

# Molecular Characterization of Mouse CREB3 Regulatory Factor in Neuro2a Cells

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## Short Report

**Keywords:** CREB3, GAL4/UAS system, CREBRF

**Posted Date:** February 10th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-180376/v1>

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# Abstract

We performed expression and functional analysis of mouse CREB3 regulatory factor (CREBRF) in Neuro2a cells by constructing several expression vectors. Overexpressed full-length CREBRF protein was stabilized by MG132; however, the intrinsic CREBRF expression in Neuro2a cells was negligible under all conditions. On the other hand, N- or C-terminal deletion of CREBRF influenced its stability. Cotransfection of CREBRF together with GAL4-tagged full-length CREB3 increased luciferase reporter activity, and only the N-terminal region of CREBRF was sufficient to potentiate luciferase activity. Furthermore, this positive effect of CREBRF was also observed in cells expressing GAL4-tagged cleaved CREB3, although CREBRF hardly influenced the protein stability of NanoLuc-tagged cleaved CREB3 or intracellular localization of EGFP-tagged one. In conclusion, this study suggests that CREBRF, a quite unstable proteasome substrate, positively regulates the CREB3 pathway, which is distinct from the canonical ER stress pathway in Neuro2a cells.

## Introduction

CREB3, which is also called Luman and LZIP, was first identified as a factor associated with herpes simplex virus-related host cell factor 1 (HCF1) [1], and it was demonstrated that nuclear localization of the CREB3/HCF1 complex plays a role in viral reactivation. CREB3 is categorized in the ATF6/CREB3 family and consists of an N-terminal transactivation region, a basic leucine zipper (bZIP) structure and a single-pass transmembrane region [2, 3]. However, the precise target genes of CREB3 family are not fully characterized, although transcriptional regulation of a variety of ATF6-regulating genes has been well characterized [2–7]. CREB3 regulatory factor (CREBRF), also called Luman regulatory factor (LIF), was identified by two-hybrid screening using CREB3 as bait, and it was reported that CREBRF negatively regulated CREB3 expression [8]. CREBRF-CREB3 pathways have been reported to play roles in the regulation of ER stress and autophagy [8, 9, 10]. However, our recent studies on CREB3 showed that its processing is triggered by the ER/Golgi stress inducer brefeldin A (BFA) but not by other typical ER stress inducers, including thapsigargin and tunicamycin [11, 12]. Considering these findings, we applied a GAL4/UAS reporter system to evaluate the relationship between CREBRF and CREB3 in Neuro2a cells in detail. Unexpectedly, it was found that CREBRF positively regulated GAL4-CREB3 reporter activity. We therefore characterized the positive regulatory effect of CREBRF by preparing several types of CREB3 and CREBRF expression constructs.

## Materials And Methods

### Construction of plasmids

For preparation of mouse CREBRF constructs, the mouse CREBRF gene was obtained from DNAFORM (RIKEN), and the full-length coding region with a myc epitope or EGFP tag at the N-terminus was cloned into the pcDNA3.1 vector (Life Technologies). Each truncated CREBRF gene with a myc epitope or EGFP tag was amplified by PCR and inserted into the pcDNA3.1 vector. Mouse CREB3 (NM\_013497) was

cloned from cDNA derived from the Neuro2a mouse neuroblastoma cell line [11]. Constructs encoding the full-length (1–379 aa) or N-terminal region (1–236 aa) of mouse CREB3 with GAL4, EGFP, or NanoLuc (NL) at the N-terminus and a myc epitope at the C-terminus were inserted into a pcDNA3.1 vector. The firefly luciferase gene was also inserted into a pcDNA3.1 vector. The GAL4 and NanoLuc genes were amplified by PCR and inserted into the pcDNA3.1 Myc/his vector.

gRNAs against mouse CREB3 (5'-GAGAGGAAAGCGGAGATTTGT-3') aligned with tracer RNA were inserted into a pcDNA3.1-derived vector with a U6 promoter [13]. To prepare the donor genes, a DNA fragment encoding the N-terminal region of CREB3 (1–141 bp from the translation start site) was fused with a hygromycin resistance gene via IRES and inserted into a pGL3-derived vector. The hCas9 construct (#41815) used in this study was obtained from Addgene [14].

## Cell culture and treatment

Neuro2a cells were maintained in Dulbecco's modified Eagle's minimum essential medium containing 5% fetal bovine serum. Transfection of the indicated constructs was performed using the PEI-MAX reagent (Polysciences) as previously described [11, 12, 13]. To establish CREB3-deficient cells, Neuro2a cells were transfected with the indicated constructs; cells transfected with gRNA, hCas9 and donor genes were cultured with hygromycin, and the resultant cells were used in this study. During selection, the normal parental cells were maintained with normal culture medium and were used as wild-type control cells for the following experiments. In each experiment, parental and deficient cells were seeded in 12- or 48-well plates with nonhygromycin-containing culture medium. Then, the cells were treated with or without brefeldin A (BFA, 0.5 µg/ml) (Sigma-Aldrich) or MG132 (10 µM) (Peptide Institute) for the indicated time period. For observation of fluorescent images, cells were seeded on poly-D-lysine-coated glass bottom dishes. After transfection of the indicated genetic constructs into the cells, fluorescence images were obtained by fluorescence microscopy (KEYENCE).

