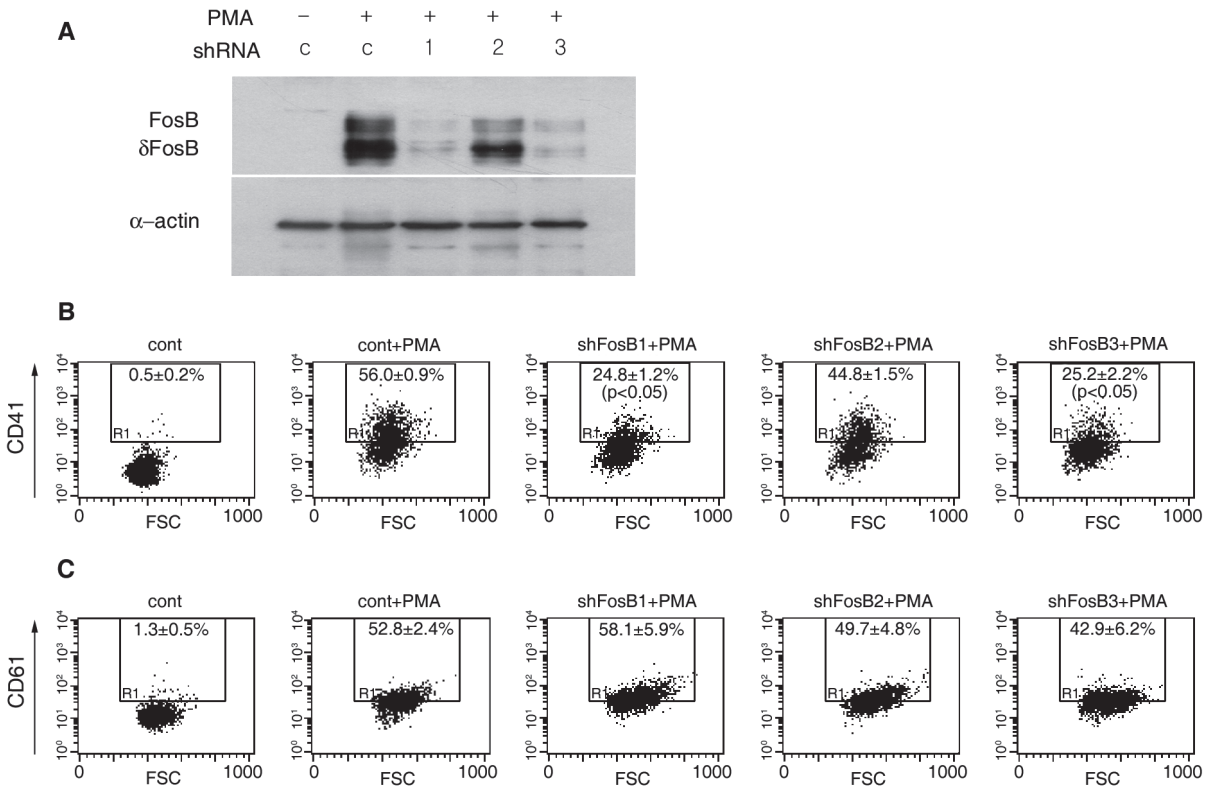

Figure 3. FosB silencing leads inhibition of CD41 but not of CD61 expression. K562 cells transfected with shFosB1, 2, 3, or shLuciferase were sorted based on GFP expression and incubated in the absence or presence of 1 nM PMA for 48 h. (*A*) Immunoblotting to examine down-regulation of FosB expression by shFosB1, 2, and 3. Cells were electroporated with one of the shFosB plasmids or control plasmid, sorted, and PMA treated (see Materials and methods). FosB protein level was dramatically down-regulated by shFosB1 and 3. (*B*) FACS analysis of CD41 expression after FosB silencing. Induction of CD41 was inhibited over 50 % by down-regulation of FosB with shFosB1 and 3. The percentage of CD41 positive cells falling within the gate is shown. Pairwise statistical analyses were performed for each shFosB transfection with shLuciferase transfection by Student's t-test. Significant changes ($^*p < 0.05$) were seen with shFosB1 and shFosB3. (*C*) Expression of the CD61 was evaluated by flow cytometry as for CD41. CD61 induction was not affected by shRNAs targeting FosB. Data are shown as mean ± SD of three independent experiments for each combination of cell surface markers and shRNA vectors.

