Cell Research (2012) 22:1140-1154.

www.nature.com/cr

ORIGINAL ARTICLE

Misfolded Gβ is recruited to cytoplasmic dynein by Nudel for efficient clearance

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The Gβγ heterodimer is an important signal transducer. Gβ, however, is prone to misfolding due to its requirement for Gγ and chaperones for proper folding. How cells dispose of misfolded Gβ (mfGβ) is not clear. Here, we showed that mfGβ was able to be polyubiquitinated and subsequently degraded by the proteasome. It was sequestered in aggresomes after the inhibition of the proteasome activity with MG132. Sustained activation of Gβγ signaling further elevated cellular levels of the ubiquitinated Gβ. Moreover, Nudel, a regulator of cytoplasmic dynein, the microtubule minus end-directed motor, directly interacted with both the unubiquitinated and ubiquitinated mfGβ. Increasing the levels of both mfGβ and Nudel promoted the association of Gβ with both Nudel and dynein, resulting in robust aggresome formation in a dynein-dependent manner. Depletion of Nudel by RNAi reduced the dynein-associated mfGβ, impaired the MG132-induced aggresome formation, and markedly prolonged the half-life of nascent Gβ. Therefore, cytosolic mfGβ is recruited to dynein by Nudel and transported to the centrosome for rapid sequestration and degradation. Such a process not only eliminates mfGβ efficiently for the control of protein quality, but may also help to terminate the Gβγ signaling.

Keywords**:** protein degradation; dynein-mediated transport; Gβ; protein misfolding; signaling
Cell Research (2012) **22**:1140-1154. doi:10.1038/cr.2012.41; published online 20 March 2012

Introduction

The heterotrimeric G proteins are signal transducers of the G protein-coupled receptors (GPCRs). The activation of GPCR by an agonist leads to the dissociation of Gβγ, a tightly bound dimer formed by the Gβ and Gγ subunits, from Gα and the subsequent activation of downstream effectors [1-4]. The G proteins also function as transducers of polarity information in a GPCR-independent manner. For instance, during asymmetric cell division, the activator of the G-protein signaling (AGS) family proteins, including LGN (also named AGS5), can bind directly to Gα, leading to the dissociation of Gβγ from Gα [5-7]. After the G protein signaling is terminated, free Gα may be recycled back to the plasma membrane [8]. However, the fate of free Gβγ is not clear. The long-term activation of μ-opioid receptor, a GPCR, has been shown to induce the proteasome-dependent degradation of Gβ [9]. Furthermore, the turnover rate of Gβ is positively correlated with Gβγ levels [10]. Therefore, there may be a feedback degradation mechanism to avoid excessive Gβγ signaling.

Gβ is prone to misfolding. Its proper conformation and subcellular localization depend on Gγ [2, 3, 8]. Only 30%-50% of *in vitro* translated Gβ and Gγ form functional Gβγ dimmers [4]. In fact, efficient formation of Gβγ from newly synthesized Gβ and Gγ also requires the CCT chaperone complex and the phosducin-like protein (PhLP1) [4, 8]. A stoichiometric excess of Gβ over Gγ, as in the case of Gβ overexpression, should also lead to misfolding. However, the mechanism of how the cells dispose of misfolded Gβ (mfGβ) remains elusive.

The proteasome is a large, multi-subunit protein complex that can degrade unwanted proteins into small peptides. Before degradation, the substrates for the proteasome are subjected to polyubiquitination. It is estimated that ~20% of nascent polypeptides are degraded, presumably due to a failure to satisfy the cellular quality control machinery [11]. As misfolding often results in

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Received 30 October 2011; revised 29 January 2012; accepted 12 Februry 2012; published online 20 March 2012

the exposure of hydrophobic regions that are normally buried inside the protein, misfolded proteins tend to aggregate. Excess protein aggregates are sometimes transported by cytoplasmic dynein, a microtubule (MT)-based motor, to the MT-organizing center (MTOC), where the centrosome resides. There, they form a large juxtanuclear inclusion, termed an aggresome. Aggresome formation sequesters protein aggregates from the cytosol to possibly reduce their potential cytotoxic effects. Aggresomes can also trigger autophagy, an intracellular "engulfing" process that degrades membrane organelles and large protein inclusions. Components of the ubiquitin (Ub)-proteasome system (UPS) and several chaperones are usually enriched in aggresomes to facilitate the elimination of misfolded proteins by degradation or refolding [11-14].

The mechanism by which different protein aggregates are loaded onto the dynein motor is still poorly understood. Cytoplasmic dynein is a very large protein complex containing two heavy chains (DHC), several intermediate (DIC), light intermediate, and light chains. Its association with many membrane cargos or target sites requires another protein complex, dynactin [15, 16]. Huntingtin-associated protein may mediate the association between huntingtin aggregates and the dynein motor by binding to the $p150^{Glued}$ subunit of dynactin [17]. Histone deacetylase 6 (HDAC6) has been proposed to regulate aggresome formation by linking ubiquitinated proteins to dynein [18], though whether HDAC6 directly interacts with dynein or dynactin is not clear.

Nudel (also called Ndel1) is a dynein-interacting protein that is critical for a variety of dynein functions *in vivo* [19-24]. In this report, we show that in addition to its regulatory function, Nudel also serves as an adaptor for mfGβ on dynein to facilitate the sequestration of mfGβ. We also provide evidence for the negative regulation of Gβγ signaling via the degradation of Gβ through the ubiquitin proteasome pathway.

Results

Nudel interacts directly with Gγ-free Gβ

To further understand Nudel function, a yeast two-hybrid screen was performed to identify novel Nudel-associated proteins. The bait plasmid, pAS2-Nudel, was constructed to express full-length Nudel fused with the DNA-binding domain of Gal4. This plasmid was transformed with a pACT2-based human placenta cDNA library (Clontech) into the yeast strain Y190. The plasmid isolated from a positive clone contained a full-length cDNA for Gβ2, one of the five Gβ subtypes in mammals [3]. The result of the screen was further confirmed by the co-transfection of pACT2-Gβ2 with either pAS2-Nudel or the vector (pAS2-1) alone (Figure 1A). Direct interaction of His-tagged Gβ2 with Flag-tagged Nudel, but not with the control protein, E2F1, was then confirmed by *in vitro* binding assays using bacterially expressed proteins (Figure 1B). The latter result also suggests that Gγ is not required for the Nudel-Gβ2 interaction.

We then performed co-immunoprecipitation (co-IP) analysis with HEK293T cells. As the anti-Gγ antibodies in our hands were not sensitive enough to detect the low-level expressions of endogenous Gγ in the HEK293T cell lysate (Supplementary information, Figure S1), we overexpressed Gγ2, which has a strong affinity to Gβ2 and is also able to heterodimerize with all of the other Gβ subtypes, except Gβ3 [25, 26], to facilitate its detection by the anti-Gγ2 antibody. When Flag-Nudel was co-expressed (Figure 1C, lane 1), co-IP using the anti-Flag M2 resin indicated an association of endogenous Gβ with Flag-Nudel (Figure 1C, lane 3). As expected, Gγ2, Gαi and Gαs were not detected in the immunocomplex (Figure 1C, lane 3). We further demonstrated the association between endogenous Nudel and Gβ *in vivo* by co-IP with either anti-Nudel IgY or anti-Gβ IgG (Figure 1D, lanes 3 and 5). Together, these results revealed that Nudel directly interacts *in vivo* with Gγ-free Gβ, which is presumably unfolded or misfolded [2, 3].

To map interaction regions, a series of Nudel or Gβ2 mutants were constructed (Figure 1E-1F). Co-IP showed that the N-terminal 201 residues of Nudel ($Nudel^{P2N}$) associated with Gβ2, whereas further depletion of the first 55 ($Nudel^{P2N2}$) or the last 35 residues ($Nudel^{P2N3}$) abolished the interaction (Figure 1E and Supplementary information, Figure S2A). $Nudel^{P2N}$ contains the "QXXER" motif at residues 145-149, which is highly conserved in Nudel family proteins from fungi, such as *Aspergillus*, to vertebrates (Supplementary information, Figure S2A). This motif was originally identified in several Gβγ effectors and is important for the association of adenylyl cyclase 2 (AC2) with Gβ [27, 28]. When the motif in $Nudel^{P2N}$ was mutated (QAIER→AAILV), the resultant protein, $Nudel^{P2Nm}$, showed a weaker association with His-Gβ2 (Figure 1E and Supplementary information, Figure S2A). Conversely, Nudel associated with Gβ2M and Gβ2C, which contained the 2nd-4th and 5th-7th WD repeats, respectively, but not with Gβ2N, which contained residues 1-68 (Figure 1F and Supplementary information, Figure S2B). As all Gβ subtypes in mammals share similar structural characteristics [2, 3], we further overexpressed HA-tagged Gβ1, β4, or β5 and found that, like HA-Gβ2, they also associated with Flag-Nudel in the co-IP assays (Figure 1G).