## Reverse transcription polymerase chain reaction

To estimate the expression level of each gene by RT-PCR, total RNA was extracted from cells lysed with TRI reagent (Molecular Research Center), and equal amounts of total RNA from each sample were converted to cDNA by reverse transcription using random nine-mer primer with SuperScript III Reverse Transcriptase (RT) (Life Technologies) as previously described [12, 13]. Each cDNA was added to a PCR mixture for amplification (Taq PCR Kit, Takara). The PCR primers used in this study were as follows: CREBRF sense primer, 5'-AAGTCAAGATCAACCCTGTG-3', CREBRF antisense primer, 5'-TTGGTTGGCTGTTCTCTCAT - 3'; G3PDH sense primer, 5'-ACCACAGTCCATGCCATCAC-3', G3PDH antisense primer, 5'-TCCACCACCCTGTTGCTGTA-3'. The typical reaction cycling conditions were as follows: 30 sec at 96°C, 30 sec at 58°C and 30 sec at 72°C. The results represent 21 or 28 cycles of amplification. The products were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. The expression level of each mRNA was analyzed using the ImageJ software (National Institutes of Health), and the relative amount of CREBRF mRNA was calculated based on the

G3PDH value obtained from the identical cDNA. The CREBRF mRNA expression of each cDNA was normalized to the values obtained from the untreated control cells [13].

## Western blotting analysis

We detected the amounts of each protein in the cell lysates as previously described [11, 12, 13]. The cells were lysed with homogenization buffer (20 mM Tris-HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml pepstatin A). After the protein concentration was determined using Bradford Protein Assay Dye Reagent (Bio-Rad), each cell lysate was dissolved in an equal amount of 2× sodium dodecyl sulfate (SDS)-Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 10% glycerol), and equal amounts of cell lysate were prepared. Equal amounts of protein were separated on 10 or 12.5% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (GE Healthcare) and identified by enhanced chemiluminescence (GE Healthcare) using antibodies against CREB3 (Proteintech and Santa Cruz), CREBRF (Abcam), EGFP (Roche), a myc-epitope and CREB3 (Santa Cruz Biotechnology) and G3PDH (Acris Antibodies). The expression level of each protein was analyzed using ImageJ software (National Institutes of Health), and the relative amount of cleaved CREB3 was calculated based on the G3PDH value obtained from the same lysate. The cleaved CREB3 protein expression levels of each lysate were normalized to the value obtained from the BFA-treated Neuro2a cells [13].

## Luciferase assay

To evaluate CREB3 activation in Neuro2a cells, cells in 48-well plates were transfected with pG5 (firefly luciferase) (Promega) or pG4.70 (an internal control, Renilla luciferase) (Promega) together with GAL4 empty vector (GAL4-MH) or the indicated GAL4-tagged CREB3 (GAL4-CREB3) or CREBRF constructs. After GAL4 reporter transfection and incubation for the indicated time, cells were lysed with 1× Passive Lysis Buffer (Promega) for 15 min at room temperature and briefly centrifuged. Luciferase activity in each lysate was measured using the Dual-Luciferase Assay System (Promega) [11]. The luciferase reporter activity in each lysate was normalized to the activity of the cotransfected Renilla luciferase by dividing the raw luciferase values by the Renilla luciferase values. In all figures, we show the relative luciferase activities, which were calculated from the values of the control cells as described in the figure legends. To measure the intracellular amounts of NanoLuc-tagged proteins, cells were transfected with the NanoLuc-tagged gene together with the firefly luciferase gene as an internal control. After incubation for the indicated time, cell lysates were prepared as described above. Luciferase activity in each lysate was measured using the Nano-Glo Dual-Luciferase Reporter Assay System (Promega). The Nanoluciferase activity in each lysate was also normalized to the activity of the cotransfected firefly luciferase by dividing the raw luciferase values by the firefly luciferase values. We show the relative Nanoluciferase activities, which were calculated from the values of the cells transfected with each empty vector (mock).

## Results And Discussion

CREBRF was first identified as a factor that negatively regulates CREB3; however, its expression has not yet been fully investigated [8, 15]. In particular, CREBRF in neuronal cells has hardly been characterized. Recently, we reported the expression and processing of CREB3 via transfection of several types of mouse CREB3 genes into the Neuro2a mouse neuroblastoma cell line [11]. Based on this knowledge, we first evaluated the expression of endogenous CREBRF mRNA and protein in Neuro2a cells in the presence or absence of BFA or MG132. BFA triggers the cleavage of CREB3, and MG132, a proteasome inhibitor, stabilizes both full-length and cleaved CREB3 [11, 12]. In Neuro2a cells, CREBRF mRNA was detected under resting conditions; however, neither reagent influenced CREBRF mRNA expression (Fig. 1A). We then investigated CREBRF protein expression in mock- or myc-tagged mouse CREBRF-transfected Neuro2a cells. As shown in Fig. 1B, CREBRF protein expression was quite low even in the CREBRF-transfected cells under resting conditions and upon BFA treatment. On the other hand, MG132 treatment dramatically increased the myc-tagged CREBRF protein level, although the level of intrinsic CREBRF expression was below the detection limit. Under these conditions, we evaluated the expression of cleaved CREB3 protein in both mock- and myc-tagged CREBRF-transfected Neuro2a cells. Consistent with previous findings, BFA treatment induced the expression of cleaved CREB3 protein in parallel to the disappearance of its full-length form. On the other hand, MG132 treatment increased the expression of both forms. However, the amount of cleaved CREB3 expression unexpectedly did not differ between the mock- and myc-tagged CREBRF-transfected Neuro2a cells.

