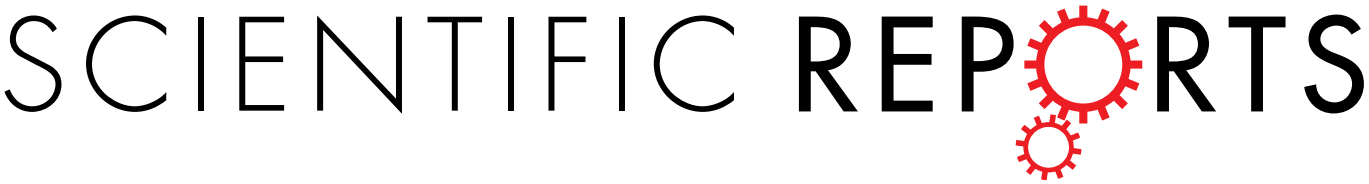

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Identification of residues crucial for the interaction between human neuroglobin and the α-subunit of heterotrimeric G_i protein

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Nozomu Takahashi & Keisuke Wakasugi

Mammalian neuroglobin (Ngb) protects neuronal cells under conditions of oxidative stress. We previously showed that human Ngb acts as a guanine nucleotide dissociation inhibitor (GDI) for the α-subunits of heterotrimeric $G_{i/o}$ proteins and inhibits the decrease in cAMP concentration, leading to protection against cell death. In the present study, we used an eukaryotic expression vector driving high-level expression of human wild-type Ngb or Ngb mutants that either exhibit or lack GDI activities in human cells. We demonstrate that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity. We further demonstrate that Glu53, Glu60, and Glu118 of human Ngb are crucial for both the neuroprotective activity and interaction with $G\alpha_{i1}$. Moreover, we show that Lys46, Lys70, Arg208, Lys209, and Lys210 residues of $G\alpha_{i1}$ are important for binding to human Ngb. We propose a molecular docking model of the complex between human Ngb and $G\alpha_{i1}$.

Neuroglobin (Ngb) is a globin widely expressed in the brain and which binds reversibly to oxygen (O_2)[1–6]. Mammalian Ngb proteins can protect neurons from hypoxic-ischemic insults and protect the brain from experimentally induced stroke *in vivo*[7–11]. To investigate the neuroprotective mechanism of human Ngb under conditions of oxidative stress, we previously performed yeast two-hybrid screening using human Ngb as a bait and identified flotillin-1, a lipid raft microdomain-associated protein, as a binding partner[12]. We demonstrated that human Ngb is recruited to lipid rafts by interacting with flotillin-1 only during oxidative stress and that lipid rafts are crucial for neuroprotection by Ngb[11]. Moreover, we found that human ferric Ngb, which is generated under oxidative stress conditions, binds exclusively to the GDP-bound form of the α-subunits of heterotrimeric $G_{i/o}$ proteins ($G\alpha_{i/o}$), which are present in lipid rafts and inhibit adenylate cyclase activity[13], thereby acting as guanine nucleotide dissociation inhibitor (GDI) for $G\alpha_{i/o}$ and inhibiting the reduction of intracellular cAMP concentration to protect against cell death[10,11,14–16].

Although Ngb was originally identified in mammalian species, it is also present in non-mammalian vertebrates[17,18]. We found that zebrafish Ngb does not exhibit GDI activity and cannot protect mammalian or zebrafish cells against oxidative stress-induced cell death[10,15,19,20]. In order to identify residues of human Ngb that are crucial for its GDI and neuroprotective activities, we prepared human Ngb mutants with a focus on residues differing between human and zebrafish Ngb and on exposed residues with positive or negative charges on the protein surface[15]. Protein transfection was achieved by using the protein delivery reagent Chariot, which can efficiently deliver a variety of proteins into several cell lines in a fully biologically active form[10,11,16,19,21,22]. We showed that human E53Q, R97Q, E118Q, and E151N Ngb mutants, which did not function as GDI proteins, did not rescue cell death under oxidative stress conditions[10,15], indicating that Glu53, Arg97, Glu118 and Glu151 of human Ngb are crucial residues for its GDI activity and that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity. The analysis using E60Q Ngb mutant revealed that Glu60 is also an essential residue for GDI and neuroprotective activities[16]. Furthermore, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis of tryptic peptides derived from a cross-linked complex between human wild-type (WT) Ngb and $G\alpha_{i1}$, which is a member of the $G\alpha_{i/o}$ family[13], revealed cross-linking between Glu60 (Ngb) and Ser206 ($G\alpha_{i1}$), and between Glu53 (Ngb) and Ser44 ($G\alpha_{i1}$)[23].

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan. Correspondence and requests for materials should be addressed to K.W. (email: wakasugi@bio.c.u-tokyo.ac.jp)

