CR6-interacting factor 1 is a key regulator in Aβ-induced mitochondrial disruption and pathogenesis of Alzheimer's disease

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Mitochondrial dysfunction, often characterized by massive fission and other morphological abnormalities, is a well-known risk factor for Alzheimer’s disease (AD). One causative mechanism underlying AD-associated mitochondrial dysfunction is thought to be amyloid-β (Aβ), yet the pathways between Aβ and mitochondrial dysfunction remain elusive. In this study, we report that CR6-interacting factor 1 (Crif1), a mitochondrial inner membrane protein, is a key player in Aβ-induced mitochondrial dysfunction. Specifically, we found that Crif1 levels were downregulated in the pathological regions of Tg6799 mice brains, wherein overexpressed Aβ undergoes self-aggregation. Downregulation of Crif1 was similarly observed in human AD brains as well as in SH-SY5Y cells treated with Aβ. In addition, knockdown of Crif1, using RNA interference, induced mitochondrial dysfunction with phenotypes similar to those observed in Aβ-treated cells. Conversely, Crif1 overexpression prevented Aβ-induced mitochondrial dysfunction and cell death. Finally, we show that Aβ-induced downregulation of Crif1 is mediated by enhanced reactive oxygen species (ROS) and ROS-dependent sumoylation of the transcription factor specificity protein 1 (Sp1). These results identify the ROS-Sp1-Crif1 pathway to be a new mechanism underlying Aβ-induced mitochondrial dysfunction and suggest that ROS-mediated downregulation of Crif1 is a crucial event in AD pathology. We propose that Crif1 may serve as a novel therapeutic target in the treatment of AD.

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Alzheimer’s disease (AD) is the most common and rapidly growing dementia among the aged population worldwide. Representative pathologies of AD include the accumulation of amyloid β (Aβ) plaques, the formation of neurofibrillary tangles, and massive neuronal loss in the brain.1,2 Although many studies have focused on the amyloid-β precursor protein (APP) or Aβ as a therapeutic target, the mechanisms by which Aβ aggravates AD are not yet fully known. Many possible scenarios have been suggested based on several lines of evidence related to mitochondrial dysfunction, oxidative stress, cerebrovascular damage, and inflammation.3-7 Among these hypotheses, abnormal mitochondrial function in AD is known as a primary causative factor in AD pathogenesis.4,5 In this study, therefore, we focused on a possible mechanism of mitochondrial dysfunction in the progression of AD.

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Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid beta; APP, amyloid beta precursor protein; Crif1, CR6-interacting factor 1; Crif1 KD cell, Crif1 knock-downed cell; Crif1 o/e cell, Crif1 overexpressed cell; Co-IP, co-immunoprecipitation; Calcein-AM, acetomethoxy derivative of Calcein; DAB, 3,3'-Diaminobenzidine; DPI, diphenyleneiodonium; EMSA, gel electrophoresis mobility shift assay; Fis1, mitochondrial fission 1 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HD, Huntington’s disease; HSP60, Heat-shock protein 60; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MIM, mitochondrial inner membrane; Mfn, mitofusins; NAC, N-acetylcysteine; NADPH, nicotine adenine diphosphate oxidase; OPA1, optic atrophy type 1; OXPHOS, oxidative phosphorylation; PD, Parkinson’s disease; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; RuR, Ruthenium Red; Sp1, specificity protein 1; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; WB, western blotting; 3-MA, 3-methyladenine

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Figure a: Western blot analysis of Crif1 in the cerebral cortex and cerebellum of Littermate and Tg6799 mice.

Figure b: Quantification of Crif1 levels in the cerebral cortex and cerebellum. No significant difference (n.s.) was observed in the cerebellum.

Figure c: Immunofluorescence images of Crif1 in the cerebral cortex of Littermate and Tg6799 mice.

Figure d: Average intensity of Crif1 in the cerebral cortex.

Figure e: Area fraction of Crif1-expressing cells in the cerebral cortex.

Figure f: Quantification of Crif1 mRNA expression in normal and AD hippocampus.

Figure g: Quantification of Crif1 protein expression in normal and AD hippocampus.

Figure h: Histopathological images of hippocampus (CA2 and CA3 regions) in control and AD mice.
Huntington’s disease (HD). As in other neurodegenerative diseases, mitochondrial fragmentation has also been observed in the brains of AD patients. Furthermore, elevated oxidative stress levels were detected in the brains of AD patients. In addition, reduced amounts of mitochondrial DNA, destroyed mitochondrial cristae, and inner/outer mitochondrial membrane structure are also directly linked to the increased incidence of AD. However, the molecular mechanisms underlying impaired mitochondrial dynamics and function in AD are unclear.