CREB3 has been reported to regulate some ER stress-inducible genes (e.g., *Herp* and *Edem1*) [8, 16]; however, our recent studies on CREB3 showed that its processing is triggered by the ER/Golgi stress inducer BFA but not by other typical ER stress inducers, including thapsigargin and tunicamycin [11, 12]. It therefore seems that the precise target genes of CREB3 are obscure. Thus, we applied a GAL4/UAS reporter system to evaluate the relationship between CREBRF and CREB3 in Neuro2a cells (Fig. 2). First, we measured the luciferase reporter activity in wild-type Neuro2a cells expressing GAL4-MH or GAL4-fused full-length CREB3 and found that luciferase activity in cells expressing GAL4-fused full-length CREB3 was higher than that in cells expressing GAL4-MH under resting conditions and markedly increased in response to BFA (Fig. 2B). Since full-length CREB3 is predominantly localized in the ER and its cleaved form was hardly detected in both untransfected cells and cells expressing the GAL4-tagged full-length form (Fig. 2A) [11, 16, 17], it is possible that only a small amount of full-length CREB3 was spontaneously cleaved to elevate this luciferase activity. These results also indicate that this system effectively monitors CREB3 activation in this cell line. Furthermore, we established CREB3-deficient cells using a CRISPR/Cas9 system to exclude the effects of endogenous CREB3 (Supplementary Fig. 1). In our preliminary experiments, CREB3 deficiency hardly influenced the expression of typical ER stress-induced genes (e.g., *GADD153*, *GRP78* and *sXBP1*) in response to BFA (unpublished data). As shown in Fig. 2C, the GAL4-CREB3 reporter activity was responsive to BFA in these CREB3-deficient cells, and cotransfection of myc-tagged CREBRF markedly increased the GAL4-CREB3 reporter activity in the presence or absence of BFA.

Since this phenomenon was also observed in the parental wild-type cells as well as our CREB3-deficient cells, we next constructed CREBRF serial deletion constructs (Fig. 3ABD) and examined their effects by

cotransfection with GAL4-MH or GAL4-tagged full-length CREB3 into wild-type Neuro2a cells (Fig. 3CE). The stability of each myc-tagged CREBRF deletion mutant was quite different, although full-length (FL) CREBRF was an unstable proteasome substrate. Interestingly, the mouse CREBRF lacking the C-terminal half of the protein, including the glutamate-rich and basic leucine zipper (bZIP) regions ( $\Delta$ C1 and  $\Delta$ C2), still increased the GAL4-CREB3 reporter activities, but deletion of the N-terminal 180 aa of mouse CREBRF ( $\Delta$ N1) abolished this unique effect on GAL4-CREB3 (Fig. 3C). We then focused on this N-terminal region and prepared additional myc-tagged CREBRF genetic constructs. As shown in Fig. 3DE, the 200-aa ( $\Delta$ C3) but not the 100-aa ( $\Delta$ C4) N-terminal CREBRF construct increased the GAL4-CREB3 reporter activity. We also constructed another myc-CREBRF construct encoding CREBRF aa 90–200 ( $\Delta$ NC); however, its expression was negligible even in the presence of MG132 (Supplementary Fig. 2). Instead, we prepared this CREBRF deletion construct (containing aa 90–200) ( $\Delta$ NC) with the EGFP gene at the N-terminus (Fig. 4A). This EGFP-tagged CREBRF deletion mutant was successfully expressed and detected without MG132, although the EGFP-tagged and myc-tagged full-length (FL) CREBRF constructs were unstable. We then investigated the intracellular localization of each EGFP gene in Neuro2a cells (Fig. 4B). Under our experimental conditions, the untagged EGFP protein was expressed in almost all cells; however, a fluorescence signal derived from EGFP-tagged full-length CREBRF was detected only in a small number of cells, and the fluorescence intensity in these cells was quite weak and distributed throughout the cell. Interestingly, point-like fluorescence within the nucleus was observed in some cells, and the number of cells with this punctate fluorescence was markedly increased by treatment with MG132. This localization of EGFP-tagged full-length (FL) CREBRF in the nuclear foci was similar to a previous report, although this distribution in our cells was prominent only in the presence of MG132 [8, 15]. On the other hand, the EGFP-tagged CREBRF deletion construct (90–200 aa) ( $\Delta$ NC) was detected in most cells, and no unique distribution was observed even in the presence of MG132 (data not shown). Then, we cotransfected EGFP and each EGFP-tagged CREBRF together with each GAL4 reporter into Neuro2a cells and found that both the full-length (FL) and deleted CREBRF (90–200 aa) ( $\Delta$ NC) increased GAL4-CREB3 reporter activity to the same extent (Fig. 4C).