Taken together, we conclude that Nudel directly in-

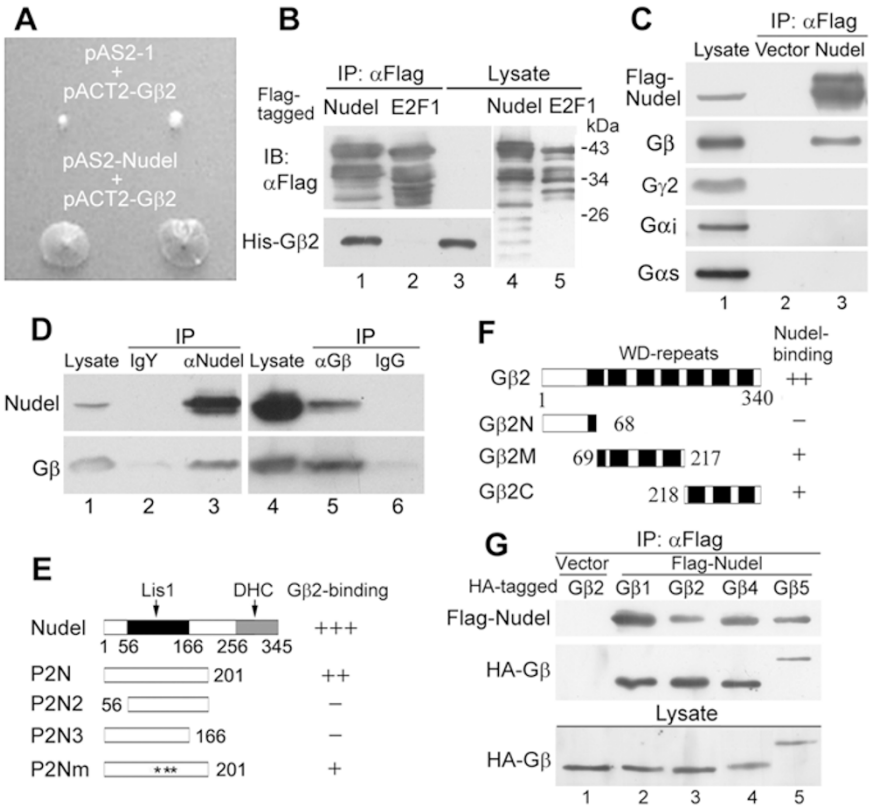

Figure 1 Interaction of Gγ-free Gβ with Nudel. **(A)** The interaction assessed in a yeast two-hybrid system. pAS2-1 and pACT2 are vectors harboring the DNA-binding domain and activation domain of Gal4, respectively. Yeast cells transformed with the indicated plasmids were initially selected on synthetic dropout medium lacking leucine and tryptophan at 30 °C. Colonies were subsequently inoculated on medium further lacking histidine. The growth of colonies suggests protein interaction. **(B)** *In vitro* binding assays using the bacterially expressed proteins. Flag-E2F1 was used as a negative control. IP, immunoprecipitation; IB, immunoblotting. **(C)** The interaction of Flag-Nudel with Gγ2-free Gβ *in vivo*. HEK293T cells transfected to overexpress Flag-Nudel and Gγ2 (lane 1) or Gγ2 alone were lysed in co-IP buffer (see Materials and Methods) and subjected to co-IP (lanes 2-3) using anti-Flag M2 resin. IB was then performed to visualize the indicated proteins. **(D)** The *in vivo* interaction between endogenous Nudel and Gβ. The HEK293T cell lysates (lanes 1, 4) were subjected to co-IP using a control IgY (lane 2), anti-Nudel IgY (lane 3), anti-Gβ antibody (lane 5), or normal IgG (lane 6). **(E**, **F)** The mapping of the binding domains. The diagrams of Nudel, Gβ2, and the mutants, together with their binding abilities as assessed by co-IP, are shown. Also see Supplementary information, Figure S2 for the corresponding IB results. The asterisks in P2Nm indicate that the QAIER motif was mutated to AAILV. **(G)** The association of Flag-Nudel with HA-tagged Gβ subtypes. Co-IP was performed with the lysates of HEK293T cells overexpressing the indicated proteins. Gβ3 was not examined due to the lack of its cDNA.

teracts with the WD repeats of Gβ that is free of α and γ subunits. As Nudel differs from other Gβ interactors that bind to the Gβγ heterodimer [1, 4], we speculate that it may regulate the clearance of mfGβ rather than physiological functions of Gβγ.

Nudel promotes the accumulation of mfGβ at the centrosome region

To gain insights into the biological significance of the Nudel-Gβ interaction, we examined their potential interplays in COS-7 cells. As shown in previous studies [29-31], GFP-Nudel localized at the centrosome (Figure 2A, panel 1). His-Gβ2 exhibited centrosome localization in 43.2% ± 1.6% of transfected cells (n = 317, two experiments) (Figure 2A, panel 2, arrow), though it also formed aggregates of varying sizes and numbers (Figure 2A, panel 2). In addition to His-Gβ2, RFP-Gβ2 also showed the similar centrosomal localization (Supplementary information, Figure S3A). Moreover, although endogenous Gβ is typically plasma membrane-localized [5-7], permeabilization briefly with Triton X-100 (0.1%) prior to the fixation facilitated the detection of its centrosome localization in COS-7 and HeLa cells (Supplementary information, Figure S3B), indicating that such

a centrosomal localization is an intrinsic property of Gβ. Surprisingly, overexpression of both GFP-Nudel and His-Gβ2 (Figure 2B) led to the formation of a large aggregate containing both proteins at the centrosome region (Figure 2A, panels 3-4) in approximately 60% of cells (n = 356), reminiscent of aggresomes in morphology [32]. As Nudel interacted with all the Gβ isoforms that we examined (Figure 1G), we coexpressed GFP-Nudel with HA-tagged Gβ1, Gβ2, Gβ4, or Gβ5 and observed the similar aggregate formation at the centrosomal region (Supplementary information, Figure S4).

We then investigated whether the large centrosomal aggregates were the aggresomes. The aggresome is surrounded by cage-like vimentin structures, due to the rearrangement of vimentin-containing intermediate filaments [12, 32], and is often rich in ubiquitin, due to the polyubiquitination of misfolded proteins [18, 32]. We coexpressed GFP-Nudel and RFP-Gβ2 to facilitate multi-color fluorescence microscopy and found that they formed centrosomal aggregates, which were indeed surrounded by the cage-like vimentin structures (Figure 2C, panels 1-2, arrows). Moreover, the aggregates were labeled by anti-Ub antibody (Figure 2C, panels 3-4, arrows), suggesting the enrichment of ubiquitinated protein(s). When Gγ2 was coexpressed, it predominantly displayed membrane localization and was absent in the aggregates (Figure 2C, panels 5-6), indicating that the Gβ2 in the aggregates is free of Gγ and thus misfolded. Together, these

