

RESEARCH ARTICLE

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Role of an ER stress response element in regulating the bidirectional promoter of the mouse *CRELD2* - *ALG12* gene pair

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Abstract

Background: Recently, we identified *cysteine-rich with EGF-like domains 2* (*CRELD2*) as a novel endoplasmic reticulum (ER) stress-inducible gene and characterized its transcriptional regulation by ATF6 under ER stress conditions. Interestingly, the *CRELD2* and *asparagine-linked glycosylation 12 homolog* (*ALG12*) genes are arranged as a bidirectional (head-to-head) gene pair and are separated by less than 400 bp. In this study, we characterized the transcriptional regulation of the mouse *CRELD2* and *ALG12* genes that is mediated by a common bidirectional promoter.

Results: This short intergenic region contains an ER stress response element (ERSE) sequence and is well conserved among the human, rat and mouse genomes. Microarray analysis revealed that *CRELD2* and *ALG12* mRNAs were induced in Neuro2a cells by treatment with thapsigargin (Tg), an ER stress inducer, in a time-dependent manner. Other ER stress inducers, tunicamycin and brefeldin A, also increased the expression of these two mRNAs in Neuro2a cells. We then tested for the possible involvement of the ERSE motif and other regulatory sites of the intergenic region in the transcriptional regulation of the mouse *CRELD2* and *ALG12* genes by using variants of the bidirectional reporter construct. With regards to the promoter activities of the *CRELD2*-*ALG12* gene pair, the entire intergenic region hardly responded to Tg, whereas the *CRELD2* promoter constructs of the proximal region containing the ERSE motif showed a marked responsiveness to Tg. The same ERSE motif of *ALG12* gene in the opposite direction was less responsive to Tg. The direction and the distance of this motif from each transcriptional start site, however, has no impact on the responsiveness of either gene to Tg treatment. Additionally, we found three putative sequences in the intergenic region that antagonize the ERSE-mediated transcriptional activation.

Conclusions: These results show that the mouse *CRELD2* and *ALG12* genes are arranged as a unique bidirectional gene pair and that they may be regulated by the combined interactions between ATF6 and multiple other transcriptional factors. Our studies provide new insights into the complex transcriptional regulation of bidirectional gene pairs under pathophysiological conditions.

Background

Among eukaryotes, analyses of the human and mouse genomes revealed that more than 10% of the genes are arranged as bidirectional gene pairs that are separated by less than only 1 kb of genomic DNA [1-3]. Some of these gene pairs could have evolved from a common ancestral gene during its duplication. Other gene pairs,

however, do not have any genetic relationship between each other, and they are thought to play different biological functions within cells. It has been reported that the human *PACPG-PARK2* gene pair [4], the human *PREPL-C2ORF34* gene pair [5], the mouse surfeit *Surf1-Surf2* gene pair [6] and the mouse *Sars2-Mrps12* gene pair [7] are co-regulated by distinctive transcriptional factors such as NRF-2, YY-1 or NF-Y. The transcriptional regulation of many other eukaryotic bidirectional gene pairs, however, remains to be determined.

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Recently, we identified *CRELD2* as a novel ER stress-inducible gene by a microarray analysis of Tg-sensitive genes in Neuro2a cells and characterized the 5'-upstream promoter region of the mouse *CRELD2* gene [8]. Some pathophysiological conditions are reported to disrupt ER functions (e.g., the folding and modifying of newly synthesized transmembrane and secretory proteins) due to an accumulation of unfolded proteins [9,10]. The accumulation of unfolded proteins activates the expression of various genes through three resident ER stress sensors, PERK [11], IRE [12] and ATF6 [13]. The activation of these genes results in various outcomes (e.g., the refolding and/or degradation of accumulated proteins in the ER, and the activation of apoptotic signaling cascades). One of these ER stress sensors, ATF6, directly regulates the transcription of the *CRELD2* gene via the ERSE motif, an ATF6 consensus sequence, located in its promoter [8]. The nucleotide sequence around the ERSE in the *CRELD2* promoter is highly conserved within the mouse, rat and human genes. Interestingly, further genomic analyses reveal that the *ALG12* gene, one of the mannosyltransferase genes [14], is adjacent to the *CRELD2* gene in a head-to-head configuration on the chromosome in some species. In this study, we first investigated the transcriptional regulation of the bidirectional *CRELD2-ALG12* gene pair as ER stress-inducible genes. We especially focused on evaluating the role of the ERSE motif, which is located within the 360-bp intergenic region, in regulating the expression of both genes under ER-stress conditions.

Results

ER stress induced the expression of both *CRELD2* and *ALG12* mRNAs in Neuro2a cells

Microarray analyses revealed that both *CRELD2* and *ALG12* mRNAs are induced in Tg-treated cells as well as *GADD153* [15], *Tib3* [16] and *Herpud1* [17] mRNA, which are known ER stress-inducible genes (Table 1). To verify the Tg-induced expression of *CRELD2* and *ALG12* mRNAs in detail, Neuro2a cells were exposed to 0.1 μ M Tg for 4, 8, or 12 h, and the expression of *CRELD2*, *ALG12*, *GRP78* and *GADD153* mRNAs were

measured by RT-PCR. As shown in Figure 1A, *CRELD2* and *ALG12* mRNAs, as well as *GRP78* [18] and *GADD153* mRNAs, were up-regulated from 4 to 12 h after Tg-treatment. Next we examined the effects of other ER stress-inducing reagents (Tm and BFA), as well as serum withdrawal, on *CRELD2* and *ALG12* mRNA expression in Neuro2a cells. Like Tg treatment, those with Tm and BFA, but not serum withdrawal, induced *CRELD2*, *ALG12*, *GRP78* and *GADD153* mRNA expression similarly (Figure 1B).

Comparison of the intergenic sequences of the *CRELD2-ALG12* gene pair within the mouse, rat and human genes

Next we analyzed the intergenic sequences of the *CRELD2-ALG12* gene pair within the mouse, rat and human genes. As shown in Figure 2, the nucleotide sequence of the mouse gene pair is highly homologous to that of the rat gene pair. The proximal promoter regions of the human and mouse *CRELD2* genes, especially around the ERSE motif, are also well conserved. We then measured the basal promoter activities of the mouse *CRELD2-ALG12* gene pair by using luciferase reporter constructs inserted with either the entire intergenic region (-396 ~ +11) or the intergenic region containing various deletion mutations in either direction. As shown in Figure 3A, reporter constructs containing the entire intergenic region in either direction (+11/-396; *CRELD2* promoter and -396/+11; *ALG12* promoter) showed the higher basal promoter activity. The activity of *ALG12* promoter (-211/+11) is still high in the absence of the ERSE motif, however a further deletion from position -211 to -108 in this promoter remarkably decreased its basal activity in Neuro2a cells. Furthermore, a deletion from position -136 to -228 in the *CRELD2* promoter dramatically decreased *CRELD2* promoter activity even though the ERSE motif is present. The deletion of a region around the ERSE motif (-229 ~ -254) further decreased the promoter activity.