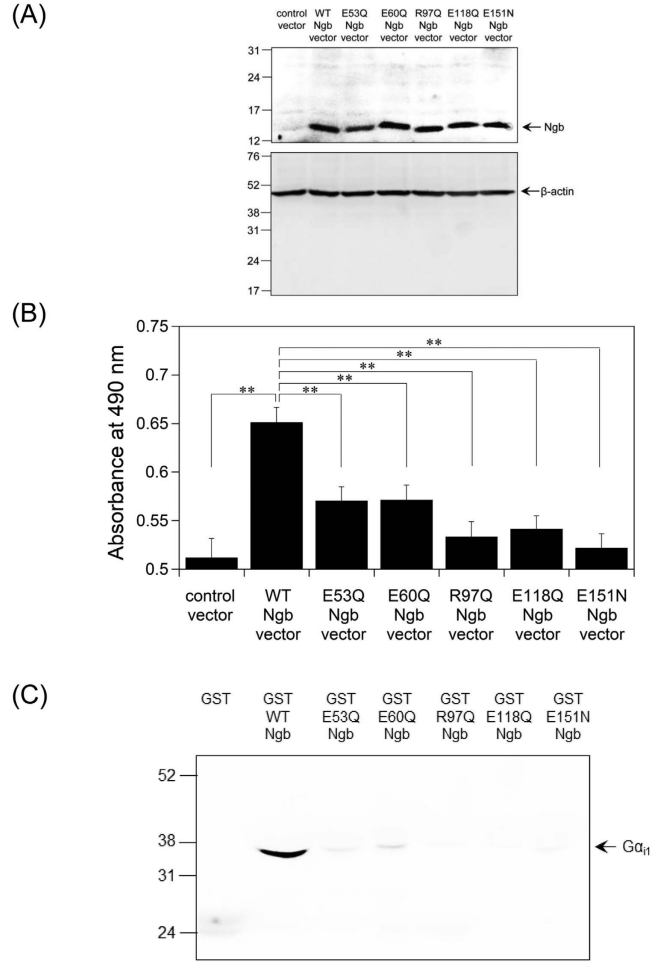

Figure 1. Effects of E53Q, E60Q, R97Q, E118Q, or E151N mutation in human Ngb on its neuroprotective activity and interaction with $G\alpha_{i1}$. (**A**) Western blot analyses of SH-SY5Y cell lysates after transfection. Control vector, human WT or Ngb mutant expression vector was transfected into differentiated SH-SY5Y cells with Lipofectamine. The cells were then incubated for 24 h. Cell lysates were analyzed on 15.0% or 12.5% polyacrylamide-SDS gels and by Western blot analyses using rabbit anti-Ngb polyclonal antibody or mouse anti-β-actin monoclonal antibody, respectively. The arrow indicates the position expected for Ngb or β-actin. Molecular size markers (in kilodaltons) are shown at the left. (**B**) Effect of the mutation in human Ngb on SH-SY5Y cell death caused by hydrogen peroxide. Differentiated SH-SY5Y cells transfected with control vector, human WT or Ngb mutant expression vector with Lipofectamine were treated with hydrogen peroxide, and cell viability was measured by MTS assays. All data are expressed as means ± standard error of means (SEM) from four independent experiments, each carried out in triplicate. Data were analyzed by one-way ANOVA followed by Tukey-Kramer post hoc tests. $^{**}P < 0.01$. (**C**) GST pull-down assays of human ferric WT Ngb or Ngb mutant with the GDP-bound truncated $G\alpha_{i1}$. GST, GST-human WT Ngb, or GST-human Ngb mutant was incubated with human GDP-bound $G\alpha_{i1}$ in a buffer (pH 7.4). Western blot analyses were performed with anti-$G\alpha_{i1}$ mouse monoclonal antibody. The arrow indicates the position expected for $G\alpha_{i1}$. Molecular size markers (in kilodaltons) are shown at the left.

In the present study, in order to reconfirm the results using the protein transfection reagent "Chariot", we used an eukaryotic expression vector which can provide the ability for high-level expression of human WT Ngb or Ngb mutants with or without GDI activities in human cells. We demonstrate that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity. We also prepared site-directed mutants of Ngb or $G\alpha_{i1}$ and investigated neuroprotective activities and protein-protein interactions by performing glutathione S-transferase (GST) pull-down assays. Moreover, we propose a model of the complex between human Ngb and $G\alpha_{i1}$ based on our experimental results.

Results

Neuroprotective and $G\alpha_{i1}$-binding assays of E53Q, E60Q, R97Q, E118Q, and E151Q Ngb mutants. We used SH-SY5Y cells differentiated into a neuron-like type to investigate the neuroprotective mechanism of human Ngb under oxidative stress conditions. A pcDNA3.1-human WT or Ngb mutant expression vector, or a control vector (pcDNA3.1 empty vector) was transfected into SH-SY5Y cells by Lipofectamine

