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A novel GDNF-inducible gene, *BMZF3*, encodes a transcriptional repressor associated with KAP-1

Chikage Suzuki [a], Yoshiki Murakumo [a,*], Yukari Kawase [a], Tomoko Sato [a], Takatoshi Morinaga [a,b], Naoyuki Fukuda [a,b], Atsushi Enomoto [a], Masatoshi Ichihara [a,c], Masahide Takahashi [a,d]

[a] *Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8850, Japan*
[b] *Department of Internal Medicine, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan*
[c] *Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai 487-8501, Japan*
[d] *Division of Molecular Pathology, Center for Neurological Disease and Cancer, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan*

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Abstract

The Krüppel-associated box (KRAB)-containing zinc finger proteins (ZFPs) comprise the largest family of zinc finger transcription factors that function as transcriptional repressors. In the study of glial cell line-derived neurotrophic factor (GDNF)-RET signaling, we have identified *bone marrow zinc finger 3* (*BMZF3*), encoding a KRAB-ZFP, as a GDNF-inducible gene by differential display analysis. The expression of *BMZF3* transcripts in the human neuroblastoma cell line TGW increased 1 h after GDNF stimulation, as determined by Northern blotting and quantitative reverse-transcriptase polymerase chain reaction. The BMZF3 possesses transcriptional repressor activity in the KRAB domain. BMZF3 interacts with a co-repressor protein, KRAB-associated protein 1 (KAP-1), through the KRAB domain and siRNA-mediated knockdown of KAP-1 abolished the transcriptional repressor activity of BMZF3, indicating that KAP-1 is necessary for BMZF3 function. Furthermore, siRNA-mediated silencing of *BMZF3* inhibited cell proliferation. These findings suggest that BMZF3 is a transcriptional repressor induced by GDNF that plays a role in cell proliferation.

Keywords: BMZF3; GDNF-inducible gene; KRAB-zinc finger; Transcriptional repressor; KAP-1

Gene expression and silencing are controlled by many transcriptional activators or repressors. The Cys_2-His_2 (C_2-H_2) zinc finger transcription factors comprise the largest family and approximately one third of C_2-H_2 zinc finger proteins (ZFPs) contain the Krüppel-associated box (KRAB) domain [1]. Genome-wide analysis revealed that there are 423 KRAB-ZFP-coding loci in the human genome that give rise to more than 700 predicted proteins [2]. The majority of genes encoding KRAB-ZFPs reside in a cluster on particular chromosomal regions including 19q13 [3]. The KRAB-ZFPs analyzed so far have transcriptional repressor activity, and are involved in cell differentiation, proliferation, apoptosis, neoplastic transformation, and embryogenesis [4].

KRAB-ZFPs consist of a KRAB domain at the N-terminus that contains the transcriptional repressor activity and repeated zinc finger motifs at the C-terminus that possess DNA-binding activity [4,5]. The KRAB domain is composed of a KRAB-A subdomain and either a KRAB-B, -C or divergent B subdomain or a KRAB-A subdomain alone. The KRAB-A subdomain is responsible for transcriptional repression, while the KRAB-B, -C, and divergent B subdomains are enhancer elements of the KRAB-A subdomain [6]. The transcriptional repressor activity of KRAB-ZFP requires a transcriptional co-repressor protein, KRAB-associated protein 1 (KAP-1, TIF1β, and KRIP-1), which is a member of the RING-B-box coiled-

* Corresponding author. Fax: +81 52 744 2098.
E-mail address: murakumo@med.nagoya-u.ac.jp (Y. Murakumo).

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coil (RBCC) subfamily of RING finger proteins [7–9]. The RBCC motif of KAP-1 interacts with the KRAB-A subdomain of KRAB-ZFPs and represses transcription when recruited to the promoter region [10].

We have been studying the glial cell line-derived neurotrophic factor (GDNF)-RET signaling pathway, which is essential for neural and renal organogenesis [11]. GDNF ligands bind to GDNF family receptor α1 (GFRα1) and activate the RET receptor tyrosine kinase. This ligand–receptor complex leads to activation of multiple downstream signaling pathways including RAS/ERK, PI3K/AKT, p38MAPK, and Rac/JNK pathways [11]. Disruption of GDNF-RET signaling in mice causes renal agenesis or severe dysgenesis and lack of enteric neurons [12–15], whereas gain-of-function mutations in RET cause constitutive activation of downstream signaling, resulting in the development of papillary thyroid carcinomas and multiple endocrine neoplasia type 2 [16,17].

