

## 論文の内容の要旨

論文題目 Identification of calcium and integrin-binding protein 1 (CIB1) as a novel regulator of production of Amyloid  $\beta$  peptide using CRISPR/Cas9-based screening system  
(CRISPR/Cas9 システムを用いた  $A\beta$  産生を制御する新規因子 CIB1 の同定)

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### [Introduction]

Alzheimer disease (AD) is the most common progressive neurodegenerative disorder, pathologically characterized by the deposition of the amyloid- $\beta$  peptide ( $A\beta$ ) as senile plaques in the brain. Several lines of evidence indicate that the abnormal aggregation of  $A\beta$  affects the pathological process of AD by triggering neurotoxicity and neurodegeneration, suggesting the important role of  $A\beta$  in the etiology of AD.  $A\beta$  is generated through the sequential cleavage of amyloid precursor protein (APP) by  $\beta$ -site APP cleaving enzyme 1 (BACE1) and  $\gamma$ -secretase. After the first cleavage by BACE1,  $\gamma$ -secretase cleaves the stub of APP at different positions, producing  $A\beta$  with various C-terminal lengths, such as  $A\beta_{40}$  and  $A\beta_{42}$ , the latter being more hydrophobic and the initial as well as predominant species in the senile plaques.  $\gamma$ -Secretase is an intramembrane-cleaving protease complex comprising four subunits, namely, the catalytic subunit presenilin 1 (PS1) or presenilin 2 (PS2), anterior pharynx-defective 1 (Aph-1), Nicastrin (Nct), and presenilin enhancer 2 (Pen2). Several genetic studies have demonstrated that familial AD-linked autosomal-dominant mutations in PS1 and PS2 increases  $A\beta_{42}$  production, indicating the involvement of  $\gamma$ -secretase in the pathogenesis of AD via generating the heterogeneity of  $A\beta$  species. As described, whereas the aberrant metabolism of  $A\beta$  has been implicated in the etiology of AD, the precise regulatory mechanism of  $A\beta$  generation remains unclear. To obtain a better understanding of the molecular mechanism of  $A\beta$  production, we established a genetic screening based on the CRISPR/Cas9 system to identify novel regulators of  $A\beta$  production.

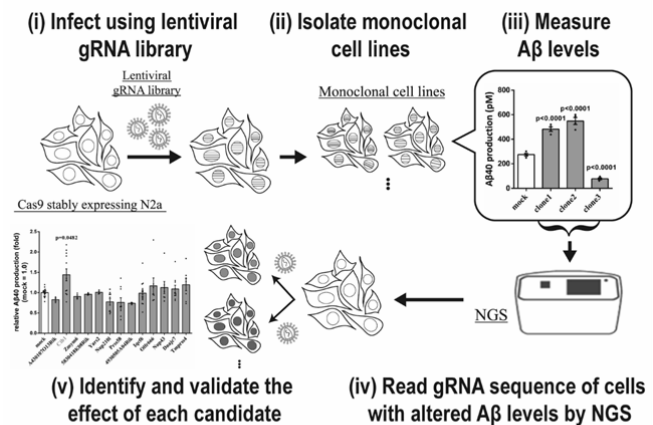


Fig. 1. The strategy of identifying novel regulators of  $A\beta$  production using CRISPR/Cas9 system.

### [Results]

#### 1. Identification of CIB1 as a negative regulator of $A\beta$ production

As shown in Fig. 1, murine neuroblastoma Neuro2a (N2a) cells were infected with a lentiviral guide-RNA (gRNA) library. The resulting cell lines were screened to identify those showing changes in  $A\beta$  production. The gRNA sequences in the obtained cell lines were amplified and

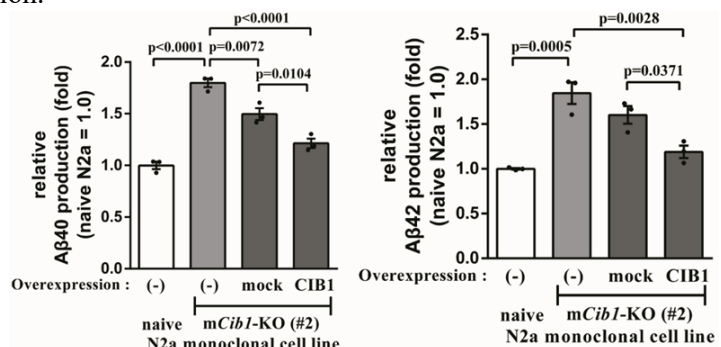


Fig. 2. CIB1 negatively regulates  $A\beta$  levels. The relative secreted  $A\beta_{40}$  and  $A\beta_{42}$  level. Mouse CIB1 was expressed in *Cib1*-KO monoclonal cell (n = 3, mean  $\pm$  SEM, P values were assessed by one-way ANOVA with Tukey's HSD post hoc analysis).

read using a next-generation sequencer (NGS) to identify candidate genes. Through this screening, we discovered 13 candidates from 3 cell lines with changes in A $\beta$  levels. Regarding these 13 candidate genes, we analyzed the effects of each candidate on A $\beta$  levels by disrupting each gene individually and finally identified calcium and integrin-binding protein 1 (CIB1) as a negative regulator of A $\beta$  production. We further confirmed that the upregulated A $\beta$  levels in *Cib1*-knockout (KO) monoclonal cell lines can be restored to basal condition by overexpressing mouse CIB1 in these cells (Fig. 2), strongly suggesting that CIB1 has the ability to negatively regulate A $\beta$  production.