ERK signaling cascade and FosB in turn functions as one of the major regulators of CD41 expression.

It is generally agreed that a given transcription factor has more than one regulatory target. Therefore, we reasoned that FosB likely has additional functions than just regulating CD41 expression and sought to identify other targets of this transcription factor. Our strategy for identifying down-stream target genes of FosB is summarized in Fig. 6A. First, we re-examined the microarray data which yielded 1779 probes that were induced or inhibited over two-fold, at least at one time point after PMA treatment. We further reasoned that down-stream targets of FosB should be induced at a later time point than or with a similar temporal profile to FosB itself. We thus used K-means clustering methods to classify the 1779 probes into 12 clusters (Fig. 5). The list of 1779 probes/genes and their cluster designations can be found in the supplemental data 1. The cluster 11 to which FosB belongs and clusters 1, 3, and 9 represented groups with potential regulatory targets of FosB. Next, the function/disease annotation data of the Ingenuity Pathway Analysis (IPA 6.0) was searched with the keyword "megakaryocyte", leading to a group of 106 genes, 23 of which were shown to be among the 1779 probes. Limiting to the four clusters above, we obtained 10 final candidate genes – CD9, INHBA, ITGA2B, IL11, TIMP1, VASP, CDKN1A, F2R, ITGB3, NOTCH1. Notable among the list were ITGA2B (integrin α_{IIb}) and ITGB3 (integrin β_3) whose protein products in fact are the megakaryocyte markers CD41 and CD61 respectively. Validation experiment was performed for these candidates using real time PCR technique with cDNA from K562 cells transfected with shRNA plasmids and PMA-treated as described above. It is seen that expression of integrin α_{IIb} but not β_3 shows the effect of shRNA expression consistent with the flow cytometric analysis (Fig. 3, Fig. 6 and data not shown). Furthermore, shFosB1 and shFosB3 were much more effective than shFosB2 in lowering integrin α_{IIb} gene mRNA level, again consistent with the results described in Figure 3. Such data validates this approach in finding additional