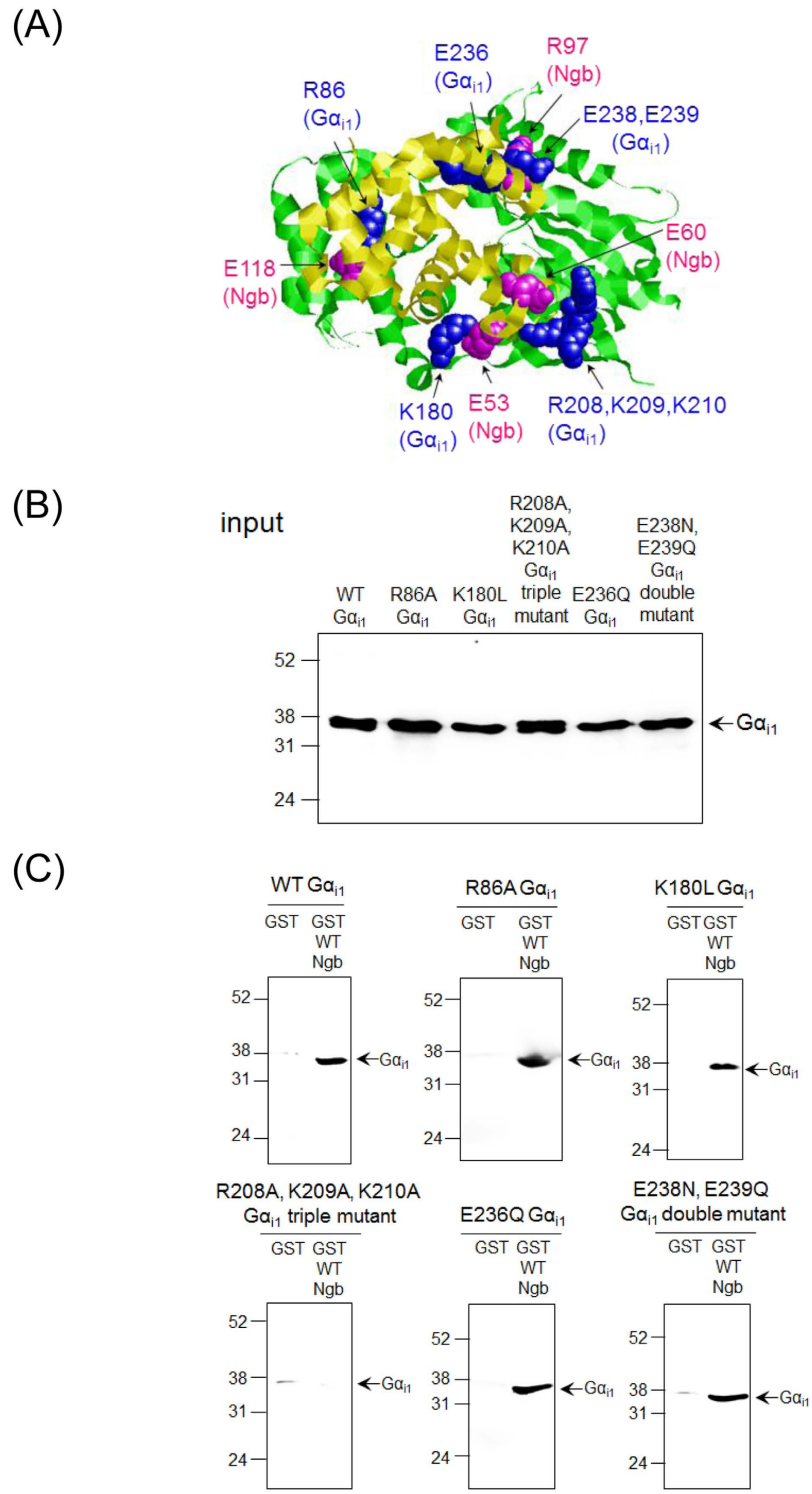

Figure 2. Effects of R86A, K180L, R208A, K209A, K210A, E236Q, E238N, or E239Q mutation in human $G\alpha_{i1}$ on its interaction with Ngb. (**A**) A molecular docking modeling of the complex of Ngb and $G\alpha_{i1}$. Tertiary structure of human Ngb (Protein Data Bank code: 4MPM) is highlighted in yellow. Residues in human Ngb crucial for its neuroprotective activity are indicated in magenta. Tertiary structure of human $G\alpha_{i1}$, which binds to the GoLoco motif of RGS14 (Protein Data Bank code: 1KJY), is highlighted in green. Residues in $G\alpha_{i1}$ are indicated in blue. (**B,C**) GST pull-down assays of human ferric WT Ngb with the GDP-bound form of truncated WT $G\alpha_{i1}$, R86A, K180L, or E236Q $G\alpha_{i1}$ single mutant, E238N, E239Q $G\alpha_{i1}$ double mutant, or R208A, K209A, K210A $G\alpha_{i1}$ triple mutant. GDP-bound WT $G\alpha_{i1}$ or $G\alpha_{i1}$ mutant was incubated with GST-human Ngb or GST in a buffer (pH 7.4). Western blot analyses of the input (**B**) and pull-down samples (**C**) were performed with anti-$G\alpha_{i1}$ mouse monoclonal antibody. The arrows indicate the positions expected for $G\alpha_{i1}$. Molecular size markers (in kilodaltons) are shown at the left.