In the study of GDNF-RET signaling, we carried out differential display analysis using the human neuroblastoma cell line TGW stimulated by GDNF. As a result, we identified the *bone marrow zinc finger 3* (*BMZF3*, *ZNF256*) gene which encodes a KRAB-ZFP as a novel GDNF-inducible gene [18]. The *BMZF3* transcript was induced 1 h after GDNF stimulation. A luciferase reporter assay showed that BMZF3 has transcriptional repressor activity that requires the KAP-1. Furthermore, cell proliferation was inhibited by siRNA-mediated *BMZF3* depletion. These findings suggest that BMZF3 is a transcriptional repressor induced by GDNF that plays a role in cell proliferation.

Materials and methods

Cell culture and reagents. TGW (human neuroblastoma), HeLa (human epitheloid carcinoma of cervix), and HEK293 (human fetal kidney) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transfection experiments, cells were transfected with expression vectors using FuGENE 6 (Roche).

Differential display analysis. Total RNAs were isolated from TGW cells treated with GDNF (100 ng/ml) for 2 h using an RNA purification kit (Qiagen). After treating the RNAs with RNase-free DNase I to eliminate contaminating genomic DNA, differential display-polymerase chain reaction (PCR) was performed using a Fluorescence Differential Display Kit (TaKaRa). The fluorescent products were resolved by electrophoresis on denaturing urea–4% polyacrylamide gels and visualized using FM-BIO II (TaKaRa). Differentially expressed bands were excised from the gels and amplified by PCR. The products were cloned into pGEM-T vector (Promega) and subjected to sequencing.

Northern blotting. Total RNAs from TGW cells were isolated using Trizol (Invitrogen) and separated on 1% agarose–formamide gels with formaldehyde and transferred onto Hybond-XL nylon membranes (Amersham Biosciences). Northern hybridization was performed using radiolabelled probes of *BMZF3* fragments using standard methods.

Quantitative real-time PCR. Real-time PCR was conducted using SYBR Green master mix (Applied Biosystems) with 0.5 μM gene-specific primers, and assays were performed with Applied Biosystems Model 7500. All data were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Vector constructs. cDNA fragments representing full-length BMZF3 (GenBank Accession Nos. AF067165 and BC001438), BMZF3 amino acid 1–98 (KRAB) and 99–627 (ΔKRAB) were placed into the vectors pEGFP-C2 or pEGFP-C3 (Clontech) in order to produce green fluorescent protein (GFP)-fused proteins (GFP-BMZF3, -KRAB, and -ΔKRAB, respectively). Construction of the expression vector pV5-HisC-GAL4DBD that contains the GAL4 DNA-binding domain (GAL4DBD) and a luciferase reporter vector containing a GAL4-binding sequence, CRE or SRE enhancer element and SV40 promoter (pGL3-GAL4-CRE-SV40 or pGL3-GAL4-SRE-SV40, respectively) were described previously [19]. cDNA fragments representing full-length BMZF3, BMZF3 amino acid 1–75 (KRAB), 70–627 (ΔKRAB), 229–627 (ZnF), and 1–238 (ΔZnF) were inserted in frame into pV5-HisC-GAL4DBD in order to express GAL4DBD-fused proteins (GAL4DBD-BMZF3, -KRAB, -ΔKRAB, -ZnF, and -ΔZnF, respectively).

Antibodies. Rabbit polyclonal anti-GFP antibody was purchased from MBL and the mouse monoclonal anti-GFP antibody was purchased from Nacalai Tesque. Rabbit polyclonal anti-KAP-1 antibody was purchased from Affinity Bioreagents.