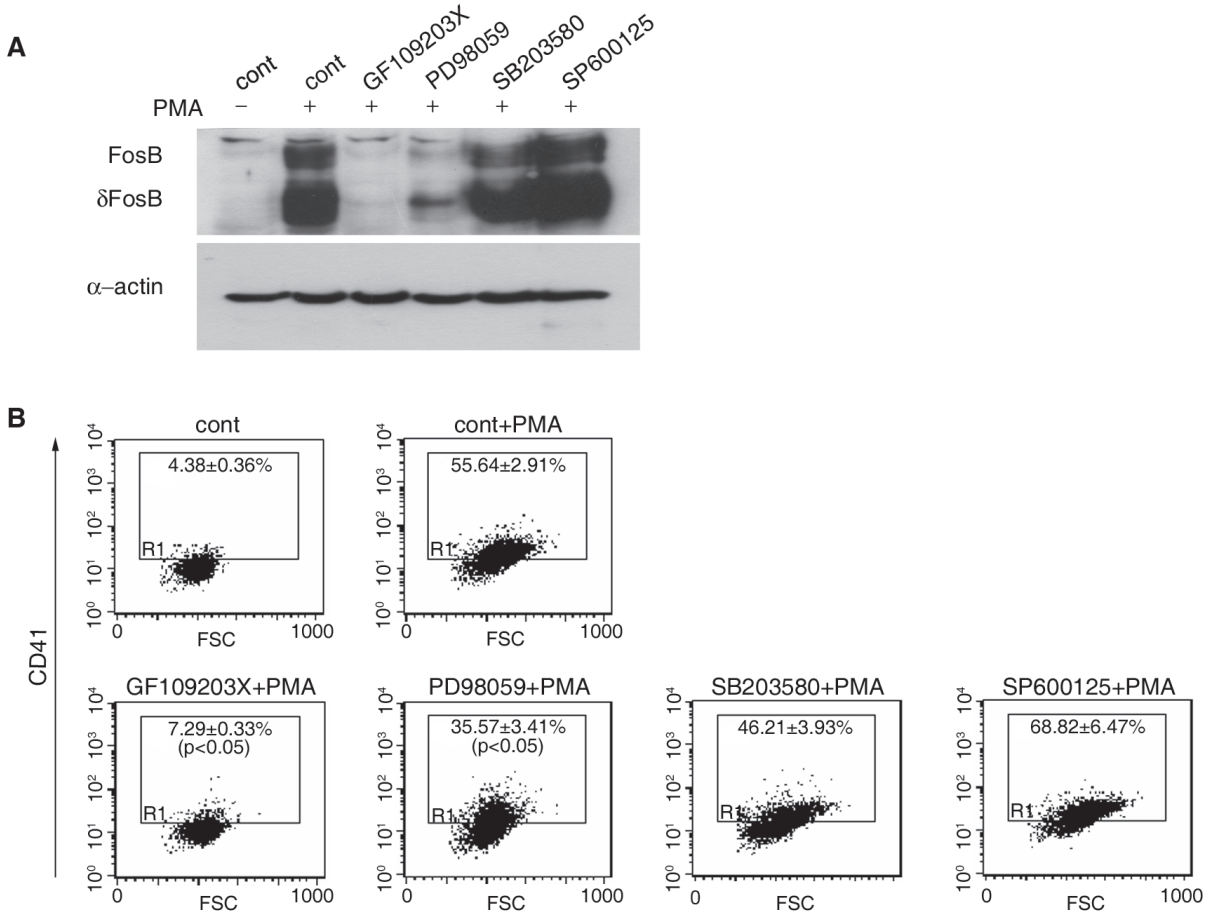

Figure 4. FosB induction is PKC-MEK-ERK signaling pathway dependent. (*A*) Immunoblot analysis was carried out using K562 cells treated with 1nM PMA for 48 h after pretreatment with various inhibitors for 1 h: 3 μM of GF109203X, 50 μM of PD98059, 10 μM of SB203580, and 10 μM of SP600125. (*B*) CD41 expressions in PMA-treated K562 after pretreatment with various inhibitors. Cells were stained with anti-CD41-FITC-Ab and analyzed by flow cytometry. Results are means ± SD (n = 3). Pairwise statistical analyses were performed for each inhibitor with PMA treatment alone by Student's t-test. Significant changes (*$p < 0.05$) were seen with GF109203X and PD98059.

targets of FosB. INHBA (inhibin beta A, or activin A) and CD9 responded to FosB specific shRNAs in a similar fashion, thereby leading in the end to three genes including integrin α_{IIb} as transcriptional regulatory targets of FosB.

In order to determine if any of these genes are directly regulated by FosB, we first carried out comparative genomics analyses and examined the entire genomic region between the two genes on either side of each of the target genes. The result showed that only two potential AP-1 sites, one located within the 2nd intron of INHBA and another within the 2nd intron of CD9, are conserved among multiple species and thus represent potential regulatory cis-elements (Fig. 7A and data not shown). Subsequent ChIP assays showed that FosB binds to the cis-element in CD9 locus but not to the one in the INHBA locus in a PMA treatment-dependent manner confirming CD9 as a direct regulatory target of FosB (Fig. 7B, C).

Discussion

Phenotypic transitions seen during cellular differentiation often result from induction and/or repression of transcriptional regulatory activities. This generalization most likely applies to the elaborate cellular events during megakaryocytic differentiation. That PMA induces megakaryocytic differentiation in K562 cells immediately suggests that AP-1 transcription factors likely play roles in the process. AP-1 has been implicated in differentiation and cell type specific gene expression of several hematopoietic lineages [24–26] including megakaryocytes [13, 27]. Specifically, it was shown that c-Fos, a member of the Fos protein family, is involved in the induction of integrin $\alpha_2\beta_1$ (CD49b) in response to PMA in an ERK-dependent manner [13, 27]. In addition, based on limited effects of over-expression and knockdown of c-Fos, it was suggested that c-Fos is not a general

