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Regulation of HOXA9 activity by predominant expression of DACH1 against C/EBPα and GATA-1 in myeloid leukemia with *MLL-AF9*

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ABSTRACT

Although *MLL-AF9* caused by the chromosomal translocation t(9;11) has a critical role in acute myeloid leukemia, the molecular pathogenesis is poorly understood. Here, we identified that the cell fate determination factor *DACH1* is directly up-regulated by MLL-AF9. Recently we showed that the forced expression of *DACH1* in myeloid cells induced $p27^{Kip1}$ and repressed $p21^{Cip1}$, which is a pivotal characteristic of the myeloid progenitor. Consistent with our previous study, ectopic expression of *DACH1* contributed to the maintenance of colonogenic activity and blocked the differentiation of myeloid progenitors. Moreover, we here identified an endogenous HOXA9–DACH1 complex mediated by the carboxyl terminus of DACH1 in t(9;11) leukemia cells. qRT-PCR revealed that DACH1 has a stronger transcription-promoting activity with HOXA9 than does PBX2 with HOXA9. Furthermore, C/EBPα and GATA-1 can directly bind to the promoter of *DACH1* and act as a transcriptional suppressor. Expression of *DACH1* is down-regulated during myeloid differentiation and shows an inverse pattern compared to *C/EBPα* and *GATA-1* expression. However, ectopic expression of *C/EBPα* and/or *GATA-1* could not abrogate the over-expression of *DACH1* induced by MLL-AF9. Therefore, we postulate that the inability of C/EBPα and GATA-1 to down-regulate *DACH1* expression induced by MLL-AF9 during myeloid differentiation may contribute to t(9;11) leukemogenesis.

1. Introduction

Recurrent chromosomal rearrangements involving the mixed lineage leukemia (*MLL*) gene at 11q23 are commonly observed in hematologic malignancies and are associated with poor outcomes. Among more than 60 different *MLL* fusion partners identified to date, *MLL-AF9* is one of the most frequent rearrangements that results mainly in AML. Although consistent expression of the *HOXA* cluster genes, including *HOXA9*, and increased expression of *MEIS1* are known to be critically involved in leukemogenesis, this knowledge is insufficient to understand the specific leukemogenic mechanism.

The human Dachshund homologue *DACH1* was initially identified from a mutant phenotype of fruit flies featuring extremely short legs relative to their body length [1]. Recently, it was reported that *DACH1* inhibited estrogen receptor alpha-mediated DNA synthesis and cellular proliferation during progression of human breast cancer [2]. In Met-1 cells, DACH1 acts as a suppressor of *Nanog* and *Sox2* through its Box-N DNA binding domain [3], and it blocks mammary tumor growth and inhibits properties involved in stem cell expansion. On the other hand, we recently showed that the knockdown of *DACH1* blocked the cell cycle progression of HL-60 promyeloblastic cells through the decrease of *cyclin D1*, *D3*, *F*, and *Cdk 1*, *4*, *6* and increase of $p21^{Cip}$ [4]. The expression of *Sox2*, *Oct4*, and *Klf4* was significantly up-regulated by the forced expression of *DACH1* in mouse myeloid progenitor cells, which shows an opposing role of *DACH1* in myeloid cells to breast cancer cells [4]. However, the specific role of *DACH1* in myeloid leukemogenesis has not been studied yet.

Committed differentiation of myeloid progenitors is regulated by combinatorial protein–protein interactions of transcription factors, including GATA-1, PU.1 and members of the CCAAT enhancer