Since certain stresses (e.g., Golgi stress) trigger the cleavage of full-length CREB3 and the translocation of its N-terminal half into the nucleus [11, 16, 17], we finally investigated whether myc-CREBRF influenced luciferase reporter activity in cells expressing GAL4-tagged cleaved CREB3. Consistent with the previous findings, EGFP-tagged cleaved CREB3 was predominantly localized within the nucleus, and cotransfection of equal amounts of myc-tagged full-length (FL) CREBRF did not affect the distribution of EGFP-tagged cleaved CREB3 (Fig. 5A). In reflection of the nuclear localization of cleaved CREB3, the luciferase reporter activity induced by GAL4-tagged cleaved CREB3 was much higher than that induced by its full-length form. We then transfected cells with more than one fiftieth of the amount of GAL4-fused cleaved CREB3 gene in comparison with its full-length form and found that cotransfection of myc-tagged full-length (FL) CREBRF also upregulated its luciferase reporter activity (Fig. 5B). In parallel, we constructed another cleaved CREB3 genetic construct with a small luciferase, NanoLuc (NL), at the N-terminus to monitor the intracellular amount of the transfected cleaved CREB3; however, cotransfection of myc-CREBRF hardly influenced the NanoLuc luciferase activities of NL-MH and NL-tagged cleaved

CREB3 (Fig. 5C and Supplementary Fig. 3). This is consistent with the finding that myc-CREBRF overexpression did not influence the amount of endogenous CREB3 (Fig. 1B). Therefore, these results imply that CREBRF overexpression positively regulates cleaved CREB3 transcriptional activity without affecting its stability or intracellular localization. Furthermore, analysis of CREBRF deletion mutants showed that a short N-terminal region including aa 90–200 ( $\Delta$ NC) plays an important role in its positive regulatory effect on cleaved CREB3. However, no structural insights around this region have been reported. Regarding the structural features of CREBRF, it possesses glutamate-rich and basic leucine zipper (bZIP) regions in its middle and C-terminal regions [8, 15], but neither region influenced the activity of cleaved CREB3 in this GAL4 reporter system. Since full-length CREBRF is an unstable proteasome substrate, we examined whether this EGFP-tagged short N-terminal region ( $\Delta$ NC) directly interacts with the cleaved CREB3 protein by a coimmunoprecipitation assay; however, we did not observe any specific interaction between the two (data not shown).

In a previous study, it was reported that EGFP-tagged CREBRF was predominantly localized within the nucleus. In contrast, this study showed that both EGFP-tagged full-length (FL) CREBRF and this short N-terminal ( $\Delta$ NC) CREBRF were distributed throughout Neuro2a cells, which indicates that they only partially overlapped with cleaved CREB3. On the other hand, MG132 treatment induced a marked accumulation of CREBRF within a certain part of the nucleus. It is therefore possible that CREBRF possesses certain roles in regulating gene expression; however, it is unclear whether CREBRF activates this CREB3 pathway directly.

CREB3, which has a structure similar to that of the well-known ER stress sensor ATF6, has been suggested to participate in ER stress responses since it was reported that thapsigargin and tunicamycin induced CREB3 cleavage and activated Herp gene transcription [16]. However, our studies on overexpressed and endogenous CREB3 clearly showed that the cleavage of CREB3 is predominantly triggered by BFA and monensin (a Na<sup>+</sup> ionophore) but not by two other ER stress inducers [11, 12]. In addition, our preliminary experiments using CREB3-deficient cells showed that CREB3 hardly contributed to BFA-induced Herp mRNA expression (unpublished data). To understand these discrepancies, a more careful search for the precise targets of CREB3 is required. Considering this knowledge, we applied a GAL4/UAS reporter system to elucidate the relationship between CREB3 and CREBRF and found a positive regulatory effect of CREBRF on the CREB3 pathway; however, its positive effect is contradictory to previous findings [8]. The precise reasons for this discrepancy are unclear for now, but it might be due to differences in cellular context between Neuro2a cells and other cell types previously reported.

Recently, it was reported that single nucleotide polymorphisms of human CREBRF are associated with body mass and energy metabolism [18, 19, 20], although it remains to be determined whether these issues are related to CREB3. In addition, some studies have suggested that CREB3 and CREBRF have roles in tumor progression [9, 10], although there are only a few studies on CREBRF in the nervous system [21, 22]. Since the precise functions of CREBRF and CREB3 are still unclear, further characterization of the relationship between CREBRF and CREB3 using various types of cells might

provide new insights into various types of disorders, including lifestyle-related diseases, cancer and neurodegenerative diseases.

## Abbreviations

CREB3 – cAMP response element binding protein 3; CREBRF – CREB3 regulatory factor, CRISPR/Cas9 – clustered regularly interspaced short palindromic repeats/ CRISPR-associated proteins 9; ER – endoplasmic reticulum; G3PDH – glyceraldehyde 3-phosphate dehydrogenase; RT-PCR – reverse transcription polymerase chain reaction.

## Declarations

### Acknowledgments

This work was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS, Japan, KAKENHI, Nos. 19H04030 and 20K21751 to K.O.).

### Author contributions

KO and YN discussed and designed the research; KO and TH performed the experiments; KO and YH confirmed the results; and KO, TH and YN prepared the manuscript.