CR6-interacting factor 1 (Crif1), also known as GADD45-associated family protein, was first identified as a molecule that regulates cell cycle and growth. Recently, some nuclear receptors, such as Nur77, STAT3, Elf3, and Nrf2, are also known to interact with Crif1. In addition, Crif1 is important for the translation of mitochondrial OXPHOS subunits and their insertion into the MIM. In this study, we investigated whether Crif1 has a role in AD pathogenesis, and found that Crif1 acts as a key player in Aβ-induced mitochondrial dysfunction. First, we found that the expression level of Crif1 was reduced in the brains of AD mouse models as well as in brain tissues from AD patients. Next, we investigated whether Aβ regulates Crif1 expression levels in vitro, and we found that Crif1 was decreased significantly in Aβ-treated SH-SY5Y cells compared with vehicle-treated cells. In addition, this process was mediated by elevated ROS levels via activation of NADPH oxidase. To examine the mechanism in detail, Aβ-induced ROS facilitated the reduced binding of the specificity protein 1 (Sp1) transcription factor on the promoter region of Crif1 by increasing the sumoylation of Sp1. Reduced Crif1 expression resulted in the disruption of mitochondrial morphology and its functions, which leads to massive cell death in AD brains. Finally, Crif1 overexpression (Crif1 o/e) rescued Aβ-induced mitochondrial alteration and neuronal cell death. These data suggest that the regulation of Crif1 could be explored as a therapeutic target for AD.

Results

Crif1 is decreased in the brains of AD patients and AD mouse models. To determine whether Crif1 expression is altered in the brains of AD mice, endogenous Crif1 levels in the frontal cortex, hippocampus, and cerebellum in 6-month-old Tg6799 mice were evaluated by western blot analysis (WB). Crif1 levels were reduced significantly in the frontal cortex, and the hippocampus of Tg6799 mice compared with non-transgenic littermate controls, respectively (Figures 1a and b and Supplementary Figures 1a and b). However, Crif1 levels in the cerebellum of Tg6799 mice were not different from those in littermate controls (Figures 1a and b), suggesting that Crif1 reduction occurs only in the pathological areas of AD. In addition, the expression of Crif1 was examined by immunohistochemistry using a specific antibody against Crif1. Both intensity and area fraction of Crif1 fluorescence signal in Tg6799 mouse brains were significantly decreased compared with those of littermate controls (Figures 1c–e). These results were also confirmed in brains of other AD model mice, such as the APP/PS1 mice (Supplementary Figure 1c). As PS1 mutations causing early-onset AD modulate protein expression by disrupting endoplasmic reticulum homeostasis, to determine whether decreased Crif1 levels in Tg6799 and APP/PS1 mouse brains were caused by PS1 mutations, we measured Crif1 levels in the brains of 12-month-old Tg2576 mice that carry only the APP mutation (Supplementary Figure 1c). The levels of Crif1 were reduced in Tg2576 mice compared with non-transgenic littermate controls, indicating that Aβ pathology-bearing mice show a reduction of Crif1 expression regardless of mutant PS1 expression. To determine whether Crif1 level is also altered in brains of AD patients, quantitative real-time PCR (qRT-PCR) and WB analyses in the superior temporal cortex of human brains, showed a reduction in Crif1 mRNA and protein levels in AD patients, as much as 35% and 21%, respectively, compared with control brains (Figures 1f and g). In addition, immunohistochemical analysis of postmortem human brain sections, containing the hippocampus, CA3, and CA1 regions, revealed that the intensity of Crif1 3′-diaminobenzidine (DAB) staining was decreased in AD patients (Figure 1h, Supplementary Table 1). Overall, these data indicate that Crif1 expression is reduced in pathological areas of AD brains.

Aβ1–42 affects Crif1 expression by modulating Crif1 mRNA. The above in vivo data showed reduced Crif1 levels in the pathological regions of AD (Figure 1 and Supplementary Figure 1), and APP mutation-bearing mouse models showed decreased Crif1 expression levels (Supplementary Figure 1b); thus, we determined whether Aβ, a major risk factor for AD, alters Crif1 expression. Exogenous Aβ1–42, but not Aβ42-1 (reverse form of Aβ1–42), decreased endogenous Crif1 levels in SH-SY5Y cells in time- (Figure 2a) and dose-dependent manners (Figure 2b). Immunofluorescence experiments, using anti-Crif1 and anti-HSP60 antibodies, the latter a mitochondria indicator, showed that Aβ decreased intracellular Crif1 levels (Figure 2c). To examine whether Aβ-induced Crif1 reduction is cell specific, Aβ was applied to HT22 cells, the mouse hippocampal neurons. HT22 cells showed decreased Crif1 levels after Aβ treatment (Supplementary Figure 2a). To examine the mechanism of downregulation of Crif1 by Aβ in SH-SY5Y cells, we checked whether Crif1 is degraded by
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Figure a: Experimental conditions: Aβ 5μM, Veh (vehicle) 0.5h, 3h, 6h, 12h, 24h. Western blots of Crif1 and β-actin.

Figure b: Aβ for 24hr, Veh 0.5, 1, 2.5, 5 μM Aβ 42, 42.1. Western blots of Crif1 and β-actin.

Figure c: Enlarged images of Merge, Crif1, Hsp60, and Merge. Veh and Aβ treated cells. Crif1 integrated density (% of Veh).