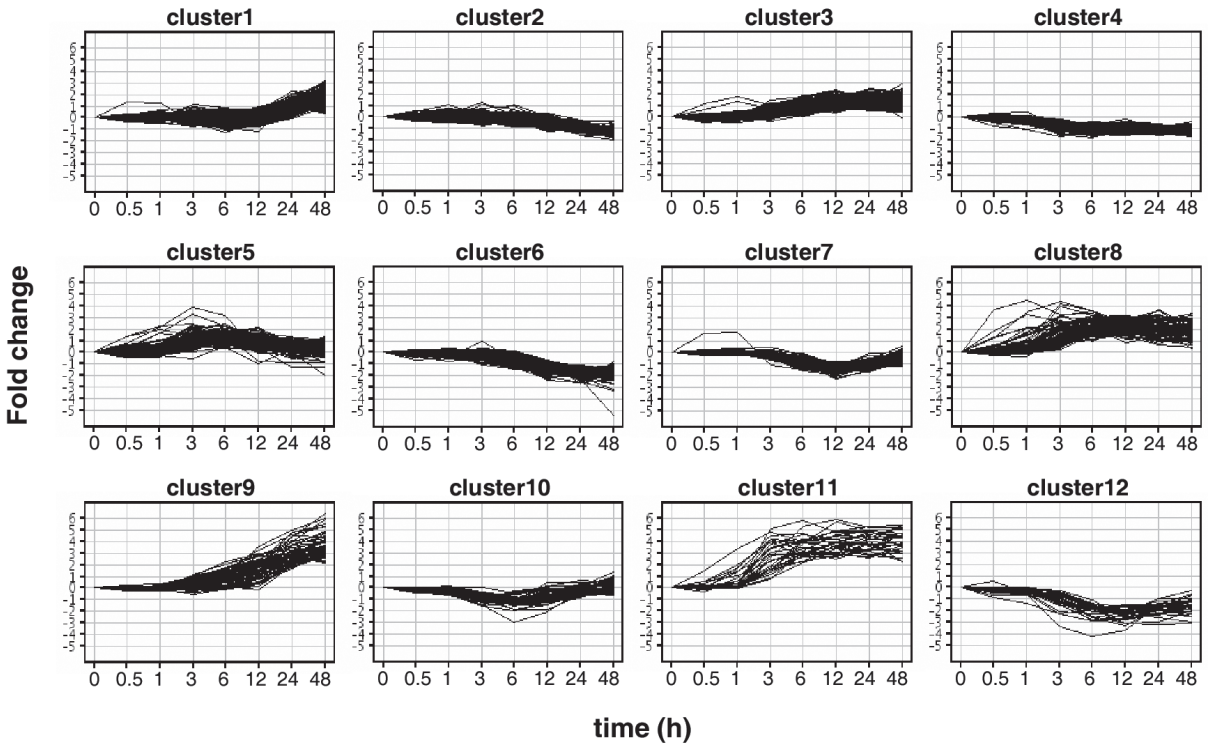

Figure 5. Clustering analysis of microarray data. 1779 probes that showed changes of over two-fold at least at one time point during PMA treatment were clustered into 12 groups using K-means clustering method (Supplemental data 1). Data shown are mean fold changes of two independent microarray experiments.

regulator of megakaryocytic differentiation but is responsible for mediating a restricted subset of events during the overall process.

Our microarray screen showed that at least one additional Fos protein family member, FosB, is strongly induced in response to PMA. A noticeable difference was the temporal profile of the induction. Specifically, unlike c-Fos which showed rapid up-regulation and down-regulation, FosB expression was delayed and sustained. Such induction in response to PMA was also seen in HEL cells, also human myelogenous leukemia cells, attesting to the generality of the FosB induction during megakaryocytic differentiation. Perhaps more importantly, a recent gene expression profiling study using primary human CD34+ hematopoietic stem cells also showed that FosB is induced during thrombopoietin-induced megakaryocytic differentiation [28].

Experiments using chemical inhibitors of kinases strongly suggest that the signaling pathway involving PKC-MEK-ERK axis, required for c-Fos induction, is also responsible for activation of FosB. We note that a general PKC inhibitor, GF109203X, had a more profound effect in inhibiting FosB and CD41 induction than an MEK inhibitor, PD98059 or any of the shRNAs for FosB. It was also seen that inhibition of FosB with shRNAs was more effective for FosB itself than for CD41. Such results are consistent with that multiple signals in addition to that mediated by FosB ultimately feed into regulating the expression of CD41. Importantly, we showed that CD41 but not CD61 is regulated by FosB, indicating that like c-Fos, FosB plays a limited role in the megakaryocytic differentiation of K562 cells. We have also examined two other aspects of megakaryocytic differentiation, cell size increase and endomitosis. Inhibition of FosB expression did not have a significant effect on these PMA-induced changes, again indicating a limited role of FosB in the overall process (data not shown). Additionally, we tested for the promotion of differentiation of K562 cells after FosB silencing toward erythrocytes, monocytes, and granulocytes using GlycophorinA, CD14, and CD16 respectively. GlycophorinA expression was inhibited by PMA treatment while CD16 expression was enhanced by PMA treatment. CD14 on the other hand showed no change. Silencing FosB did not change the expression pattern of these markers in response to PMA, indicating that FosB does not play a significant role in lineage determination process of these cell types (data not shown). The limited role not withstanding, to the best of our knowledge this is the first report on the role of FosB in megakaryocytic differentiation.

