Original Article

Title:

***EP300-ZNF384* fusion gene product up-regulates *GATA3* gene expression and induces hematopoietic stem cell gene expression signature in B-cell precursor acute lymphoblastic leukemia cells**

Short title: EP300-ZNF384 up-regulates *GATA3* expression

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Abstract It has been clarified that the *ZNF384*-related fusion genes consist of a distinct subgroup of B-cell precursor acute lymphoblastic leukemia in childhood with a frequency of approximately 3 to 4%. Among them, *EP300-ZNF384* is a novel one that we identified. Patients with the *ZNF384*-related fusion gene exhibit a hematopoietic stem cell gene expression signature and characteristic immunophenotype with negative or low expression of CD10 and aberrant expression of myeloid antigens, such as CD33 and CD13. However, its molecular basis remains completely unknown. In the present study, we examined the biological effects of EP300-ZNF384 expression in an REH B-cell precursor acute lymphoblastic leukemia cell line by retrovirus-mediated gene transduction, and observed the acquisition of the gene expression signature for hematopoietic stem cells and an up-regulation of *GATA3* gene expression as assessed by microarray analysis. In contrast, the gene expression profile induced by wild-type ZNF384 in REH cells was significantly distinct from that by EP300-ZNF384 expression. Together with the results of reporter assays that revealed the enhancement of *GATA3*-promoter activity by EP300-ZNF384 expression, EP300-ZNF384 mediates *GATA3* gene expression and might be involved in acquisition of the hematopoietic stem cell gene expression signature and characteristic immunophenotype in B-cell precursor acute lymphoblastic leukemia cells.

Keywords *EP300·ZNF384* ·*GATA3* ·CD33 ·transcription

Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common cancer diagnosed in children and a genetically heterogeneous disease. A series of well-characterized genetic abnormalities that are strongly associated with characteristic biological and clinical features can be detected by standard genetic analyses in approximately two-thirds of pediatric BCP-ALL patients [1]. On the other hand, major pathogenic or driver cytogenetic abnormalities have yet to be clarified in the remaining BCP-ALL patients, and they are called "B-others". However, recent studies using advanced analytical approaches have stratified a variety of genetic abnormalities in B-others [2-6].

In such a situation, it has been identified by several groups, including us, that fusion genes involving the *zinc-finger protein 384* (*ZNF384*) gene consist of a distinct subgroup of pediatric BCP-ALL with a frequency of approximately 3 to 4 % [4-9]. In addition to the three previously reported fusion partners: the *Ewing sarcoma breakpoint region 1* gene {*EWSR1*, t(12;22)(p13;q12)}, *TATA box binding protein-associated factor 15* gene {*TAF15*, t(12;17)(p13;q12)}, and *transcription factor 3* gene {*TCF3* or *E2A*, t(12;19)(p13;p13)} [10, 11], five other novel fusion partners for *ZNF384*: the *E1A Binding Protein P300* gene {*EP300*, t(12;22)(p13;q13)}, *CREB Binding Protein* gene {*CREBBP*, t(12;16)(p13;p13)}, *AT-Rich Interaction Domain 1B* gene {*ARID1B*, t(6;12)(q25;p13)}, *synergin gamma* gene {*SYNRG*, t(12;17)(p13; q12)}, and *BMP2 Inducible Kinase* gene {*BMP2K*, t(4;12)(q21;p13)}, have recently been

discovered [4-9]. Among the recently identified *ZNF384*-related fusion genes, *EP300-ZNF384* (A predicted structure of fusion protein is presented in Figure 1A) is the most frequent one with an incidence of approximately 1% in pediatric BCP-ALL patients [7] as well as a higher incidence in adolescent and young adult (AYA, 8.2% in Philadelphia chromosome-negative ALL) [4] or adult BCP-ALL patients (7.7%) [5].

As reported previously, BCP-ALL patients harboring the *ZNF384*-related fusion gene possess a characteristic immunophenotype of weak CD10 and aberrant CD13 and/or CD33 expression [5, 7-9]. Although the *ZNF384*-related fusion gene is observed predominantly in BCP-ALL, some of the cases are presented as mixed phenotype B/myeloid acute leukemia [12, 13] or acute myeloid leukemia [10]. In addition, cases with a lineage switch from BCP-ALL to AML have also been reported [11, 14, 15]. Therefore, it is speculated that *ZNF384*-related fusion may occur in early common progenitor cells that could differentiate into both the myeloid and lymphoid lineages [12]. Our recent observation that the signature gene expression profile in *ZNF384*-related fusion gene-positive ALL was enriched in hematopoietic stem cell (HSC) features [9] supports the above notion.

The *ZNF384* gene, also called the *nuclear matrix protein 4* (*NMP4*) or *CAS-interacting zinc finger protein* (*CIZ*) gene, encodes a C2H2-type zinc finger protein with transcription activity that can

bind to promoters through the consensus DNA sequence (G/C)AAAAA(A) and regulate extracellular matrix genes, including matrix metalloproteinases (MMPs) -1, -3, and -7 and the type I collagen α 1 polypeptide chain (COL1A1) [16, 17]. It has been shown that *TAF15*-, *EWSR1*-, and *TCF3-ZNF384* induce 3T3 fibroblast transformation and thus it has been speculated that the transformation of 3T3 fibroblasts by *ZNF384*-related fusions is dependent on DNA-binding and might involve the transactivation of ZNF384 target genes [10, 18]. Since *ZNF384* is a commonality in all of these fusion genes, the aberrant function of the ZNF384 protein may be responsible for the characteristics of the immunophenotype as well as gene expression profile, whereas the biological significance of ZNF384-fusion molecules remains largely unknown.

In order to investigate the functional role of ZNF384-related fusion molecules in the pathogenesis of BCP-ALL, we transduced BCP-ALL cells with the *EP300-ZNF384* gene and examined its biological effect. In this paper, we report that *EP300-ZNF384* gene expression leads to an acquisition of the gene expression signature for HSCs and an enhancement of *GATA-binding protein 3* (*GATA3*) gene expression in BCP-ALL cells. GATA3 plays an essential role in T lymphoid cell development and immune regulation [19-22], whereas it has been reported to induce myeloid-features in B-cell precursors under some conditions [23]. The contribution of GATA3 expression mediated by ZNF384-related

fusion molecules to the development of the characteristic gene expression profile and immunophenotypes of BCP-ALL harboring *ZNF384*-related fusion gene is discussed.

Materials and methods

Cells and reagents

REH cells [24] were maintained in RPMI 1640 medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Sigma-Aldrich). In the case of the experiments using tetracycline (Tet)-inducible gene expression, Tet System Approved FFBS (Takara Bio USA, Inc., Mountain View, CA, USA) was used. HEK-293 cells (Japanese Cancer Research Resource Bank, JCRB, National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki-shi, Osaka, Japan) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS.