### Compliance with ethical standards

### Data availability

The data generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of interest

The authors declare that they have no conflict of interest.

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## Figures

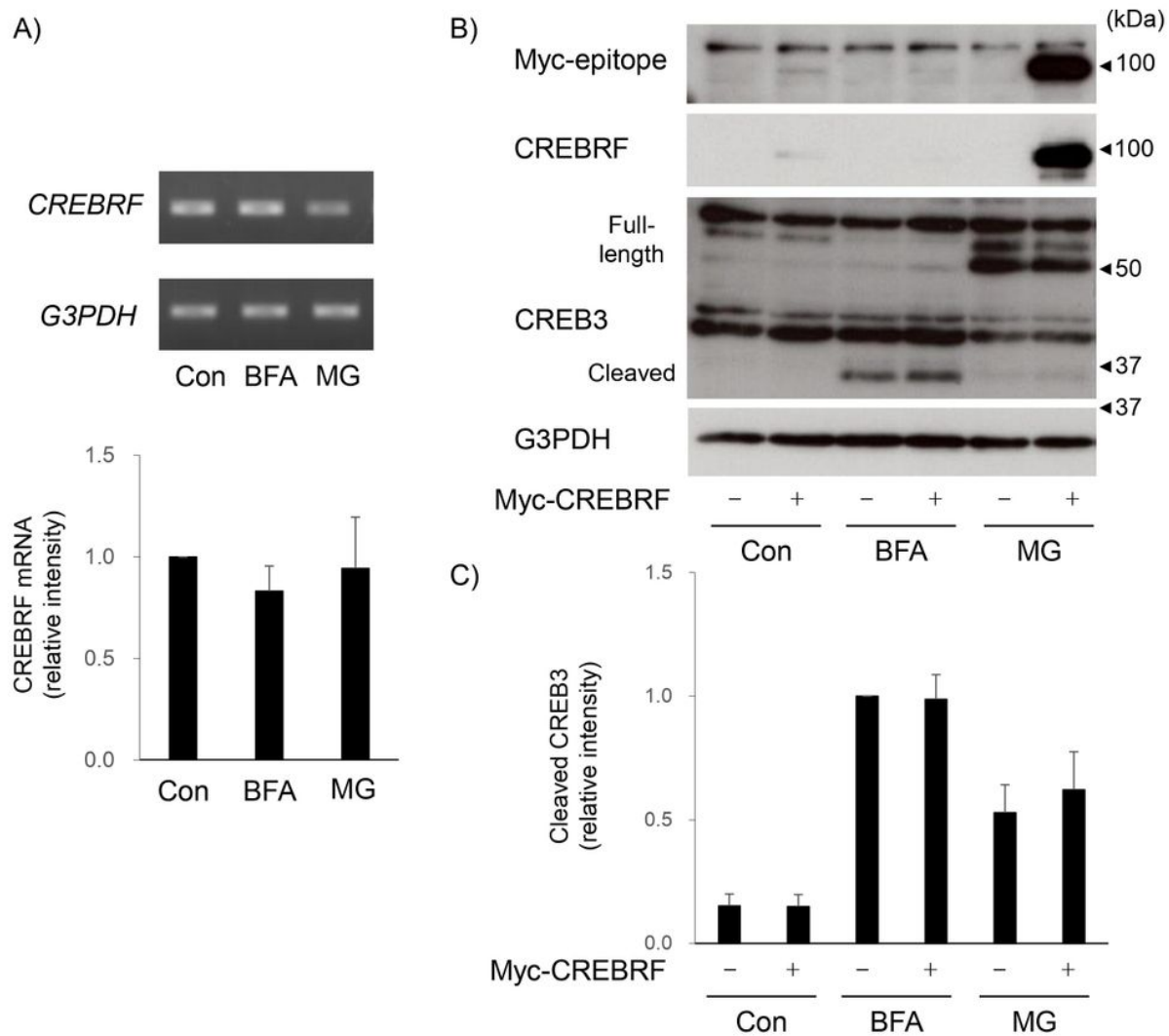


Fig. 1

## Figure 1

Expression analysis of intrinsic CREBRF in Neuro2a cells A) Neuro2a cells were treated with BFA (0.5  $\mu\text{g/ml}$ ), MG132 (10  $\mu\text{M}$ ) or vehicle for 3 h. B, C) Thirty-nine hours after the transfection of myc-tagged CREBRF or the empty vector (pcDNA3.1), Neuro2a cells were treated with the indicated reagents for an additional 3 hours. The expression of the indicated mRNA (A) and protein (B) was detected as described in the Materials and Methods. The values obtained from untreated Neuro2a cells (A) or mock-transfected cells after 6 h of treatment with BFA (C) were considered "1". Each value represents the mean  $\pm$  SEM from 4–5 independent cultures.