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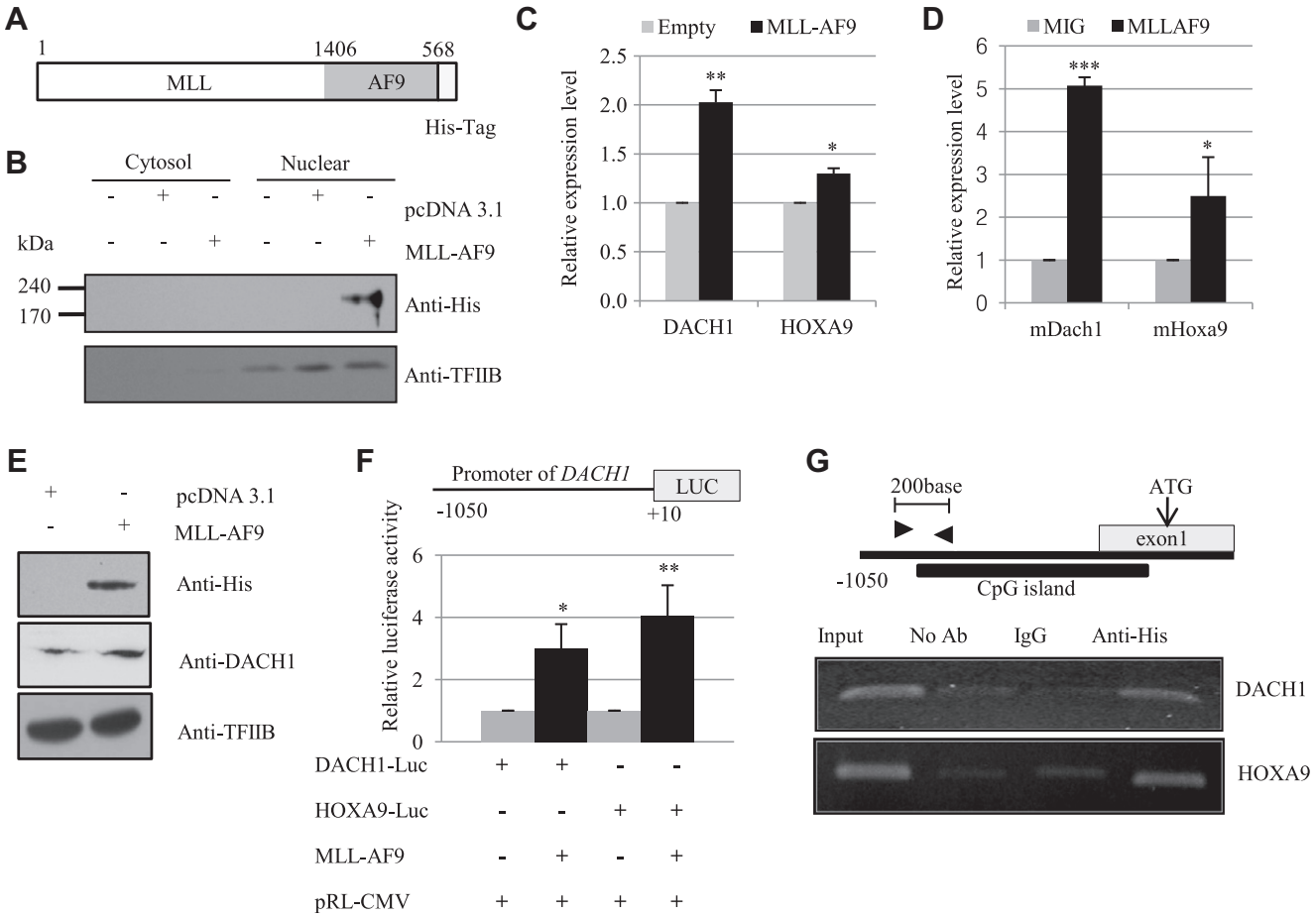

Fig. 1. (A) His-tagged MLL-AF9 fusion construct. (B) Nuclear localization of MLL-AF9. TFIIB is used as a nuclear localization marker. (C and D) Increased mRNA expression level of *DACH1*. The expression level of *DACH1* and *HOXA9* was determined by quantitative real-time PCR analysis in *MLL-AF9*-transfected HEK 293 cells (C) and mouse primary myeloid progenitor cells retrovirally transduced with *MLL-AF9* (D). (E) Western blot analysis. The protein level of DACH1 was increased after MLL-AF9 expression in HEK 293 cells compared with the non-induced cells. TFIIB is used as a loading control. (F) Dual-luciferase reporter gene assays. HEK 293 cells were transiently transfected with an *MLL-AF9*-expressing construct and a *DACH1* reporter gene construct, along with pRL-CMV as an internal control. The *HOXA9* promoter region occupied by MLL-AF9 was used as positive control. The data were normalized to the internal control and the basic vector. (G) ChIP assay was performed by using anti-6xHis antibodies, or normal rabbit serum as a negative control. Immunoprecipitated promoter region of *DACH1* was identified by PCR methods using the indicated primer set (triangles). The data were obtained from at least three independent experiments (mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control cells).

binding protein (C/EBP) family [5]. In particular, abrogation of the lineage-specific role of C/EBPα causes a block of myeloid differentiation ultimately leading to AML [6]. Furthermore, increased expression levels of C/EBPα and GATA-1 induce maturation of myeloid progenitors [7]. However, identification of the specific target gene directly regulated by C/EBPα and GATA-1 is still required for the understanding of myeloid differentiation. Here, we report that C/EBPα and/or GATA-1 directly suppress the expression of *DACH1* through binding to the promoter region. Furthermore, these studies suggest that the increased transcriptional activation of *HOXA9* caused by the abrogated regulation of *DACH1* expression might contribute to the MLL-AF9 mediated leukemogenesis.