Plasmids

The cDNAs of wild-type *EP300* and *ZNF384* were purchased from Promega Corporation (Madison, WI, USA) as the Flexi® ORF clone F1KB4967 and pF1KB5965, respectively. After digestion with *Sfg*I and *Pme*I followed by blunt ending and the attachment of protruding dATPs at the 3' ends, the coding regions of *EP300* and *ZNF384* genes were subcloned into pGEM-T easy and pGEM-T vector (Promega), respectively, as described previously [25]. Subsequent plasmids were designated as

pGEM-Teasy-*EP300* (from SP6 to T7 sites), pGEM-Teasy-*ZNF384* (from SP6 to T7 sites) and pGEM-T-*ZNF384* (from T7 to SP6 sites). A fragment of *EP300-ZNF384* cDNA including the joining region of both genes was amplified from a clinical specimen of a patient carrying the fusion gene by PCR using KOD plus ver.2 (Toyobo Co., Ltd., Osaka, Japan) with forward 5'-aacaggattgagaggccccc-3' and reverse 5'-tggtctcggtgtgtgacttg-3' primers and subcloned into the pGEM-T vector using the same methods as described above and designated as pGEM-T-*EZ*-junction (from SP6 to T7 sites). The use of clinical materials was approved by the institutional review boards and informed consent was obtained from the parents or guardians as described previously [7]. To develop full-length ORF of *EP300-ZNF384* cDNA, firstly the cDNA fragments from pGEM-T-*ZNF384* digested with the combinations of *Nco*I/*Xma*I were subcloned into the same site of pGEM-T-*EZ*-junction and then the cDNA fragments digested with *Nco*I (blunted)/*Afl*II were subcloned into the *Afl*II/*Sal*I (blunted) site of pGEM-Teasy-*EP300* by sequential ligation. The subsequent plasmid was designated as pGEM-Teasy-*EP300-ZNF384*-F. The full-length ORF of *EP300-ZNF384* cDNA was then digested with *Eco*RI and subcloned into pGEM-T vector from SP6 to T7 sites as described above and designated as pGEM-T-*EP300-ZNF384*-R.

To develop a retroviral expression vector for *EP300-ZNF384* and Wild-type *ZNF384* (pRetroX-Tight-puro-*EP300-ZNF384* and -*ZNF384*), the full-length insert of either *EP300-ZNF384* or

ZNF384 cDNA was digested with *Eco*RI, and subcloned into the same sites (treated with calf-intestinal alkaline phosphatase) of pRetroX-Tight-puro (Clontech Laboratories, Inc., Madison, WI, USA). To develop a retroviral expression vector for *EGFP* expression (pRetroX-Tight-puro-*EGFP*), the full-length insert of *EGFP* cDNA was digested with *Nhe*I (blunted) and *Eco*RI from pEGFP-C1 (Clontech), and subcloned into the *Bam*HI (blunted) and *Eco*RI sites of pRetroX-Tight-puro.

The full-length ORF of *EP300-ZNF384* and *ZNF384* cDNA fragments digested with *Not*I /*Apa*I from pGEM-T-*EP300-ZNF384*-R and pGEM-T-*ZNF384*, respectively, were subcloned into the same site of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The subsequent plasmids were designated as pcDNA3-*EP300-ZNF384* and pcDNA3-*ZNF384*.

The LightSwitch promoter reporter GoClones, including *ANPEP (CD13)*, *BLNK*, *CD19*, *CD33*, *CEBPA*, *CEBPB*, *COL1A1*, *GAPDH*, *GATA3*, *MME (CD10)*, *MMP1*, and random control (R01 prom), were purchased from SwitchGear Genomics (Carlsbad, CA, USA). To develop EGFP-expression vector driven by the *GATA3* promoter, *Renilla luciferase* (*RLuc*) gene was removed from pLightSwitch_GATA3-promoter by *Hind*III/*Eco*RI(blunted) digestion and *EGFP* gene obtained from pEGFP-N2 (Clontech) by *Hind*III/*Not*I(blunted) digestion was subcloned (designated as p*GATA3*p-EGFP).

Detection of transduced gene expression

For detection of the *EP300-ZNF384* fusion and wild-type *ZNF384* transcripts, PCR was carried out as described previously [7] and 345 and 404 bp fragments were amplified using the sets of primers: EP300-s4, 5'-tccccctcaaaaatgctggt-3'/ZNF384-as4, 5'-tgtggggatagaaggccaga-3' and ZNF384-s101, 5'-aatctgcagtcccacagacg-3'/ZNF384-as101 5'-ggagactggaagtgtggtgg-3', respectively. As an internal control, the primer set for the detection of GAPDH, GAPDH-s, 5'-gctcagacaccatggggaaggt-3' /GAPDH-as 5'-gtggtgcaggaggcattgctga-3', was also used as described previously [7].

Gene transduction and transfection

A chimera gene-inducible REH cell line was generated by employing retroviral transduction using the Retro-X™ Tet-On® Advanced Inducible Expression System (Clontech) as described previously [25]. The 2 x10^6 REH cells that introduced Tet-on Advanced were stably maintained in the presence of neomycin (250 μg/mL), and were further infected with retrovirus of pRetroX-Tight-puro-*EP300-ZNF384* in a total of 2 mL of medium and exposed for 10 hours. Cells were then washed, treated with or without 1 μg/mL of doxycycline (DOX) to induce the target proteins for 72 hours, and then harvested for the

subsequent experiments. REH cells transduced with wild-type *ZNF384* and *EGFP* expression vector and empty vector, as Mock, were similarly developed. Experiments were repeated at least three times independently and representative data are presented.

Transfection of pcDNA3-related vectors and the LightSwitch promoter reporter GoClones into HEK293 cells for the luciferase assay (described below) was performed using ScreenFect™A plus (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as instructed by the manufacturer with a DNA:reagent ratio of 1:3.

Transfection of p*GATA3*p-EGFP into REH cells with or without expression of *EP300-ZNF384*/wild-type *ZNF384* was performed using Amaxa™ 4D-Nucleofector™ System with P3 Primary Cell 4D-Nucleofector Kit (Lonza Cologne GmbH, Köln, Germany) as instructed by the manufacturer with program E0-117. As positive and negative controls, empty vector (without promoter) and pmaxGFP™ vector (Lonza), respectively, were similarly transfected.

Flow cytometric analysis

The expression of EGFP protein was quantified using flow cytometry (FC500, Beckman Coulter Inc., Indianapolis, IN, USA). Experiments were performed in triplicate, and the means and SEM were calculated. Experiments were repeated three times independently and representative data are presented.

Microarray and data analyses

The cDNAs were amplified and labeled using the GeneChip® 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA) as instructed by the manufacturer. The labeled probes were hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix). The experiments were performed in triplicate. The arrays were analyzed using GENECHIP OPERATING Software 1.2 (Affymetrix) and GENESPRING GX 14.5 software (Agilent Technologies, Santa Clara, CA, USA), and data analysis including gene set enrichment analysis (GSEA) was performed as described previously [9, 25].

Immunoblotting

Immunoblot analysis was performed as described previously [25] using the rabbit polyclonal anti-ZNF384 antibody (BMR00539, Bio Matrix Research Inc., NagareyamaCity, Chiba, Japan) and mouse monoclonal anti-β actin antibody (clone AC-40, A4700, Sigma-Aldrich), as well as horseradish

peroxidise (HRP)-conjugated secondary Abs (Dako, Glostrup, Denmark). Experiments were repeated three times independently, and representative data are presented.

Luciferase assay

The mixtures of 200 ng of the LightSwitch promoter reporter GoClone series and 300 ng of pcDNA3, pcDNA3-*EP300-ZNF384*, or pcDNA3-*ZNF384* (indicated in the figure) were used to transiently transfect HEK-293 cells, as described above. Then 100 μL each of the transfection mixture was plated on a 96-well plate and cultured for 48 hrs. The luciferase assay was performed using LightSwitch Luciferase Assay Kits (SwitchGear) according to the manufacturer's protocol. Experiments were performed in quadruplicate and the means and SEM were calculated. Experiments were repeated three times independently and representative data are presented.

