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Promoter analysis and transcriptional regulation of human carbonic anhydrase VIII gene in a MERRF disease cell model

Che-Min Lo[a], Yi-Shing Ma[b,c], Yau-Huei Wei[b,c], Benjamin Y.T. Hsieh[d], Mingli Hsieh[a,e,*]

[a] Department of Life Science, Tunghai University, Taichung, Taiwan, ROC
[b] Department of Biochemistry and Molecular Biology, School of Life Sciences, National Yang-Ming University, Taipei, Taiwan, ROC
[c] Center for Mitochondrial Medicine and Free Radical Research, Changhua Christian Hospital, Changhua, Taiwan, ROC
[d] Department of Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC
[e] Life Science Research Center, Tunghai University, Taichung, Taiwan, ROC

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ABSTRACT

Myoclonic epilepsy with ragged-red fibers (MERRF) is a maternally inherited mitochondrial neuromuscular disease. We previously reported a significant decrease of mRNA and protein levels of nuclear DNA-encoded carbonic anhydrase VIII (CA8) in MERRF cybrids harboring A8344G mutation in mitochondrial DNA (mtDNA). In this study, we established a reporter construct of luciferase gene-carrying *hCA8* promoter containing several putative transcription factor-binding sites, including GC-box, AP-2 and TATA-binding element in the 5'flanking region of the *hCA8* gene. Using a series of mutated *hCA8* promoter constructs, we demonstrated that a proximal GC-box, recognized by Sp1 and other Sp family members, may be a key cis-element functioning at the promoter. Additionally, a significant increase of the *hCA8* promoter activity was observed in the wild-type and mutant cybrids with over-expression of eGFP-Sp1, but no detectable increase in the CA8 protein expression. In contrast, over-expression of Flag-Sp1 and Flag-Sp4 significantly increased the *hCA8* promoter activity as well as endogenous CA8 protein expression in neuron-like HEK-293 T cells. However, down-regulation of Sp1, but not Sp4, in 293 T cells revealed a significant reduction of CA8 expression, suggesting that Sp1 is a predominant transcription factor for regulation of CA8 activity. Furthermore, our data indicate that chromatin structure may be involved in the expression of *hCA8* gene in MERRF cybrids. Taken together, these results suggest that Sp1 transactivates *hCA8* gene through the proximal GC box element in the promoter region. The key modulator-responsive factor to the mtDNA mutation and how it may affect nuclear *hCA8* gene transcription need further investigations.

1. Introduction

MERRF (Myoclonic Epilepsy with Ragged-Red Fibers) syndrome is a maternally inherited encephalomyopathy, with the clinical symptoms of myoclonus epilepsy, generalized seizure, cerebellar ataxia, and muscle weakness [1,2]. The molecular basis of MERRF syndrome is most commonly due to the A-to-G transition at position 8344 of mtDNA in the gene of $tRNA^{Lys}$ [3,4]. This point substitution on mitochondrial $tRNA^{Lys}$ gene leads to premature translation termination and production of truncated polypeptides in mitochondria. Hence, MERRF A8344G mutation gives rise to the dysfunction of Complexes I and IV, which encompass most of the mtDNA-encoded proteins, and thus also impairs mitochondrial respiratory function [5–11]. The heteroplasmy of pathogenic mtDNA mutation is varied in MERRF patients, and the clinical manifestation is positively correlated with the percentage of mutant mtDNA in affected tissues. The 13 functional α-carbonic anhydrase (α-CA) isozymes, which physiologically catalyze a reversible hydration of carbon dioxide into carbonate and proton functioning through the necessary Zn^{2+}-coordinating residues in the active site cavity [12], belong to α-CA family in mammals. In addition, 3 acatalytic isozymes with impaired metalloenzymatic activity are generally called CA-related proteins (CARPs) without the aforementioned CA function. CARP8, hereafter designated as CA8, is one of the acatalytic isozymes, because of a substitution of arginine for active zinc-liganded histidine in the

The abbreviations used are: AP-2, activator protein-2; CA8, carbonic anhydrase 8; CARPs, CA-related proteins; DNA, deoxyribonucleic acid; EGFP, enhanced green fluorescent protein; HEK cells, human embryonic kidney cells; HDAC, histone deacetylase; IP3, inositol trisphosphate; IP_3R1, inositol 1,4,5 trisphosphate receptor type 1; MERRF, myoclonic epilepsy with ragged red fibers; mtDNA, mitochondrial DNA; PTMs, post-translational modifications; RTG, retrograde; shRNA, short hairpin RNA; Sp1, specificity protein 1; TFBSs, transcription factor-binding sites; TSA, trichostatin A; NS, not significant

* Corresponding author. Department of Life Science, Laboratory of Molecular Medicine, Tunghai University, BS205 Basic Science Building, No.1727, Sec.4, Taiwan Boulevard, Taichung 40704, Taiwan, ROC
E-mail address: mhsieh@thu.edu.tw (M. Hsieh).

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active site binding pocket [13,14]. Car8, analogue to human CA8 protein, was firstly described in adult mouse brain with abolishing CA function. The only reported biochemical role of CA8 is to inhibit the binding of secondary messenger inositol 1, 4, 5-trisphosphate (IP_3) toward IP_3 receptor-1 (IP_3R1) on endoplasmic reticulum [15]. CA8 has been found to be highly expressed in cerebellar Purkinje cells and some parts of CNS [14,16,17], suggesting its pivotal roles in neuronal functions. A 19-bp deletion in murine *Car8* gene, which was found in *Waddles* mice that exhibited distinctive motor dysfunction and ataxia, resulted in an almost undetectable CA8 expression [18]. In addition, the S100P and G162R point mutations of hCA8 were reported in patients with ataxia, mental retardation, and disequilibrium with predisposition to quadrupedal gait [19], suggesting that CA8 abnormality may be predisposed to motor dysfunction and ataxic disorders in the human. We have previously shown that CA8 overexpression desensitizes neuronal cells to staurosporine (STS) induced apoptotic stress and increases cell migration ability in neuronal cells [20]. Additionally, it has been found that the oncogenic property of CA8 promotes tumor growth, glucose uptake and anticancer drug resistance in human osteosarcoma cells [21]. These observations suggest that CA8 may play important roles in neuronal protection, calcium homeostasis, glucose uptake and tumor oncogenicity.

MERRF is a prototypical neuromuscular disease characterized by severe cerebellar ataxia, and the symptom is also found in the neurodegenerative diseases caused by CA8 substitution mutations [19,22]. We accordingly speculated whether CA8 correlates with MERRF syndrome. Surprisingly, we observed a dramatic decrease in nuclear *hCA8* expression at both transcriptional and translational levels in MERRF cybrid cells harboring A8344G mtDNA mutation [23]. Moreover, CA8 molecule displayed a protective function in the mutant cybrids to desensitize cell apoptosis induced by STS. The intriguing cross-organelle cellular signaling from mitochondria to nucleus may offer a new perspective on the pathogenicity of MERRF syndrome. Therefore, we have attempted to elucidate the mechanism underlying the down-regulation of CA8 in a cell model of MERRF syndrome by investigating the transcriptional control of nuclear *hCA8* gene.

In the present study, we defined the *hCA8* promoter region with putative GC box-binding elements and activator protein-2 (AP-2)-binding sites analyzed by bioinformatics methods. In addition, the established full-length *hCA8* promoter-reporter construct showed a significantly decreased luciferase activity in mutant cybrids as compared with that in normal cybrids, indicating that the reporter construct is functional. By introducing deletions and motif disruptions on the *hCA8* promoter, we found that the major responsive element is located on the proximal GC box-binding site. Our results suggest that Sp1, a characteristic GC box-associating transcription factor, may play a predominant role in the regulation of *hCA8* gene activation.

2. Materials and methods

2.1. Cell culture and reagents

Cybrids that had been established were maintained as previously described [23]. Human embryonic kidney (HEK) 293 T cells from ATCC were grown in Minimum Essential Medium (SH40015-11, Hyclone, Logan, UT, USA) supplemented with 1% non-essential amino acid, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% FBS, 100 μg/ml pyruvate. Cells were cultured in a 95% air, 5% CO_2 humidified incubator. Cells were trypsinized and plated on 35 mm dishes before transfection by using PolyJet™ *In Vitro* DNA Transfection Reagent (SignaGen Laboratories, Gaithersburg, MD, USA). pEGFP-Sp1 and its empty vector were kindly provided by Dr. Jan-Jong Hung (Institute of Bioinformatics and Biosignal Transduction, National Cheng Kung University, Tainan, Taiwan, R.O.C). pCMV-DYKDDDDK(Flag)-N empty vector (#635688) was purchased from Clontech. Sp3 expression vector and its empty vector, pN3-Sp3fl and pN3, were kindly provided by Dr. G. Suske (Institute of Molecular Biology and Tumor Research, Phillipps-University Marburg, Marburg, Germany). Anti-CA8, anti-Sp1, anti-Sp3, anti-Sp4 and anti-Hsp27 antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-DYKDDDDK antibody was purchased from Clontech. Histone H3 XP Rabbit mAb and Normal Rabbit IgG were purchased from Cell Signaling Technology, Inc. Mouse monoclonal anti-β-actin and anti-α-tubulin antibodies were purchased from Novus Biologicals.

