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Zfp296 is a novel Klf4-interacting protein and functions as a negative regulator

Yuka Fujii, Madoka Kakegawa, Hiroshi Koide, Tadayuki Akagi *, Takashi Yokota *

Department of Stem Cell Biology, Graduate School of Medical Sciences, Kanazawa University, Japan

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ABSTRACT

Pluripotency and self-renewing ability of embryonic stem (ES) cells are regulated by several transcription factors, including Oct3/4, Sox2, Kruppel-like factor 4 (Klf4), and c-Myc. These transcription factors reprogram somatic cells into induced pluripotent stem (iPS) cells. Zinc finger protein (Zfp) 296 has been reported to enhance iPS cell formation. Here we found that Zfp296 interacts with Klf4. A maltose-binding protein pull-down assay demonstrated that Klf4 binds to the Zfp296 158–483 amino acid region, and that Zfp296 binds to the Klf4 DNA-binding domain (DBD). A quantitative reverse transcription-polymerase chain reaction analysis revealed that expression of Zfp296 and Klf4 decreased during differentiation of E14 and ZHBTc4 ES cells. We also found that green fluorescent protein-labeled Zfp296 and Klf4 were localized to the nucleus. Because Zfp296 bound to the Klf4 DBD, we next examined the influence of Zfp296 on Klf4 DNA-binding activity. A biotin DNA pull-down assay showed that Klf4 binds to the *Lefty1* promoter region, and that binding activity was sustained even in the presence of Zfp296. In contrast, a reporter assay showed that the *Lefty1* promoter was activated by Klf4, and that the enhanced activity was repressed by Zfp296. These findings suggest that Zfp296 is a functional regulator of Klf4 in ES cells.

1. Introduction

Pluripotency and self-renewal capacity are major characteristics of embryonic stem (ES) cells. Undifferentiated mouse ES cells are stimulated to proliferate by leukemia inhibitory factor (LIF) [1,2], and ES cells spontaneously differentiate in the absence of LIF. Several transcription factors, including STAT3, Oct3/4, and Sox2 play crucial roles in ES cell characteristics [3–5]. Artificial activation of STAT3 enables ES cells to self-renew without LIF stimulation [6]. Targeted disruption of either *Oct3/4* or *Sox2* genes results in loss of the pluripotent inner cell mass [7,8], and *Oct3/4*- and *Sox2*-deficient ES cells differentiate even in the presence of LIF [9,10]. Forced expression of Kruppel-like factor 4 (Klf4), Nanog, and Esrrb partially supports self-renewal of ES cells without LIF [11–15].

Interestingly, self-renewal related transcription factors, including Oct3/4, Sox2, Klf4, and c-Myc (OSKM) reprogram somatic cells into pluripotent stem cells [16–19], which are widely known as induced pluripotent stem (iPS) cells, and their characteristics are similar to those of ES cells. Among the four reprogramming factors, c-Myc, which enhances iPS cell tumorigenesis, is dispensable for reprogramming, and somatic cells are reprogrammed to a pluripotent state by the other three factors, although their efficiency is low [20,21]. Recent investigations have clarified additional transcription factors, including Glis1, LRH1, Esrrb, and Nanog plus Lin28 that facilitate reprogramming [19,22–24]. Zinc finger protein (Zfp) 296 is also a reprogramming factor, and Zfp296, together with OSKM, leads to effective formation of mouse iPS cells compared with the effect of OSKM alone [25]. Therefore, ES cell-specific transcription factors are involved in the maintenance of ES cell characteristics and reprogramming of somatic cells.

Zfp296 consists of 445 amino acids and contains two zinc finger motifs. Zfp296 is expressed strongly in mouse testis but weakly in mouse brain and bone marrow [26]. *In situ* hybridization analyses have shown that the inner cell mass of the mouse blastocyst and ES cells express Zfp296 mRNA [27,28]; therefore, Zfp296 is often used as a marker gene for undifferentiated ES and reprogrammed cells [14,16,29]. In the present study, we examined the protein–protein interaction of Zfp296 with reprogramming factors and found that Klf4 was a Zfp296-binding partner. Here, we report that Zfp296 functions as a negative regulator of Klf4 in ES cells.