2. Materials and methods

2.1. Retroviral transformation assays

Retroviral transformation of human *DACH1* to myeloid cells were performed as described previously [4]. Cells (1.0×10^4) were cultured in MeholCult M3234 methylcellulose medium (Stem Cell Technologies) in IMDM, 15% FBS, IL-3, IL-6, and GM-CSF, all at 10 ng/mL, and SCF at 100 ng/mL. Cell growth, colony morphology and numbers were measured after serial replating of methylcellulose cultures.

2.2. Microarray and SAGE data analysis

The microarray data analyzed in this study were obtained from the Gene Expression Omnibus (GEO), accession number GSE21973 contributed by Doulatov et al. [8]. Among 16 samples, the expression signal intensities of *C/EBPα*, *GATA-1*, *DACH1* were analyzed using three samples from HSC and two samples each from CMP, GMP, MEP. The SAGE data analyzed in this study were obtained from the report by Lee et al. [9].

Other materials and methods are given in the Supplementary data.

3. Results and discussion

3.1. MLL-AF9 directly induces the expression of DACH1

To identify highly expressed transcriptional regulators in myeloid progenitor cells with t(9;11), we analyzed differentially expressed genes in CD15+ myeloid progenitor cells from AML M5 patients using published serial analysis of gene expression (SAGE)

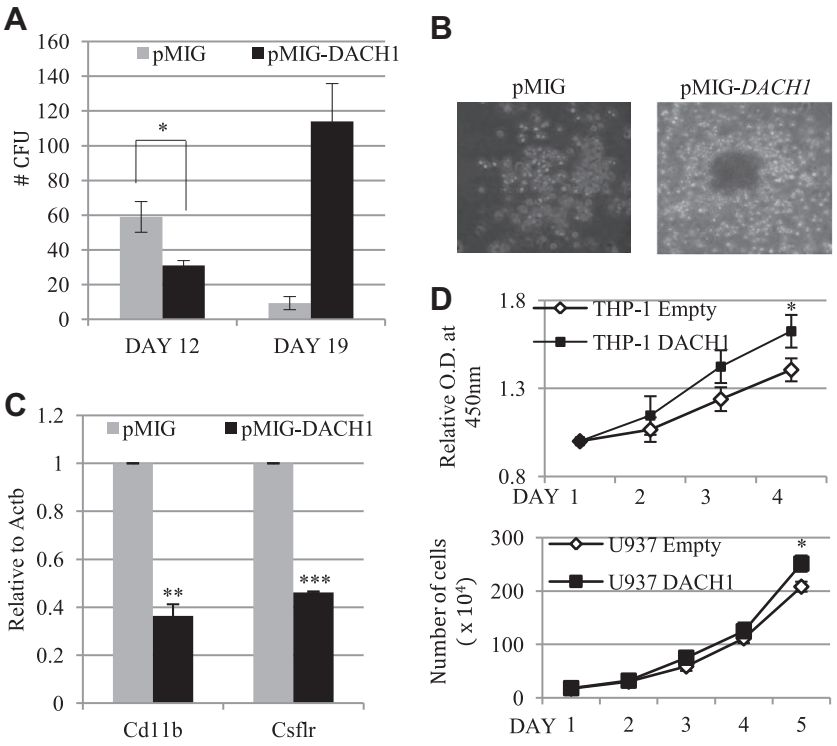

Fig. 2. (A) Number of colonies for cells transformed by empty vector or *DACH1*. Error bars represent standard deviations of three independent analyses. (B) Morphologies of representative colonies. (C) Relative expression levels of *Cd11b* and *Csf1r* in GFP-positive cells expressing either the empty vector or *DACH1*. The relative expression level was normalized with that of *ACTB*. (D) MTT assays were performed on THP-1 cells transfected with *DACH1* to assess cell proliferative activity. Graph represents the average of three separate experiments. Growth curve of U937 cells transfected with empty or *DACH1* vector. U937 cells were cultured in triplicate at 1×10^5 cells into six-well plates and counted every 24 h (mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control cells).