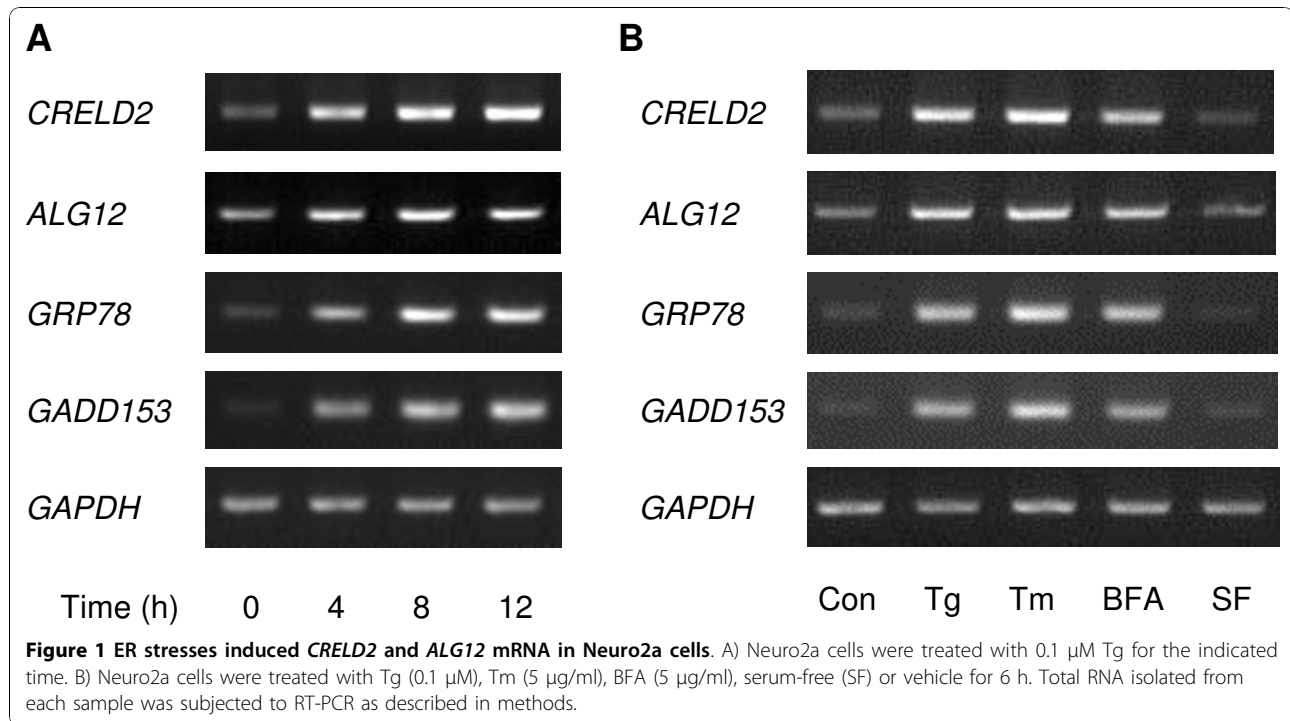
The role of the ERSE motif in *CRELD2* and *ALG12* promoter activities under ER stress condition

As shown in Figure 3B, the mouse *CRELD2* promoter containing the proximal region (-229 ~ -396), but no

Table 1 Tg-inducible genes in Neuro2a cells

Genes	Name	RefSeq No.	Fold induction	
			4 h	8 h
<i>ddit3</i> (<i>GADD153</i>)	DNA-damage inducible transcript 3	NM_007837	6.1	9.8
<i>trib3</i>	tribbles homolog 3	NM_175093	3.9	7.2
<i>herpud1</i>	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	NM_022331	4.4	6.0
<i>creld2</i>	cysteine-rich with EGF-like domains 2	NM_029720	2.9	4.3
<i>alg12</i>	asparagine-linked glycosylation 12 homolog	NM_145477	3.6	3.7

Neuro2a cells were treated with or without 0.1 μ M Tg for 4 or 8 h. Total RNA was isolated from each cells and subjected to identify Tg-inducible genes using microarray analysis as described in methods. Representative genes are shown as ratio (Tg-treated cells/control cells).



deletion mutation construct of mouse *ALG12* promoter, was significantly activated by Tg-treatment. Consistent with our previous report, the *CRELD2* promoter construct containing the longer intergenic region (-136 ~ -396) showed higher basal promoter activity but a lower responsiveness to Tg compared to the above-mentioned construct (-229/-396) (Figure 3B). The *CRELD2* promoter without the ERSE motif (-255/-396) had an even further diminished basal promoter activity and Tg-responsiveness. Next, we determined the effect of various mutations within the ERSE motif on the activity of the mouse *ALG12* promoter. Unlike the *CRELD2* promoter (-229/-396) and its point mutation constructs (-229/-396 m1 or m2; a mutation at the ATF6 or NF-Y binding site [8,13,18-20], respectively), the mutations in the *ALG12* promoter (-361/+11 m1 and m2) did not affect the basal promoter activity and the responsiveness to Tg (Figure 4). Then, we evaluated the effect of the ERSE motif's direction on the responsiveness of the mouse *CRELD2-ALG12* gene pair to Tg by using a pGL3 vector containing the SV40-promoter. The reporter constructs containing a partial intergenic region of the gene pair (-343 ~ -212) in either direction responded to Tg and ATF6-overexpression similarly (Figure 5A and 5B). Interestingly, Tg-treatment and ATF6-overexpression stimulated the luciferase activity of the *CRELD2* promoter (-229/-396) construct more effectively than the *ALG12* promoter (-361/+11) construct (Figure 5C and 5D).

To study the unresponsiveness of the *ALG12* promoter (-361/+11) to Tg, we prepared another reporter construct in which the middle intergenic region of the *ALG12* promoter (-211 ~ -108) that contributes to the basal promoter activity is deleted (Figure 3A). This construct (-343/-212 & -107/+11), however, did not respond to Tg (Figure 6A). Serial deletions of the 3'-end of the *ALG12* promoter lacking the middle intergenic region revealed that there is a suppressive site from position -75 to -16 in the *ALG12* promoter (Figure 6B). Deletion around three putative Ets family binding sites (TTCC) [21,22] from position -52 to -20 (Figure 2, site III) in the *ALG12* promoter (-343/-212 & -107/+11 m1) also restored responsiveness to Tg. Yet, this same site III deletion in the *ALG12* promoter containing the middle intergenic region (-361/+11 m3) showed unresponsiveness to Tg (Figure 6C). To determine if there are other suppressive sites in this intergenic region, we prepared various deletion mutation constructs of the *ALG12* promoter and evaluated their responsiveness to Tg. As shown in Figure 7A, we identified two additional suppressive sites (I and II in Figure 2). We also found that the deletion of all three sites (-361/+11 m7; *ALG12* promoter) was required in order to restore the responsiveness to Tg. A mutation in either the NF-Y binding site (CCAAT) of the ERSE motif (-361/+11 m8) or a site 8-bp downstream of the ERSE motif (-361/+11 m9) in the *ALG12* promoter showed that each NF-Y binding site partially participated in its basal promoter activity.