2. Materials and methods

2.1. Cell culture

The ES cell lines E14tg2a (E14) and ZHBTc4 (conditional *Oct3/4*-knockout ES cells), as well as human embryonic kidney (HEK) 293

* Corresponding authors. Address: Department of Stem Cell Biology, Graduate School of Medical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan. Fax: +81 76 234 4238 (T. Akagi).
E-mail addresses: tadayuki@staff.kanazawa-u.ac.jp (T. Akagi), tyokota@med.kanazawa-u.ac.jp (T. Yokota).

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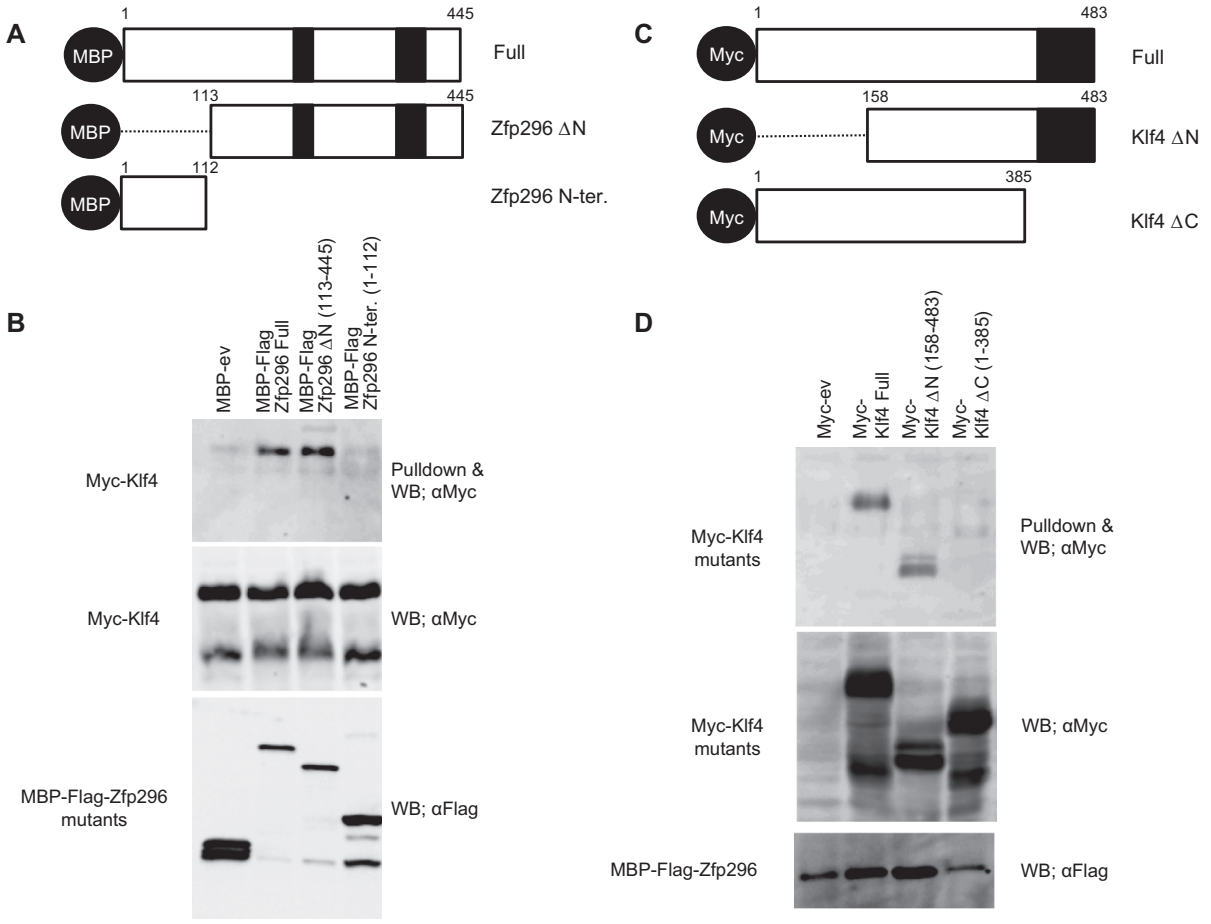