data [9]. The expression level of *DACH1* was 7.0-fold higher in CD15+ myeloid progenitor cells harboring t(9;11) compared to normal counterparts. To determine whether the expression of *DACH1* is induced by *MLL-AF9*, HEK 293 cells were transiently transfected with the *MLL-AF9* fusion construct (Fig. 1A). After 48 h, the appropriate 170-kDa MLL-AF9 fusion protein was detected in the nuclear fraction by antibodies against the carboxyl terminal 6xHis epitope (Fig. 1B). The mRNA expression level of *DACH1* was 2.0-fold higher in the *MLL-AF9*-transfected HEK 293 cells, and *HOXA9* expression was also increased (Fig. 1C). The endogenous expression levels of Dach1 and Hoxa9 were also increased in retrovirally transduced mouse mononuclear cells with MLL-AF9 (Fig. 1D). The protein level of DACH1 was also increased in *MLL-AF9*-transfected HEK 293 cells (Fig. 1E). Luciferase reporter gene assays shows that *MLL-AF9* expression enhances the promoter activity of *DACH1* by 9.4-fold compared to a control construct (Fig. 1F). Moreover, chromatin immunoprecipitation (ChIP) with anti-His antibodies determined that MLL-AF9 was bound directly to the promoter region of *DACH1*. We also found the *HOXA9* promoter region to be occupied by MLL-AF9, as previously reported (Fig. 1G) [10].

3.2. DACH1 enhances the colony-forming capacity and blocks the differentiation of mouse bone marrow progenitor cells

Recently, we showed that the forced expression of *DACH1* significantly increased the expression of *cyclin D1*, *D3*, *F*, *and Cdk 1*, *4*, and *6* in myeloid progenitor cells [4]. Furthermore, the knockdown of *DACH1* in HL-60 promyelocytic cells significantly reduced the expression levels of cyclin D-Cdk 4/6, which results in the block of cell cycle progression [4]. To evaluate the leukemogenic effect of *DACH1*, we performed colony-forming/replating assays. Mouse bone marrow progenitor cells transduced with pMIG empty vector or pMIG-*DACH1* were isolated by FACS using green fluorescent protein (GFP) expression (Supplementary Fig. 1) and cultured in methylcellulose. Forced expression of *DACH1* resulted in a reduced colony number 12 days after the first plating (Fig. 2A). To test the replating ability of bone marrow cells, primary colonies were harvested and replated. Remarkably, cells transduced with *DACH1* maintained significant colonogenic activity and formed an increased number of compact colonies with an immature morphology after 19 days (Fig. 2A, B). In contrast, cells transduced with empty vector were mostly differentiated (Fig. 2B). To confirm the inhibitory effect of *DACH1* in myeloid differentiation, expression levels of the monocytic/granulocytic cell differentiation marker *Cd11b* and the monocyte terminal differentiation marker *Csf1r* were measured using qRT-PCR (Fig. 1C) [11]. Consistent with the immature morphology of the *DACH1*-transduced cells, the levels of *Cd11b* and *Csf1r* were significantly down-regulated by forced expression of *DACH1*. Taken together, these data indicate that *DACH1* enhances the colonogenic activity and blocks the differentiation of mouse bone marrow progenitor cells. It is consistent with our previous study describing that the forced expression of *DACH1* in myeloid progenitor cells induces $p27^{Kip1}$ and represses $p21^{Cip1}$ which is a pivotal characteristic of the myeloid progenitor.

To examine the effect of *DACH1* on proliferation of other cell lines, a cell proliferation assay was performed using myeloid cells. As shown in Fig. 2D ectopic expression of *DACH1* produced a moderate enlargement in growth compared to cells transfected with empty vector alone.

Recently, we showed that the expression level of $p57^{Kip2}$ is significantly increased by the forced expression of *DACH1* in myeloid progenitor cells [4]. $p57^{Kip2}$ is the most abundant family member ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) in HSCs and has a pivotal role in