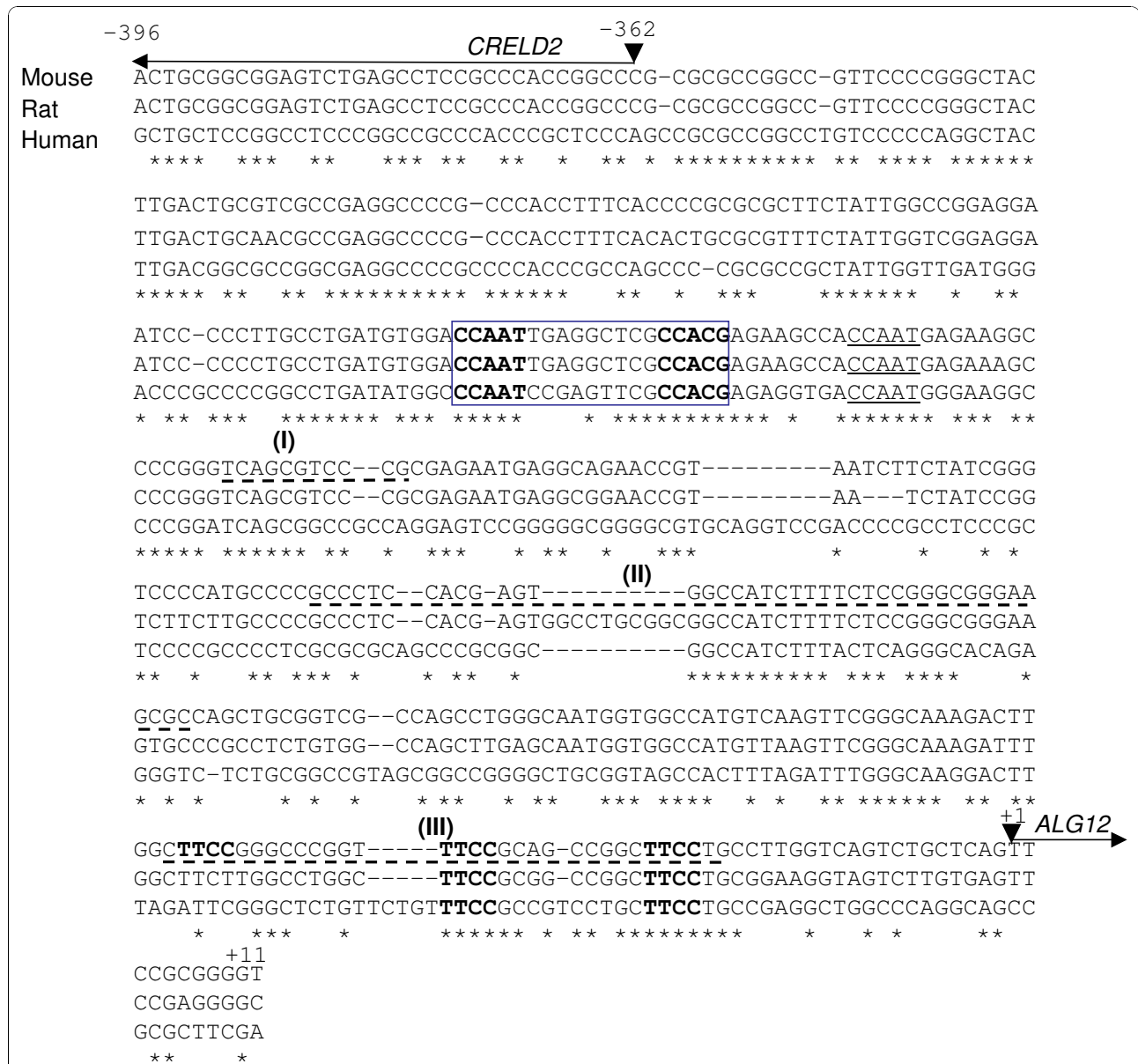


Figure 2 Nucleotide sequences of the mouse, rat and human CRELD2-ALG12 gene pairs. Nucleotide sequences conserved among the mouse, rat and human CRELD2-ALG12 gene pairs are shown with asterisks. A conserved ERSE motif and an adjacent NF-Y binding site are shown in a box and underline, respectively. The transcriptional direction and putative transcriptional start sites of the mouse CRELD2 and ALG12 genes are shown with arrows and arrowheads, respectively. Three suppressive regions identified in this study are shown with broken lines (sites I, II and III). Putative binding sequences of the Ets family (TTCC) in site III are shown with bold types.

Only the site in the ERSE motif in the ALG12 promoter (-361/+11 m8), however, are crucial to the responsiveness to Tg as well as the CRELD2 promoter (-209/+396 m1) (Figure 7B and 7C). Finally, we measured the promoter activity of the entire intergenic region of the CRELD2-ALG12 gene pair in the both direction (-396/+11; CRELD2 and ALG12 promoters) after Tg treatment or ATF6 cotransfection. Both promoter constructs only slightly responded to Tg, but the deletion of the three suppressive regions (+11/-396 m1; CRELD2 promoter

and -396/+11 m1; ALG12 promoter) restored responsiveness to Tg. Furthermore, the basal promoter activities of these mutant constructs markedly decreased (Figure 8A). ATF6-overexpression enhanced the promoter activity of all of the above-mentioned constructs. The Tg-responsive reporter constructs (+11/-396 m1 and -209/-396; CRELD2 promoters and -396/+11 m1; ALG12 promoter) also showed a further increase in their promoter activities by ATF6-overexpression (Figure 8B).

