

1 **The ATF6 β -calreticulin axis promotes neuronal survival under**
2 **endoplasmic reticulum stress and excitotoxicity**

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19 **Running title:** Neuroprotective role of the ATF6 β -calreticulin axis

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1 **Abstract**

2 While ATF6 α plays a central role in the endoplasmic reticulum (ER) stress
3 response, the function of ATF6 β is largely unknown. Here, we demonstrate that ATF6 β is
4 highly expressed in the hippocampus of the brain, and specifically regulates the expression
5 of calreticulin, a molecular chaperone in the ER with a high Ca^{2+} -binding capacity.
6 Calreticulin expression was reduced to ~50% in the central nervous system of *Atf6b*^{-/-}
7 mice, and restored by ATF6 β . Analysis using cultured hippocampal neurons revealed that
8 ATF6 β deficiency **reduced Ca^{2+} stores in the ER and** enhanced ER stress-induced death,
9 which was rescued by **ATF6 β , calreticulin, Ca^{2+} -modulating reagents such as BAPTA-**
10 AM and 2-APB, and ER stress inhibitor salubrinal. *In vivo*, kainate-induced neuronal
11 death was enhanced in hippocampi of *Atf6b*^{-/-} and *Calr*^{+//-} mice, and restored by 2-APB and
12 salubrinal. These results suggest that the ATF6 β -calreticulin axis plays a critical role **in**
13 **the neuronal survival by improving Ca^{2+} homeostasis under ER stress.**

14

1 **Introduction**

2 The endoplasmic reticulum (ER) is an intracellular organelle in which secretory
3 proteins and lipids are synthesized, and intracellular Ca^{2+} is stored. However, recent
4 studies demonstrated that a stress response occurs in the ER. When cells are exposed to
5 specific conditions such as impaired Ca^{2+} homeostasis, energy shortage, and increased
6 protein synthesis, unfolded proteins accumulate in the ER, leading to a condition generally
7 termed ER stress (Bukau *et al.*, 2006). In the central nervous system (CNS), pathological
8 situations such as brain ischemia, neurodegeneration, excitotoxicity and demyelination are
9 tightly associated with ER stress (Sokka *et al.*, 2007; Sprenkle *et al.*, 2017; Thiebaut *et al.*,
10 2019). Cells can respond to ER stress by activating the unfolded protein response (UPR).
11 There are at least three transducers of the UPR, namely, protein kinase R (PKR)-like ER
12 kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6
13 (ATF6) (Mori, 2009; Walter & Ron, 2011). Among these, ATF6 is responsible for
14 induction of the major molecular chaperones in the ER, such as glucose-regulated protein
15 78 (GRP78) and glucose-regulated protein 94 (GRP94), in addition to several ER-
16 associated degradation components such as homocysteine-responsive ER-resident
17 ubiquitin-like domain member 1 protein and ER degradation enhancing α mannosidase
18 (Yamamoto *et al.*, 2007; Mori, 2009). In mammals, there are two subtypes of ATF6,
19 called ATF6 α and ATF6 β . Both molecules are type II transmembrane proteins in the ER
20 and translocate to the Golgi apparatus for cleavage upon ER stress. Although ATF6 α
21 plays a dominant role in the transcriptional activation in response to ER stress, ATF6 α/β -
22 mediated adjustment of chaperone levels to meet the increased demands in the ER is

1 essential for the development of the notochord (Yamamoto *et al.*, 2007; Ishikawa *et al.*,
2 2013).

3 We previously reported that ATF6 α contributes to both neuronal survival and glial
4 activation in different neuropathological situations. Deletion of *Atf6* α or that of a
5 downstream molecular chaperone, *Hyou1*, sensitizes hippocampal neurons to glutamate-
6 induced toxicity most likely via Ca²⁺ overload and neuronal hyperactivity *in vivo* (Kitao *et*
7 *al.*, 2001; Kezuka *et al.*, 2016). *Atf6* α deficiency is also associated with reduced astroglial
8 activation and glial scar formation in mouse models of Parkinson's disease (Hashida *et al.*,
9 2012) and stroke (Yoshikawa *et al.*, 2015), respectively, both of which are associated with
10 an enhanced level of neuronal death. By contrast, in experimental autoimmune
11 encephalomyelitis mice, a model of multiple sclerosis, and cultured microglia, *Atf6* α
12 deficiency suppresses microglial activation, clinical symptoms and demyelination via a
13 mechanism involving rapid degradation of NF- κ B p65 by the proteasome (Ta *et al.*, 2016).

14 In contrast with the role of ATF6 α in the ER stress response/UPR and ER stress-
15 related pathophysiologies, the function of ATF6 β is largely unknown, especially in the
16 CNS. Transcriptional activity of ATF6 β is reportedly much weaker than that of ATF6 α
17 (Haze *et al.*, 2001). However, recent reports demonstrated that ATF6 α and ATF6 β may
18 have overlapping and differential functions in the mouse heart (Lynch *et al.*, 2012; Correll
19 *et al.*, 2019). We therefore sought to investigate the expression and possible roles of
20 ATF6 β in the CNS under normal and ER stress conditions. Here, we demonstrate that
21 calreticulin (CRT), a molecular chaperone in the ER with a high Ca²⁺-binding capacity, is

- 1 a unique target of ATF6 β in the CNS, and the ATF6 β -CRT axis plays a critical role in the
- 2 neuronal survival **by improving** Ca^{2+} homeostasis under ER stress.

1 **Results**

2 ***Expression of ATF6 β in the CNS and other tissues***

3 We first verified the tissue distribution of ATF6 β in mice. Quantitative real-time
4 PCR (qRT-PCR) revealed that *Atf6b* mRNA was broadly expressed, but was most highly
5 expressed in the hippocampus of the brain among the tissues analyzed (Figure 1A).

6 Further analysis of cultured cells revealed that expression of *Atf6b* mRNA was higher in
7 hippocampal neurons than in cortical neurons and astrocytes under normal conditions
8 (Figure 1B). Consistently, *in situ* hybridization revealed that *Atf6b* mRNA was highly
9 expressed in hippocampal neurons (Figure 1C). These patterns were in contrast with those
10 of *Atf6a* mRNA, which was more ubiquitously expressed (Figure S1 A, B). **There was no**
11 **significant difference in *Atf6b* mRNA levels between male and female mice (Figure S1 C).**

12 We next analyzed expression of *Atf6b* mRNA under ER stress. Treatment of
13 cultured hippocampal neurons with the ER stressors tunicamycin (Tm) and thapsigargin
14 (Tg) significantly increased expression of *Atf6b* mRNA (1.5–2-fold increase) (Figure 1D),
15 although these increases were smaller than those in expression of *Atf6a* mRNA (5–6.5-
16 fold increase) (Figure S1 D). **At the protein level**, both the full-length 110 kDa protein
17 (**FL**) and a cleaved N-terminal 60kDa fragment (**NTF**) of **ATF6 β** were detected in primary
18 **hippocampal neurons**. The level of this fragment was low under normal conditions, but
19 increased as early as 2h after Tg treatment **or 1h after dithiothreitol (DTT) treatment**, the
20 **latter also causes ER stress**. These results suggest that ATF6 β functions in neurons
21 especially under ER stress.

22

1 **Calr is a unique target gene of ATF6 β in the CNS**

Table 1 Differentially expressed genes in *Atf6b*^{-/-} brain (q<0.05)

Downregulated genes in <i>Atf6b</i> ^{-/-} brain					Upregulated genes in <i>Atf6b</i> ^{-/-} brain				
Gene	WT	<i>Atf6b</i> ^{-/-}	log2 (fold-change)	q-value	Gene	WT	<i>Atf6b</i> ^{-/-}	log2 (fold-change)	q-value
<i>Atf6b</i>	31.633	7.66511	-2.04505	0.0465921	<i>Gm47283</i>	13.354	43.6105	1.70741	0.0465921
<i>Calr</i>	203.013	123.236	-0.720147	0.0465921	<i>Igf2</i>	19.0054	39.9251	1.07089	0.0465921
					<i>Ptgds</i>	452.8	942.637	1.05783	0.0465921
					<i>Mgp</i>	20.9161	35.9567	0.781646	0.0465921

2 To identify downstream molecules of ATF6 β in the CNS, RNA-sequencing was
3 performed using hippocampal brain samples from wild-type (WT) and *Atf6b*^{-/-} mice. A
4 total of 55,531 genes were examined. **We filtered genes in two ways. When** filtering genes
5 with FPKM values in WT mice higher than 10 and **q** values smaller than 0.05, only 2
6 downregulated genes and 4 upregulated genes were identified in *Atf6b*^{-/-} mice (Table 1).
7 Although expression of *Atf6b* mRNA was observed to some extent in *Atf6b*^{-/-} mice, this
8 may be due to the presence of the 5' *Atf6b* transcript **with exon 1-9** in these mice, **in which**
9 **exon 10 and 11 were deleted by homologous recombination** (Yamamoto *et al.*, 2007)
10 (Figure S2). Besides *Atf6b*, only *Calr*, which encodes CRT, a molecular chaperone in the
11 ER with a high Ca²⁺-binding capacity, was downregulated in *Atf6b*^{-/-} mice (Table 1). **By**
12 **contrast, in case filtering** genes with FPKM values in WT mice higher than 10 and **p**
13 values smaller than 0.05, 22 downregulated genes and 27 upregulated genes were
14 identified in *Atf6b*^{-/-} mice (Table S1). **Calr was again identified as a gene downregulated in**
15 ***Atf6b*^{-/-} mice, and interestingly, six ER stress-responsive genes**, namely, *Hpsa5* (GRP78),
16 *Pdia4* (ERP72), *Dnajb11*, *Atf4*, *Wfs1*, and *P4ha1* were upregulated in *Atf6b*^{-/-} mice (Table

1 **S1** right columns). RNA-sequencing also indicated that expression of *Calr* was highest
2 among the major molecular chaperones in the ER in WT brains (Table S2). **Taken together**
3 **with previous reports demonstrating possible roles of ATF6 β in the expression of**
4 **molecular chaperones in the ER (Yamamoto *et al.*, 2007; Ishikawa *et al.*, 2013; Correll *et***
5 ***al.*, 2019), we decided to focus on ATF6 β -CRT axis in further experiments.**

6 Consistent with the RNA-sequencing data, RT-qPCR using hippocampi of WT,
7 *Atf6a*^{-/-}, and *Atf6b*^{-/-} mice revealed that expression of *Calr* mRNA was reduced to ~50% in
8 *Atf6b*^{-/-} brains, but not in *Atf6a*^{-/-} brains (Figure 2A). This was in contrast with expression
9 of *Hspa5* (GRP78) mRNA, which was reduced in *Atf6a*^{-/-} brains, but increased in *Atf6b*^{-/-}
10 brains (Figure 2A). Similar differences in expression of *Hsp90b1* (GRP94) mRNA were
11 observed, although these were not significant. Expression of *Canx*, which encodes
12 calnexin, another molecular chaperone in the ER with similarities to CRT, was unaffected
13 by *Atf6a* and *Atf6b* deficiency (Figure 2A).

14 The effect of *Atf6b* deletion on CRT expression was next analyzed in different
15 tissues under normal conditions (Figure 2B, C). Both qRT-PCR (Figure 2B) and western
16 blotting (Figure 2C) revealed that CRT expression was significantly lower in the CNS, but
17 not in other tissues tested, in *Atf6b*^{-/-} mice than in WT mice.

18

19 ***The role of ATF6 β in CRT promoter activity***

20 To analyze the role of ATF6 β in CRT expression at the promoter level, reporter
21 assays were performed with a chloramphenicol acetyltransferase (CAT) plasmid
22 containing 1763bp and 415bp of the mouse CRT promoters, pCC1 and pCC3, respectively

1 (Waser *et al.*, 1997) and luciferase plasmids containing 459bp of the WT (huCRT(wt)) or
2 mutant (huCRT(mut)) human CRT promoter, with the latter containing mutated sequences
3 of two ER stress-responsive elements (ERSEs) (Yoshida *et al.*, 1998) (Figure 3A). Upon
4 transfection of pCC1 or pCC3, promoter activity was lower in *Atf6b*^{-/-} hippocampal
5 neurons (59% in pCC1 and 58% in pCC3, respectively) than in WT hippocampal neurons
6 (Figure 3B). Overexpression of ATF6 β cDNA restored the promoter activity in *Atf6b*^{-/-}
7 neurons (103% in pCC1 and 132% in pCC3). Similarly, upon transfection of huCRT(wt),
8 CRT promoter activity was lower (59%) in *Atf6b*^{-/-} neurons than in WT neurons, and
9 overexpression of ATF6 β cDNA restored promoter activity to 87% in *Atf6b*^{-/-} neurons
10 (Figure 3C). Interestingly, overexpression of ATF6 α cDNA restored CRT promoter
11 activity to a greater extent in the same condition (187% in pCC1, 201% in pCC3, and
12 109% in huCRT(wt)) in *Atf6b*^{-/-} neurons. By contrast, upon transfection of huCRT(mut),
13 CRT promoter activity was not detected in either WT or *Atf6b*^{-/-} neurons and was not
14 restored at all by overexpression of ATF6 β or ATF6 α cDNA (Figure 3C), suggesting that
15 ERSEs are essential for ATF6 β -mediated CRT promoter activation.