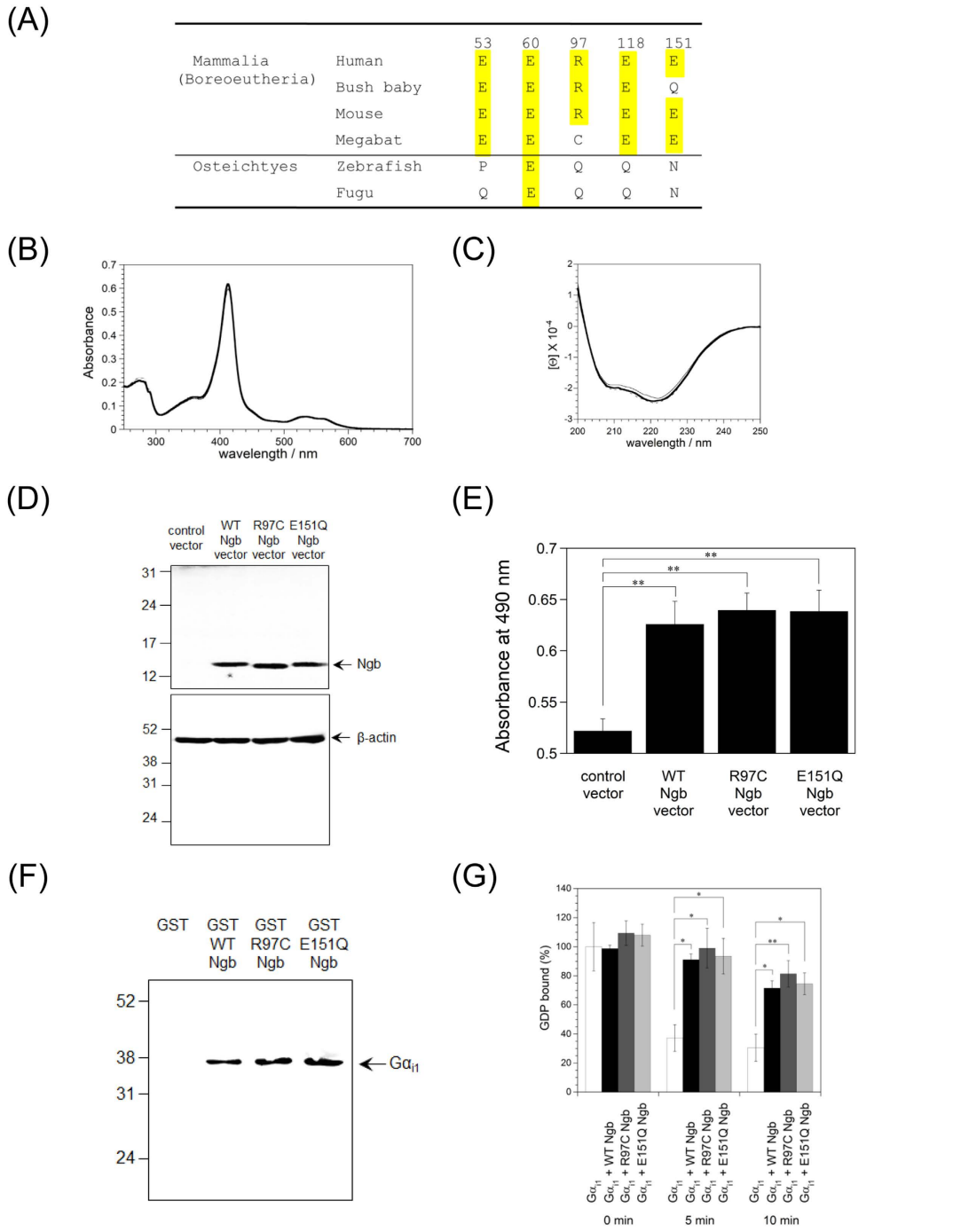

Figure 3. Effects of R97C or E151Q mutation in human Ngb on its structure, GDI and neuroprotective activities. (**A**) Partial sequence alignment among human, bush baby, mouse, megabat, zebrafish and fugu Ngb proteins. Identical residues to human Ngb are highlighted in yellow. Numbers above the sequences correspond to those of the residues of human Ngb. (**B**) Electronic absorption spectra of the ferric form of human WT Ngb (bold line), R97C Ngb (fine line), and E151Q Ngb (dotted line). (**C**) CD spectra in the far UV region of the ferric form of human WT Ngb (bold line), R97C Ngb (fine line), and E151Q Ngb (dotted line). (**D**) Western blot analyses of SH-SY5Y cell lysates after transfection. Control vector, human WT or Ngb mutant expression vector was transfected into differentiated SH-SY5Y cells with Lipofectamine. The cells were then incubated for 24 h. Cell lysates were analyzed on 15.0% or 12.5% polyacrylamide-SDS gels and by Western blot analyses using rabbit anti-Ngb polyclonal antibody or mouse anti-β-actin monoclonal antibody, respectively. The arrow indicates the position expected for Ngb or β-actin. Molecular size markers (in kilodaltons) are shown at the left. (**E**) Effect of the mutation in human Ngb on SH-SY5Y cell death caused by hydrogen peroxide. Differentiated SH-SY5Y cells transfected with control vector, human WT or Ngb mutant expression vector with Lipofectamine were treated with hydrogen peroxide, and cell viability was measured by MTS assay. All data are expressed as means ± SEM from four independent experiments, each carried out in triplicate. Data were analyzed by one-way ANOVA followed by Tukey-Kramer post hoc tests. $^{**}P < 0.01$. (**F**) GST pull-down assays of the human

Ngb mutant with GDP-bound $G\alpha_{i1}$. GST, GST-human WT Ngb, or GST-human Ngb mutant was incubated with human GDP-bound $G\alpha_{i1}$ in a buffer (pH 7.4). Western blot analyses were performed with anti-$G\alpha_{i1}$ mouse monoclonal antibody. The arrow indicates the position expected for $G\alpha_{i1}$. Molecular size markers (in kilodaltons) are shown at the left. (**G**) The effect of the R97C or E151Q mutation on dissociation of GDP from human GDP-bound $G\alpha_{i1}$. The amount of [^{3}H]GDP bound to $G\alpha_{i1}$ in the absence of human ferric Ngb at 0 min was defined as 100%. All data are expressed as means ± SEM from three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey-Kramer post hoc tests. $*P < 0.05$, $**P < 0.01$.

and the protective effects of Ngb proteins against hydrogen peroxide-induced cell death was tested. Expression of human Ngb proteins was confirmed by Western blot analyses (Fig. 1A). Cell viability was measured by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). The absorbance at 490 nm was directly proportional to the number of living cells (Supplementary Fig. S1). As shown in Fig. 1B, MTS assays showed that human WT Ngb enhanced cell survival. By contrast, human E53Q, E60Q, R97Q, E118Q, and E151N Ngb single mutants, which lack GDI activity, did not protect SH-SY5Y cells against cell death (Fig. 1B). These results are consistent with previous results using the protein transfection reagent "Chariot"[10,16]. Taken together, we reconfirmed that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity.

Next, to characterize the protein-protein interaction between human Ngb and $G\alpha_{i1}$ *in vitro*, we performed GST pull-down assays employing human Ngb fused to GST (GST-human Ngb). We previously demonstrated that GST-human ferric Ngb bound to the GDP-bound form of $G\alpha_{i1}$[11,24]. These data are consistent with those obtained by surface plasmon resonance of non-tagged Ngb[14], suggesting that the GST tag has no effect on protein-protein interactions between Ngb and $G\alpha_{i1}$. In the present study, because the truncated $G\alpha_{i1}$ protein, which lacks the N-terminal disordered region (25 amino acids), was more stable than the full-length enzyme[25,26], GST-human ferric Ngb, or GST was purified (Supplementary Fig. S2), incubated with human truncated $G\alpha_{i1}$ for GST pull-down assays and Western blot analyses were performed using antibody against $G\alpha_{i1}$. As shown in Fig. 1C, GST-human WT Ngb bound to the GDP-bound form of the truncated $G\alpha_{i1}$. Moreover, we found that human E53Q, E60Q, R97Q, E118Q, and E151N Ngb mutants did not, suggesting that Glu53, Glu60, Arg97, Glu118 and Glu151 residues of human Ngb are all indispensable for its interaction with $G\alpha_{i1}$.

Analyses based on a possible model of the complex between human Ngb and $G\alpha_{i1}$. To gain further insight into the structure of the complex between human Ngb and $G\alpha_{i1}$, we created a molecular docking model of the complex based on the following two experimental results: i) Glu53, Glu60, Arg97, Glu118, and Glu151 of human Ngb are all crucial residues for its neuroprotective effect and interaction with $G\alpha_{i1}$; ii) Glu53 and Glu60 of human Ngb were cross-linked to Ser44 and Ser206 of $G\alpha_{i1}$, respectively[23]. The molecular docking model was created by manually adjusting the strucutres of Ngb and $G\alpha_{i1}$ to avoid steric hindrance between Ngb and $G\alpha_{i1}$ and is shown in Fig. 2A.