Figure d: Aβ treated conditions: Veh, +MG, +3MA, +Baf. Western blots of Crif1 and β-actin.

Figure e: mRNA levels (Fold changes) with Crif KD (knockdown).
degradation pathways such as the proteasome and/or autophagy-lysosomal pathways. We found that MG132, a potent proteasome inhibitor,\textsuperscript{18} and/or 3-methyladenine (3-MA) and bafilomycin, inhibitors of the autophagy-lysosomal system,\textsuperscript{19} failed to rescue Aβ-induced *Crif1* reduction (Figure 2d), suggesting that Aβ-mediated *Crif1* regulation did not occur via protein degradation. Next, we checked for changes in the transcriptional levels of *Crif1* after Aβ treatment by using qRT-PCR. We found that Aβ reduced *Crif1* mRNA levels without reducing the mRNA levels of other mitochondrial proteins, such as TOM20 (translocase of outer mitochondrial membranes 20 kDa) and TIM50 (translocase of inner mitochondrial membrane 50 kDa), indicating that Aβ disturbs the transcriptional processing of *Crif1* (Figure 2e). In addition, the reduction of *Crif1* mRNA levels lasted for 24 h after Aβ treatment (Supplementary Figure 2b). These data indicate that Aβ induced the reduction of *Crif1* levels at the transcriptional level.

**Crif1** mRNA is downregulated by Aβ-induced ROS by facilitating sumoylation of the Sp1 transcription factor. Previous studies showed that Aβ increased ROS production via the activation of several pathways, and the increased ROS has been proposed to have a harmful role in AD pathogenesis.\textsuperscript{3,5} To check the effect of ROS on *Crif1* levels, treatment with H$_2$O$_2$ reduced *Crif1* levels significantly (Figure 3a). To determine specifically whether Aβ-induced ROS generation reduced *Crif1* levels, treatment with the ROS scavenger, N-acetylcysteine (NAC), could rescue *Crif1* levels in Aβ-treated SH-SY5Y cells (Figure 3b), indicating that *Crif1* levels are affected by ROS levels, which might be excessively increased by Aβ.\textsuperscript{5} ROS are formed as byproducts of mitochondrial respiration or by the action of oxidases, including nicotine adenine diphosphate (NADPH) oxidase, and xanthine oxidase.\textsuperscript{20} Because Aβ is known to accelerate ROS generation by activating NADPH oxidase,\textsuperscript{21} and Aβ-induced *Crif1* reduction is mediated by ROS, we examined whether NADPH oxidase mediates Aβ-induced *Crif1* regulation. When treatment of Aβ with apocynin and diphenyleneiodonium (DPI), well-known NADPH oxidase inhibitors,\textsuperscript{22,23} was applied to SH-SY5Y cells, *Crif1* levels showed an increase compared with Aβ-treated cells. These data indicate that Aβ-induced activation of NADPH oxidase generates ROS, thereby reducing *Crif1* levels. Because Aβ-induced reduction of *Crif1* expression levels is mediated by transcriptional regulation in Figure 2e, to further investigate the mechanisms of *Crif1* reduction by Aβ, we used a software program to search for transcription factor-binding sites with the promoter region sequence of *Crif1* (http://www.genetools.us/genomics/). Through the binding motif analysis, we identified several candidate transcription factors, including AML-1A, SRY, and Sp1. Among them, Sp1 is a ubiquitous transcription factor, and has been previously reported to be an ROS-sensitive transcription factor for other proteins.\textsuperscript{24,25} To determine whether Sp1 binds to the *Crif1* gene promoter region, gel electrophoresis mobility shift assay (EMSA) was performed (Figure 3d). There was a reduction in the moving distance in a non-denaturing gel because of the binding of Sp1 to the probe, the *Crif1* gene promoter sequence (Figure 3d, arrowhead). We also found that 5 μM of Aβ treatment resulted in reduced binding between Sp1 and *Crif1* promoter region by detecting a less intense signal on the gel (Figure 3d, arrowhead). According to previous studies, sumoylation of Sp1 blocks the cleavage for the negative regulatory domain of Sp1 and decreases Sp1-dependent transcription.\textsuperscript{26} As increased ROS facilitates sumoylation of many proteins, and high ROS levels have been demonstrated in AD,\textsuperscript{5,24,25} we tested the possibility that abnormal over-production of ROS in AD might cause sumoylation of Sp1, thereby decreasing Sp1-dependent transcription of *Crif1*. To determine whether Aβ increased sumoylation of Sp1, we performed co-immunoprecipitation (Co-IP) experiments with Sp1- and SUMO-1-specific antibodies. We found that Aβ enhanced the interaction between Sp1 and SUMO-1, which indicates that Aβ-induced Sp1 sumoylation (Figures 3e and f). To test the effects of Sp1 sumoylation on *Crif1* levels, Sp1 wild type and Sp1 K16A, a sumoylation-deficient mutant of Sp1,\textsuperscript{27} cDNAs were transfected to SH-SY5Y cells. When Aβ was added to cells, Sp1 K16A mutant-transfected cells showed less decrease in *Crif1* levels compared with Sp1 wild-type-transfected cells (Figure 3g). These data indicate that Aβ-induced reduction of *Crif1* is mediated by sumoylation of Sp1, which negatively affects binding of Sp1 to the *Crif1* gene promoter following gene expression.

