**Supplemental Figures**

**Figure legends**

**Figure S1.** Specificity and selectivity of the antibodies used in this study. *A*) Both anti-Flag and anti-$\beta_3$ antibodies recognize identical "ladder"-like Flag-$\beta_3$ bands. Flag-$\beta_3$ was expressed in Cos-7 and the cell lysate was subject to SDS-PAGE and immunoblotting (IB) under conditions described in Fig. 1A. *B*) The antibodies to tags and $\beta_3$ antibody in immunoblotting or immunoprecipitation (IP) show no cross-reactivity. Flag-, V5-His- and Venus-tagged $\beta_3$ subunits were each expressed in Cos7 cells, and aliquots of the cell lysate were subject to Flag immunoprecipitation and His-pull down, and then immunoblotted with the indicated antibodies. Flag immunoprecipitation and His-pull down recognized only the corresponding tagged proteins, and anti-tags antibodies recognized only the fusion proteins with their specific tags, while an anti-$\beta_3$ antibody recognized all the fusion proteins. Notice that non-specific (n.s.) band recognized by a His antibody (marked with *) in the lysate fraction disappeared after His-pull down or Flag immunoprecipitation. *C)* Specificity of Flag-IP to study protein oligomerization. Oligomerization of bacterial alkaline phosphatase (BAP) sensitive to DDT treatment was used as an example. Flag-BAP (0.5 µg, Sigma) was expressed in Cos7 cells and immunoprecipitated and immunoblotted with an anti-Flag antibody after standard heat denaturation (5 min, 95˚C) with or without a prior DTT treatment. Non-transfected (NT) cells were used as negative control. Only a monomeric Flag-BAP was detected on Western blot after DTT treatment (arrow marked with *), while dimers and trimers (arrows) were also observed in the absence of DTT treatment consistent with previous reports (1, 2). No reactivity was seen with the non-transfected cells (right panel). *D)* The immunoblot shown in Fig. 1*F* is $\beta_3$-specific. Samples collected by the same procedures as described in Fig. 1*F* were subject to immunoblotting with an anti-$\beta_3$ antibody, and all $\beta_3$ bands were confirmed by this antibody.

**Figure S2.** Flag-$\beta_3$ oligomerizes at the expression level of mouse brain tissue. *A)* 200 mg of C57BL/6J mouse brain tissue was treated by a Cellytic MT (Sigma) lysis solution and 15 mg protein was loaded on the gel (left lane). Cos7 cells were transfected with the indicated amounts of Flag-$\beta_3$ plasmid and 15 mg of total protein with increasing expression of Flag-$\beta_3$ loaded on other lanes. Aliquots of the protein samples were treated with DTT as described in Methods,

subjected to immunoblotting with an anti-$\beta_3$ antibody, and the $\beta_3$ bands intensities were normalized to their respective GAPDH levels (shown on the bottom). Amount of Flag-$\beta_3$ in the samples was relatively close to that of mouse brain tissue endogenous level. *B)* A "ladder"-like pattern of Flag-$\beta_3$ was observed when immunoblotting of the same samples was performed without the pre-treatment by DTT. The "ladders" were recognized by both a polyclonal anti-$\beta_3$ antibody and a monoclonal anti-Flag antibody. The monomer of Flag-$\beta_3$ is marked by (*).

**Figure S3.** Plasma membrane localization of $\alpha_{1C}$ and its mutants. Flag-$\alpha_{1C}$, $\alpha_{1C}$AID and $\alpha_{1C}$AID/IQ (1.2 μg DNA each) were co-expressed with $\alpha_2\delta$ (1.0 μg) and Venus-$\beta_{2d}$ (0.8 μg) in Cos7 cells. The membrane-bound complexes were labeled by cell surface biotinylation and isolated by avidin pull-down under conditions described in Fig. 4*A*, and 5% of each lysate was used as expression input. Non-transfected cells (NT) were used as negative control. Cytosolic proteins did not contaminate the membrane proteins fraction as confirmed by the absence of GADPH.