Next, to evaluate this model, we prepared site-directed $G\alpha_{i1}$ mutants at residues with positive or negative charges at the protein binding interface between human $G\alpha_{i1}$ and Ngb: R86A, K180L, and E236Q $G\alpha_{i1}$ single mutants, an E238N, E239Q $G\alpha_{i1}$ double mutant, and a R208A, K209A, K210A $G\alpha_{i1}$ triple mutant. For example, Lys180 of human $G\alpha_{i1}$ was mutated to Leu because the corresponding residue of Lys180 of $G\alpha_{i1}$ in human $G\alpha_s$ is Leu. An E238N, E239Q $G\alpha_{i1}$ double mutant was generated because the corresponding residues of Glu238 and Glu239 of $G\alpha_{i1}$ in human $G\alpha_s$ are Asn and Gln, respectively. As shown in Fig. 2B,C, R208A, K209A, K210A $G\alpha_{i1}$ triple mutant did not interact with human ferric WT Ngb, whereas R86A, K180L, and E236Q $G\alpha_{i1}$ single mutants, and the E238N, E239Q $G\alpha_{i1}$ double mutant bound to Ngb to an extent similar to that of WT $G\alpha_{i1}$. These data suggest that Arg208, Lys209, Lys210, but not Arg86, Lys180, Glu236, Glu238, Glu239, of $G\alpha_{i1}$ are crucial for the interaction with Ngb and that the binding model in Fig. 2A is not appropriate.

Functional analyses of Ngb mutants prepared on the basis of amino acid sequence alignment among various species of Ngb. We previously reported that Glu53, Arg97, Glu118, and Glu151 of human Ngb are conserved only among boreoeutheria of mammalia, and not among afrotheria, maetatheria, prototheria, aves, reptillia, amphibia, or osteichtyes[27], suggesting that boreoeutheria Ngb proteins may protect neurons against oxidative stress-induced cell death. In the present study, we performed more detailed sequence comparisons by using sequence data of Ngb proteins from a greater number of species. As shown in Fig. 3A and Supplementary Fig. S3, the residue corresponding to Arg97 of human Ngb is a Cys in megabat Ngb. And the residue corresponding to Glu151 of human Ngb is a Gln in bush baby Ngb (Fig. 3A and Supplementary Fig. S3). Therefore, we created human R97C and E151Q Ngb mutants and investigated their structure, neuroprotective activity and interaction with $G\alpha_{i1}$.

Initially, we evaluated the effects of the R97C or E151Q mutation on the electronic state of the heme group by measuring the absorption spectra. As shown in Fig. 3B and Table 1, the wavelength of the Soret peak of human ferric R97C or E151Q Ngb was the same as those of human ferric WT Ngb, demonstrating that the R97C or E151Q mutation of human Ngb did not perturb the electric state of the heme group. Next, to examine the effect of the R97C or E151Q substitution upon secondary structure, we measured the far-UV circular dichroism (CD) spectra. As shown in Fig. 3C, human WT, R97C and E151Q Ngb proteins exhibited two negative broad peaks around 222 and 208 nm, which are characteristic of an α-helical structure. The α-helical contents of the R97C and E151Q Ngb proteins were estimated to be 68.9% and 73.4%, which is almost identical to that of human WT Ngb (71.4%) (Table 1). These results showed that the secondary protein structure is not affected by the amino acid substitution.

	WT	WT	E53Q	E60Q	R97Q	R97C	E118Q	E151N	E151Q
Electronic absorption spectra									
Soret (nm)	413	413	413	413	413	413	413	413	413
CD spectra									
$[\Theta]_{222\,nm} \times (10^{-4})$ (deg cm^2 dmol^{-1})	−2.32	−2.40	−2.40	−2.29	−2.26	−2.32	−2.34	−2.36	−2.46
α-helical content (%)	68.9	71.4	71.5	67.9	66.9	68.9	69.5	70.2	73.4
References	Ref. 16	this study	Ref. 16	Ref. 16	Ref. 16	this study	Ref. 16	Ref. 16	this study

Table 1. Structural data of human ferric WT Ngb and Ngb mutants.

Next, we tested whether R97C and E151Q Ngb can protect SH-SY5Y cells against oxidative stress. We first confirmed that human WT, R97C, and E151Q Ngb proteins were expressed at a similar level in SH-SY5Y cells by Western blot analyses (Fig. 3D). MTS assays showed that the expression of human R97C or E151Q Ngb mutant in the cells rescued cell death under oxidative stress condition, as did that of human WT Ngb (Fig. 3E). Moreover, GST-human ferric Ngb, or GST was purified (Supplementary Fig. S2), incubated with human truncated $G\alpha_{i1}$ and Western blot analyses were performed using antibody against $G\alpha_{i1}$. As shown in Fig. 3F, GST pull-down assays showed that R97C and E151Q Ngb bound to the truncated $G\alpha_{i1}$ as did the WT Ngb. Furthermore, to examine the effect of the R97C or E151Q mutation of human Ngb upon the release of GDP from $G\alpha_{i1}$, we measured the rates of GDP dissociation in the absence or presence of human Ngb. In the presence of an excess amount of unlabeled GTP, [^{3}H]GDP release from [^{3}H]GDP-bound $G\alpha_{i1}$ was inhibited by human ferric WT Ngb (Fig. 3G). As shown in Fig. 3G, human ferric R97C and E151Q Ngb functioned as the GDI for $G\alpha_{i1}$ as did human WT Ngb. These results suggest that the positive charge of Arg97 and the negative charge of Glu151 of human Ngb are not essential for the GDI and neuroprotective activities. Taken together, we conclude that Glu53, Glu60, and Glu118 of human Ngb are the only crucial residues for its activities.

Search for residues of $G\alpha_{i1}$ crucial for the interaction with human Ngb. Next, because we found that Arg97 and Glu151 residues of Ngb are not involved in the interface with $G\alpha_{i1}$, we created a new molecular docking model of the complex between human Ngb and $G\alpha_{i1}$, which is depicted in Fig. 4A. To clarify binding sites in the complex between human Ngb and $G\alpha_{i1}$, we prepared K46A, K67A, and K70A $G\alpha_{i1}$ single mutants. As shown in Fig. 4B,C, K46A and K70A $G\alpha_{i1}$ single mutants did not interact with Ngb. Moreover, the R208A, K209A, K210A $G\alpha_{i1}$ triple mutant also did not interact with Ngb (Fig. 2B,C). These results suggest that the model of Fig. 4A is correct. Moreover, K67A $G\alpha_{i1}$ bound to Ngb as did WT $G\alpha_{i1}$ (Fig. 4B,C), indicating that Glu118 of human Ngb interacts with Lys70, but not Lys67, of $G\alpha_{i1}$ in a site-specific manner.