Fig. 1. Identifying the protein-interaction domains between Zfp296 and Klf4. (A) Schematic view of Flag-MBP-Zfp296 and its truncated mutants. Three Flag-tagged MBP-fused constructs, including full-length Zfp296 (full, amino acids 1–445), Zfp296 ΔN (amino acids 113–445), and the N-terminal region (amino acids 1–112) were designed. Zinc finger motifs are indicated by black boxes. (B) Klf4 binds to the Zfp296 zinc finger motifs. HEK293 cells were transfected with Myc-Klf4, together with an either empty control vector (MBP-ev), Flag-MBP-Zfp296 Full, Flag-MBP-Zfp296 ΔN (113–445), or Flag-MBP-Zfp296 N-terminal (1–112). MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis with anti-Myc antibody. Expression of each protein was confirmed with anti-Myc and anti-Flag antibodies, respectively. (C) Schematic view of Myc-tagged Klf4 and its truncated mutants. Three Myc-tagged constructs, including full-length Klf4 (full, amino acids 1–483), Klf4 ΔN (amino acids 158–483), and Klf4 ΔC (amino acids 1–385) were designed. The DNA-binding domain (DBD) is indicated by black boxes. (D) Zfp296 binds to the Klf4 DBD. HEK293 cells were transfected with MBP-fused Flag-tagged Zfp296 and either control empty vector (Myc-ev), Myc-Klf4 Full, Myc-Klf4 ΔN (158–483), or Myc-Klf4 ΔC (1–385). MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis with anti-Myc antibody. Expression of each protein was confirmed with anti-Myc and anti-Flag antibodies. All results are representative of several independent experiments.

cells were cultured as described previously [6,9,30,31]. E14 ES cells were cultured for 3–6 days without LIF to prepare the LIF depletion culture. ZHBTc4 ES cells were cultured with or without 1 μg/ml tetracycline (Tet; Sigma–Aldrich, St. Louis, MO, USA) for 24–48 h to control Oct3/4 expression. The Tet-treated cell culture medium was changed to a Tet-free medium to restore Oct3/4 expression, and the cells were cultured for another 24 h.

2.2. Plasmid construction, plasmid transfection, and luciferase assay

Construction of mammalian expression vectors, including pCMV5-Flag-maltose-binding protein (MBP), pCAGIP-enhanced green fluorescent protein (GFP), pCAGIP-Myc, and pCAGIP-Flag was described previously [32]. Zfp296 and Klf4 coding regions and their truncated mutants were amplified by polymerase chain reaction (PCR) and cloned into expression vectors. The *Lefty1* gene promoter region (from +66 to −1314, transcription start site is +1) was amplified by PCR to construct the reporter plasmids, cloned into pGL4.10 (Promega, Madison, WI, USA), and called pGL4.10-Lefty1 (−1.3 k). The primer sequences are listed in Table S1.

Plasmids were introduced into cultured cells using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). Cell extracts for the luciferase assay were prepared 48 h after transfection, and luciferase activity was measured using a luciferase assay kit (Promega) using an AB-2200 luminometer (ATTO, Tokyo, Japan).

2.3. Preparation of mRNA, cDNA synthesis, and quantitative reverse transcription-PCR (qRT-PCR) assay

mRNA was prepared, and cDNA was synthesized as described previously [32]. Gene expression was determined by qRT-PCR using Sybr green (MxPro Mx3005P; Stratagene, La Jolla, CA, USA), and target gene expression levels were normalized with glyceraldehyde-3-phosphate dehydrogenase. The primer sequences are listed in Table S1.

2.4. MBP pull-down assay and Western blot analysis

An MBP pull-down assay was performed as described previously [32,33]. HEK293 cell lysates and pull-down samples were subjected to Western blot analysis using anti-Myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Flag (F3165; Sigma–Aldrich) antibodies followed by horseradish peroxidase-conjugated antimouse antibody (Millipore, Billerica, MA, USA). The blot was visualized using enhanced chemiluminescence