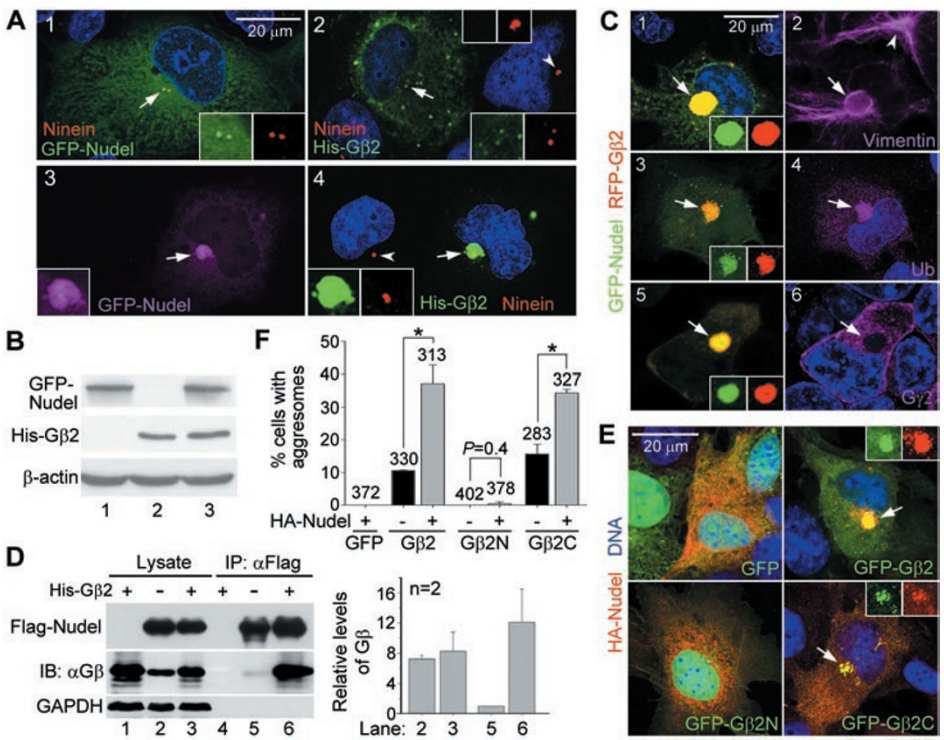

Figure 2 Gβ2 forms aggresomes when overexpressed with Nudel. **(A)** GFP-Nudel and His-Gβ2 were overexpressed either separately (panels 1-2) or together (panels 3-4) in COS-7 cells. The centrosomes marked by ninein staining are indicated by the arrows in the transfected cells or arrowheads in the untransfected cells. Typical centrosome regions are magnified 2× to show details. Nuclear DNA is shown in blue. **(B)** The relative expression levels of GFP-Nudel and His-Gβ2 in COS-7 cells. **(C)** The centrosomal aggregates formed by exogenous Nudel and Gβ2 (arrows) were aggresomes. COS-7 cells were transfected to express GFP-Nudel and RFP-Gβ2. The cells were immunostained to visualize vimentin (panels 1-2) or Ub (panels 3-4). In panels 5-6, Gγ2 was further overexpressed to facilitate its detection by the anti-Gγ2 antibody, which did not have enough affinity to detect endogenous Gγ2 in cultured cells (see Supplementary information, Figure S1). The arrows point to the positions of the aggresomes. The arrowhead in panel 2 denotes vimentin staining in an untransfected cell. The insets show the fluorescence of individual proteins. Nuclear DNA is shown in blue. **(D)** Overexpression of Gβ2 dramatically stimulated the Nudel-Gβ interaction. Flag-Nudel was overexpressed alone or with His-Gβ2 in HEK293T cells. 1/40 of input or 1/10 of the co-IP eluate was loaded in each lane. **(E**, **F)** The aggresome formation of exogenous Gβ2 required the Nudel-Gβ interaction. HA-Nudel was coexpressed with the indicated GFP fusion proteins in COS-7 cells. The arrows indicate the aggresomes. The statistical results were from two independent experiments. The total numbers of counted cells are listed above the histograms. *indicates $P < 0.05$ in Student's t-test.

results indicate that Nudel and mfGβ form aggresomes upon their co-overexpression.

To understand whether the aggresome formation was correlated with increased Nudel-Gβ interaction, we used Flag-Nudel to immunoprecipitate Gβ from HEK293T cells. We found that, although overexpression of His-Gβ2 did not significantly increase the total levels of Gβ (Figure 2D, lane 3 vs 2), the amount of Gβ associated with Flag-Nudel increased by an average of 12.1-fold (lane 6 vs 5). Such a result is consistent with the stoichiometric requirement of both Nudel and Gβ2 for the aggresome formation (Figure 2A-2C) and also suggests that the overexpression of His-Gβ2 markedly increases the total levels of mfGβ. Provided that all the mfGβ was precipitated in the co-IP experiments, the overexpression of His-Gβ2 was estimated to increase the levels of mfGβ by approximately 10-fold, from 0.3% ± 0.1% to 3.1% ± 0.3% of the total Gβ.

To further confirm the specificity of the aggresome formation, we coexpressed HA-Nudel with GFP or GFP-tagged Gβ2 or its mutants (Figure 2E). Statistical analyses indicated that GFP-Gβ2 or GFP-Gβ2C, a deletion mutant capable of binding to Nudel (Figure 1F and Supplementary information, Figure S2B), alone formed the aggresome-like aggregates in an average of 10.6% or 15.7% of cells, the coexpression of HA-Nudel increased the incidence of the aggresome formation to 37.1% and 34.4%, respectively (Figure 2E and 2F). However, neither GFP nor GFP-Gβ2N, a truncation mutant showing no interaction with Nudel (Figure 1F and Supplementary information, Figure S2B), exhibited the aggresome formation, regardless of the expression of HA-Nudel (Figure 2E and 2F). Taken together, we conclude that Nudel facilitates the centrosome accumulation of mfGβ in their interaction-dependent manner.

The aggresome formation of Gβ2 requires cytoplasmic dynein activity

To understand how the Gβ2 aggresome was formed, we examined the dynamics of RFP-Gβ2 in the presence of GFP-Nudel or GFP in live cells. To monitor early events, COS-7 cells that were transfected for 12 h, at which time the accumulation of RFP-Gβ2 at the juxtanuclear region was still not significant (Figure 3A), were imaged at 3-min intervals for at least 240 min. In the control cells overexpressing GFP, RFP-Gβ2 failed to show an enrichment at the juxtanuclear region over time, whereas in the presence of GFP-Nudel, small RFP-Gβ2 aggregates gradually accumulated at the centrosome (Figure 3A, arrows; Figure 3B).

As Nudel is a dynein regulator, we examined whether dynein contributed to the Gβ2 aggresome formation. We found that both dynein (DIC) and dynactin ($p150^{Glued}$) were enriched at the aggresomes (Figure 3C, arrows). Moreover, when GFP-Nudel and His-Gβ2 were co-overexpressed with Flag-tagged p50, a dynactin subunit that inactivates dynein upon its overexpression [33], the aggresome formation was abolished in 91% (n = 448, two experiments) of the triple positive cells. In these cells, exogenous Nudel and Gβ2 only exhibited basal-level centrosome localizations (Figure 3D, arrow). Immunoblotting (IB) indicated that p50 overexpression had no effect on the protein levels of Nudel and Gβ (Supplementary information, Figure S5). Therefore, the loss of Gβ aggresome formation (Figure 3D) was attributed to the inactivation of dynein instead of decreased Nudel or Gβ levels. The His-Gβ2 remaining at the centrosome (Figure 3D) may be due to residual dynein activity or to the direct association of Gβ2 with centrosomal Nudel, which is dynein-independent [31].

To corroborate the above results, we used erythro-9-(3-(2-hydroxynonyl))adenine (EHNA), a chemical inhibitor of dynein, to block dynein-mediated transport [34-36] and observed a dose-dependent reduction in aggresome formation in cells overexpressing both RFP-Gβ2 and GFP-Nudel (Figure 3E).

The aggresome formation of Gβ2 requires the interaction of Nudel with dynein

We further clarified whether the interaction between Nudel and dynein is important for the aggresome formation of Gβ. To do so, we made use of the mutant $Nudel^{N20/C36}$, which cannot interact with either Lis1 or dynein and thus fails to affect dynein function upon overexpression [19]. Co-IP showed that Flag- $Nudel^{N20/C36}$ still associated with Gβ, though with reduced affinity as compared with Flag-Nudel (Figure 4A, lane 6 vs 5). When GFP- $Nudel^{N20/C36}$ was overexpressed with RFP-Gβ2, it failed to facilitate aggresome formation (Figure 4B). These results confirmed that Nudel recruited Gβ to dynein.

Tctex1, a dynein light chain, is known to associate with Gβγ [37]. If dynein could transport Gβγ to the centrosome, overexpression of Tctex1 with Gβ might also form aggresomes. Nevertheless, when Flag-tagged Nudel or Tctex1 was overexpressed with RFP-Gβ2, only Flag-Nudel stimulated aggresome formation (Figure 4C). Such a result further supports the idea that the Gβ-containing aggresome is formed by mfGβ, not Gβγ.

Endogenous mfGβ is enriched in aggresomes upon inhibition of the proteasome activity

Many proteins are disposed into aggresomes upon inhibition of their proteasome-mediated degradation [12,