**Crif1** is crucial for maintaining mitochondrial morphology in SH-SY5Y cells. Previous studies have demonstrated the disruption of mitochondrial morphology and impaired mitochondrial function in AD.\textsuperscript{28} Aβ seems to contribute to these phenomena, by inducing excessive mitochondrial fission and failure of the OXPHOS system.\textsuperscript{29} Disrupted mitochondrial morphology was consistently observed in the cortex of Tg6799 mouse brains upon EM analyses (Figure 4a).
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Because previous studies show that Crif1 has a critical role in maintaining mitochondrial function via the integration of OXPHOS polypeptides into the mitochondrial membrane, and Crif1 is mainly located in mitochondria, we investigated whether reduced Crif1 expression causes the disruption of mitochondrial morphology. We transfected SH-SY5Y cells with Crif1 siRNA (Crif1 KD), and the expression of Crif1 was reduced by as much as about 60% in relation to the control siRNA-transfected group (Figure 2e and Supplementary Figure 4a). Using a Mito-DoRed expressing SH-SY5Y cell line, we analyzed alterations in mitochondrial morphology in control siRNA (Veh), Aβ-treated control siRNA(Aβ), Crif1 siRNA (Crif1 KD), and Aβ-treated Crif1 siRNA-transfected cells (Crif1 KD+Aβ; Figures 4b and c). Consistent with previous studies, mitochondria were shortened (represented by aspect ratio) and more circular (represented by form factor) after treatment with 5 μM of Aβ (Figure 4c). Interestingly, Crif1 knockdown itself (Crif1 KD) could disrupt mitochondrial morphology resembling that of Aβ-treated control siRNA-transfected cells (Aβ; Figures 4b and c). In addition, Aβ-treated Crif1 KD cells (Crif1 KD+Aβ) did not show further mitochondrial disruption compared with Crif1 KD cells, indicating that reduced Crif1 levels are sufficient to disrupt the mitochondrial structure (Figures 4b and c). Furthermore, massive mitochondrial fission and loss of cristae were observed in Crif1 KD cells using EM analysis (Figure 4d). To determine whether Crif1 interacts with mitochondrial fission/fusion protein, Co-IP with Crif1 and fission/fusion protein-specific antibodies was performed. We found no interaction between Crif1 and mitochondrial fission/fusion proteins (Supplementary Figure 5). These data suggest that Crif1 is important for maintaining mitochondrial morphology and that Aβ-induced mitochondrial disruption is mediated by attenuating Crif1 levels.

Loss of endogenous Crif1 leads to severe defects in various mitochondrial functions in SH-SY5Y cells. We determined whether Crif1 regulates mitochondrial function as well as mitochondrial morphology under Aβ-treated conditions. When Crif1 siRNA was transfected into SH-SY5Y cells, the mitochondrial membrane potential (MMP) was significantly attenuated using JC-1 and TMRM assays (Figure 4e and Supplementary Figure 6a). In addition, Crif1 KD cells showed decreased ATP levels by ATP-luciferase assays (Figure 4f). To check ROS generation, we used the dichloro-dihydrofluoresceindiacetate and MitoSOX assays, which showed increased ROS generation in Crif1 KD cells (Figure 4g, and Supplementary Figure 6b). Overall, these data indicate that downregulated Crif1 expression induced mitochondrial dysfunctions as shown in AD condition.

Overexpression of Crif1 rescues Aβ-induced disruption of mitochondrial morphology. To examine whether Crif1 is a key molecule for maintaining mitochondrial integrity, either scrambled or Crif1 cDNA was transfected into SH-SY5Y cells. When Aβ was administered to the cells, Crif1 overexpression (o/e) cells showed healthy mitochondria in terms of length and shape (Figures 5a and b). To further confirm the role of Crif1 in mitochondrial function, mitochondrial functions were evaluated after scrambled or Crif1 cDNA transfection followed by Aβ treatment. Decreased MMPs and ATP synthesis were not fully, but were significantly, rescued as much as 50% and 20% in Crif1 o/e cells after evaluation with JC-1 (Figure 5c) and ATP-luciferase assays (Figure 5d), respectively. Strikingly, Aβ-induced ROS generation was completely blocked by Crif1 o/e (Figures 5e and f). These data show that Aβ decreases Crif1 levels, and enhances ROS generation, causing impairment in mitochondrial functions. In summary, our data suggest that Crif1 levels are essential to maintain mitochondrial homeostasis and Aβ-induced mitochondrial disruption is mediated by decreased Crif1 expression.