**Figure S4.** Mutagenesis study of $\beta_3$ segmental oligomerization. Amino acid residues for mutagenesis were identified by deletion analysis of GK fragments NN (*A*), CN (*B*) and CC (*C*) inhibiting their binding to GKN and/or GKC (see Fig. 3). Panel 1, Schematic maps and amino acid residues of the tested GK fragments. The inactive fragments NC (*A*, *B*) and V2 (*C*) were used as carriers. The identified critical amino acid residues are shown in green and introduced amino acid mutations are shown in red. The GK deletion fragments and constructed mutants (M) were labeled with mVenus, co-expressed with Flag-GKN or Flag-GKC, and analyzed by anti-Flag-IP/Western blot (IB) with indicated antibodies to identify amino acid residues critical for the interaction between GKN and NN (*A*, 2), GKC and NN (*A*, 3), GKC and CN (*B*, 2), GKN and CC (*C*, 2) and GKC and CC (*C*, 3). Alanine mutation of the identified amino acid residues disrupted binding of the fragments to GKN and/or GKC as compared to the respective wild type fragments (panels *A*, 4; *B*, 3 and *C*, 4) but did not fully inhibit the oligomerization when incorporated in different combinations into $\beta_3$ (see Supplemental Table S1).

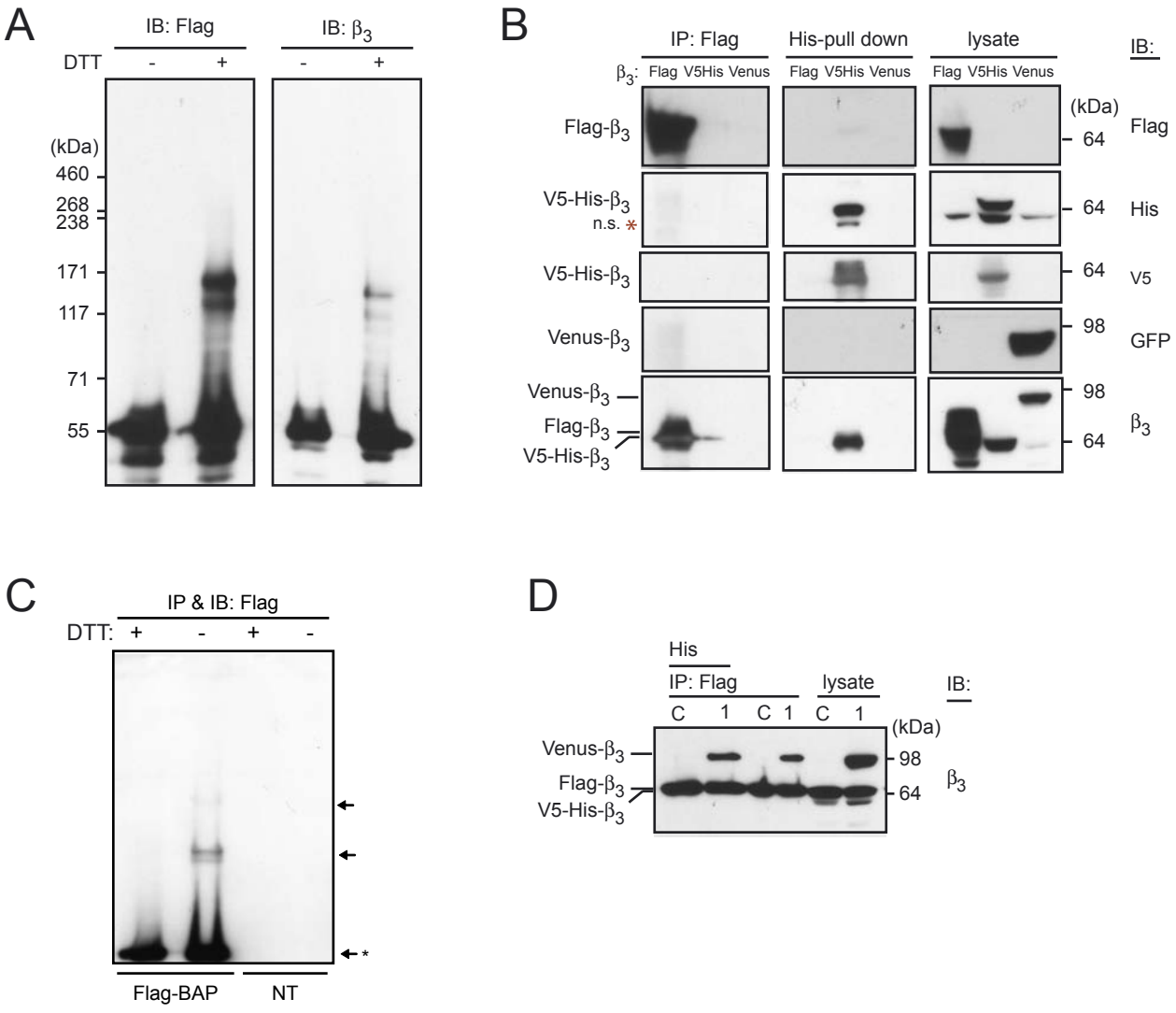
**Figure S5.** Effects of $\beta_3$ fragments on $\beta_3$ oligomerization. Venus-tagged $\beta_3$ fragments (see Fig. 3 for details) (0.4 μg each) were co-expressed with Flag-$\beta_3$ (0.3 μg) and Venus-$\beta_3$ (0.5 μg). Flag