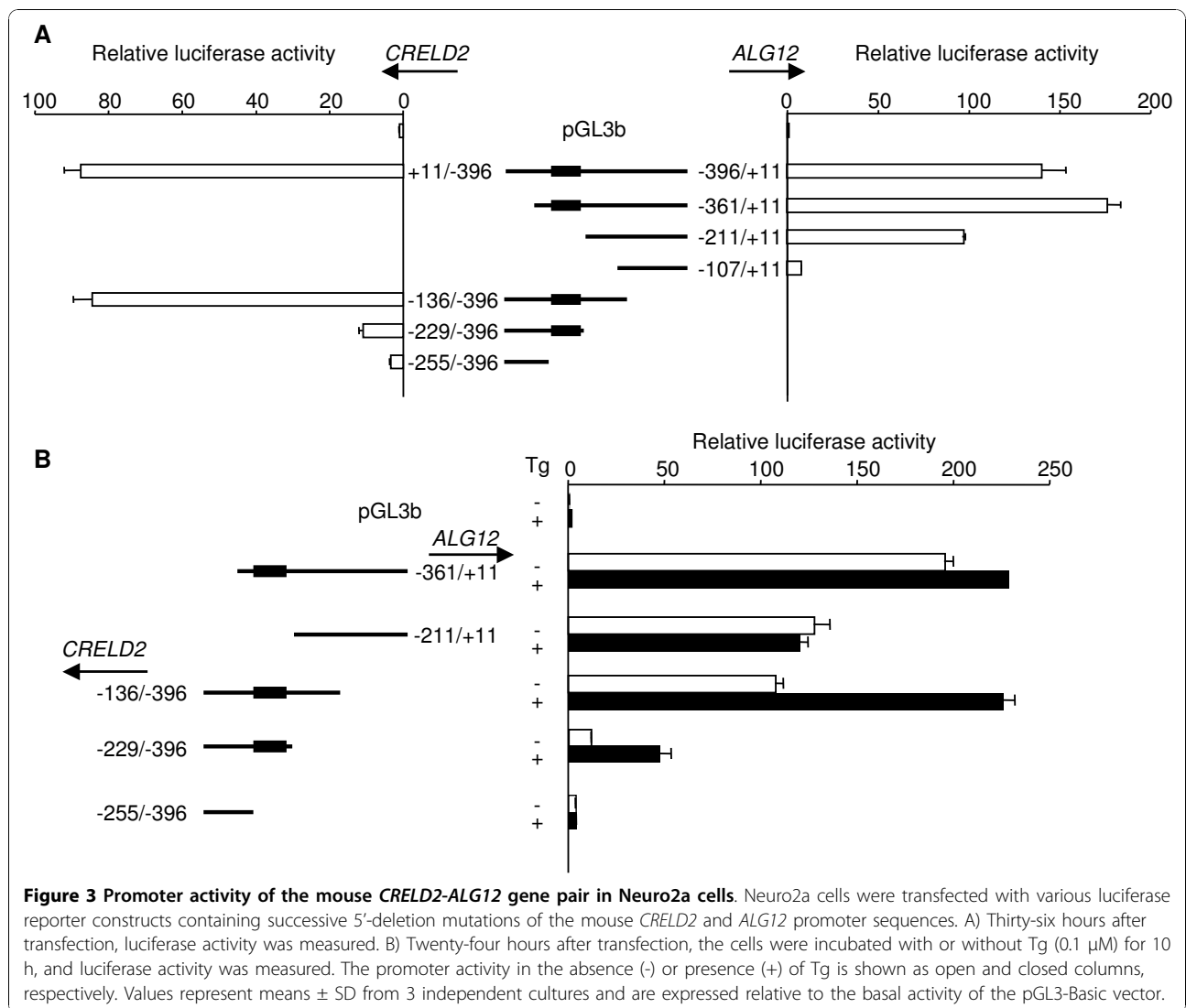


Figure 3 Promoter activity of the mouse *CRELD2-ALG12* gene pair in Neuro2a cells. Neuro2a cells were transfected with various luciferase reporter constructs containing successive 5'-deletion mutations of the mouse *CRELD2* and *ALG12* promoter sequences. A) Thirty-six hours after transfection, luciferase activity was measured. B) Twenty-four hours after transfection, the cells were incubated with or without Tg (0.1 μ M) for 10 h, and luciferase activity was measured. The promoter activity in the absence (-) or presence (+) of Tg is shown as open and closed columns, respectively. Values represent means \pm SD from 3 independent cultures and are expressed relative to the basal activity of the pGL3-Basic vector.

Discussion

Recently, we identified *CRELD2* as a novel ER-stress inducible gene and characterized its ATF6-dependent transcriptional regulation using constructs containing the proximal region of the mouse *CRELD2* promoter [8]. Genomic analyses reveal that the *ALG12* gene [14] is adjacent to the *CRELD2* gene in a head-to-head configuration on the chromosome in some species. *CRELD2* and *ALG12* genes are a bidirectional gene pair arranged less than 400 bp apart. The nucleotide sequences of this intergenic region are moderately conserved among the mouse, rat and human genes. Furthermore, those regions around an ERSE motif in the *CRELD2-ALG12* gene pair are highly conserved (Figure 2). In this study, we demonstrate that the expression of *CRELD2* and *ALG12* mRNAs, and *GRP78* and *GADD153* mRNAs, which are well known ER stress-inducible genes, was induced by three distinct ER stress inducers (Figure 1). In regards to

the promoter activity of the mouse *CRELD2-ALG12* gene pair, only the *CRELD2* promoter containing just the proximal region (-229/-396 and -209/-396; *CRELD2* promoter) significantly responded to Tg. Additionally, the *CRELD2* promoter containing the full intergenic region (+11/-396; *CRELD2* promoter) decreased in responsiveness to Tg, whereas its basal promoter activity markedly increased. In contrast, the *ALG12* promoters only slightly responded to Tg even though some of the reporters contained the ERSE motif, which is 300-bp apart from the transcription start site of the mouse *ALG12* gene. The direction of the ERSE motif and its distance from each of the transcription start sites for the mouse *CRELD2* or *ALG12* genes, however, appear to have no influence in these findings. Therefore, it seems that the full intergenic region contains one or more unknown suppressive sites that interfere with the ERSE-mediated enhancement of the *ALG12* and *CRELD2* promoter activities.

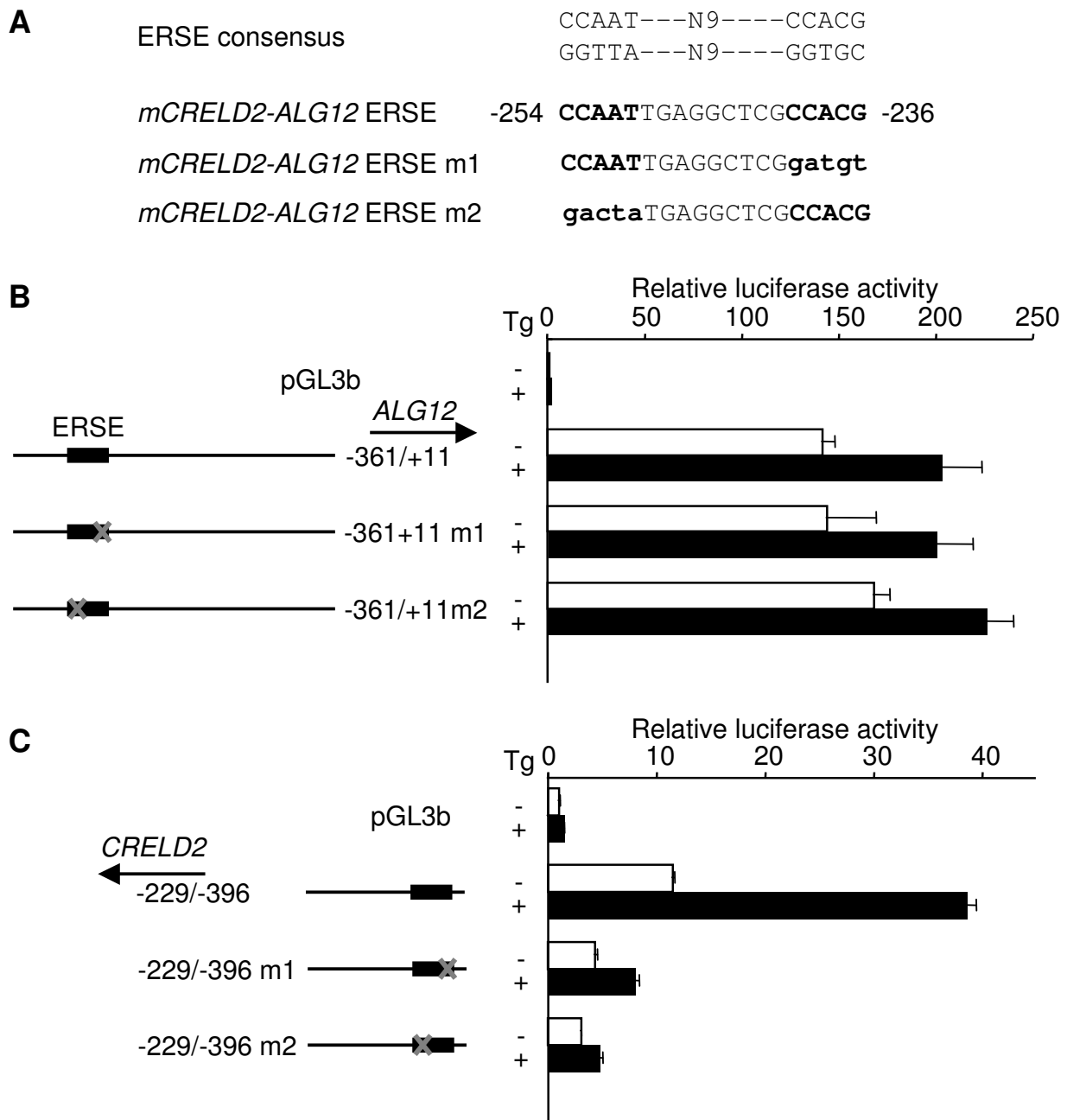


Figure 4 Role of the ERSE motif in mouse *CRELD2* and *ALG12* promoter activities in Neuro2a cells. A) The nucleotide sequence of a common ERSE motif in the mouse *CRELD2-ALG12* gene is shown. NF-Y and ATF6 binding sequences in the ERSE motif and their mutated sequences are shown with bold types and small letters, respectively. Neuro2a cells were transfected with the indicated *CRELD2* or *ALG12* reporter constructs. Twenty-four hours after transfection, the cells were incubated with or without Tg (0.1 μ M) for 10 h (B and C). After incubation, the cells were lysed and luciferase activity was measured as described in methods. The activities obtained from control and Tg-treated cells are shown as open and closed columns, respectively. Values represent means \pm SD from 3 - 4 independent cultures and are expressed relative to the basal activity of the pGL3-Basic.