16 Similar results were obtained in mouse embryonic fibroblasts (MEFs) transfected
17 with pCC1, huCRT(wt), or huCRT(mut), although ATF6 β cDNA seemed to have slightly
18 higher restoring effects than ATF6 α cDNA in *Atf6b*^{-/-} cells (Figure S3). In contrast to CRT
19 promoter activity, GRP78 promoter activity, which was measured using a luciferase
20 plasmid containing 132 bp of the human GRP78 promoter (Yoshida *et al.*, 1998), did not
21 differ between WT and *Atf6b*^{-/-} neurons under normal or ER stress conditions (data not

1 shown). These results suggest that ATF6 β specifically **enhances** CRT promoter activity in
2 a similar manner to ATF6 α via ERSE motifs.

3

4 ***Effect of ATF6 β deletion on expression of molecular chaperones in the ER in primary***
5 ***hippocampal neurons***

6 The effect of ATF6 β deletion on expression of molecular chaperones in the ER
7 was next examined under normal and ER stress conditions using WT and *Atf6b* $^{-/-}$
8 hippocampal neurons. Consistent with the results obtained using mouse tissues under
9 normal conditions, RT-qPCR revealed that *Calr* expression was significantly lower in
10 *Atf6b* $^{-/-}$ neurons than in WT neurons under both control and ER stress conditions, with the
11 latter induced by Tg (Figure 4A upper row) and Tm (Figure 4A lower row). By contrast,
12 expression of other molecular chaperones in the ER such as *Canx* (calnexin), *Hsp90b1*
13 (GRP94), and *Hspa5* (GRP78) was temporally lower in *Atf6b* $^{-/-}$ neurons than in WT
14 neurons after stimulation with Tg (Figure 4A upper row) or Tm (Figure 4A lower row).
15 Similarly, western blot analysis revealed that expression of CRT protein was
16 constitutively lower in *Atf6b* $^{-/-}$ neurons than in WT neurons, while protein expression of
17 other molecular chaperones in the ER was similar in *Atf6b* $^{-/-}$ and WT neurons under both
18 normal and ER stress conditions (Figure 4B).

19 Finally, gene-silencing experiments were performed to exclude the possibility that
20 the effect of *Atf6b* deletion on CRT expression was indirect due to the long-term absence
21 of *Atf6b*. Transfection of Neuro 2a cells with two sets of ATF6 β -targeting siRNAs
22 (ATF6 β -siRNA1 and ATF6 β -siRNA2) reduced *Atf6b* expression to 30% and 43% and

1 reduced *Calr* expression to 62% and 66%, but did not affect *Hspa5* (GRP78) expression,
2 compared with that in control-siRNA-transfected cells (Figure S5 A).

3

4 ***Effect of ATF6 β deletion on Ca $^{2+}$ homeostasis in primary hippocampal neurons***

5 Consistent with the reduced level of *Calr* expression in *Atf6b* $^{-/-}$ neurons, Ca $^{2+}$
6 levels in the ER, which were measured by the green fluorescence-Ca $^{2+}$ -measuring
7 organelle-entrapped protein indicator 1 in the ER (G-CEPIA1er) (Suzuki *et al.*, 2014),
8 were lower in the in *Atf6b* $^{-/-}$ neurons under both normal and ER stress conditions (Figure
9 4C left). By contrast, Ca $^{2+}$ levels in the cytosol, which were measured by GFP-based Ca $^{2+}$
10 calmodulin probe 6f (GCaMP6f)(Chen *et al.*, 2013) were higher in *Atf6b* $^{-/-}$ neurons under
11 both normal and ER stress conditions (Figure 4C middle). Ca $^{2+}$ levels in the mitochondria,
12 which were measured by CEPIA2 in the mitochondria (CEPIA2mt)(Suzuki *et al.*, 2014),
13 were at similar levels between two genotypes under both normal and ER stress conditions
14 (Figure 4C right).

15

16 ***The neuroprotective role of the ATF6 β -CRT axis against the ER stress-induced***
17 ***neuronal death***

18 To evaluate whether ATF6 β has a neuroprotective role against ER stress, WT and
19 *Atf6b* $^{-/-}$ hippocampal neurons were treated with Tg or Tm and the cell death/survival was
20 evaluated in two ways. Staining of living and dead cells with the fluorescent dyes, calcein-
21 AM (green) and ethidium homodimer-1 (EthD-1, red), respectively, revealed that almost
22 all cells are alive in normal conditions. Treatment with ER stressors induced death and

1 reduced viability of both WT and *Atf6b*^{-/-} neurons, but this effect was more pronounced in
2 *Atf6b*^{-/-} neurons (Figure 5A, Figure S4). Consistently, immunocytochemistry using an
3 antibody against the apoptosis marker cleaved caspase-3 (red) and neuronal marker β III
4 tubulin (green) indicated the caspase-3 activation and loss of β III tubulin expression were
5 higher in *Atf6b*^{-/-} neurons than in WT neurons under ER stress (Figure 5B). Similarly,
6 transient silencing of *Atf6b* gene using siRNA enhanced ER stress-induced death of Neuro
7 2a cells (Figure S5 B, C).

8 To confirm the neuroprotective role of the ATF6 β -CRT axis against ER stress in
9 hippocampal neurons, rescue experiments were performed by transfecting ATF6 β and
10 ATF6 α cDNAs (Figure 5C), or by using a lentivirus-mediated CRT overexpression system
11 (LV-CRT) (Figure 6A, B). When cells were co-transfected with ATF6 β and GFP cDNAs,
12 the number of cleaved caspase-3-positive cells in GFP-positive cells were reduced in
13 *Atf6b*^{-/-} neurons. However, this rescuing effect was not observed with ATF6 α cDNA
14 (Figure 5 C). Consistent with the rescuing effect of ATF6 β , overexpression of CRT in
15 *Atf6b*^{-/-} neurons restored its expression to a similar level as that in control WT neurons
16 (Figure 6A), and restored the survival of WT and *Atf6b*^{-/-} neurons upon Tm treatment, but
17 this effect was greater in *Atf6b*^{-/-} neurons than in WT neurons (Figure 6B).

18

19 **Effects of Ca^{2+} -modulating reagents and an ER stress inhibitor on ER stress-induced**
20 **neuronal death**

21 The CRT level and Ca^{2+} signaling are critical for modulating neuronal death in a
22 neurodegeneration model (Taguchi *et al.*, 2000; Bernard-Marissal *et al.*, 2012); therefore,

1 the effects of the Ca^{2+} -modulating reagents O,O'-bis(2-aminophenyl)ethyleneglycol-
2 N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM), a cell-permeable Ca^{2+}
3 chelator, and 2-aminophosphate borate (2-APB), an inhibitor of IP3Rs and store-operated
4 channels, and the ER stress inhibitor salubrinal were analyzed in our model.

5 Immunocytochemical analysis revealed that both BAPTA-AM and 2-APB significantly
6 improved survival of *Atf6b*^{-/-} neurons, but not of WT neurons, under ER stress (images are
7 shown in Figure S6 A and quantified data are shown in Figure 6C). By contrast, salubrinal
8 enhanced survival of both WT and *Atf6b*^{-/-} neurons under ER stress (images are shown in
9 Figure S6 A and quantified data are shown in Figure 6C). These results suggest that
10 temporal impairment of Ca^{2+} homeostasis in *Atf6b*^{-/-} neurons enhances ER stress, leading
11 to increased ER stress-induced neuronal death. Because it was reported that BAPTA-AM
12 alone caused ER stress and neuronal death at the concentrations higher than 13 μM
13 (Paschen *et al.*, 2003), we confirmed no toxicity of BAPTA-AM and 2-APB at the
14 concentrations in this study (5 μM and 2 μM , respectively) (Figure S6 B). Similarly, we
15 analyzed the effect of salubrinal with different concentrations. Significant neuroprotection
16 was observed at 5 and 10 μM , and higher concentration (50 μM or higher) enhanced ER
17 stress-induced neuronal death in our model (Figure S6 C).

18

19 **Effect of *ATF6 β* deletion on molecular chaperones in the ER in kainite (KA)-injected**
20 **mice**

21 Kainate (KA), an agonist of glutamate receptors, causes Ca^{2+} -dependent
22 hyperactivation of neurons, followed by the induction of ER stress and neuronal death in

1 the hippocampus (Kezuka *et al.*, 2016). We and other groups have demonstrated the
2 protective role of UPR signaling in KA-injected mice (Kitao *et al.*, 2001; Sokka *et al.*,
3 2007; Kezuka *et al.*, 2016). In this study, we investigated the role of ATF6 β -CRT axis and
4 expression of its components in these mice. RT-qPCR revealed that expression of *Atf6b*,
5 *Calr*, *Canx*, and *Hspa5* mRNAs mildly, but significantly, increased after injection of KA
6 into the mouse hippocampus (Figure 7A). Consistent with the results in cultured neurons,
7 the level of *Calr* mRNA, but not of other mRNAs, was reduced to ~50% in *Atf6b*^{-/-} mice
8 under both sham and KA-injected conditions (Figure 7A). Western blot analysis
9 confirmed that the level of CRT protein was decreased in the *Atf6b*^{-/-} hippocampus under
10 both sham and KA-injected conditions (Figure 7B).

11

12 ***The neuroprotective role of ATF6 β -CRT axis against KA-induced neuronal death***
13 The neuroprotective effect of ATF6 β *in vivo* was evaluated using KA-injected
14 mice. Consistent with our previous reports (Kitao *et al.*, 2001; Kezuka *et al.*, 2016), Nissl
15 staining and immunohistochemical staining for cleaved caspase-3 revealed that KA caused
16 neuronal death in the CA3 region of the hippocampus, which is one of the most KA-
17 sensitive areas. The level of neuronal death was significantly higher in *Atf6b*^{-/-} mice than
18 in WT mice at 1 and 3 days after KA injection (Figure 8A, B). To analyze the involvement
19 of CRT in KA-induced neuronal death, *Calr*^{+/+} mice, which developed normally and
20 showed no gross phenotypes with a reduced level of *Calr* expression (Figure S7), were
21 injected with KA and neuronal death was evaluated. Consistent with the results obtained

1 with *Atf6b*^{-/-} mice, the level of neuronal death in the hippocampus was significantly higher
2 in *Calr*^{+/−} mice than in WT mice (Figure 8C).

3

4 ***ATF6 β -mediated regulation of neuronal activity and Ca²⁺homeostasis after KA
5 administration***

6 To elucidate the mechanism underlying the enhanced level of neuronal death in
7 *Atf6b*^{-/-} hippocampus after KA injection, earlier events following KA injection were
8 investigated. qRT-PCR (Figure S8 A) and immunohistochemistry (Figure S8 B) revealed
9 that expression of the immediate-early genes such as *Fos* (c-Fos), *Fosb* and *Bdnf* was
10 induced in both genotypes after KA-injection, but was higher in *Atf6b*^{-/-} mice, suggesting
11 that hyperactivity is involved in the enhanced level of neuronal death in the *Atf6b*^{-/-}
12 hippocampus. The effects of the Ca²⁺-modulating reagent 2-APB and the ER stress
13 inhibitor salubrinal were next analyzed. **They did not cause neuronal death at the doses**
14 **used in this study (Figure S8 C).** Immunohistochemical analysis revealed that both
15 reagents significantly improved neuronal survival in the *Atf6b*^{-/-} hippocampus after KA
16 injection (Figure 9A, B), suggesting that temporal dysregulation of Ca²⁺ homeostasis in
17 *Atf6b*^{-/-} neurons enhances ER stress, leading to increased ER stress-induced neuronal
18 death.

1 **Discussion**

2 The major findings of the current study are that ATF6 β specifically regulates CRT
3 expression in the CNS and that the ATF6 β -CRT axis plays an important role in survival of
4 hippocampal neurons upon exposure to ER stress and excitotoxicity.

5 CRT is a Ca²⁺-binding molecular chaperone in the ER that functions in diverse
6 cellular processes such as Ca²⁺ homeostasis, protein folding, gene expression, adhesion,
7 and cell death (Michalak *et al.*, 2009; Wang *et al.*, 2012). It is also important for
8 organogenesis especially in the heart, brain, and ventral body wall (Rauch *et al.*, 2000).