2.2. Luciferase reporter and expression constructs

The pBluescriptII (KS +) vector containing a fragment of luciferase reporter gene (pBSII(KS +)-Luc) was kindly provided from Dr. Linda Boxer (Department of Medicine, Stanford University School of Medicine, Stanford, California, USA). The human CA8 promoter reporter construct was established by PCR amplification of genomic DNA with *Pst*I- and *Hind*III-anchored primers and the 993 bp digested amplicon was subcloned into the *Pst*I and *Hind*III sites of pBSII(KS +)-Luc vector. This construct was named full-length *hCA8* promoter (FL-P) reporter construct (− 971/ + 22, + 1 denotes the translation start site of *hCA8* gene). Two further 5′ truncated reporter constructs, − 417/ + 22 (Deletion-1 promoter, D1-P) and − 302/ + 22 (Deletion-2 promoter, D2-P), were generated by inverse PCR of FL-P with two forward primers and one shared reverse primer. To introduce mutations to the AP-2 and Sp1 binding motifs, the following primer sets were prepared, and site-directed mutagenesis PCR was performed using FL-P as the template. The Sp1 and Sp4 cDNA fragments were PCR amplified from human cDNA library using *Hind*III- and *Eco*RI-anchored Sp1 primers and *Hind*III- and *Xho*I-anchored Sp4 primers, respectively. The resultant

Table 1
The list of primers used in PCR amplification for plasmid constructions.

Amplicon	primer	Sequence 5'→3′	Annotation
hCA8 promoter reporter construct	Forward	ACAGCCAGGTGAGCCTGCAG	Bold denotes PstI
	Reverse	CGATAAGCTTCGATGAAGCTCAGGTCCGCC	Bold denotes HindIII
Deletion 1 of *hCA8* promoter	Forward	GGCGGGGATGCGCCGAGGTTTC	
	Reverse	CGCTGCTGCAGCCCGGGGGA	
Deletion 1 of *hCA8* promoter	Forward	CCAGACACGCCCCGGTCGGG	
	Reverse	CGCTGCTGCAGCCCGGGGGA	
Mutated proximal AP-2 site	M-AP-2	GCATTCTAACTGTCGGCTCaaaTGGCCCCAACGGCC	Lowercase denotes the mutated sequence
Mutated proximal GC box	M-GC box	GCCAGAGAGAGAGAAGGGaatGGATCCTCGCGTACCAGACACG	Lowercase denotes the mutated sequence
human Spl cDNA	Forward	TTCAACAAGCTTATGAGCGACCAAGATCACTCCATGG	Bold denotes HindIII
	Reverse	CTACATGAATTCTCAGAAGCCATTGCCACTGATATTAATGGAC	Bold denotes EcoRI
human Sp4 cDNA	Forward	TTCAACAAGCTTATGAGCGATCAGAAGAAGGAGGAGGAG	Bold denotes HindIII
	Reverse	CTACATCTCGAGTCAGAATTCTTCCATGTTGGTTGAAACATTGGG	Bold denotes XhoI
ChIP-*hCA8* promoter	Forward	AGGCGCATTCTAACTGTCGGCTCC	
	Reverse	TGCGTTCGGACCGAGTGTTCC	

products were endonuclease-digested and then ligated into pCMV-Flag-N vector to generate pFlag-Sp1 and pFlag-Sp4 expression plasmids, respectively. The list of primers used in this study is shown in Table 1.

2.3. Transient transfection and luciferase reporter assays

For reporter assay, 2.5×10^5 cells/well were seeded on 35 mm dishes. Cells were transfected the next day with 600 ng of each of the indicated luciferase reporter plus 400 ng of β-galactosidase expression plasmid, pcDNA3.1/myc-His/LacZ (Invitrogen), for total 1 μg plasmid DNA. For overexpression assay, cells were co-transfected with 100 ng full-length *hCA8* promoter reporter construct, 300 ng expression constructs alone or together, and 300 ng β-galactosidase expression plasmid. The total amount of transfected DNA was kept equal by adding pCMV-Flag-N vector. After 48hr expression, luciferase activities were measured by GloMax-20/20 Single-Tube Luminometer (Promega), and β-galactosidase activities were measured at Abs 420 nm. Data were normalized by the activity of β-galactosidase to account for transfection efficiency.

2.4. Western blot analyses

In brief, cell lysates containing 20 μg or 30 μg of proteins were loaded onto 10 or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Resolved proteins were electrophoretically transferred onto 0.2 μm PVDF membranes. After blocking the membrane with 5% nonfat milk in 1× TTBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl. 0.1% tween-20) buffer for 1 h at room temperature, the antibody-binding reactions were performed in the same buffer supplemented with 1% nonfat milk at 4 °C overnight or at room temperature for 1 h in order for secondary antibodies to couple to horseradish peroxides (HRP)-conjugated anti-mouse, anti-rabbit or anti-goat IgG. Pre-stained high molecular weight markers were included in this study. Signals were visualized with the enhanced chemiluminescence. A positive signal was detected with an ECL substrate (Millipore). Densitometric quantification was performed using Multi gauge version 2.2 (Fuji photo film co.) and normalized to the corresponding β-actin or α-tubulin levels.

2.5. Small-hairpin RNA (shRNA) -based specific gene knockdown

pLKO.1 and pLKO_TRC005 plasmids expressing shRNA for the knockdown of Sp1 and Sp4 were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Approximately 2.5×10^5 cybrids with or without A8344G mtDNA mutation and HEK-293 T cells were seeded in 35 mm dishes, and were transfected the next day respectively with Sp1-shRNA (TRCN0000020448), Sp4-shRNA (TRCN0000431990), and Luciferase-shRNA (pLKO.1, TRCN0000072249 and pLKO_TRC005, TRCN0000231719) as a negative control. After 24 h expression, transfected cells were selected for 7 days using 1 μg/ml Puromycin for the cybrids and 3 μg/ml Puromycin for HEK-293 T cells. Stable cells expressing shRNA plasmids were validated by Western blot analysis.

2.6. Statistical analysis

All values are expressed as mean ± SEM. We assessed statistical significance using *t*-test and used ANOVA when multiple samples were compared. A *P*-value of less than 0.05 is considered to be significant.

3. Results

3.1. Representative human CA8 promoter-reporter construct reveals impaired transcriptional activity in mutant cybrids

In a previous study, we found a decrease in the CA8 expression at the mRNA and protein levels in cybrids harboring A8344G mutation of mtDNA [23], which suggests impaired nuclear CA8 gene transcription in the presence of the mtDNA mutation. In order to investigate whether there is a differential transcriptional activity of *hCA8* gene between D5-1 (wild-type) and C5 (MERRF mutant) cybrids, a full-length *hCA8* promoter-reporter construct was established (Fig. 1). We first searched 5-kb upstream of 5′ flanking region of the *hCA8* gene from the NCBI (National Center for Biotechnology Information) human genome database. The sequence was then analyzed for the putative transcription factor-binding sites (TFBSs) by using the PROMOTER SCAN program [24]. Several calculated possible TFBSs were located in the 1-kb region upstream of ATG site (Fig. 1A). Besides TATA-binding element, we found three GC boxes and two activator protein-2 (AP-2)-binding sites on the *hCA8* promoter. To construct the *hCA8* promoter reporter system, the promoter fragment was amplified from human genomic DNA and then subcloned into a luciferase gene-carrying vector (Fig. 1B). The reporter construct and its vehicle control were sequence-verified and transfected into the wild-type and mutant cybrids, and the luciferase assay showed a significantly decreased activity in C5, compared with the D5-1 cybrid (Fig. 1C), indicating that the *hCA8* promoter-reporter construct was functional and representative of the impaired transcriptional activity in the cell model of MERRF syndrome.

3.2. Proximal GC box is the key cis-acting element functioning on the hCA8 promoter region in cybrids

We next examined which the key cis-element is functioning in the *hCA8* promoter region. To this end, we established two promoter deletion mutants and then transfected them into the D5-1 cybrid to assay for luciferase activity. As the data shown in Fig. 2A, the 555 bp-deleted *hCA8* promoter (D1-P, −417/+22) acquired the promoter activity similar to the full-length *hCA8* promoter (FL-P, −971/+22). Moreover, the promoter was further deleted to become TATA-binding element only (D2-P, −302/+22), and the promoter activity showed a 3.5-fold decrease to near the vehicle control, as compared with that of the FL-P. Similar results were also obtained in C5 cybrids (Fig. 2B). These data suggest that the functional promoter activity was responsive to the region between −417 and −302 on the *hCA8* promoter, which contains one AP-2 and one GC box-binding sites. Next, by introducing motif disruptions of these two putative transcription factor-binding elements, our analysis revealed that the proximal AP-2 site was not the key cis-element, evidenced by no detectable change of luciferase activity in cells transfected with mutated AP-2 site (M-AP-2), compared with the wild-type *hCA8* promoter. On the other hand, the motif-disrupted GC box site (M-GC box) showed a 10-fold decrease in the promoter activity (Fig. 3), indicating that the proximal GC box-binding motif is a prerequisite to the *hCA8* promoter activity.