Discussion

In the present study, by using eukaryotic expression vectors for human Ngb proteins we demonstrated that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity. GST pull-down assays using GST-fused human Ngb demonstrated that Glu53, Glu60, Arg97, Glu118 and Glu151 residues of human Ngb are all indispensable for its interaction with $G\alpha_{i1}$. However, R97C and E151Q Ngb single mutants, generated based on sequence alignment among various species of Ngb, showed that the positive charge of Arg97 and the negative charge of Glu151 of human Ngb are dispensable for both the GDI and neuroprotective activities. We therefore speculated that the lack of GDI and neuroprotective activities of the R97Q and E151N Ngb mutants may arise from structural alterations induced by the mutations and concluded that only the negative charges of Glu53, Glu60, and Glu118 of human Ngb are crucial for the GDI and neuroprotective activities. We measured the UV-visible and CD spectra of the WT and mutants but could not detect any significant structural differences among them. Further studies are in progress to clarify structural alterations between the WT and R97Q or E151N Ngb mutant.

Moreover, we identified residues of $G\alpha_{i1}$ crucial for the interaction with human Ngb; Lys46, Lys70, Arg208, Lys209, and Lys210 residues of $G\alpha_{i1}$ are important for its binding to human Ngb. The present results imply that electrostatic interactions between the negative charges of Glu53, Glu60, and Glu118 of human Ngb and the positive charges of Lys46, Lys70, Arg208, Lys209, and/or Lys210 of $G\alpha_{i1}$ are crucial for the formation of a complex between Ngb and $G\alpha_{i1}$. This is consistent with the previous observation that electrostatic complementarity is an important factor for the interaction of Gα with its regulator[28]. Further more detailed studies are in progress to investigate the significance of Arg208, Lys209, and/or Lys210 of $G\alpha_{i1}$. Moreover, because the residue corresponding to Arg97 of human Ngb is a Gln in yak Ngb (Supplementary Fig. S3), it is interesting to investigate whether yak Ngb has the GDI and neuroprotective activities.

The present results support that a molecular docking model of the complex between human Ngb and $G\alpha_{i1}$ in Fig. 4A. In this model, Glu60 (Ngb), which was cross-linked to Ser206 ($G\alpha_{i1}$) by chemical cross-linking[23], interacts with Arg208, Lys209, and/or Lys210 of $G\alpha_{i1}$. The amino acid sequence surrounding Glu60 in human Ngb has a motif homologous to those of the R6A-1 peptide and the KB-752 peptide, which interact with GDP-bound $G\alpha_{i1}$[16]. It has previously been reported that the R6A-1 and KB-752 peptides interact with the switch II (a.a. 199–219) of $G\alpha_{i1}$[26,29,30]. The molecular docking model of the complex between human Ngb and $G\alpha_{i1}$ shows that Glu60 of human Ngb is located near the switch II of $G\alpha_{i1}$, as are R6A-1 and KB-752 peptides, suggesting that the motif including Glu60 in human Ngb functions as the core motif for the binding with $G\alpha_{i1}$.