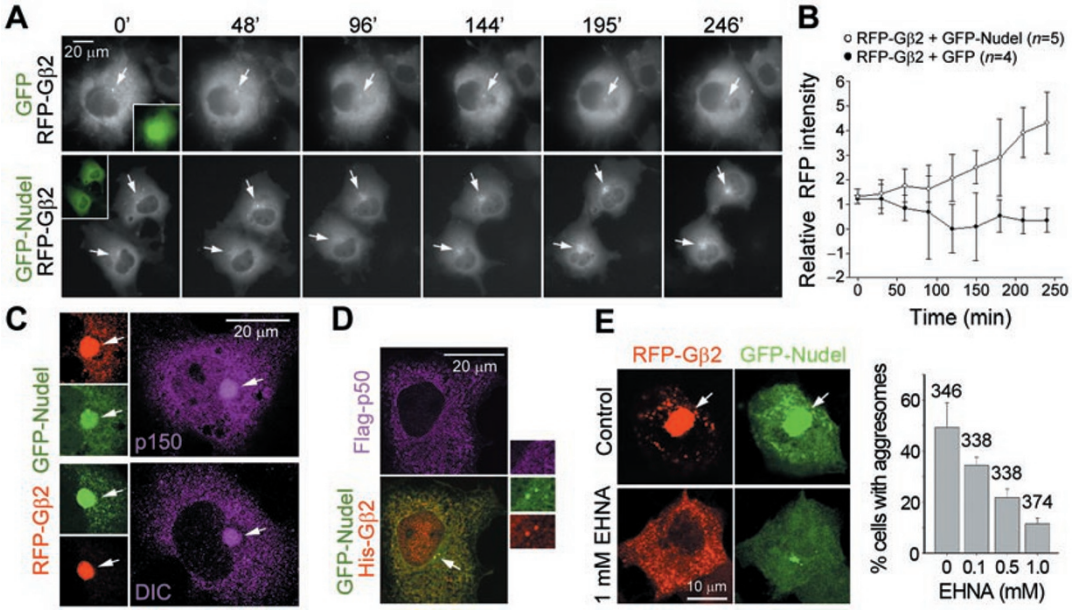

Figure 3 The formation of the Gβ2 aggresomes requires the dynein-mediated transport. **(A)** The representative RFP image sequences for COS-7 cells overexpressing the indicated proteins. The insets show the expression of GFP or GFP-Nudel. Cells were imaged at 3-min intervals for at least 240 min at 12 h post transfection. The arrows indicate the centrosome regions. **(B)** The statistical analysis for relative RFP intensities at the centrosome regions. The quantification was performed using the images with 30-min intervals. The relative intensity was obtained by normalizing the centrosome/aggresome intensity with the total cell intensity to reduce the influences of quenching or the protein level changes during the imaging. **(C)** The enrichment of dynactin and dynein in the Gβ2 aggresomes. COS-7 cells overexpressing GFP-Nudel and RFP-Gβ2 were subjected to immunostaining to visualize $p150^{Glued}$ or DIC. DIC was detected with the mAb 70.1. The arrows indicate the aggresomes. **(D)** Overexpressing p50 abolished the Gβ aggresome formation. COS-7 cells were co-transfected for 48 h to overexpress the indicated proteins. The immunostaining was performed using antibodies to Flag and polyhistidine (His). Centrosome regions that were magnified 2× are also shown. **(E)** The dynein inhibitor EHNA repressed the Gβ aggresome formation. The HEK293T cells were transfected to overexpress GFP-Nudel and RFP-Gβ2 for 24 h and treated with EHNA for another 24 h prior to fixation. The statistical results for the aggresome formation were from two independent experiments. The total numbers of counted cells are listed above the histograms.

13]. To understand whether endogenous Gβ could accumulate in such aggresomes, we treated COS-7 cells for 8 h with MG132, a proteasome inhibitor, to induce aggresome formation [38]. In contrast to mock-treated cells, aggresomes formed in MG132-treated cells, as indicated by the appearance of cage-like structures of vimentin (Figure 5A, panel 4 vs 2) [12, 32]. Gβ was enriched in these aggresomes (Figure 5A, panel 3, arrowheads) compared to its punctate centrosomal localization in mock-treated cells (panel 1, arrowheads). Such data suggest that endogenous Gβ is degraded by the proteasome in cells.

MfGβ is degraded via the UPS pathway

We next examined whether Gβ was subjected to polyubiquitination prior to degradation. HEK293T cells were treated with MG132 for 24 h, lysed in the RIPA buffer to disrupt protein-protein interactions, and subjected to IP using an anti-Gβ antibody (Figure 5B, lanes 1-4; Supplementary information, Figure S6). Compared to the mock-treated cells, the ubiquitination levels of endogenous Gβ markedly increased after MG132 treatment (Figure 5B, lanes 5-6; Supplementary information, Figure S6). When Gβ1, Gβ2, Gβ4, and Gβ5 were ectopically expressed, polyubiquitination was observed for all of the Gβ isoforms (Supplementary information, Figure S7, lanes 7-10). Moreover, the overexpression significantly increased the ubiquitination levels of the Gβ because polyubiquitinated Gβ was detected in the absence of MG132 (Figure 5C, lane 5 vs 4; Supplementary information, Figure S7). Further overexpression of Gγ2 to assist the proper folding of excess Gβ [2, 3, 8] largely reduced the ubiquitination levels of exogenous Gβ2 (Figure 5C, lane 6 vs 5), again suggesting that the polyubiquitination occurs to mfGβ. Consistently, when Flag-Nudel was used to immunoprecipite mfGβ, Nudel-associated Gβ was re-

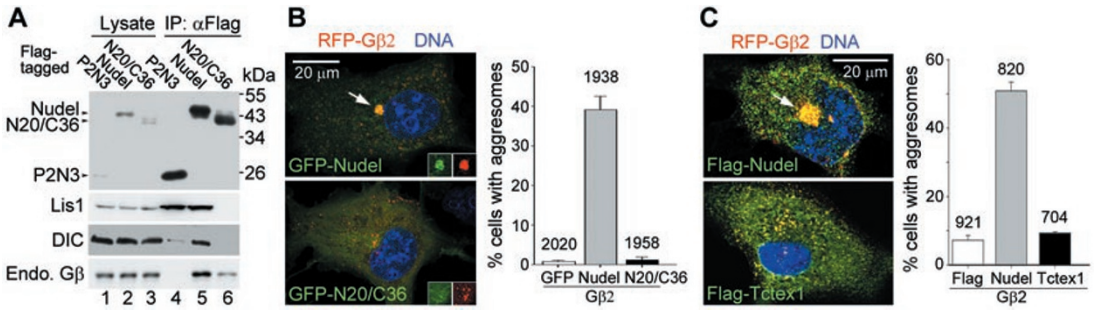

Figure 4 The formation of the Gβ2 aggresomes requires the Nudel-dynein association. **(A)** The association of endogenous Gβ with Nudel$^{N20/C36}$. HEK293T cells overexpressing Flag-tagged Nudel or Nudel mutants were lysed in co-IP buffer and subjected to IP. IB was then performed to detect the indicated proteins. DIC was detected with the mAb 70.1. **(B, C)** The effect of Nudel$^{N20/C36}$ or Tctex1 on the aggresome formation of RFP-Gβ2. The COS-7 cells were transfected to co-overexpress the indicated fusion proteins. The arrow points to an aggresome. The insets in **B** show the fluorescence patterns of each protein at the centrosome regions. The statistical results for the aggresome formation were from two independent experiments. The total numbers of cells are listed above histograms.

duced by 50.0% on average in cells overexpressing Gγ2 (Figure 5D, lane 6 vs 5). Interestingly, in contrast to the situation of the Gβ overexpression (Figure 5D), the overexpressed Gγ2 had little effect on the levels of Nudel-associated endogenous mfGβ (Figure 5E, lane 6 vs 7), suggesting that the majority of endogenous mfGβ is probably no longer reversible to the native conformation and is thus destined for degradation.

Nudel can associate with ubiquitinated Gβ

We then performed double IP experiments to examine whether Nudel could interact with ubiquitinated Gβ (Figure 5F and 5G). We overexpressed His-Gβ2 in HEK293T cells to enrich the polyubiquitinated Gβ (Figure 5C) and then mixed the cell lysate with those containing Flag-tagged Nudel, Nudel^{P2N3}, or Gγ2. Co-IP was then performed using anti-Flag M2 resin to precipitate associated proteins. As expected, Gβ co-immunoprecipitated with both Flag-Nudel (Figure 5G, lane 2) and Flag-Gγ2 (lane 3), but not Flag-Nudel^{P2N3} (lane 1). When the immunocomplexes were diluted in the RIPA buffer and subjected to the second round of IP with an anti-Gβ antibody to isolate Gβ, we found that polyubiquitinated Gβ was highly enriched in the immunoprecipitates of Flag-Nudel (Figure 5G, lane 5) compared to those of Flag-Gγ2 (lane 6). Therefore, Nudel is able to interact with both unubiquitinated and ubiquitinated mfGβ (Figure 5F and 5G, lane 5).

MfGβ associates with dynein in a Nudel-dependent manner

To further confirm that mfGβ was loaded onto dynein, we examined whether endogenous Gβ was able to associate with dynein. We found that overexpressing low levels of Flag-GFP-DIC2 in HEK293T cells using an internal ribosome entry site (IRES) [39] followed by co-IP with anti-Flag M2 resin efficiently isolated dynein (marked by DHC and DIC), dynactin (marked by p150^{Glued}), and Nudel (Figure 6A and Supplementary information, Figure S8, lane 7). Endogenous Gβ was detected in association with Flag-GFP-DIC2 as well (Figure 6A, lane 3). In contrast, Flag-GFP-p50 expressed in the similar way mainly precipitated dynactin (Supplementary information, Figure S8, lane 6), whereas co-IP with anti-DIC antibody (74.1) only enriched dynein (lane 9) presumably due to disruption of the DIC-p150^{Glued} and DIC-Nudel interactions [40, 41]. We thus used Flag-GFP-DIC2 in the following experiments.

First, we examined whether reducing the levels of mfGβ could affect dynein-associated Gβ. Since endogenous mfGβ was hardly restored by extra Gγ2 (Figure 5E), possibly due to fetal structural defects, we overexpressed His-Gβ2 to induce restorable mfGβ (Figures 2D and 5D). Co-IP with anti-Flag resin followed by IB indicated that dynein-associated Gβ decreased in the presence of exogenous Gγ2 (Figure 6B, lane 8 vs 7). Gγ2 itself, however, did not associate with dynein (Figure 6B, lane 8), again suggesting an association of mfGβ, but not the Gβγ dimer, with dynein.