3.3. Sp1 and Sp4 are involved in the control of hCA8 gene expression

Specificity protein 1 (Sp1), so-called Krüppel-like factor [25], and other Sp family members are the well-known GC box-associating transcription factors. Sp1, Sp3, and Sp4 in the Sp family evidently acquire higher affinity to recognize conserved GGGCGG sequence, same as the proximal GC box on the *hCA8* promoter (Fig. 1A). In addition, Sp1 has been shown to regulate the expression of thousands of genes necessary for cellular processes, and act as a transactivator alone or with other recruited co-activators to upregulate the expression of some specific genes [26–28]. Thereby, we next investigated the endogenous expression of Sp1 in the wild-type and mutant cybrids. The data showed no detectable difference of the Sp1 protein levels between D5-1 and C5 cybrids, although a manifest decrease of CA8 expression was observed in the cybrids harboring the A8344G mutation of mtDNA (Fig. 4A). Additionally, we also examined the endogenous protein expression of Sp3 and Sp4. Again, the results showed no altered expression of these two Sp family members between the wild-type and mutant cybrids (Fig. 4B and C). To examine the role of Sp1 in regulating *hCA8* gene transcription, we performed luciferase reporter assay by transfecting

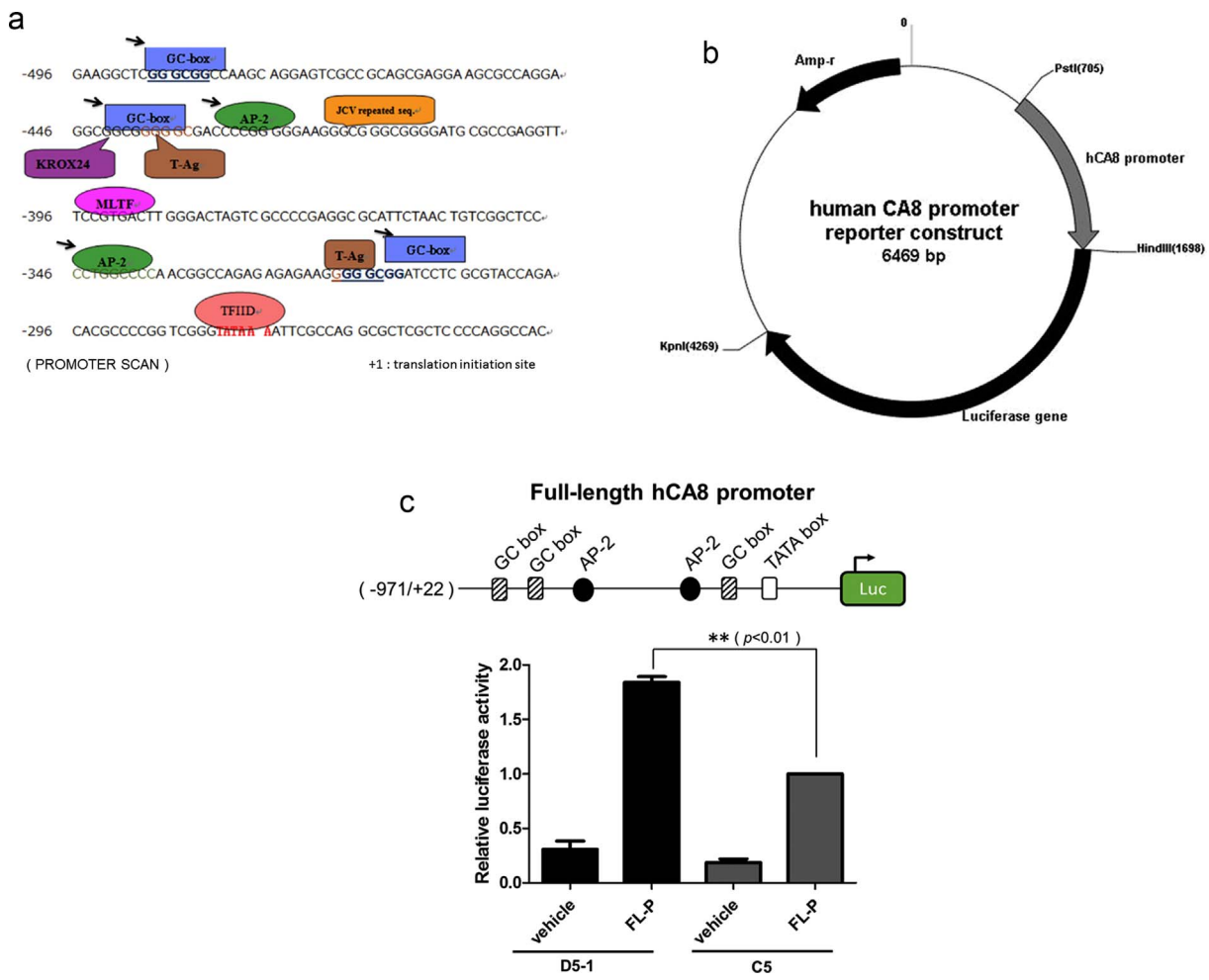


Fig. 1. The human CA8 promoter reporter construct represented a decreased transcriptional activity in mutant cybrids. (A) Nucleotide sequence of the 5′ flanking region of human *CA8* gene analyzed by promoter-scanning program. +1 represents A of ATG start codon. Arrowhead indicates the mammalian transcription factors (TFs). (B) Schematic representation of luciferase gene-carrying human CA8 promoter reporter construct. (C) Reporter activity of *hCA8* gene promoter in D5-1 and C5 cybrids. The 993 bp full-length (FL) putative *hCA8* promoter region (−971/+22) containing GC-box (twill box), AP-2 (solid circle), and TATA-binding element (hollow circle) was amplified by PCR and shown in the upper panel. FL-hCA8-P or empty vector were co-transfected with β-galactosidase expression vectors into cybrids. Luciferase activity was normalized to β-galactosidase activity and expressed as fold increase over the vehicle control. Data are the mean values ± SEM of at least 3 independent experiments. $^{**}P < 0.01$.

the FL-*hCA8* promoter construct alone or with increasing amounts of pEGFP-Sp1 expression vectors into the cybrids. The results showed a significant increase of the *hCA8* promoter activity with increasing eGFP-Sp1 expression, suggesting that the *hCA8* transcriptional activity was responsive to Sp1 in both wild-type and mutant cybrids (Fig. 4D). Furthermore, we overexpressed eGFP-Sp1 and then examined its effects on the endogenous CA8 expression level. Surprisingly, we did not observe any significant change of the expression of CA8 in the MERRF cell model (Fig. 4E). To eliminate the possible steric effect due to the fusion of the bulky eGFP, we then constructed smaller flag-tagged (1-KDa) Sp1 expression plasmids. For comparison, we also constructed flag-tagged Sp4 expression plasmids as well (see “Materials and Methods”). However, overexpression of pFlag-Sp1 and pFlag-Sp4 in the cybrids still failed to alter the expression level of endogenous CA8 (Fig. 5). Meanwhile, to ensure the overexpression of Flag-Sp1 and Sp4 were functionally effective in C5 and D5-1 cells, we also examined the expression level of B-cell lymphoma 2 (Bcl-2) gene, which is known to be regulated by Sp family proteins [29]. Our results showed that the expression levels of Bcl-2 were definitely increased in cybrids with overexpression of Flag-Sp1 and Sp4 (Fig. 5), demonstrating that Sp1 and Sp4 were functionally effective in fusion protein forms.