Crif1 levels are important for Aβ-induced cell death in SH-SY5Y cells. Previous studies have demonstrated that mitochondrial dysfunction leads to cell death via intrinsic apoptotic pathways. To determine whether the Aβ-induced decrease in Crif1 can influence cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Calcein-AM assays were performed (Figures 6a and b). We found that Crif1 KD alone, as well as Aβ treatment, caused significant cell death. Interestingly, overexpression of Crif1 significantly increased cell viability of Aβ-treated conditions (Figures 6c and d). To confirm the role of Crif1 on Aβ-induced cell death, TUNEL (TdT-mediated dUTP Nick-End Labeling) assays were performed (Figure 6e). Consistent with the results of MTT and...
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Calcein-AM assays, o/e of Crif1 diminished Aβ-induced cell death. These data indicate that Crif1 levels are crucial for cell survival in the presence of Aβ.

Discussion

Although many studies have reported that Aβ induces severe mitochondrial dysfunction, which is believed to have a central role in AD pathogenesis,12,28-32 pow Aβ affects mitochondrial impairment is not yet fully understood. Previous findings demonstrated that several MIM structural proteins contribute to the maintenance of mitochondrial morphology, including cristae structures.33-35 In a recent report, it was shown that Crif1 is important for maintaining mitochondrial function, and alterations in Crif1 levels cause severe mitochondrial dysfunction.16 However, there were no data regarding the role of Crif1 in the brain and its relationship to pathological conditions. Here, we demonstrate the role of Crif1 in the brain and its regulation, particularly in the pathological condition of AD. This study provides in vitro and in vivo evidence that shows that reduced Crif1 levels, mediated by Aβ, cause mitochondrial defects, resulting in cell death (Figure 7). Recently, Crif1 was reported to be important for maintaining mitochondrial function by aiding the insertion of OXPHOS subunits and stabilizing the MIM integrity.16 In our imaging data, using super-resolution microscopy, we confirmed that Crif1 is mainly located in MIM (Supplementary Figure 3); thus, the reduction in Crif1 levels might affect the stability of the OXPHOS system in MIM and the energy production required for cell survival. Furthermore, reduced Crif1 expression in AD brains would contribute to AD pathology by impairing the OXPHOS system and mitochondrial functions.

There is increasing evidence that ROS accelerate the progression of AD pathology during the early period of the disease.36,37 However, the precise role of ROS in Aβ-induced neuronal loss has not yet been unraveled. In an effort to identify signaling molecules underlying the role of Crif1 in AD pathogenesis, we also found that Aβ-induced downregulation of Crif1 requires increased levels of ROS. These results were extended to an additional mechanism that ROS-dependent sumoylation of the Sp1 transcription factor has a role in Aβ-induced downregulation of Crif1 expression. Until now, there was no known transcription factor regulating the expression levels of Crif1, and we first identified that the ROS-sensitive transcription factor Sp1, regulates Crif1 levels directly after Aβ treatment. Previous studies showed that sumoylation of Sp1 attenuates protein stability and decreases the Sp1-dependent transcription,26 whereas oxidative stress regulates Sp1 activity.38 In addition, Sp1 dysregulation has been reported in AD brains,39 suggesting that dysregulation of Sp1, by excessive ROS, might contribute to AD progression. In this study, we showed that Aβ increased Sp1 sumoylation by using Co-IP, indicating that Aβ might reduce Crif1 expression through increased Sp1 sumoylation (Figures 3e and f). Data obtained using a sumoylation-deficient mutant form of Sp1 confirmed these results (Figure 3g). In addition, co-treatment of Aβ with NADPH oxidase inhibitors (apocynin and DPI) rescued Aβ-induced Crif1 reduction in SH-SY5Y cells, suggesting that Aβ activates NADPH oxidase, resulting in ROS generation (Figure 3c). Previous reports showed that Aβ could induce pathological insults, including an increase in intracellular Ca2+, inflammation, and endoplasmic reticulum stress, as well as ROS generation.3,5,40 We also examined whether treatment with a Ca2+ chelator or chemical chaperone could block Aβ-induced Crif1 reduction. However, these blockers could not reverse the decrease in Crif1 levels (data not shown). These data indicate that Aβ induced downregulation of Crif1 by elevating ROS level. It is well known that in vivo ROS levels are enhanced in vulnerable regions that exhibit AD pathology, such as the cortex and the hippocampus. Xie et al. reported that oxidative stress was more dramatic in neurites near plaques, and rapid cell death by amyloid plaque-mediated oxidative stress was shown in AD brains.41 In previous studies, no plaques are shown in the cerebellum of AD brains,42,43 so ROS-induced Crif1 reduction is mainly demonstrated in the cortex and the hippocampus (Figure 1 and Supplementary Figure 1), but not in the cerebellum (Figure 1a). Further studies on the Crif1 levels in many other neurodegenerative diseases, including PD and HD that are associated with increased ROS levels, should be investigated.