Transient-transfection reporter assays. HEK293 cells grown in 24-well plates were co-transfected with the indicated amount of expression vector for GAL4DBD-fused protein, 200 ng of pGL3-GAL4-CRE-SV40 or pGL3-GAL4-SRE-SV40, and 50 ng of pRL-TK (Promega) using FuGENE 6. 48 h after transfection, cell lysates were prepared using the Dual-Luciferase Reporter Assay System (Promega) and luciferase activity was measured with a Lumicounter 700 (MicroTech Nition). Co-transfection of pRL-TK was used to normalize luciferase values.

siRNA transfection. siRNAs against *KAP-1* and *BMZF3* were purchased from Dhamacon and MBL, respectively. Their target sequences are; siKAP-1: GAACGAGGCCUUCGGUGACUU, siBMZF3-9: UUAUCCCUUGUGAACGUUCUGAUGUUU, siBMZF3-12: GGACACUUCUCAGUCCGUAACAGCCCU. Cells grown on a 60-mm culture dish were transfected with siRNA at a final concentration of 10 nM using Lipofectamine™ RNAiMAX (Invitrogen). The cells were seeded in 24-well or 96-well plates 24 h after transfection for use in the luciferase reporter assay or the cell proliferation assay.

Western blotting. Cells were lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 2% 2-mercaptoethanol, and 0.01% bromophenol blue) by sonication. After boiling, the lysates were subjected to SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with 5% bovine serum albumin, membranes were probed with the primary antibody, followed by incubation with the secondary antibody conjugated to horseradish peroxidase (Dako). After washing, antigen–antibody complexes were detected by the enhanced chemiluminescence system (ECL; GE Healthcare).

Immunoprecipitation. Cells were lysed in lysis buffer (20 mM Hepes, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.05% Tween 20, and 1 mM PMSF) by repeated freeze and thaw cycles. After centrifugation, supernatants were incubated with protein A–sepharose beads (Sigma) for 30 min to eliminate the non-specific binding of proteins to the beads. After brief centrifugation, supernatants were incubated with 2 μg of rabbit polyclonal anti-GFP antibody or normal rabbit IgG for 4 h, followed by an overnight incubation with protein A–sepharose beads. After washing the beads, the bound proteins were eluted by boiling in SDS sample buffer and subjected to Western blotting.

Cell proliferation assay. HeLa cells transfected with BMZF3 siRNA were seeded in 96-well plates at a density of 1000 cells/well 20 h after transfection. The cell proliferation assay was commenced 10 h after seeding using WST-1 Reagent (Roche). Absorbance was measured at a wavelength of 450 nm with a reference wavelength of 620 nm using a microplate reader (Tecan).

Results

Identification of BMZF3 as a GDNF-inducible gene

To identify GDNF-inducible genes, we performed differential display analysis using RNAs from the human

neuroblastoma cell line TGW expressing RET and GFRα1. As a consequence, we found a gene whose expression significantly increased after GDNF stimulation. Sequence analysis revealed that this gene was *BMZF3*, which encodes a KRAB-ZFP whose function had not yet been analyzed [18]. The predicted protein product of *BMZF3* is composed of 627 amino acids that contain a KRAB A domain in the N-terminal region and 15 C_2-H_2-type zinc finger motifs in the middle and C-terminal regions (Fig. 1A). Bioinformatic analysis showed that the *BMZF3* genomic locus resides on chromosome 19q13, a major cluster region of genes encoding ZFPs, and contains three exons (Fig. 1B). *BMZF3* induction in TGW cells by GDNF stimulation was confirmed by Northern blotting of a time course analysis and showed a rapid increase of the *BMZF3* transcript 1 h after GDNF stimulation (Fig. 1C). Furthermore, *BMZF3* induction was assessed by quantitative real-time PCR, which showed a 2.5-fold elevation of *BMZF3* transcripts 1 h after GDNF stimulation, when compared to non-stimulated cells (Fig. 1D), indicating that *BMZF3* is a novel GDNF-inducible gene.

Intracellular localization of BMZF3

To characterize the BMZF3 protein, we first analyzed the intracellular localization of BMZF3. Mammalian expression vectors for GFP-BMZF3, -KRAB and -ΔKRAB were constructed (Fig. 1E). HeLa cells were transfected with the vectors and the intracellular localization of the GST-fused BMZF3 proteins were determined 48 h after transfection. The GFP-BMZF3 was localized to the nucleus, whereas GFP alone distributed in both the nucleus and the cytoplasm (Fig. 1F). GFP-ΔKRAB was also localized in the nucleus, however GFP-KRAB was dispersed in both the nucleus and the cytoplasm in a similar manner to GFP alone. These results indicate that BMZF3 is a nuclear protein and that the KRAB domain is not required for nuclear localization of the BMZF3 protein.