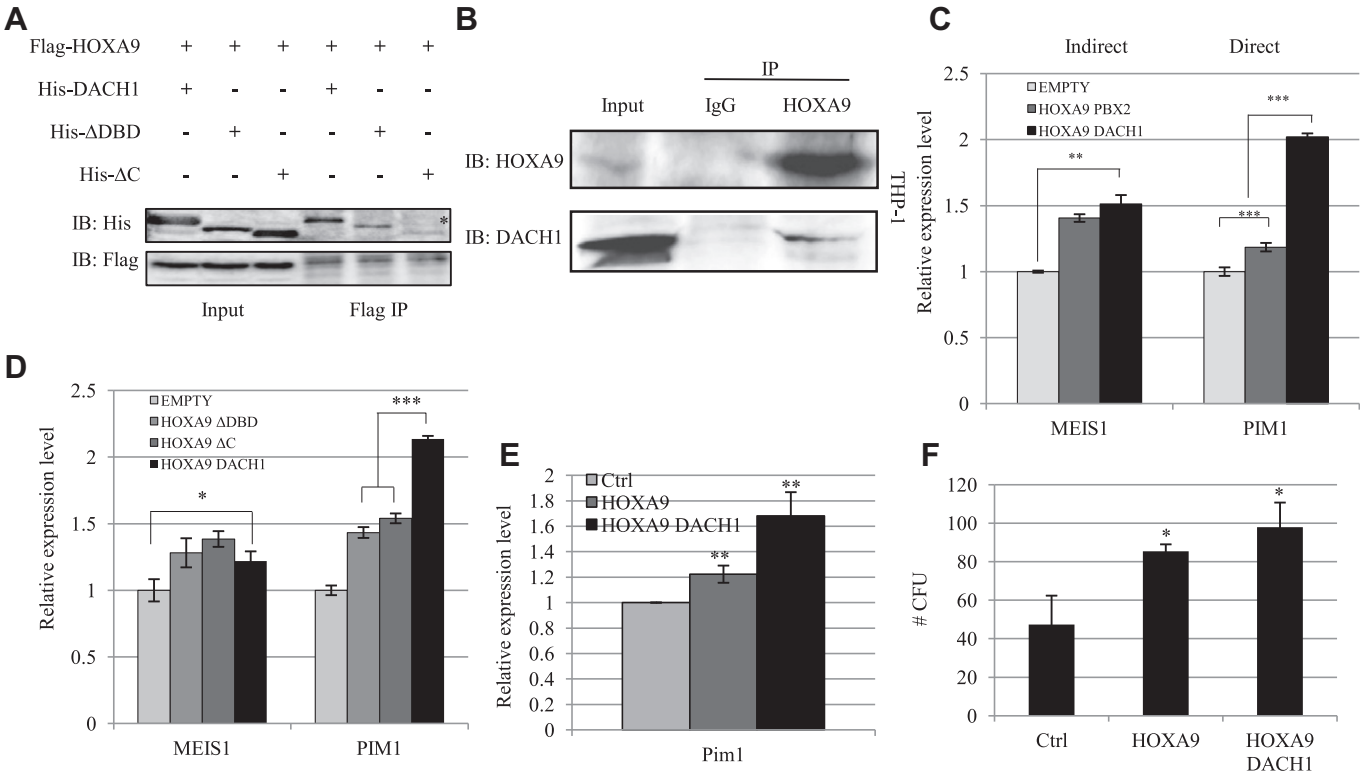

Fig. 3. (A) Immunoprecipitation (IP) of cells transfected with His-tagged DACH1 deletion mutants and Flag-tagged HOXA9 used to identify the domain on DACH1 needed for binding HOXA9. The weak signal generated in the case of the carboxyl terminal deletion mutant is indicated by an asterisk (∗). (B) Immunoprecipitation (IP) of myeloid cells with antibody specific for HOXA9 to detect the endogenous interaction between HOXA9 and DACH1. Non-specific antibodies were used as a negative control. (C and D) Investigation of the quantitative relative transcript levels of *MEIS1* and *PIM1* in HEK 293 cells overexpressing the indicated proteins as determined by qRT-PCR. The relative expression level was obtained by normalization to that of GAPDH. (E) Increased mRNA expression level of *Pim1*. The expression level of *Pim1* was determined by qRT-PCR analysis in mouse primary myeloid progenitor cells retrovirally transduced with *HOXA9* genes either individually or in combination with *DACH1* as indicated. The relative expression level was obtained by normalization to that of *Sdha*. (F) Colony forming assay of *HOXA9* and/or *DACH1* transduced mouse primary myeloid progenitor cells. Error bars represent standard deviations of three independent analyses Ctrl, control (mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control cells).

self-renewal capacity [12]. Interestingly, the predominant expression of *DACH1* in long-term hematopoietic stem cells (LT-HSC) as a potential regulator of HSC self-renewal and proliferation [13] was consistent with the expression of $p57^{Kip2}$. Therefore, the maintenance of colonogenic activation by *DACH1* suggests that the abnormally increased $p57^{Kip2}$ expression by *DACH1* in myeloid leukemia cells harboring t(9;11) may be associated with maintaining the self-renewal capacity and survival advantage. Because the role of transcription factors governing the hematopoiesis could be dependent on the cell type and differentiation state from LT-HSCs to mature myeloid cells, precise roles of *DACH1* regulating self-renewal and proliferation of hematopoietic cells should be elucidated.