Nevertheless, it was noted that the exogenous Sp1 protein expression was relatively weak in the cybrids, which may be the reason for the undetectable effect of Sp1 on *hCA8* gene activation. Therefore, we also chose the HEK-293 T (abbreviated as 293 T) cell line, which is easily transfected and has many neuronal progenitor properties, to study the transcriptional regulation of *hCA8* gene. We first examined whether the proximal GC box-binding element is also necessary for the *hCA8* promoter activity in HEK-293 T cells. The data showed a significant decrease of the promoter activity accompanying with M-GC box, compared with the wild-type FL-P (Fig. 6A). Additionally, we found that the fold of reduction of the activity of proximal GC box-mutated promoter in 293 T cells was more dramatic than that in the cybrids (Fig. 3), suggesting that *hCA8* promoter was highly responsive to GC box-binding protein in 293 T cells. We thereby studied the transcriptional control of *hCA8* gene in this cell model. To confirm that Sp1 is involved in regulating CA8 expression in 293 T cells, the effects of different recombinant Sp1 proteins were assessed. Interestingly, our analysis revealed that overexpressing smaller flag- or HA-tagged (1-kDa) Sp1 proteins significantly elevated endogenous CA8 protein levels (Fig. 6B and C). In contrast, exogenous expression of larger eGFP-tagged (27-KDa) Sp1 did not show any detectable increase of the CA8 protein expression (Fig. 6B). Meanwhile, to understand whether Sp3 and Sp4, which also recognize the same GGGCGG box as Sp1, have any impact on *hCA8* gene expression in 293 T cells, we then transiently transfected pN3-Sp3fl, full-length Sp3 expression plasmids [30], and pFlag-Sp4 with the corresponding empty vectors into 293 T cells. Our data showed a statistically significant increase of CA8 protein under exogenous Sp4

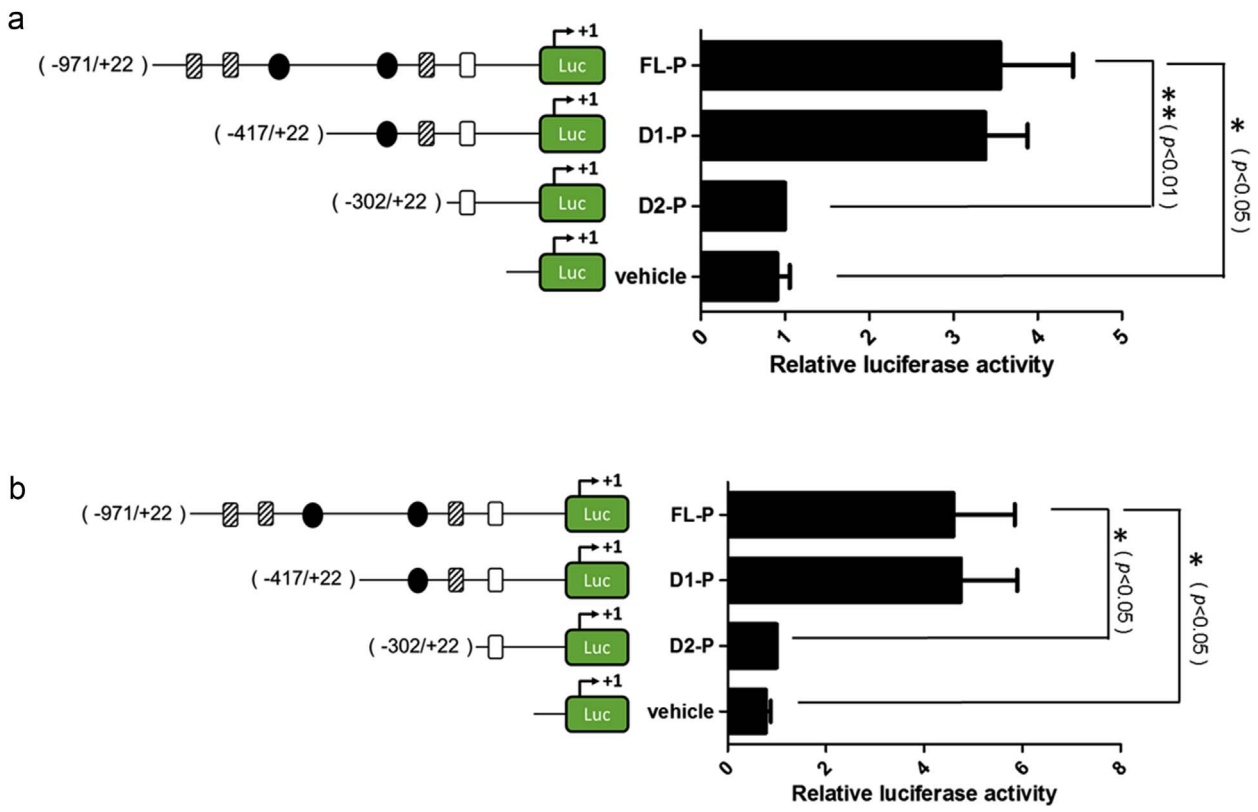


Fig. 2. Identification of *hCA8* minimal promoter region in wild-type and mutant cybrids.
Deletion mutants or empty vector were co-transfected with β-galactosidase expression vectors into wild-type D5-1 (A) and mutant C5 (B) cybrids. On the left, schematic representation of deletion mutants of *hCA8* promoter. Numbers indicate the relative positions with respect to the ATG start codon. Besides FL-P, D1-P (deleted promoter 1) is in the range between −471 and + 22, and D2-P (deleted promoter 2) is between −302 and + 22. Luciferase activity was normalized to β-galactosidase activity and expressed as fold increase over the vehicle control. Data are the mean values ± SEM of at least 3 independent experiments. $*P < 0.05$. $**P < 0.01$.

expression (Fig. 6D). However, we failed to detect a significant difference of CA8 expression between cells with and without Sp3 overexpression (Fig. 6E). Meanwhile, to avoid false positive results due to heavy overexpression of Sp1 and Sp4 in 293 T cells, we performed a dosing experiment by using either 0.5 or 2 μg of Sp protein expression plasmids for the transfection assay. As shown in Fig. 6F, a clear increase of CA8 expression was detected with using a small amount of plasmids in the assay. Taken together, our results suggest that both transcription factors Sp1 and Sp4 may be involved in activating *hCA8* gene expression *in vitro*.

3.4. Sp1 predominantly transactivates hCA8 gene expression functioning by binding to the proximal GC box on the promoter

Sp1 has the characteristics of cooperatively interplaying with other Sp family members to co-activate specific gene expression [31,32]. To address the relationship between Sp1 and Sp4 in *hCA8* gene regulation, we examined the effects of co-expressing Sp1 and Sp4 on the *hCA8* promoter activity in 293 T cells. The data showed a significant increase of *hCA8* promoter activity with the transfection of Sp1 or Sp4 expression vector alone, compared with that of the empty vector in 293 T

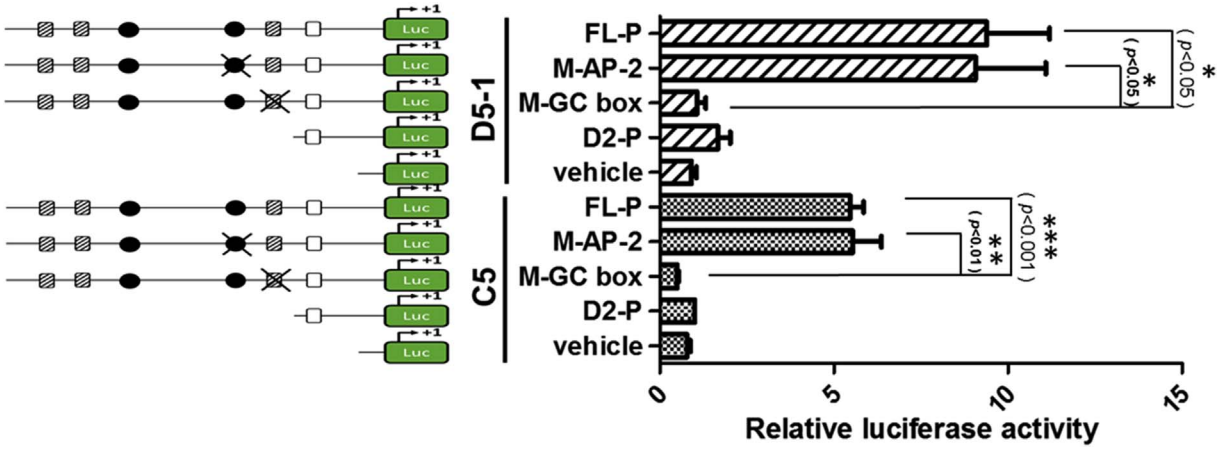


Fig. 3. GC-box-binding protein is involved in the activation of *hCA8* gene in cybrids. Motif-disrupted mutants (M-GC box and M-AP-2) or empty vector were co-transfected with β-galactosidase expression vectors into D5-1 (twill bars) and C5 (lozenge bars) cybrids. Luciferase activity was normalized to β-galactosidase activity and expressed as fold increase over the vehicle control. Cross sign represents the mutation of TFBS. M-GC box represents mutation of GC box-binding site. M-AP-2 represents mutation of AP-2-binding site. Data are the mean values ± SEM of at least 3 independent experiments. $*P < 0.05$. $**P < 0.01$. $***P < 0.001$.