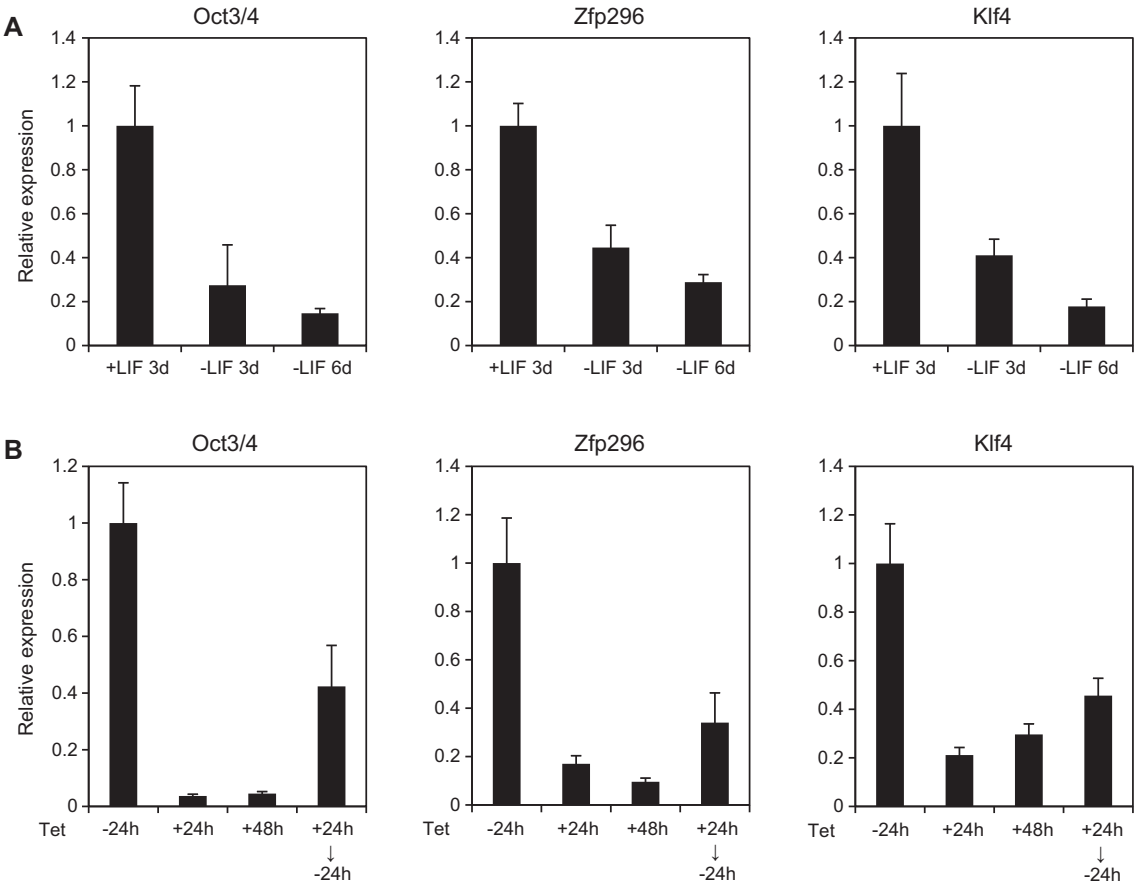

Fig. 2. Zfp296 and Klf4 mRNA expression in ES cells. (A) Zfp296 and Klf4 are expressed in self-renewing ES cells. E14 ES cells were cultured with (+) or without (−) leukemia inhibitory factor (LIF) for 3 (3d) to 6 days (6d), and Oct3/4, Zfp296, and Klf4 expression was examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (B) Decreased Zfp296 and Klf4 mRNA expression was observed after repressing Oct3/4 expression. ZHBTc4 ES cells were cultured with (+) or without (−) tetracycline [Tet (1 μg/ml)] for 24–48 h in the presence of LIF. The Tet-treated cell culture medium was charged to Tet-free medium to recover Oct3/4 expression, and the cells were cultured for another 24 h. Oct3/4, Zfp296, and Klf4 expression was examined by qRT-PCR. All samples were analyzed in triplicate, and the data were normalized to GAPDH expression.

reagents (PerkinElmer, Waltham, MA, USA) with an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

2.5. Biotin-labeled DNA pull-down assay

A biotin-labeled DNA pull-down assay was performed as described previously [32,33]. The biotin-labeled oligonucleotide was incubated with HEK293 cell extracts transfected with Flag-Klf4, Myc-Zfp296, or Flag-Klf4 plus Myc-Zfp296 in the presence of streptavidin–agarose (Novagen, Darmstadt, Germany). Twenty-five-fold nonlabeled oligonucleotide (either wild-type or mutant) was added for the competition assays. The beads were washed 3 times with a washing buffer, the bound proteins were eluted by boiling in 2× sodium dodecyl sulfate sample buffer, and the signals were detected by Western blot analysis as described above.