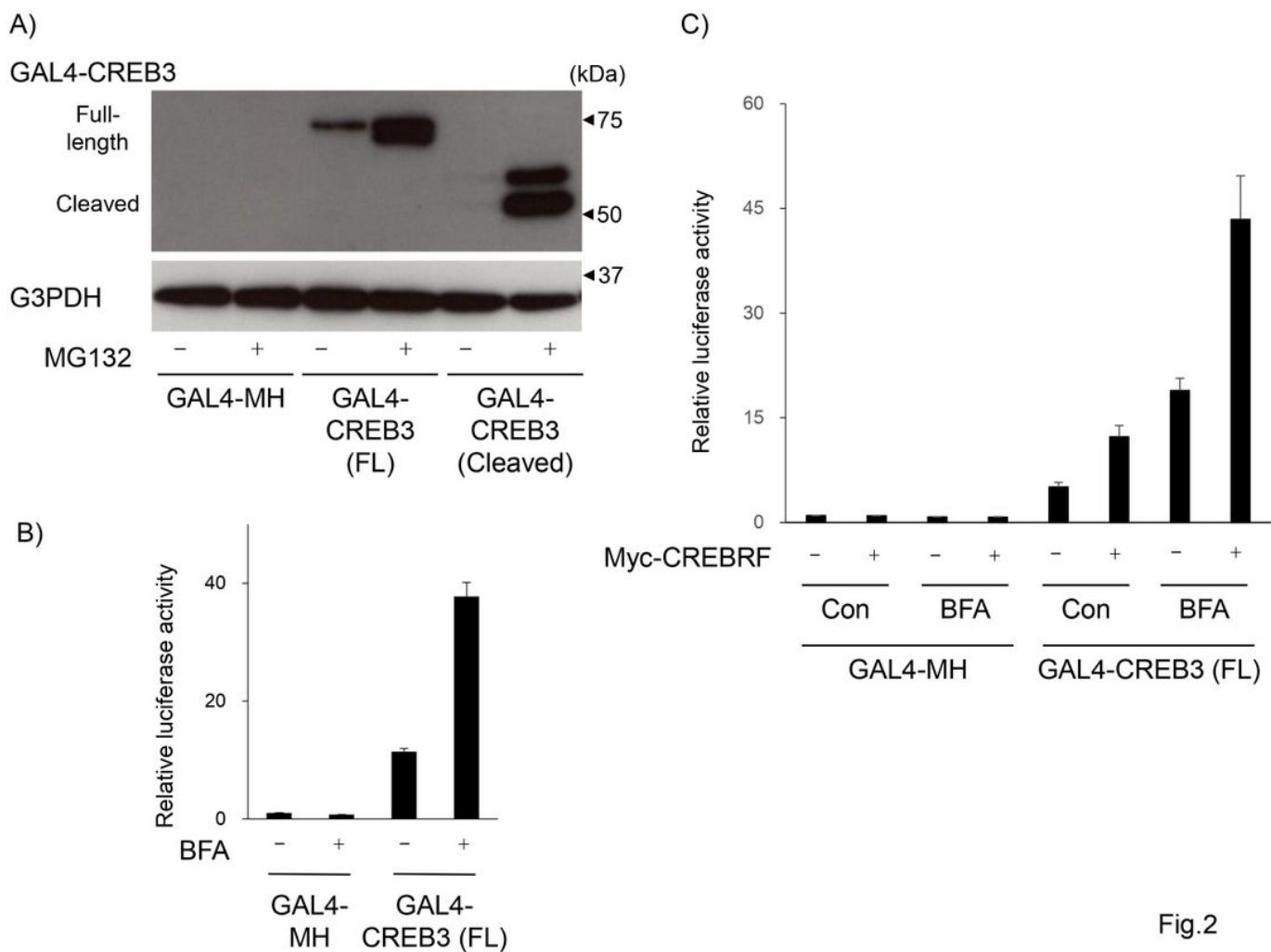
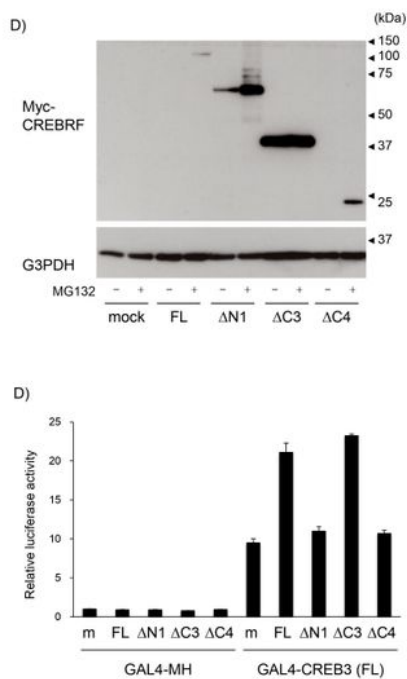
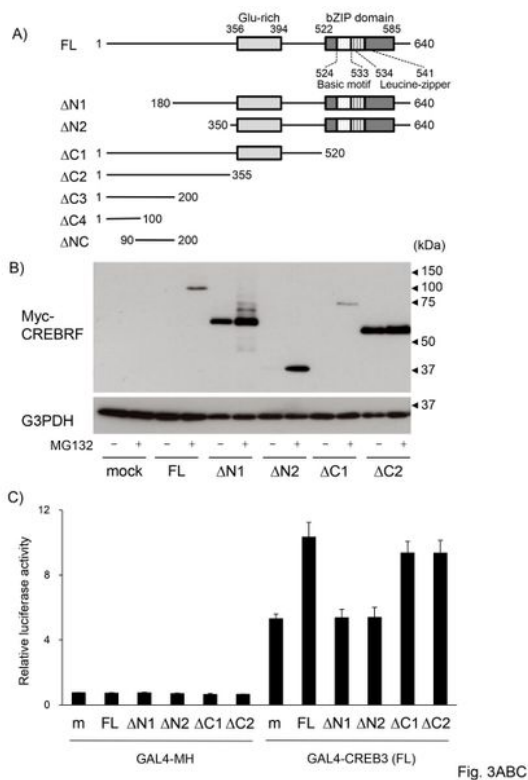