3.3. DACH1 interacts with HOXA9 in myeloid leukemia cells, mainly through the carboxyl terminal domain of DACH1

Furthermore, we identified the interaction between DACH1 and HOXA9 using Co-Immunoprecipitation (Supplementary Fig. 2A). To determine which region of DACH1 mediates the interaction with HOXA9, DNA-binding domain and carboxyl terminal deletion mutants of DACH1 (ΔDBD, ΔC) were tested by immunoprecipitation assay. The DNA-binding domain deletion mutant was precipitated along with HOXA9. DACH1 lacking the carboxyl terminal region had a significantly reduced interaction with HOXA9 (Fig. 3A). These results indicate that the carboxyl terminus, a predicted α-helical coiled-coil, is critical for HOXA9 binding. This is consistent with results of a previous study, which demonstrates the carboxyl terminus of DACH1 is necessary for binding to TCERG1 (Transcription Elongation Regulator 1) [14]. However, the presence of a weak signal detected for the DACH1 mutant lacking the carboxyl terminus suggested that a region of DACH1 outside its carboxyl terminus is also involved in the interaction with HOXA9. Immunoblotting analysis revealed that endogenous DACH1–HOXA9 complex was present in THP-1 myeloid leukemia cells (Fig. 3B).

3.4. DACH1 acts as a coactivator with HOXA9

To explore the oncogenic relevance of the DACH1 interaction with *HOXA9*, the mRNA levels of HOXA9 target genes (*PIM1*, *MEIS1*) were measured in HEK 293 cells transiently transfected with *HOXA9* alone or in combination with *PBX2* (PBX2 is a transcriptional activator that interacts with HOXA9) and *DACH1*, or a control empty vector. The mRNA expression level of the *PIM1* oncogene, which is significantly up-regulated in U937 myelomonocytic leukemia cells and is a direct target of HOXA9 in hematopoietic cells [15], was significantly increased in the *HOXA9/PBX2/DACH1* transfectants compared to the control and *HOXA9* transfectants (Supplementary Fig. 3A). Furthermore, the mRNA expression level of *MEIS1*, which is essential in the initiation and maintenance of *MLL* leukemia stem cell potential [16], was also induced by *DACH1* expression. To determine whether the coactivation effect of DACH1 is independent of PBX2, HEK 293 cells were transiently transfected with either *HOXA9/PBX2* or *HOXA9/DACH1*. The mRNA expression of both *PIM1* and *MEIS1* was significantly induced by co-expression of *HOXA9* and *DACH1*. Moreover, *DACH1* significantly enhanced the