Statistical analysis

Data were analyzed using Student's t-test. Values of p less than 0.05 were considered significant.

Results

The transduction of *EP300-ZNF384* fusion induces HSC signature and up-regulates the *GATA-3* gene in the REH BCP-ALL cell line

Firstly, we examined the biological effects of EP300-ZNF384 fusion as well as wild-type ZNF384 molecule expression on the BCP-ALL cell line REH by employing a tetracycline-inducible gene expression system. When we transiently introduced the EGFP expression vector accompanied by DOX-treatment to assess the transduction efficiency of our system, more than 95% of the cells expressed EGFP as assessed by flow cytometry (Figure 1B), indicating the significantly high transduction efficiency of our system. We therefore expressed the *EP300-ZNF384* or wild-type *ZNF384* gene in REH cells by transient transduction. As shown in Figure 1C and D, RT-PCR and immunoblot analysis specifically detected expressions of *EP300-ZNF384* fusion gene and EP300-ZNF384 fusion protein, respectively, in REH cells transduced with *EP300-ZNF384* expression retrovirus vector after DOX-treatment, but not the DOX-treated Mock REH transduced with empty vector. We also detected enforced gene and protein expressions of wild-type ZNF384 in REH cells transduced with *ZNF384* expression retrovirus vector (Figure 1C and D). It is noteworthy that the expression of small amount of wild type ZNF384 was also detected in REH cells (Figure 1C and D). Although we examined the cell proliferation, apoptosis-induction, and morphological change, no significant differences were observed in

EP300-ZNF384- or *ZNF384*-expressing REH cells in comparison with Mock REH cells (data not shown). Furthermore, immunophenotypic changes such as down- and up-regulation of CD10 and CD13/CD33, respectively, were not observed (data not shown).

Therefore, we next examined the effects of *EP300-ZNF384* expression on the gene expression in REH cells by employing an oligonucleotide microarray. To test whether the expression of EP300-ZNF384 induces changes in the gene expression profile related to hematopoietic cell differentiation, we employed GSEA, as described previously [9]. As shown in Figure 2A, GSEA revealed the significant enrichment of HSC, common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor cell (MEP), and multi-lymphoid progenitor (MLP) signatures in *EP300-ZNF384*-expressing REH cells compared with Mock REH cells. The signatures of granulocyte-macrophage progenitor (GMP)/Pro-B cells and early T-cells (ETP) were enriched in *EP300-ZNF384*-expressing and Mock REH cells, respectively, but significance was not observed.

As shown in Table 1 and Figure 2B, fold change analysis identified the differential expression of 55 genes (up: 51, down: 4, >2.0) in *EP300-ZNF384*-expressing REH cells compared with Mock REH cells. Interestingly, transcription factors such as *GATA3*, *Pre-B-Cell Leukemia Homeobox 1* (*PBX1*), and

Thymocyte selection-associated high mobility group box protein (*TOX*) were listed as more highly expressed genes in *EP300-ZNF384*-expressing REH cells.

To further evaluate the biological significance of differentially expressed genes after *EP300-ZNF384*-transduction, we performed pathway analysis and identified significant enrichment of the up-regulated genes in the pathways, including the "Transcriptional regulation of pluripotent stem cells" (Supplementary Table 1), indicating that *EP300-ZNF384*-transduction up-regulates genes related to pluripotency.

Next, we similarly examined the effects of wild-type *ZNF384* overexpression on the gene expression in REH cells. As shown in Figure 3A, GSEA revealed that the signatures of HSC, MLP and Pro-B, but not ETP, CMP, GMP, and MEP, were enriched in REH cells after ZNF384 overexpression, while statistical significance was not observed. As shown in Figure 3B and Supplementary Table 2, fold change analysis identified the differential expression of 4,120 genes (up: 2,686, down: 1,434, >2.0) in *ZNF384*-overexpressing REH cells compared with Mock REH cells and the number of genes that expression changed after *ZNF384*-overexpression was overwhelmingly larger. Importantly, only 7 of up-regulated and none of down-regulated genes were overlapped with those of *EP300-ZNF384*-expressing REH cells (Figure 3C). Furthermore, none of *GATA3*, *PBX1*, and *TOX* was

listed as more highly expressed genes in *ZNF384*-overexpressing REH cells. Together with the results of pathway analysis (Supplementary Table 3), above data indicate that the effects of wild-type *ZNF384* overexpression on the gene expression in REH cells are distinct from those of *EP300-ZNF384* expression.

EP300-ZNF384 enhances the promoter activity of *GATA3* gene

We then examined whether the expression of EP300-ZNF384 and wild-type ZNF384 affect the promoter activity of transcription factors related to hematopoietic cells and others by employing the luciferase assay in HEK293 cells. As shown in Figure 4A, the transient transfection of the EGFP expression vector by lipofection resulted in 89.1 ± 3.3% expression in HEK-293 cells, indicating that our system has an evaluable transfection efficiency. As we mentioned above, promoters of *COL1A1* and *MMP1* reported to be regulated by ZNF384. In the case of the *COL1A1* gene, both EP300-ZNF384 and ZNF384 exhibited significant enhancement of promoter activity (Figure 4B). In the case of the *MMP1* gene, on the contrary, promoter activity itself was not significant in HEK-293 cells, and thus the effects of EP300-ZNF384 and ZNF384s were not clear.

When we examined the effect of EP300-ZNF384 on promoter activities of the indicated genes using the cell systems above, we observed that EP300-ZNF384 significantly enhanced the *GATA3* promoter activity in HEK-293 cells (Figure 4B). On the other hand, EP300-ZNF384 very slightly, but significantly, enhanced CD33 promoter activity. Inconsistent with the results of oligonucleotide microarray, when we examined the effect of wild-type ZNF384, we observed that ZNF384 also significantly enhanced the *GATA3* promoter activity in HEK-293 cells (Figure 4B). In the case of *CEBPB* genes, ZNF384 slightly enhanced their promoter activities. Neither wild-type ZNF384 nor EP300-ZNF384 affected the promoters of *MME* (*CD10*) and *BLNK*, and promoters of *CEBPA*, *ANPEP (CD13)* or *CD19* exhibited no significant activity in HEK-293 cells.

Next we examined whether EP300-ZNF384 and wild-type ZNF384 also enhance the *GATA3* promoter activity in REH cells or not. As shown in Figure 5A, electroporation successfully introduced positive control vector (pmaxGFP) into REH cells with or without expression of EP300-ZNF384 or wild-type ZNF384 by efficiency of approximately 80%. However, when we similarly transfected luciferase assay reporter vectors, we could not detect any luciferase activity even if the case of a positive control (pLightSwitch_GAPDH-promoter, data not shown). Therefore, we selected EGFP as a reporter for *GATA3* promoter assay in REH cells. As shown in Figure 5A and B, although the expression level

was not so strong, *GATA3*-promoter expressed EGFP more frequently in *EP300*-*ZNF384*- or wild-type *ZNF384*-expressing REH cells than Mock REH cells.

Discussion

We previously reported that the signature gene expression profile in *ZNF384*-related fusion-positive ALL was enriched in HSC compared with that of either *TCF3-PBX1*-positive ALL or B-others without *ZNF384*-fusions [9]. In this report, we further showed that the transduction of the *EP300-ZNF384* gene led to an acquisition of the gene expression signature for HSCs in the REH BCP-ALL cell line, as assessed by GSEA. Consistently, pathway analysis further revealed that *EP300-ZNF384* transduction up-regulates the genes related to pluripotency, as presented above. The data indicate that EP300-ZNF384 directly mediates the expression of genes related to the properties of pluripotent stem cells.