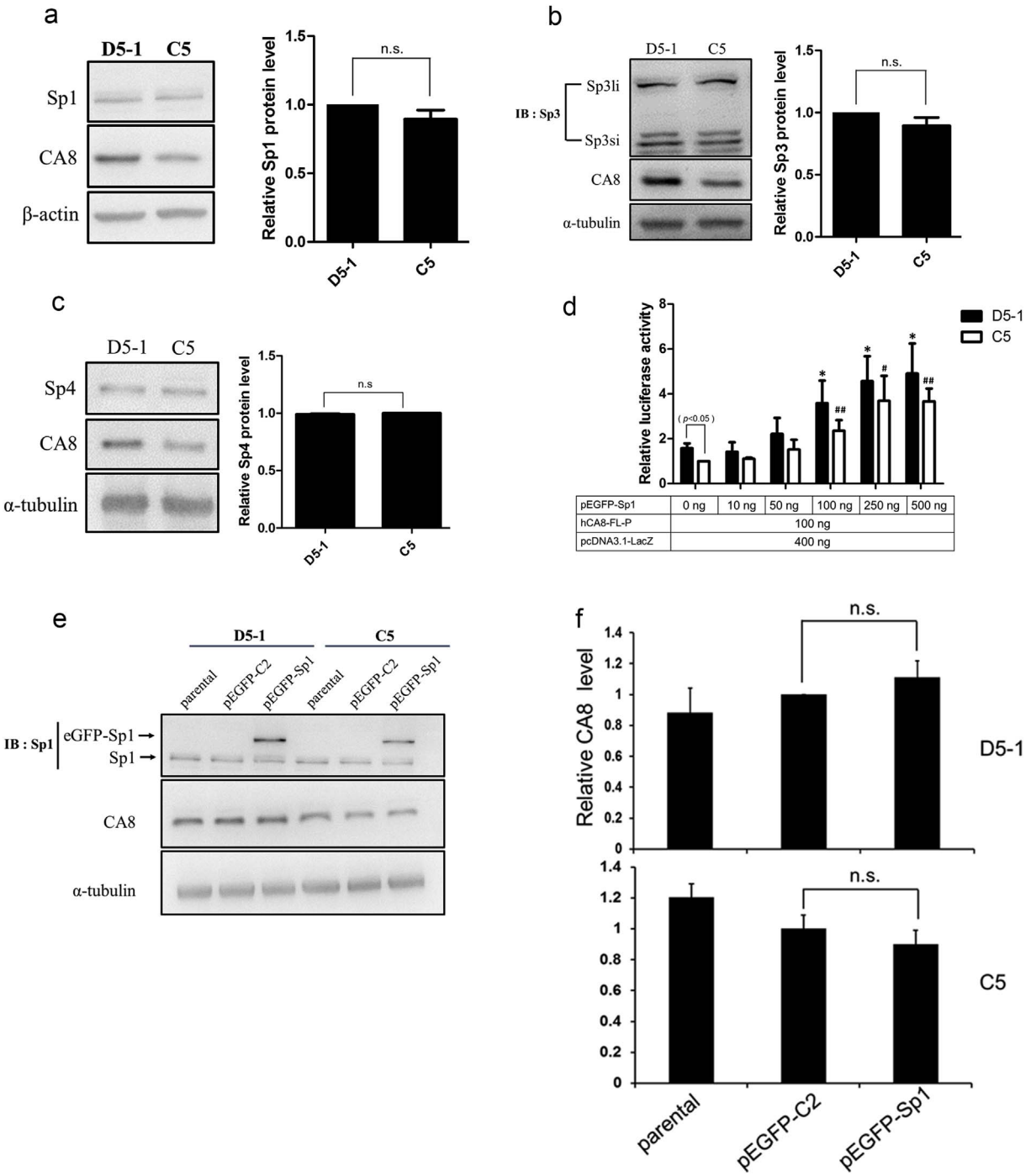


Fig. 4. Effects of Sp1 expression on controlling *hCA8* gene. Western blot analysis and quantitative analysis of endogenous Sp1 (A), Sp3 (B) and Sp4 (C) protein expression in C5 and D5-1 cells. (D) Cybrids were co-transfected with FL-hCA8-P, increasing amounts of Sp1 vector (eGFP-Sp1) and control β-galactosidase expression vector. Luciferase activity was normalized to β-galactosidase and expressed as fold increase over the control vector. The *hCA8* luciferase activity without Sp1 transfection in the C5 cybrid is taken as the reference. * is compared with 0 ng Sp1 transfection in the D5-1 cybrid; # is compared with 0 ng Sp1 transfection in the C5 cybrid. (E) Western blot analysis of CA8 protein levels in the presence or absence of eGFP-Sp1 overexpression. (F) Quantitative analysis of CA8 protein levels with or without Sp1 overexpression in D5-1 and C5 cybrids. Data are the mean values ± SEM of 3 independent experiments. n.s = non-significant.

cells. However, there was no synergistic effect of Sp1 and Sp4 together on *hCA8* gene activation, suggesting that there is no cooperative binding between Sp1 and Sp4 on the *hCA8* promoter (Fig. 7A). The same phenomena were also observed in wild-type and mutant cybrids (Fig. 7B). Furthermore, we used small-hairpin RNA-based shRNA for the specific knockdown of the endogenous Sp1 or Sp4. In 293 T cells, a 50% decrease of endogenous CA8 protein level was observed under Sp1 knockdown, while down-regulation of Sp4 did not significantly reduce the endogenous CA8 expression (Fig. 7C and D). Additionally, knockdown of Sp1 showed an apparent increase of endogenous Sp4 expression, which is consistent with the previous report [33] that Sp1 could bind to Sp4 promoter and then transcriptionally inactivate Sp4 expression. The results suggest that Sp1 may play a predominant role in the upregulation of CA8 expression. However, we could not exclude the possibility that Sp4 may be also involved in *hCA8* gene activation, but the down-regulation of Sp4 may be compensated for by the presence of Sp1. Next, we performed the down-regulation of Sp1 or Sp4 in the wild-type and mutant cybrids. Nevertheless, the data showed no detectable

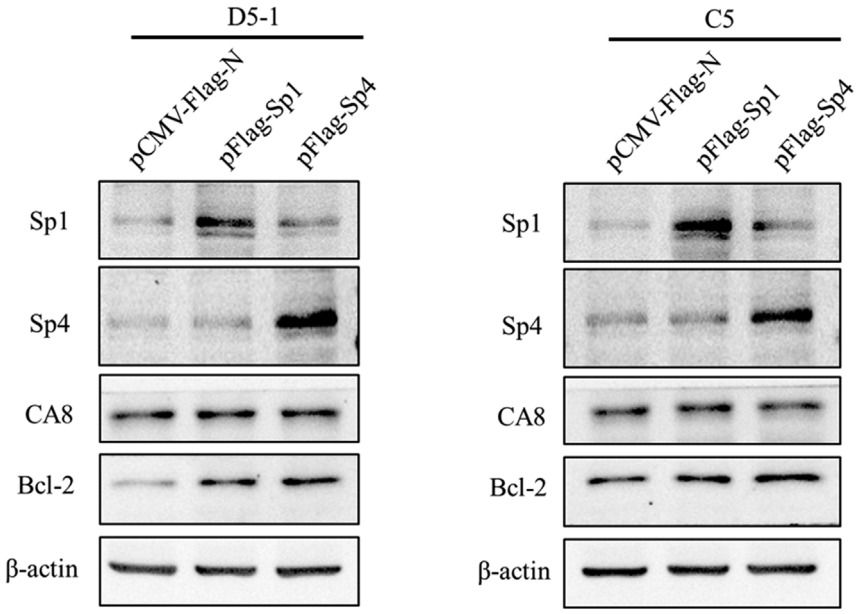


Fig. 5. No altered expression of CA8 in the wild-type and mutant cybrids with or without Sp1 and Sp4 overexpression. Western blot analysis of CA8 and Bcl-2 protein levels in the wild-type D5-1 (left) and mutant C5 (right) cybrids with or without Sp1 and Sp4 overexpression. The cybrid cells were transfected with 2 μg pFlag-Sp1, pFlag-Sp4, or pCMV-Flag-N and cell lysates were harvested 48 h after transfection. Endogenous CA8 and Bcl-2 proteins and overexpressed Sp1 and Sp4 were examined by immunoblotting. No detectable increase of endogenous CA8 protein was observed under exogenous Sp1 or Sp4 overexpression, compared with that of the empty vector controls.

alteration of CA8 protein levels under Sp1 or Sp4 down-regulation (Fig. 7E and F). It is worth noting that a slow turnover of CA8 proteins has been observed in the cybrids with or without A8344G mutation [23].

To examine whether the chromatin structure plays a specific role in controlling the transcriptional activities of Sp transcription factors on the *hCA8* promoter, cybirds with and without Sp overexpression were treated with trichostatin A (TSA), a well-known histone deacetylase inhibitor, and mRNA was assessed by real time PCR. Interestingly, our data showed that an increase of at least 4-fold of CA8 mRNA signal was accompanied by treatment of cybrids with TSA (data not shown), suggesting that the chromatin structure may play an important role in regulating *hCA8* gene expression on the *hCA8* promoter *in vivo*.