Next, we investigated how Nudel levels impacted the dynein-Gβ interaction. We increased the total levels of Nudel by 5.7±0.6-fold via overexpression of HA-Nudel (Figure 6C, lane 3 vs 2) and found an increased associations of Gβ (by 2.4-fold on average) and Nudel with dynein in co-IP experiments (Figure 6C, lane 8 vs 7). Conversely, knockdown of Nudel expression by RNAi (Figure 6C, lane 5 vs 4) attenuated dynein-associated Gβ (lane 10 vs 9). These data confirm that the Gβ-dynein interaction depends on Nudel.

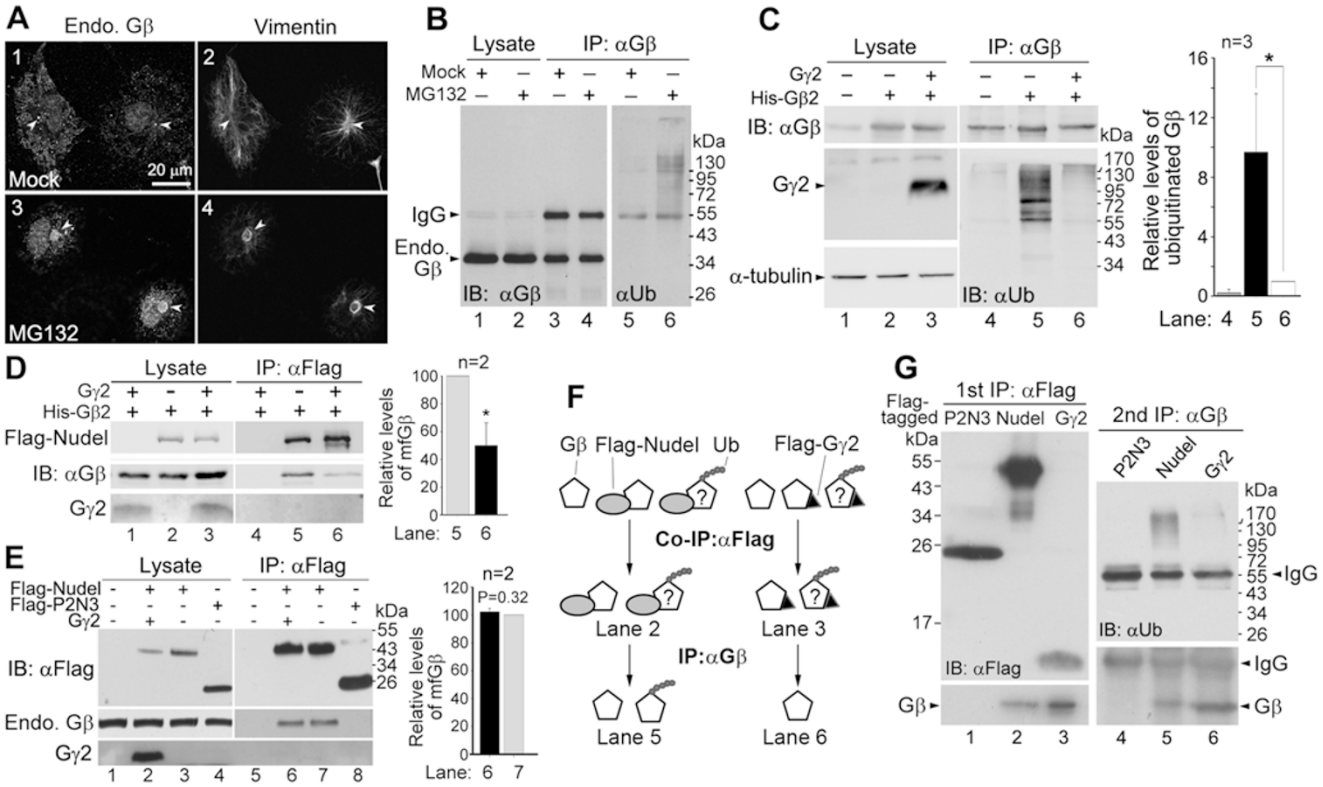

Figure 5 mfGβ can be ubiquitinated and then degraded through the UPS pathway. **(A)** Endogenous (endo) Gβ was enriched in the aggresomes after the inhibition of proteasome activity. The COS-7 cells treated with MG132 (5 μM) or solvent (DMSO) for 8 h were immunostained to visualize Gβ and vimentin. The arrowheads point to the centrosome regions. **(B)** Endogenous Gβ was able to be polyubiquitinated. The HEK293T cells were treated with MG132 (5 μM) or DMSO for 24 h (lanes 1-2), lysed in the RIPA buffer, and subjected to IP using an anti-Gβ antibody. The IB was then performed using an antibody against either Gβ (lanes 3-4) or Ub (lanes 5-6). **(C)** Exogenous Gβ2 exhibited misfolding-dependent ubiquitination. HEK293T cells were either untransfected (lane 1) or transfected for 48 h to overexpress Gγ2 and/or His-Gβ2 (lanes 2-3). The cells were lysed in the RIPA buffer and subjected to IP using an anti-Gβ antibody. The histogram shows the quantification results of three independent experiments. The intensities of the ubiquitinated Gβ were normalized with those of corresponding Gβ. *indicates $P < 0.05$ in Student's *t*-test. **(D, E)** The effect of Gγ2 overexpression on the Gβ-Nudel interaction. HEK293T cells overexpressing the indicated proteins were subjected to co-IP using anti-Flag M2 resin. The band intensities of Gβ were normalized with those of corresponding Flag-Nudel and are presented in histograms. **(F, G)** Ubiquitinated Gβ was able to associate with Nudel. Approximately 1×10^8 HEK293T cells overexpressing His-Gβ2 were lysed in the co-IP buffer, mixed with an equal amount of lysate containing Flag-tagged Nudel, Nudel^{P2N3}, or Gγ2, respectively, and co-immunoprecipitated using anti-Flag M2 resin (lanes 1-3). The immunocomplexes were then diluted in the RIPA buffer and subjected to the second round of IP using an anti-Gβ antibody (lanes 4-6). The experiments are diagrammed in **F**.

Dynein-mediated transport promotes the degradation of mfGβ

To confirm that endogenous mfGβ was indeed transported to the centrosome in dynein and Nudel-dependent manner, we knocked down Nudel in HeLa cells using three different siRNAs (Figure 7A), which was expected to both inactivate dynein-mediated protein transport [22, 31] and prevent mfGβ from loading onto dynein (Figures 5 and 6). To facilitate the detection of mfGβ, we treated cells with MG132 for 12 h to increase the levels of endogenous polyubiquitinated mfGβ (Figure 5A and 5B) and performed immunostaining (Figure 7B). Similar to untransfected cells (Figure 5A), aggresome formation was observed in 39.6% of cells transfected with control oligo on average (Figure 7B and 7C). By contrast, aggresomes were seen in only 2.4%-8.8% of cells after the depletion of Nudel (Figure 7B and 7C).

To understand whether the dynein-mediated transport affects Gβ turnover, we compared the half-life of nascent Gβ in control or Nudel RNAi cells. When nascent Gβ in control cells was pulse-labeled with ^{35}S-Met/Cys and then chased for up to 3 h, its half-life was estimated to

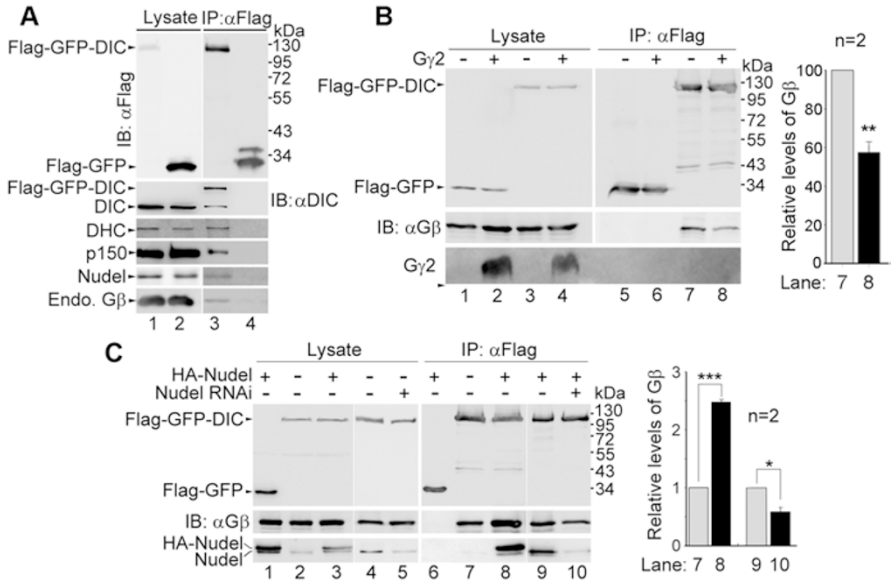

Figure 6 mfGβ associates with dynein in a Nudel-dependent way. **(A)** The association of endogenous Gβ with dynein. The HEK293T cells overexpressing Flag-GFP-DIC or Flag-GFP were subjected to co-IP using anti-Flag M2 resin. IB was then performed to detect the indicated proteins. Please note that Flag-GFP-DIC was not detected in the lysate with the antibody to DIC due to its low levels (lane 1), unless the blot was overexposed (data not shown). **(B)** Decreasing the levels of mfGβ by overexpression of Gγ2 reduced the Gβ-dynein association. HEK293T cells overexpressing His-Gβ2 and the indicated proteins were subjected to co-IP using anti-Flag M2 resin. **indicates $P < 0.01$ in Student's *t*-test. **(C)** The Gβ-dynein association depended on Nudel. HEK293T cells were transfected to overexpress His-Gβ2 and the indicated tagged proteins or to knock down Nudel by RNAi. Co-IP was then performed using anti-Flag M2 resin. * and *** indicates $P < 0.05$ and 0.001, respectively.

be ~0.8 h (Figure 7D-7F). However, in Nudel-depleted cells, the protein was markedly stabilized, with a half-life of more than 3 h (Figure 7D-7F). Therefore, the dynein-mediated transport of Gβ mainly facilitates the degradation of Gβ.