Reporter constructs used in this study contain 5'-untranslated regions (5'-UTR) of *CRELD2* and/or *ALG12* gene. Especially, reporter constructs containing the entire intergenic region of *CRELD2* - *ALG12* gene pair contain the UTR regions at both ends. However,

the deletion of three suppressive sites (sites I, II and III) in each construct recovered the responsiveness to Tg (-343/-212 - -107/+11 m1 in Figure 6C and -361/+11 m7, +11/-396 m1 and -396/+11 m1 in Figure 7A and 8A). Therefore, it seems likely that each 5'-UTR hardly

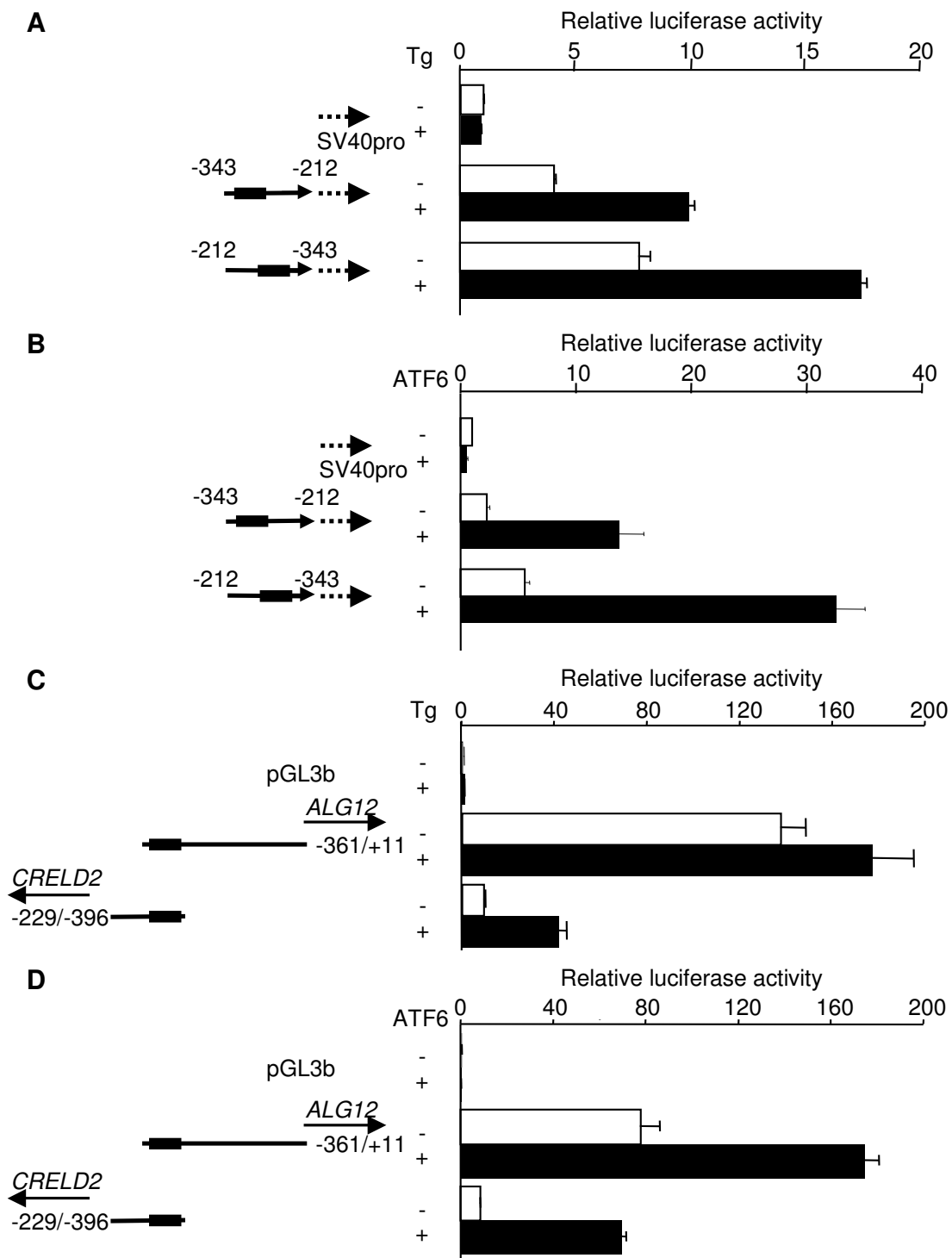


Figure 5 Role of nucleotide sequence around the ERSE motif in mouse *CRELD2* and *ALG12* promoters in regulating the transcriptional activity of both genes. Neuro2a cells were transfected with the indicated *CRELD2* or *ALG12* reporter constructs. Twenty-four hours after transfection, the cells were incubated with or without Tg (0.1 μ M) for 10 h (A and C). The expression vector for ATF6 or an empty vector (mock) was co-transfected with each reporter construct into Neuro2a cells and cultured for 36 h (B and D). After incubation, the cells were lysed and luciferase activity was measured as described in methods. Control and Tg-treated or ATF6-stimulated activities are shown as open and closed columns, respectively. Values represent means \pm SD from 3 - 4 independent cultures and are expressed relative to the basal activity of the pGL3-Promoter (A and B) and pGL3-Basic (C and D).