3. Results

3.1. Interaction domains between Klf4 and Zfp296

Transcription factors, including Oct3/4, Sox2, Klf4, and c-Myc, are involved in reprogramming of somatic cells and self-renewal of ES cells. Among transcription factors, we discovered that Klf4 associated with Zfp296.

We prepared three Flag-tagged MBP-fused constructs, including full-length Zfp296 (full, amino acids 1–445), Zfp296 ΔN (amino acids 113–445), and the N-terminal region (amino acids 1–112) to identify the Klf4-binding region of Zfp296 (Fig. 1A). The MBP pull-down assay revealed that Klf4 precipitated strongly with full-length Zfp296 and Zfp296 ΔN but did not precipitate with the N-terminal region, indicating that Klf4 binds to the Zfp296 ΔN form (Fig. 1B).

We generated three Myc-tagged constructs to identify the Zfp296-binding region of Klf4, including full-length Klf4 (full, amino acids 1–483), Klf4 ΔN (amino acids 158–483), and Klf4 ΔC (amino acids 1–385) (Fig. 1C). As shown in Fig. 1D, full-length Klf4 and Klf4 ΔN precipitated strongly with Zfp296, whereas Klf4 ΔC did not precipitate with Zfp296, suggesting that Zfp296 associates with the Klf4 amino acid region 386–483, where the Klf4 DNA-binding domain (DBD) is located.

3.2. Zfp296 and Klf4 mRNA expression in ES cells

Next, we examined whether Zfp296 expression correlates with Klf4 expression in ES cells. E14 ES cells were cultured with or without LIF for 3–6 days, and gene expression, including Zfp296, Klf4, and Oct3/4 as undifferentiated markers, was examined. qRT-PCR analyses revealed that Zfp296 and Klf4 expression was detected in undifferentiated ES cells, and that their expression decreased upon differentiation induced by depleting LIF (Fig. 2A).

We also examined Zfp296 expression in ZHBTc4 ES cells (Tet-inducible Oct3/4 conditional knockout ES cells) [9]. Tet stimulation completely repressed Oct3/4 expression, which recovered by removing Tet (Fig. 2B). Zfp296 and Klf4 expression decreased