immunoprecipitation followed by GFP immunoblotting revealed that only GKC fragement binds to the $\beta_3$ oligomeric complex and augments its oligomerization.

**Figure S6.** Interaction of $\beta_3$ and its GK fragments with I-II linker. *A)* The presence of GK fragments does not affect the interaction of $\beta_3$ with I-II linker. Venus-$\beta_3$ was co-expressed with V5-His-I-II linker in the absence (lane 1) or presence of the indicated GK fragments (lanes 2-9). The Venus-$\beta_3$ complexes with V5-His-I-II linker and GK fragments were revealed by immunoblotting with an anti-GFP antibody after His-pull down. *B)* Binding of GK fragments to I-II linker. V5-His-I-II linker was co-expressed with mVenus (lane 1) or the indicated Venus-labeled GK fragments (lanes 2-7). His-pull down followed by immunoblotting with an anti-GFP antibody revealed binding of GK, GKN, GKC, NN and CN to the I-II linker.

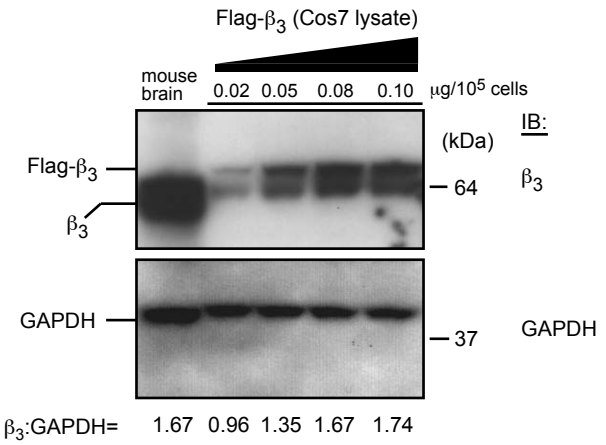
References


1. Akiyama, Y., and Ito, K. (1993) Folding and assembly of bacterial alkaline phosphatase in vitro and in vivo. *J. Biol. Chem.* **268**, 8146-8150
2. Yeh, M. F., and Trela, J. M. (1976) Purification and characterization of a repressible alkaline phosphatase from Thermus aquaticus. *J. Biol. Chem.* **251**, 3134-3139