Long-term activation of Gβγ signaling increases Gβ ubiquitination

To assess whether proteasome-mediated degradation could serve as negative feedback to quench Gβγ signaling, we traced ubiquitination levels of Gβ after the activation of a typical GPCR, β2-adrenergic receptor (β2AR). We initially treated HEK293T cells with isoproterenol (Iso), a βAR agonist [42], for 10 h in the presence of MG132, but we failed to see a clear change in Gβ ubiquitination levels. We thus overexpressed HA-β2AR (Figure 8A, lane 2) to enhance the cellular response to Iso stimulation. In cells overexpressing HA-β2AR, Iso induced ERK1/2 activation as well as an accumulation of the polyubiquitinated Gβ (Figure 8A, lane 2 vs 1).

We then tested whether the activation of Gβγ in a GPCR-independent manner could also promote Gβ ubiquitination. HEK293T cells were transfected to overexpress Flag-tagged LGN to activate Gβγ signaling in a GPCR-independent manner [5]. Cells were collected at 10, 20, or 30 h post transfection to achieve different LGN expression levels (Figure 8B, lanes 2-4). To enrich ubiquitinated Gβ, cells were also treated with MG132 for 10 h prior to lysis with the RIPA buffer. IP with an anti-Gβ antibody indicated that the ubiquitinated Gβ positively correlated with LGN levels (Figure 8B, lanes 1-4). These data indicate that the sustained activation of Gβγ signaling is accompanied by the elevated ubiquitination of Gβ.

Discussion

Nudel selectively interacts with mfGβ

We showed that Nudel distinguishes itself from all other known Gβ-interacting proteins [1, 4] in that it only interacts with Gγ-free Gβ, which is generally believed to be unfolded or misfolded [2, 3, 8]. First, Nudel associated with Gγ-free Gβ both *in vitro* and *in vivo* (Figures 1, 5D and 5E). In addition to the full-length Gβ, Nudel also associated with deletion mutants of Gβ2 that contained only portions of the seven WD repeats (Figure 1). Second, Nudel was able to interact with polyubiquitinated Gβ (Figure 5F and 5G). Such forms of Gβ are most likely misfolded because they were enriched after the MG132

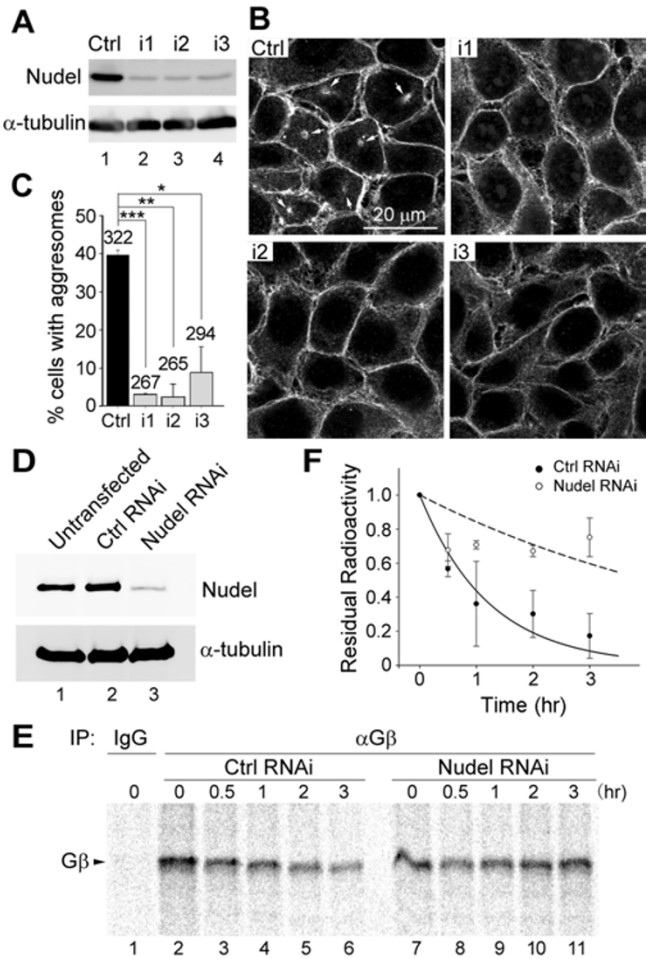

Figure 7 Nudel RNAi impairs the centrosomal accumulation of mfGβ and its turnover. **(A)** Confirmation of the Nudel RNAi efficiencies of individual siRNAs by IB. HeLa cells were transfected with a control oligo or one of the three siRNAs (i1-i3) for 72 h. **(B**, **C)** Nudel RNAi impaired the centrosomal accumulation of endogenous mfGβ. HeLa cells transfected with the indicated oligos for 60 h were treated with MG132 (5 mM) for 12 h prior to the fixation. Immunostaining was then performed with an anti-Gβ antibody. The statistical data were obtained from two independent experiments. The total numbers of cells are listed above the histograms. **(D)** Nudel RNAi efficiency in pulse-chase experiments. HEK293T cells were transfected with the control oligo or a mixture of i1-i3 for 48 h. A set of sample was then collected for IB. **(E**, **F)** Nudel RNAi markedly stabilized nascent Gβ. Cells transfected with siRNAs for 48 h were pulse-labeled with ^{35}S-Met/Cys for 1 h and then chased for the indicated time. Gβ was immunoprecipated with anti-Gβ antibody and subjected to SDS-PAGE and autoradiography. Its relative radioactivity was averaged from the results of two independent experiments. The curve fitting of the pulse chase data was performed using SigmaPlot.

treatment and were accumulated in the aggresome for degradation and sequestration (Figures 2C, 5B and 5C). Third, the aggresome formed by the overexpression of both Gβ2 and Nudel was devoid of Gγ2 (Figure 2C). This piece of data further confirmed the interaction between the Gγ-free Gβ and Nudel. Finally, the amount of Nudel-associated Gβ is positively correlated with the levels of Gγ-free Gβ. Overexpression of Gβ2 only slightly increased the total steady-state levels of Gβ but dramatically stimulated the Nudel-Gβ interaction (Figure 2D). Such a phenomenon is attributed to the marked increase in mfGβ levels, as judged by the robust polyubiquitination of Gβ (Figure 5C). The overexpression of Gγ2 attenuated the levels of both polyubiquitinated and Nudel-associated Gβ (Figure 5C and 5D). Interestingly, we found that overexpressed Gγ2 was unable to strongly attenuate the levels of endogenous mfGβ (Figure 5E), possibly because such mfGβ was already destined for degradation and thus irreversible to the native conformation. It should be noted that, although the overexpression of Gβ2 markedly elevated the levels of mfGβ (Figure 2D, lane 6 vs 5; Figure 5C, lane 5 vs 6), possibly due to increased nascent Gβ over endogenous Gγ, the overexpression only led to a mild increase in the total levels of Gβ (Figure 2D, lane 3 vs 2; Figure 5C, lanes 2-3 vs 1; Supplementary information, Figure S7, lanes 2-5 vs 1). Thus, we used the overexpression to increase the levels of mfGβ in this study to facilitate the biochemical and cytological assays. We also confirmed our major results with endogenous proteins.

Domain mapping suggests that Nudel uses its N-terminal region to bind to the WD repeats of Gβ (Figure 1E, 1F and Supplementary information, Figure S2). Interestingly, Nudel contains the "QXXER" motif that is found in many Gβγ effectors [27]. A peptide from AC2 that covers this motif can bind to multiple regions in the WD repeats of Gβ1 [27, 28]. Since this motif also contributes to the Nudel-Gβ interaction (Figure 1 and Supplementary information, Figure S2), it may mediate the interaction of Nudel with the WD repeats of Gβ as well.