3.5. Investigation of chaperone Hsp27 in controlling the expression of Sp1-regulated hCA8 gene

In a previous study, we found a significant decrease of chaperone Hsp27 protein expression in a cell model of MERRF syndrome [34]. It was also noted that Hsp27 plays a potential role as a co-activator in the transcriptional control on Sp1-dependent gene expression. Friedman and colleagues [35] reported the protein-protein interaction between endogenous Sp1 and Hsp27 in HEK-293 cells. Based on these observations, we attempted to inspect whether the reduced Hsp27 expression is involved in the impaired transcription of Sp1-regulated *hCA8* gene in the cell model of MERRF syndrome. We examined the effects of dominant-negative form (Hsp27-S3A) and phosphomimicking form (Hsp27-S3D) of Hsp27 on CA8 expression in the cybrids with or without MERRF mutation and in 293 T cells (Fig. 8). We found that the effect of exogenous Sp1 expression was consistent with the foregoing data (Fig. 6). However, we did not observe any detectable change in the endogenous expression of CA8 protein under the exogenous expression of dominant-negative or phosphomimicking Hsp27 mutants in both cybrids and 293 T cells. This suggests that the transient overexpression of Hsp27-S3A or Hsp27-S3D has no effect on the regulation of *hCA8* expression.

4. Discussion

The proper mitochondrial function for energy supply is critical for the maintenance of cell survival. The robust scaffold for mitochondrial energy production is virtually dependent upon the oxidative phosphorylation machinery. Moreover, the majority of OXPHOS polypeptides are encoded by the nuclear DNA, and only a limited number of the subunits are encoded by the mtDNA. Thus, the anterograde regulation, by which the nuclear or cytoplasmic materials transfer into the mitochondria, is fundamental to the integrity of OXPHOS assembly. Some of human mitochondrial respiratory chain disorders are caused by the abnormalities of nuclear DNA-encoded mitochondrial proteins [36–39]. Besides nuclear DNA mutations, mitochondrial disease could be also caused by mtDNA mutations, such as MERRF, MELAS and CPEO syndromes [40,41]. In the recent decades, a term of mitochondrial retrograde (RTG) regulation has been defined, and is characterized by several cellular changes in response to the mitochondrial defects [42–46]. MERRF syndrome is a prototypical mitochondrial disease rendered by the mtDNA-encoded OXPHOS dysfunction and with the symptoms including cerebellar ataxia. In the study of human retrograde regulations, Arnould and colleagues [47] showed that upregulation of *CREB*-dependent luciferase activities in the 143B-based ρ^0 cells, MERRF cybrids and L929 mouse fibrosarcoma ρ^0 cells was resulted from respiratory chain defects of the mitochondria. Blocking the cytosolic Ca^{2+} by BAPTA, a specific intracellular Ca^{2+} chelator, inhibited the Ca^{2+}-mediated CREB activation in L929 cells, which confirmed the role of Ca^{2+} in the mitochondrial retrograde signaling.

Recently, our laboratory found a significantly reduced CA8 expression in 143B osteosarcoma cell-based cybrids harboring MERRF A8344G mutation [23]. CA8 is known for binding to IP_3 receptor and then interfering the association of ligand IP_3 with its receptor, which physically controls the ER-dependent cytosolic Ca^{2+} homeostasis [15]. Increased cytosolic Ca^{2+} signals in the mutant cybrids, as compared with those in the normal cybrids, suggest that altered CA8 expression may be associated with mitochondrial stress (retrograde) signaling caused by the A8344G mutation. The dramatic decreases on both mRNA and protein levels of the nuclear *hCA8* gene implied an impaired *hCA8* transcriptional activity resulted from the A8344G mutation of mtDNA. To address the *hCA8* transcriptional activity in the MERRF cell model, we searched the 5′ flanking sequence of *hCA8* gene with the putative TFBSs and established the *hCA8* promoter-driven reporter construct (Fig. 1A and B). The full-length *hCA8* promoter-reporter construct was transfected into the cybrids with or without the MERRF mutation, and the analysis showed a significant decrease of *hCA8* promoter activity in the mutant cybrids (Fig. 1C). The data suggested that an altered transcriptional programming of *hCA8* gene occurs in the presence of A8344G mtDNA mutation. Therefore, to further analyze the

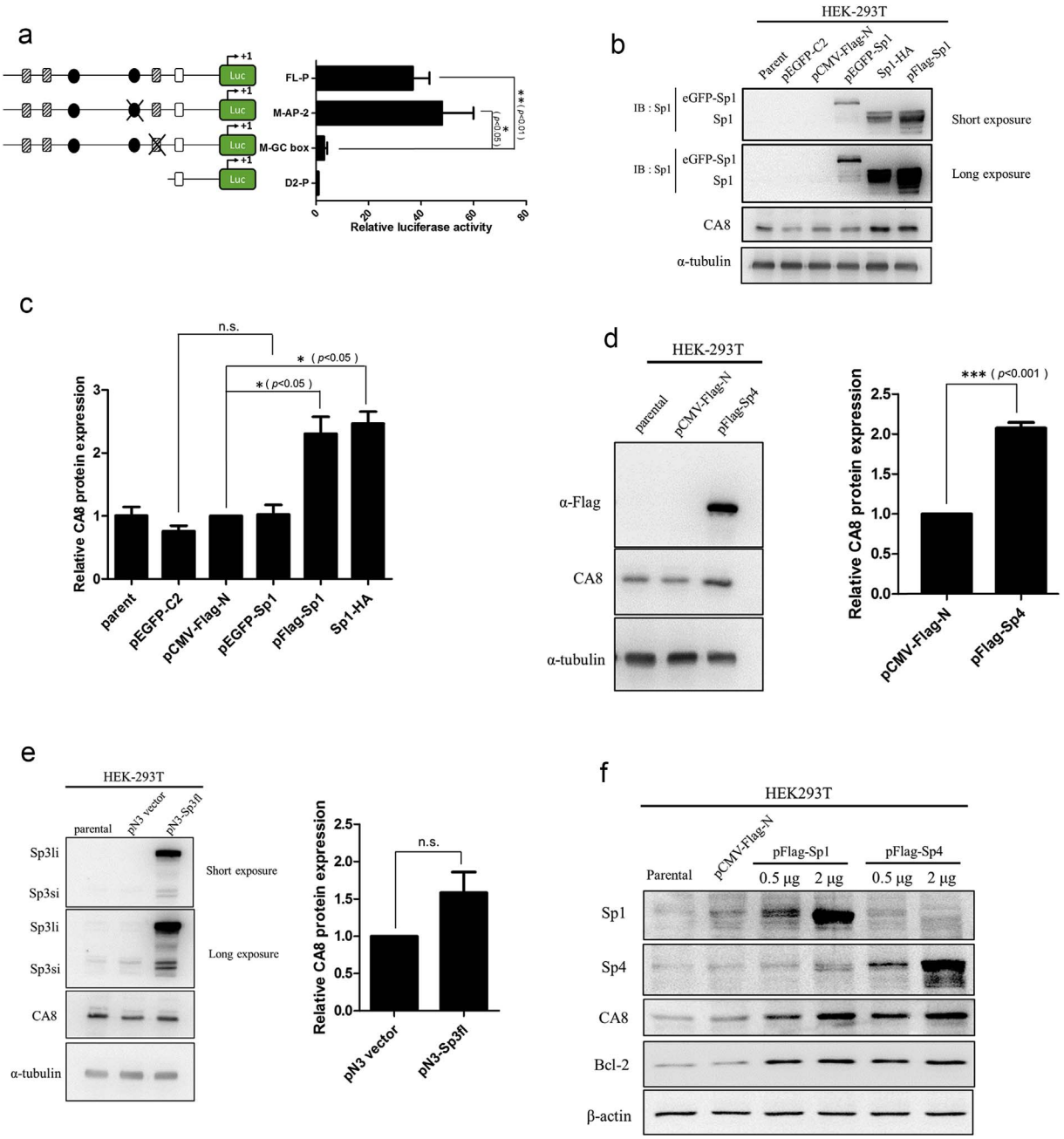
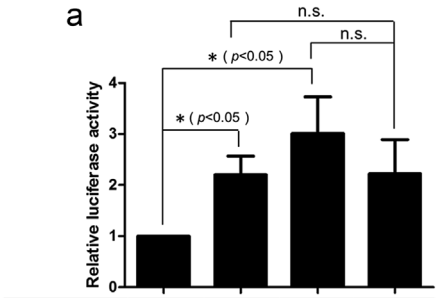