Our data showed that the dysregulation in mitochondrial dynamics (such as abnormal mitochondrial fission) mediated by Crif1 KD and/or the treatment with Aβ, induced mitochondrial dysfunction, resulting in cell death (Figure 4). However, in Figures 5 and 6, there is only a slight rescue in MMP and ATP production by overexpression of Crif1, yet almost we see a complete reversal of ROS production and apoptosis. There is an explanation for the results; in previous studies, Aβ is known...
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A

Veh

Aβ

Crif1 o/e

B

Aspect Ratio

Form Factor

C

Green/Red ratio (% of Veh)

D

AIF level (% of Veh)

E

Veh

Aβ

Crif1 o/e

Crif1 o/e + Aβ

F

Veh

Aβ

Crif1 o/e

Crif1 o/e + Aβ

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to interact with ATP synthase and cyclophilin D in the mitochondria, and then results in decreased ATP production and the opening of the mitochondrial permeability transition pore. Because overexpression of Crif1 cannot block Aβ binding to its interacting partners, ATP levels and MMP might be partially, but significantly, rescued in Crif1-overexpressing cells. However, partially restored ATP, MMP, and inhibition of ROS might be enough to maintain cell survival as shown in Figures 6c–e.

Previous studies indicated that Aβ can enter the cells, and mediate excitotoxicity by enhancing Ca²⁺ influx, which eventually leads to cell death. Aβ has been found to induce mitochondrial Ca²⁺ overload through a mitochondrial Ca²⁺ uniporter (MCU). To determine whether Aβ-induced mitochondrial Ca²⁺ overload, induced mitochondria abnormalities and cell death, Ruthenium red (RuR), a nonspecific inhibitor of the MCU, and Ru360, a highly specific inhibitor of the MCU, were administered to SH-SY5Y cells, and then MTT and Calcein-AM assays were performed. We found that treatment with MCU inhibitors (RuR and Ru360) along with Aβ failed to rescue Aβ-induced mitochondrial dysfunction and cell death (Supplementary Figures 7a and b). This result indicates that MCU-dependent Ca²⁺ influx into mitochondria, have no role in Aβ-induced mitochondrial dysfunction. Treatment with a ROS scavenger rescued Aβ-induced mitochondrial dysfunction and cell death. Our data indicate that Aβ-induced mitochondrial dysfunction and cell death are mainly mediated by ROS (Supplementary Figures 7a and b).

There are important MIM proteins that regulate mitochondrial morphology, including chchd3, mitofilin, ATP synthase, and prohibitin. Abolition of these proteins is known to cause the disruption of mitochondrial morphology as well as severe mitochondrial dysfunction, which was also observed in Crif1 KD MEF cells. Massive neurodegeneration and hyper-phosphorylated tau were observed in prohibitin-deficient neurons in the forebrain of phb²knf mice, implying that disruption of mitochondrial morphology by loss of MIM proteins is closely related to the development of AD pathology. Thus far, there have been no detailed reports suggesting a close relationship between other MIM proteins and AD. Therefore, it is highly likely that dysregulation of these proteins in AD might induce mitochondrial dysfunction, and their role in AD should be further investigated in future studies.

Collectively, our results identify the ROS-Sp1-Crif1 pathway to be a new mechanism underlying Aβ-induced mitochondrial dysfunction and suggest that ROS-mediated downregulation of Crif1 is a crucial event in AD pathology (Figure 7). To investigate the relationship between other transcription factors besides Sp1, and Crif1 levels, inhibitors for several other transcription factors, including AP-1 and NF-κB, were administered together with Aβ into cells, and we found that these inhibitors did not block the reduction of Crif1 levels by Aβ (data not shown). Therefore, we hypothesize that the ROS-Sp1-Crif1 pathway regulates Aβ-induced mitochondrial dysfunction, which is a crucial event in AD pathology (Figure 7).

Materials and Methods

Animals and human brain samples. We used three different types of AD model mice: Tg2576 mice (expressing the APP Swedish mutation), APP/PS1 mice (expressing Swedish APP and Presenilin1 delta exon 9 mutations), and Tg6799 mice. The Tg6799 mice (formerly JAX Stock No. 002720) overexpress both mutant human APP695 form along with the Swedish (K670N, M671L), Florida (I176V), and London (V717I) familial AD (FAD) mutations and human PS1 harboring two FAD mutations, M146L and L286V. All mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and the animals maintained in Seoul National University’s mouse facility. All experiments were approved by the Institute of Laboratory Animal Resources of Seoul National University. Human brain tissues for WB and qRT-PCR were obtained from Boston University, Boston, MA, USA, and postmortem tissue blocks, containing the hippocampal formation from AD patients (Braak V-VI) and healthy control donors for immunohistochemistry, were obtained from the Harvard Brain Tissue Resource Center, Belmont, MA, USA (see Supplementary Table 1 for additional information of human brain tissues).