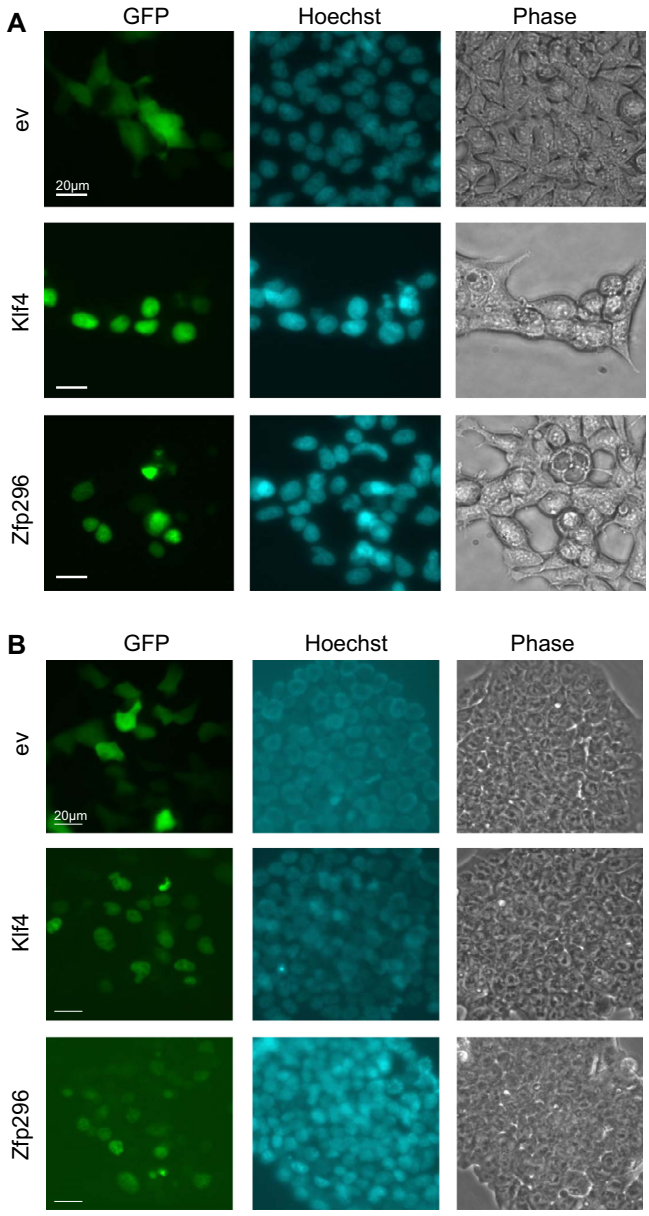

Fig. 3. Nuclear localization of Zfp296 and Klf4. (A) Nuclear localization of Zfp296 and Klf4 in HEK293 cells. HEK293 cells were transfected with either control GFP empty vector, GFP-Klf4, or GFP-Zfp296. Cells were photographed 2 days after transfection. (B) Nuclear localization of Zfp296 and Klf4 in E14 ES cells. E14 ES cells were transfected with either control GFP empty vector, GFP-Klf4, or GFP-Zfp296. Cells were photographed 2 days after transfection. Nuclei were stained with Hoechst stain. All results are representative of several independent experiments. Bars, 20 μm.

dramatically following Tet stimulation and was restored after removing Tet (Fig. 2B). These results show that Zfp296 and Klf4 are expressed in undifferentiated E14 and ZHBTc4 ES cells, and that their expression decreases after differentiation following LIF removal or Oct3/4 depletion, indicating that Zfp296 mRNA expression correlates with that of Klf4 in ES cells.

3.3. Cellular localization of Zfp296 and Klf4

We also investigated cellular localization of Zfp296 and Klf4 using GFP-tagged Zfp296 and Klf4. As shown in Fig. 3A, GFP-Zfp296 and GFP-Klf4 signals were found in HEK293 cell nuclei (Fig. 3A). We also examined their localization in E14 ES cells. Similar to HEK293 cells, Zfp296 and Klf4 GFP signals were found in E14 ES cell nuclei (Fig. 3B). These observations suggest that both Zfp296 and Klf4 are localized to the nucleus.

3.4. Effects of Zfp296 upon DNA binding and Klf4 transcriptional activity

Because Zfp296 binds to the Klf4 DBD, we examined whether Klf4 DNA-binding activity was affected by Zfp296. Here, we focused on the *Lefty1* gene, because the *Lefty1* gene promoter region contains a putative Klf4-binding site, and promoter activity is enhanced by Klf4 [34]. DNA-binding activity was assessed from the results of a biotin-labeled DNA pull-down assay using a biotinylated oligonucleotide of the *Lefty1* gene Klf4-binding site. Flag-Klf4 was precipitated with biotinylated oligonucleotide, and competitive binding was generated by adding 25-fold nonlabeled oligonucleotide of the same sequence but not the mutated oligonucleotide (Fig. 4A), indicating that the association is sequence specific. Next, the biotin-labeled DNA pull-down assay was performed using HEK293 cells expressing both Flag-Klf4 and Myc-Zfp296 or not. As shown in Fig. 4B, Flag-Klf4 was precipitated with the biotin-labeled DNA in the absence of Myc-Zfp296. Notably, Flag-Klf4 was precipitated even in the presence of Myc-Zfp296. Taken together, these data suggest that Klf4 binds to DNA even in the presence of Zfp296.

These findings prompted us to examine whether Klf4 transcriptional activity is influenced by Zfp296. The luciferase reporter assay results revealed that *Lefty1* gene promoter activity was strongly activated by Klf4 in HEK293 cells, and that the enhanced activity was repressed by Zfp296 (Fig. 4C). Strong reporter activity was also detected in E14 ES cells and was decreased by Zfp296 (Fig. 4D). These findings indicate that Zfp296 inhibits Klf4 transcriptional activity in both HEK293 and E14 ES cells.

4. Discussion

We demonstrated here that Zfp296 interacts with Klf4 and that Zfp296 and Klf4 expression are correlated in ES cells. Furthermore, both molecules are localized to the nucleus, and Zfp296 functions as a negative regulator of Klf4 in ES cells.