BMZF3 is a transcriptional repressor

Since the domain structure of the BMZF3 protein suggested that it might function as a transcriptional repressor, we performed a luciferase reporter assay to assess the tran-

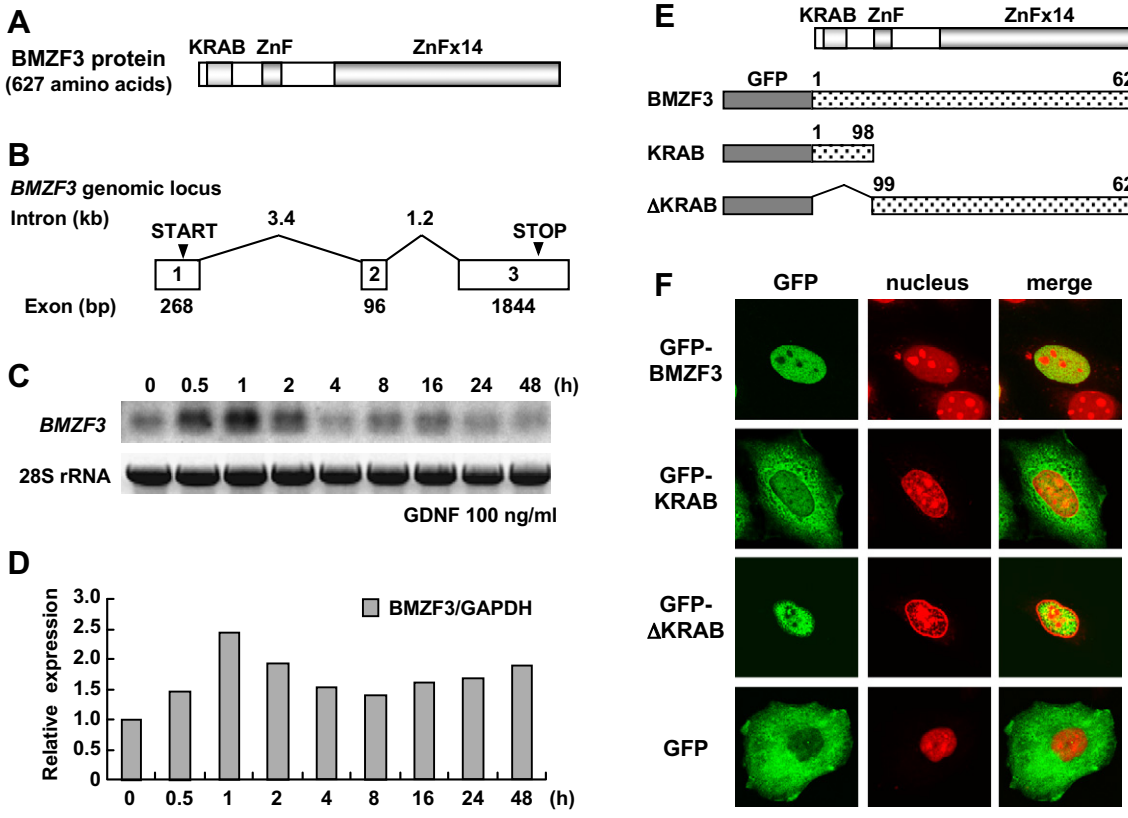

Fig. 1. Identification of *BMZF3* as a GDNF-inducible gene. (A) Domain structure of the BMZF3 protein. ZnF: zinc finger motif. (B) Exon–intron structure of the *BMZF3* genomic locus. (C,D) Induction of *BMZF3* transcript by GDNF stimulation. (C) TGW cells were stimulated by GDNF (100 ng/ml) for the indicated times, and their total RNAs were subjected to Northern blotting using a human *BMZF3* cDNA fragment as a probe (upper panel). 28S ribosomal RNAs are shown (lower panel). (D) Total RNAs from the TGW cells were subjected to quantitative real-time PCR to estimate the relative expression of *BMZF3* transcripts. Averages of three independent experiments are shown. (E,F) Intracellular localization of BMZF3. (E) GFP-fused BMZF3 proteins are illustrated. The amino acid numbers of the N- and C-terminal residues of each BMZF3 fragment are indicated. (F) Fluorescence microscopic images of the GFP-fused BMZF3 proteins in HeLa cells. Left, middle and right panels show localization of GFP-fused BMZF3, the nuclei stained with propidium iodide, and merged images, respectively.