Fig.2

## Figure 2

Effect of CREBRF overexpression on GAL4-tagged full-length CREB3 reporter activities in Neuro2a cells. A) Thirty-nine hours after the transfection of GAL4-tagged CREB3 (full-length (FL) or cleaved forms) or empty vector (mock, GAL4-MH), wild-type Neuro2a cells were treated with MG132 (MG, 10  $\mu$ M) or vehicle for an additional 3 hours. Expression of the indicated protein was detected as described in the Materials and Methods. B) Twenty-four hours after the transfection of GAL4/UAS reporter constructs containing GAL4-tagged full-length CREB3 or GAL4-MH into wild-type Neuro2a cells, the cells were treated with BFA (0.5  $\mu$ g/ml) or vehicle for an additional 18 hours. C) Twenty-four hours after the transfection of the indicated GAL4/UAS reporter constructs together with myc-CREBRF or empty vector into CREB3-deficient cells, the cells were treated with BFA (0.5  $\mu$ g/ml) or vehicle for an additional 18 hours. The cells were lysed, and their luciferase activities were measured as described in the Materials and Methods. The values represent the means  $\pm$  SEM from 3 independent cultures and are expressed relative to the luciferase activity of mock-transfected untreated cells.



### Figure 3

Effect of truncated CREBRF on GAL4-full-length CREB3 reporter activity in Neuro2a cells. A) Schematic representation of the mouse CREBRF constructs used in this study. The myc-epitope was fused at the N-terminus of full-length (FL) CREBRF and the CREBRF deletion mutants. B, D) Thirty-nine hours after the transfection of each myc-tagged CREBRF or the empty vector (mock (m), pcDNA3.1), Neuro2a cells were treated with MG132 (10  $\mu$ M) or vehicle for an additional 3 hours. The expression of the indicated protein

was detected as described in the Materials and Methods. C, E) Forty-two hours after the transfection of the indicated GAL4/UAS reporter constructs together with each myc-CREBRF or the empty vector (mock (m)) into wild-type Neuro2a cells, the cells were lysed, and their luciferase activities were measured as described in the Materials and Methods. The values represent the means  $\pm$  SEM from 3 independent cultures and are expressed relative to the luciferase activity of mock-transfected cells.

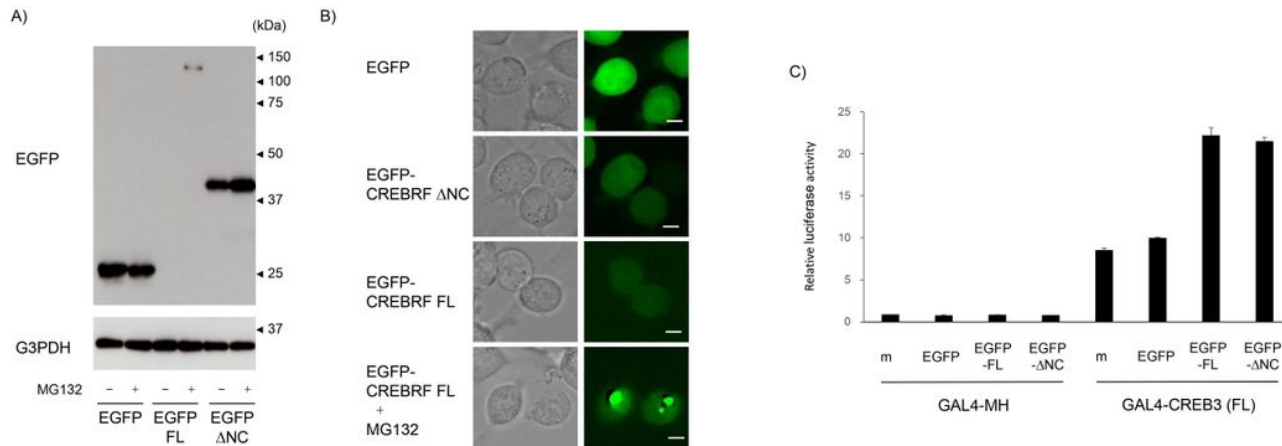


Fig. 4

Fig.4C

## Figure 4

Effect of EGFP-tagged full-length and N-terminal CREBRF on GAL4-full-length CREB3 reporter activity in Neuro2a cells. A) Thirty-nine hours after the transfection of each EGFP-tagged CREBRF (FL or  $\Delta$ NC) or EGFP construct, wild-type Neuro2a cells were treated with MG132 (10  $\mu$ M) or vehicle for an additional 3 hours. The expression of the indicated protein was detected as described in the Materials and Methods. B) Forty-two hours after the transfection of each EGFP-tagged CREBRF (FL or  $\Delta$ NC) or EGFP construct, fluorescent images were observed as described in the Materials and Methods. Cells expressing EGFP-tagged full-length (FL) CREBRF were treated with MG132 (10  $\mu$ M) for an additional 3 hours to monitor its nuclear accumulation. Scale bar is 5  $\mu$ m. C) Forty-two hours after the transfection of the indicated GAL4/UAS reporter constructs together with each EGFP-CREBRF, EGFP or the empty vector (mock (m), pcDNA3.1) into wild-type Neuro2a cells, the cells were lysed, and their luciferase activities were measured as described in the Materials and Methods. The values represent the means  $\pm$  SEM from 3 independent cultures and are expressed relative to the luciferase activity of mock-transfected cells.