We also showed that the transduction of the *EP300-ZNF384* gene up-regulates *GATA3* expression in REH BCP-ALL cells. Furthermore, EP300-ZNF384 enhances promoter activity of the *GATA3* gene. It is in agreement with previous observations from several groups including us that the *GATA3* gene is up-regulated in patients with *ZNF384*-related fusion gene-positive BCP-ALL [5, 9]. Since the *GATA3* promoter region possesses several consensus sequences for ZNF384-binding sites, EP300-ZNF384 protein should enhance *GATA3* promoter activity directly.

In contrast, although wild-type ZNF384 also possess the significant ability to enhance the *GATA3* promoter activity, the effects of wild-type ZNF384 expression on the gene expression in REH cells are markedly distinct from those of EP300-ZNF384 and up-regulation of *GATA3* gene expression was not observed in wild-type *ZNF384*-introduced REH cells. Although the detailed mechanism is unknown, overall effect of EP300-ZNF384 fusion protein on the gene expression in BCP-ALL cells should be significantly different from that of wild-type ZNF384 protein.

As mentioned above, GATA3 is essential transcription factor for early T-cell development [19-22] and it was also reported that the transcriptional repression of *GATA3* was essential for early B cell commitment [26]. However, it has been reported that GATA3 strongly promoted *in vitro* macrophage differentiation and myeloid colony formation of Pax5(-/-) pro-B cells, and that *GATA3* expression also resulted in efficient engraftment and myeloid development of Pax5(-/-) pro-B cells *in vivo*, indicating that GATA3 exhibits myeloid-inducing activity in the absence of PAX5 that is dominant over the activity of other lineage-specific transcription factors on committed B-lymphocytes [23]. Since we reported that BCP-ALL cases harboring fusion genes involving *ZNF384* revealed low-level expression of *PAX5* [9], it is possible that the expression of *EP300-ZNF384* in BCP-ALL with suppressed *PAX5* expression induces myeloid activity via GATA3 function, revealing the aberrant expression of myeloid antigens. As we

described above, however, ectopic expression of EP300-ZNF384 failed to induce immunophenotypic changes in REH cells, such as down- and up-regulation of CD10 and CD13/CD33, respectively, indicating that EP300-ZNF384 dose not affect the expression of these antigens directly and it should be required the effects of molecules downstream of GATA3. Although our data suggest direct effect of EP300-ZNF384 on *CD33*-promoter activity in HEK-293 cells, it should not be sufficient to induce CD33 antigen expression in REH cells.

There are several lines of evidence that GATA3 is related to the maintenance of HSC potency. First, the overexpression of GATA3 in HSCs results in the cessation of cell expansion [27]. GATA3 has also been reported to be expressed in multi-potent HSCs and is required for the regulation of the balance between self-renewal and differentiation in HSCs [28,29]. Therefore, it is possible that the acquisition of the gene expression signature for HSCs in REH BCP-ALL cell line after transduction of *EP300-ZNF384* gene might be mediated, at least in part, by the GATA3 function.

It has been reported recently that specific germline variant of *GATA3* that closely related to significantly increased *GATA3* mRNA level was associated with a higher incidence of BCP-ALL in adolescents and young adults (AYA) and a higher risk of relapse of childhood BCP-ALL with Ph-like phenotype [30, 31], and thus the novel mechanisms of ALL etiology and also previously unrecognized

function of GATA3 in leukemogenesis was speculated [30]. On the other hand, repressing expression of GATA3 defines a novel stem cell-like subgroup of ETP-ALL [32]. These reports should indicate that up-regulation of GATA3 expression related to the development of BCP-ALL as well as Ph-like phenotype of BCP-ALL, but not T-ALL, while the detailed mechanism must be elucidated in future.

In conclusion, the transduction of *EP300-ZNF384* enhances *GATA3* expression and leads to the acquisition of the gene expression signature for HSCs in the REH BCP-ALL cell line. Although further investigation is needed to assess the precise mechanisms, clarification of the effect of the GATA3 function in BCP-ALL with suppressed PAX5 expression should shed light on the pathogenesis and molecular basis of the specific phenotype of *ZNF384*-related fusion gene-positive ALL.

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Competing interests We have no conflicts of interest regarding this study.

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Table 1. High (A) and low (B) expression genes in *EP300*-*ZNF384*-expressing REH cells in comparison with Mock.

Supplementary Table 1. Up-regulated pathways in *EP300*-*ZNF384*-expressing REH cells in comparison with Mock.

Supplementary Table 2. High (A) and low (B) expression genes in wild-type *ZNF384*-expressing REH cells in comparison with Mock.

Supplementary Table 3. Up- (A) and down- (B) regulated pathways in wild-type *ZNF384*-expressing REH cells in comparison with Mock.

Legends for Figures

Figure 1. Expression of *EP300-ZNF384* and wild-type *ZNF384* in REH Cells. (**A**) A predicted structure of fusion protein of EP300-ZNF384 is schematically presented. (**B**) REH cells stably expressing Tet-On® Advanced were transduced with *EGFP*-expressing retroviral vector (REH/EGFP) or empty vector (REH/Mock). After 72-hour treatment with doxycycline (Dox), cells were harvested and EGFP-expression was examined by flow cytometry. Experiments were performed in triplicate and the mean ± SEM of positivity (%) are presented with a typical cytogram. Data shown are representative of three independent experiments. X-axis, fluorescence intensity; Y-axis, relative cell number. (**C**) REH cells were transduced with *EP300-ZNF384*- (R/*EP300-ZNF384*), *ZNF384*-expressing retroviral vector (R/*ZNF384*), or empty vector similarly to (B) and total RNA was extracted from $1x10^7$ of cells. The expressions of *EP300-ZNF384*, *ZNF384* and *GAPDH*, as an internal control, were examined by RT-PCR. Of note, since complete *ZNF384* gene is retained in *EP300-ZNF384* fusion gene, the sets of primers for detection of the *ZNF384* transcripts also recognize *EP300-ZNF384* fusion gene (third lane from the right). The φ/*Hae*III DNA markers were applied to the same gel. (**D**) The protein expressions of EP300-ZNF384 (solid arrow, predicted to be a 110

kDa protein with 1,027 amino acids) and ZNF384 (broken arrow, 63 kDa) were examined by immunoblotting. As an internal control, β actin was detected. Some nonspecific bands (arrow head) were also detected.

Figure 2. Effects of *EP300-ZNF384* expression on the gene expression in REH cells. (**A**) Gene set enrichment analysis (GSEA) for curated gene sets of hematopoietic precursors was performed of the differentially expressed genes between R/*EP300-ZNF384* cells (red) and REH/Mock cells (blue). Enrichment plots for the hematopoietic stem cell (HSC), multi-lymphoid progenitor (MLP), pro-B cell (Pro-B), early T-cell precursor (ETP), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), and megakaryocyte-erythroid progenitor cell (MEP) signatures are presented. Bold lines represent significant enrichments {false discovery rate (FDR) q-value<0.25 and/or nominal (NOM) p-value<0.05}. NES, normalized enrichment score. (**B**) Two-way hierarchical clustering was performed on filtered microarray probes listed in Table 1 up- or down-regulated by 2.0-fold or more in R/*EP300-ZNF384* cells (red) and REH/Mock cells (blue). The results are displayed using a heat map as a dendrogram.