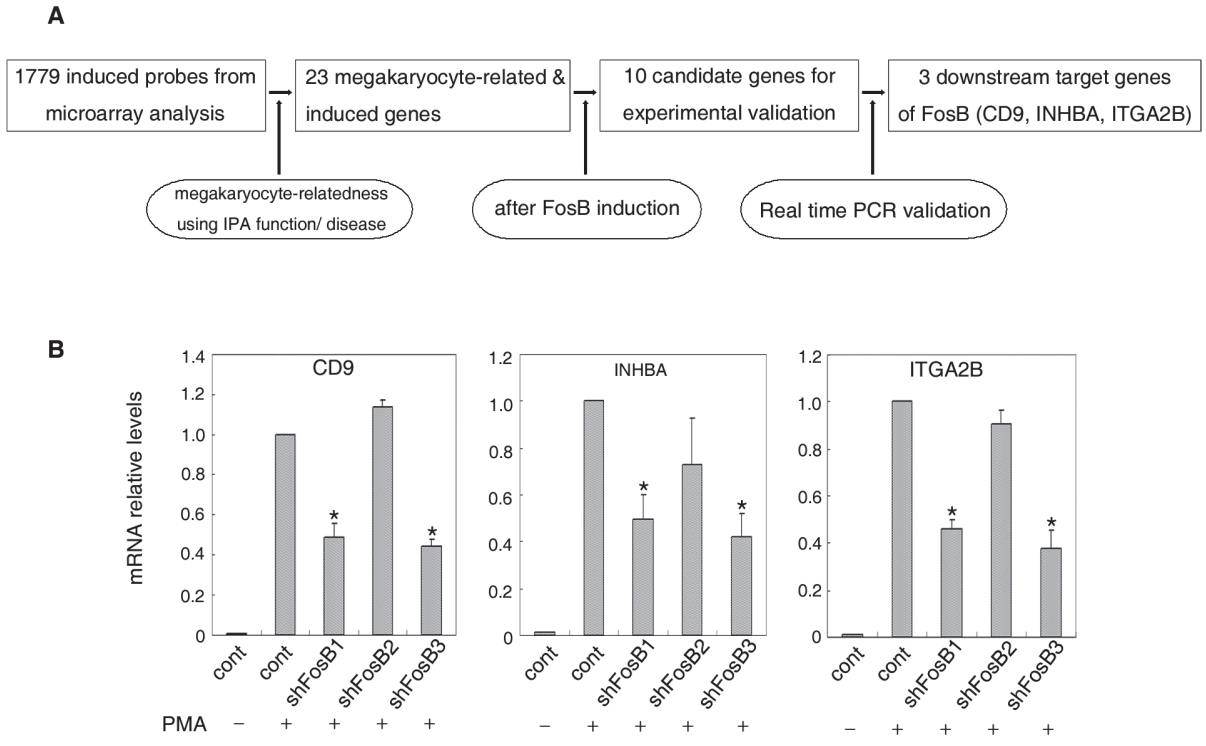

Figure 6. Targets of FosB. (*A*) Schematic pipeline showing filtering strategy for identification of FosB target genes. (*B*) Down-regulation of FosB by shFosBs results in down-regulation of three genes, CD9, INHBA, and ITGA2B. Real time PCR was performed with cDNA preparations from K562 cells treated with indicated combinations of PMA and shRNA vectors. The values shown are the relative ratios of expression levels normalized to β-actin. Data are the average of three independent experiments, and error bars represent standard deviations. Pairwise statistical analyses were performed for each shFosB transfection + PMA treatment with shLuciferase transfection + PMA treatment by Student's t-test. Significant changes (*$p < 0.01$) were seen with shFosB1 and shFosB3.