Cell culture, transfection, and drug treatment. SH-SY5Y, a human neuroblastoma cell line, and HT22, an immortalized mouse hippocampal neuronal cell line, were cultured as previously described. Cells were transfected with cdNA for Crif1 (gifted from Dr. Shong (Chungnam University, Korea), Sp1 (mM000598; provided from Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea), Sp1 K16A or Mito-DsRed using Lipofectamine and Plus reagents provided from Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea), and Crif1 siRNA (Santa Cruz Biotechnology, Santa Clara, CA, USA) and Crif1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using RNAiMax reagents (Invitrogen) for 48 h according to the manufacturer’s instructions. Cells were grown to 60% confluency and treated with vehicle (dimethyl sulfoxide) or Aβ42–43 (American Peptide, Sunnyvale, CA, USA), Aβ42–43 (Bachem, Bubendorf, Switzerland) and various reagents; MG132 (10 μM; M7449, Sigma-Aldrich, St. Louis, MO, USA), 3-MA (2 mM; M9281, Sigma-Aldrich), bafilomycin (5 μM; B1793, Sigma-Aldrich), NAC (1 mM; A7250, Sigma-Aldrich), apocynin (10 μM; A1089, Sigma-Aldrich), DPI (10 μM; D2626, Sigma-Aldrich), and H₂O₂ (216763, Sigma-Aldrich).

Western blotting. Harvested cell pellets and mouse brain tissues were prepared as previously described. The antibodies used for WB were: anti-Crif1 (sc-134882), TOM20 (sc-17764), TOM40 (sc-11414), Dp1 (sc-32898), F1st (sc-99900), Mfn1 (sc-50330), and OPA1 (sc-30573) antibodies from Santa Cruz Biotechnology (1:500 for WB); anti-TIM50 (ab23938; 1:1000); GAPDH (ab8485; 1:3000) antibodies from Abcam; anti-Sp1 antibody (07-645) from Millipore (Billerica, MA, USA; 1:1000); anti–Sumo-1 (4930); HSP60 (4890), and OPA1 antibodies from Cell Signaling Technology (Beverly, MA, USA; 1:1000 for WB; 1:200 for IP); anti–β-actin antibody from Sigma-Aldrich (1:3000). Immunoreactive bands were photographed and quantified on an LAS-3000 with Multi Gauge (Fuji Film Inc., Tokyo, Japan).

Mitochondrial morphology analysis. Mitochondrial morphology was investigated using Mito-Tracker Green-transfected SH-SY5Y cells. Briefly, 5 μM of Aβ was administered to the cells for 12 h before live-cell imaging or staining process.
Crif1 levels are important for \( \beta \)-induced cell death in SH-SY5Y cells. (a and b) Crif1 KD induced cell death by MTT assay (a) and Calcein-AM assay (b). Cell viability assays showed that Crif1 KD enhanced cell death as much as \( \beta \)-treated condition (5 \( \mu \)M, 24 h). * \( P < 0.05 \), ** \( P < 0.01 \) versus vehicle-treated control siRNA-transfected cells (c and d). Increased Crif1 expression blocked \( \beta \)-induced cell death. After cDNA transfection for 24 h, 5 \( \mu \)M \( \beta \) was administered for 24 h, and then MTT (c) and Calcein-AM assay (d) were performed. * \( P < 0.05 \) versus vehicle-treated mock vector-transfected cells. Data were obtained from at least five replicates per group (\( N = 5 \) experiments). (e) To confirm cell death in Crif1 KD or Crif1 o/e cells, TUNEL assay was performed. Scale bar represents 20 \( \mu \)m. Data are shown as mean ± S.E.M. of three independent experiments (\( n = 200 \) each groups, \( N = 3 \) experiments). ** \( P < 0.01 \) versus vehicle-treated mock vector-transfected cells; ## \( P < 0.01 \) versus \( \beta \)-treated mock vector-transfected cells; NS indicates no significant difference.

Figure 6  
Crif1 levels are important for \( \beta \)-induced cell death in SH-SY5Y cells. (a and b) Crif1 KD induced cell death by MTT assay (a) and Calcein-AM assay (b). Cell viability assays showed that Crif1 KD enhanced cell death as much as \( \beta \)-treated condition (5 \( \mu \)M, 24 h). * \( P < 0.05 \), ** \( P < 0.01 \) versus vehicle-treated control siRNA-transfected cells (c and d). Increased Crif1 expression blocked \( \beta \)-induced cell death. After cDNA transfection for 24 h, 5 \( \mu \)M \( \beta \) was administered for 24 h, and then MTT (c) and Calcein-AM assay (d) were performed. * \( P < 0.05 \) versus vehicle-treated mock vector-transfected cells. Data were obtained from at least five replicates per group (\( N = 5 \) experiments). (e) To confirm cell death in Crif1 KD or Crif1 o/e cells, TUNEL assay was performed. Scale bar represents 20 \( \mu \)m. Data are shown as mean ± S.E.M. of three independent experiments (\( n = 200 \) each groups, \( N = 3 \) experiments). ** \( P < 0.01 \) versus vehicle-treated mock vector-transfected cells; ## \( P < 0.01 \) versus \( \beta \)-treated mock vector-transfected cells; NS indicates no significant difference.
were transfected with control and Crif1 siRNA or scrambled and Crif1 cDNA for 24 h. Expression resulted in disruption of mitochondrial morphology and mitochondrial reduction of Crif1 mRNA levels by increasing the sumoylation of Sp1. Reduced Crif1 mRNA in SH-SY5Y cells, RNA was isolated using the RNeasyPlus Mini Kit (Qiagen, Valencia, CA, USA) and reverse transcription of the isolated RNA was performed in vehicle or 5 μM Aβ1-42-treated SH-SY5Y cells. The following sense and antisense primers were used for human Crif1: 5′-GACAGGCAAGC AGCCTACTA-3′ (sense), 5′-ATCATCTGTGGCATCTTG-3′ (antisense).