9 Deficiency of CRT leads to defects in myofibrillogenesis and thinner ventricular walls in
10 the heart (Mesaeli *et al.*, 1999). Interestingly, overexpression of CRT in the heart also
11 causes severe phenotypes such as arrhythmias and sudden heart block following birth
12 (Nakamura *et al.*, 2001). Therefore, the transcription of CRT in the heart is strictly
13 controlled by several transcriptional factors such as Nkx2.5, COUP-TF1, GATA6 and
14 Evi-1 (Qiu *et al.*, 2008). CRT is also highly expressed in the developing brain and retina,
15 and its deficiency leads to a defect in closure of neural tubes (Rauch *et al.*, 2000).

16 Although it is unclear whether overexpression of CRT is toxic in the CNS, it is possible
17 that the similar strict regulation of CRT expression is required and that ATF6 β is utilized
18 in addition to ATF6 α for this purpose.

19 Our results indicate an **important, but a bit puzzling** role for ATF6 β in CRT
20 expression in the CNS. All the data from RNA-sequence to the promoter analysis
21 suggested that CRT expression was ATF6 β -dependent in primary hippocampal neurons.
22 However, overexpression of ATF6 α and ATF6 β both enhanced CRT promoter activity,

1 and those effects were severely diminished when the CRT promoter contained mutated
2 ERSEs (Figure 3B, Figure S3). These results were consistent with a previous report
3 demonstrating that both homo- and heterodimer of ATF6 α and ATF6 β bind to ERSEs in a
4 similar manner (Yoshida *et al.*, 2001). In fact, the amino acid sequences of the basic
5 regions of ATF6 α and ATF6 β , which are required for DNA binding, are 91% identical (21
6 of 23 amino acid) (Yoshida *et al.*, 1998). In contrast to CRT expression, those of other
7 molecular chaperones in the ER such as GRP78 and GRP94 were more ATF6 α -dependent
8 (Figure 2A) and the deletion of ATF6 β only temporally affected their expressions under
9 ER stress (Figure 4A). These results may raise a scenario that, in the CNS, expression of
10 molecular chaperones in the ER is generally governed by ATF6 α as previously described
11 (Yamamoto *et al.*, 2007) and that ATF6 β functions as a booster if their levels are too low.
12 However, expression of CRT is somewhat governed by ATF6 β , and ATF6 α functions as a
13 booster. The underlying mechanism for this scenario is not clear yet, but neurons may
14 require a high level of CRT expression even under normal condition, as described in Table
15 S2, which may lead to the development of a unique biological system to constitutively
16 produce CRT in neurons. Further studies are required to clarify the molecular basis how
17 this unique system is constructed and regulated.

18 The current study demonstrated that ATF6 β has neuroprotective roles against ER
19 stress and KA-induced neurotoxicity, both of which are associated with CRT. Deletion of
20 *Atf6b* reduced CRT expression (Figure 4A, B), impaired intracellular Ca^{2+} homeostasis
21 (Figure 4C), and enhanced ER stress-induced death of cultured hippocampal neurons
22 (Figure 5) and Neuro 2a cells (Figure S5). Overexpression of ATF6 β or CRT, but not

1 **ATF6 α , rescued *Atf6b* $^{-/-}$ hippocampal neurons against ER stress-induced cell death (Figure**
2 **5C, Figure 6A, B).** The lack of rescuing effect of ATF6 α may be due to the fact that this
3 molecule enhances the expression of different genes including cell death-related molecule
4 **CHOP in addition to molecular chaperons in the ER (Yoshida *et al.*, 2000).** Consistent
5 **with the role of ATF6 β -CRT axis in the Ca $^{2+}$ homeostasis and neuroprotection,** treatment
6 with the Ca $^{2+}$ -modulating reagents BAPTA-AM and 2-APB or the ER stress inhibitor
7 salubrinal restored the survival of neuronal cells **under ER stress conditions** (Figure 6C,
8 **Figure S6 A, C).** Interestingly, the effects of the Ca $^{2+}$ -modulating reagents were observed
9 only in *Atf6b* $^{-/-}$ neurons, while that of the ER stress inhibitor was observed in both WT and
10 *Atf6b* $^{-/-}$ neurons (Figure 6C). These results suggest that the ATF6 β -CRT axis maintains
11 intracellular Ca $^{2+}$ level, which contributes to suppression of ER stress and prevention of
12 apoptosis. Similarly, our *in vivo* results suggest that a reduced level of CRT, Ca $^{2+}$ -
13 mediated neuronal hyperactivity, and subsequent ER stress underlie enhanced neuronal
14 death in the hippocampus of KA-injected *Atf6b* $^{-/-}$ mice (Figure 8 and Figure 9).

15 Accumulating evidence suggests that a reduced level of CRT is associated with the
16 pathologies of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS)
17 (Bernard-Marissal *et al.*, 2012; Bernard-Marissal *et al.*, 2015) and Alzheimer's disease
18 (AD) (Taguchi *et al.*, 2000; Lin *et al.*, 2014). In a mutant superoxide dismutase (mSOD1)
19 model of ALS, activation of the Fas/nitric oxide (NO) pathway reduces CRT expression in
20 motoneurons, which further activates Fas/NO signaling on one hand and enhances ER
21 stress and neuronal death on the other hand (Bernard-Marissal *et al.*, 2012). Consequently,
22 the level of CRT is drastically decreased in 50% of fast fatigable motoneurons (Bernard-

1 Marissal *et al.*, 2015). Although it is unclear whether ATF6 β is involved in the Fas/NO-
2 mediated reduction of the CRT expression, it will be intriguing to analyze the expression
3 profile of ATF6 β and the phenotype of *Atf6 α* ^{-/-} mice in these models.

4 Consistent with the observations in ALS, the level of CRT is reduced in the brains
5 (Taguchi *et al.*, 2000) and sera (Lin *et al.*, 2014) of AD patients, raising the possibility that
6 CRT is a good biomarker of AD (Lin *et al.*, 2014). However, it was also reported that a
7 portion of CRT is located on the cell surface membrane, and acts as a receptor for C1q, the
8 recognition subunit of the first component of complement. The C1q-CRT complex
9 induces oxidative neurotoxicity (Luo *et al.*, 2003); therefore, CRT may also have a
10 pathological role in AD. Further analysis is required to elucidate the precise role of CRT
11 and involvement of ATF6 β in AD.

12 Although the function of ATF6 β has been considered to be very limited or
13 redundant compared with that of ATF6 α , our results emphasize the critical and beneficial
14 roles of ATF6 β in neuropathological conditions. A recent study also demonstrated that
15 ATF6 β is functional in the heart, especially during the pressure overload-induced cardiac
16 hypertrophic response (Correll *et al.*, 2019). The role of ATF6 β may be determined by the
17 need for specific molecular chaperones in the ER such as CRT, which may differ between
18 tissues. Further studies dissecting the cell- and tissue-specific roles of ATF6 β will help to
19 elucidate the function of the UPR in pathophysiological conditions.

20

1 **Materials and Methods**

2 ***Animals***

3 All animal experiments were conducted according to the guidelines of the Animal
4 Care and Use Committee of Kanazawa University (Approval No. AP-184013). *Atf6a*^{+/−} and
5 *Atf6b*^{+/−} mice were generated as previously described (Yamamoto *et al.*, 2007), and
6 backcrossed with the C57BL/6 strain for more than eight times at the Institute of Laboratory
7 Animals, Graduate School of Medicine, Kyoto University. *Calr*^{+/−} mice were generated as
8 previously described (Tokuhiro *et al.*, 2015), and provided by the RIKEN BioResource
9 Research Center (Tsukuba, Ibaraki, Japan). *Atf6a*^{+/−} and *Atf6b*^{+/−} mice were intercrossed to
10 obtain wild-type (WT), *Atf6a*^{−/−}, and *Atf6b*^{−/−} mice. These lines were maintained by mating
11 mice of the same genotype at the Institute for Experimental Animals, Advanced Science
12 Research Center, Kanazawa University. *Calr*^{+/−} mice were maintained by mating mice with
13 WT mice in the C57BL/6 background. WT, *Atf6a*^{−/−}, *Atf6b*^{−/−}, and *Calr*^{+/−} mice (male; age, 10–
14 12 weeks; weight, 25–30 g) were used for experiments.

15 To develop a KA injection model, mice were anesthetized, and saline or KA (0.2
16 µg/µl, 0.5 µl in total; Sigma, St Louis, MO, USA) was injected unilaterally into the
17 hippocampus (from Bregma: dorso-ventral, -2.0; medio-lateral, -2.4; anterior-posterior, -1.8),
18 as previously described (Kezuka *et al.*, 2016). In some cases, 2-APB (12 µM, 0.5 µl in total;
19 FUJIFILM Wako Pure Chemical Co., Osaka, Osaka, Japan) or salubrinal (1mg/kg; Cayman
20 Chemical, Ann Arbor, MI, USA) was co-injected with KA into the hippocampus, or

1 intraperitoneally injected 30 min before KA administration, as previously described (Sokka *et*
2 *al.*, 2007; Kim *et al.*, 2014; Ikebara *et al.*, 2017). Mice were sacrificed at the indicated
3 timepoints after KA injection, and brain samples were prepared for histological and
4 biochemical analysis.

5

6 ***Cell cultures***

7 Primary hippocampal and cortical neurons were isolated from embryonic day 17.5
8 (E17.5) WT and *Atf6b*^{-/-} mice, as previously described, with minor modifications (Kaech &
9 Banker, 2006). Briefly, hippocampi and cerebral cortices were harvested from prenatal mice,
10 and digested using neuron dissociation solution (FUJIFILM Wako Pure Chemical Co.). After
11 isolation, neurons were plated into 24-well culture plates precoated with poly-L-lysine
12 (10 μ g/ml; Sigma) at a density of 8 \times 10⁵ cell/well, and cultured in Neurobasal Medium (Life
13 Technologies, Carlsbad, CA, USA) supplemented with 2% B-27 serum free supplement (Life
14 Technologies), 0.4 mM L-glutamine (Sigma), 5% fetal bovine serum (FBS)(Sigma), 100U/ml
15 penicillin and 100 μ g/ml streptomycin (Nacalai Tesque, Kyoto, Kyoto, Japan). After 3 days,
16 neurons were used for experiments. Hippocampal neurons were treated with the ER stressors
17 Tg (300nM; Sigma), **DTT (1mM; Nacalai Tesque)** and Tm (1 μ g/ml; FUJIFILM Wako Pure
18 Chemical Co.). In some cases, they were treated with BAPTA-AM (5 μ M; Dojindo Molecular
19 and Technologies Inc., Mashiki-machi, Kumamoto, Japan), 2-APB (2 μ M) or salubrinal
20 (5 μ M) in addition to Tm for the indicated durations.

1 Astrocytes were isolated from the cerebral cortex of postnatal day 1–3 WT mice, as
2 previously described (McCarthy & de Vellis, 1980), and cultured in Dulbecco's Modified
3 Eagle Medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. Cells
4 were used for experiments after achieving full confluence.

5 MEFs were isolated from the skin of E15.5 WT and *Atf6b*^{-/-} mice, as previously
6 described (Yamamoto *et al.*, 2007), and were cultured in DMEM supplemented with 20%
7 FBS and penicillin/streptomycin. Cells were used for experiments after achieving full
8 confluence.

9 Neuro 2a cells were plated at a density of 5 x10⁴ cells/well in 24- or 12-well culture
10 plates, and cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin.
11 Cells were used for experiments after achieving 70% of confluence.

12

13 ***Preparation and transfection of plasmids***

14 Plasmids expressing full length mouse ATF6 α and ATF6 β were constructed by
15 inserting ATF6 α and ATF6 β cDNAs into the pCDF1-MCS2-EF1-Puro expression vector
16 (System Biosciences, Palo Alto, CA, USA). **pcDNA3.1(+)** GFP was obtained from
17 **Invitrogen/Thermo Fisher Scientific (Waltham, MA, USA)**. CAT plasmids containing the
18 mouse CRT promoter (pCC1 and pCC3) were provided by Dr. Marek Michalak (University
19 of Alberta) (Waser *et al.*, 1997). Both plasmids contain ERSE, a consensus of
20 **CCAATN₉CCACG** (Yoshida *et al.*, 1998) (Figure 3A). Luciferase plasmids containing
21 huCRT(wt) and huCRT(mut), with the two ERSEs mutated in the latter, and a plasmid
22 containing the WT human GRP78 promoter (huGRP78) were constructed as previously

1 described (Yoshida *et al.*, 1998). The pRL-SV40 plasmid was obtained from Promega
2 (Madison, WI, USA). Plasmids for Ca^{2+} imaging such as pCMV G-CEPIA1er, pGP-CMV-
3 GCaMP6f and pCMV CEPIA2mt were obtained from Addgene (Watertown, MA, USA).
4 Cells were transfected with each plasmid for 5 h using Lipofectamine 2000 (Life
5 Technologies) and further incubated for 24-48 h. In our model, transfection efficiency was
6 approximately 5% in primary neurons.