scriptional activity of BMZF3. HEK293 cells were co-transfected with the expression vector for GAL4DBD-BMZF3 or GAL4DBD alone and the luciferase reporter vector (Fig. 2A), and luciferase activity in the cells was measured. As shown in Fig. 2B, luciferase activity was significantly suppressed by transfection of the GAL4DBD-BMZF3 vector in a dose-dependent manner, compared with transfection of the control GAL4DBD vector, indicating that BMZF3 suppressed transcription of the luciferase reporter gene.

Next, we determined the functional domain of BMZF3 required for transcriptional repression. HEK293 cells were co-transfected with expression vector for GAL4DBD-BMZF3, -KRAB, -ΔKRAB, -ZnF, -ΔZnF, or GAL4DBD

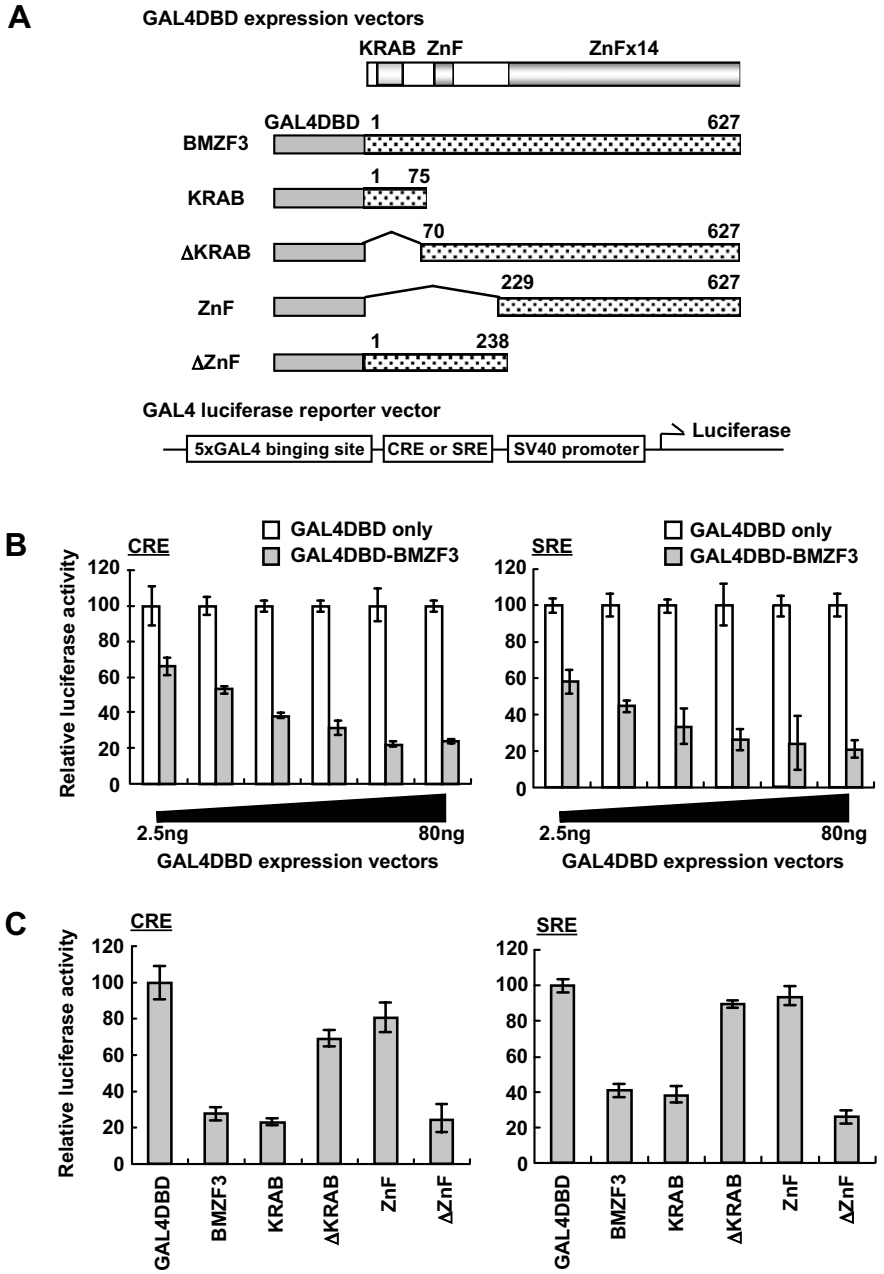

Fig. 2. BMZF3 possesses transcriptional repressor activity which requires the KRAB domain. (A) BMZF3 fragments fused with the GAL4 DNA-binding domain (GAL4DBD) are illustrated. The amino acid numbers of the N- and C-terminal residues of each BMZF3 fragment are indicated. The GAL4 luciferase reporter vectors are also illustrated. (B) BMZF3 suppresses luciferase activity. HEK293 cells were co-transfected with increasing amounts of GAL4DBD-BMZF3 or GAL4DBD expression vectors and GAL4 luciferase reporter vector; luciferase activity was determined 48 h after transfection. Luciferase activity of cells transfected with the GAL4DBD vector was defined as 100. (C) The KRAB domain is necessary for the transcriptional repressor activity of BMZF3. HEK293 cells were co-transfected with 100 ng of expression vectors for GAL4DBD-BMZF3, -KRAB, -ΔKRAB, -ZnF, -ΔZnF, or GAL4DBD and a GAL4 luciferase reporter vector; luciferase activity was determined 48 h after transfection. Luciferase activity in cells transfected with the GAL4DBD vector was defined as 100. Left and right graphs in (B) and (C) show results for the GAL4 luciferase reporter vector with CRE and SRE enhancers, respectively. Each luciferase value represents means ± standard deviation (SD) of three independent experiments.