Functional analysis of Klf4 has been thoroughly examined during normal homeostasis, cell differentiation, and cancer formation. The function of Klf4 during reprogramming of somatic cells into pluripotent cells and self-renewal of ES cells has been investigated extensively. Notably, EpiStem cells, which are established from post implantation epiblasts, can be reprogrammed into naïve pluripotent stem cells by a single Klf4 transfection [35]. Klf4 is thought to be a direct downstream target of STAT3 in ES cells, and Klf4-overexpressing ES cells enhance ES cell self-renewal in a LIF-independent manner [36,37]. Klf4 regulates expression of several self-renewal-related genes, including Nanog, telomerase, and Lefty1 and prevents ES cell differentiation [12,34,38]. Our results demonstrated that Klf4 transcriptional activity in the *Lefty1* gene promoter region is repressed by Zfp296; thus, Zfp296 regulates Klf4 activity and may contribute to self-renewal of ES cells. We did not observe endogenous downregulation of Lefty1 mRNA expression by transient transfection of Zfp296 in ES cells (data not shown). This result probably occurred because endogenous Lefty1 expression is maintained by epigenetic regulation and other transcription factors including Oct3/4 and Sox2 [34].

Klf4 interacts with various factors, including Oct3/4, Sox2, and Glis1 [22,39], and these interactions promote somatic cell reprogramming [22,39]. Here, we identified Zfp296 as a novel Klf4-interacting protein. As described above, Zfp296 acts as a functional repressor of Klf4. Dax1 binds to either Oct3/4 or Esrrb, inhibits their