7

8 ***Preparation and infection of lentivirus vectors***

9 The lentivirus vector expressing full-length mouse CRT under the control of the
10 human eukaryotic translation elongation factor 1 $\alpha 1$ promoter and the lentivirus vector alone
11 was purchased from VectorBuilder (Chicago, IL, USA). Viral stocks had titers of $\sim 10^9$
12 plaque-forming units/ml. Hippocampal neurons were infected with the CRT-expressing (LV-
13 CRT) or control (LV-control) lentivirus vector at a multiplicity of infection 10 for 16 h and
14 further incubated for 48-72 h.

15

16 ***Preparation and transfection of ATF6 β -targeting siRNAs***

17 ATF6 β -specific siRNAs, namely, ATF6 β -siRNA1 (SASI_Mm01_00110468) and
18 ATF6 β -siRNA2 (SASI_Mm01_00110470), and control-siRNA were obtained from Sigma.
19 Neuro2a cells were transfected with each siRNA for 5 h using Lipofectamine RNAiMAX
20 (Life Technologies) and further incubated for 24-48 h.

21

22 ***qRT-PCR***

1 Total RNA was extracted from the indicated mouse tissues and cultured cells using
2 RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription reactions
3 containing 1 µg of total RNA were performed using PrimeScript™ (Takara, Otsu, Shiga,
4 Japan). Individual cDNAs were amplified with THUNDERBIRD™ SYBR qPCR Mix
5 (TOYOBO CO, LTD, Osaka, Osaka, Japan) using specific primers for *Atf6b*, *Atf6a*, *Calr*,
6 *Canx*, *Hspa5*, *Hsp90b1*, *Fos*, *Fosb*, *Bdnf* and *Gapdh*. The primers are listed in Table S3. The
7 comparative Ct method was used for data analyses with MxPro 4.10 (Agilent Technologies,
8 Santa Clara, CA, USA). Values for each gene were normalized against the *Gapdh* expression
9 level.

10

11 ***Western blotting.***

12 Samples from the indicated mouse tissues and cultured cells were solubilized in
13 RIPA buffer, which contained 10 mM Tris (pH 7.6), 1 mM EDTA, 150 mM NaCl, 1% NP-40,
14 0.1% SDS, 0.2% sodium deoxycholate, 1 mM PMSF, 1µg/ml aprotinin, 10 mM NaF, and 1
15 mM Na₃VO₄. To detect endogenous ATF6β protein in primary hippocampal neurons, RIPA
16 buffer without sodium deoxycholate was used. Membranes were incubated with 3% bovine
17 serum albumin for 1 h and then with the primary antibodies for overnight at 4°C. The primary
18 antibodies included those against ATF6β (853202; Biolegend, San Diego, CA, USA; 1:500),
19 CRT (SPA-600; Enzo Life Sciences Inc., Farmingdale, NY, USA; 1:2000 and 10292-1-AP;
20 Proteintech, Rosemont, IL, USA; 1:1000), calnexin (SPA-865; Enzo Life Sciences Inc.;
21 1:1000), KDEL (PM-059; Medical & Biological Laboratories, Nagoya, Aichi, Japan; 1:1000),

1 and GAPDH (016-25523; FUJIFILM Wako Pure Chemical Co.; 1:2000). Sites of primary
2 antibody binding were determined using an enhanced chemiluminescence system (GE
3 Healthcare, Pittsburgh, PA, USA). Horseradish peroxidase (HRP)-conjugated secondary
4 antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were used to detect
5 immunoglobulins from mouse or rabbit. The intensity of each band was, quantified by using
6 Image J software (version 1.50i, Wayne Rasband, National Institutes of Health, Bethesda,
7 MD, USA, <https://imagej.nih.gov/ij/>).

8

9 ***Histology and immunohistochemistry***

10 At the indicated timepoints after KA injection, mice (WT, *Atf6b*^{-/-}, and *Calr*^{+/−}) were
11 deeply anesthetized with isoflurane and transcardially perfused with phosphate-buffered
12 saline (PBS) followed by 4% paraformaldehyde prepared in 0.1 M phosphate buffer (pH 7.4).
13 Brains were harvested, post-fixed with 4% paraformaldehyde for 8 h, and cryoprotected in
14 30% sucrose for at least 24 h. Cortical sections (10 µm-thick coronal sections containing the
15 hippocampus (between Bregma -1.5 and -2.1 mm)) were cut on a cryostat (Leica Biosystems,
16 Wetzler, Germany). Sections were processed for Nissl staining (Cresyl violet staining) or
17 immunohistochemistry with antibodies against cleaved caspase-3 (Asp175; Cell Signaling
18 Technology, Inc. Danvers, MA, USA; 1:500) and c-Fos (PC05; Merck; 1:200). Nuclei were
19 visualized with 4',6-diamidino-2-phenylindole (DAPI; Sigma). Anti-rabbit and anti-mouse
20 Alexa Fluor 488-conjugated (Life Technologies; 1:200) and Cy3-conjugated (Jackson

1 ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:200) secondary antibodies
2 were used to visualize immunolabeling. Imaging was performed using a laser scanning
3 confocal microscope (Eclipse TE200U; Nikon, Tokyo, Japan) and Nikon EZ-C1 software or
4 using a light and fluorescence microscope (BZ-X700; KEYENCE, Osaka, Japan).

5

6 ***In situ hybridization-immunohistochemistry***

7 *In situ* hybridization was performed as previously described (Hattori *et al.*, 2014). In
8 brief, a ~600 bp mouse ATF6 β cDNA fragment was PCR-amplified using 5'-
9 AACAGGAAGGTTGTCTGCATCAT-3' and 5'-GTATCCTCCCTCCGGTCAAT-3'
10 primers and inserted into the pGEM-T vector (Promega). The plasmid was linearized using
11 EcoRV and ApaI to synthesize the antisense and sense probe, respectively. Brains were
12 removed from mice after perfusion with PBS and immediately placed at -80°C. Serial 14 μ m-
13 thick coronal sections were obtained using a cryostat and hybridized with a digoxigenin-
14 labeled ATF6 β RNA probe. After development and thorough washing with PBS, brain
15 sections were subjected to immunohistochemistry using a mouse anti-NeuN antibody
16 (MAB377; Merck, Kenilworth, NJ, USA; 1:500) followed by incubation with an anti-mouse
17 IgG antibody (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were
18 developed in peroxidase substrate solution (ImmPACT DAB, Vector Laboratories, Inc.).
19 Imaging was performed using a light and fluorescence microscope (BZ-X700, KEYENCE).

20

21 ***RNA-sequencing***

1 Total RNA purified from hippocampi of WT and *Atf6b*^{-/-} mice (n=2 per group) was
2 used to prepare RNA libraries using a TruSeq Stranded mRNA Sample Preparation Kit
3 (Illumina, Inc., San Diego, CA, USA), with polyA selection for ribosomal RNA depletion.
4 The RNA libraries were generated from 500 ng of total hippocampal RNA and sequenced on
5 an Illumina HiSeq 2000 to obtain paired-end 101 bp reads for each sample.

6 RNA-sequencing reads were mapped to the Mouse genome (GRCm.38.p6/mm10)
7 using STAR v2.7.0f (Dobin *et al.*, 2013). Aligned reads were counted and assigned to genes
8 using Ensembl release 99 gene annotation (Cunningham *et al.*, 2019). Gene expression levels
9 were quantified using Cufflinks v2.2.1 (Trapnell *et al.*, 2013), and denoted by fragments per
10 kilobase of exon per million reads mapped (FPKM) values, which were normalized by the
11 number of RNA fragments mapped to the reference genome and the total length of all exons
12 in the respective transcripts. Differentially expressed genes between WT and *Atf6b*^{-/-}
13 hippocampi were identified by Cuffdiff, which is part of the Cufflinks toolkit. Two replicates
14 per group were combined as the input of Cuffdiff, and **q- and p-values were** reported to show
15 the significance of differentially expressed genes. **The raw reads are available in the DNA**
16 **Data Bank of Japan (DDBJ) with DDBJ Sequence Read Archive (DRA) accession number,**
17 **DRA011345.**

18

19 ***Ca*²⁺ measurement in the intracellular organelles and in the cytosol**

1 Ca²⁺ levels in the ER, cytosol and mitochondria of hippocampal neurons were
2 measured using G-CEPIA1er, GCaMP6f and CEPIA2mt, respectively. Forty-eight hours after
3 transfection, Ca²⁺ imaging was performed using a light and fluorescence microscope (BZ-
4 X700, KEYENCE) under normal and ER stress conditions. The intensity of the fluorescence
5 in each cell was measured using ImageJ software for 200 neurons in each condition.

6

7 ***Immunocytochemistry***

8 Cultured hippocampal neurons and Neuro 2a cells were fixed in 4%
9 paraformaldehyde for 15 min at room temperature, and permeabilized in 0.3% Triton-X100
10 for 10 min. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling
11 Technology, Inc; 1:800) and βIII tubulin (MAB1637; Merck; 1:500) were used. Nuclei were
12 visualized with DAPI (Sigma). Imaging was performed using a light and fluorescence
13 microscope (BZ-X700, KEYENCE).

14

15 ***LIVE/DEAD viability assay***

16 Living and dead neurons and Nuro 2a cells were evaluated using a LIVE/DEAD
17 Viability Assay kit (Life Technologies). In brief, cells were washed in PBS and incubated
18 with calcein-AM (1μM), EthD-1 (2μM) and Hoechst 33342 (1 μg/ml, Dojindo Molecular and
19 Technologies Inc.) in regular medium. Imaging was performed using a fluorescence
20 microscope (BZ-X700, KEYENCE).

1

2 ***Reporter assay***

3 *Luciferase assay*: At 48 h after transfection, cells were lysed in 100 µl of Passive
4 Lysis Buffer (Promega). Firefly luciferase and Renilla luciferase activities were measured
5 using the Dual-Luciferase Reporter Assay System (Promega) and analyzed as previously
6 describe (Yoshida *et al.*, 1998).

7 *CAT ELISA*: At 48 h after transfection, cells were lysed in 125 µl of lysis buffer
8 provided with a CAT ELISA kit (Sigma). CAT expression and Renilla luciferase activities
9 were measured and the ratio was calculated.

10

11 ***Image quantification***

12 **For the** LIVE/DEAD viability assay and immunocytochemistry, four images per well
13 were acquired and the numbers of EthD-1-positive cells/Hoechst 33342-positive cells and
14 cleaved caspase-3-positive cells/DAPI-positive total cells were counted using ImageJ
15 software, respectively. For Nissl staining, three brain sections containing the hippocampal
16 CA3 area close to the KA injection site were selected per mouse and the number of Nissl-
17 positive neurons was counted using ImageJ software. For immunohistochemistry, two brain
18 sections with the highest numbers of cleaved caspase-3-positive cells and c-Fos-positive cells
19 were selected per mouse, and the numbers of these cells were counted using ImageJ software.

1

2 ***Statistical analyses***

3 Statistical analyses were performed using the Mann-Whitney U test or a one-way or
4 two-way analysis of variance (ANOVA) followed by the Tukey/Bonferroni test. GraphPad
5 Prism software 5.0 was used for statistical analyses. A p-value less than 0.05 was considered
6 statistically significant.

7

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9 .

10

11

12 **Conflict of interest disclosure**

13 The authors declare no conflicts of interest.

14

1 **Figure Legends**

2 **Figure 1. Expression and activity of ATF6 β .** A, B, Expression of *Atf6b* mRNA in normal
3 tissues (n=5 mice) (A) and in cultured cells (n=3–8) (B). HPC: hippocampus, Cx: cerebral
4 cortex, SC: spinal cord. Total RNA was isolated from the indicated samples and qRT-PCR
5 was performed. Data are shown as mean \pm SEM. *p < 0.05, ***p < 0.001 by a one-way
6 ANOVA followed by the Tukey test. C, *In situ* hybridization (upper panel) and *in situ*
7 hybridization-immunohistochemistry (lower panel) of *Atf6b* mRNA in the normal brain.
8 Images in the right panels are enlarged views of the CA3 area. Scale bars: 200 μ m (left
9 panels) and 25 μ m (right panels). Typical images from three independent experiments are
10 shown. D, qRT-PCR analysis of expression of *Atf6b* mRNA in primary hippocampal neurons
11 under ER stress. Cells were treated with Tg (300nM) or Tm (1 μ g/ml) for 8 h and then qRT-
12 PCR was performed. n=3. Data are shown as mean \pm SEM. *p < 0.05, **p < 0.01 by a one-
13 way ANOVA followed by the Tukey test. E, Activation of ATF6 β by ER stress **in primary**
14 **hippocampal neurons.** Cells were treated with Tg (300nM) **for 2h or DTT for 1h.** Extracted
15 proteins were subjected to western blotting. The typical data from two independent
16 experiments are shown. **FL: full length, NTF: N-terminal fragment**

17

18 **Figure 2. Reduces CRT expression in the CNS of *Atf6b* $^{-/-}$ mice.** A, Expression of molecular
19 chaperones in the ER in WT, *Atf6a* $^{-/-}$ and *Atf6b* $^{-/-}$ hippocampi. Total RNA was isolated from
20 the CA3 region of the hippocampus and qRT-PCR was performed. n=5–6 mice. Data are
21 shown as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by a one-way ANOVA followed
22 by the Tukey test. B, Expression of *Calr* mRNA in WT and *Atf6b* $^{-/-}$ tissues. Total RNA was

1 isolated from the indicated tissues of each mouse and qRT-PCR was performed. n=5 mice.
2 Data are shown as mean \pm SEM. **p < 0.01 by the Mann-Whitney U test. HPC:
3 hippocampus, Cx: cerebral cortex, SC: spinal cord. C, Expression of CRT protein in WT and
4 *Atf6b*^{-/-} tissues. Protein samples were extracted from the indicated tissues of WT and *Atf6b*^{-/-}
5 mice, and subjected to western blotting. n=5–7 mice. Data are shown as mean \pm SEM. **p <
6 0.01 by the Mann-Whitney U test.