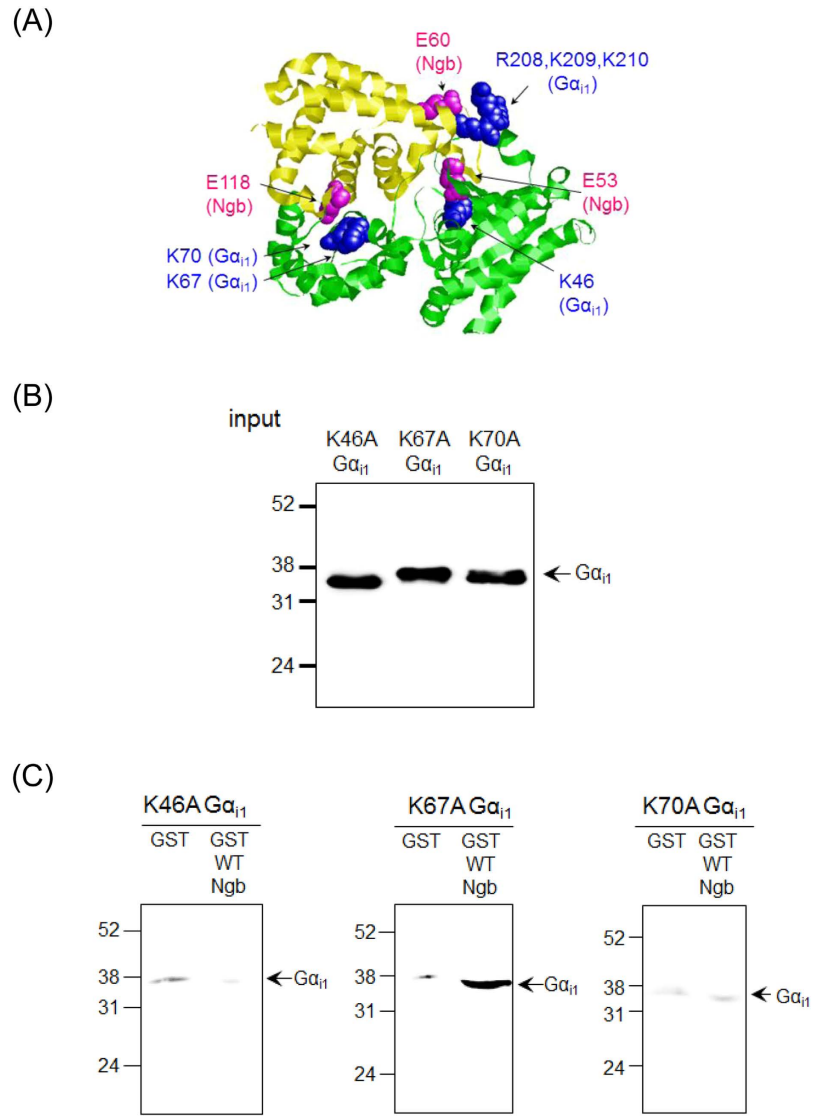

Figure 4. Effects of K46A, K67A, or K70A mutation in human $G\alpha_{i1}$ on its interaction with Ngb. (**A**) A molecular docking model of the complex of Ngb and $G\alpha_{i1}$. Tertiary structure of human Ngb (Protein Data Bank code: 4MPM) is highlighted in yellow. Residues in human Ngb crucial for its neuroprotective activity are indicated in magenta. Tertiary structure of human $G\alpha_{i1}$, which binds to the GoLoco motif of RGS14 (Protein Data Bank code: 1KJY), is highlighted in green. Residues in $G\alpha_{i1}$ are indicated in blue. (**B,C**) GST pull-down assays of human ferric WT Ngb with the GDP-bound form of truncated WT $G\alpha_{i1}$, K46A, K67A, or K70A $G\alpha_{i1}$ single mutant. GDP-bound form of WT $G\alpha_{i1}$ or $G\alpha_{i1}$ mutant was incubated with GST-human Ngb or GST in a buffer (pH 7.4). Western blot analyses of the input (**B**) and pull-down samples (**C**) were performed with anti-$G\alpha_{i1}$ mouse monoclonal antibody. The arrows indicate the positions expected for $G\alpha_{i1}$. Molecular size markers (in kilodaltons) are shown at the left.

As shown in Fig. 4A, Glu53 (Ngb), which was cross-linked to Ser44 ($G\alpha_{i1}$) by chemical cross-linking[23], binds to Lys46 of $G\alpha_{i1}$. X-ray structural data of $G\alpha_{i1}$ show that Ser44 and Lys46 of $G\alpha_{i1}$ are located in the vicinity of GDP[25]. This implies that Ngb could be positioned near the GDP-binding site in the Ngb-$G\alpha_i$ complex, which would inhibit the dissociation of GDP from the binding site. Moreover, it should be also noted that Glu53 of Ngb is conserved among boreotheria except for the harp or hooded seal (Supplementary Fig. S3). In seals Ngb is mainly localized in astrocytes, whereas other boreotheria Ngb exists in neuron[31,32]. The different localization of Ngb suggests that the function of seal Ngb may be different from that of other boreotheria Ngb.

Figure 4A shows that Glu118 of Ngb interacts with Lys70 of $G\alpha_{i1}$. We previously carried out chemical cross-linking of the Ngb and $G\alpha_{i1}$ complex by treatment with zero-length cross-linkers to identify the sites of interaction between Ngb and $G\alpha_{i1}$[23]. In the peptide map of tryptic peptides derived from the cross-linked Ngb-$G\alpha_{i1}$ complex, the MS peak of a Ngb peptide (amino acids 103–119 of Ngb), which was observed in the tryptic peptide map of Ngb, was missing or significantly decreased in intensity[23], suggesting that the 103–119 amino acid residues of Ngb are involved in the Ngb-binding site for $G\alpha_{i1}$, but information about the detailed residues

cross-linked in the complex of Ngb and $G\alpha_{i1}$ remained unclear. The present results suggest that Glu118, which is located in the 103–119 region, of human Ngb interacts with Lys70 residue, which is located in the α-helical domain of $G\alpha_{i1}$, as shown in Fig. 4A. Strikingly, crystal structure of GoLoco motifs of LGN protein, which act as GDI for $G\alpha_{i1}$, elucidated that they interact with the switch II region and Tyr69 and Val72, which are close to Lys70, in the α-helical domain of $G\alpha_{i1}$[33]. It is also worth noting that RGS domains, which bind to $G\alpha_i$ selectively, interact with some residues in the α-helical domain of Gα as well as the switch regions[28].

Lys46, Arg208, Lys209, and Lys210 residues, which are crucial for the interaction with Ngb, and their sequential neighborhoods of $G\alpha_{i1}$ are conserved among $G\alpha_{i/o}$ and $G\alpha_s$. In contrast, Lys70 residue of $G\alpha_{i1}$ is conserved among $G\alpha_{i/o}$, but corresponds to Val in $G\alpha_s$ based on sequence alignment among members of the Gα family[13]. Moreover, sequential neighborhoods around Lys70 of $G\alpha_{i/o}$ are very different from those of $G\alpha_s$. Therefore, the positive charge of the 70th residue of Gα, which interacts with the negative charge of carboxylic group of Glu118 through electrostatic interaction, may be crucial for the $G\alpha_{i/o}$–specific binding property of Ngb.

Methods

Cell culture. SH-SY5Y cells (CRL-2266) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture containing 2.5 mM glutamine, supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed every 4 days, and the cultures were split at a 1:20 ratio once a week. Cultured cells were induced to differentiate into a neuronal phenotype by treatment with 10 μM retinoic acid (Sigma-Aldrich, St. Louis, MO) over a period of 6 days (media were exchanged every 3 days during sub-culture). Differentiation was verified by monitoring macroscopic changes to the cells.

Transfection of Ngb expression vector into SH-SY5Y cells and treatment of cells with hydrogen peroxide. The eukaryotic expression vector pcDNA3.1 (Invitrogen) for human Ngb was prepared as described previously[11]. A QuikChange™ site-directed mutagenesis system (Stratagene, La Jolla, CA) was used for site-directed mutagenesis and the constructs were confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services, Atsugi, Japan). Differentiated SH-SY5Y cells were plated on poly-D-lysine coated 96-well plates at a density of 5.0×10^5 cells/mL for 24 h. The pcDNA3.1-human WT or Ngb mutant expression vector or control vector (pcDNA3.1 empty vector) was transfected by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, hydrogen peroxide (100 μM) was added and cells were incubated for 24 h.

Western blot analyses. After cell lysates were resolved by electrophoresis on polyacrylamide-SDS gels, proteins were electroblotted onto Hybond-P PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ), which were then blocked with phosphate-buffered saline (PBS) and 5% skim milk (Wako Pure Chemical Industries, Osaka, Japan) and incubated with rabbit anti-Ngb (FL-151) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti-β-actin monoclonal antibody (Sigma-Aldrich). After washing, membranes were incubated with an HRP-linked $F(ab')_2$ fragment of donkey anti-rabbit IgG or an HRP-linked whole antibody of sheep anti-mouse IgG (GE Healthcare Biosciences). Proteins were visualized using ECL™ Western blotting detection reagents (GE Healthcare Biosciences). Chemiluminescent signals were detected using a LAS-4000 mini luminescent image analyzer (GE Healthcare Biosciences).