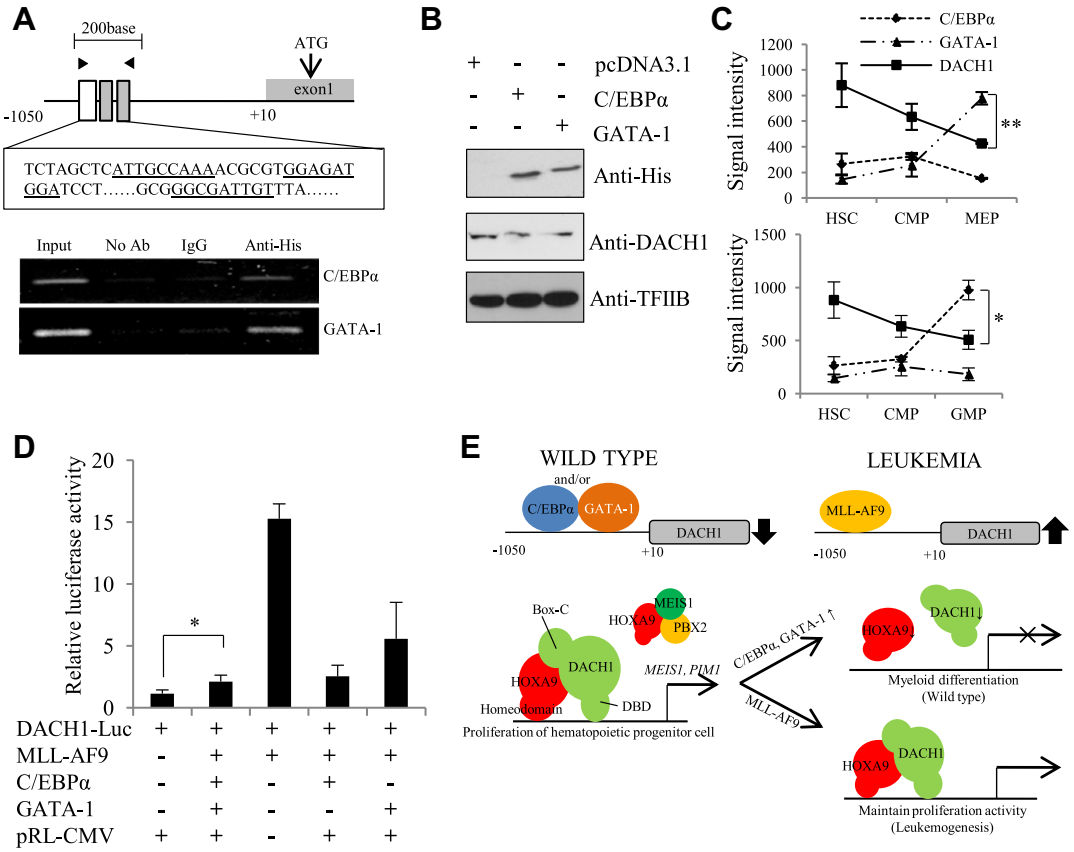

Fig. 4. (A) ChIP assay. Immunoprecipitated promoter region of DACH1 containing putative C/EBPα and GATA-1 binding sites was amplified. HEK 293 cells were transfected with 3-terminal His-tagged C/EBPα or GATA-1. After crosslinking of the cultured cells, chromatin was sheared and immunoprecipitated with anti-6xHis antibody. (B) Western blot analysis. HEK 293 cells were transiently transfected with His-tagged C/EBPα or GATA-1 as indicated. The expression levels of C/EBPα and GATA-1 were detected by anti-His antibodies; DACH1 was detected by using anti-DACH1 antibodies. (C) Inverse expression pattern of DACH1 transcript with C/EBPα and GATA-1 during myeloid differentiation. The signal intensities of DACH1, C/EBPα and GATA-1 in three samples from HSC and two samples each from CMP, GMP, and MEP were analyzed. (D) Dual-luciferase reporter gene assay. HEK 293 cells were transfected with empty vector, MLL-AF9, C/EBPα and/or GATA-1 and DACH1-luciferase reporter construct and co-transfected with pRL-CMV as an internal control. Relative luciferase activity was calculated from three different experiments. (mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control cells.) (E) A model for MLL-AF9 and C/EBPα, GATA-1 and DACH1–HOXA9 heterodimer-mediated acute myeloid leukemogenesis. During myeloid differentiation, C/EBPα and GATA-1 occupy the DACH1 promoter and down-regulate its expression. In the presence of MLL-AF9, the down-regulation of DACH1 is altered. In myeloblasts, HOXA9 with MEIS1 and PBX co-factors regulate transcription of their target genes. During myeloid differentiation, DACH1 and HOXA9 are gradually down-regulated. In the presence of MLL-AF9, however, induced DACH1 forms a heterodimer with HOXA9, which enhances HOXA9 transcriptional activity.

expression of direct target genes of HOXA9 compared with the traditional complex of HOXA9 and PBX2 (Fig. 3C). However, expression of *DACH1* without *HOXA9* could not induce the expression of HOX target genes (Supplementary Fig. 3B). *HOXA9* and *MEIS1* are highly expressed in MLL-rearranged leukemia and have been reported to have a central role in the pathogenesis of leukemia. Therefore, these results suggest that *PBX2*-independent and more potent oncogenic effects of *DACH1* could contribute to *MLL*-mediated leukemogenesis. To confirm the coactivation effect of *DACH1*, mouse primary myeloid cells activated with SCF, IL-3, and IL-6 were retrovirally transduced with *HOXA9* gene either alone or in combination with *DACH1*. The mRNA expression of endogenous *Pim1* oncogene was significantly increased in the presence of *HOXA9/DACH1* compared to *HOXA9* alone after incubation with SCF, IL-3, and IL-6 for one week from retroviral transduction (Fig. 3E). To evaluate the colonogenic effect of HOXA9/DACH1, colony-forming assays were performed. Consistent with previous study [17], the forced expression of *HOXA9* significantly increased the colony number at 7 days after retroviral transduction. Furthermore, cotransduction of *HOXA9* and *DACH1* also showed the increased colony number compared with negative control (Fig. 3F). Until now, several studies described a correlation between the reduced expression of *DACH1* and poor prognosis of breast cancer [18] and the loss of *DACH1* in prostate and endometrial cancer [19,20]. However, this discrepancy of oncogenic effect of *DACH1* could be explained by the different roles of *HOXA9* in MECs (Mammary Epithelial Cell) versus lymphocytes [21]. Interestingly, it was reported that *HOXA9* promotes breast tumor cell differentiation and inhibits breast cancer progression by directly regulating the expression of the *BRCA1* tumor suppressor gene [21]. These findings suggest that the exact role of *HOXA9* could depend on its co-regulator, which is consistent with our data demonstrating the synergic effect of HOXA9–DACH1 complex.