7

8 **Figure 3. Reduced CRT promoter activity in Atf6b-deleted neurons.** A, Schematic
9 representation of the promoters used. Triangles indicate the locations and orientations of
10 ERSE motifs that completely or considerably match the consensus **CCAATN₉CCACG**
11 (*Yoshida et al.*, 1998). Numbers indicate nucleotide positions from transcription start site.
12 ERSE2 and ERSE3 of the human CRT promoter were disrupted by mutating their sequences
13 (marked by crosses). B, **C**, Reporter assays using cultured hippocampal neurons. The CAT
14 ELISA and luciferase assay were performed using cells transfected with the mouse CRT
15 promoter pCC1 **and** pCC3 (B), or with the human CRT promoter huCRT (C). n=4. Data are
16 shown as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001. by a two-way ANOVA followed
17 by the Bonferroni test. Note that both ATF6 α and ATF6 β cDNAs increased CRT promoter
18 activity in *Atf6b*^{-/-} cells.

19

20 **Figure 4. Expression of molecular chaperones in the ER **and** Ca²⁺ homeostasis in Atf6b^{-/-}**
21 **hippocampal neurons.** A, Total RNA was isolated from WT and *Atf6b*^{-/-} hippocampal

1 neurons (n=3–8), and qRT-PCR was performed with the indicated primers. Data are shown as
2 mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001 between two genotypes by a two-way
3 ANOVA followed by the Bonferroni test. Note that expression of *Calr* mRNA was
4 significantly lower in *Atf6b*^{-/-} neurons than in WT neurons under both normal and ER stress
5 conditions. B, Protein samples extracted from WT and *Atf6b*^{-/-} hippocampal neurons exposed
6 to control or ER stress conditions for 16h (n=5–6) were analyzed by western blotting using
7 antibodies against the indicated proteins. Data are shown as mean \pm SEM. ***p < 0.001
8 between two genotypes and # p < 0.05, ## p < 0.01, ### p < 0.001 compared to normal
9 conditions by a two-way ANOVA followed by the Bonferroni tests. Note that expression of
10 CRT protein was significantly lower in *Atf6b*^{-/-} neurons than in WT neurons under both
11 normal and ER stress conditions. C, **Ca²⁺ measurement.** Ca²⁺ levels in the ER, cytosol and
12 mitochondria of hippocampal neurons were measured using G-CEPIA1er, GCaMP6f and
13 CEPIA2mt, respectively under normal and ER stress (Tm for 3h) conditions. n=60–250 cells
14 in each condition from two independent experiments. Data are shown as mean \pm SEM. ***p <
15 0.001 between two genotypes and # p < 0.05, ## p < 0.01, ### p < 0.001 compared to normal
16 conditions by a two-way ANOVA followed by the Bonferroni tests.

17
18 **Figure 5. Protection of the primary hippocampal neurons by ATF6 β .** A, Primary
19 hippocampal neurons were treated with Tg (300nM) or Tm (1 μ g/ml) for 24 h, and cell
20 survival/death was evaluated by the LIVE/DEAD viability assay. Representative fluorescent
21 microscopic images from four independent experiments are shown. The graph depicts the
22 percentages of dead cells. n=4 experiments. Data are shown as mean \pm SEM. ***p < 0.001 by a

1 two-way ANOVA followed by the Bonferroni test. Scale bar: 20 μ m. B, Apoptosis was
2 evaluated by immunocytochemical staining of cleaved caspase-3 (red), β III tubulin (green)
3 and DAPI staining (blue). Representative fluorescent microscopic images from four
4 independent experiments are shown. The graph depicts the percentages of cleaved caspase-3-
5 positive cells. n=4 experiments. Data are shown as mean \pm SEM. ***p<0.001 by a two-way
6 ANOVA followed by the Bonferroni test. Scale bar: 20 μ m. C, Apoptosis was evaluated by
7 immunocytochemical staining of cleaved caspase-3 (red) and GFP (green) using primary
8 hippocampal neurons co-transfected with vector, ATF6 β cDNA or ATF6 α cDNA together
9 with GFP cDNA, followed by Tm treatment for 24h. Representative fluorescent microscopic
10 images of vector-transfected cells are shown. Arrows indicate caspase-3-positive and GFP-
11 positive cells. The graph depicts the percentages of cleaved caspase-3-positive cells out of
12 GFP-positive cells. n=400 cells per condition from two independent experiments. Data are
13 shown as mean \pm SEM. **p<0.01, ***p<0.001 by a two-way ANOVA followed by the
14 Bonferroni test. Scale bar: 10 μ m. Note that ATF6 β cDNA, but not ATF6 α cDNA, rescued
15 *Atf6b*^{-/-} neurons.

16

17 **Figure 6. Protection of the primary hippocampal neurons by CRT overexpression or by**
18 **treatment with Ca²⁺/ER stress-modulating compounds.** A, B, WT and *Atf6b*^{-/-}
19 hippocampal neurons were infected with a control or CRT-expressing lentiviral vector, and
20 the expression levels of CRT and calnexin were measured by western blotting (A). n=3
21 experiments. Data are shown as mean \pm SEM. *p < 0.05, **p < 0.01 by a two-way ANOVA
22 followed by the Bonferroni tests. Cells were then treated with Tm (1 μ g/ml) for 24 h, and cell

1 death was evaluated by immunocytochemical staining for cleaved caspase-3 (B). Scale bar: 20
2 μ m. n=3 experiments. Data are shown as mean \pm SEM. *p < 0.05, ***p < 0.001 by a two-way
3 ANOVA followed by the Bonferroni tests. C, WT and *Atf6b*^{-/-} hippocampal neurons were
4 treated with Tm (1 μ g/ml) together with BAPTA-AM (5 μ M), 2-APB (2 μ M) or salubrinal
5 (5 μ M). Cell death was evaluated by immunocytochemical staining for cleaved caspase-3. n=3
6 experiments. Typical images are shown in Figure S5 Data are shown as mean \pm SEM. *p <
7 0.05, **p < 0.01, ***p < 0.001 by a two-way ANOVA followed by the Bonferroni tests.
8

9 **Figure 7. Expression of molecular chaperones in the ER in KA-injected mice.** A, Total
10 RNA was isolated from the CA3 region of hippocampi from KA-injected WT and *Atf6b*^{-/-}
11 mice and qRT-PCR was performed. n=4 mice. Data are shown as mean \pm SEM. ***p < 0.001
12 between two genotypes and # p < 0.05, ## p < 0.01, ### p < 0.001 compared to normal
13 conditions by a two-way ANOVA followed by the Bonferroni tests. B, Protein samples were
14 extracted from the CA3 region of hippocampi from KA-injected WT and *Atf6b*^{-/-} mice and
15 subjected to western blotting using antibodies against CRT, calnexin, GRP94 and GRP78.
16 n=4–5 mice. Data are shown as mean \pm SEM. **p < 0.001, ***p < 0.001 between two
17 genotypes and # p < 0.05, ## p < 0.01, ### p < 0.001 compared to normal conditions by a two-
18 way ANOVA followed by the Bonferroni tests.
19

20 **Figure 8. Neuroprotection by ATF6 β and CRT in KA-injected mice.** A, Brain sections
21 containing the CA3 area of the hippocampus from WT and *Atf6b*^{-/-} mice were subjected to
22 Nissl staining. The right graph depicts the number of surviving CA3 neurons. n=6–8 mice.

1 Data are shown as mean \pm SEM. **p < 0.01, ***p < 0.001 by a two-way ANOVA followed
2 by the Bonferroni tests. Scale bar: 100 μ m. B, C, Brain sections including the CA3 area of the
3 hippocampus from WT and *Atf6b*^{-/-} mice (B) (n=6–7 mice) or WT and *Calr*^{+/+} mice (C) (n=6
4 mice) were subjected to immunohistochemical staining for cleaved caspase-3. The right
5 graphs depict the number of cleaved caspase-3-positive CA3 neurons. Data are shown as
6 mean \pm SEM. **p < 0.01, ***p < 0.001 by a two-way ANOVA followed by the Bonferroni
7 tests in (B) and by the Mann-Whitney U test in (C). Scale bar: 50 μ m.

8

9 **Figure 9. Ca^{2+} dysregulation in *Atf6b*^{-/-} mice after KA-injection.** Brain sections including
10 the CA3 area of the hippocampus obtained from WT and *Atf6b*^{-/-} mice at 3 days after injection
11 with KA, KA plus 2-APB and KA plus salubrinal were subjected to Nissl staining (A) or
12 immunohistochemical staining for cleaved caspase-3 (B). The right graphs depict the number
13 of surviving CA3 neurons (A) and cleaved caspase-3-positive cells (B), respectively. n=6
14 mice. Data are shown as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by a two-way
15 ANOVA followed by the Bonferroni tests.

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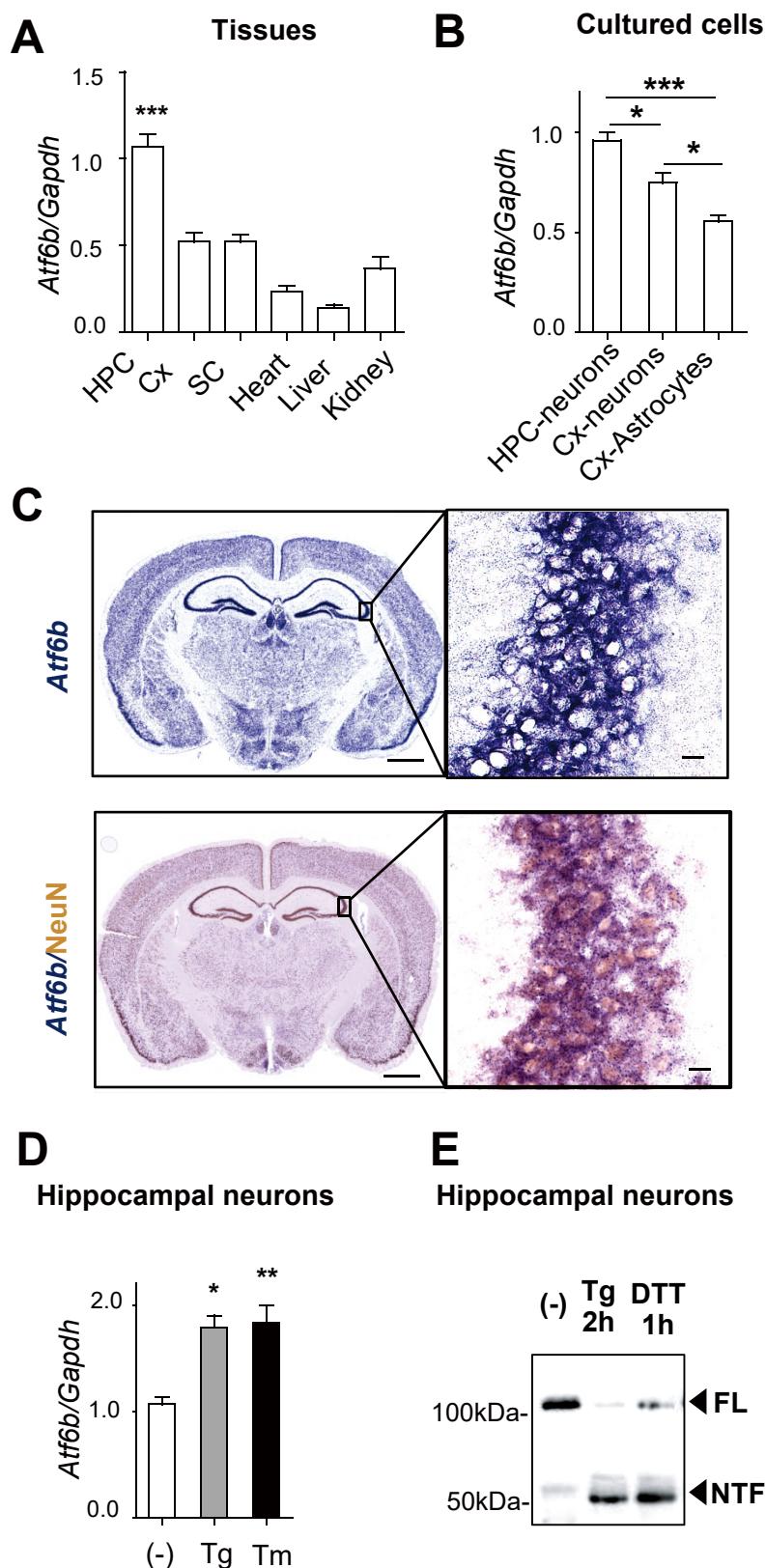