Cell viability assay. Cell viability was measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay Reagent (Promega, Madison, WI), containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carb oxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. Cultured cells were incubated with the MTS reagent at 37 °C for 4 h in a humidified, 5% CO_2 atmosphere. The amount of colored formazan dye formed was then quantified by measuring absorbance at 490 nm with a Beckman Coulter DTX880 plate reader (Beckman Coulter, Fullerton, CA).

Preparation and purification of GST and a fusion protein of GST and Ngb. Human Ngb cDNA was cloned into the pGEX-4T-1 vector (GE Healthcare Biosciences) to produce the fusion protein GST-Ngb[11,24]. A QuikChange™ site-directed mutagenesis system (Stratagene) was used for site-directed mutagenesis. The constructs were confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services). Overexpression of GST-Ngb and GST alone (as a control) was induced in the *Escherichia coli* strain BL21 (DE3) (Novagen, Madison, WI) by treatment with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Both GST-ferric Ngb and GST were purified by using glutathione-Sepharose 4B beads (GE Healthcare Biosciences) according to the manufacturer's instructions.

Preparation of recombinant human truncated $G\alpha_{i1}$ protein. The DNA fragment containing the human truncated $G\alpha_{i1}$ subunit (residues 26–354) was amplified by PCR and cloned into the pET151/D-TOPO® vector (Invitrogen) to be expressed as human WT truncated $G\alpha_{i1}$ protein (residues 26–354) fused to a TEV protease recognition site directly after an N-terminal tag of six histidine residues (His_6-tag). A QuikChange™ site-directed mutagenesis system (Stratagene) was used for site-directed mutagenesis. The constructs were confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services). The resulting $G\alpha_{i1}$ was expressed in *E. coli* strain BL21 (DE3) by induction with IPTG and purified by using a nickel affinity column (His·Bind® resin; Novagen), as described previously[11]. Then, the sample was incubated with His_6-tagged TEV protease (MoBiTec GmbH, Göttingen, Germany) and loaded onto a His·Bind® column to separate the cleaved $G\alpha_{i1}$ from the cleaved His_6-tag, any uncleaved protein, and His_6-tagged TEV protease, as described previously[11].

GST pull-down assays using truncated $G\alpha_{i1}$. Truncated $G\alpha_{i1}$ was incubated with either GST alone or GST–ferric Ngb immobilized on glutathione-Sepharose 4B beads (GE Healthcare Biosciences) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 10 μM GDP, 0.1% Tween20, pH 7.4) for 1 h at 4 °C. The beads were washed extensively three times with the buffer, and the samples were then resuspended in Laemmli sample buffer, heated for 5 min at 95 °C, and separated on 12.0% polyacrylamide-SDS gels. For Western blot analyses, the proteins were transferred onto Hybond-P PVDF membranes (GE Healthcare Biosciences), which were then blocked with PBS and 5% skim milk (Wako Pure Chemical Industries) and incubated with mouse anti-$G\alpha_{i1}$ (Ab-3; clone R4.5) monoclonal antibody (Thermo Fisher Scientific, Fremont, CA). After washing, the membranes were incubated with an HRP-linked whole antibody of sheep anti-mouse IgG (GE Healthcare Biosciences). Proteins were visualized using ECL™ western blotting detection reagents (GE Healthcare Biosciences). Chemiluminescent signals were detected using a LAS-4000 mini luminescent image analyzer (GE Healthcare Biosciences).

Preparation of non-tagged recombinant human Ngb proteins. Plasmids for human Ngb were prepared as described previously[14,15]. A QuikChange™ site-directed mutagenesis system (Stratagene) was used for site-directed mutagenesis. The constructs were confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services). Overexpression of each Ngb was induced in *E. coli* strain BL 21 (DE 3) by treatment with IPTG for 4 h, and each Ngb protein was purified as described previously[10,11,14–16]. Briefly, soluble cell extracts were loaded onto DEAE sepharose anion-exchange columns equilibrated with buffer A (20 mM Tris-HCl, pH 8.0). Ngb proteins were eluted from columns with buffer A containing 150 mM NaCl, and further purified by passage through Sephacryl S-200 HR gel filtration columns. The protein concentration of human ferric Ngb was determined spectrophotometrically using an extinction coefficient of 122 $mM^{-1}cm^{-1}$ at the Soret peak.

UV-visible spectra. Electronic absorption spectra of purified proteins were recorded with a UV-visible spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan) at ambient temperature (~20 °C). Spectra were recorded in PBS (pH 7.4).

CD spectra. CD spectra in the far-UV region were measured with a spectropolarimeter (J-805; JASCO Co., Tokyo, Japan) at 20 °C. The samples were measured at a concentration of approximately 5 μM in 50 mM sodium phosphate buffer (pH 7.4). The path length of the cells used for the measurements was 1 mm. The molar ellipticity (deg cm^2 $dmol^{-1}$) was determined on the mean residue basis. The α-helix content (f_H) was calculated according to Chen *et al.*[34] by the following equation:

$$f_H = -\left([\Theta]_{222nm} + 2340\right)/30300$$

[^{3}H]GDP dissociation assays. GDP dissociation assays were performed, as described previously[11,16]. In brief, $G\alpha_{i1}$ complexed with [^{3}H]GDP (0.3 μM) was prepared by incubating 0.3 μM $G\alpha_{i1}$ with 2 μM [8,5′-^{3}H]GDP (PerkinElmer Life Sciences, Boston, MA) in buffer B [20 mM Tris-HCl, 100 mM NaCl and 10 mM $MgSO_4$ at pH 8.0] for 1.5 h at 25 °C. Excess unlabeled GTP (2 mM) was added to monitor dissociation of [^{3}H]GDP from $G\alpha_{i1}$ in the absence or presence of non-tagged ferric Ngb (10 μM). Aliquots were withdrawn at 0, 5, and 10 min and were passed through nitrocellulose filters (0.45 μm) (Advantec Toyo, Tokyo, Japan). The filters were then washed three times with 1 ml of ice-cold buffer B and were counted in a liquid scintillation counter (LS6500; Beckman Coulter).

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Author Contributions

N.T. and K.W. designed the study and wrote the paper. N.T. performed the experiments. N.T. and K.W. analyzed the results and approved the final version of the manuscript.

Additional Information

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