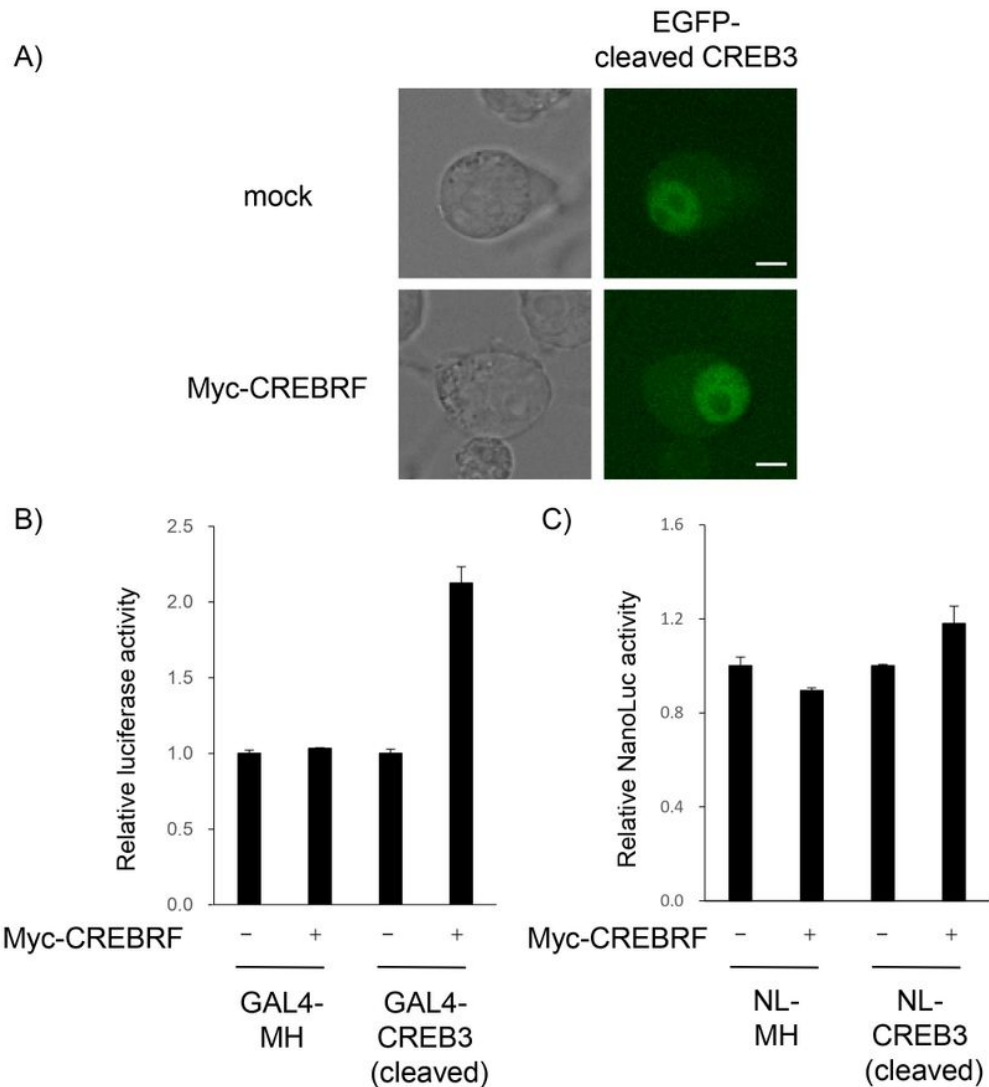


Fig. 5

## Figure 5

Effect of myc-tagged full-length CREBRF on GAL4-tagged cleaved CREB3 reporter activities in Neuro2a cells. A) Forty-two hours after the transfection of each EGFP-tagged cleaved CREB3 together with myc-tagged full-length CREBRF or the empty vector (mock, pcDNA3.1), fluorescent images were observed as described in the Materials and Methods. Scale bar is 5  $\mu$ m. B, C) Forty-two hours after the transfection of the indicated GAL4/UAS reporters (B) or NanoLuc-tagged cleaved CREB3 (C) together with myc-tagged full-length CREBRF or the empty vector (mock, pcDNA3.1) into wild-type Neuro2a cells, the cells were lysed, and their luciferase activities were measured as described in the Materials and Methods. The values represent the means  $\pm$  SEM from 3 independent cultures, and each value in the myc-CREBRF transfected cells was expressed relative to the luciferase activity of each mock-transfected cells, respectively.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 210121CREBRFSupplementaryFigs.pptx