Figure 3. Effects of wild-type *ZNF384* expression on the gene expression in REH cells. (**A**) Gene set enrichment analysis (GSEA) for curated gene sets of hematopoietic precursors was performed of the differentially expressed genes between R/*ZNF384* cells (yellow) and REH/Mock cells (blue) similarly as in Figure 2A. (**B**) Two-way hierarchical clustering was performed on filtered microarray probes listed in Table 3 up- or down-regulated by 2.0-fold or more in R/*ZNF384* cells (yellow) and REH/Mock cells (blue) similarly as in Figure 2B.

Figure 4. Effect of EP300-ZNF384 on *GATA3* promoter activity. (**A**) HEK-293 cells were transfected with *EGFP*-expressing vector (HEK-293/EGFP) or empty vector (HEK-293/Mock) by lipofection. After 48-hour cultivation, cells were harvested, EGFP-expression was examined by flow cytometry, and the data are presented in Figure 1A. (**B**) The mixtures of the LightSwitch promoter reporter GoClone series and pcDNA3, pcDNA3-*EP300-ZNF384*, or pcDNA3-*ZNF384* as indicated were used to transiently transfect HEK-293 and the luciferase assay was performed as described in Materials and Methods. Experiments were performed in quadruplicate and the means and SEM of subsequent signal values (Luciferase activity) were calculated and presented by bar graph.

Figure 5. Effect of EP300-ZNF384 on *GATA3* promoter activity in REH cells. (**A**) REH cells were transfected with *EGFP*-expressing vector driven by *GATA3*-promoter (p*GATA3*p-EGFP, *GATA3*p), empty vector, or pmaxGFP by electroporation. After 36-hour cultivation, cells were harvested, EGFP-expression was examined by flow cytometry, and the data are presented as in Figure 1B. (**B**) The transfections with *GATA3*p presented (A) were performed in triplicate and the means and SEM of subsequent positivity (%) were calculated and presented by bar graph.

Table

Table 1

A) High expression genes in EP300-ZNF384-expressing REH cells in comparison with Mock

Notes : UP - FC ([EP300-ZNF384] vs [Empty])
#Only up-regulated entities from parent entity list :
#Fold change >= 2.0
#Entity List : Moderated T-Test p cut-off = 0.05
#Fold-Change cut-off : 2.0
Technology : Affymetrix.GeneChip.HG-U133_Plus_2