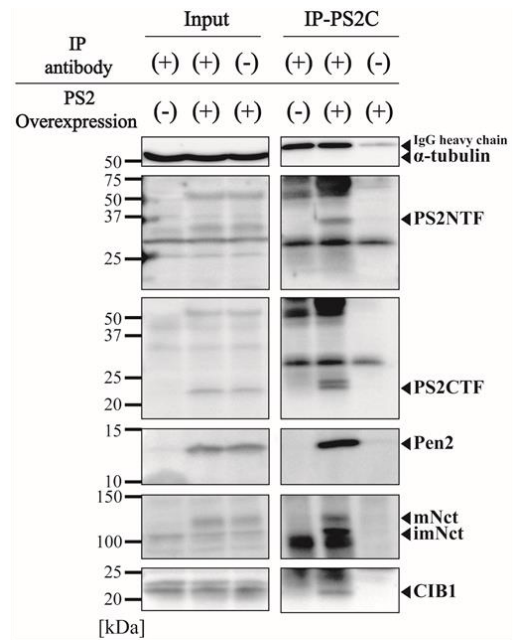
### 2. Direct interaction between CIB1 and $\gamma$ -secretase

We first analyzed the expression levels of the proteins related to the A $\beta$  production machinery. However, no significant difference was found in the expression levels of APP, BACE1, and the  $\gamma$ -secretase components in *Cib1*-KO cells. Since CIB1 has been previously identified as a PS2-binding protein, we next hypothesized the possibility of CIB1 in regulating  $\gamma$ -secretase activity through their interaction. To clarify this, we performed immunoprecipitation using the anti-PS2 C terminus antibody. After assembly in the endoplasmic reticulum, the  $\gamma$ -secretase complex is transported to the plasma membrane and activated. During this process, PS undergoes endoproteolysis to generate the N- and C-terminal fragments (i.e., NTF and CTF, respectively), and Nct is fully glycosylated as mature Nct (mNct). When we overexpressed PS2 in PS1/PS2 double-knockout (DKO) mouse embryonic fibroblasts (MEFs), sufficient active  $\gamma$ -secretase with fragmented PS and mNct was detected. Notably, together with the components of  $\gamma$ -secretase, endogenous CIB1 was coimmunoprecipitated, indicating the interaction between CIB1 and the  $\gamma$ -secretase complex (Fig. 3). This result suggested the possibility that CIB1 regulates A $\beta$  production by controlling the  $\gamma$ -secretase through their direct interaction.

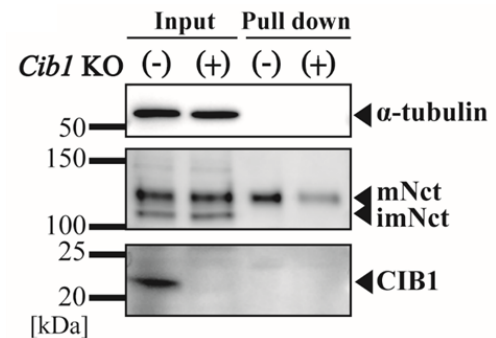
### 3. CIB1 regulates the subcellular localization of $\gamma$ -secretase

To assess whether the direct binding of CIB1 to the  $\gamma$ -secretase affects its intrinsic enzymatic activity, we carried out an *in vitro* assay using detergent-solubilized membrane fractions derived from *Cib1*-disrupted cells. The  $\gamma$ -secretase activity was preserved in this membrane fraction to produce A $\beta$  species upon coinubation with the recombinant  $\gamma$ -secretase substrate C99. However, we observed no difference in the *de novo* generation of A $\beta$  levels, suggesting the intrinsic enzymatic activity of  $\gamma$ -secretase was not affected by the downregulation of CIB1.

Previous reports indicated that CIB1 regulates the intracellular translocation of its binding



**Fig. 3. Direct interaction between CIB1 and  $\gamma$ -secretase.** Representative immunoblot for immunoprecipitation with anti-PS2 C terminus antibody.