Quantitative real-time PCR. To examine the levels of mRNA of Crif1 or mitochondrial protein in human brain samples and SH-SY5Y cells, qRTPCR was performed as previously described.57 The following sense and antisense primers were used: 5′-GTGCCCAATGATGGTGA-3′ (sense), 5′-CGTCTTCTGTT TCCCTCTCCT-3′ (antisense) for Crif1; 5′-GACGCGAAAGAAGGATGAC-3′ (sense), 5′-GCTTCAGACATTTAAGGTCAAG-3′ (antisense) for TOM20; 5′-AGCA CTATGCCCTGAGGATG-3′ (sense), 5′-GAGCCAGAAGGATTCTGC-5′ (antisense) for TIM50; 5′-ACAGCGGCTCTCTTGTGACGTG-3′ (sense), 5′-GGCC TTGACTGTGCCGTTGAATT-3′ (antisense) for GAPDH.

Electrophoretic mobility shift assay. To examine the interaction with the promoter region of Crif1 and Sp1 transcription factor, we performed an EMSA in vehicle or 5 μM Aβ1-42-treated SH-SY5Y cells for 24 h using the Light Shift Chemiluminescent EMSA kit (Thermo Scientific, Hudson, NH, USA) following the manufacturer’s protocol. A double-stranded probe was generated by annealing two biotin-labeled oligonucleotides against the putative Sp1-binding site (5′-ACCCGCC-3′) within the human Crif1 gene promoter region; forward probe (5′-biotin-TTRGACGCC CCCACC-3′) and reverse probe (5′-biotin-GGTT GGCGCGGTTGATTAAA-3′).

Co-immunoprecipitation. For Co-IP experiments, vehicle or SH-SY5Y cells treated with Aβ1-42 for 24 h were lysed with M-PER mammalian protein extraction reagent (Thermo Scientific) and processed as previously described.58

Immunostaining. Immunocytochemical staining was performed as previously described.59 Images were obtained using a confocal laser scanning microscope (FV10α-w, Olympus). For histological analyses, Tg6799 mice were killed at 6 months of age (n = 6 per group), brains were sliced and were stained for Crif1 (Santa Cruz Biotechnology, 1:250),40 Immunohistochemistry on human brain tissues was performed as previously described.59 Briefly, postmortem tissue blocks were cut into 40 μm-serial sections on a freezing sliding microtome (Model 860; American Optical Company, Buffalo, NY, USA). Sections were stored in cryoprotectant solution (30% glycerol, 30% ethylene glycol, 0.1% sodiumazide in PB; pH 7.4) at −20 °C. Tissue sections were incubated for 48 h with anti-Crif1 (Santa Cruz Biotechnology; 1:100), then the sections were incubated in biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA; 1:500) for 2 h at room temperature. Subsequently, the sections were incubated with streptavidin (Invitrogen; 1:5000) for 2 h at room temperature. Nickel-enhanced DAB/peroxidase reaction (0.02% DAB (Sigma-Aldrich), 0.08% nickel-sulfate, 0.06% hydrogen peroxide) was used to visualize the reaction product.

Aβ1-42 preparation. Aβ1-42 peptide (American peptide, Sunnyvale, CA, USA) was prepared as previously described.55 Aβ1-42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich) to a concentration of 1 mM. The solution was aliquoted and 1,1,1,3,3,3-hexafluoro-2-propanol was evaporated by vacuum (SpeedVac Concentrator; Savant Instruments, Hyderabad, India). The dry peptide was maintained at −80 °C and dissolved with anhydrous dimethyl sulfoxide to 1 mM following dilution in Opti-MEM (Invitrogen). Most of Aβ1-42 consisted predominantly of oligomers, and some monomers.56

Statistical analysis. For WB, protein levels were normalized to pan forms or a housekeeping protein, such as β-actin or GAPDH. All data were expressed as means ± standard error of the mean (S.E.M.). Student’s t-test was used for two-group comparisons, and analysis of variance, followed by Fisher’s LSD post-hoc test, was used to compare three or more groups using SigmaStat for Windows Version 3.10 (Systat Software, Inc., Point Richmond, CA, USA). P values < 0.05 were considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.
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Author contributions

JB and SM performed research, collected data, and wrote the manuscript. YH, JK, MM and K-SK performed research and collected data for human brain tissues. M-YC, MS and SKP collected data. IM-J supervised the study and reviewed and edited the manuscript.


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