Figure 1

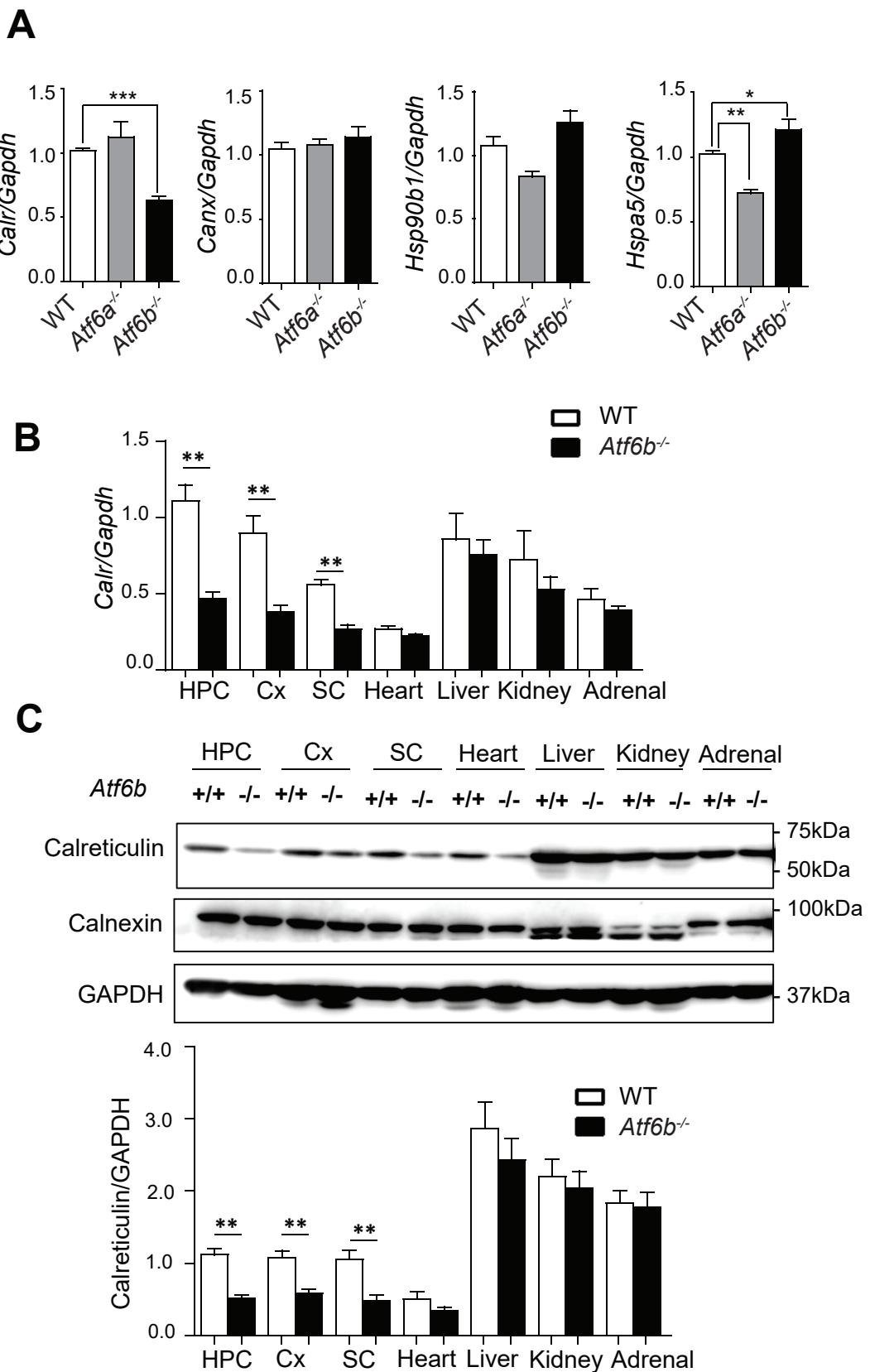


Figure 2

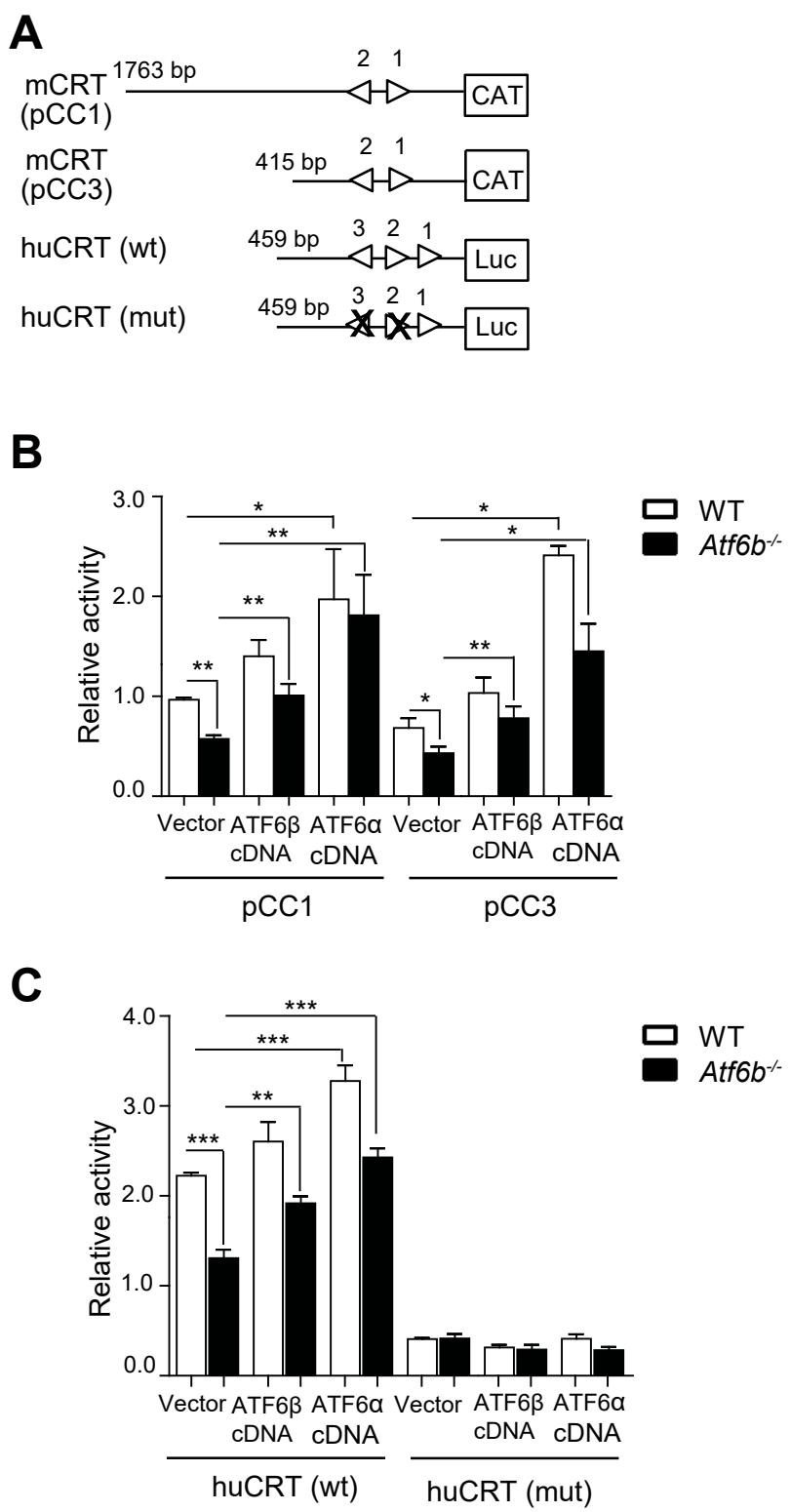
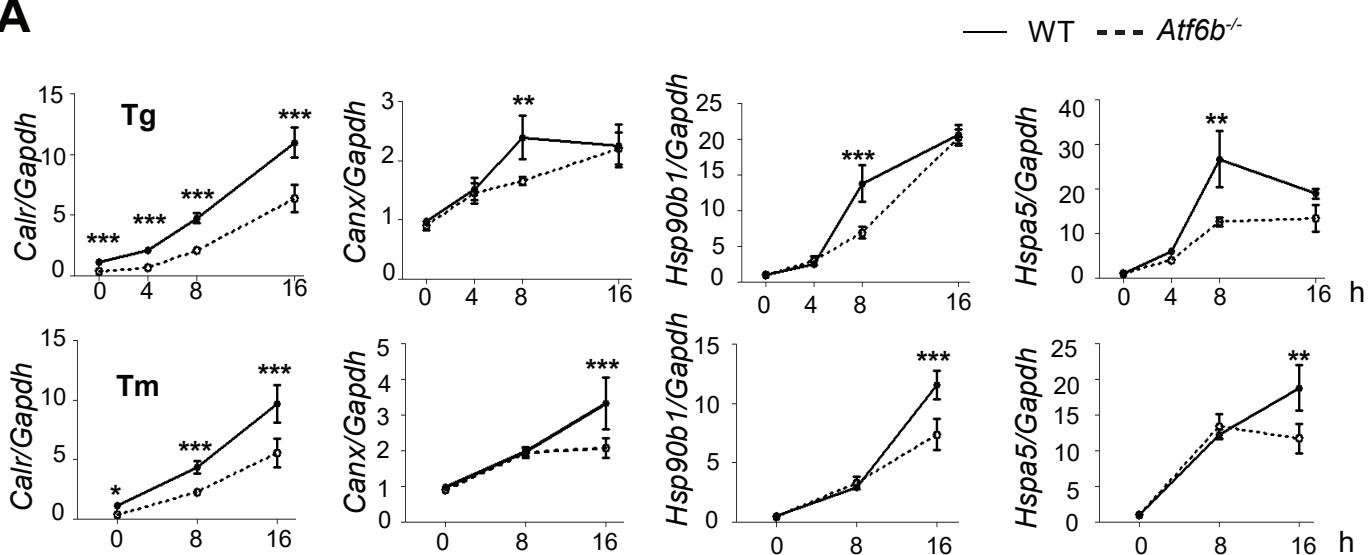
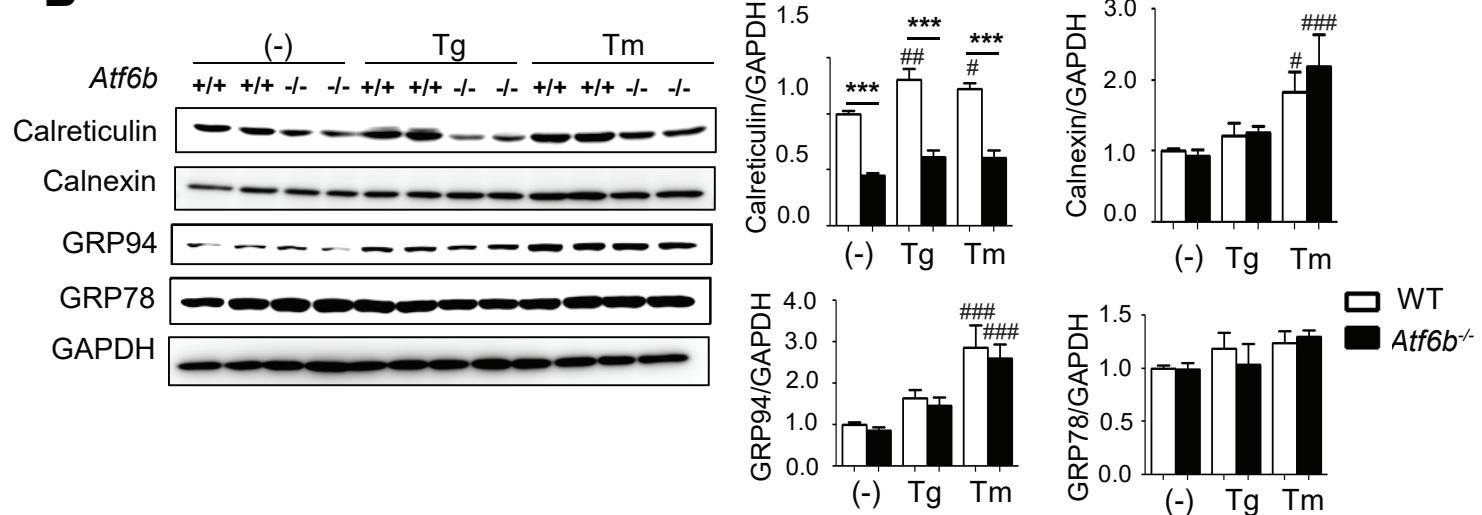