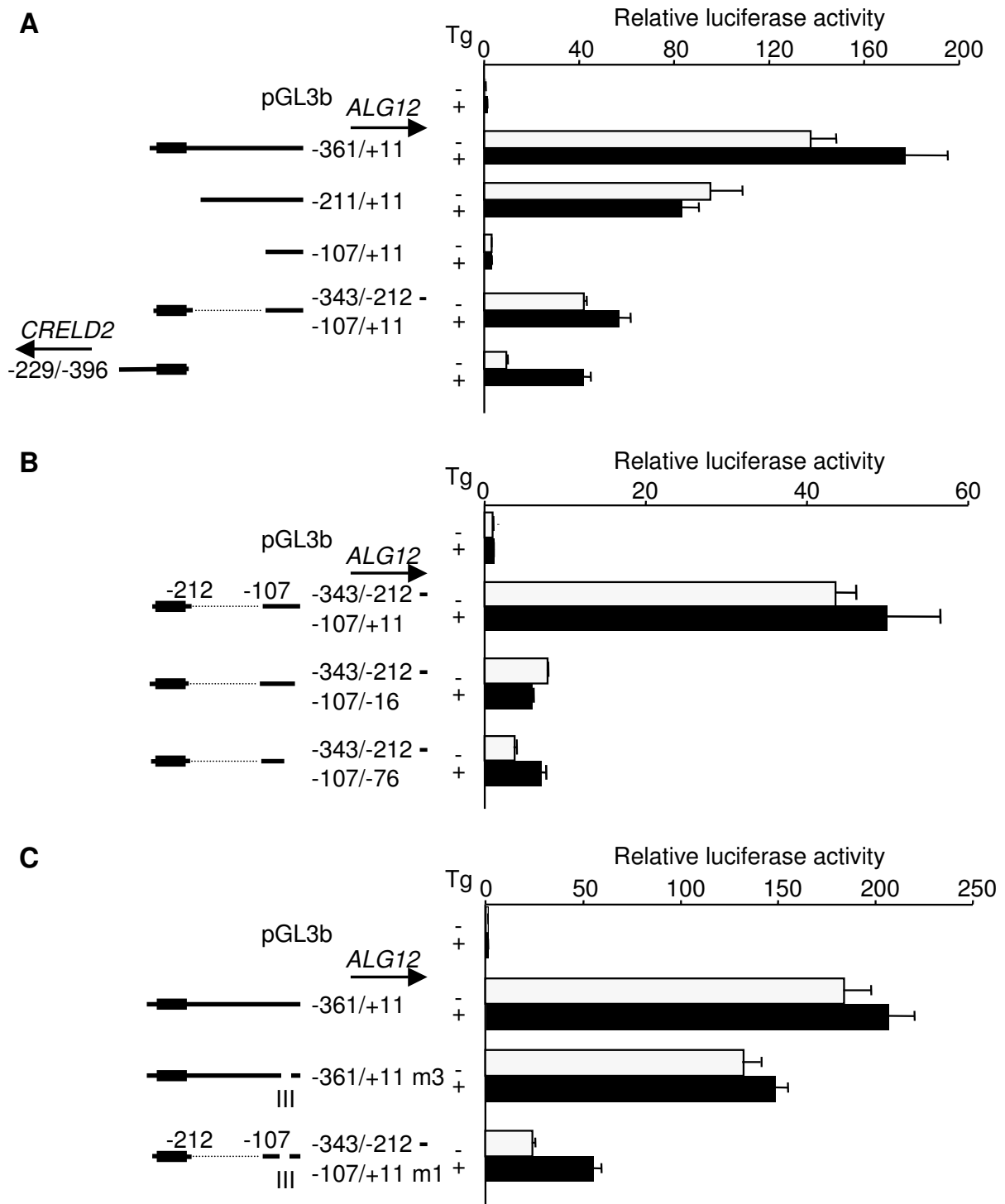
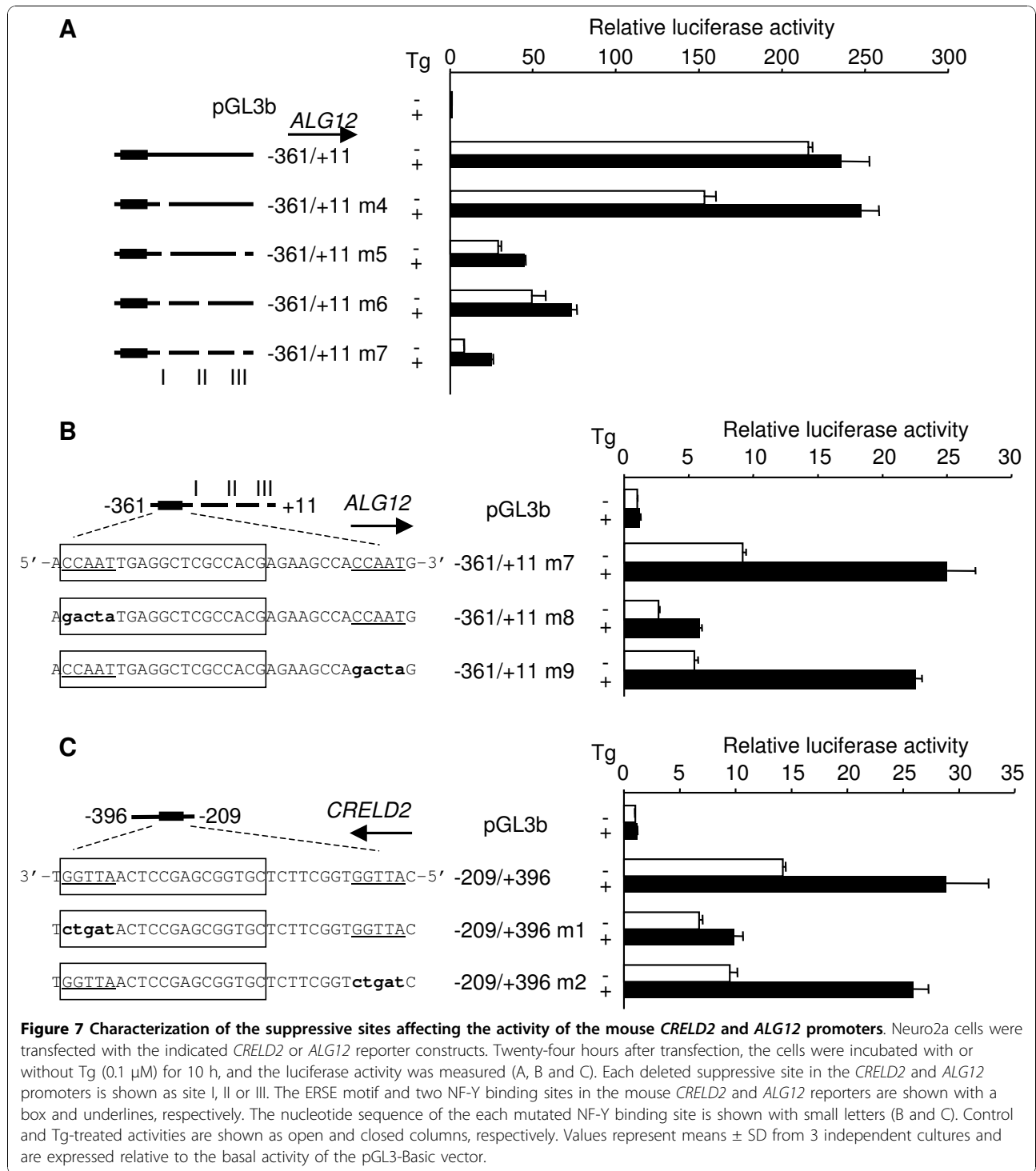