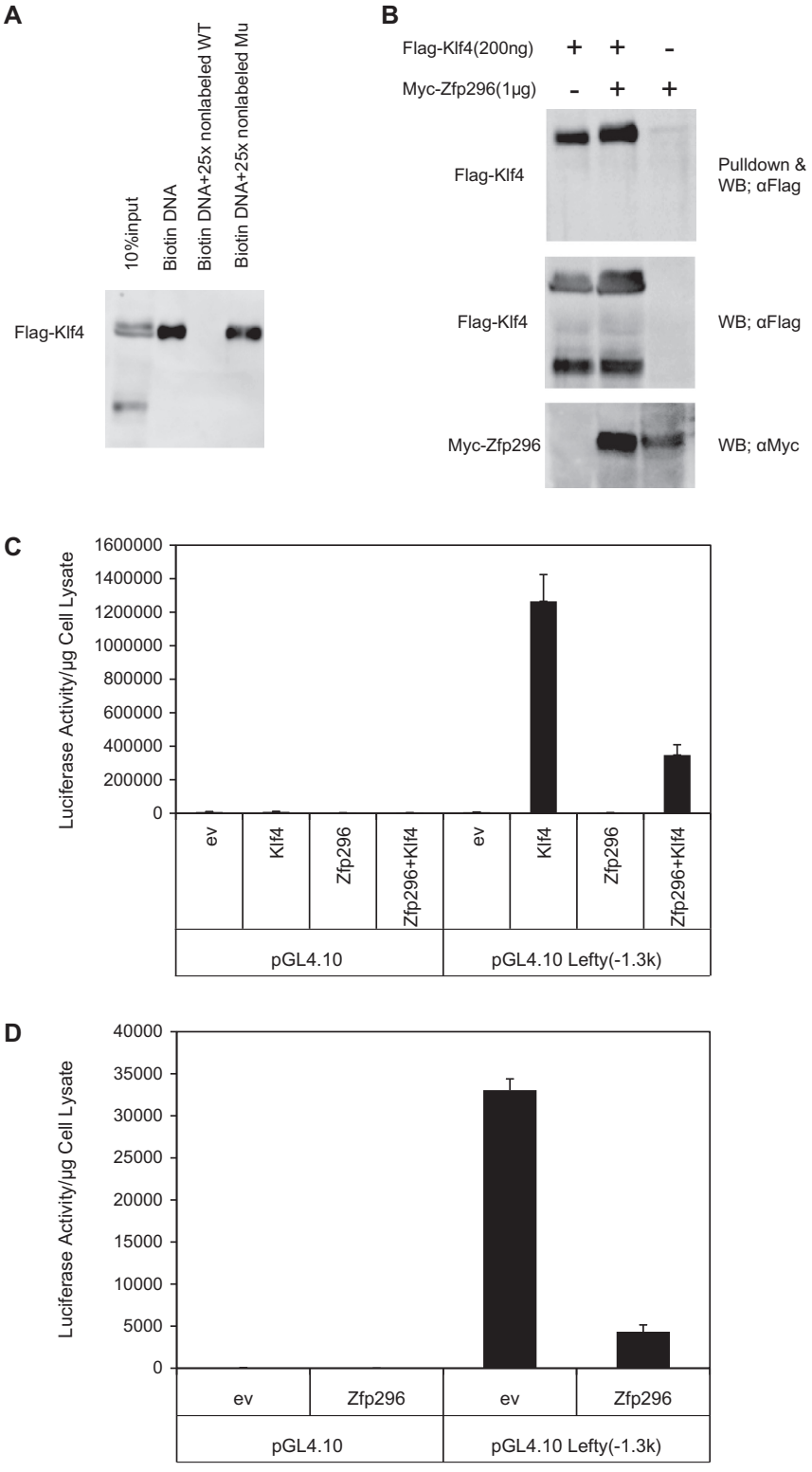

Fig. 4. Effects of Zfp296 on Klf4 DNA binding and transcriptional activity. (A) Klf4 bound to the *Lefty1* gene promoter region. Biotin-labeled oligonucleotide containing the *Lefty1* gene Klf4-binding site was incubated with Flag-Klf4-transfected HEK293 cell extracts either with or without 25-fold nonlabeled wild-type (WT) or mutated (Mu) oligonucleotide. The precipitates and cell lysates were analyzed by Western blot analysis with anti-Flag antibody. (B) Klf4 bound to the *Lefty1* gene promoter region in the presence of Zfp296. Lysates from HEK293 cells expressing Flag-Klf4 and/or Myc-Zfp296 were subjected to pull-down assay with biotinylated DNA. The precipitates were analyzed by Western blot analysis with anti-Flag antibody. Klf4 and Zfp296 expression levels were examined by Western blot analysis with anti-Flag or anti-Myc antibodies, respectively. All results are representative of several independent experiments. (C) *Lefty1* gene promoter activity upon Klf4 expression was repressed by Zfp296 in HEK293 cells. HEK293 cells were transfected with control empty vector (ev), Myc-Klf4 (Klf4), Myc-Zfp296 (Zfp296), or Myc-Zfp296 plus Myc-Klf4 (Zfp296 + Klf4) together with either pGL4.10 or pGL4.10-Lefty1 (−1.3 k). (D) *Lefty1* gene promoter activity was repressed by Zfp296 in E14 ES cells. E14 ES cells were transfected with ev or Myc-Zfp296 (Zfp296) together with either pGL4.10 or pGL4.10-Lefty1 (−1.3 k). The cells were harvested 2 days after transfection in all experiments. Data are representative of several independent experiments, and values shown are the means plus standard deviations (error bars) ($n = 3$).

transcriptional activities, and contributes to maintain self-renewal of ES cells [32,33]. As expression and cellular localization of Zfp296 were consistent with Klf4, these two molecules function together in ES cells. Although Zfp296 associated with the Klf4 DNA-binding domain, Klf4 DNA-binding activity was intact even in the presence of excess Zfp296. Therefore, Zfp296 binds to Klf4 without interfering with its DNA-binding activity.

Zfp296 seems to have both oncogenic and tumor suppressive functions. The *Zfp296* locus was first identified as a proviral insertion site in BHX2 mice with retrovirally induced myeloid leukemia [40], and Zfp296 cDNA was cloned later [26]. The retroviral insertion site was found in an upstream region of the *Zfp296* gene in a multiple leukemic mice model [41]. A translocation t(17;19)(q23;q13.32) was found in a pediatric patient with acute myeloid leukemia. Interestingly, the *myeloperoxidase* gene promoter region and the *ZNF342* gene, a human homologue of Zfp296, were fused in the translocation, and a higher level of ZNF342 expression is associated with a poor outcome [42], indicating that Zfp296 is associated with tumorigenesis in hematopoietic cells. In contrast, the *ZNF342* gene is often deleted or methylated in patients with oligodendroglioma, a subtype of primary brain tumor; and ZNF342 expression is repressed in these cells, indicating that Zfp296 may act as a tumor suppressor [43]. Zfp296 appeared to have a cytotoxic effect, as Zfp296-overexpressing ES cell colonies were not obtained (data not shown), suggesting that Zfp296 may have growth suppressive activity in ES cells.

Given these findings, Zfp296 functions as a negative regulator of Klf4 with the *Lefty1* promoter and may be involved in the maintenance of ES cell self-renewal. Further studies, including generation of *Zfp296*-deficient ES cells and a functional investigation of iPS cell generation will be required to completely understand Zfp296 function.

Acknowledgments

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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.2013.10.073.

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