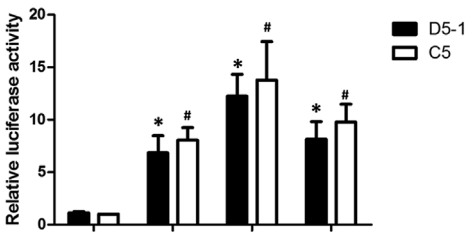
Fig. 6. Overexpression of Sp family members significantly or partially increases the endogenous CA8 protein expression in HEK-293 T cells. (A) GC box-binding protein is involved in the activation of *hCA8* gene in HEK-293 T cells. Motif-disrupted mutants (M-GC box and M-AP-2) or empty vector were co-transfected with β-galactosidase expression vectors into 293 T cells. Luciferase activity was normalized to β-galactosidase activity and expressed as fold increase over the vehicle control. (B, C) Western blot and quantitative analysis of CA8 protein levels in cells with overexpression of a series of Sp1 expression vectors (pEGFP-Sp1, pFlag-Sp1, and Sp1-HA). Transient transfection assays were performed by using 2 μg of plasmid DNA. (D, E) Western blot and quantitative analysis of CA8 protein levels under Sp4 and Sp3 overexpression, compared with the corresponding empty vector controls. Data are the mean values ± SEM of 3 independent experiments. $^{*}P < 0.05$. $^{***}P < 0.001$. n.s = non-significant. (F) Western blot analysis of CA8 protein levels under different amounts of Sp1 and Sp4 overexpression, compared with the corresponding empty vector controls. HEK-293 T cells were transfected with 0.5 and 2 μg of pFlag-Sp1 or pFlag-Sp4 plasmid DNA. The total amount of transfected DNA (2 μg) was kept equal by adding pCMV-Flag-N vector and cell lysates were harvested 48 h after transfection. Endogenous CA8 protein and overexpressed Sp1or Sp4 were examined by immunoblotting.

hCA8 gene transcription regulation, we introduced the deleted mutants and DNA-binding motif-disruptions on the *hCA8* promoter, and the results indicated that the proximal GC box was necessary to the promoter activity of *hCA8* gene (Figs. 2 and 3). These data from the *hCA8* promoter analyses revealed that the key cis-acting element was located on the proximal GC box of the promoter.

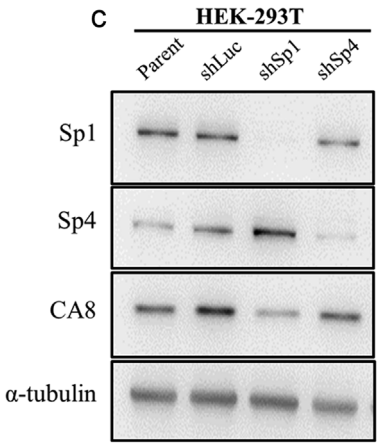
To examine whether the steadily reduced *hCA8* transcriptional activity in the mutant cybrids is caused by the differential protein expression of Sp proteins, we next investigated the endogenous expression of Sp1, Sp3 and Sp4 in the cybrids. However, there was no detectable difference of the three Sp proteins between the wild-type and mutant cybrids (Fig. 4A, B and C), indicating that the expression levels of Sp proteins may not be responsible for the reduced expression of hCA8 in C5 cells. However, it is well-documented that the post-translational modifications (PTMs) of Sp1 and other Sp proteins contribute to their transcriptional effects on gene expression [26,27,48]. For instance, it is reported that there are 23 putative phosphorylation sites on the amino acid sequence of Sp1, and that different phosphorylated Sp1 proteins

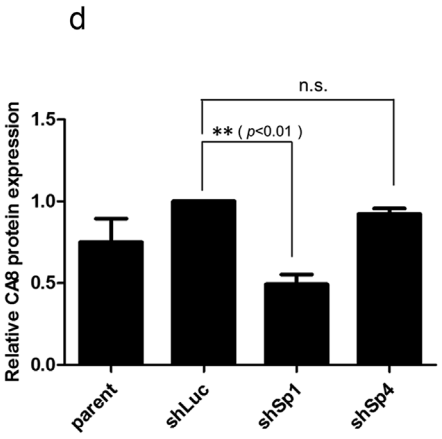


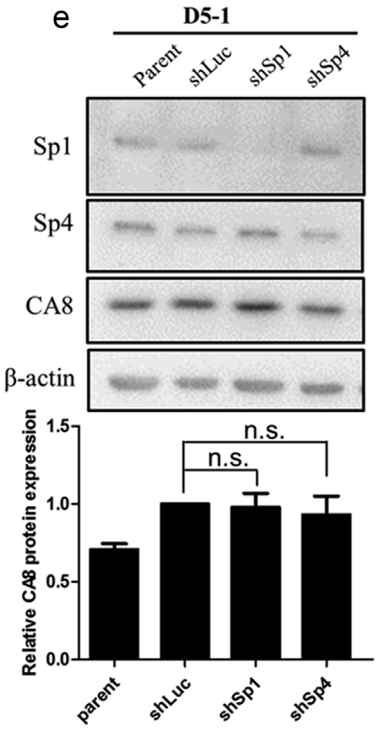
pFlag-vector	600 ng	300 ng	300 ng	-
pFlag-Sp1	-	300 ng	-	300 ng
pFlag-Sp4	-	-	300 ng	300 ng
hCA8-FL-P	100 ng			
pcDNA3.1-LacZ	300 ng			



pFlag-vector	600 ng	300 ng	300 ng	-
pFlag-Sp1	-	300 ng	-	300 ng
pFlag-Sp4	-	-	300 ng	300 ng
hCA8-FL-P	100 ng			
pcDNA3.1-LacZ	300 ng			







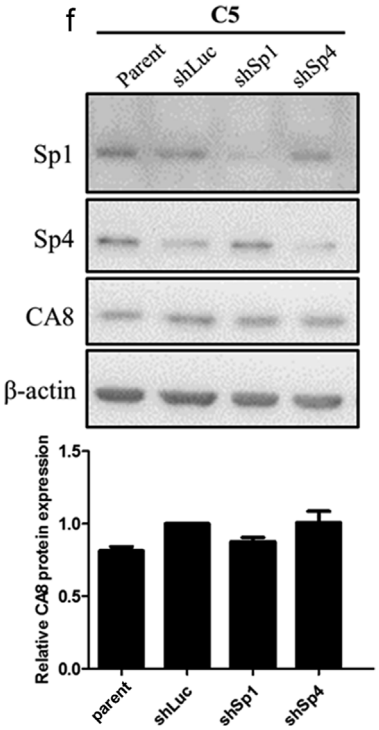


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Fig. 7. Effects of down-regulating endogenous Sp1 and Sp4 on CA8 expression. (A) HEK-293 T cells were co-transfected with hCA8-FL-P, with or without Sp1 and Sp4 expression vectors, and control β-galactosidase expression vector. Luciferase activity was normalized to β-galactosidase and expressed as fold increase over the control vector. (B) Luciferase activity of *hCA8* promoter in the cybrids transfected with Sp1, Sp4 or both. The wild-type (black bar) and mutant (white bar) cybrids were co-transfected with hCA8-FL-P, with or without Sp1 and Sp4 expression vectors, and control β-galactosidase expression vector. Luciferase activities were normalized to β-galactosidase and expressed as fold increase over the control vector. Data are the mean values ± SEM of 3 independent experiments. * or $^{\#}P < 0.05$. * is compared with vehicle control in D5-1. $^{\#}$ is compared with vehicle control in C5. (C) Western blot analysis of CA8 protein expression in 293 T cells with Sp1 or Sp4 knockdown. (D) Quantitative analysis of CA8 protein levels with or without knockdown of endogenous Sp1 or Sp4. (E, F) Western blot analysis and quantitative assessment of CA8 protein levels in cybrids with down-regulation of endogenous Sp1 and Sp4. Data are the mean values ± SEM of 3 independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$. n.s = non-significant.

may contribute positively or negatively to gene transcription by altering the DNA-binding, transactivation, or total protein level of Sp1 [28]. We do not know whether the PTMs of Sp transcription factors are altered in the cybrids with or without the MERRF mutation, and further investigations are warranted to clarify this point.

To verify the roles of Sp1, we examined whether the representative *hCA8* promoter is responsive to the effect of Sp1. The analysis showed that *hCA8* promoter activities were highly responsive to the increasing amounts of eGFP-Sp1 in the cybrids (Fig. 4D). Consistently, we found the same phenomenon in the HEK-293 T cells (Fig. 7A), where the stronger *hCA8* promoter activity than that in the cybrids was observed (Fig. 6A). However, overexpression of eGFP-Sp1 did not upregulate the endogenous CA8 protein in cybrids and 293 T cells (Figs. 4E and 6B), suggesting that the plasmid DNA-based *hCA8* reporter effectively responding to eGFP-Sp1 may be an *in vitro* artificial effect. Interestingly, further analyses showed an apparent increase of CA8 protein levels by overexpression of small tag-carrying Sp1 plasmids (Fig. 6B and C), indicating that the native conformation of endogenous Sp1 may be required for its function on the chromatin architecture. We suspect that the structurally interfering recombinant eGFP-Sp1 may disrupt the intrinsic association between Sp1 and its interacting factors *in vivo*. On the other hand, we also performed overexpression of pFlag-Sp1 and pFlag-Sp4 in the wild-type and mutant cybrids. It is noted that an increase of Bcl-2 was accompanied by the overexpression of Sp1 and Sp4 in the cybrids, indicating that the fusion Sp1 and Sp4 are functionally active. Nevertheless, we did not observe detectable change of endogenous CA8 protein level, (Fig. 5). The results may be due to, at least partly, the slow turnover of CA8 protein in the 143B-based cybrids [23]. Taken together, we suggest that Sp1 is involved in upregulating *hCA8* gene expression in HEK-293 T cells, and its proper binding alone or with co-activator(s) on the promoter may contribute to *hCA8* gene activation *in vivo*.