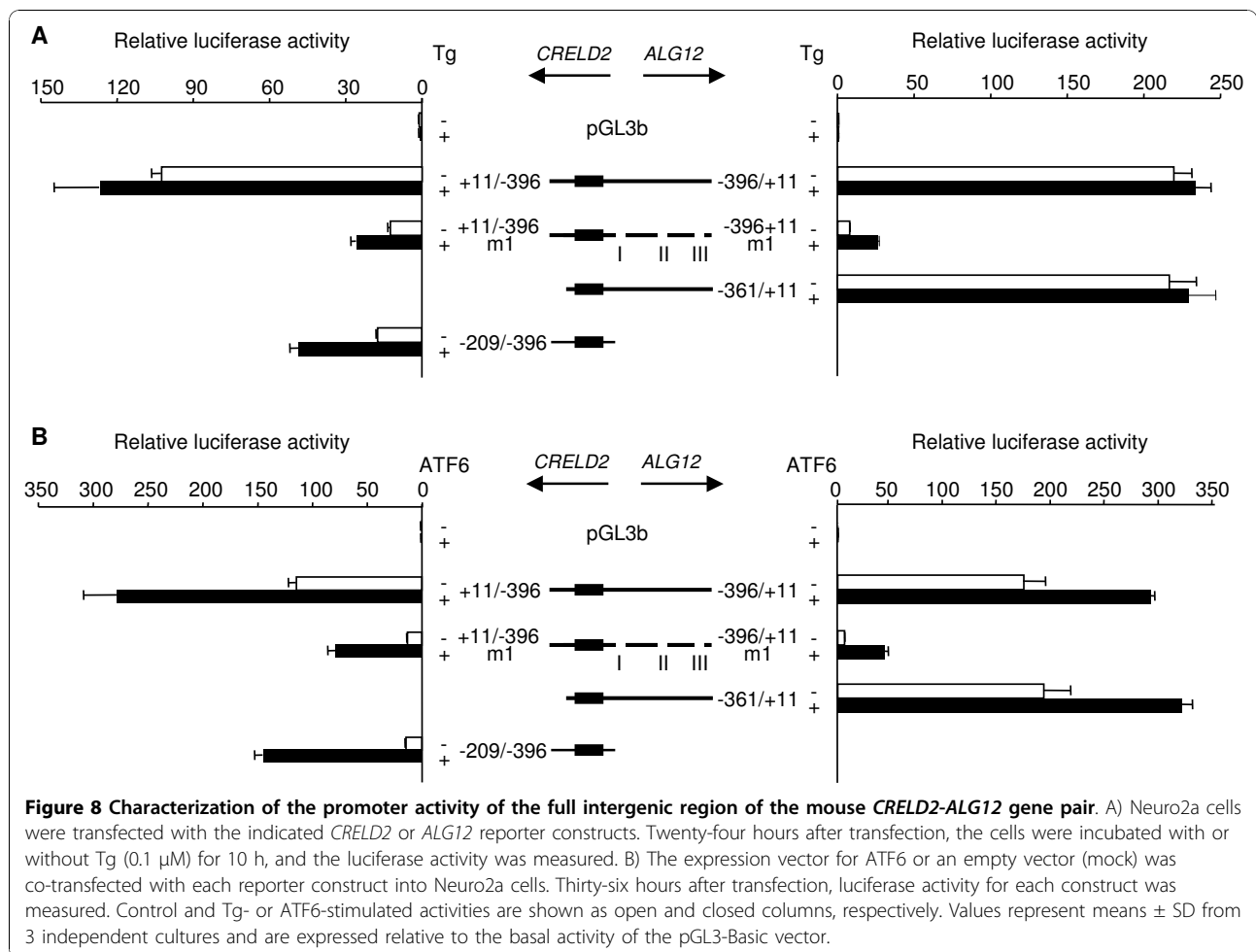
Figure 6 Role of the proximal region of the mouse *ALG12* promoter in regulating the transcriptional activity of the gene. Neuro2a cells were transfected with the indicated *CRELD2* or *ALG12* reporter constructs. Twenty-four hours after transfection, the cells were incubated with or without Tg (0.1 μ M) for 10 h, and luciferase activity was measured. A deleted site in the most proximal region of the *ALG12* promoter is shown as site III. Control and Tg-treated activities are shown as open and closed columns, respectively. Values represent means \pm SD from 3 independent cultures and are expressed relative to the basal activity of the pGL3-Basic vector (A, B and C).



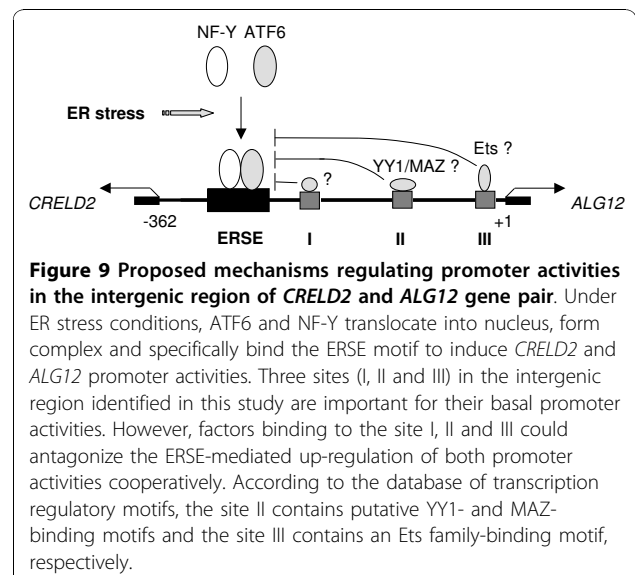
influenced the corresponding promoter activity of the CRELD2 and ALG12 promoter constructs in our assay system. CRELD2 (NM_029720.2) and ALG12 (NM_145477.1) genes possess 5'-UTR (66 and 55 bp) and 3'-UTR (245 and 584 bp) respectively though their effects on transcription are not elucidated yet. Further

characterization of these regions would reveal regulations of CRELD2 and ALG12 mRNA expression.

Using various deletion mutation constructs, we showed that three suppressive sites in the CRELD2-ALG12 gene pair play a crucial role in interfering with Tg responsiveness. Interestingly, the deletion of all three



of these suppressive sites was required in order to restore the responsiveness to Tg (Figure 2, 7 and 8). These results imply that these suppressive sites are not only important in maintaining basal promoter activity, but that they synergistically counteract the ERSE-mediated transcriptional activity (Figure 9). Among these sites, the most proximal to the *ALG12* promoter contains a conserved response element that Ets-family transcriptional factors recognize [21]. Ets transcription factors consist of approximately 30 family members and share a highly conserved DNA-binding domain. It has been reported that these factors are involved in regulating a variety of biological processes including development, differentiation and inflammation [21,22]. In the site II, there are putative YY1- (GCCATC) [23] and MAZ- (CCCCGCCCT) [24] binding sites judged from some databases such as SwissRegulon (a database of genome-wide annotations of regulatory sites), but the precise roles remain to be determined. On the contrary,



we are unable to find any unique sequences in the sites I. Further studies characterizing each of these suppressive sites (site I, II, and III) are required in order to understand the complex transcriptional regulation of the *CRELD2-ALG12* gene pair. Jones PL *et al.* reported that murine manganese superoxide dismutase gene is regulated through a complex intronic enhancer involving C/EBP- β and NF- κ B [25]. Donati G *et al.* demonstrated that ER stress triggers dynamic modification of chromatin components and transcriptional factors under ER stress [20]. Therefore, we should focus on other aspects such as local chromatin remodeling and histone modifications (e.g., phosphorylation, acetylation and methylation of histones) within the *CRELD2* and *ALG12* genes in addition to the 5'-flanking sequences in this intergenic region. Furthermore, other approaches should be employed to elucidate the discrepancy between the expression levels of both intrinsic mRNAs and the promoter activities of their full intergenic region under ER stress conditions.

Among the bidirectional gene pairs characterized in mammalian cells, *Surf1-Surf2* [6], *Reql4-Lrrc14* [26], *PDCD10-SERPINI1* [27] and *Thox-DUOXA* [28] gene pairs seem to share their intergenic region equally because mutations in the transcription factor binding sites decline those promoter activities equally. In contrast, the transcriptional regulations of *C2ORF34-PREPL* [5], *Sarsm-Mrps12* [7] and *HAND2-DEIN* [29] are asymmetric. According to the present study, the transcriptional regulatory pattern of the mouse *CRELD2-ALG12* gene pair belongs to the latter group. Analyses of these bidirectional gene pair sharing a common intergenic region have mostly consisted of characterization without any stimuli. Recently, Zanutto E *et al.* reported that the *Sarsm-Mrps12* promoter activity is modulated by mitochondrial stresses, especially mitochondrial reactive oxygen species, in a complex manner [30]. At this time, however, the significance and relevance of many bidirectional gene pairs under pathophysiological conditions are not well understood.

The mammalian *ALG12* gene is the ortholog of the yeast gene that encodes the dolichyl-P-Man₇-GlcNAc₂-PP-dolichyl α 6-mannosyltransferase, and its mutation causes a congenital disorder affecting glycosylation in the ER [14,31,32]. Clinically, a child suffering from a point mutation in the *ALG12* gene has been reported to show severe symptoms such as psychomotor retardation, hypotonia, growth retardation, dysmorphic features and anoxia [31]. Sequential protein glycosylation in the ER is important in maintaining the quality control of glycoproteins through folding and ER-associated protein degradation. Moreover, its defects could also interfere with the intracellular trafficking and secretion of glycoproteins. Therefore, suitable regulation of

the *ALG12* gene should be required in order to maintain ER homeostasis.