FosB, as a member of AP-1 transcription factor family, likely functions in cooperation with co-regulators of AP-1 responsive genes. C/EBP, ATF, Maf, Ets, SMAD, and NFAT gene family members have been reported to play such co-regulatory roles [16]. Some of these genes are represented on the microarray we used, and among them, C/EBP-β and MafB showed significant up-regulation as the result of PMA treatment (Supplemental data 1 and data not shown). It will be of interest to see if either or both of these genes co-regulate certain aspects of megakaryocytic differentiation in conjunction with FosB or with other AP-1 transcription factors. We note that only two other AP-1 transcription factors, JunD and Fra-1, are represented on the microarray in addition to c-Fos and FosB. They show little change in the expression levels, but constitutively expressed AP-1 members nevertheless may possess important regulatory roles in conjunction with dynamically expressed AP-1 members or with co-regulators.

The functions of AP-1 genes need to be ultimately integrated with those of other transcription factors known to be regulators of megakaryopoiesis [29–31]. Among the GATA family members, GATA1 showed a transient inhibition of up to four-fold while GATA2 showed a sustained three-fold up-regulation in response to PMA (Supplemental data 1 and data not shown). Among Ets gene family members, only Ets2 is represented on the microarray we used, and this gene showed no significant changes. In contrast, NF-E2, an essential regulator of megakaryocytic differentiation, showed a transient down-regulation of up to 20-fold followed by a full recovery by the 48^{th} h point (Supplemental data 1 and data not shown). The significance of the expression pattern of these genes is not clear. One possibility is that differentiation of K562 cells in response to PMA corresponds to a distinct phase of megakaryopoiesis and the dynamics of the gene expression in K562 cells thus do not necessarily reflect their function in the overall process. Future efforts should at least in part be focused on identifying portion of megakaryocytic differentiation for which K562 cells can serve as a faithful model.

It is broadly accepted that a single transcription factor regulates multiple target genes. Here, we propose 3 targets of FosB: ITGA2B, INHBA, and CD9. In fact, we demonstrated that CD9 is a direct regulatory target of FosB. ITGA2B (Integrin α_{IIb}) is the gene for CD41,