It is known that the characteristic trans-acting factors for GC box recognition are Sp1 and other Sp family members [27]. Because Sp4 is a neuron specific Sp family member, the roles of Sp4 on controlling *hCA8* gene expression in neuronal-like HEK-293 T cells were also

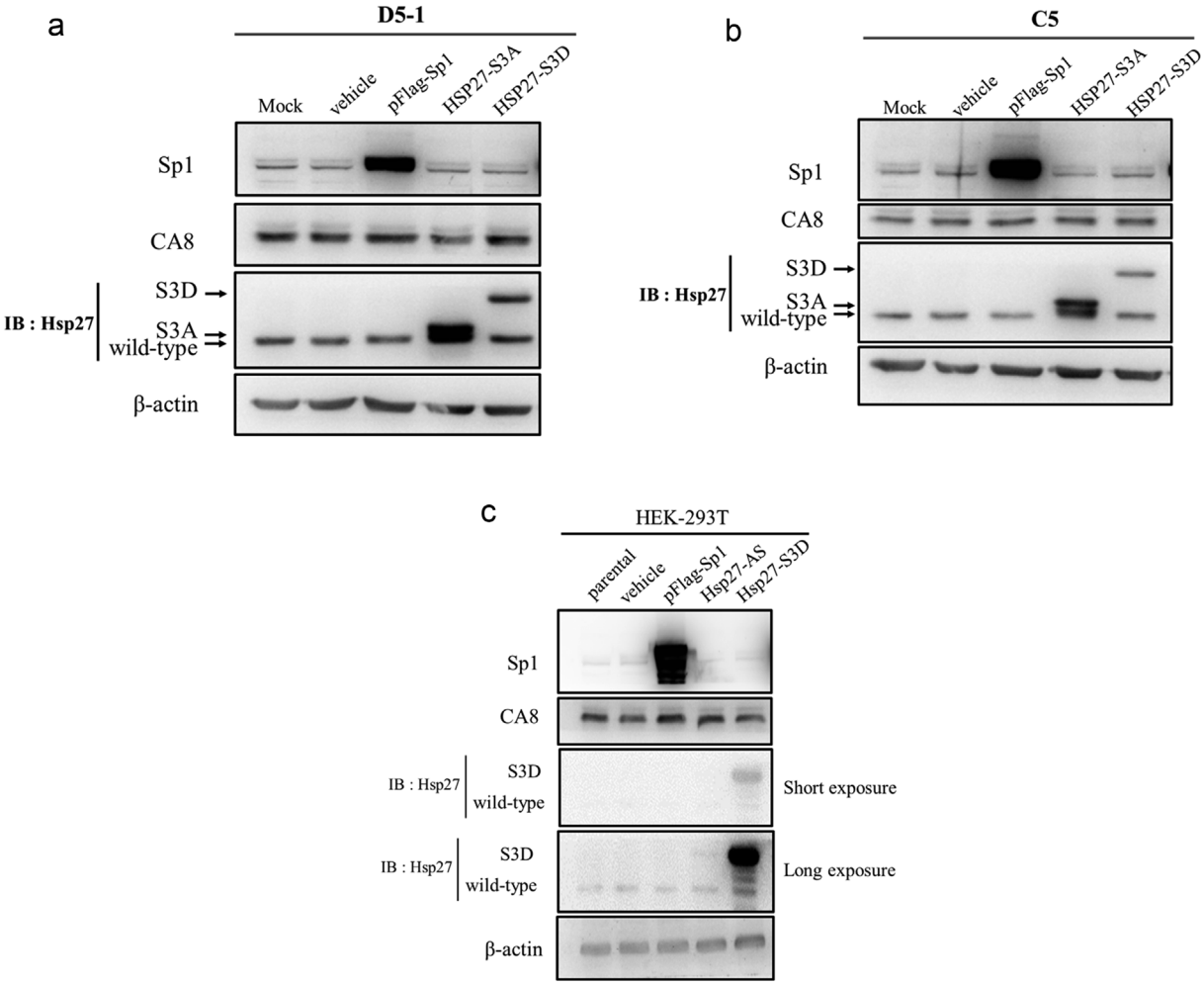

Fig. 8. Over-expression of Sp1 or different Hsp27 mutants does not change the endogenous CA8 protein expression. Western blot analysis of the endogenous CA8 protein level under overexpression of pFlag-Sp1, Hsp27-S3A, Hsp27-S3D, Hsp27-AS or empty vehicle control in (A, D5-1), (B, C5) and (C, HEK-293 T) cells.

investigated. We showed the relevant upregulations of both *hCA8* promoter activity and CA8 protein level in 293 T cells harboring the exogenous expression of Sp4 (Figs. 6D and 7A). However, Sp1 and Sp4 seemed to have no synergistic effect on regulating *hCA8* transcription in both 293 T and cybrid cells (Fig. 7A and B), indicating that Sp1 and Sp4 may bind independently to the proximal GC box on the promoter. To further confirm the roles of Sp1 and Sp4, knockdown of these two Sp proteins revealed that down-regulation of Sp1 rather than Sp4 showed a significantly reduced CA8 protein level in 293 T cells (Fig. 7C and D). The data suggest that Sp1 plays a predominant role on controlling *hCA8* gene transcription in 293 T cells. Unexpectedly, we failed to observe the same reduction of CA8 expression in cybrids with down-regulation of Sp1. It is noted that CA8, which is degraded via autophagy-lysosomal pathway, is moderately stable in the cybrids [23]. Given the fact that the effects of down-regulated Sp1 on CA8 expression was assayed 48 h after transient transfection, we cannot rule out the possibility that the slow turnover of CA8 may account for the non-detected decrease of endogenous CA8 in the cybrids (Fig. 7E and F).

On the other hand, it is known that the histone modifications play critical roles in the chromatin structure formation [49], such as acetylation, methylation and phosphorylation may lead to altered transcriptional regulation of target genes. By using trichostatin A (TSA), an antifungal antibiotics used as a potent and specific inhibitor of histone deacetylase (HDAC) [50], our data indicated that an increase of at least 4-fold of CA8 mRNA signal was accompanied by treating cybrids with TSA, suggesting that histone modifications may exist on the *hCA8* promoter regions in the MERRF cybrids. It is worth noting that defects in mtDNA maintenance and translation have been demonstrated to regulate transcripts that encode proteins that are involved in the regulation of lipid and glucose metabolism [46]. Considering our previous observation that CA8 promotes glucose uptake in human osteosarcoma cells [21], we speculated that the integrated mitochondrial stress response due to the A8344G mutation of mt DNA may be responsible for the altered transcriptional regulation of the *hCA8* gene. However, whether the decreased *hCA8* transcription observed in the mutant cybrids is due to altered chromatin modifications on the promoter region needs further investigations.

In one of our previous studies [51], a reduced p-Hsp27 expression in the cybrids harboring MERRF mutation was reported. Recently, a similar trend of CA8 alteration was also demonstrated in a mitochondrial disease cell model [23]. Because Hsp27 has been reported to play a potential role of co-activator interplaying with Sp1 in upregulating Sp1-responsive gene expression [35], we attempted to resolve whether Hsp27 is involved in controlling *hCA8* gene in the cybrids. However, our data showed no detectable change of endogenous CA8 protein level by expression of dominant-negative or phosphomimicking Hsp27 (Fig. 8). It is noted that the potent role of Hsp27 as a transcriptional activator is determined by its translocation into the nucleus [35]. It was demonstrated that p-Hsp27 was accumulated in the nuclei during the recovery time after heat shock treatment in wild-type and mutant cybrids [51]. Therefore, the reason why we failed to observe any alteration of CA8 expression in the presence of overexpressing Hsp27 may be due to the absence of Hsp27 nuclear localization under our experimental conditions. To understand whether the nuclear localization of Hsp27 is critical for CA8 expression regulation, overexpression of NLS-Hsp27 or inducing Hsp27 translocation by heat shock treatment may provide further information.

In summary, our results have unraveled the transcriptional regulation of the novel *hCA8* gene. The analyses demonstrated that Sp1 plays a predominant role in regulating *hCA8* gene transcription. In addition, the results from investigating Sp4 also showed its potential roles on controlling *hCA8* gene. Furthermore, our results indicate that the chromatin structure influences the effects of Sp transcription factors on the proximal *hCA8* promoter, which may be involved in the mitochondrial retrograde signaling. Although the detailed underlying mechanisms of controlling hCA8 transcription regulation may be more complicated than expected, our findings advance the understanding of the regulation of novel CA8 in the MERRF disease model.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2018.01.012.

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