As the CRELD proteins have multiple EGF-like domains, they are considered to be cell adhesion molecules [33-35]. It has been reported that missense mutations in the *CRELD1* gene increases an individual's susceptibility to atrioventricular septal defects [33], but the physiological roles of these family members remain poorly understood. In contrast to *CRELD1*, *CRELD2* lacks a transmembrane domain in the C-terminal region. Ortiz *et al.* reported that the overexpression of *CRELD2* impairs the membrane transport of acetylcholine receptor α 4/ β 2 in *Xenopus laevis* oocytes [36]. We recently demonstrated that the *CRELD2* gene is one of the downstream targets of ATF6 and that its product is predominantly localized in the ER-Golgi apparatus [8]. Interestingly, the mouse model for multiple epiphyseal dysplasia, which specifically expresses a mutation in matrilin-3, was reported to induce *CRELD2* mRNA expression and other ER-stress inducible genes as the symptoms progressed [37]. According to these reports, *CRELD2* seems to be involved in the folding, processing and transport of some proteins under pathophysiological conditions, though the precise role of *CRELD2* remains to be determined. Furthermore, we believe that the sharing of the ERSE motif in the *CRELD2-ALG12* gene pair may be advantageous in regulating ER homeostasis under various ER-stress conditions, even though it is unlikely that the *CRELD2* and *ALG12* proteins function by directly interacting with each other.

Conclusion

In this study, we first demonstrate that both the *CRELD2* and *ALG12* genes, which form a bidirectional gene pair, are potent ER stress-inducible genes. Our present results indicate that the *CRELD2-ALG12* gene pair could be asymmetrically regulated by multiple transcriptional factors in addition to ATF6. Because the *CRELD2-ALG12* gene pair contains an evolutionally conserved ERSE motif, the cooperative induction of these genes may play important roles in confronting ER stresses and in appropriately regulating ER homeostasis and cell fates, together with other ER stress-inducible genes (e.g., *GRP78* and *GADD153*). Therefore, further characterization of the *CRELD2-ALG12* gene pair may provide new insights into the complex transcriptional regulation of ER stress-inducible genes as well as into the onset and progression of various ER stress-associated diseases.

Methods

Cell culture and treatment

Neuro2a cells were maintained in Dulbecco's Modified Eagles minimum essential Medium containing 8% fetal bovine serum. Transfection of each construct used in

this study was performed using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's instructions. For stimulation, Neuro2a cells were treated with Tg (0.1 μ M), Tm (5 μ g/ml), BFA (5 μ g/ml) or serum-free medium (SF) for the indicated time.

Construction of plasmids

For preparation of reporter constructs for the mouse *CRELD2* and *ALG12* promoters, genomic DNA from Neuro2a cells was extracted, and the mouse *CRELD2* and *ALG12* promoters were amplified by polymerase chain reaction (PCR) and cloned into the pGL3-Basic vector (Promega). To evaluate the promoter activity of the intergenic region of the mouse *CRELD2* and *ALG12* genes, the position of the putative transcriptional start site of mouse *CRELD2* or *ALG12* is defined as -362 and +1, respectively. The promoter region was defined using a database of the NIH full-length cDNA project and RIKEN functional annotation of a full-length mouse cDNA collection (FANTOM). To characterize the enhancer activity of the partial intergenic region containing ERSE (-343 ~ -212), it was inserted into the pGL3-Promoter vector (Promega). We also constructed various other bidirectional reporter construct carrying point and deletion mutations. Mouse ATF6 was amplified by PCR using cDNA from Neuro2a cells and cloned into the pFlag-CMV vector.

Reverse transcription polymerase chain reaction (RT-PCR)

To estimate the expression level of each gene by RT-PCR, total RNA was extracted from cells lysed with Trizol and converted to cDNA by reverse transcription using random ninemers to prime superscript III RNase⁻ reverse transcriptase (RT) (Invitrogen) as previously described [8]. Specific cDNAs were mixed and amplified with a PCR reaction mixture (Taq PCR kit, Takara). The RT-PCR primers used in this study were as follows: *CRELD2* sense primer 5'-ACTGAAGAAGGAGCACCCCAAC-3', *CRELD2* antisense primer 5'-GCACACTCATCCACATC-CACACA-3', *ALG12* sense primer 5'-GTGATTTCTG-GACTCTGGAC-3', *ALG12* antisense primer 5'-GGGGT ATGAAGAGAAGGCTGCA-3', *GADD153* sense primer 5'-GAATAACAGCCGGAACCTGA-3', *GADD153* antisense primer 5'-GGACGCAGGGTCAAGAGTAG-3', *GRP78* sense primer 5'-ACCAATGACCAAAACCGCCT-3', *GRP78* antisense primer 5'-GAGTTTGCTGATAAT TGGCTGAAC-3', *GAPDH* sense primer 5'-ACCACAG TCCATGCCATCAC-3', *GAPDH* antisense primer 5'-TC CACCACCCTGTTGCTGTA-3'. The typical reaction cycle conditions were 30 sec at 96°C, 30 sec at 60°C and 30 sec at 72°C. The results represent 18 ~ 33 cycles of amplification, after which cDNAs were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. Experiments were repeated to confirm reproducibility.

GeneChip analysis

After Neuro2a cells were incubated in the absence or presence of Tg for the indicated time, total RNA was extracted as described in the above methods. After measuring the quantity and quality of the RNA, biotin-labeled cRNAs were generated from 5 μ g of each total RNA using a GeneChip[®] One-Cycle Target Labeling and Control Reagents package (Affymetrix) according to the manufacturer's protocol. Afterwards, 15 μ g of the purified cRNAs were mixed with 3 nM Control Oligo B2, and the hybridization cocktail was denatured at 99°C for 5 min in a heat block, followed by incubation at 45°C for 5 min, and centrifugation for 5 min in order to remove any insoluble material. Hybridization to a mouse DNA array (GeneChip[®] Mouse Genome 430 2.0 Array) was carried out at 45°C for 16 h using a hybridization oven 640 (Affymetrix). After hybridization, the arrays were washed and stained with the GeneChip[®] Hybridization Wash and Stain Kit (Affymetrix) using the GeneChip[®] Fluidics Station 450 (Affymetrix) according to the manufacturer's protocol. The signal intensities were quantified using a GeneArray Scanner 3000 (Affymetrix), and the raw data obtained were converted into MAS (Microarray Suite) files using the GeneChip[®] Operating Software (GCOS). After normalization, the identification of the temporal expression patterns of genes was performed using the Spotfire[®] DecisionSite. In this analysis, the mean signal intensity of gene expression in each group included in the study (three samples/group) was used. As a selection criteria to present only the most relevant genes, a cutoff of a 2.0-fold increased/decreased expression and a $p < 0.01$ were arbitrarily chosen.

Reporter gene assay

Reporter constructs and the pRL-TK vector, an internal control, were transfected into Neuro2a cells in a 48-well plate. Twenty-four hours after transfection, the cells were treated with Tg (0.1 μ M) or vehicle for 10 ~ 12 h. To determine the effects of ATF6 on reporter activity, the ATF6 expression vector or empty vector (mock) was co-transfected with the reporter construct into the cells and cultured for 36 h. After incubation under each condition, the cells were lysed and the luciferase activity in each lysate was measured using a Dual-Luciferase assay system (Promega). Reporter activity in each lysate was normalized to the co-transfected Renilla luciferase activity, and the results are shown as relative luciferase activity.

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Authors' contributions

KO conceived of this study, carried out the molecular genetics studies, participated in the sequence alignment and drafted the manuscripts. HK, SI and KS participated in microarray analysis. YH and KK participated in its design and coordination. All authors read and approved the final manuscript.

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