**Fig. 4. Disruption of *Cib1* decreased  $\gamma$ -secretase membrane localization.** Upper: Representative immunoblotting of cell surface biotinylation for Nct. Bottom: Quantification of band intensities of mNct and cell surface mNct (n = 4, mean  $\pm$  SEM, P values were assessed by Student's t test)

partners. Intriguingly, the endo-lysosomal localization of the  $\gamma$ -secretase enhances its activity to overproduce A $\beta$ , and may contribute to the pathogenesis of AD. Thus, we hypothesized that CIB1 regulates the A $\beta$  production by affecting the subcellular localization of  $\gamma$ -secretase. To test this hypothesis, the cell surface biotinylation assay was performed using *Cib1*-disrupted cells. We found that the levels of mNct at the cell surface significantly decreased upon KO of *Cib1* without changes in the total level of mNct (**Fig. 4**). In contrast, we did not observe significant differences in the cell surface expression levels of other membrane proteins, including APP and BACE1. This suggested that the disruption of *Cib1* specifically decreased the surface localization of  $\gamma$ -secretase. Since the colocalization of internalized APP and  $\gamma$ -secretase facilitates the A $\beta$  generation, CIB1 would assist the translocation of the  $\gamma$ -secretase to the plasma membrane and reduce the encounter of  $\gamma$ -secretase with its substrate, thereby suppressing the A $\beta$  production.

#### 4. *CIB1* mRNA level decreased in AD patients

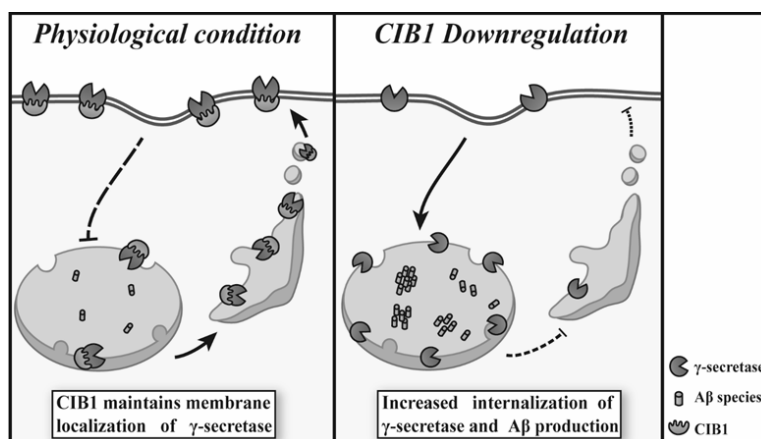
To investigate the relationship between CIB1 and AD pathology in humans, we referred to a single-cell RNA-seq analysis of AD patients. The level of *CIB1* transcripts in excitatory neurons significantly decreased in the early stage of AD compared with control without pathology, suggesting that the downregulation of CIB1 is involved in the pathogenesis of AD (**Table.1**).

No-pathology vs Early-pathology differential expression								
mRNA	IndModel. adj.pvals	no.pathology. mean	early.pathology. mean	Ind-Model. FC	Mixed- Model.z	Mixed- Model.p	DEGs.Ind- Model	DEGs.Ind- Mix.models
CIB1	1.64E-46	0.0502	0.03026	-0.7302	-5.4569	4.84E-08	TRUE	TRUE

**Table 1. Single cell RNA-seq data showed that the *CIB1* mRNA level significantly decreased in the excitatory neurons in AD patients with the early-pathology.** (DEGs.Ind-Model and DEGs.Ind.Mix.models showed true, indicating the significant difference between two groups). IndModel.adj.pvals, FDR-adjusted p-values, two-sided Wilcoxon rank-sum test. IndModel.FC, log2 fold change of pathology mean value relative to no-pathology mean value. MixedModel.z, z-score of Poisson mixed model. MixedModel.p, p-value of Poisson mixed model. DEGs.Ind.Model, logical indication of whether a gene meets the criteria FDR-adjusted p-value < 0.01 and absolute log2 fold change > 0.25. DEGs.Ind.Mix.models, logical indication of whether a gene meets the criteria FDR-adjusted MixedModel.p < 0.05 (fdr-adjustment over genes meeting criteria in DEGs.Ind.Model).

#### [Summary and Discussion]

In this study, we established an innovative approach using the CRISPR/Cas9 system and successfully identified CIB1 as a negative regulator of A $\beta$  production. In addition, we demonstrated that CIB1 regulates A $\beta$  production via controlling the subcellular localization of  $\gamma$ -secretase (**Fig. 5**). However, the precise mechanism whereby CIB1 regulates the  $\gamma$ -secretase activity is not well understood. As one possibility, CIB1 might promote the membrane localization by directly shuttle  $\gamma$ -secretase from the cytosol to the plasma membrane. Additionally, CIB1 could retain  $\gamma$ -secretase at an early endosome by interacting with other vesicles transporting proteins. It is also possible that CIB1 suppresses the further internalization of  $\gamma$ -secretase by competing for the binding with the molecules helping  $\gamma$ -secretase internalization. Whereas further molecular and cellular studies will be needed to clarify the mechanistic role of CIB1 in the intracellular trafficking of  $\gamma$ -secretase, our data demonstrated CIB1 is involved in the development of AD.



**Fig. 5. Scheme of mechanism on regulation of A $\beta$  production by CIB1.**

#### [References]

1. Chiu, Y. W\*, Hori Y\*, Ebinuma I, Sato H, Hara N, Ikeuchi T. & Tomita, T. *FASEB J.* 2020 Jun;34(6):7661-7674. \*Co-first authors.