alone and the luciferase reporter vector, and luciferase activity in the cells was assessed. As shown in Fig. 2C, transfection of the expression vectors for GAL4DBD-BMZF3, -KRAB, and -ΔZnF, which contained the KRAB domain, suppressed luciferase activity, whereas introduction of the expression vectors for GAL4DBD-ΔKRAB and -ZnF, which did not contain the KRAB domain, showed significantly less suppression of luciferase activity. These findings indicate that BMZF3 is a transcriptional repressor, whose activity is mediated by the KRAB domain.

The KAP-1 co-repressor is required for BMZF3-mediated transcriptional repression

Since it is proposed that KRAB-mediated gene silencing requires an interaction with KAP-1, we analyzed the ability of BMZF3 to interact with KAP-1. HEK293 cells were transfected with the expression vector for GFP, GFP-BMZF3, -KRAB or -ΔKRAB, and their cell lysates were subjected to immunoprecipitation using rabbit polyclonal anti-GFP antibody, followed by Western blotting with anti-KAP-1 antibody and mouse monoclonal anti-GFP antibody (Fig. 3A). Endogeneous KAP-1 co-immunoprecipitated with GFP-BMZF3 or GFP-KRAB, but not with GFP nor GFP-ΔKRAB, indicating that BMZF3 interacts with KAP-1 via the KRAB domain.

Next, we assessed the transcriptional repressor activity of BMZF3 in KAP-1-depleted HEK293 cells treated with KAP-1 siRNA. The level of endogeneous KAP-1 apparently decreased in cells transfected with KAP-1 siRNA compared with that of control cells (Fig. 3B). Then, the HEK293 cells treated with KAP-1 siRNA or control siRNA were used for luciferase reporter assay. As shown in Fig. 3C, the luciferase activity in cells treated with control siRNA was effectively suppressed by transfection of GAL4DBD-BMZF3 vector in a dose-dependent manner.