Dynein transports Nudel-associated mfGβ to the centrosome for degradation

Our results suggested that mfGβ is subjected to dynein-mediated transport to the centrosome (Figure 8C). Dynein associated with Gγ-free Gβ (Figure 6B). Reducing the levels of mfGβ by overexpressing Gγ2 attenuated the levels of dynein-associated Gβ (Figure 6A and 6B). More importantly, inhibition of dynein activity by either p50 overexpression or EHNA treatment repressed the aggresome formation of mfGβ2 (Figure 3). The knockdown of Nudel by RNAi also disrupted the aggresome formation of endogenous mfGβ (Figure 7A-7C), though depletion of Nudel may impair both the dynein activity [22, 31] and the loading of Gβ onto dynein (see below).

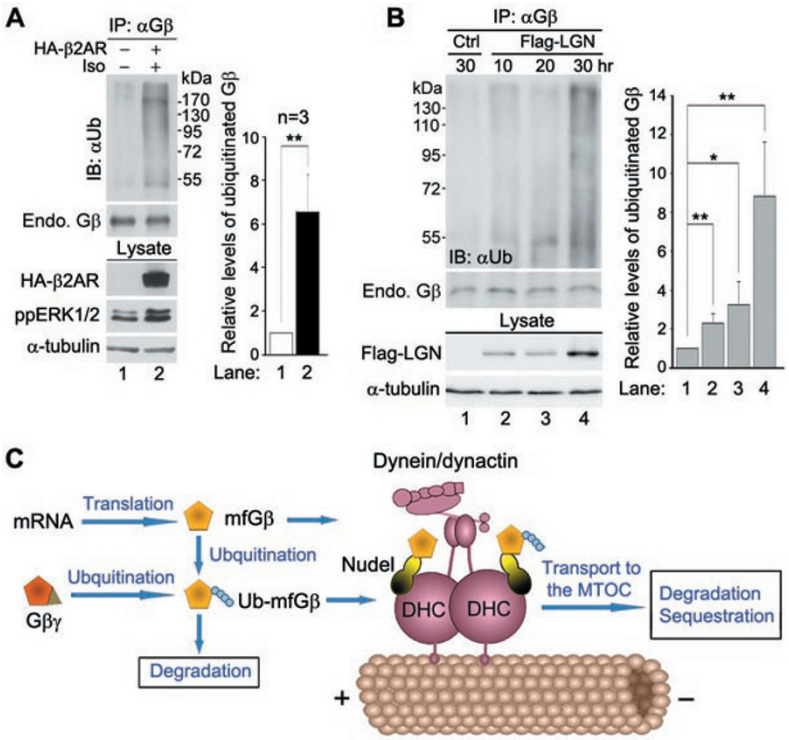

Figure 8 The polyubiquitination of Gβ can be stimulated through the sustained activation of Gβγ signaling. **(A)** The activation of Gβγ signaling via β2AR. HEK293T cells transfected for 38 h to overexpress HA-β2AR were treated with Iso and MG132 for an additional 10 h (lane 2), whereas the mock-transfected cells were treated only with MG132 (lane 1). The cells were then lysed in the RIPA buffer. The activation of Gβγ signaling was assessed by the increase in phosphorylated ERK1/2 (lane 2 vs 1). The lysates were subjected to IP using an anti-Gβ antibody (lanes 3-4). The intensities of ubiquitinated Gβ were normalized with those of the corresponding Gβ. **(B)** The activation of Gβγ signaling via LGN. Flag-LGN was overexpressed in HEK293T cells to induce the dissociation of Gβγ from Gα. The transfected cells were collected at the indicated time points after MG132 treatment for 10 h. The cell lysates in the RIPA buffer (lanes 1-4) were subjected to IP using an anti-Gβ antibody (lanes 5-8). The histograms show the quantification results of three independent experiments. **(C)** A model for the role of Nudel in the disposal of mfGβ. mfGβ may be generated after either the translation or the Gβγ signaling. It may either be degraded in the cytoplasm or be recruited to dynein by Nudel, followed by the transport to the MTOC for degradation and sequestration.

The failure of Tctex1 [37] overexpression to promote the formation of Gβ-containing aggresomes further suggests that dynein transports mfGβ, not Gβγ (Figure 4C).

We demonstrated that Nudel mediates the mfGβ-dynein association (Figure 8C). Dynein activity at basal levels is already sufficient for aggresome formation because mutant cystic fibrosis transmembrane regulator ($CFTR^{F508}$) or GFP-250 alone can form aggresomes upon overexpression [32, 43]. The overexpressed Gβ2 or its WD repeat-containing mutant Gβ2C also formed the aggresome-like structures in a fraction of cells (Figure 2F). Therefore, the positive effect of the Nudel overexpression on the Gβ aggresome formation (Figure 2 and Supplementary information, Figure S4) is attributed to a more efficient loading of mfGβ onto dynein due to the increased Nudel-mfGβ interaction (Figure 2). Consistently, the levels of dynein-associated mfGβ (Figure 6B) were correlated with Nudel levels in the co-IP experiments (Figure 6C). The aggresome formation was independent of the expression tags that were used for the expressions of the exogenous Gβ and Nudel. Besides, GFP or GFP-Gβ2N, which was incapable of binding Nudel, failed to form the aggresomes regardless of the Nudel levels (Figure 2E and 2F), indicating the specificity of the aggresome formation. Specifically blocking the loading of mfGβ onto dynein through the overexpression of $Nudel^{N20/C36}$, a Nudel mutant that was able to interact with Gβ but not dynein (Figure 4A) [19, 22], also repressed the aggresome formation of the overexpressed Gβ2 (Figure 4B). The knockdown of Nudel by RNAi abolished the Gβ-dynein association as well (Figure 6C).

The centrosome is thought to be a site of protein sequestration, degradation, and refolding [44-46]. Since the half-life of nascent Gβ was markedly prolonged in cells

deprived of Nudel (Figure 7D and 7F), we concluded that mfGβ is transported to the MTOC for degradation (Figure 8C). The centrosomal localization of endogenous Gβ in intact cells (Supplementary information, Figure S3B) may reflect the steady-state levels of mfGβ, as a result of the dynamic balance between its accumulation and degradation. When the dynein-mediated transport of mfGβ surpasses the protein degradation rate at the centrosome, as in the case of MG132 treatment (Figure 5A) or Gβ overexpression (Figure 2 and Supplementary information, Figure S4), the accumulated misfolded protein(s) then trigger the aggresome formation and is thus sequestered from the cytosol. Interestingly, Gβ2 has recently been shown to exhibit a Gγ-independent function in mitochondrial fusion via the interaction with mitofusin 1 [47]. Whether this unique function of Gβ2 requires its dynein-mediated transport, however, is currently not known.

Degradation of mfGβ is important for the protein quality control and possibly the negative regulation of Gβγ signaling

Like many other proteins [11], mfGβ, possibly resulting from translational errors [11] or the failure to form a Gβγ dimer [4, 8], is also a target of the cellular protein quality control machinery and is thus subjected to degradation by the UPS system (Figure 8C). Overexpression of Gβ markedly increased the levels of mfGβ (Figure 2D) and of polyubiquitinated Gβ (Figure 5C and Supplementary information, Figure S7). The existence of Nudel-associated endogenous Gβ that was free of Gγ2 (Figures 1C, 1D, 2D and 5E) indicates the presence of mfGβ in intact cells. The enrichment of polyubiquitinated Gβ after the MG132 treatment (Figure 5B) indicates that the proteasome-mediated degradation of the protein occurs as a constitutive process in cells.

Emerging lines of evidence suggest that the degradation of Gβ is also used to negatively regulate Gβγ signaling. Excess Gβγ results in increased Gβ degradation [10]. Chronic stimulation of a GPCR leads to Gβ degradation as well [9]. We found that long-term activation of Gβγ signaling through either a GPCR (β2AR) or LGN increased the levels of polyubiquitinated Gβ (Figure 8A and 8B). These results strongly suggest the existence of a cellular mechanism to prevent excessive Gβγ signaling by degrading Gβ (Figure 8C). Such a negative feedback mechanism could apparently provide more precise control over Gβγ signaling, though further investigation is needed for a more detailed understanding of the mechanism. In addition to the Gβγ signaling, the functions of AGS family may also be regulated by the aggresome pathway because Gαi has been shown to prevent the aggresome localization of AGS3 promoted by Inscuteable or MG132 [7].

Because the proteasome-mediated degradation can occur both in the cytosol and at the centrosome [11, 44, 46], the dynein-mediated transport appears to function to efficiently clear mfGβ from the cytosol, allowing its rapid sequestration and degradation at the centrosome to possibly reduce the deleterious effects of misfolded proteins in the cytosol [12, 48]. Interestingly, in addition to Gβ, the Nudel-binding proteins Lis1 and DIC are also WD repeat-containing proteins [16, 29, 41]. Thus, Nudel might bind other WD-repeat proteins as well to either carry out physiological functions (similar to Lis1 and DIC) or promote the clearance of their misfolded forms (like Gβ).