3.5. The coactivation effect of DACH1 requires both its DNA binding and carboxyl terminal domains

To characterize the functional roles of the domains of DACH1, we evaluated the mRNA levels of HOXA9 target genes from HEK 293 cells that were transfected with *HOXA9* and co-transfected with wild-type *DACH1* or the two deletion mutants ΔDBD and ΔC (Fig. 3D). The impaired interaction of DACH1 ΔC with HOXA9 resulted in significantly reduced co-transcriptional activity. In addition, the reduced expression level of *PIM1* by DACH1 ΔDBD suggests that the DNA binding ability of DACH1 is also required to enhance the transcriptional activity of HOXA9.

3.6. DACH1 is down-regulated in inverse proportion to the expression of C/EBPα and GATA-1 during myeloid differentiation

To further investigate the molecular mechanism of *DACH1* regulation, we analyzed the transcription factor binding sites on the promoter region of the *DACH1* gene using TFSEARCH: Searching Transcription Factor Binding Sites (version 1.3). We identified a single C/EBPα (TCATTGCCAAA) and two GATA-1 (GGAGATGGA, GGCGATTGT) putative binding sites located ~929 bp upstream of the initiation ATG (Fig. 4A). To confirm that C/EBPα and GATA-1 directly associate with the promoter region of *DACH1*, we performed a Chromatin immunoprecipitation (ChIP) assay in HEK 293 cells that were transfected with 6xHis-tagged C/EBPα and GATA-1. Both C/EBPα and GATA-1 bound to their respective putative binding sites of the *DACH1* promoter (Fig. 4A). Moreover, immunoblotting of lysates of HEK 293 transfectants expressing C/EBPα or GATA-1 showed down-regulation of endogenous *DACH1* compared to the non-induced control (Fig. 4B). To investigate the expression levels of *DACH1*, *C/EBPα* and *GATA-1* during myeloid differentiation, we analyzed submitted microarray data set from the Gene Expression Omnibus GEO (accession number GSE21973). As reported previously in many studies [8], the expression of *C/EBPα* was significantly increased during differentiation from HSCs (hematopoietic stem cell) to GMPs (granulocyte–macrophage progenitor), while the expression of *GATA-1* was increased during MEP (Megakaryocyte–erythrocyte progenitor) differentiation. Furthermore, the expression level of *DACH1* was significantly down-regulated during both GMP and MEP differentiation and inversely correlated with the expression level of *C/EBPα* or *GATA-1* (Fig. 4C). It has been reported that C/EBPα and GATA-1 bind to their consensus sites and regulate the transcription of their target genes through combinatorial interactions with each other [5]. In particular, the previously described localization of synergistically acting consensus binding sites for C/EBPα and GATA-1 is similar to what we found in the *DACH1* promoter. Thus, it is possible that C/EBPα and GATA-1 cooperate synergistically in the regulation of *DACH1*.

Because C/EBPα, GATA-1 and MLL-AF9 likely co-exist in leukemia stem cells harboring t(9;11), we assume that substantial up-regulation of *DACH1* by MLL-AF9 could contribute to leukemogenesis despite significant down-regulation by C/EBPα and GATA-1. To support this hypothesis, we performed luciferase reporter gene assays (Fig. 4D) and showed that the expression level of *DACH1* induced by MLL-AF9 was 2.1-fold higher than its basal expression level, even though both C/EBPα and GATA-1 could significantly suppress transcription of *DACH1*. This result indicates that activation of *DACH1* by MLL-AF9 fusion protein is not abrogated by the inhibitory effects of C/EBPα and GATA-1 during myeloid differentiation, a situation that may contribute to myeloid leukemogenesis (Fig. 4E).

3.7. Concluding remarks

Our data support the epochal concept of oncogenes which also play roles as a tumor suppressor dependent on the expressed tissue or partner molecules. *HOXA9* has been known as critical oncogene in myeloid leukemogenesis, however, roles of *HOXA9* involved in tumor suppressor in breast cancer was previously described [21]. Interestingly, *DACH1* also act as a tumor suppressor in breast cancer. However, we recently demonstrated the opposite roles of *DACH1* associated with the regulation of the cell cycle machinery in myeloid cells. Here, our data show that *DACH1* is a novel coactivator of HOXA9 and has an oncogenic effect in hematopoietic malignancy. Moreover, we identified DACH1 as the target of general differentiation-related transcription factor *C/EBPα* and *GATA1*. Despite extensive investigations, the specific mechanisms by which *MLL* chimeras contribute to leukemogenesis remain unclear due to the complex function of *MLL* fusion proteins. Here, we report a novel mechanism of myeloid leukemogenesis mediated by *DACH1*, which may help us understand the development of myeloid leukemia with *MLL*-fusion genes and lead to new therapeutic approaches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.048.

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