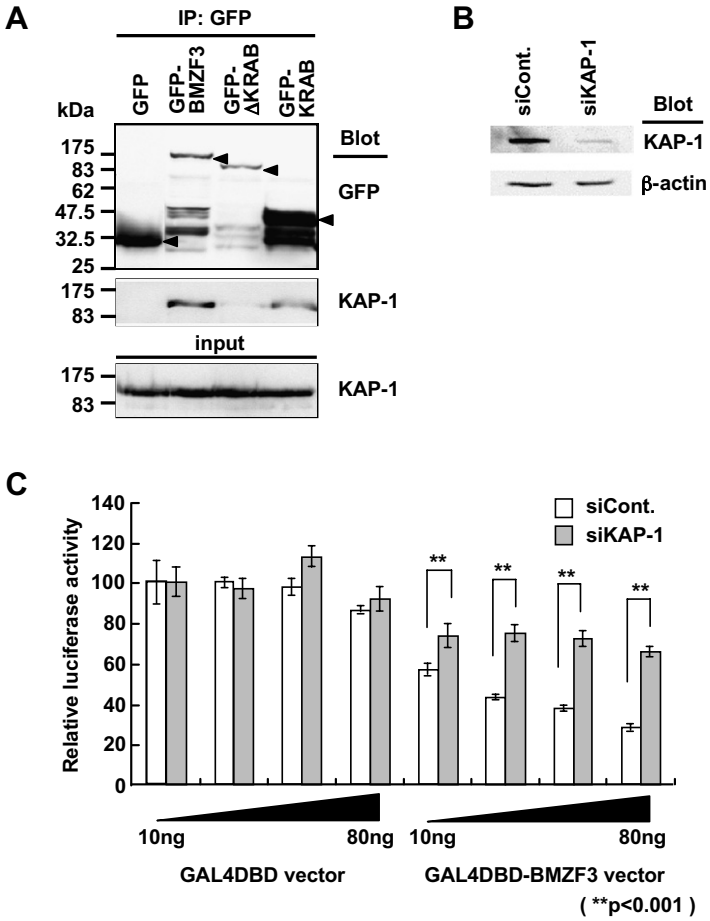

Fig. 3. KAP-1 is required for BMZF3-mediated transcriptional repression. (A) BMZF3 interacts with KAP-1. HEK293 cells were transfected with expression vector for GFP, GFP-BMZF3, -ΔKRAB, or -KRAB, and their cell lysates were subjected to immunoprecipitation with rabbit polyclonal anti-GFP antibody, followed by Western blotting with mouse monoclonal anti-GFP antibody or anti-KAP-1 antibody. Arrowheads indicate the GFP and GFP-fusion proteins. (B) siRNA-mediated knockdown of KAP-1. HEK293 cells were transfected with KAP-1 siRNA (siKAP-1) or control siRNA (siCont.), and their lysates were subjected to Western blotting with anti-KAP-1 antibody (upper panel) and anti-β-actin antibody (lower panel). (C) KAP-1 is required for the transcriptional repressor activity of BMZF3. HEK293 cells were transfected with KAP-1 or control siRNA and then co-transfected with increasing amounts of GAL4DBD or GAL4DBD-BMZF3 expression vector and GAL4 luciferase reporter vector with SRE enhancer. Luciferase activity was determined 48 h after transfection. Luciferase activity in cells transfected with 10 ng of the GAL4DBD vector was defined as 100. Each luciferase value represents means ± SD of three independent experiments.