Materials and Methods

Plasmids and siRNAs

The expression plasmids for Flag or GFP-tagged p50 (dynamitin), human Nudel and its mutants, Nudel^{C36}, Nudel$^{N20/C36}$, and Nudel^{P2N}, were described previously [19, 22, 30]. Other deletion or point mutants of Nudel described in this study were generated by polymerase-chain reaction (PCR). pEGFP-C1 (Clontech) was used to express GFP-Gβ2 or its mutants, whereas RFP-Gβ2 was expressed using a modified vector [22]. His- or HA-tagged Gβ2 was expressed using pcDNA3 (Invitrogen). HA-tagged Gβ1, Gβ4, and Gβ5, whose cDNAs were kindly provided by Dr N Gautam (Washington University at St. Louis, USA), were expressed using pcDNA3. pcDNA3-Gγ2 from Dr P Wedegaertner (Thomas Jefferson University, USA) was used to amplify the Gγ2 cDNA for construction of a Flag-Gγ2-expression plasmid using pUHD30F [49]. pXJ40-Flag-LGN was kindly provided by Dr S Bahri (Institute of Molecular and Cell Biology, Singapore). The expression plasmids for HA-β2AR and Flag-Tctex1 were from Drs G Pei and S Yang, respectively (Institute of Biochemistry and Cell Biology, China). pLV-IRES-Flag-GFP was described previously [39] and was used to construct pLV-IRES-Flag-GFP-DIC2 to express low levels of mouse DIC2. pFlag1 (IBI) and pTrc-HisA (Invitrogen) were used to express Flag- or His-tagged proteins in *E. coli*. All the PCR-amplified DNA sequences were verified by sequencing.

The siRNAs against human Nudel mRNA were purchased from Invitrogen. Their sequences are 5′-GAGCAUCAAUAUGCACAGAGCUAUA-3′; 5′-GGUCUCAGUGUUAGAAGAUGAUUUA-3′; and 5′-CCAGGCCAUUGAACGAAAUGCAUUU-3′.

Antibodies and reagents

Mouse monoclonal antibodies to α-tubulin, γ-tubulin, DIC (70.1), and to Flag, HA, and His tags were purchased from Sigma. Mouse monoclonal antibodies to p150^{Glued} and DIC (74.1) were from BD Biosciences and Chemicon, respectively. Mouse antibody to ninein was kindly provided by G Chan (University of Alberta, Canada). Rabbit anti-phospho-Erk1/2 (Thr202/Tyr204) antibody was purchased from Cell Signaling Technology. Rabbit antibodies to GFP, Ub, DHC, Gαi, Gαs, Gγ2, and pan-Gβ were from Santa Cruz Biotechnology. Rabbit antibodies to the Flag tag

and γ-tubulin were from Sigma. Goat polyclonal antibody to LGN (also named GPSM2) and rabbit polyclonal to LIS1 were from Abcam. Anti-Nudel IgY was generated from chicken [22]. Rabbit polyclonal antibodies to Gγ and Centrin1 were from ProteinTech Group. Secondary antibodies conjugated with Alexa 405, 488, 546, or 633 were from Molecular Probes. Cy5-conjugated antibodies were from Rockland.

Isoproterenol (Iso), 4′,6-diamidino-2-phenylindole (DAPI), EHNA, and MG132 were purchased from Sigma.

Yeast two-hybrid screen

The MATCHMAKER GAL4 two-hybrid system 2 and a human placenta cDNA library in pACT2 were from Clontech. Human Nudel was expressed using pAS2-1 as the bait. The screening was performed following the manufacturer's manuals.

Cell culture and transfection

HEK293T, COS-7, or HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% bovine serum (Sijiqing Company, Hangzhou, China) in 5% CO_2 at 37 °C. The plasmid transfection was performed using the conventional calcium phosphate method, whereas the siRNAs were transfected using Lipofectamine 2000 (Invitrogen). In overexpression experiments, the cells were harvested approximately 48 h post transfection for biochemical assays or fixed for microscopy unless otherwise described. Usually, HEK293T cells were used for the biochemical assays due to their high transfection efficiency, while COS-7 and HeLa cells were used for the microscopic analyses due to their large cell sizes.

Fluorescence microscopy

Generally, cells grown on coverslip were fixed in 4% paraformaldehyde for 15 min and then permeabilized with either 0.5% Triton X-100 in PBS or cold methanol for 5 min prior to immunofluorescence staining. Proper antibody combinations were chosen for multi-color staining, whereas fluorescent proteins were visualized via their autofluorescence. Nuclear DNA was stained with DAPI. To stain for endogenous centrosomal Gβ, cells were permeabilized with 0.1% Triton X-100 in PEM buffer (80 mM PIPES at pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$) for 1 min, followed by fixation in PEM buffer containing 4% paraformaldehyde. Fluorescence images were captured by using an Olympus BX51 microscope with a cold CCD camera (SPOT II, Diagnostic), or a laser confocal microscope (Leica TCS SP2 or SP5). For confocal microscopy with fixed cells, optical sections were scanned at 0.1-0.5 μm intervals. Z stack images were then formed by maximal projection.

For live-cell imaging, pEGFP-C1 (Clontech) or pEGFP-Nudel was cotransfected with pRFP-Gβ2 at 1:1 ratio into COS-7 cells. Cells were imaged at 37 °C using Leica AS MDW microscope system equipped with a cool CCD camera (SNAP HQ, Roper Scientific).

Quantitation for fluorescence intensities was done as described [50] with minor modifications. Statistic results were obtained in a blind fashion and presented as mean ± SD.

Immunoprecipitation and immunoblotting

For immunoprecipitation (IP) of protein complexes, HEK293T cells were lysed in co-IP buffer (20 mM Tris-Cl, pH 7.4, 150 mM KCl, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 1% NP-40, 10% glycerol, and 10 mM sodium pyrophosphate, together with protease inhibitors). For IP of the target protein alone, cells were lysed in the RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 1 mM DTT, together with protease inhibitors). The Flag-tagged proteins were precipitated using anti-Flag M2 resin (Sigma) as described [30]. HA-tagged proteins were precipitated by EZview™ Red Anti-HA Affinity Gel (Sigma) according to the instructions. Other proteins were precipitated using ~2 μg of antibody and 25 μl 50% slurry of protein G (Invitrogen) or protein A (Phamacia) Sepharose.

The bacterially expressed proteins were induced by IPTG as described [51]. For *in vitro* binding assay, *E. coli* was lysed in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 10 mM EDTA, 5 mM DTT) by sonication. Flag-tagged proteins expressed in *E. coli* were each immobilized on 20 μl of anti-Flag M2 beads (Sigma) and incubated with 400 μl of bacterial lysate containing His-Gβ2 for 2 h at 4 °C. After three times of wash, bound proteins were eluted using Flag peptide.

Proteins were resolved by conventional SDS-PAGE, except that Gγ was separated with Tris-Tricine-SDS-PAGE [52]. Immunoblots were developed in Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences) and exposed to X-ray films (Kodak) or with Luminescent Image Analyzer (Fujifilm LAS4000). Blots were stripped for re-probing when necessary.

Pulse-chase analysis

Pulse-chase experiments were performed as described [53] with minor modifications. Briefly, HEK293T cells transfected with siRNAs for 48 h and grown in 60-mm Petri dishes were pre-incubated in Dulbecco's modified Eagle's medium minus Met and Cys (Invitrogen) for 1 h. Each plate of cells was then labeled with 1 ml of the medium containing 18 μl (200 μCi) of EasyTag™ EXPRESS ^{35}S Protein Labeling Mix (11 mCi/ml, Perkin Elmer) for 1 h. Cells were rinsed three times with the medium supplemented with 10% of fetal bovine serum and 2 mM of cold Met and Cys (Sigma-Aldrich), chased in the same medium for different time, and lysed in the RIPA buffer. Gβ was immunoprecipitated with anti-Gβ antibody from lysates with equal total radioactivity and subjected to 15% SDS-PAGE. Autoradiography was performed with Fluorescent Image Analyzer (FLA-9000, FujiFilm). Band intensities were quantified with Multi Gauge software (FujiFilm). Dynamic Curve Fitting of the pulse chase data was performed with SigmaPlot software.

Acknowledgments

We thank Dr Yixian Zheng (Carnegie Institution for Science, USA) and Dr Ronggui Hu (Institute of Biochemistry and Cell Biology (IBCB), Chinese Academy of Sciences (CAS)) for helpful suggestions and discussions, Qiongping Huang, Wei Yu and Yan Li for technical assistance. We also thank Drs Narasimhan Gautam (Washington University at St. Louis, USA) for Gβ cDNAs, Phillip B Wedegaertner (Thomas Jefferson University, USA) for pcDNA3-Gγ2, Sami Bahri (Institute of Molecular and Cell Biology, Singapore) for pXJ40-Flag-LGN, Gordon Chan (University of Alberta, Canada) for the ninein antibody, Gang Pei (IBCB, CAS) for pcDNA3-HA-β2AR, and Shuo Yang (ICBC, CAS) for pFlag-Tctex1. This work was supported by the National Basic Re-

search Program of China (2010CB912102 and 2012CB945003), the National Natural Science Foundation of China (30830060 and 31010103910), Chinese Academy of Sciences (XDA01010107), and Science and Technology Commission of Shanghai Municipality (09JC1416200).

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