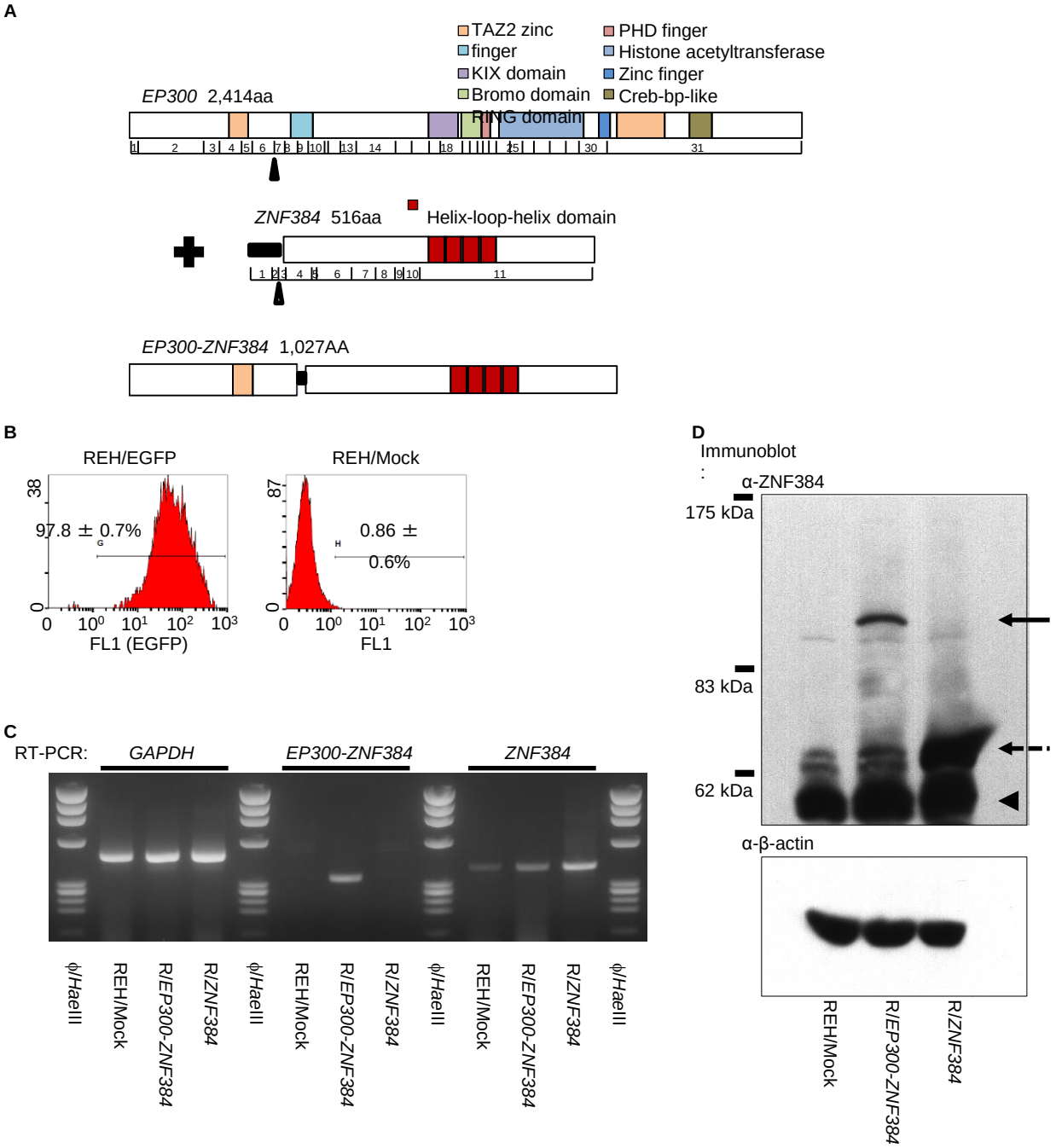
Probe Set ID	Gene Symbol	FC	LogFC	Regulation	Alignments
205363_at	BBOX1	54.11	5.76	up	chr11:27062997-27149354 (+) // 92.56 // p14.2
210432_s_at	SCN3A	44.81	5.49	up	chr2:165944039-166060553 (-) // 98.62 // q24.3
229327_s_at		38.56	5.27	up	chr16:79631220-79632352 (+) // 94.61 // q23.2
209348_s_at	MAF	38.52	5.27	up	chr16:79630369-79634611 (-) // 87.31 // q23.2
224242_at	GALP	15.49	3.95	up	chr19:56687388-56697144 (+) // 98.73 // q13.43
227484_at	SRGAP1	9.36	3.23	up	chr12:64539620-64540679 (+) // 93.13 // q14.2
204439_at	IFI44L	8.93	3.16	up	chr1:79086148-79108069 (+) // 88.1 // p31.1
1566324_a_at	MAF	7.47	2.90	up	chr16:79629404-79629760 (-) // 99.44 // q23.2
224259_at	WNT8A	5.95	2.57	up	chr5:137419678-137428054 (+) // 99.94 // q31.2
239292_at		5.85	2.55	up	chr3:31687797-31688290 (-) // 24.58 // p23
229273_at	SALL1	5.82	2.54	up	chr16:51169897-51170415 (-) // 86.43 // q12.1
206893_at	SALL1	5.57	2.48	up	chr16:51170328-51185152 (-) // 98.91 // q12.1
204437_s_at	FOLR1	4.92	2.30	up	chr11:71900601-71907341 (+) // 100.0 // q13.4
241789_at	RBMS3	4.84	2.27	up	chr3:30048084-30048527 (+) // 99.77 // p24.1
205419_at	GPR183	4.19	2.07	up	chr13:99946795-99948417 (-) // 96.96 // q32.3
48031_r_at	FAXDC2	4.18	2.07	up	chr5:154198051-154199638 (-) // 96.34 // q33.2
211959_at	IGFBP5	4.14	2.05	up	chr2:217536827-217540879 (-) // 95.63 // q35
204035_at	SCG2	3.96	1.99	up	chr2:224461726-224467079 (-) // 99.87 // q36.1
202481_at	DHRS3	3.80	1.93	up	chr1:12627939-12677406 (-) // 98.0 // p36.22
212148_at	PBX1	3.76	1.91	up	chr1:164816353-164821067 (+) // 91.39 // q23.3
238447_at	RBMS3	3.61	1.85	up	chr3:30045651-30046619 (+) // 94.96 // p24.1
205922_at	VNN2	3.20	1.68	up	chr6:133065009-133079033 (-) // 94.43 // q23.2
212151_at	PBX1	3.19	1.68	up	chr1:164816353-164821067 (+) // 91.39 // q23.3
226482_s_at	TSTD1	3.06	1.61	up	chr1:161007420-161008726 (-) // 92.77 // q23.3
202524_s_at	SPOCK2	2.98	1.57	up	chr10:73818792-73848767 (-) // 97.61 // q22.1
204529_s_at	TOX	2.85	1.51	up	chr8:59717976-60031767 (-) // 98.36 // q12.1
207761_s_at	METTL7A	2.81	1.49	up	chr12:51318801-51326288 (+) // 89.79 // q13.12
209602_s_at	GATA3	2.79	1.48	up	chr10:8096669-8117213 (+) // 95.62 // p14
203650_at	PROCR	2.78	1.47	up	chr20:33759933-33765159 (+) // 96.42 // q11.22
232517_s_at	HELZ2	2.57	1.36	up	chr20:62190598-62193265 (-) // 99.25 // q13.33
235570_at	RBMS3	2.54	1.34	up	chr3:30050779-30051877 (+) // 94.62 // p24.1
219073_s_at	OSBPL10	2.47	1.30	up	chr3:31702317-31918006 (-) // 99.23 // p23
209875_s_at	SPP1	2.41	1.27	up	chr4:88896869-88904284 (+) // 99.69 // q22.1
207480_s_at	MEIS2	2.38	1.25	up	chr15:37183285-37391922 (-) // 98.56 // q14
209676_at	TFPI	2.37	1.24	up	chr2:188331284-188419050 (-) // 99.02 // q32.1
244611_at	MED13	2.35	1.23	up	chr17:60106364-60107055 (-) // 79.02 // q23.2
201427_s_at	SEPP1	2.34	1.23	up	chr5:42799989-42808468 (-) // 97.45 // p12
207969_x_at	ACRV1	2.32	1.22	up	chr11:125542229-125550742 (-) // 99.88 // q24.2
219580_s_at	TMC5	2.29	1.20	up	chr16:19485506-19510078 (+) // 92.22 // p12.3
226806_s_at	NFIA	2.27	1.18	up	chr1:61927710-61928460 (+) // 82.1 // p31.3
214043_at	PTPRD	2.26	1.18	up	chr9:8314246-8314734 (-) // 77.35 // p24.1
203232_s_at	ATXN1	2.21	1.15	up	chr6:16299343-16761687 (-) // 97.3 // p22.3
206767_at	RBMS3	2.21	1.15	up	chr3:29323024-30032755 (+) // 97.54 // p24.1
64064_at	GIMAP1-GIMAP5/// GIMAP5	2.20	1.14	up	chr7:150439581-150440734 (+) // 84.91 // q36.1
1556834_at	LOC100652770	2.17	1.12	up	chr18:29122108-29136874 (-) // 59.41 // q12.1
200878_at	EPAS1	2.14	1.10	up	chr2:46524581-46613836 (+) // 96.6 // p21
222173_s_at	TBC1D2	2.14	1.10	up	chr9:100961320-101017493 (-) // 95.56 // q22.33
202499_s_at	SLC2A3	2.13	1.09	up	chr12:8071826-8088871 (-) // 89.96 // p13.31
213060_s_at	CHI3L2	2.09	1.06	up	chr1:111772332-111786062 (+) // 92.13 // p13.3
219257_s_at	SPHK1	2.04	1.03	up	chr17:74380745-74383941 (+) // 98.49 // q25.1
235376_at		2.01	1.00	up	chr3:49357728-49358882 (-) // 37.91 // p21.31

B) Low expression genes in EP300-ZNF384-expressing REH cells in comparison with Mock

Notes : DOWN - FC ([EP300-ZNF384] vs [Empty])
#Only down-regulated entities from parent entity list :
#Fold change >= 2.0
#Entity List : Moderated T-Test p cut-off = 0.05
#Fold-Change cut-off : 2.0
Technology : Affymetrix.GeneChip.HG-U133_Plus_2

Probe Set ID	Gene Symbol	FC	Log FC	Regulation	Alignments
1562353_x_at	LOC101928583	-3.42	-1.77	down	chr3:170374350-170379346 (+) // 38.31 // q26.2
228022_at	LOC101928583	-2.74	-1.46	down	chr1:93621939-93645927 (+) // 99.81 // p22.1
1563296_at	LINC00572	-2.29	-1.20	down	chr13:30492787-30500788 (-) // 89.59 // q12.3
219845_at	BARX1	-2.19	-1.13	down	chr9:96713908-96717602 (-) // 86.64 // q22.32