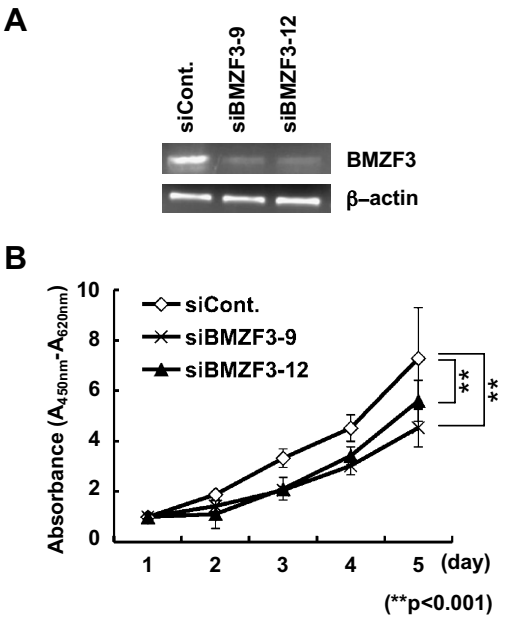

Fig. 4. BMZF3 depletion inhibits cell proliferation. (A) siRNA-mediated knockdown of BMZF3. HeLa cells were transfected with BMZF3 siRNA (siBMZF3-9, -12) or control siRNA (siCont.), and total RNA was extracted 72 h after transfection. *BMZF3* and *β-actin* expression was assessed by RT-PCR. (B) Proliferation analysis of cells treated with BMZF3 and control siRNAs. HeLa cells transfected with BMZF3 or control siRNA were seeded onto 96-well plates 20 h after transfection and the cell proliferation assay commenced 10 h after seeding. The absorbance value for day 1 was defined as 1.0. The means ± SD of three independent experiments is shown.

By contrast, suppression of the luciferase activity was significantly ameliorated in cells treated with KAP-1 siRNA, indicating that KAP-1 is necessary for effective transcriptional repression by BMZF3.

Knockdown of BMZF3 impairs cell proliferation

To assess the significance of BMZF3 in cell biology, we checked whether knockdown of *BMZF3* affected cell proliferation. HeLa cells transfected with *BMZF3* siRNA, which showed a significant decrease in *BMZF3* transcripts, were analyzed for cell proliferation (Fig. 4A). As shown in Fig. 4B, proliferation of the *BMZF3*-knockdown cells was significantly inhibited compared with that of control cells, suggesting that BMZF3 is required for cell proliferation.

Discussion

KRAB-ZFPs represent one of the largest family of transcription factors, which are involved in cellular activities such as signaling and cell proliferation. Recently, new members of the KRAB-ZFPs have been identified and their roles in cellular processes have been analyzed as well as their transcriptional repressor activities.

BMZF3 was originally identified in a study that attempted to find zinc finger genes expressed in the hematopoietic system by homologous PCR-based cloning of Krüppel-like gene family [18]; in this report, tissue expression of *BMZF3* was analyzed by semi-quantitative RT-PCR, however, characterization of the *BMZF3* gene and protein function have not yet been elucidated. In the present study, we identified the *BMZF3* gene as a novel GDNF-inducible gene by differential display analysis using TGW cells with or without GDNF stimulation. We show that BMZF3 is a transcriptional repressor whose activity requires the KAP-1 co-repressor. GDNF is a ligand of the RET receptor tyrosine kinase, and signaling via the GDNF-RET pathway is essential for renal organogenesis and development of the enteric nervous system. Previously, we identified several GDNF-inducible genes that encode transcription factors by differential display analysis, including *c-FOS*, *CREM*, and *GDNF-inducible zinc finger protein 1* (*GZF1*) [20]. Among these genes, *GZF1* encodes a BTB/POZ (broad complex, tramtrack, and bric-a-brac)/(pox virus and zinc finger) domain-containing ZFP, and possesses transcriptional repressor activity. We found that the *GZF1* transcript increased 1 h after GDNF stimulation, and is required for renal branching morphogenesis and cell proliferation [20,21]. *BMZF3* is also a GDNF-inducible gene and is required for cell proliferation as observed for GZF1. Therefore, there is a possibility that BMZF3 plays a role in cell functions or organogenesis under the control of GDNF-RET signaling.

To analyze biological activities of BMZF3, we tried to establish stable cell lines overexpressing BMZF3 using HEK293 cells and HeLa cells. However, it was unsuccessful. A possible reason why we could not obtain stable cell lines overexpressing BMZF3 is that an excess amount of exogenous BMZF3 may impair cell homeostasis or inhibit endogeneous BMZF3 function. It was reported that transcriptional repression by KOX1, a KRAB-ZFP, was abrogated by overexpression of wild-type KOX1 [7]. Considering these findings, it may be that physiological levels of the BMZF3 protein are required for its transcription factor activity and for maintaining cellular activities such as cell proliferation.

To further elucidate the roles of BMZF3 in GDNF-RET signaling, it is necessary to identify target genes of BMZF3-mediated transcriptional repression. GDNF stimulation upregulates the expression of *BMZF3*, which may repress transcription of other genes. Further analysis, including microarray screening is necessary to clarify the role of BMZF3 in GDNF-RET signaling.

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