Supplemental Figure S1

A

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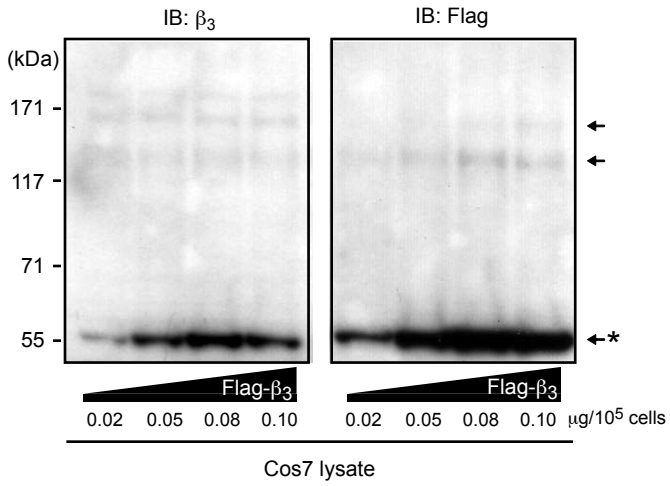
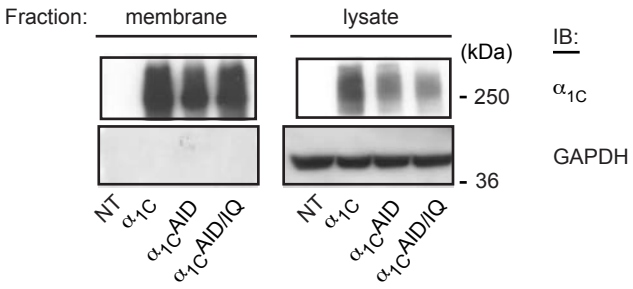
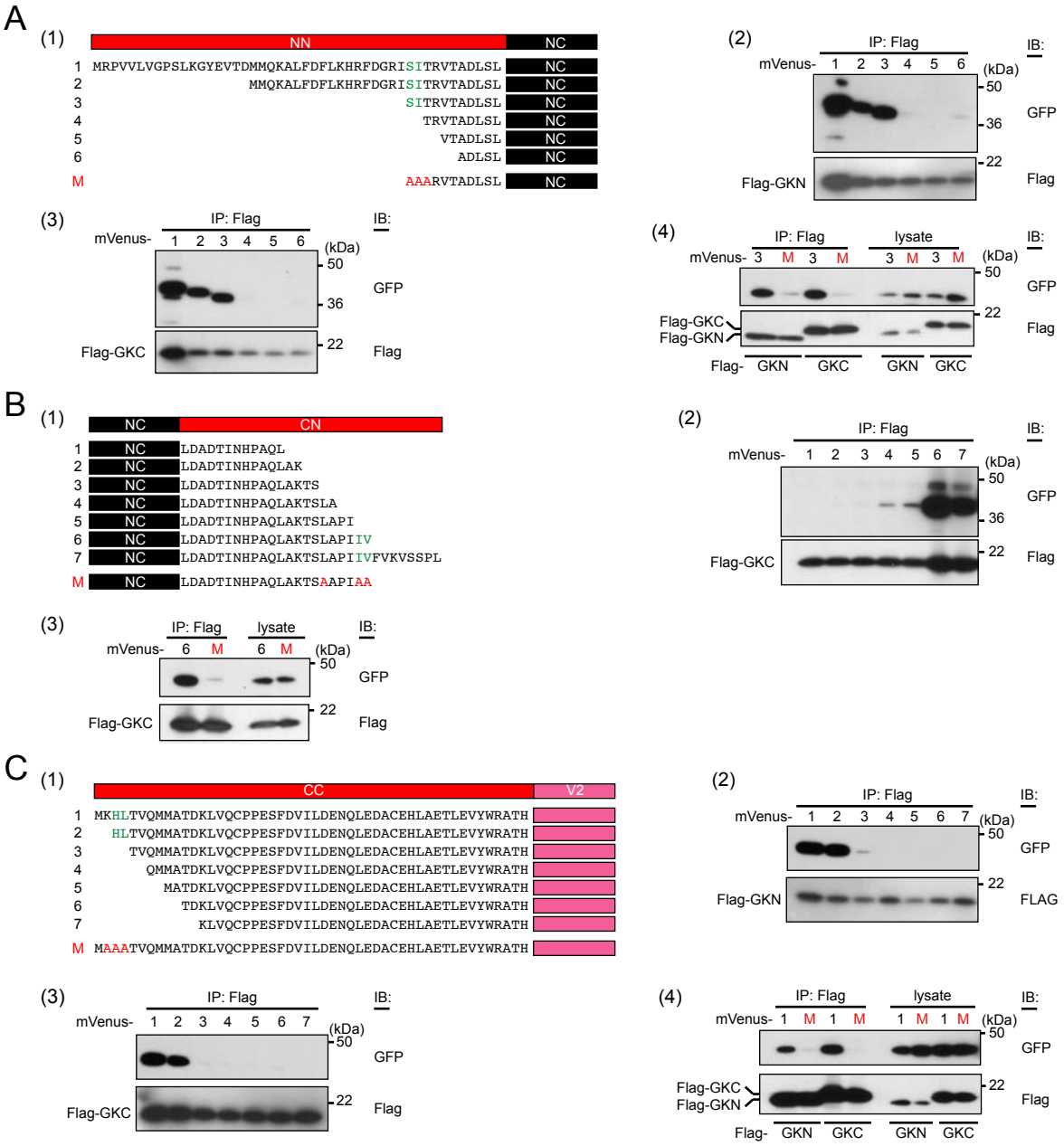