Figure-1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KIX domain
Bromo domain
RING domain
PHD finger
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Zinc finger
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ZNF384 516aa
Helix-loop-helix domain
EP300-ZNF384 1,027AA
B
REH/EGFP
REH/Mock
97.8 ± 0.7%
0.86 ± 0.6%
FL1 (EGFP)
FL1
C
RT-PCR:
GAPDH
EP300-ZNF384
ZNF384
ϕ/HaeIII
REH/Mock
R/EP300-ZNF384
R/ZNF384
D
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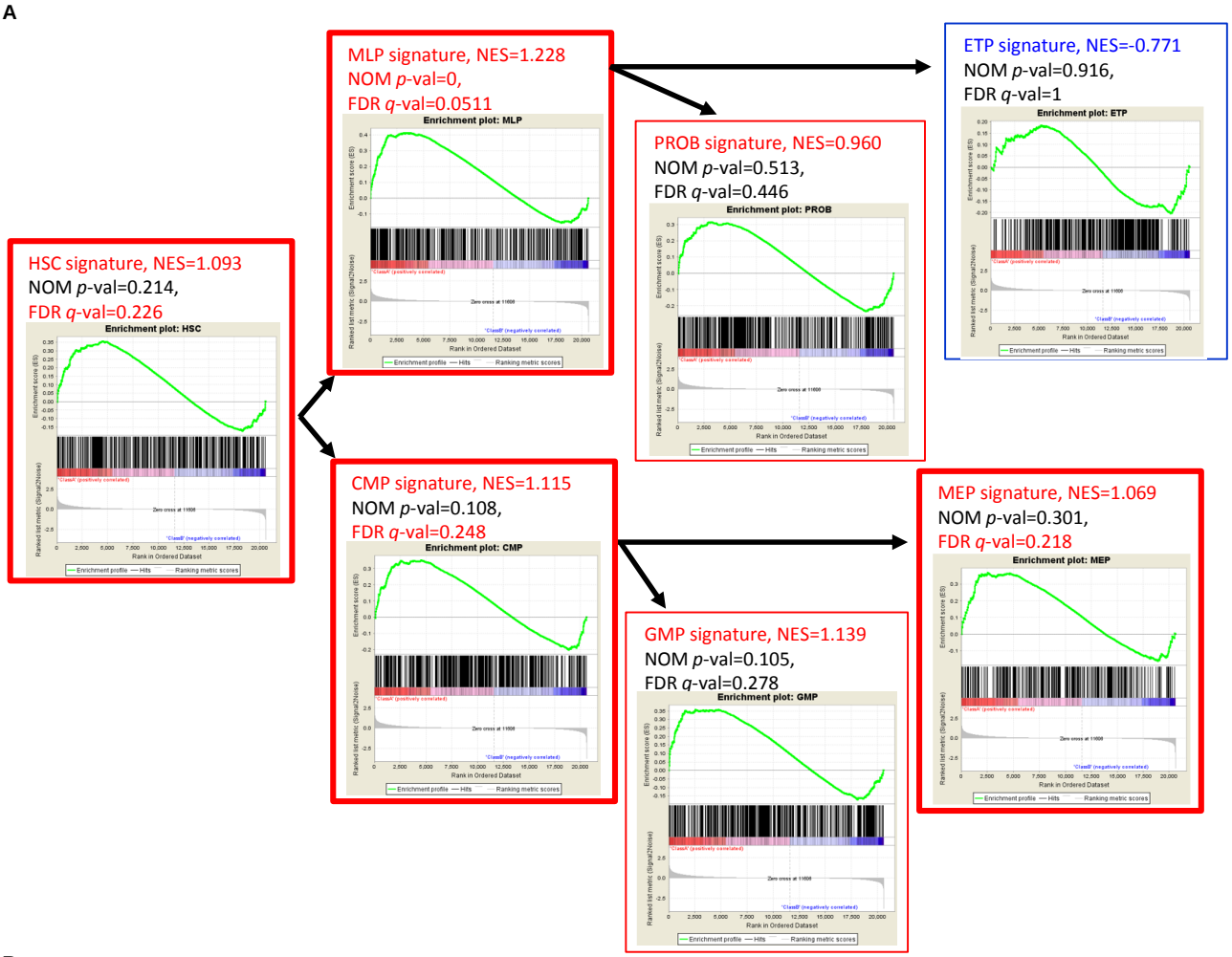
83 kDa
62 kDa
α-β-actin
REH/Mock
R/EP300-ZNF384
R/ZNF384

Figure-2

A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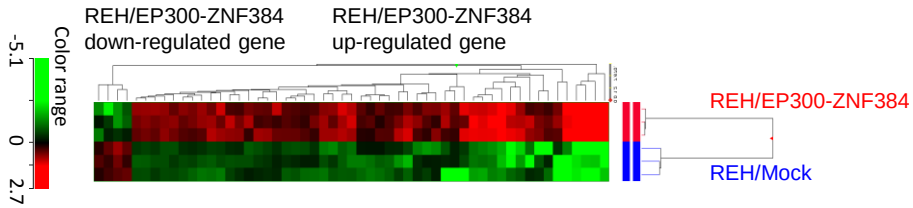
B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down-regulated gene
REH/EP300-ZNF384
up-regulated gene
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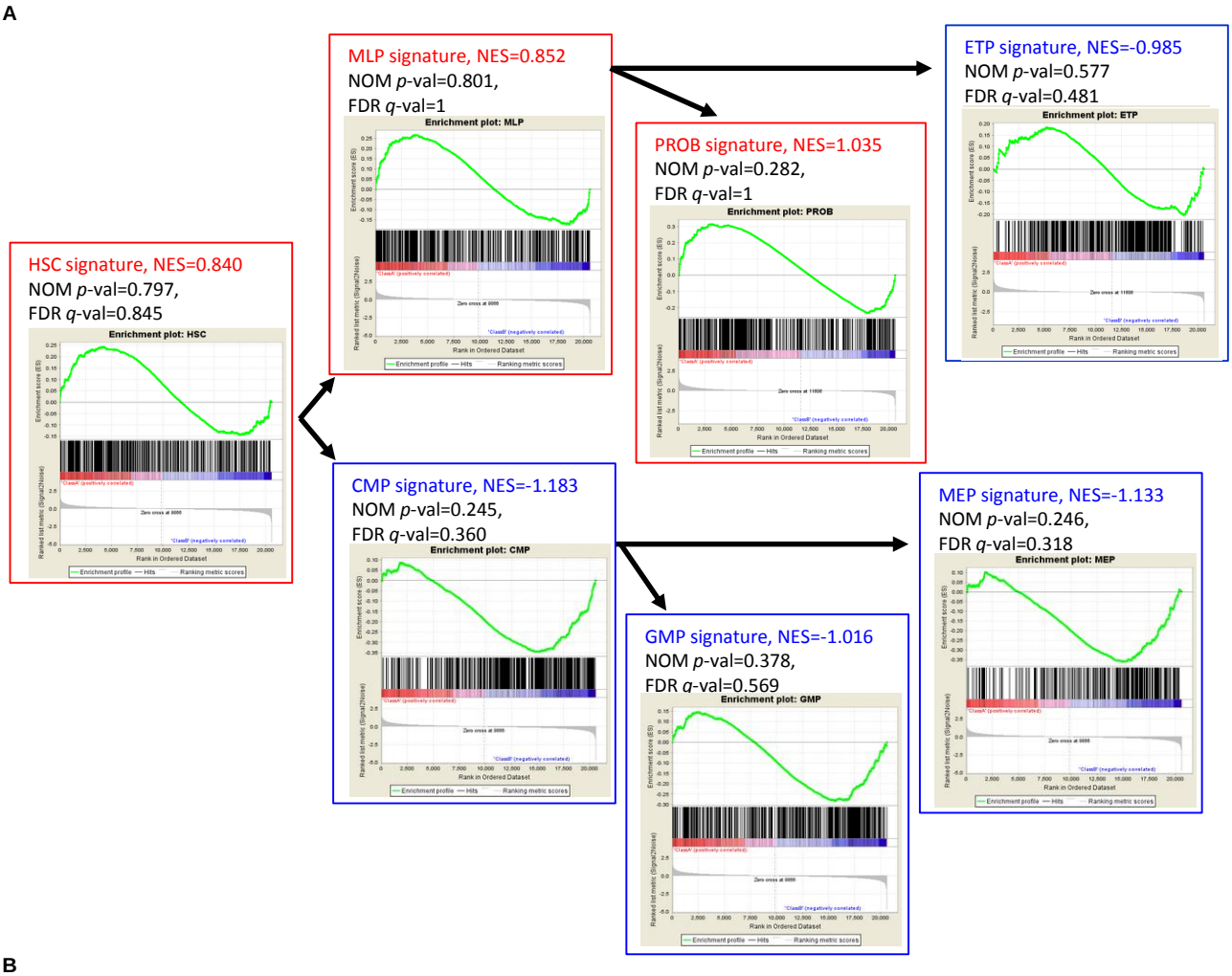
2.7
REH/EP300-ZNF384
REH/Mock