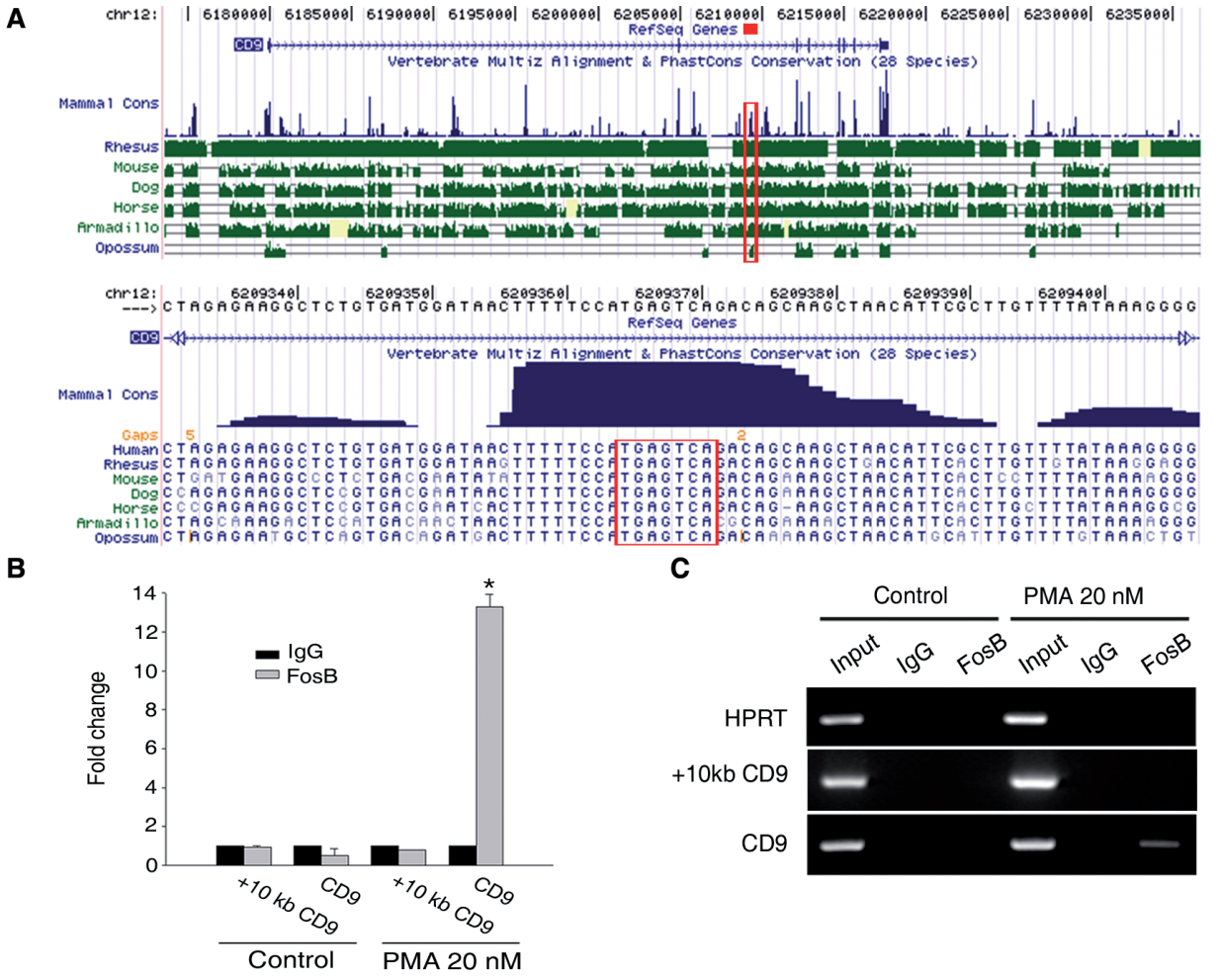

Figure 7. (*A*) CD9 locus on human chromosome 12; adapted from the University of California at Santa Cruz (UCSC) Genome Browser. The location of putative FosB binding site within the genomic locus of CD9 is indicated by red boxes in the upper panel at two different zoom levels. The nucleotide sequences surrounding the conserved binding site are shown in detail in the lower panel. Within the red box in the lower panel is the conserved core AP1 sequence, 5'TGAGTCA3'. (*B* and *C*) Chromatin immunoprecipitations assays were carried out with K562 cells. Immunoprecipitations were performed with anti-FosB antibody and control IgG antibody. DNA from cellular lysates was used as the input control. (*B*) Values from immunoprecipitation with anti-FosB antibody represent the fold differences relative to those from IgG control antibody after normalization with HPRT expression levels. Data are the average of two independent experiments each with two measurements, and error bars represent standard deviations. Student's t-test shows statistically significant difference between 0 and 20 nM PMA treatments in terms of FosB binding to the proposed conserved target site (*$p < 0.05$) but not to cis-control site 10 kb away. (*C*) Gel electrophoresis analysis of ChIP assay. Immunoprecipitated and PCR amplified DNA product is seen only for the proposed candidate FosB binding site in conjunction with PMA treatment and with the use of anti-FosB antibody.

one of the representative specific cell surface markers for differentiating megakayrocytes [32]. CD41 is also responsible for the so-called inside-out signaling mediated conformational change endowing platelets with affinity for fibrinogen, which in turn promotes aggregation. The expression of activin A (INHBA) in K562 cells in response to PMA has been reported previously [33]. However, the role of activin A in megakaryocytic differentiation is unclear. Although its effect has been shown using murine megakaryoblastic leukemia cell line [34], a more recent report indicated that activin A had little effect on the megakaryocytic differentiation of primary CD34+ human cells [35]. It is possible that other activin dimers than activin A function in human megakaryocytic differentiation. It is also possible that megakaryocytes express activin as a paracrine or endocrine rather than as an autocrine. CD9 expression in hematopoietic lineages has been recently reported [36, 37]. Consistent with our finding, cells committed to megakaryocytic lineages express relatively high level of CD9 [36]. Furthermore, it was suggested that CD9 regulates differentiation of megakaryocytes by participating in the remodeling of the membrane [36]. It is highly likely that additional targets of FosB exist. We restricted the scope of the search to the group of 106 genes that had previously been connected with megakaryocytes. It should be noted that the genes that

do not belong to this subset but nevertheless have shown changes in the expression during PMA treatment far outnumber those that do. Some of these genes are likely to be regulated by FosB and play a role in megakaryocytic differentiation. Also, genes that were down-regulated during PMA treatment of K562 cells were not examined in our current study. FosB may directly or indirectly repress some of these genes which may be required for proper differentiation. Perhaps a more exhaustive study using a combination of RNAi–mediated FosB inhibition and microarray gene expression profiling will reveal the role of FosB in regulating these genes and thereby the megakaryocytic differentiation.

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