Figure 3

A



B



C

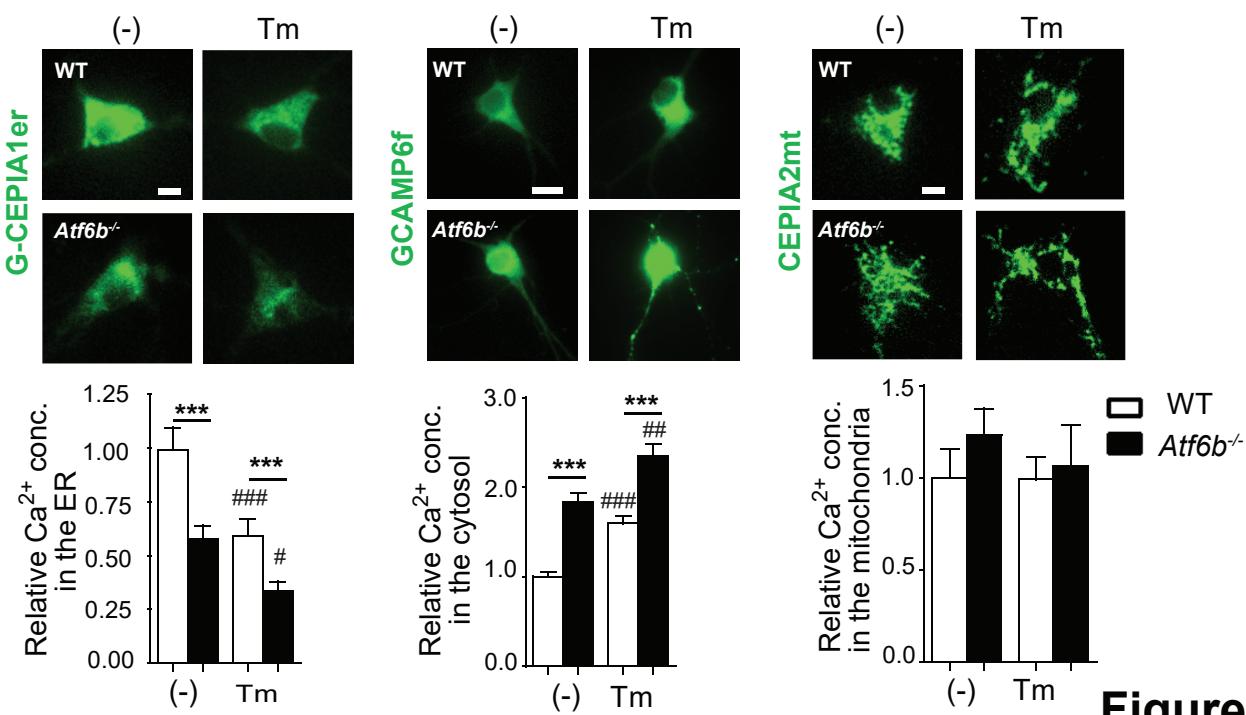


Figure 4

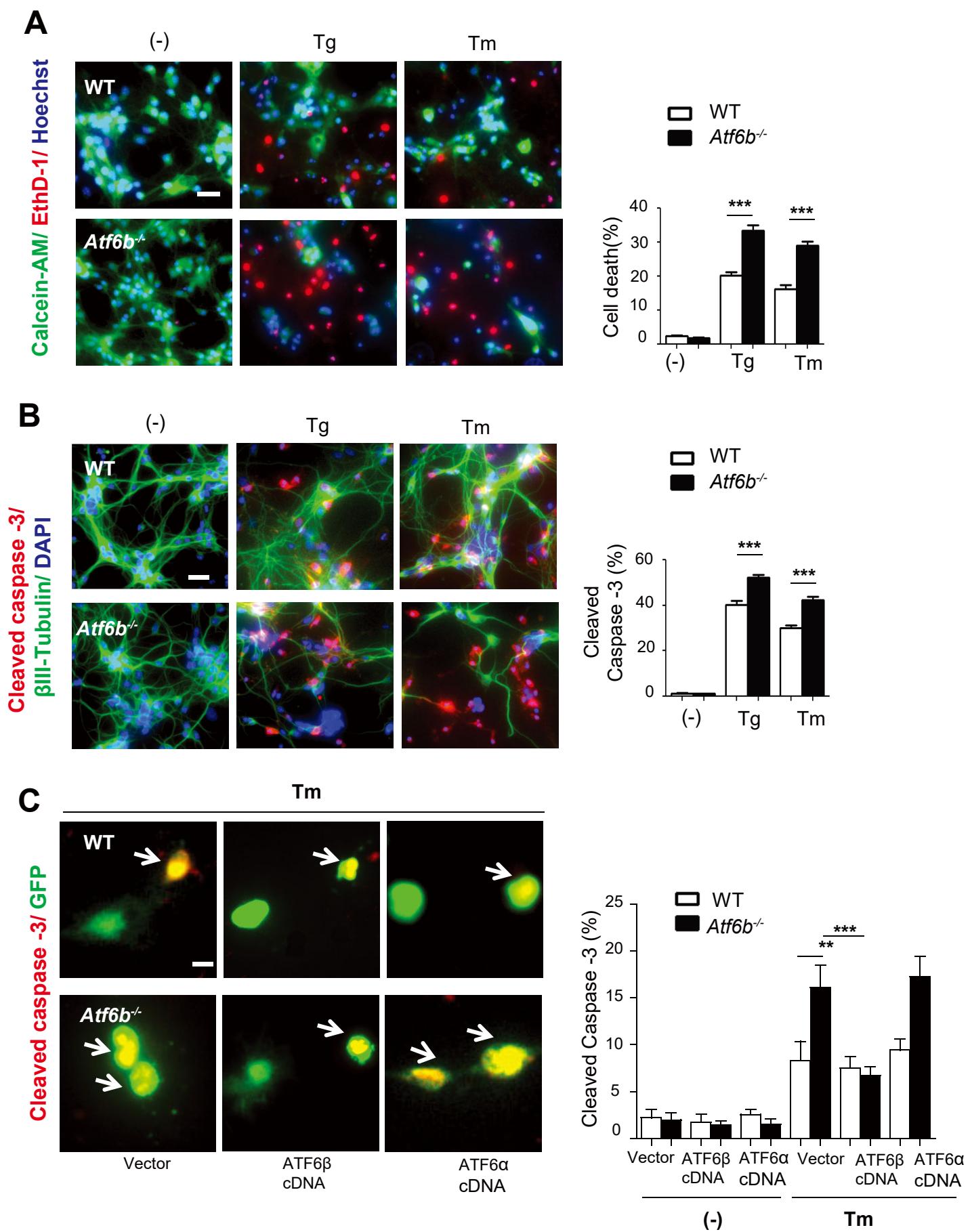


Figure 5

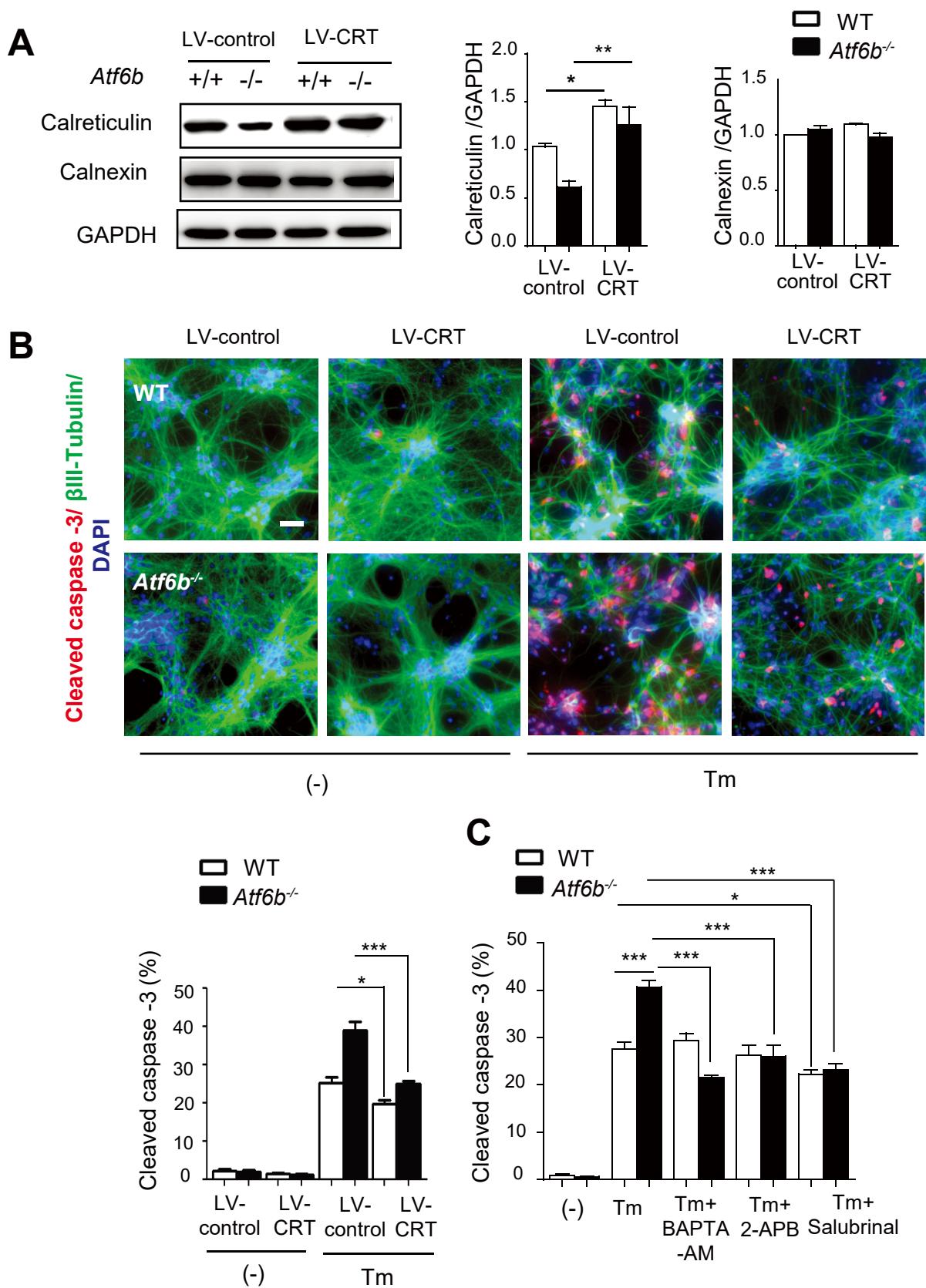


Figure 6

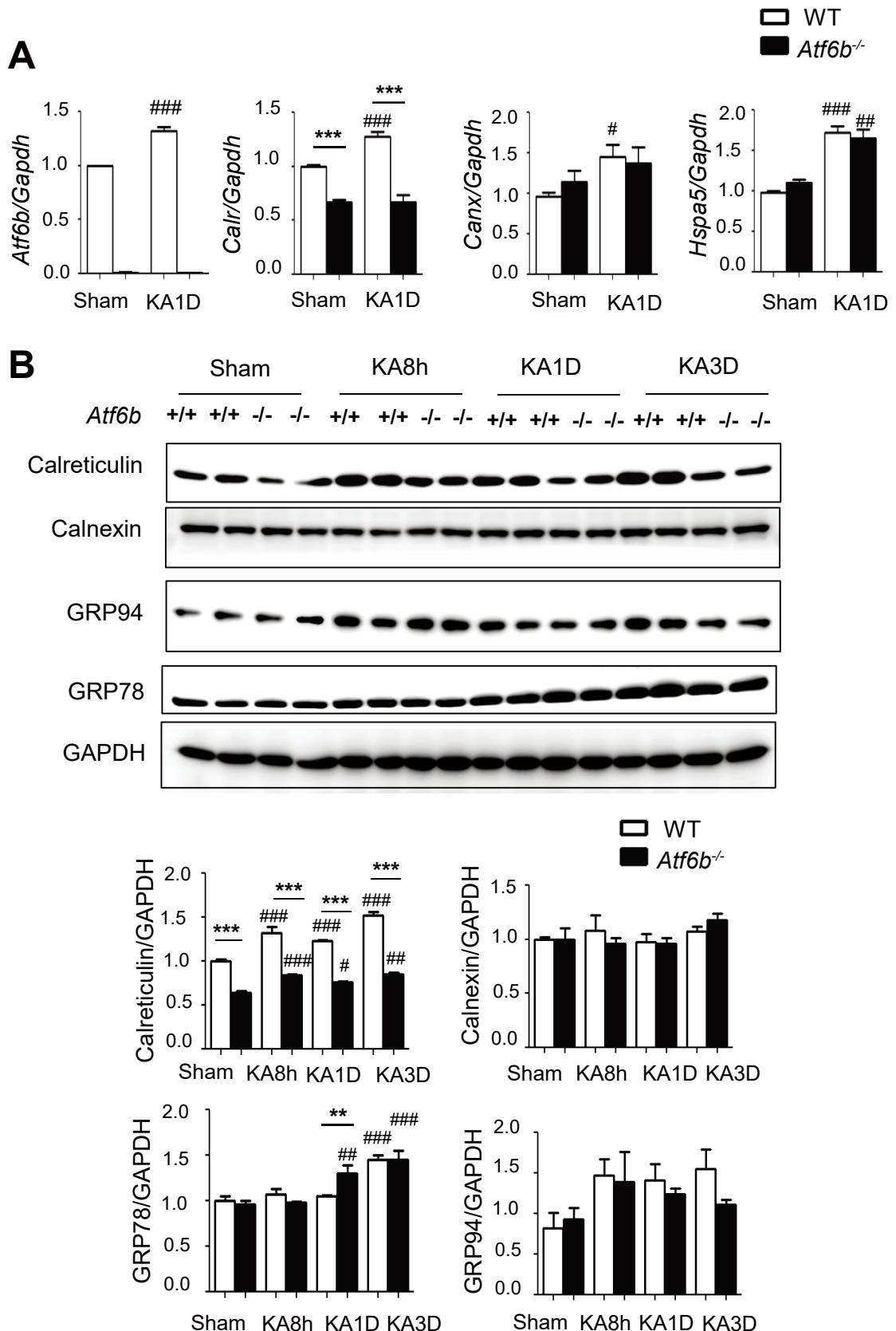