Figure-3

A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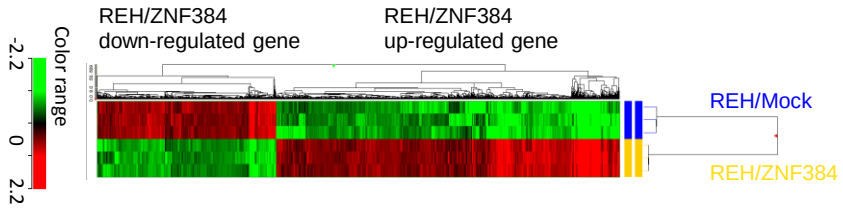
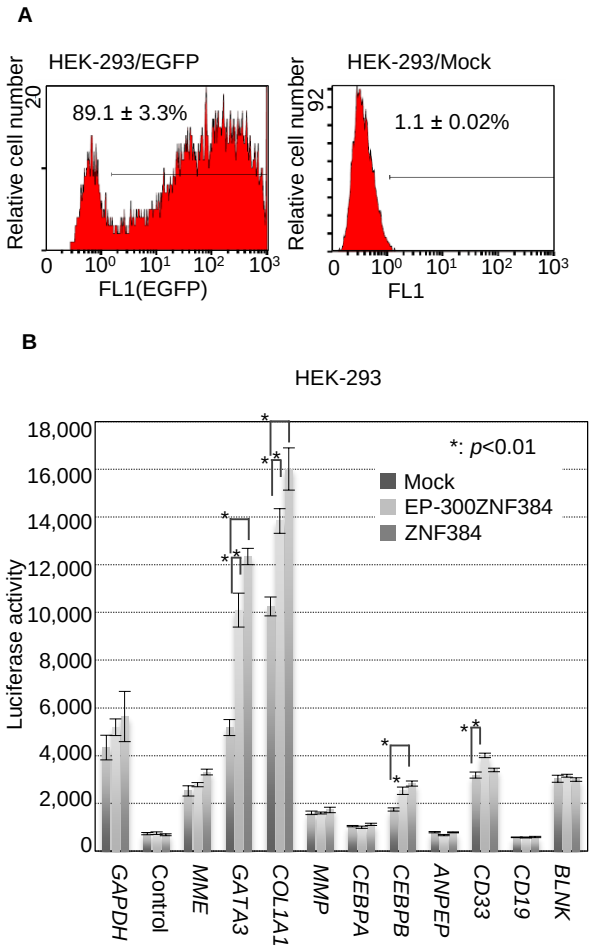
B


REH/ZNF384
down-regulated gene
REH/ZNF384
up-regulated gene
Color range
-2.2
0
2.2
REH/Mock
REH/ZNF384

C


Up-regulated gene
44
7
2679
Down-regulated gene
4
1434
REH/EP300-ZNF384
REH/ZNF384

Figure-4


A
HEK-293/EGFP
89.1 ± 3.3%
Relative cell number
20
0
10^0 10^1 10^2 10^3
FL1(EGFP)
HEK-293/Mock
1.1 ± 0.02%
92
FL1
B
HEK-293
*: p<0.01
Mock
EP-300ZNF384
ZNF384
Luciferase activity
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
GAPDH
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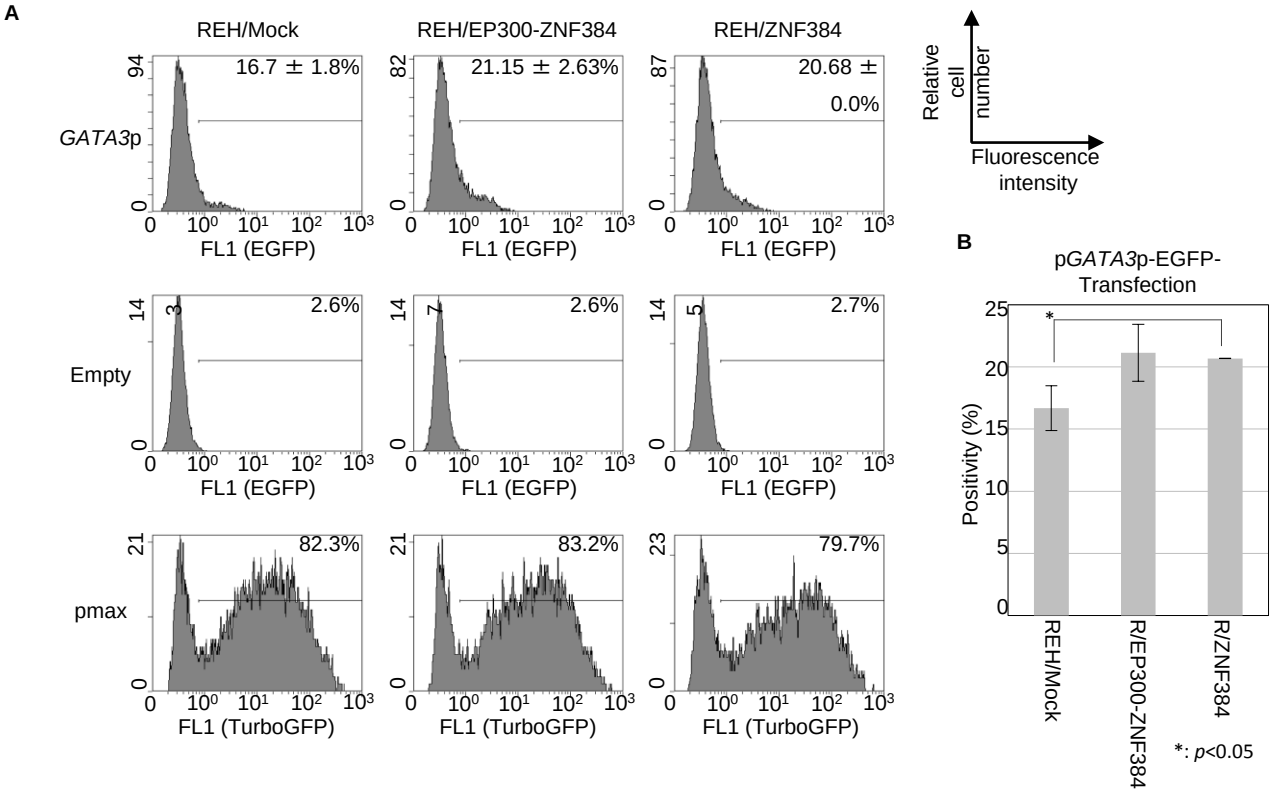
MME
GATA3
COL1A1
MMP
CEBPA
CEBPB
ANPEP
CD33
CD19
BLNK

Figure-5


A
REH/Mock
REH/EP300-ZNF384
REH/ZNF384
GATA3p
Empty
pmax
16.7 ± 1.8%
21.15 ± 2.63%
20.68 ± 0.0%
2.6%
2.6%
2.7%
82.3%
83.2%
79.7%
FL1 (EGFP)
FL1 (TurboGFP)
Relative cell number
Fluorescence intensity
B
pGATA3p-EGFP-Transfection
Positivity (%)
REH/Mock
R/EP300-ZNF384
R/ZNF384
*: p<0.05