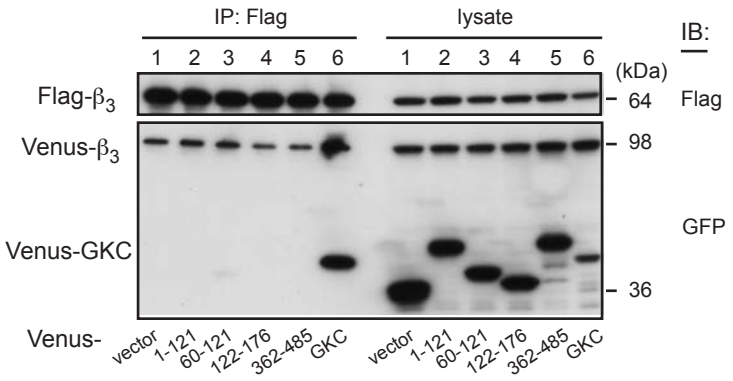

Supplemental Figure S2



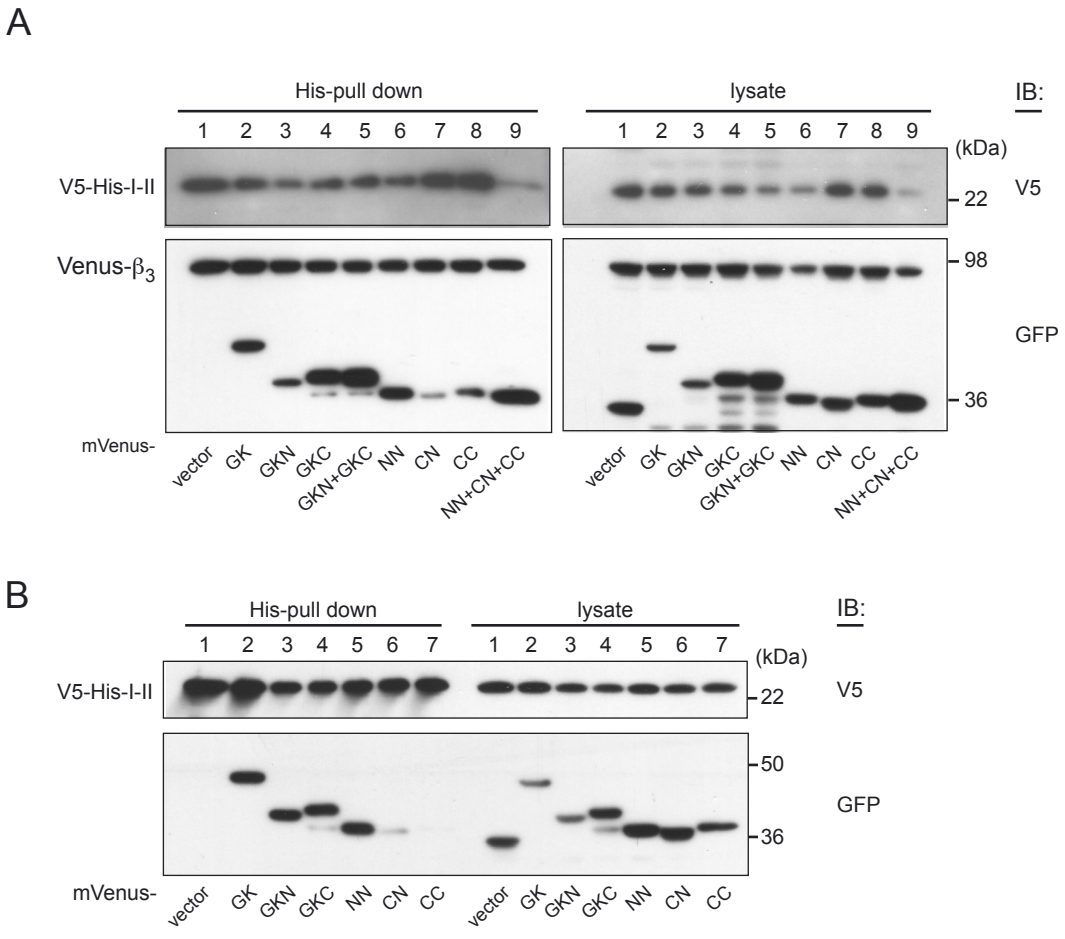

Supplemental Figure S3




Supplemental Figure S4




# Supplemental Figure S5




Supplemental Figure S6

**Table S1. List of tested $\beta_3$ mutants**

| | NN | NC | CN | CC |
|---|---|---|---|---|
| 1 | S213A/I214A/T215A | | | |
| 2 | | | L285A/I289A/V290A | |
| 3 | | | | K313A/H314A/L315A |
| 4 | S213A/I214A/T215A | | L285A/I289A/V290A | K313A/H314A/L315A |
| 5 | S213A/I214A/T215A | | | K313A/H314A/L315A |
| 6 | I212K/S213K/I214K/T215K | | | K313E/H314E/L314E |
| 7 | L187A/G189A/Y190A | | | K313A/H314A/L315A |
| 8 | | N231K/V267E | | |
| 9 | D220Y | | | |
| 10 | L200Y | | I274F | L315Q |
| 11 | | E255V | Q301L | K355N |
| 12 | S213A/I214A/T215A | | L285A/I289A/V290A | K313A/H314A/L315A C328A/C34A |

Shown are mutated sites in GK domain. Clones 1-5 carry the alanine mutations of the sensitive sites identified in Supplemental Fig. S3. Clone 6 carries the mutations that confer different charge on the regions. Clone 7 carries the alanine mutations on the sites showing some homology with the I-II linker AID domain in NN region together with the alanine mutations in the CC region. Clones 8-11 are the mutants created by random mutagenesis targeting on GK domain in full size Flag-$\beta_3$. Ninety clones of random mutagenesis were labeled by Flag and screened by expression in Cos7 cells with the subsequent anti-Flag immunoblotting. Among the tested random mutants, clones 8-11 exhibited decreased tendency to show the "ladder"-like pattern on the blot in the absence of DTT treatment. These clones were selected for the anti-Flag co-immunoprecipitation screening. Clone 12 has two cysteine-to-alanine mutations in addition to the mutations listed in clone 4 to eliminate the possible disulfide bonds. All listed $\beta_3$ mutants were labeled with Flag and mVenus, respectively, and differentially labeled pairs of the mutants were co-expressed for anti-Flag IP-Western blot analysis. None of the mutants exhibited decrease in oligomerization compared to that of Flag- and mVenus-labeled wild-type $\beta_3$ pair.