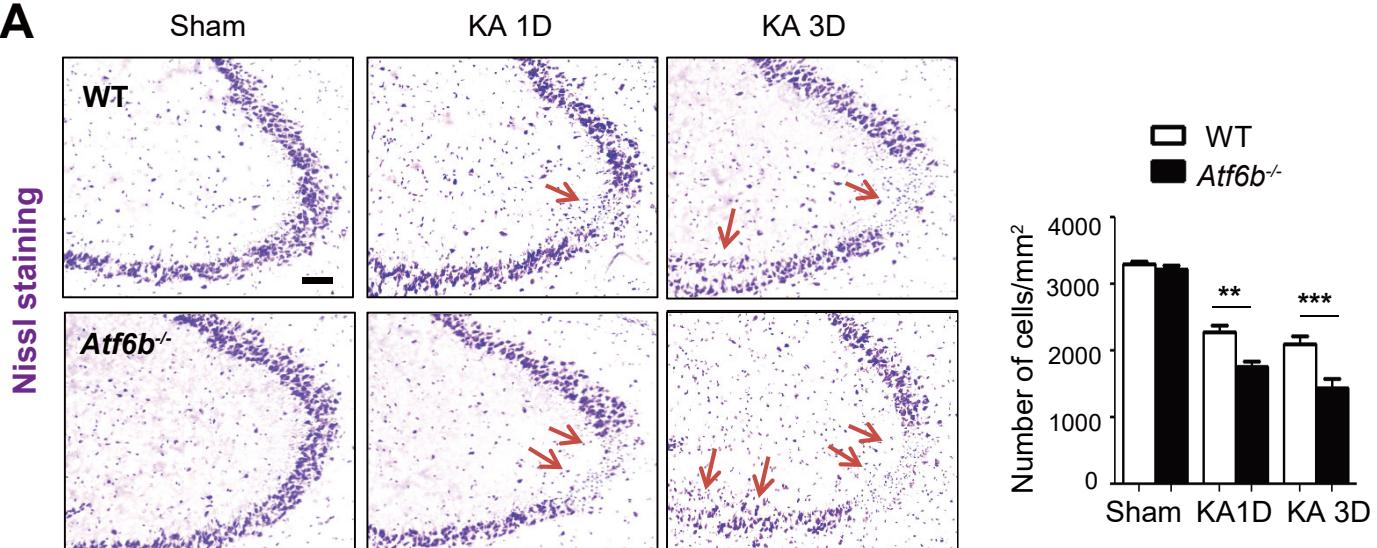
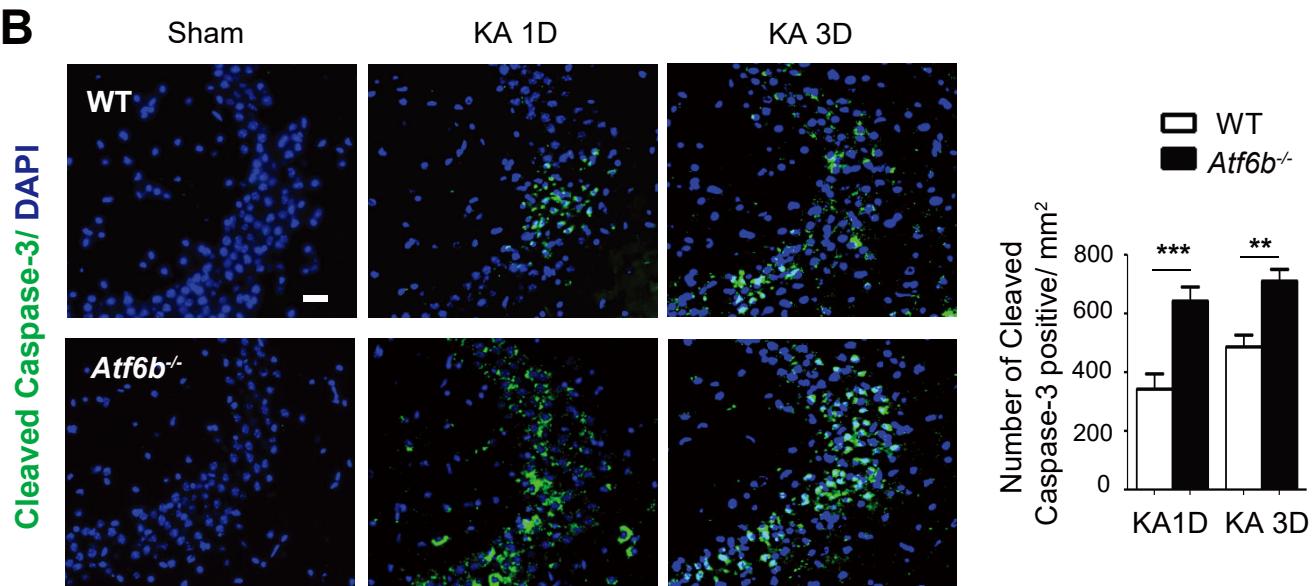


Figure 7

A



B



C

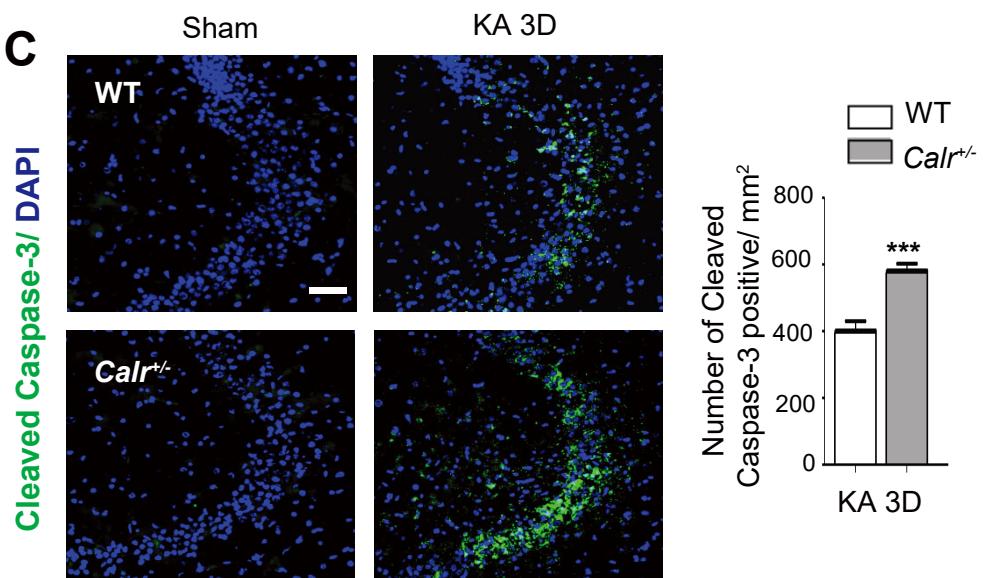


Figure 8

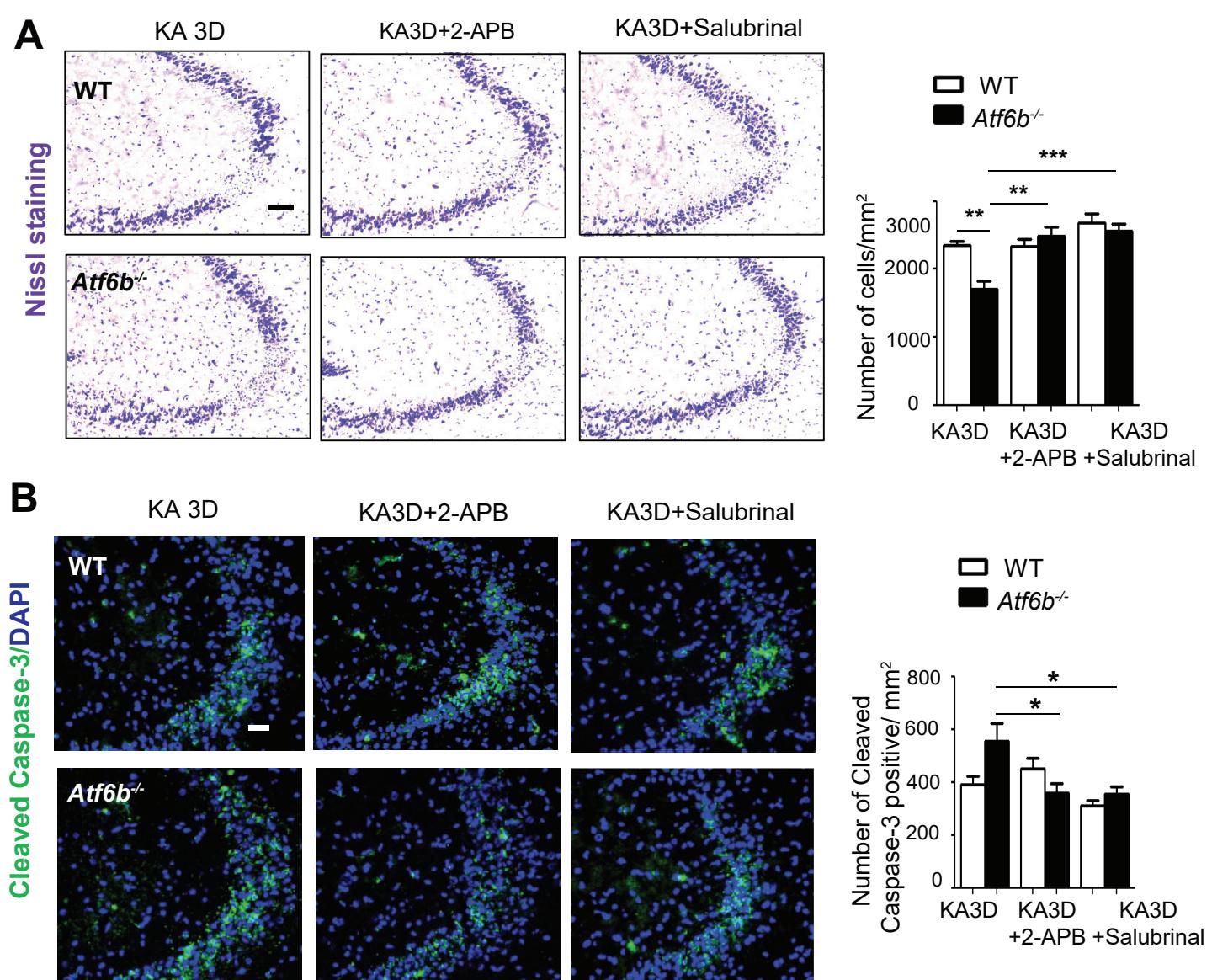


Figure 9